PUBLISHED PAPERS

"MECHANISM OF ACTION OF THE CARDIAC GLYCOSIDES" AND RELATED AREAS OF RESEARCH

Submitted by

JOHN S. CHARNOCK, B.Sc., Ph.D. (Adelaide 1962)

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To Barbara

who has consistently encouraged my efforts
and shared my travails through the long journeys
and the lonely years, and without whom this work
could never have been accomplished.

Preface

The material submitted in this volume represents those of my scientific publications in the field of Biochemical Pharmacology which are concerned with the Mechanism of Action of the Cardiac Glycosides or closely related areas of research. The work was carried out in various laboratories in Canada, United States of America, United Kingdom and Australia between January 1963 and December 1977; either directly by myself or in collaboration with colleagues whose various contributions are acknowledged in the accompanying explanatory notes to this submission.

The aid of my various technicians and graduate students over the years is also gratefully acknowledged as is the financial support I have received from diverse government agencies and private foundations.

I hereby certify that none of the material collected here has been previously submitted by me or any other person to the University of Adelaide or any other University for the Degree of Doctor of Science.

I further certify that to the best of my knowledge, and except where expressly indicated, no portion of this work has been submitted by any other person for any other degree at this or any other university.

Dated this 12th day of October, 1977 at Edmonton, Alberta, Canada.

Signed

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EXPLANATORY NOTES

Paper No.

PART I

1.	Sole authorship:	A review of the literature and discussion of recent experimental findings in my own laboratory.
2.	Joint authorship:	Mr. L.P. Simonson, M.Sc., is a research assistant working under my direct supervision in my laboratory. The project was initiated, designed and supervised by J.S.C. The data was collated and the manuscript prepared by J.S.C.
3.	Joint authorship:	As in (2) above.
4.	Joint authorship:	Mr. A.F. Almeida, M.Sc. is a research assistant working under my direct supervision in my laboratory. The project was initiated, designed and supervised by J.S.C. The data was collated and the manuscript prepared by J.S.C. in collaboration with A.F.
5.	Joint authorship:	As in (3) and (4) above.
6.	Joint authorship:	A collaborative study carried out in the laboratories of Dr. J.C. Ellory at the Institute of Animal Physiology, Cambridge, U.K. and Dr. G. Radda, Department of Biochemistry, University of Oxford, U.K. All authors contributed equally to the design, implementation and reporting of the project. The manuscript was prepared by J.S.C.
7.	Joint authorship:	A collaborative study carried out with C.L.B. in the laboratory of Dr. G.K. Radda, Department of Biochemistry, University of Oxford, England. The project was initiated and designed by J.S.C. but carried out with the assistance of Dr. C.L. Bashford who had considerable experience in the spectrophotofluoremetric polarization procedures employed. The manuscript was prepared by J.S.C.
8.	Joint authorship:	Mr. Almeida, M.Sc. and Mrs. To, B.Sc., were research assistants working under my direct supervision. The project was initiated, designed and supervised by J.S.C. who also prepared the manuscript.

9. Joint authorship: Dr. D.A. Cook is a colleague in the Department of Pharmacology at the University of Alberta. His contribution was the development of a computer programme used in the statistical analysis of the experimental data. Mr. Almeida and Mrs. To and other information as in (8) above.

10. Joint authorship: Mr. Frankel was a graduate student registered for the degree of M.Sc. at the time the work was carried out under the direct supervision of J.S.C. The data was collated by J.S.C. who also prepared the manuscript for publication. The experimental findings of this paper were <u>not</u> submitted by D.F. for the degree of M.Sc.

11. Joint authorship: Miss Trebilcock was a graduate student registered for the degree of M.Sc. at the University of Adelaide. She carried out the majority of the laboratory experiments. Dr. Casley-Smith was a colleague who carried out the majority of the quantitative electron microscopy involved. J.S.C. initiated and designed the project, collated and analysed the experimental findings, supervised the work of H.A.T. and prepared the manuscript. Some of the experimental findings of this paper were submitted by H.A.T. for the degree of M.Sc.

12. Joint authorship: A collaborative effort in which the project was initiated and designed by my colleagues, Drs. Glow and Opit, in consultation with J.S.C. The analytical work was carried out in my laboratory. The data was collated by J.S.C. who also prepared the manuscript.

13. Joint authorship: Mr. Almeida was a graduate student registered for the degree of M.Sc. at the time the work was carried out under the direct supervision of J.S.C. The data was collated by A.F. who collaborated in the preparation of the manuscript. Some of the experimental findings of this paper were submitted by A.F. for the degree of M.Sc.

14. Joint authorship: Dr. D.A. Cook was a junior colleague in the Department of Pharmacology at the time the work was initiated and carried out under my supervision. Mrs. Casey, B.Sc., was employed as a technician. The data was collated and analysed by J.S.C. who also prepared the manuscript for publication.

15. Joint authorship: Dr. Cook was a junior colleague in the Department of Pharmacology at the time the work was initiated and carried out under my supervision. Dr. Opit was a colleague with whom I had actively collaborated previously. They participated in discussion of the experimental results and assisted in the preparation of the manuscript. The work is a collaborative effort in interpretation.

16. Joint authorship: Dr. Russel was a colleague at the University of Alberta at the time. He contributed the experimental technique which was utilized in the enzyme assays employed in this particular project. Miss Doty was our technician in this work. Data collate and analysed by J.S.C. Manuscript prepared by J.S.C.

17. Joint authorship: Miss Potter, B.Sc., and Mr. McKee were technical assistants in my laboratory at the Queen Elizabeth Hospital. The project was initiated, designed and supervised by J.S.C. who also collated the material and prepared the manuscript.

18. Joint authorship: As in (17) above.

19. Joint authorship: A review of the experimental results available at the time. A completely collaborative effort with a respected colleague. Manuscript prepared jointly by J.S.C. and L.J.O.

20. Joint authorship: An experimental project initiated and designed by J.S.C. Experimental work carried out by J.S.C. with the assistance of Miss Potter, B.Sc., who was employed as a technician in my laboratory at the Queen Elizabeth Hospital. Manuscript prepared by J.S.C.

21. Joint authorship: A project initiated by J.S.C. but designed and conducted with the active collaboration of Dr. Casely-Smith who carried out the semi-quantitative electron microscopy. Dr. Opit participated in the discussion and interpretation of the results. Manuscript prepared by J.S.C.

22. Joint authorship: Project initiated by J.S.C., experimental work carried out by Miss Potter under my supervision. Dr. Opit collaborated in the discussion and interpretation of the results. Manuscript prepared by J.S.C.

23. Joint authorship: Project initiated by L.J.O. and carried out by himself and Miss Potter under the supervision of J.S.C. J.S.C. was an active consultant throug out the work and during the preparation of the manuscript by L.J.O.

24. Sole authorship: Early development of a hypothesis.

25. Joint authorship: A collaborative development of the hypothesis referred to in (24) above. A totally joint effort.

26. Joint authorship: Project initiated and designed by L.J.O. in collaboration with J.S.C. Experiments carried out by L.J.O. under supervision of J.S.C. Manuscript prepared by L.J.O.

27. Joint authorship: Experimental work carried out by J.S.C. at Vanderbilt University during a post-doctoral fellowship under the supervision of R.L.P. Mr. Rosenthal was a medical student who had attempted some experimental work the year before, but whose contribution Dr. Post wished to acknowledge. Manuscript prepared by J.S.C.

28. Joint authorship: Experiments suggested by R.L.P. but designed and carried out by J.S.C. who also prepared the manuscript.

29. Joint authorship: Description of experimental work initiated and carried out by J.S.C. in the laboratory of R.L.P. at Vanderbilt University, U.S.A. Manuscript prepared by J.S.C.

30. Sole authorship: Carried out by J.S.C. at McGill University, Montreal.

31. Sole authorship: Carried out by J.S.C. at McGill University, Montreal.

PART II

32. Joint authorship: As in (2) above.

33. Joint authorship: As in (4) above.

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PART III

35.	Joint authorship:	Project initiated, designed and supervised by J.S.C. Paper prepared by and delivered by J.S.C.
36.	Joint authorship:	As in (35) above.
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40.	Joint authorship:	As in (35) above. Paper prepared and delivered by A.F.A.
41.	Sole authorship:	
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48.	Joint authorship:	Project initiated by R.L.P. Experimental work designed and carried out by J.S.C. Paper prepared by J.S.C. but presented by R.L.P.

PART I

In: 'Strategies in Cold: Natural Torpidity and Thermogenesis'. Academic Press, London and New York. Publication date, December 1977.

MEMBRANE LIPID PHASE-TRANSITIONS: A POSSIBLE BIOLOGICAL RESPONSE TO HIBERNATION?

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ABSTRACT

The effect of temperature upon the physical properties of membrane lipids and proteins is briefly reviewed as is the case for interpreting non-linear Arrhenius plots of enzyme activity in terms of lipid phase-transitions in biological membranes.

The thermal profiles of cytoplasmic, mitochondrial, plasma and endoplasmic reticular enzymes from poikilotherms, homeotherms and hibernators are compared.

It is concluded that whereas lipid phase-transitions play no part in the temperature dependence of the cytoplasmic enzymes lactic dehydrogenase and malic dehydrogenase, the evidence for pyruvate kinase is not yet clear. There can be little doubt of the important role of the lipid phase-transitions in modulating the temperature dependence of mitochondrial succinate oxidation in homeotherms, poikilotherms and during hibernation. Although there is strong evidence that lipid phase-transitions occur in the plasma and endoplasmic reticular membranes from a wide variety of species, and that in some instances these thermionic transitions occur at temperatures which correspond to marked changes in the apparent activation energy of Ca^{++}-ATPase and $(Na^{+} + K^{+})$-ATPase, there does not appear to be convincing evidence that these parameters undergo significant seasonal variation in hibernating species.

Thus no unifying concept of the role of lipid phase-transitions in the biological response to hibernation can be developed at the present time as it appears to vary with the particular enzyme system and its subcellular membrane localization.

I. INTRODUCTION

A. Membrane Structure

There has been much interest in the control of metabolism at low temperature, particularly the low body and organ temperatures encountered in some poikilotherms and during the hibernation state in mammals. Quite recently the idea has been growing that enzyme velocity is powerfully modulated by the physical properties of membrane lipids, and that so-called phase-changes in which the fluidity of membranes is greatly altered may provide the means for continuing enzyme function when the organ temperature falls far below the range at which many biochemical processes are expected to cease.

It is therefore my purpose in this review to examine the experimental evidence available in an attempt to decide what role phase-changes in membrane lipids may play in the process of hibernation. However, before looking at some recent experimental evidence from animal studies we should remember that as early as 1962, Luzzati and Husson had noted that the transition temperature (T_c°) of many solid-gel to liquid-crystalline phase-changes in lipoprotein systems seemed to occur very close to the body temperature of the animals from which the lipoproteins were obtained. They speculated that this observation was not coincidence, but that they were observing a very fine biological control mechanism for electrical potential or ion permeability changes in biological membranes (51).

This observation has much greater significance today than it did in 1962 as there has been great progress in recent years in our understanding of membrane structure.

That biological membranes are basically composed of an oriented bilayer of lipids interspersed with proteins has been known for many years (30,31,68), yet it has only been since the work of Singer and Nicolson in 1972 that a really satisfactory description of membrane architecture has been available (71). From the information obtained from a very wide variety of techniques which include chemical and immunological analyses as well as such biophysical procedures of freeze-fracture electron microscopy, X-ray diffraction, circular-dichroism and electron paramagnetic resonance and fluorescent membrane probes, we can be reasonably confident that the now common picture (Fig. 1) of globular protein marcomolecules embedded into a bimolecular lipid matrix properly represents the general membrane structure (17,70).

It is also quite reasonable to suppose that specificity of function takes place not only because of the diversity of chemical composition that can be shown in the lipid and protein components of the system, but also because of the non-homogeneous and asymmetric distribution of these components on either side of the membrane itself (69). In addition, some larger proteins appear to traverse the entire distance of the membrane and possess inherent orientation characteristics of their own (43) which permit vectorial processes to occur.

Furthermore, the work of Edidin and his colleagues (34,36) at Johns Hopkins has dramatically confirmed the dynamic state of the membrane conglomerate in that lateral diffusion of the protein globules in the plane of the membrane is commonplace, while even more recently the work of Chapman has suggested that rotational movement of the protein

macromolecules occurs in the microsecond time range (18). In fact, the only molecular movements which appear unlikely are those of the so-called "flip-flop" variety in which either lipids or proteins are translocated from one side of the membrane to the other. These "flip-flop" translocations either do not occur, or the time course of their translocation is so great as to exclude them from consideration in most, but not all, biological processes.

We therefore have a very dynamic view of membrane proteins and hence membrane enzymes functioning in a fairly mobile lipid matrix whose viscosity is subject to many changes in its immediate environment - pressure, temperature, ionic strength and pH, as well as variations which arise as a consequence of changes in the nature (class) and fatty acid composition of the lipid components themselves. Changes in any of these factors are all capable of influencing the physical and chemical properties of the membrane.

In addition, because of the non-homogeneous and asymmetric distribution of membrane components we can now see how one area of a biological membrane may be functioning in a certain mode, whilst another area not too far distant may be exhibiting quite different properties in terms of either permeability, electrical potential and/or enzyme function. It is therefore hardly surprising that vast differences in behaviour are encountered in various membranes from a wide variety of tissues and species as these membranes are required to meet the extraordinary range of situations encountered during the lifetime of the organism, whether it be a relatively simple bacterium or a highly complex multicellular assembly

like man himself.

Let us therefore consider only what happens in this complex membrane system when the temperature is varied through the relatively narrow "Physiological" range of 0°-40°C. One of the most striking features of the behaviour of the lipids in this range is the frequent observation that many of the individual components undergo a change in phase from a highly ordered state in which the hydrocarbon chains of the fatty acids are considered to be relatively rigid with a reduced protein mobility (the so-called solid-gel phase) to a much more fluid liquid-crystalline phase with enhanced protein mobility when the temperature is raised above a certain critical transition temperature (T_c°) (18,58).

This endothermic transition occurs over a somewhat broader range in biological membranes than in single phospholipid model systems (because of the variety in acyl-chain components as well as polar head-groups of the phospholipids) and is accompanied by a lateral expansion and a decrease in the thickness of the bilayer (3,61).

In this more fluid state the hydrocarbon chains maintain an average orientation perpendicular to the plane of the bilayer but are relatively disordered and undergo rapid rotational isomerization along the chains. This liquid-crystalline state is characterised by the onset of rapid lateral diffusion of both lipid and protein molecules in the plane of the bilayer. One should not forget, however, that both ordered and fluid domains can co-exist at a given temperature and that membrane proteins will tend to preferentially partition into the more fluid parts of the membrane.

B. Arrhenius Analysis - Activation Energy

What of the behaviour of the enzyme proteins embedded in this lipid matrix? How does it alter from the activity of the so-called soluble or cytoplasmic enzymes when the temperature changes from 0°-40°C? Before attempting to answer these questions let me briefly review the effect of temperature upon the velocity of any chemical reaction. That the velocity of any chemical reaction is reduced when the temperature falls is taken today as a self-evident axiom, although it was only during the latter half of the nineteenth century when this observation was formally established by van't Hoff who documented that the velocity of a variety of organic reactions increased by a factor of about 2 for every 10°C rise in temperature (74).

However, by 1889 Arrhenius had observed that a number of biological reactions - referred to by him as "life-processes" like the conversion of cane-sugar into glucose and fructose by the enzyme mixture "invertase" - did not properly obey the earlier van't Hoff relationship (5). He therefore studied the invertase reaction in great detail - that is, at a large number of different temperatures - and devised an empirical relationship for the nearly exponential increase in reaction velocity (K) which occurred with the change in absolute temperature (T°) (6).

When the rate constants for the reaction (usually V_{max}) are converted to a log form and the reciprocal of the absolute temperature is employed, a straight line plot (Fig. 2) can be obtained from the Arrhenius equation:

$$Ea = \frac{2.303\ T_1.T_2\ (\log K_1 - \log K_2)\ R}{T_1 - T_2}$$

where Ea = apparent energy of activation, R is the gas constant (1.987 cals mole $^{-1}$ deg^{-1}) and K_1 and K_2 are the maximal velocities of the

reaction at absolute temperatures T_1 and T_2. Thus the slopes of these plots are equal to -Ea/2.303R and give a measure of the Arrhenius activation energy of the reaction under study. This value is not quite the same as that obtained from direct measurement of the heat content of the reaction, but usually only differs from this value by a few hundred calories/mole (64).

For many years this type of Arrhenius analysis of the effect of temperature upon a biological process was thought to consistently yield a straight line Arrhenius plot and hence a single unique value for the activation energy (Ea), even if it became necessary to disregard some aberrant values or to use what Drost-Hansen (33) has so aptly called "brute force" methods to drive a straight line through fairly obviously non-linear data points. Generally departures from linearity were regarded as deficiencies in experimental data and were not assigned any biological significance (10,46).

However, by the early 1960's sufficient experimental data (Table I) had been produced for some workers to seriously question the absolute truth of the linear "Arrhenius law" and to suggest alternative explanations for the non-linearity of many Arrhenius plots (56). A typical example of a non-linear Arrhenius plot is shown in Fig. 3. Naturally, the early heretics who produced such plots were vigorously attached by the scientific establishment of the day (10,32,46).

Without pursuing this story in detail, it is clear today that

in at least some instances, these discontinuities in Arrhenius plots of enzyme activity which lead to more than one value for the activation energy of an enzyme process, are not experimental artifacts but are due to the existence of two different stable conformational forms of the enzyme which can be identified by different sedimentation characteristics, or electrophoretic patterns (56) as well as changes in their thermodynamic properties. In addition, the elegant studies of Raison and Lyons and their colleagues (66) using electron spin probes and those of many others using fluorescent probes of membrane lipid mobilities have shown that quite frequently there is a good correlation between the thermal relationships of enzyme activity and the physical state of associated membrane lipids (8,20,63).

Although there are still many who do not unequivocally accept this view (32,41,78), there is a great deal of experimental and theoretical evidence to suggest that discontinuities in Arrhenius plots represent thermal transitions between two distinct isothermal phases, which correspond to order-disroder transitions in the mobility of membrane lipids surrounding membrane proteins. In fact, in reviewing the effect of temperature upon Cellular Metabolism at a symposium on Low Temperature Physiology in 1972 Lyons stated that ' one of the most useful and consistent lines of evidence for phase-changes in biological systems has been the presentation of data relating the influence of temperature and reaction velocity (--of enzymes--) as an Arrhenius plot' (52)!

Today there is considerable biochemical evidence that the lipids of biological membranes exert a powerful modulating effect upon membrane enzyme function, and this is particularly noticeable when the temperature of the reaction is changed. By invoking the property of mutual exclusion (49) - that is, that two separate phases exist in the membrane as two separate domains each of constant activity and independent of the amount in each state, we can see how the same enzyme process can function with two markedly different energy requirements in two respective temperature ranges (49,78). The "break" in an Arrhenius plot (T_c°) therefore represent an isokinetic point, that is the temperature where the value for ΔF^* is the same for the two different transition states (49,64).

With this background let us now look briefly at a number of important enzymes generally involved in energy transduction, and whose temperature profiles have been examined in preparations isolated from homeotherms, poikilotherms and hibernators. Although not an exhaustive list, I will review the information relating to several cytoplasmic enzymes involved in glucose utilization and gluconeogenesis; some membrane-bound enzymes of mitochondrial oxidative phosphorylation and some plasma membrane enzymes involved in active cation transport.

(A) Cytoplasmic Enzymes of Glucose Utilization

1. Lactic Dehydrogenase (LDH)

In 1968 Hochachka and Somero reported that fish muscle LDH gave

linear Arrhenius plots between 5°-35°C even when the variety of fish ranged from long-term cold adapted Antarctic fish (-2°C) through lake and brook trout and bluefin tuna (presumably between 10°-20°C), to long-term warm adapted South American lungfish whose ambient temperature is normally between 27°-30°C (42). In addition, the apparent energies of activation that were obtained for these various muscle LDH preparations were all about 12 kcals/mole and did not correlate with the environmental temperatures of the fish (Table 2).

Similarly, Baldwin and Aleksiuk (7) obtained linear Arrhenius plots for liver and skeletal muscle LDH in the Australian platypus and echidna, both of which are reported to have unusual thermoregulatory properties. The Ea value for the platypus was 19 kcals/mole; Ea value for the echidna was 17 kcals/mole.

However, in an earlier study Aleksiuk (1) had noted an unusual 'inverted break' in muscle LDH from a warm-adapted garter-snake collected in Florida, which was not apparent in preparations from similar reptiles collected in Manitoba (see Table 2). Interestingly the Ea value from these latter cold-adapted snakes was again close to 12 kcals/mole.

More recently Borgmann and Moon have examined the kinetics of LDH function from homeotherms (beef heart and muscle) and poikilotherms (flounder muscle) in great detail. Although their purpose was not to obtain data for the usual Arrhenius equation of log V_{max} vs $\frac{1}{T°}$, it is apparent that no major changes were observed in this parameter between 5°-30°C, although there were important differences in other thermodynamic properties of these systems between homeotherms and poikilotherms (12, 13).

Finally, in another recent study Olsson (60) compared the Arrhenius function of LDH from several organs of a homeotherm (guinea pig), poikilotherms (frog and cod-fish), and the hibernating hedgehog and bat. Irrespective of the organ (heart, brain, muscle), the species or the season, the LDH Arrhenius plots were all linear between 0°-30°C and continued in this fashion for another 20°C in the case of the homeotherm preparations.

The values for Ea obtained by Olsson ranged from 12 kcals/mole for the frog muscle up to 19 kcals/mole for the hibernating bat heart. Again there was no obvious correlation between this thermodynamic parameter and environmental temperature.

2. Malic Dehydrogenase (MDH)

Although less information is available regarding this enzyme than LDH, what is known is very similar to that for LDH (Table 2).

Baldwin and Aleksiuk (7) had found linear Arrhenius plots for liver MDH between 10°-40°C for both platypus and echidna, with identical Ea values of 16.5 kcals/mole. This data is very similar to that obtained by Aleksiuk (2) for cytoplasmic MDH from the livers of two species of birds (the common crow and the pintail), where the Arrhenius plots were again linear between 5°-35°C, and the values for Ea were 12 kcals/mole and 15 kcals/mole respectively.

In Olsson's major comparative study discussed above for LDH (60) he also found linear Arrhenius plots for MDH between 0°-30°C for all the various organs and species examined. Because of heat denaturation of homeothermic enzymes many of his studies were not continued much above 30°C.

3. Pyruvate Kinase (PK)

The data for this enzyme is different for that of LDH and MDH just

discussed.

In 1968 Somero and Hochachka (72) showed that while muscle PK from fish (adult rainbow trout) gave linear Arrhenius plots between 5°-30°C, with Ea values of 30 kcals/mole, i.e. much higher than the Ea values reported for LDH or MDH, the data for PK from rat skeletal muscle PK between 10°-40°C was not linear. There was a sharp "break" in the plot at 25°C with a value for Ea_I of 10 kcals/mole above this temperature, and a value for Ea_{II} of 20 kcals/mole below this temperature! Interestingly these workers also reported an 'inverse break' in the data they obtained for the cold adapted (-2°) Antarctic fish where T_c° was about 10°C, and the values for Ea_I and Ea_{II} were the exact converse of those obtained for the rat (Table 3).

More recently, Borgmann and Moon (11) studied the temperature dependence of liver and flight muscle PK from a species of hibernating bat. These workers also found non-linear Arrhenius plots with PK from both organs whether the bat was hibernating or not. However, there was a marked shift in the T_c° from 17°C down to 5°C when the animal was in hibernation! It should also be noted that while the values for Ea_I above and Ea_{II} below the T_c° of 17°C in the non-hibernating bat were 8.6 kcals/mole and 19.5 kcals/mole respectively, the value for Ea above the T_c° at 5°C for the hibernating bat was 13.7 kcals/mole. That is, a value intermediate between the two values for the non-hibernating animal.

(B) Mitochondrial Membrane Enzymes of Oxidative Phosphorylation:

1. Succinate Oxidation

Let us turn now from the enzymes of glucose utilization to those of

oxidative phosphorylation located in the mitochondrial membrane.

As early as 1969 Kemp, Groot and Reitsma (47) had demonstrated non-linear Arrhenius plots for succinate oxidation by rat liver mitochondria. The T_c° they reported was 17°C with values for Ea_I above this of 8.9 kcals/mole and 18.8 kcals/mole for Ea_{II} below the temperature (Table 4). The following year Lyons and Raison (53) confirmed this non-linearity for succinate oxidation in rat liver mitochondria although their value for T_c° was 23°C, while that for Ea_I above the transition was 2.5 kcals/mole and that for Ea_{II} below the transition was 23.4 kcals/mole. That is, values very different from those of Kemp, et al. (47). Lyons and Raison also studied the temperature dependence of succinate oxidation from mitochondria obtained from the livers of poikilotherms (rainbow trout and channel catfish). Both these preparations gave linear Arrhenius plots without any thermal transitions in the 5°-35°C temperature range, and with a single Ea value of 8.3 kcals/mole for the trout and 12.2 kcals/mole for the catfish.

All these values were later confirmed in a subsequent paper (67) where it was shown that the treatment with detergent converted the discontinuous homeotherm plot of the rat into a linear plot similar to that obtained from poikilotherms.

Very soon after this Raison and Lyons (65) also reported that the nonhibernating ground squirrel gave a non-linear plot for liver succinate oxidation with a T_c° at about 20°C and values of 9.1 kcals/mole and 16.7 kcals/mole for Ea_I above and Ea_{II} below the transition respectively. However, preparations obtained from a hibernating animal gave a linear plot

with no thermal transition between 5°-35°C and again an intermediate value of 13.8 kcals/mole for a single Ea. Later, this work was extended by McMurchie and Raison (57) to show similar non-linear data for rabbit heart mitochondria (T_c° about 21°C; Ea_I 3.4 kcals/mole and Ea_{II} 12.6 kcals/mole) while that from the heart of the poikilotherm cane toad gave a linear response to temperature and hence a single value of 15 kcals/mole for the Ea.

Lenaz, et al. (50) have also reported non-linear Arrhenius data for beef heart mitochondrial succinate oxidation with a somewhat higher T_c° at 27°C and values for Ea_I of 9.1 kcals/mole and Ea_{II} of 17.0 kcals/mole above and below this transition temperature.

More recently, Wodtke (76) has reported non-linear Arrhenius plots for both mitochondrial succinate oxidation and cytochrome oxidase activity from the livers and muscle of a species of hatchery-bred carp (Cyprinus carpino) which had been maintained at an environmental temperature between 18°-20°C. When these fish were exposed to colder water at 10°C for 28 days the value for T_c° was 15°C, while it rose to 23°C when the fish were warm-adapted to water at 26°C for 21 days. Interestingly the values for Ea_I and Ea_{II} under both cold and warm adapted situations did not change significantly but remained near 5 kcals/mole above and 10 kcals/mole below the T_c°.

There is some experimental evidence which does not fit this general scheme for mitochondrial enzymes. It is the data of Woodard and Zimny (77) who measured succinic dehydrogenase activity in the hearts and brains of non-hibernating and hibernating ground squirrels. These workers reported only linear Arrhenius plots in both organs and under both

physiological states. Unfortunately these investigators studied only four temperatures in their experiments (5°, 13°, 21° and 35°C), and there were huge variations in the standard errors of the mean data points they obtained. Therefore, I do not feel that the data can be regarded as definitive. However, although not the interpretation favoured by the authors, my own assessment of their data from brain preparations is that the non-hibernator probably gave a non-linear Arrhenius plot which became linear during hibernation!

(C) Membrane Enzymes of Active Transport

Let us now look at enzymes of another form of energy tranduction, those involved in the active transport of ions across biological membranes.

1. Ca^{++}-ATPase

In an early study Inesi and Watanabe (45) observed a linear temperature dependence, i.e. a single straight line Arrhenius plot of Ca^{++}-activated ATPase of sarcoplasmic reticulum (SR) preparations from rabbit leg muscle examined between 5°-20°C. This plot showed good correlation with $^{45}Ca^{++}$-uptake by the same system. About the same time Yamamoto and Tonomura (79) also reported a linear Arrhenius plot obtained from SR of rabbit dorsal muscle over the wider temperature range of 0°-37°C, however these latter workers used a substrate concentration well below the optimum necessary for maximal Ca^{++} uptake (Table 5).

At the 1971 Perth Conference on Muscle Disease I reported that under experimental conditions of maximal $^{45}Ca^{++}$-uptake by rabbit leg muscle SR preparations (23), we could observe a non-linear Arrhenius plot

for Ca^{++}-ATPase activity between 0°-37°C with the T_c° at about 10°C. The values for Ea_I and Ea_{II} were 16.3 kcals/mole and 33.4 kcals/mole respectively. It should be noted that this data was obtained under experimental conditions which yielded a linear Arrhenius plot for Ca^{++}-uptake. That is, the temperature dependence of the enzyme process was different from that of the transport process. Presumably these different thermal profiles reflect two different but very closely associated protein functions in the overall Ca^{++} pump.

Subsequently, in a very detailed study of $^{45}Ca^{++}$-uptake and Ca^{++}-ATPase activity of white skeletal muscle of rabbit hind leg, Inesi, Millman and Eletr (44) confirmed that these preparations have non-linear Arrhenius characteristics. They determined a T_c° at 20° with values for Ea_I and Ea_{II} of 16 and 29 kcals/mole respectively, in their preparations. Similar findings were reported by Madeira and Antunes-Madeira (54) who also observed non-linear Arrhenius characteristics in rabbit skeletal muscle preparations with a T_c° at about 17°. However, the activation energies reported by these latter workers were somewhat lower in that the values for Ea_I and Ea_{II} were 9 and 22 kcals/mole respectively. A transition temperature at 11.5° has also been reported for sarcoplasmic reticulum preparations of the lobster (55) suggesting that non-linear Arrhenius characteristics for Ca^{++}-ATPase can also be observed in poikilotherms.

However, Becker and Willis (9) have reported linear Arrhenius plots for sarcoplasmic reticulum Ca^{++}-ATPase obtained from the leg muscles of the

homoethermic rat (Rattus norvegicus) and an awake hibernator - the thirteen-lined ground squirrel (Citellus tridecemlineatus). Although these observations were made between 5°-37°C there were only six temperature points through this range and inspection of the data shows that a non-linear fit could be made to the rat preparation (Fig. 4) with a T_c° at about 27°C, while that from the ground squirrel was probably linear near this temperature. In a related study Cossins and Bowler (28) have reported that they did not find any differences in the Arrhenius activation energy for thermal inactivation of Ca^{++}-ATPase activity of sarcoplasmic reticulum from the abdominal muscles of freshwater crayfish which had been temperature adapted to either 4°C or 25°C for several weeks.

Obviously the situation with this particular enzyme is not completely clear and more detailed comparative studies are required before we can finally decide what are the characteristic thermal profiles for Ca^{++}-ATPase in a variety of species, and at variable environmental temperatures.

2. $(Na^+ + K^+)$-ATPase

There is considerably more information available about the other major cation-transporting enzyme system of the plasma and endoplasmic reticular membranes - the $(Na^+ + K^+)$-ATPase of the "Sodium Pump".

Firstly, it is well known that the specific activity of this enzyme is subjected to very marked seasonal variations. In poikilotherms (frogs) both Kennedy and Nayler (48) and Csaky and Gallucci (29) have shown dramatic falls in enzyme activity of both the heart and intestine during the winter months. This fall correlates with either the loss of sensitivity to some cardiovascular active agents or to a decrease in sugar and amino-acid

transport. Conversely, Fang and Willis (35) reported a significant increase in the specific activity of $(Na^+ + K^+)$-ATPase in the renal cortex of Syrian hamsters during hibernation, and we have recently observed an equally marked increase in brain $(Na^+ + K^+)$-ATPase from ground squirrels during the hibernation state (25).

In addition to these seasonal variations in enzyme activity, there have been many studies of this enzyme's temperature profile from a large variety of animals and tissues. For example, as early as 1966 Gruener and Avi-Dor (40) reported a non-linear Arrhenius plot for $(Na^+ + K^+)$-ATPase obtained from rat brain (Table 6). This non-linearity in $(Na^+ + K^+)$-ATPase activity of enzymes prepared from the brains of homeotherms has been confirmed repeatedly (15,59,73). In my laboratory we have observed the same phenomena in enzymes prepared from guinea pig, rabbit, sheep and pig kidney (19,20,21,22). By recalculating the data of Willis and Li (75) obtained from an awake hamster, we could also obtain a non-linear Arrhenius plot for the renal cortical $(Na^+ + K^+)$-ATPase of this hibernating species.

McMurchie, Raison and Cairncross (57) have also reported non-linear temperature dependence for $(Na^+ + K^+)$-ATPase preparations from rabbit heart at the same time as they detected only linear temperature dependence with an enzyme preparation from the poikilotherm toad. Unfortunately, the enzyme assay system employed by these investigators contained only 40 mM Na^+ and 40 mM K^+, and did not include ouabain or other specific enzyme inhibitors, thus making the assertion that they were measuring

$(Na^+ + K^+)$-ATPase equivocal. However, a similar but not identical change had been reported some years before by Bowler and Duncan (14) who showed that in a brain preparation from a frog kept at 9°C for 1 week prior to their experiment, the microsomal $(Na^+ + K^+)$-ATPase had a very much lower value for the energy of activation below T_c° than that of a rat. This change in temperature profile they described as representing a characteristic "poikilotherm pattern" which was different from that found in enzyme preparations from homeotherms.

Some studies have also been carried out with several hibernating species both awake and during torpor. Bowler and Duncan (16) demonstrated markedly non-linear Arrhenius plots for brain $(Na^+ + K^+)$-ATPase from the hedgehog, which by our calculations from their data did not change greatly between non-hibernation and hibernation states. Similarly, when we recalculated the effect of temperature from the brain $(Na^+ + K^+)$-ATPase data of Goldman and Willis (38) and Goldman and Albers (39) for hamster preparations, we also obtained markedly non-linear Arrhenius plots whether the animals were awake and active, or whether they had been in hibernation for various periods of time.

We have recently extended these observations in a series of experiments in our own laboratory. Firstly, we compared the temperature dependence of ATP hydrolysis by brain and kidney $(Na^+ + K^+)$-ATPase preparations obtained from several common species of homeotherms (rabbit, sheep and beef), an awake hibernating species (the Richardson's ground squirrel, Spermophilus richardsonii) common to this area of Alberta, and from the leg nerves of two species of poikilotherms the Pacific crab (Cancer magister) and the Atlantic lobster (Homarus vulgaris) (24).

When the Arrhenius plots and the Ea values of all these preparations are examined (Table 7), it is apparent that all the temperature plots are non-linear and there are no striking differences in the temperatures of the thermionic transitions, nor are the values for Ea_I and Ea_{II} from a particular tissue very different. The awake hibernator could not be distinguished from a homeotherm on these grounds, although the enzyme preparations from the axons of the poikilotherms gave values for Ea_I and Ea_{II} which are lower than any obtained from either homeotherms or an awake hibernator (24).

Apparently the enzyme preparations from poikilotherms are the most thermodynamically efficient when judged on this basis, although they did not produce the straight-line pattern of Arrhenius activity previously reported by McMurchie, et al. (57) for their toad heart preparations. Perhaps we should recall that Bowler and Duncan (14) did not observe a linear Arrhenius plot with their frog brain preparations either!

In this particular study we also labelled the membrane enzyme preparations with the fluorescent probe 12-(9-anthroyl) stearic acid (12-AS) which we had used previously to determine the effect of temperature on the molecular mobility of the membrane lipids of sheep and pig kidney $(Na^+ + K^+)$-ATPase preparations (20).

Like our previous findings with the sheep and pig enzyme there was a strong correlation between the non-linear plots of molecular mobility of the lipids of homeotherm brains and non-linear Arrhenius plots of their $(Na^+ + K^+)$-ATPase activity. The T_c° values were about 25°C for all preparations (Fig. 5).

The results with poikilotherm membranes were quite different in that the change in fluorescence polarization was only linear between 8°-32°C as a thermal transition could be observed above this temperature! That is, the previously observed close correlation between thermal effects on lipid mobility and $(Na^+ + K^+)$-ATPase activity in membranes from kidney and brain of homeotherms could not be so readily observed in membranes from poikilotherms. Whereas there is a marked change in the fluidity of the lipids of homeotherm membranes at about 25°C, no such change could be detected by 12-AS labelling in the fluidity of the membranes from poikilotherms at this temperature, although a change did occur above 32°C.

However, when we compared the slopes of the fluorescence polarization vs temperature plots from the two species (Fig. 5), it is apparent that the crab membranes are more fluid than those of beef brain over their normal physiological temperature ranges. This of course is in complete agreement with the lower energies of activation that were found for poikilotherm $(Na^+ + K^+)$-ATPase preparations than for beef brain enzyme preparations at all temperatures studied (24).

In some subsequent experiments we also found that the markedly <u>non-linear</u> Arrhenius pattern of ground squirrel brain $(Na^+ + K^+)$-ATPase did not change even after more than 100 days into their hibernation season (25). Furthermore, the values for T_c°, and Ea_I above and Ea_{II} below this critical temperature were not significantly different from those previously reported for enzyme preparations from homeotherms (21) (Table 8).

What we did find which was of great interest to us was that there was a very marked fall in both the rate and amount of inhibitor (^{3}H-ouabain)

binding to these membrane enzyme preparations after prolonged periods (30-100 days) into the hibernation season. Clearly there were very marked changes in both the rate of [^{3}H]-ouabain binding (affinity) and the total amount of binding, i.e. the number of inhibitor binding sites in this brain tissue during hibernation (27) (Table 9).

Very recently we have completed a similar study of the ($Na^+ + K^+$)-ATPase of the ground squirrel renal cortex (26). Firstly, and unlike a previous study by Fang and Willis (35) using cold adapted and hibernating Syrian hamsters, we found a highly significant fall in ($Na^+ + K^+$)-ATPase in the ground squirrel preparations after both short term (< 30 days) and long term (> 75 days) hibernation at 5°C (Table 10). This fall in enzyme activity was matched by a fall in both the rate and amount of ouabain-binding to the membrane enzyme preparations. Thus the fall in kidney enzyme activity during hibernation is unlikely to be due to a change in the functional ability of the enzyme, but is propably due to a loss in enzyme protein under these conditions.

As we have repeatedly seen already in this discussion, another measure of an enzyme's thermodynamic efficiency is to determine the Ea of the system via an Arrhenius analysis. For comparative purposes alone this was most suitable in this case. From the data we obtained in thirteen separate experiments with awake and hibernating ground squirrels, it is also apparent that their renal cortical ($Na^+ + K^+$)-ATPase gave <u>non-linear</u> Arrhenius plots under all experimental conditions (Table 11). In addition, the values for T_c° and Ea_I and Ea_{II} during hibernation were not significantly different from those obtained in the awake summer control group.

Thus it is clear that no changes occurred in any of these thermodynamic parameters during the hibernation season, and that no changes in thermodynamic efficiency of the enzyme could be inferred from this data.

Rather, these findings strongly support our earlier proposal that in ground squirrel kidney ($Na^+ + K^+$)-ATPase preparations there is a marked loss of activity due to a significant fall in the amount of "pumping enzyme" available during hibernation. These results are in marked contrast to the concept of "cold resistance" in this tissue, which has been proposed by Willis and his colleagues (35,75).

V. DISCUSSION

In discussing these experimental results I think it is clear from Table 2 that for the cytoplasmic enzymes LDH and MDH there is overwhelming evidence that their Arrhenius plots are linear, irrespective of the tissue or species of origin, and apparently the environmental temperature. For pyruvate kinase (PK) the situation may be different in that while it is possible that enzyme preparations from poikilotherms give linear Arrhenius plots, it seems that enzyme preparations from both hibernators and homeotherms could yield non-linear Arrhenius plots (Table 3). In addition, it also seems that biological adaption to reduced environmental temperature results in a marked reduction in the transition temperature of this enzyme. As PK is part of the complete enzyme mechanism for the breakdown of glycogen which is found in the "soluble" fraction of cells, the non-linearity of PK Arrhenius plots is presumably independent of any membrane effects.

For the succinate oxidation system of the mitochondrial membranes of either liver or heart, there is some evidence that the enzyme systems

from poikilotherms display linear Arrhenius plots, while those mitochondrial enzymes from homeotherms yield non-linear plots. With mitochondrial enzymes from the hibernating ground squirrel there is evidence that the temperature profile seems to change from the non-linearity of a homeotherm in the summer to the linear form of a poikilotherm enzyme during hibernation (Table 4). In vitro modification of the lipid content of isolated mitochondrial membranes by detergents strongly suggests that this is a lipid mediated phenomena (64,67). There is also good evidence that the fatty acid content of mitochondria undergoes major changes during hibernation although the effects seem to be confined to young animals (62).

While there is not yet a great deal of evidence concerning thermal profiles for Ca^{++}-ATPase of either poikilotherms or hibernator preparations, it seems probable that membrane preparations from homeotherms display non-linear Arrhenius plots with a variety of transition temperatures, possibly depending upon the experimental conditions under which the enzyme was assayed (Table 5).

Fortunately, there is a wealth of data concerning the $(Na^{+} + K^{+})$-ATPase of coupled sodium and potassium transport across biological membranes. Only the toad heart enzyme has been reported to yield a linear Arrhenius plot (57), and for technical reasons this must be regarded as a somewhat doubtful result. To my knowledge no fish heart $(Na^{+} + K^{+})$-ATPase enzymes have been studied in this way. All homeotherm preparations which have been examined yield non-linear Arrhenius plots irrespective of the tissue source of the enzyme (kidney, brain and heart). Even the frog brain preparation of Bowler and Duncan (14) did not yield a

linear Arrhenius plot although we should remember that the animal was collected during the spring and had been maintained at 9°C for a week prior to preparation of the enzyme (Table 6).

By recalculating the experimental data of Bowler and Duncan (16), Goldman and Willis (38) and Goldman and Albers (39) obtained from both awake and hibernating hedgehogs and hamsters, we also found non-linear Arrhenius plots for $(Na^+ + K^+)$-ATPase preparations under all these conditions, a result which agrees with our own recent findings for the ground squirrel (25,26). It seems very probable then that these non-linear temperature profiles for $(Na^+ + K^+)$-ATPase are not altered by seasonal changes in the temperature of the environment.

This was certainly true in our own experiments with ground squirrel whether the $(Na^+ + K^+)$-ATPase enzyme was derived from brain or renal cortex (Table 8), as we could not detect any significant change in T_c° during hibernation. However, we did find major decreases in inhibitor binding to the enzyme preparations (Tables 9 and 10) similar to the changes in ligand and substrate binding to cytoplasmic enzymes during hibernation reported by Borgmann, et al. (11,12,13).

There is as yet little evidence that the enzymes of different organs in a particular animal display markedly different forms of temperature profiles, although until further work is done it may be that the heart will prove to be different from brain or kidney in this regard.

VI. SUMMARY

Generally linear profiles are obtained with the soluble enzymes LDH and MDH, yet PK is clearly different in that there seems to exist a characteristic pattern for poikilotherms which differs from that of

homeotherms. This type of characteristic pattern change is even more apparent with mitochondrial enzymes of oxidative phosphorylation where there is also good evidence for correlation between patterns of activation energy and the molecular mobility of lipids in the membranes.

With the $(Na^+ + K^+)$-ATPase of the plasma and endoplasmic reticular membranes the situation is different again. Experimental evidence is strongly in favour of non-linear Arrhenius plots for enzyme preparations from all tissues and species so far examined. We do not believe that this characteristic temperature profile changes during hibernation.

Thus there is no unifying concept for the role of phase-changes in biological membranes being responsible for the changes in enzyme function during hibernation, for while the organ may not be important in this regard, it seems very likely that the subcellular localization of the enzyme in either the mitochondrial or the plasma membrane makes a most important difference. While there can be little doubt about the profound changes that occur in the lipid composition of both whole brains and hearts of animals during hibernation (4,62), it is therefore not yet clear what effect this has upon the enzymatic function of the organs.

Presumably very detailed reconstitution studies of purified enzymes into liposomes of variable phospholipid composition obtained from both hibernators and euthermic controls will be necessary before this question can be answered, as this would allow us to rigorously control and test the effects of changing membrane lipid composition upon the temperature dependence of selected enzyme systems thought to be important in hibernation.

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FIGURE LEGENDS

Fig. 1: The Singer-Nicholson fluid mosaic model of membrane structure. Globular membrane proteins are shown embedded in the lipid bilayer (white) which has occasional cholesterol molecules (black) interspersed between the fatty-acid "tails" of the phospholipids. Some proteins are embedded on only one side of the lipid bilayer with their hydrophobic ends deep within the hydrocarbon core of the membrane, while their hydrophilic ends protrude into the aqueous phase. Other larger trans-membrane proteins pass entirely through the lipid bilayer. Illustration adapted from Singer (70).

Fig. 2: Typical linear Arrhenius plot of log initial rate of reaction velocity (K) versus reciprocal of absolute temperature (T°) in degrees Kelvin.

Fig. 3: Typical non-linear Arrhenius plot of $(Na^+ + K^+)$-ATPase activity from 5°-37°C. Adapted from Charnock, et al. (21,22).

Fig. 4: Arrhenius plot of rat skeletal muscle sarcoplasmic reticulum Ca^{++}-ATPase.

(●); experimental data taken directly from Becker and Willis (reference 9) with a single value for the apparent activation energy of about 25 kcals/mole derived from a straight line fit of the datum points.

(▩); identical experimental data offset by 0.5 log units and analysed by the computer assisted method described by

Charnock, Cook and Casey (reference 22). The calculated transition temperature (T_c°) is near 18°C. The values for Ea_I above and Ea_{II} below the transition are 19.4 and 32.1 kcals/mole respectively.

Fig. 5: Temperature dependence of fluorescence polarization (P) of membrane preparations of ($Na^+ + K^+$)-ATPase labelled with the lipid probe 12-(9-anthroyl) stearic acid (12-AS). Results are given as $\frac{1}{P}$ versus temperature (°C) and are the means of four determinations at each temperature on crab nerve (▭) and beef brain (o) enzyme preparations. The transition temperature (T_c°) for beef membranes is 25°C while that for crab nerve membranes is at 32°C. The temperature dependence of crab nerve preparations is linear below 32°C.

Full experimental details are given in Charnock and Simonson (24).

TABLE 1: PARTIAL LIST OF ENZYMES DEMONSTRATED TO HAVE NON-LINEAR ARRHENIUS PLOTS PRIOR TO 1965

Enzyme	Critical Temperature °C	Reference*
Invertase	0	Sizer, et al. (1942)
Trypsin	0	Sizer, et al. (1942)
Pancreatic lipase	0	Sizer, et al. (1942)
D-amino-acid oxidase	8	Massey, et al. (1966)
Phosphorylase b	13	Graves, et al. (1965)
Aldolase	16	Massey, et al. (1966)
Myosin ATPase	16	Levy, et al. (1959)
Fumarase	17	Massey (1953)
β-amylase	20	Piguet, et al. (1952)
Cytochrome C reductase	21	Vernon, et al. (1952)
Salivary amylase	24	Schneyer (1952)
Phosphorylase a	31	Helmreicht, et al. (1964)
Chymotrypsin	34	Havsteen, et al. (1963)
Chymotrypsinogen	44	Brandts (1964)
Ribonuclease	63	Harrington (1956)

* For full details of the references cited see Massey, et al. (56).

TABLE 2: ACTIVATION ENERGIES OF CYTOPLASMIC ENZYMES LDH AND MDH DISPLAYING LINEAR ARRHENIUS CHARACTERISTICS

Enzyme	Species	Tissues	Activation Energy kcals/mole	Refs*
	Salmonids	Muscle	10 - 13	42
	Cod	------, liver, heart	15 - 17	60
	Frog	Muscle, liver, heart	11 - 17	60
	#Snake (cold adapted)	Muscle	12	1
LDH	Platypus	Muscle, liver	19	7
	Echidna	Muscle, liver	17	7
	Hedgehog	------, liver, heart, brain	13 - 18	60
	Bat	Muscle, -----, heart	17 - 19	60
	Guinea pig	------. liver, heart, brain	12-- 16	60
	Frog	Muscle, liver, heart	12 - 13	60
	Cod	------, liver, heart	10 - 13	60
	Platypus	------, liver	16.5	7
MDH	Echidna	------, liver	16.5	7
	Avian	------, liver	12 - 15	2
	Hedgehog	------, liver, heart, brain	12 - 14	60
	Bat	Muscle, liver, heart	13	60
	Guinea pig	------, liver, heart, brain		

* Full details of these references are given in the Reference Section to this paper.

\# The author also describes a non-linear Arrhenius plot having an "inverse break" at 28°C and Ea_I 15.5 kcals/mole and Ea_{II} 6 kcals/mole for a warm adapted variety of the same reptile.

TABLE 3: ARRHENIUS CHARACTERISTICS OF PYRUVATE KINASE FROM VARIOUS SOURCES

Enzyme	Species	Tissue	Arrhenius Plot	T_c°	Activation Energy kcals/mole Ea_I	Ea_{II}	Refs*
PK	Rainbow trout	Muscle	Linear	none	30	none	72
	Rat	Muscle	Non-linear	25	10	20	72
	Antarctic fish	Muscle	Non-linear#	10	20	10	72
	Bat (euthermic)	Muscle, liver	Non-linear	17	9	20	13
	Bat (hibernating)	Muscle, liver	Non-linear	5	14	44	13

* Full details of these references are given in the Reference Section to this paper.

\# This non-linearity is described by the authors as an "inverse break" in which the value for $Ea_I > Ea_{II}$.

TABLE 4: ARRHENIUS CHARACTERISTICS OF MITOCHONDRIAL SUCCINATE OXIDATION FROM A VARIETY OF SOURCES

Class	Animals	Tissue	Arrhenius Plot	T°_{c}	Activation Energy kcals/mole Ea_{I}	Ea_{II}	Refs*
Poikilotherms	Rainbow trout	Liver	Linear	none	8.3	none	53
	Catfish	Liver	Linear	none	12.2	none	53
	Carp (cold adapted)	Liver	Non-linear	15	4.8	9.8	76
	Carp (warm adapted)	Liver	Non-linear	23	5.7	8.9	76
	Cane toad	Heart	Linear	none	15	none	57
Homeotherms	Rat	Liver	Non-linear	17	8.9	18.8	47
	Rat	Liver, heart	Non-linear	21 - 24	2.5 - 2.9	19.5 - 23.4	53
	Beef	Heart	Non-linear	27	9.1	17.0	50
Hibernators	Ground squirrel (awake)	Liver	Non-linear	22 - 23	9.1	16.7	65
	Ground squirrel (hibernating)	Liver	Linear	none	13.8	none	65

* Full details of these references are given in the Reference Section of this paper.

TABLE 5: ARRHENIUS CHARACTERISTICS OF SARCOPLASMIC RETICULUM Ca^{++}-ATPase FROM A VARIETY OF SOURCES

Class	Animal	Tissue	Arrhenius Plot	$T°_c$	Activation Energy kcals/mole Ea_I	Ea_{II}	Refs*
Poikilotherms	Lobster	Skeletal muscle	Non-linear	11.5	10	19.5	54
	Crayfish (cold adapted)	Abdominal muscle	Linear	None	--	--	28
	Crayfish (warm adapted)	Abdominal muscle	Linear	None	--	--	28
Homeotherms	Rabbit	Leg muscle	Linear	None	13.8 - 16.0	none	45
	Rabbit	Dorsal muscle	Linear	None	24	none	79
	Rabbit	Leg muscle	Non-linear	10	16.3	33.4	23
	Rabbit	Skeletal muscle	Non-linear	20	16	29	44
	Rabbit	Skeletal muscle	Non-linear	17	9	22	55
	Rat	Leg muscle	Linear	None	25	none	9
	Rat	Leg muscle	?Non-linear	18.6	19.6	31.6	#
Hibernator	Ground squirrel (awake)	Leg muscle	Linear	None	28	none	9
	Ground squirrel (awake)	Leg muscle	?Non-linear	22.8	19.8	32.1	#

* Full details of these references are given in the Reference Section of this paper.

--, indicates that values were not given by the original authors.

TABLE 6: ARRHENIUS CHARACTERISTICS OF MEMBRANE BOUND $(Na^+ + K^+)$-ATPase FROM A VARIETY OF SOURCES

Class	Animal	Tissue	Arrhenius Plot	T_c°	Activation Energy kcals/mole Ea_I	Ea_{II}	Refs*
Poikilotherms	Toad	Heart	Linear	none	22	none	57
	Frog	Brain	Non-linear	10	9.7	22	14
	Crab	Leg nerve	Non-linear	16	12.5	28.5	24
Homeotherms	Rat	Brain	Non-linear	20	7.8	15.2	40
	Rat	Brain	Non-linear	?	11	80	15
	Beef	Brain	Non-linear	18	18.1	37.0	27
	Rabbit	Kidney	Non-linear	17	15.4	27.5	19
	Rabbit	Heart	Non-linear	22	10	17	57
	Sheep	Kidney	Non-linear	22	15.7	34.4	20
Hibernators	Hedgehog (control)	Brain	Non-linear	?	13.3	37	16
	Hedgehog (hibernating)	Brain	Non-linear	?	13.8	31	16
	Hamster (awake)	Kidney	Non-linear	17	18.7	43.6	75 #
	Hamster (control)	Brain	Non-linear	17	19	42	38 #
	Hamster (hibernating)	Brain	Non-linear	17	18	34	38 #
	Hamster (control)	Brain	Non-linear	16.5	24.5	38.1	39 #
	Hamster (hibernating)	Brain	Non-linear	13.6	21.1	40.8	39 #

* Full details of these references are given in the Reference Section of this paper.

\# Recalculated from data supplied by authors, using the computer assisted analysis of Charnock, Cook and Casey (22).

TABLE 7: APPARENT ACTIVATION ENERGIES OF $(Na^+ + K^+)$-ATPase PREPARATIONS OBTAINED FROM HOMEOTHERMS, AWAKE HIBERNATOR AND POIKILOTHERMS$^{\Delta}$

Tissue	n	Ea_I*	Ea_{II}*	T°_c	Ea_I:Ea_{II}
Beef brain	9	18.1 ± 1.1	37.0 ± 3.4	20.0 ± 1.0	0.49
Ground squirrel brain	6	14.2 ± 1.2	32.4 ± 1.4	19.8 ± 0.4	0.44
Ground squirrel kidney	5	21.4 ± 1.4	45.1 ± 6.2	16.7 ± 2.2	0.47
Rabbit kidney	10	20.4 ± 1.0	44.5 ± 3.4	18.2 ± 1.7	0.46
Sheep kidney	3	16.8 ± 0.2	41.1 ± 7.1	18.2 ± 3.2	0.41
Lobster axon #	5	13.2 ± 0.9	23.7 ± 3.4	22.0 ± 3.3	0.56
Crab axon #	3	12.5 ± 1.5	28.5 ± 1.9	16.2 ± 2.3	0.44

Δ Data taken from Charnock and Simonson (24).

* Values for Ea_I and Ea_{II} were given as kcals/mole.

0.4 mM ouabain was used to inhibit $(Na^+ + K^+)$-ATPase from homeotherm and awake hibernator preparations, 2.5 mM ouabain was used for the preparations from poikilotherms.

TABLE 8: SEASONAL VARIATION IN THE APPARENT ENERGIES OF ACTIVATION AND TRANSITION TEMPERATURE OF $(Na^+ + K^+)$-ATPase FROM THE BRAINS OF AWAKE AND HIBERNATING GROUND SQUIRRELS[Δ]

	Awake animals (June - August) n = 6	After short-term hibernation (Nov - Dec) n = 2	After long-term hibernation (Feb - March) n = 4
Ea_I	14.9 ± 0.91	15.8 ± 1.67	14.3 ± 1.62
Ea_{II}	26.8 ± 0.92	31.3 ± 3.46	30.9 ± 1.68
T_c°	21.2 ± 0.30	18.6 ± 2.22	20.2 ± 1.04

Δ Adapted from Charnock and Simonson (25)

Values for activation energy (Ea) are given as mean kcals/mole ± S.E., while those for transition temperature (T_c°) are given as the mean °C ± S.E.

TABLE 9: SEASONAL VARIATION IN THE RATE AND AMOUNT OF [^{3}H]-OUABAIN BINDING TO GROUND SQUIRREL BRAIN ($Na^+ + K^+$)-ATPase COMPARED TO THAT OF OTHER SPECIES

	Species	n	Conditions	Specific Activity (mean ± SE)	Rate [^{3}H]-ouabain binding (mean ± SE)	Amount [^{3}H]-ouabain binding (mean ± SE)
Δ	Ground squirrel	5	awake controls	71.3 ± 3.2	5.15 ± 0.30	201 ± 9.7
Δ	Ground squirrel	4	short-term hibernation	62.6 ± 6.3	5.42 ± 0.49	230 ± 19.1
Δ	Ground squirrel	5	long-term hibernation	84.6 ± 3.0	3.97 ± 0.30	166 ± 6.0
*	Beef brain	4	---	31.3 ± 1.5	2.42 ± 0.15	133 ± 8.6
*	Crab nerve	3	---	12.7 ± 4.2	.041 ± 0.01	6.00 ± 1.15

(Δ) Data for ground squirrel taken from Charnock and Simonson (25); data for beef brain and crab nerve (*) taken from Charnock and Simonson (24). Specific activity is given as μmoles ATP hydrolysed/mg protein/hour. Rate [^{3}H]-ouabain binding is given as pico moles/mg protein sec^{-1}. Amount [^{3}H]-ouabain bound is given as pico moles/mg protein. All determinations were done at 37°C.

Short-term hibernation is 30 days or less, long-term hibernation is 75 days or more, after onset of hibernating season.

TABLE 10: SEASONAL VARIATION IN THE SPECIFIC ACTIVITY AND RATE AND AMOUNT OF $[^3H]$-OUABAIN BINDING TO RENAL CORTEX $(Na^+ + K^+)$-ATPase PREPARATIONS FROM THE GROUND SQUIRREL$^{\Delta}$

Condition of Animals	n	Specific Activity	Rate $[^3H]$-ouabain Binding	Amount $[^3H]$-ouabain Binding	Ouabain Binding/ Specific Activity
Awake controls	5	32.2 ± 1.9	2.53 ± 0.24	123.1 ± 6.9	3.82 ± 0.14
< 30 days hibernation	5	12.9 ± 1.6	1.25 ± 0.32	46.9 ± 8.4	3.64 ± 0.17
> 75 days hibernation	3	13.6 ± 1.6	1.31 ± 0.23	53.6 ± 14.4	3.94 ± 0.26

Δ Data taken from Charnock and Simonson (26).

All experiments carried out at 37°C according to the procedures described in detail by Charnock, Simonson and Almeida (27).

S.A. given as µmoles Pi liberated from ATP/mg enzyme protein/hour.

Values for $[^3H]$-ouabain binding are given in pico moles/mg enzyme protein/sec for the rate and pico moles/mg enzyme at equilibrium for the amount.

TABLE 11: SEASONAL VARIATION IN THE APPARENT ENERGIES OF ACTIVATION, TRANSITION TEMPERATURE AND ARRHENIUS ANALYSIS OF OUABAIN-SENSITIVE $(Na^+ + K^+)$-ATPase OF THE GROUND SQUIRREL RENAL CORTEX$^{\Delta}$

Condition of Animals	n	Ea_I	Ea_{II}	T_c°	Arrhenius Analysis
Awake controls	5	15.7 ± 1.3	31.7 ± 2.4	20.4 ± 0.6	non-linear
< 30 days hibernation	3	14.0 ± 1.6	27.4 ± 2.9	20.8 ± 2.5	non-linear
> 75 days hibernation	5	16.2 ± 1.1	30.7 ± 1.8	19.8 ± 0.9	non-linear

Δ Data taken from Charnock and Simonson (26).

Values for the apparent energies of activation are given as kcals/mole and are the means ± standard errors of the individual experiments.

THE FLUID MOSAIC MODEL OF MEMBRANES

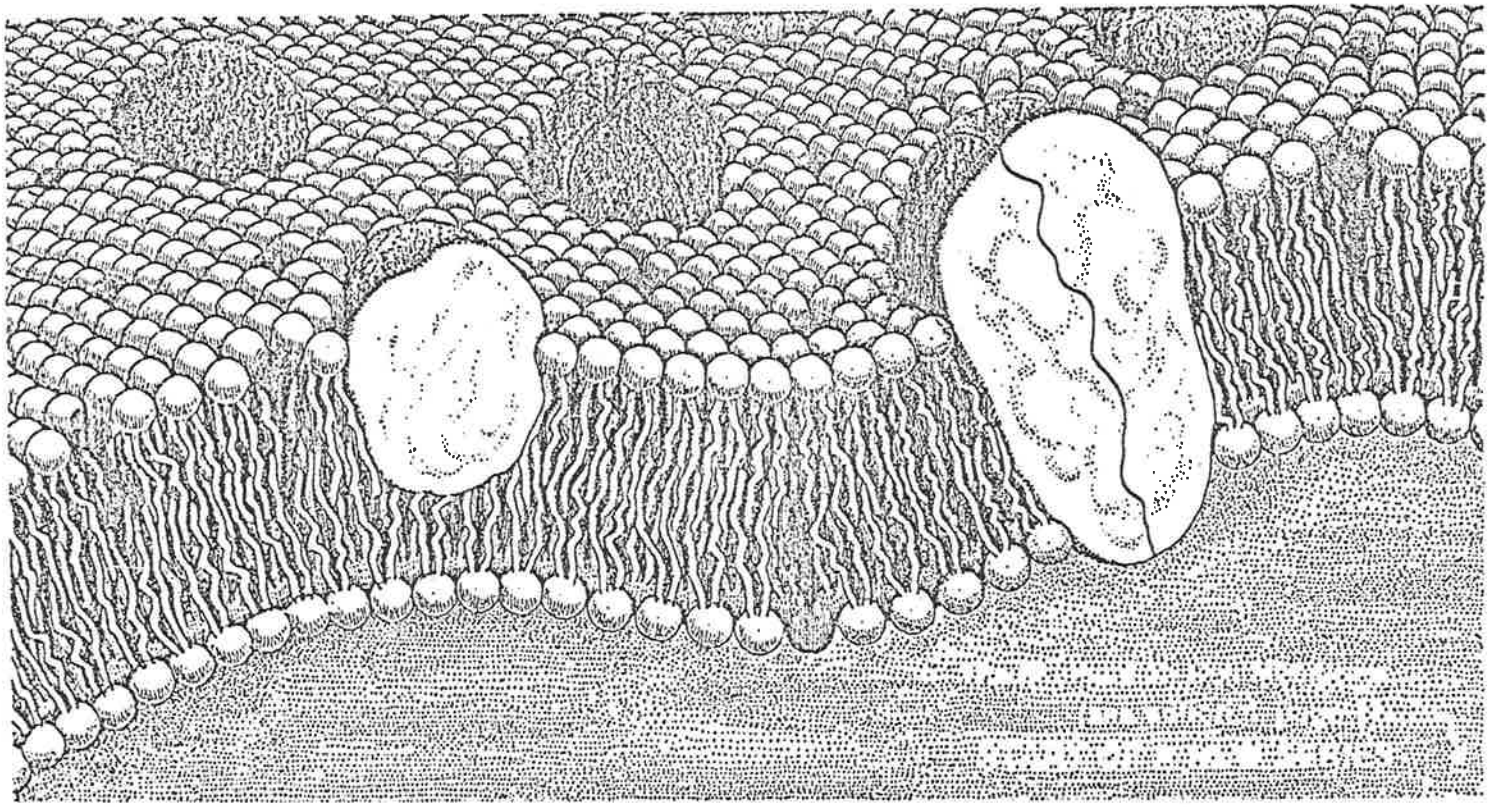


FIG. 1

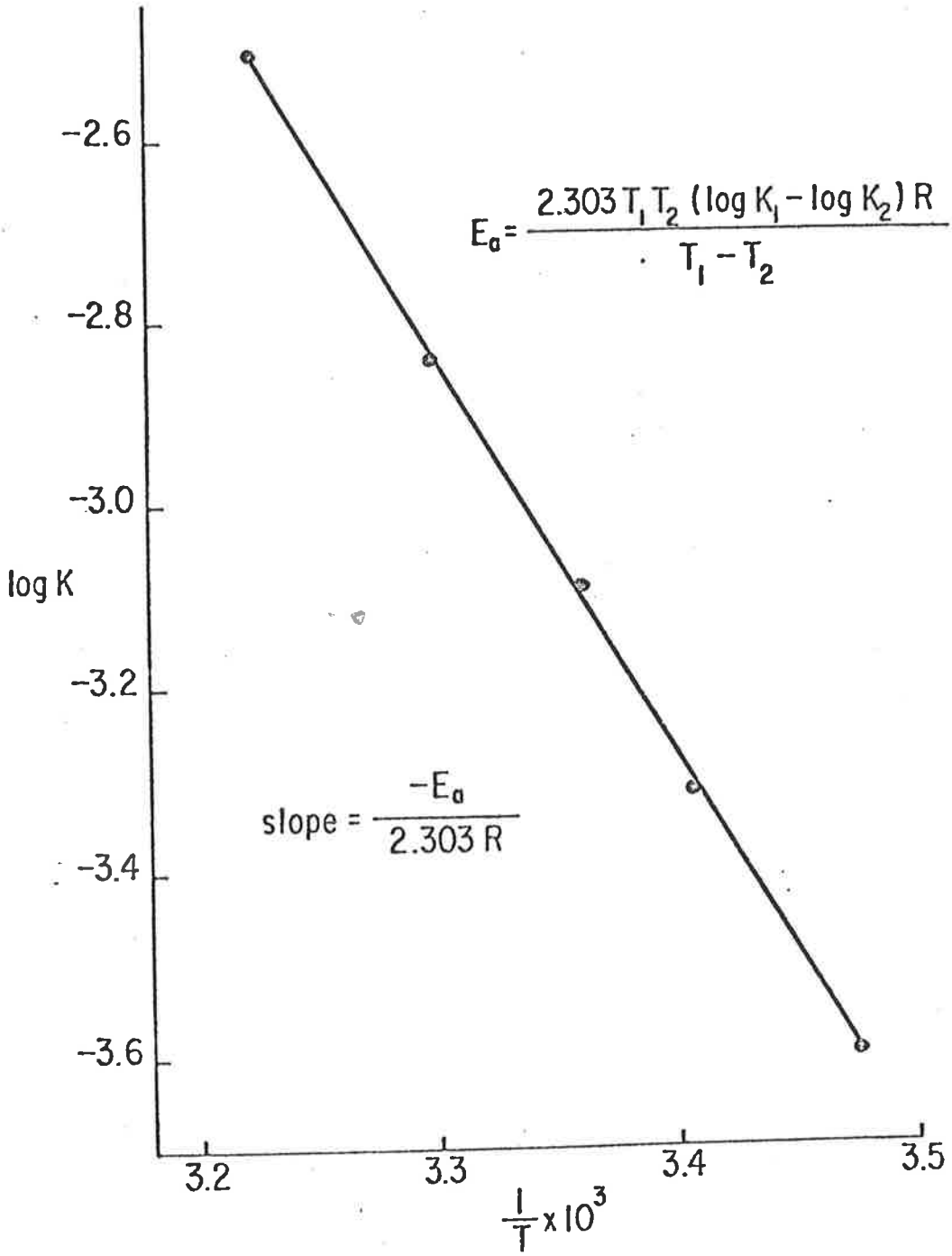
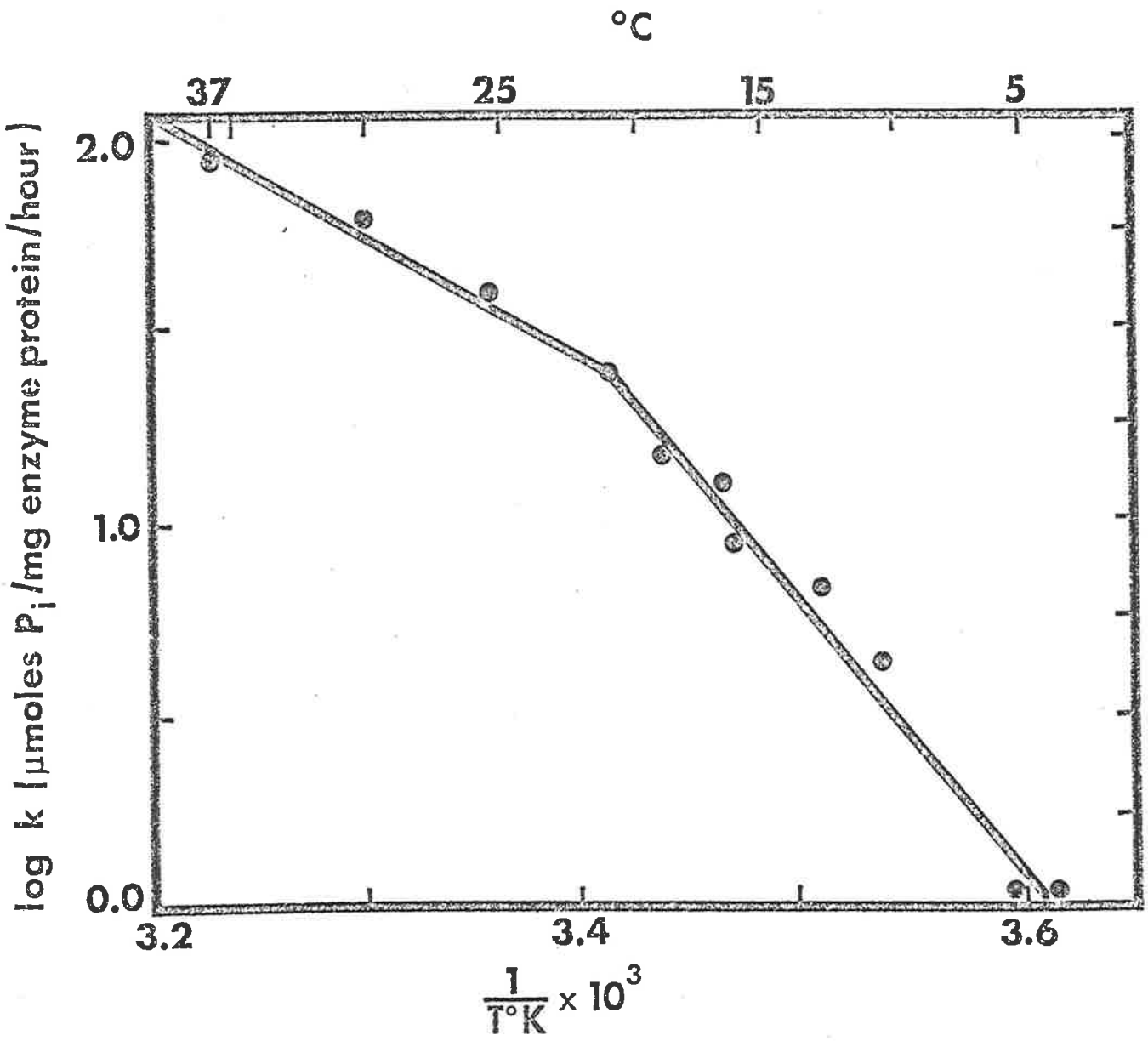

-2.6
-2.8
-3.0
-3.2
-3.4
-3.6
log K
3.2
3.3
3.4
3.5
$\frac{1}{T} \times 10^3$
$E_a = \frac{2.303\, T_1 T_2 (\log K_1 - \log K_2) R}{T_1 - T_2}$
$\text{slope} = \frac{-E_a}{2.303\, R}$

FIG. 2

FIG. 3


°C
37
25
15
5
2.0
1.0
0.0
log k (μmoles P_i/mg enzyme protein/hour)
3.2
3.4
3.6
$\frac{1}{T°K} \times 10^3$

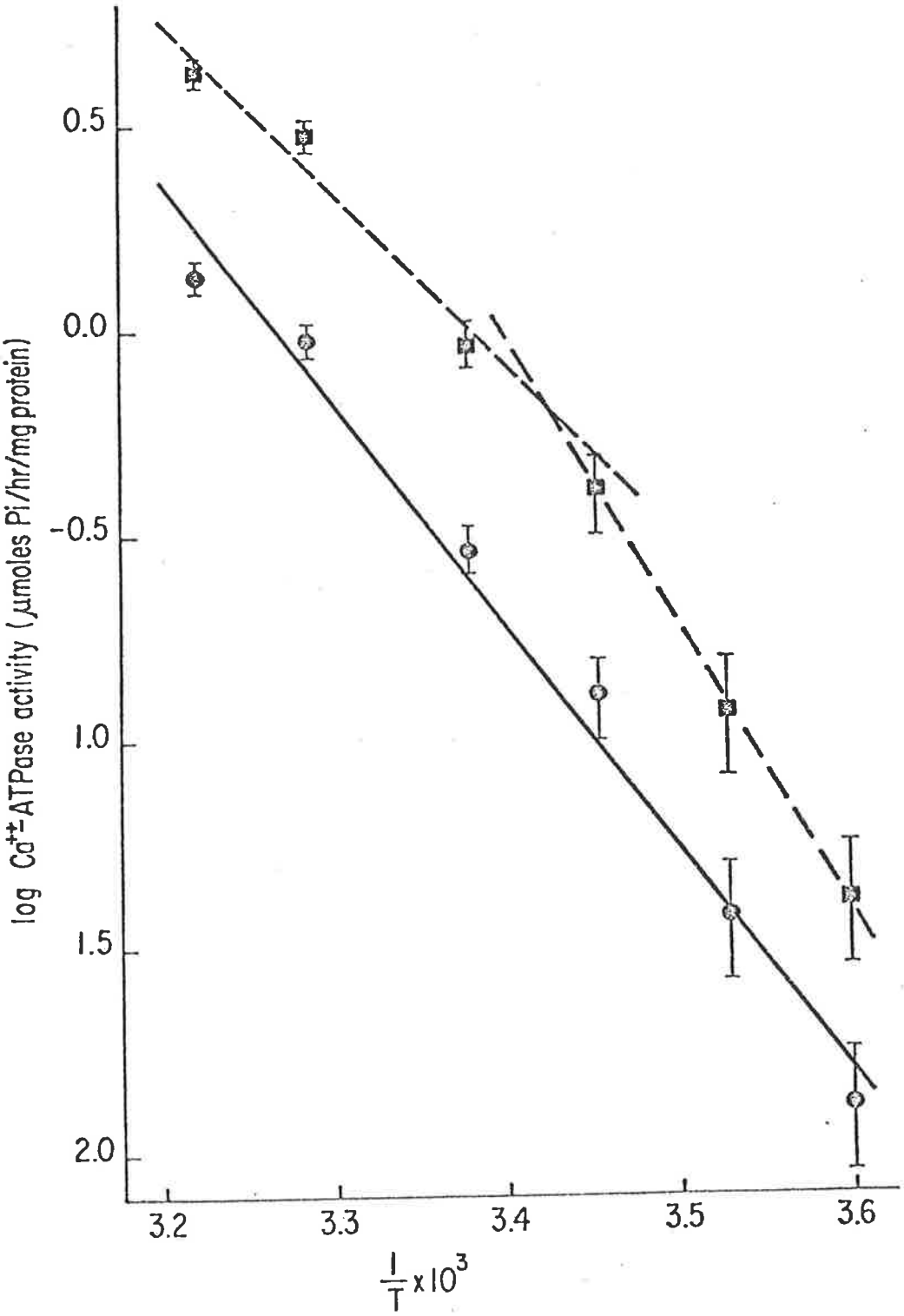

0.5
0.0
-0.5
1.0
1.5
2.0
log Ca++-ATPase activity (μmoles Pi/hr/mg protein)
3.2
3.3
3.4
3.5
3.6
1/T x 10^3

FIG. 4

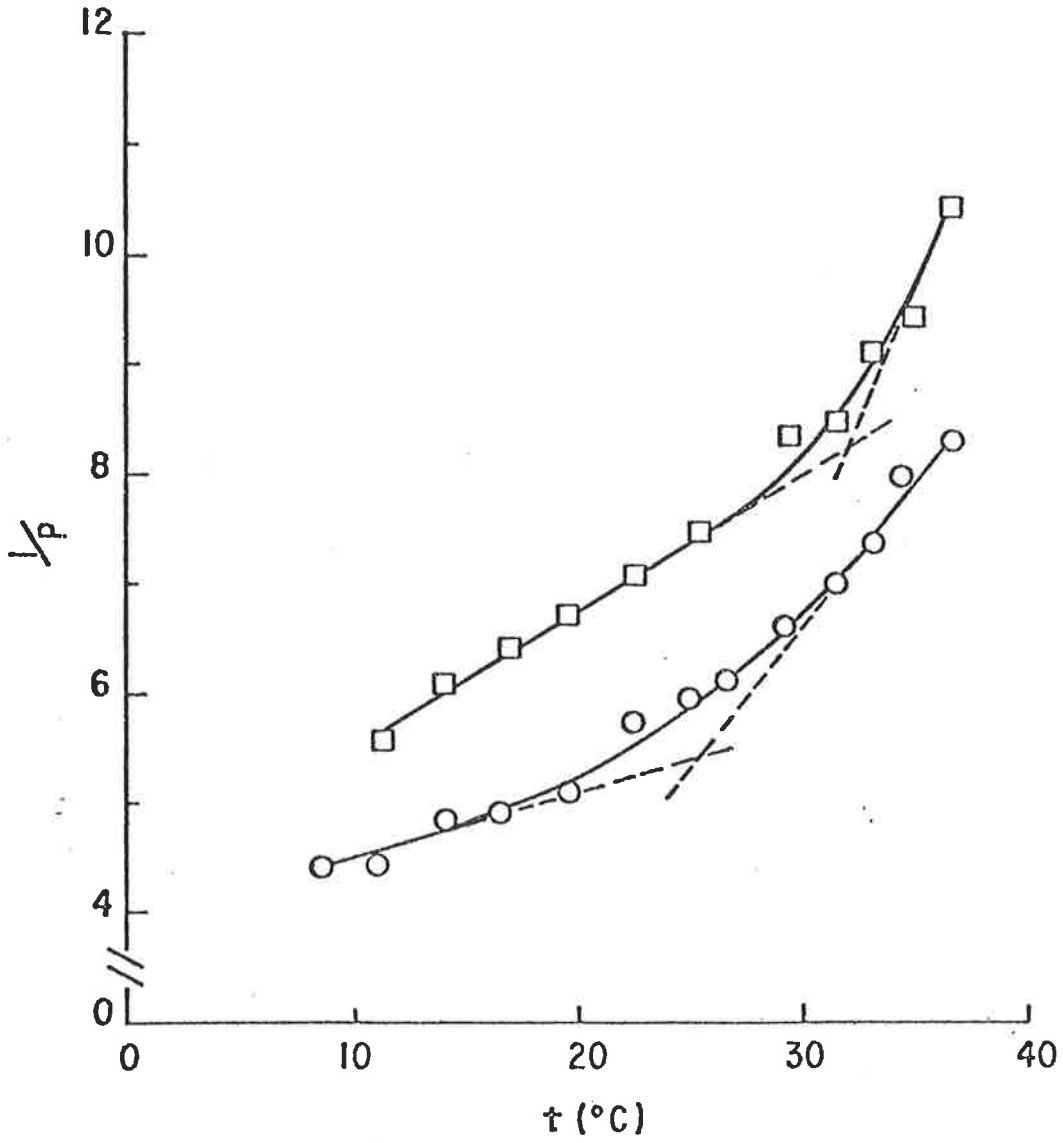


FIG. 5

Comp. Biochem. Physiol. (Accepted for publication June, 1977).

VARIATIONS IN $(Na^+ + K^+)$-ATPase AND Mg^{++}-ATPase ACTIVITY OF THE GROUND SQUIRREL BRAIN DURING HIBERNATION

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Variation in brain ATPases during hibernation.

ABSTRACT

1. The specific activity of brain ($Na^+ + K^+$)-ATPase and Mg^{++}-ATPase of the ground squirrel (Spermophilus richardsonii) is significantly increased after long-term hibernation.
2. The markedly non-linear thermal dependence of ($Na^+ + K^+$)-ATPase is unchanged during hibernation whereas the near linear thermal dependence of Mg^{++}-ATPase undergoes minor alteration after prolonged hibernation.
3. The sensitivity of ($Na^+ + K^+$)-ATPase to inhibition by ouabain is significantly decreased after 100 days of hibernation as is both the rate and amount of [^{3}H]-ouabain binding.
4. These changes may be related to alteration in the phospholipid matrix of the membrane rather than alteration in the protein structure of the enzyme.

INTRODUCTION

Although the body temperature of many hibernating species falls below 5°C during hibernation, it is generally believed that sufficient neural function is maintained by the brain to provide for both minimal information transfer and metabolic activity (Kayser, 1961). This phenomenon has frequently been described as "cold resistance during hibernation" and has been extensively studied in a variety of species.

Because the membrane enzyme $(Na^+ + K^+)$-ATPase plays such an integral role in sodium transport (Skou, 1965; Dahl & Hokin, 1974) and thus in neural activity, several investigators have studied the activity of this enzyme and the Mg^{++}-ATPase with which it is so closely associated, in the brains of both awake and hibernating mammals. For example, Bowler & Duncan (1969) reported that the markedly non-linear temperature dependence of the hedgehog (Erinaceus europaens) brain $(Na^+ + K^+)$-ATPase was greatly reduced in hibernation and that during this state its characteristics were similar to those of a poikilotherm preparation. The Mg^{++}-ATPase of these preparations demonstrated essentially linear temperature dependence with no seasonal variations.

Conversely, neither Goldman & Willis (1973) or Goldman & Albers (1975) observed such seasonal variation in the brain $(Na^+ + K^+)$-ATPase of Syrian hamsters (Mesocricetus auratus) although they did observe a relative increase in the activity of this enzyme at low experimental temperatures when the brains of hibernating animals were compared to summer controls. However, Goldman & Albers (1975) used enzyme preparations which had been treated with both a detergent and the chaotropic agent NaI during

preparation, rather than the untreated microsomal preparations of Bowler & Duncan (1969).

As many membrane bound enzyme systems are modulated by lipid-protein interactions (Lyons, 1972; Weiss, 1973; Raison, 1973) it is possible that the isolation procedures used to obtain ($Na^+ + K^+$)-ATPase *in vitro* may significantly influence the experimentally observed characteristics of this enzyme. Recent work by Whittam *et al.* (1976) and by Charnock *et al.* (1977) have demonstrated that this is true for its ouabain-binding characteristics.

We have therefore re-examined the temperature dependence of both ($Na^+ + K^+$)-ATPase and Mg^{++}-ATPase from the brains of a local species of hibernators - the Richardson ground squirrel (*Spermophilus richardsonii*) both during the summer months of July and August as well as after 30 days and 100 days of hibernation. In addition to an evaluation of the apparent energy of activation of ATP hydrolysis by these enzymes, we have sought further information about the possible cold resistance of ($Na^+ + K^+$)-ATPase by determining both the rate and extent of inhibitor binding to the enzyme throughout the period of observation.

MATERIALS AND METHODS

The Richardson ground squirrel (*Spermophilus richardsonii*) which are abundant in this area of Alberta, were collected from the fields in small traps during May and June of 1976. After a period of quarantine they were weighed and transferred to single cages in the vivarium where they were fed ad libitum on a standard diet of pelleted laboratory chow (Vitamite

cubes; 25% protein, 5% fat and 6% fibre supplied by North West Feeds of Edmonton) which was supplemented by sunflower seeds and some green feed. Weight gain was continuous over this period so that all animals exceeded 500 gm live weight on October 1st.

The animals were provided with nesting material for one week prior to transferring them to an environmentally controlled room during October where the temperature was 5°C and the light-dark cycle was changed from 12-12 hr to 0-24 hr respectively. The animals were deprived of all food and water; all animals which did not enter a sleep cycle within four days were removed from the 5°C room and feeding was resumed for at least one week before they were returned to the cold. After a further two weeks (early November 1976) all animals to be used in the experiment were in hibernation and a constant dark cycle of 24 hr/day was maintained for the remainder of the experimental period (March 1977).

The animals were killed by decapitation with a small guillotine and their brains removed in the cold and immediately frozen in liquid N_2. The method for enzyme preparation was similar to that described for beef brain (Charnock & Simonson, 1977). Both Na^+-activation and ouabain-inhibition of the $(Na^+ + K^+)$-ATPase and the ouabain-insensitive Mg^{++}-ATPase were routinely determined by the coupled enzyme procedure we have employed before which uses a temperature controlled Gilford 2400 recording spectrophotometer (Charnock, Simonson & Almeida, 1977). Mg^{++}-ATPase was determined as the residual ATPase measured in the absence of Na^+ or in the presence of 0.4 mM ouabain. The thermal dependence of ATPase activity was determined in 3°C steps between 8°C-37°C and the data obtained analysed by

the computer assisted procedure we have discussed previously (Charnock, Cook, Almeida & To, 1973; Charnock & Simonson, 1977).

The method for determining both the rate and extent of [^{3}H]-ouabain binding to ($Na^+ + K^+$)-ATPase containing membranes has also been described in full in a previous publication (Charnock, Simonson & Almeida, 1977). However, because the enzyme preparations were deliberately not subjected to any further purification steps by either detergents or chaotropic agents, additional controls were always included without ATP or Na^+ to determine the extent of non-specific [^{3}H]-ouabain binding. In these experiments it was always less than 1% of the total drug uptake.

RESULTS

One of the major biochemical characteristics of ($Na^+ + K^+$)-ATPase is its sensitivity to the cardiac glycoside ouabain (Skou, 1957, 1960; Post, Merritt, Kinsolving & Albright, 1960; Charnock & Post, 1963) which is frequently used to distinguish this enzyme from other membrane-bound ATPase reactions with which it is closely associated in isolated membrane fragments. For this reason we began our study by determining the ouabain sensitivity of our ($Na^+ + K^+$)-ATPase preparations over the complete period of investigation in order to determine whether or not there was any seasonal variation in this important characteristic.

Our results are shown in Fig. 1 where it can be seen that a typical dose-response curve was obtained for ouabain inhibition of ground squirrel brain ($Na^+ + K^+$)-ATPase during the summer collection period (June-August) when the animals are awake and active. A value for 50% inhibition by

ouabain (K_i) of 4×10^{-7}M is obtained under these conditions. While this is not altered appreciably after 30 days of hibernation (data not shown) there is a clear shift to the right in this curve after 100 days of hibernation. Although the magnitude of this shift is relatively small it is significantly different from the summer controls and the value for K_i is now 8×10^{-7}M. Thus the brain $(Na^+ + K^+)$-ATPase preparations from long-term hibernators have become slightly less sensitive to ouabain. However, as we routinely used 4×10^{-4}M ouabain in all standard assays there can be no doubt that maximum inhibition was obtained under all experimental conditions.

Examination of the enzyme activity ± ouabain at 37°C permits the comparison of the specific activity of both $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase over the period of study. The data are given in Table 1 which shows that there is a significant increase in the specific activity of both enzymes ($p < .01$) after one hundred days of hibernation. This effect was not seen after only thirty days of hibernation.

The temperature dependence of $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase of the brains of ground squirrels was now examined for seasonal variation by assay at 3° intervals between 8°C-37°C. The rate data was first displayed as Arrhenius plots which were analysed by the computer assisted procedure we have described before (Charnock, Cook, Almeida & To, 1973). This procedure yields the confidence limits for single or two-line fits to the plots as well as the values for the apparent energies of activation for the process under study above and below a single inflection point in non-linear plots.

The data we obtained for $(Na^+ + K^+)$-ATPase could always yield non-linear plots with much greater statistical confidence than could be obtained for a single straight line fit whether the enzyme was obtained from an awake animal or after any period of hibernation. This finding was not altered whether the activity was determined by inhibition by 0.4 mM ouabain or by stimulation by 80 mM Na^+ in the presence of 20 mM K^+. The calculated values for Ea_I above the transition temperature T_c° and for Ea_{II} below this temperature are given in Table 2. It is clear that no statistically significant changes occurred in these parameters due to either short-term or long-term hibernation. These results are in agreement with the findings of Goldman and his colleagues for the hamster brain (Goldman et al., 1973, 1975) but do not agree with the conclusions of Bowler & Duncan (1969) for hedgehog brain. However, subjecting the reported data of these latter investigators to our method of analysis also reveals non-linear temperature dependence for both awake and hibernating hedgehog brain enzyme preparations with activation energies quite comparable to those reported here, although the mean values for T_c° which we calculated from their data were several degrees lower than our values for the ground squirrel.

For comparison we have tabulated the comparative data we obtained when the reported values of Goldman & Willis (1973) for Syrian hamsters and that of Bowler & Duncan (1969) for hedgehog were submitted to the same computer assisted analysis that we used for the data for ground squirrel obtained in these present experiments (Table 3). From this comparison it may be seen that the data for the hedgehog brain do not

necessarily conflict with either our findings for the ground squirrel nor the findings of Goldman & Willis (1973) or Goldman & Albers (1975) for the Syrian hamster.

In a previous report from this laboratory (Charnock, Cook, Almeida & To, 1973) we reported that the ouabain-insensitive Mg^{++}-ATPase of the renal cortex of New Zealand white rabbits (Oryctolagus cuniculus) did not demonstrate a temperature dependence which could be adequately described statistically as either a linear or non-linear Arrhenius plot having two intersecting slopes. The data obtained with brain Mg^{++}-ATPase preparations from the ground squirrel is quite similar in this regard (Table 4).

With either the summer control animals or those having a short-term period of hibernation, the Arrhenius plots were nearly linear but could be fitted by two intersecting straight lines thus yielding quite small differences in activation energies above and below calculated transition temperatures.

Conversely, enzyme preparations obtained from animals after long-term hibernation of more than one hundred days gave Arrhenius plots which could not be statistically described as a single straight line but could more easily be fitted by two intersecting straight lines again yielding two values for the apparent energy of activation of the process above and below a single thermal transition at 26°C. However, the difference in activation energies above and below the transition point was now greater than that seen in either the summer controls or short-term hibernators. It should be appreciated that the difference between these values for

Ea_I and Ea_{II} were very much less for Mg^{++}-ATPase than the difference found in $(Na^+ + K^+)$-ATPase (see Table 2). For comparison Table 4 also shows the values for Mg^{++}-ATPase we calculated for the Syrian hamster and the hedgehog brain from the data of Goldman & Willis (1973) and Bowler & Duncan (1969) respectively.

It is clear that there are considerable differences in these findings for which no ready explanation is available. A controlled experiment involving all three species examined after comparable periods of hibernation would be necessary before the reported differences could be clarified.

It is of interest that while Bowler & Duncan (1969) reported a linear relationship between the activity ratio of $(Na^+ + K^+)$-ATPase to Mg^{++}-ATPase of their hedgehog brain preparations with varying temperatures, Goldman & Willis (1973) found a relative increase in the proportion of $(Na^+ + K^+)$-ATPase in the brains of hibernating hamsters below 10°C. We therefore examined this property in ground squirrels and our results are given in Fig. 2 where it can be seen that a linear relationship was not obtained throughout the whole temperature range. In general the rate of increase in the activity ratio of the two enzymes was only linear between 10°C-25°C, above which there was much less change in the activity ratio with temperature. This confirms our previous observation with rabbit kidney preparations in which we also showed that above 25°C the rate of increase in Mg^{++}-ATPase activity parallels that of $(Na^+ + K^+)$-ATPase (Charnock, Doty & Russel, 1971). The apparent marked decline in the activity ratio below 10°C in the group of short-term hibernating animals may be seriously influenced by the very small number of animals in this

group. However the major difference between our findings with ground squirrel brain preparations and those reported for Syrian hamsters and hedgehogs is that at all temperatures the ratio of activity of $(Na^+ + K^+)$-ATPase : Mg^{++}-ATPase for long-term hibernators is less than for the summer controls while that of the short-term hibernators is greater. While this apparent difference might arise from unexplained species differences, it may also originate in the very different assay procedures which have been employed in these three different studies.

A number of investigators have demonstrated the stoichiometric relationship between inhibitory action of ouabain upon ATP hydrolysis by $(Na^+ + K^+)$-ATPase and the binding of $[^3H]$-ouabain to isolated membrane preparations of this enzyme (Erdman & Schoner, 1974; Wallick & Schwartz, 1974). Recent work in this laboratory has confirmed these observations in $(Na^+ + K^+)$-ATPase preparations from beef brains (Bos taurus) using a procedure which permits the determination of both the initial rate of drug binding as well as the amount of drug bound under equilibrium conditions (Charnock, Simonson & Almeida, 1977). We therefore examined both these parameters of $[^3H]$-ouabain binding in preparations of ground squirrel brain $(Na^+ + K^+)$-ATPase obtained from awake summer control animals and after short- and long-term hibernation. These results were compared to data we had previously obtained for $(Na^+ + K^+)$-ATPase preparations from beef brain and from the peripheral nerve of the cold water crab Cancer magister (Charnock & Simonson, 1977). Our results are given in Table 5 which shows that both the rate and amount of drug binding to untreated preparations of ground squirrel brain $(Na^+ + K^+)$-ATPase is

much greater than that to either beef brain or crab nerve enzyme preparations. This finding is not changed whether the preparations are obtained from awake or hibernating animals and is in keeping with the increased specific activity of ground squirrel brain preparations that was consistently found throughout this work. However, it should be noted that while there was no apparent change in any of these parameters after 30 days of hibernation, there was about a 20% reduction in both the rate and amount of drug binding after 100 days hibernation when compared to summer controls. This reduction in drug binding was accompanied by an equivalent increase in enzyme specific activity.

DISCUSSION

A seasonal examination of the thermal dependence of brain $(Na^+ + K^+)$-ATPase activity has been employed by several previous investigators as a measure of degree of "cold resistance" of the brain during hibernation.

In the present study we have shown that not only is the thermal dependence of $(Na^+ + K^+)$-ATPase of the brain of an awake hibernator similar to that of several non-hibernating species we have studied before (Charnock, Cook, Almeida & To, 1973; Charnock, Simonson & Almeida, 1977) but that this function does not undergo any marked change during either short-term or long-term hibernation. In this regard our findings with the Richardson ground squirrel are in agreement with those of Goldman & Willis (1973) for the Syrian hamster. However the apparent activation energies for $(Na^+ + K^+)$-ATPase that we calculated both above and below the so-called transition temperature were less than those calculated for

both Syrian hamsters and hedgehogs (Bowler & Duncan, 1969). From other studies from this laboratory (Charnock & Bashford, 1975; Almeida & Charnock, 1977) it is clear that this finding indicates a relatively more fluid membrane matrix for $(Na^+ + K^+)$-ATPase of the ground squirrel brain when compared to either the hamster or the hedgehog at any experimental temperature or during any season examined. As these results were obtained with enzyme preparations which had not been subjected to treatment with either detergents or such chaotropic agents as NaI, it seems unlikely that any differences reported in the literature could arise from the different methods of preparation that have been employed. Perhaps it is this constancy of behavior which should be regarded as "cold resistance" rather than the type of thermal dependence discussed by some previous investigators (Goldman & Willis, 1973; Goldman & Albers, 1975).

Nevertheless detailed examination of some of the other properties of brain ATPases indicates that some important changes in biochemical behaviour do occur during hibernation. Firstly, there is a marked increase in the specific activity of both $(Na^+ + K^+)$-ATPase and Mg^{++}-ATPase after 100 days of hibernation. This increase is equal to 137% and 241% of the non-hibernating control values of these enzymes respectively and has been seen in both hamster and hedgehog brain preparations by previous investigators.

Whereas Goldman & Albers (1975) found that both the K^+-activated para-nitro-phenyl-phosphatase and Na^+-dependent nucleotide transphosphorylation exchange reaction - that is two partial reactions of $(Na^+ + K^+)$-ATPase (Albers, 1967; Dahl & Hokin, 1974) - were increased

concomitantly with the increase in ATPase activity during hibernation, our findings with [^{3}H]-ouabain inhibitor binding were quite different. There was a small but significant decrease in the sensitivity (K_i) of the system to ouabain during hibernation (8×10^{-7}M compared to 4×10^{-7}M for the summer controls). This shift in sensitivity was accompanied by about a 20% decrease in the affinity for drug binding and a similar decrease in the amount of drug bound (using 5×10^{-7}M ouabain) after 100 days of hibernation. These effects were not seen after relatively short periods of hibernation.

These changes demonstrate that during hibernation there is a progressive loss of affinity for ouabain binding, and presumably for the number of ouabain sites as the ratio of amount of ouabain bound at equilibrium : enzyme specific activity falls from 2.8:1 in the summer controls to 1.9:1 after 100 days of hibernation. That is, the fall in ouabain binding sites is not paralleled by a fall in enzyme activity. This must arise from some qualitative change in enzyme function similar to that referred to by Goldman & Albers (1975) and in which it seems probable that membrane lipids are involved as these are known to both modulate ($Na^+ + K^+$)-ATPase activity and its cardiac glycoside receptor characteristics (Charnock, Simonson & Almeida, 1977) and to change during hibernation (Roelofsen & van Deenen, 1973; Aloia, Pengellay, Bolen & Rouser, 1974; Goldman, 1975).

However it should be noted that despite the high specific activity of the ground squirrel brain (even after 100 days of hibernation) the ratio of amount of ouabain bound : enzyme specific activity is less than that found in preparations of beef brain ($Na^+ + K^+$)-ATPase (4.3:1). On the

other hand, the values for crab nerve preparations are almost an order of magnitude less than those of the beef or ground squirrel.

We believe that these major differences are related to changes in the phospholipid components of the membrane in which the $(Na^+ + K^+)$-ATPase protein is embedded (Hilden & Hokin, 1976; Palatini, Dabbeni-Sala, Pitotti, Bruni & Mandersloot, 1977).

The data obtained for membrane Mg^{++}-ATPase of the ground squirrel brain is more difficult to interpret. The lack of strong evidence for a transition temperature during the summer months and the far less striking evidence for this phenomenon during hibernation than was found for $(Na^+ + K^+)$-ATPase (where the difference in Ea values above and below the transition temperature is much more marked), does suggest a degree of "cold resistance" for this enzyme which was not found for the cation transport enzyme system. There can be no doubt that there was no change in the activation energy of Mg^{++}-ATPase between 5°C-25°C in both hibernating and summer control animals; a finding which is very similar to that reported earlier for summer and hibernating hedgehogs (Bowler & Duncan, 1969). However as the physiological role of Mg^{++}-ATPase remains unknown, the significance of this finding must also await an explanation.

ACKNOWLEDGEMENT

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TABLE 1. Seasonal variation in specific activity* of ouabain-sensitive $(Na^+ + K^+)$-ATPase and ouabain-insensitive Mg^{++}-ATPase of ground squirrel cerebral cortex

	n	$(Na^+ + K^+)$-ATPase	Mg^{++}-ATPase
Awake controls	6	63.4 ± 1.6	14.8 ± 1.1
< 30 days hibernation	2	68.7 ± 0.9	10.7 ± 0.8
> 100 days hibernation	3	86.6 ± 7.7	35.8 ± 4.8

* Specific activity given as umoles Pi liberated from ATP/mg protein/hour at 37°C.

TABLE 2. Seasonal variation in the apparent energies of activation and transition temperature of $(Na^+ + K^+)$-ATPase from the brains of awake and hibernating ground squirrels

	Awake animals (June - August) n = 6		After short-term hibernation (Nov - Dec) n = 2		After long-term hibernation (Feb - March) n = 4	
	Na^+ Stimulation	Ouabain Inhibition	Na^+ Stimulation	Ouabain Inhibition	Na^+ Stimulation	Ouabain Inhibition
Ea_I	14.8 ± 0.86	14.9 ± 0.91	15.6 ± 1.50	15.8 ± 1.67	14.7 ± 1.94	14.3 ± 1.62
Ea_{II}	26.7 ± 1.34	26.8 ± 0.92	30.7 ± 2.78	31.3 ± 3.46	29.9 ± 1.30	30.9 ± 1.68
T_C°	20.8 ± 0.61	21.2 ± 0.30	19.1 ± 1.82	18.6 ± 2.22	19.9 ± 0.41	20.2 ± 1.04

Values for activation energy (Ea) are given as mean kcals/mole ± S.E., while those for transition temperature (T_C°) are given as the mean °C ± S.E.

TABLE 3. Comparison of the seasonal variation in the apparent energies of activation and the transition temperature of ouabain sensitive ($Na^+ + K^+$)-ATPase from the brains of three species of hibernators

Species	Summer			Winter		
	Ea_I	Ea_{II}	T_c°	Ea_I	Ea_{II}	T_c°
Ground squirrel (1)	14.9	26.8	21.2	14.3	30.9	20.2
Syrian hamster (2)	19.2	41.7	17.0	18.3	34.4	16.9
Hedgehog (3)	18.2	31.9	14.8	19.1	30.5	15.5

(1) Present work.

(2) Calculated from data of Goldman & Willis (1973).

(3) Calculated from data of Bowler & Duncan (1969).

Units for Ea and T_c° are as in Table 2.

TABLE 4. Comparison of the seasonal variation in thermal dependence of ouabain-insensitive Mg^{++}-ATPase from the brain of ground squirrels and some other species of hibernators

Species	Experimental Conditions	T_c°	Ea_I	Ea_{II}
Ground squirrel	non-hibernating	30°	14.4 ± 0.9	17.3 ± 1.5
Ground squirrel	< 30 day hibernators	--	12.4 ± 1.8	13.9 ± 1.6
Ground squirrel	> 100 day hibernators	26.3	11.4 ± 0.6	16.9 ± 1.5
* Syrian hamster	non-hibernating	17.9	13.6	24.0
Syrian hamster	hibernating	15.6	14.1	26.1
# Hedgehog	non-hibernating	--	11.8	11.8
Hedgehog	hibernating	--	12.3	12.3

* From Goldman & Willis (1973).

\# From Bowler & Duncan (1969).

Units for T_c° and Ea_I and Ea_{II} are as in Table 2; -- indicates no value obtained.

TABLE 5. Seasonal variation in the rate and amount of [^{3}H]-ouabain binding to ground squirrel brain ($Na^+ + K^+$)-ATPase compared to that of other species

Species	n	Conditions	Specific Activity (mean ± SE)	Rate [^{3}H]-ouabain binding (mean ± SE)	Amount [^{3}H]-ouabain binding (mean ± SE)
Ground squirrel	5	awake controls	71.3 ± 3.2	5.15 ± 0.30	201 ± 9.7
Ground squirrel	4	short-term hibernation	62.6 ± 6.3	5.42 ± 0.49	230 ± 19.1
Ground squirrel	5	long-term hibernation	84.6 ± 3.0	3.97 ± 0.30	166 ± 6.0
* Beef	4	---	31.3 ± 1.5	2.42 ± 0.15	133 ± 8.6
* Crab nerve	3	---	12.7 ± 4.2	.041 ± 0.01	6.00 ± 1.15

* Taken from Charnock & Simonson (1977). Specific activity is given as μmoles ATP hydrolysed/mg protein/hour. Rate [^{3}H]-ouabain binding is given as pmoles/mg protein sec^{-1}. Amount [^{3}H]-ouabain bound is given as pmoles/mg protein. All determinations were done at 37°C.

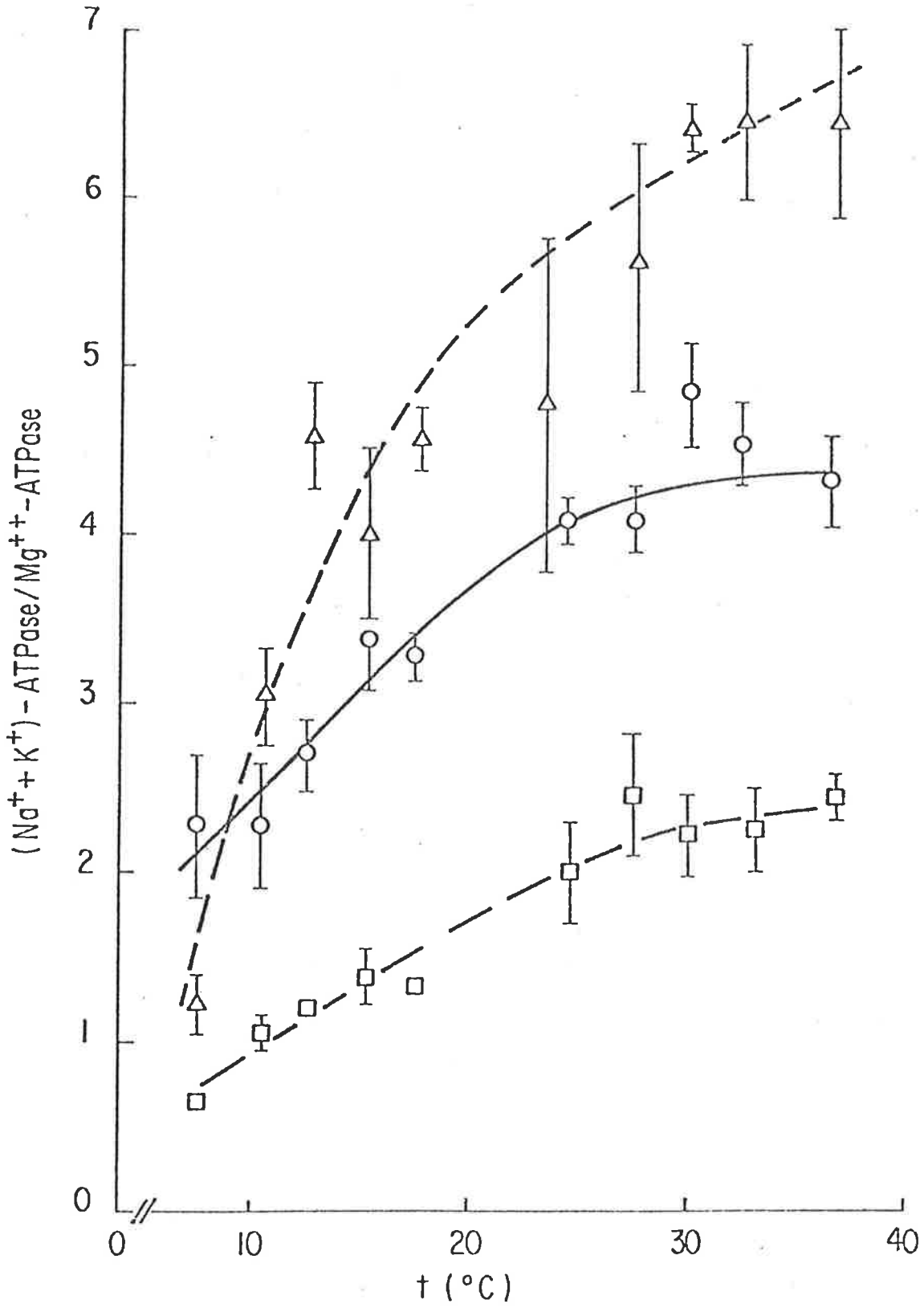
FIGURE LEGENDS

Fig. 1. Seasonal variation in the dose response curves for ouabain inhibition of brain ($Na^+ + K^+$)-ATPase prepared from summer (July - August) control (o) and 100 day hibernating (□) Richardson ground squirrels. Results are the means ± S.E. of three separate enzyme preparations. Assays were in duplicate and were carried out with 1.5 mM Tris-ATP at varying concentrations of ouabain. 100% inhibition of ($Na^+ + K^+$)-ATPase occurs when the inhibition by ouabain is ≥ than the stimulation of ATPase activity on the addition of 80 mM Na^+ to a medium which contained 20 mM K^+. The concentrations of ouabain giving one-half maximal inhibition of ($Na^+ + K^+$)-ATPase, (i.e. K_i), were 4.4×10^{-7}M and 8.6×10^{-7}M for enzyme preparations from summer and hibernating animals respectively.

Fig. 2. Seasonal variation in the effect of temperature on the activity ratios of ($Na^+ + K^+$)-ATPase v. Mg^{++}-ATPase for brain preparations from summer (o), < 30 day hibernating (Δ) and > 100 day hibernating (□) Richardson ground squirrels. The results are given as the means ± S.E. of six, two and four separate enzyme preparations respectively.

% inhibition of ($Na^+ + K^+$)-dependent ATPase
0
20
40
60
80
100
Ouabain (M)
0
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}

FIG. 2



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DIFFERENTIAL LIPID CONTROL OF $(Na^+ + K^+)$-ATPase IN HOMEOTHERMS AND POIKILOTHERMS

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ABSTRACT

1. $(Na^+ + K^+)$-ATPase from homeotherms and poikilotherms demonstrate non-linear thermal dependence for ATP hydrolysis. Apparent energies of activation from crab nerve preparations are less than those of brain or kidney preparations from beef, sheep or ground squirrel.
2. Crab nerve $(Na^+ + K^+)$-ATPase is less sensitive to inhibition by ouabain than that from beef or ground squirrel; lower rates of [^{3}H]-ouabain binding and reduced amount of drug bound at equilibrium are found.
3. K^+-activated acyl-phosphatase is similar in all preparations.
4. Fluorescence polarization of 12-AS labelled membranes demonstrate greater mobility of crab nerve lipids compared to beef brain which has a thermal transition at 20-25°C. Crab nerve is linear in this range.

INTRODUCTION

There is a considerable body of evidence which suggests that the lipids of biological membranes exert a modulating effect upon the behaviour of integral membrane enzyme systems (Overath, Schairer & Stoffel, 1970; Lyons, 1971; Raison, 1973). This is particularly true for the process of active cation translocation where the enzyme system ($Na^+ + K^+$)-ATPase (E.C.3.6.1.3) actually requires closely associated membrane lipids for full hydrolytic activity and presumably full transport function. Many investigators have shown that removal of lipids during isolation procedures frequently leads to a significant reduction in the hydrolytic activity of this enzyme which in some cases can be restored by the reintroduction of negatively charged phospholipids to the system (Tanaka & Strickland, 1965; Tanaka, Sakamoto & Sakamoto, 1971; Kimelberg & Papahadjopolous, 1972).

In this laboratory we have demonstrated that there is a good correlation between the non-linear thermal response of ($Na^+ + K^+$)-ATPase isolated from a number of mammalian systems and the physical properties of the associated lipids in the membrane fragments examined (Charnock & Bashford, 1975). In addition, we have been able to alter the thermal behaviour of ($Na^+ + K^+$)-ATPase by modification of the membrane lipids by treatment with phospholipase-A, followed by partial reconstitution of the membranes by the addition of phosphatidyl serine (Charnock, Cook, Almeida & To, 1973).

In the experiments discussed in this paper, we have examined the effect of temperature on the hydrolytic activity and other biochemical characteristics of ($Na^+ + K^+$)-ATPase containing membranes isolated from

both homeotherms and poikilotherms. By using the fluorescent probe 12-(9-anthroyl) stearic acid (12-AS) which we had employed in previous studies (Charnock & Bashford, 1975) we have been able to examine the relationship between some physical properties of the membrane lipids of these different enzyme preparations with the activity of their $(Na^+ + K^+)$-ATPase. The results reinforce the view that the lipids of biological membranes exert a modulating action upon membrane enzyme behaviour.

MATERIALS AND METHODS

Untreated preparations of $(Na^+ + K^+)$-ATPase were obtained from the heavy microsomal fractions of the renal cortex of rabbits (Oryctolagus cuniculus), sheep (Ovis aries) and ground squirrel (Spermophilus richardsonii) as well as the cerebral cortex of beef (Bos taurus) and the ground squirrel using a procedure of homogenisation and differential centrifugation first employed by Charnock & Post (1963) but recently modified in this laboratory (Charnock, Simonson & Almeida, 1977). A membrane preparation containing $(Na^+ + K^+)$-ATPase activity was also obtained from the axon of the walking legs of the lobster (Homarus vulgaris) obtained from a commercial supplier in the United Kingdom, and the Dungeness crab (Cancer magister) collected from a depth of 14-17 fathoms off Barkley Sound, Vancouver Island. The ambient temperature of these waters is reported to be 4-8°C; the crabs were maintained alive in sea water in the aquarium at the Bamfield Marine Biology Station at 8-9°C for a period of ten days prior to dissection.

Enzyme activity was determined by the coupled optical assay procedure described previously (Schoner, von Ilberg, Kramer & Seubert, 1967; Charnock,

Simonson & Almeida, 1977), except that activation by cations was always measured in addition to inhibition of the system by the cardiac glycoside ouabain. This important difference was necessary because the crab enzyme was found to be much less sensitive to inhibition by ouabain than is the enzyme derived from mammalian sources.

Our assay medium therefore contained all ingredients described previously (Charnock, Simonson & Almeida, 1977) with the exception of NaCl. Non-sodium salts of phospho(enol)-pyruvate and EDTA were used. Since one of the coupling enzymes, pyruvate kinase, requires K^+ for activity (Bergmeyer, 1965) 20 mM KCl was present in all assays unless specified otherwise in the text; 0.4 mM tris-ATP was used as substrate.

Cation activation of the enzyme was determined as the stimulation from the basal ATPase activity with the addition of 80 mM NaCl to the medium. Ouabain inhibition was determined by the decrease in activity which occurred when the drug was added to the media which contained both 80 mM Na^+ and 20 mM K^+. The concentrations of ouabain which were used are given in the legends to the tables and figures. Units of specific activity are defined as μmoles of substrate hydrolysed/mg protein/hour.

The methods for determining the initial rates of enzyme activity at various temperatures, the binding of [^{3}H]-ouabain to the preparations and the computer assisted analysis of the data have all been described previously (Charnock, Cook, Almeida & To, 1973; Charnock, Simonson & Almeida, 1977).

K^+ stimulated acyl phosphatase (E.C.3.6.1.7) was also determined on these preparations by a method based upon that described by Bergmeyer

(1965) and Tanaka, Sakamoto & Sakamoto (1971) which we modified to utilize a Guilford 2400 recording spectrophotometer in order to obtain the initial rates of p-nitro-phenol formation from 10 mM p-nitro-phenylphosphate in the presence and absence of 10 mM KCl. These concentrations of substrate and K^+ gave optimal rates of product formation at 37°C in an assay medium containing 50 mM glycylglycine, 5 mM $MgSO_4.7H_2O$ and 0.5 mM EDTA (pH 7.6). Where required, 2 mM ouabain was included in the assay medium (Walker & Wheeler, 1975).

The effect of temperature on the fluorescence polarization of ($Na^+ + K^+$)-ATPase containing membranes labelled with 12-(9-anthroyl) stearic acid (12-AS) was determined using a Hitachi-Perkin Elmer Spectrophotofluorometer Model MPF-4 fitted with the commercially available polarizer accessory and with the temperature of the sample chamber controlled to ± 0.2°C by an external heating/cooling system. The fluorescent probe was added to the membrane suspension to give a concentration of probe of 20 pico moles/µg membrane protein. Labelling was carried out for 30 mins at 37°C by which time maximum incorporation as determined by increase in fluorescence intensity had occurred. With the excitation wavelength at 385 nm and the emission wavelength at 437 nm a series of measurements were obtained with the polarizers set at (1) 90°,90°; (2) 0°,90°; (3) 0°,0° and (4) 90°,0° for the excitation and emission positions respectively. The values so obtained were used to calculate the fluorescence polarization P as defined by Weber (1953,1973) and used by others in several recent studies (Radda, 1971; Charnock & Bashford, 1975; Bashford, Morgan & Radda, 1976,

Vanderkooi, Landesberg, Selick & McDonald, 1977) where

$$P = \frac{(3) - (2)\left[\frac{4}{1}\right]}{(3) + (2)\left[\frac{4}{1}\right]}$$ from the notation used above.

The reciprocal values of P were then plotted against temperature to yield an index of membrane mobility.

RESULTS

Effect of temperature upon $(Na^+ + K^+)$-ATPase

The initial rates of cation activation of $(Na^+ + K^+)$-ATPase were determined at 3°C intervals between 5-37°C using membrane enzyme preparations obtained from the cerebral cortex of beef and ground squirrels, the renal cortex of rabbit, sheep and ground squirrel and the axons of the walking legs of cold water crabs and lobster. The rate data were first plotted according to Arrhenius (1902, 1912) and the slopes of the lines calculated to yield values for the apparent energies of activation above and below a so-called critical temperature (T_c°) which could be derived from the computer assisted analysis we have employed before (Charnock, Cook, Almeida & To, 1973). All of the Arrhenius plots we obtained could be fitted by two intersecting straight lines with an acceptable statistical confidence, and yielded calculated values for T_c° which lay well within the experimental range of temperature. These data are given in Table 1.

The values for T_c° which were obtained showed no statistically significant variation between groups ($p > 0.1$). As T_c° can only represent some mean value approaching the mid-point of a thermal transition (Singer, 1976)

which presumably ranges over at least 5°C, it is clear that there is no evidence from this type of analysis for a markedly different range of transition temperatures between any of these preparations. A more direct form of study using membrane probes might however reveal some differences.

The values for the apparent energies of activation of $(Na^+ + K^+)$-ATPase that could be calculated above (Ea_I) and below (Ea_{II}) the thermal transition do reveal some differences between the preparations. The highest values were obtained for the homeotherm kidney preparations, intermediate values were obtained from the brains of both beef and awake ground squirrel with the lowest values obtained from the crab and lobster nerve preparations.

It is of interest that all preparations showed values of Ea_{II} which were about double that of Ea_I irrespective of the actual values for Ea_I.

During the course of this study we observed that poikilotherm $(Na^+ + K^+)$-ATPase was much less sensitive to inhibition by the cardiac glycoside ouabain than were preparations obtained from either the kidneys or brains of homeotherms. We therefore examined this situation systematically by constructing dose-response curves for ouabain inhibition using $(Na^+ + K^+)$-ATPase preparations from both beef and ground squirrel brain and compared these data to that obtained from crab axon preparations. The results are shown in Fig. 1 where it can be seen that complete inhibition of $(Na^+ + K^+)$-ATPase from beef and ground squirrel brain is obtained with 100 μM ouabain and the concentration for 50% inhibition (K_i) is near 2 μM which is similar to the values for guinea pig and rabbit cortex we have reported earlier (Charnock & Opit, 1968).

The $(Na^+ + K^+)$-ATPase preparations from crab axon differ from this

quite markedly in that the dose-response curve is shifted by more than two log units to the right. Nearly 3 mM ouabain was required to produce complete inhibition and the K_i is increased from 2 μM to 130 μM. The K_i for crab axon preparations was not changed when the concentration of Na^+ in the medium was increased from 80 mM to 200 mM, although there was both a small loss in ATPase activity and an increase in sensitivity to ouabain when the concentration of K^+ was reduced from 20 mM to 2 mM (data not shown). Thus although the inhibition of crab axon ($Na^+ + K^+$)-ATPase can be influenced by the concentration of K^+ as in preparations from homeotherms (Charnock & Post, 1963; Repke, 1965; Charnock & Opit, 1968), these crab preparations are about one hundred fold less sensitive to ouabain. This finding extends the original observation of Skou (1960) who reported only partial inhibition of the ($Na^+ + K^+$)-ATPase of the leg nerve of preparations from the shore crab (Carcinus maenas) with 1 mM G-strophanthin.

Since ouabain inhibition of ($Na^+ + K^+$)-ATPase reflects the specific binding of the cardiac glycoside to the enzyme receptor (Schwartz, Allen & Harigaya, 1969; Erdmann & Schoner, 1974; Charnock, Simonson & Almeida, 1977) a study of the binding characteristics of [^{3}H]-ouabain to the crab preparations might reveal further information concerning the decreased sensitivity of these systems. The results of a comparison of both the rate and equilibrium level of binding of [^{3}H]-ouabain to beef and crab preparations of ($Na^+ + K^+$)-ATPase are given in Table 2.

In a previous study with beef brain ($Na^+ + K^+$)-ATPase (Charnock, Simonson & Almeida, 1977) we have described in detail the linear relationship which exists between [^{3}H]-ouabain binding and the specific activity of

the enzyme. The same relationship was found with beef brain preparations in these experiments where 5×10^{-7}M ouabain produced a 19% inhibition of $(Na^+ + K^+)$-ATPase and resulted in 3-4 pmoles [^{3}H]-ouabain bound at equilibrium for every "unit" of specific activity measured at 37°C.

The same concentration of ouabain only produced about a 4% inhibition of $(Na^+ + K^+)$-ATPase in the crab preparations and there was a very marked decrease in both the rate of drug binding and the amount of [^{3}H]-ouabain bound at equilibrium. These results indicate that the affinity for drug binding in crab enzyme preparations is much less than is the case with $(Na^+ + K^+)$-ATPase preparations from homeotherms.

With the finding of these comparative differences in the properties of $(Na^+ + K^+)$-ATPase preparations between a poikilotherm and some homeotherms, it was of interest to us to compare the properties of a ouabain-sensitive partial reaction of $(Na^+ + K^+)$-ATPase, that is the K^+ stimulated acyl phosphatase (E.C.3.6.1.7) which is generally believed to represent the terminal acyl-phosphatase of stepwise ATP hydrolysis by $(Na^+ + K^+)$-ATPase (Wheeler & Whittam, 1970; Askari & Rao, 1971; Dahl & Hokin, 1974; Whittam & Chipperfield, 1975; Wheeler & Walker, 1975; Schwartz, Lindenmayer & Allen, 1975).

Fig. 2 shows the results of our experiments to determine the temperature dependence of this partial reaction of $(Na^+ + K^+)$-ATPase by measuring the initial rates of conversion of p-nitro-phenylphosphate to p-nitrophenol between 8°-37°C. The data from crab nerve enzyme was superimposeable on a linear plot of rate v. temperature with that obtained from beef brain enzymes. However both sets of data yielded non-linear Arrhenius plots

(Fig. 2 insert) which on computer assisted analysis yielded slightly different values for the so-called transition temperatures discussed above in relation to ATPase activity. The calculated values for T_c° for crab and beef preparations were 24.3°C and 20.8°C respectively. The values for the apparent energies of activation for both preparations were much greater below this transition temperature than were the values calculated above this temperature.

In recent years there have been numerous reports which suggest that marked changes in the apparent energies of activation of membrane bound enzymes can be correlated with changes in the physical properties of associated membrane lipids (Raison, 1973; Charnock, Cook, Almeida and To, 1973; Charnock & Bashford, 1975; Wodtke, 1976). A direct measure of lipid behaviour can be achieved by a variety of biophysical techniques including the use of fluorescent probes which preferentially intercalate with the lipids of biomembranes (Radda & Vanderkooi, 1972; Bashford, Morgan & Radda, 1976).

We have used the fluorescent probe 12-AS to study the effect of temperature on the fluorescence polarization (P) of membrane preparations of $(Na^+ + K^+)$-ATPase from both beef brain and crab nerve. Our results are shown in Fig. 3 which gives plots of $\frac{1}{P}$ v. t°. After labelling with 12-AS both preparations gave non-linear thermal responses with transition temperatures calculated as 25°C for the beef preparation and 32°C for the crab preparation by our method of analysis. That is that whereas there is a marked change in the fluidity of the homeotherm membranes containing $(Na^+ + K^+)$-ATPase within their normal physiological range, no such change occurs in the fluidity of membranes containing $(Na^+ + K^+)$-ATPase in the

crab nerve preparation. In addition, the slopes of the fluorescence polarization plots suggest that the crab membranes are more fluid than those of beef brain at their respective physiological ranges.

DISCUSSION

We have compared the temperature dependence of ATP hydrolysis by $(Na^+ + K^+)$-ATPase preparations obtained from several common species of homeotherms (rabbits, sheep and beef), an awake hibernating species of ground squirrel common to this area of Alberta, and two species of poikilotherms (the Pacific crab and the Atlantic lobster).

When the apparent energies of activation (Ea) of these various preparations are calculated over the temperature range 5°-37°C, it is found that $(Na^+ + K^+)$-ATPase preparations from the kidney of rabbit, sheep and ground squirrel give values which are very similar and are all greater than those for the brain of ground squirrel or beef. On the other hand the values for both the crab and the lobster nerve preparations are less than any of those found with either homeotherms or an awake hibernator. Apparently the poikilotherm preparations are the most thermodynamically efficient when judged on this basis alone.

Another difference between poikilotherm $(Na^+ + K^+)$-ATPase preparations and those of either homeotherms or the awake hibernator was the marked decrease in sensitivity to ouabain, the common cardiac glycoside inhibitor of this enzyme. The crab nerve preparations we examined were nearly one

hundred-fold less sensitive to ouabain than were the enzymes from beef or ground squirrel brain studied here or that of rabbit and guinea pig kidney which we had reported previously (Charnock & Opit, 1968). This reduced sensitivity was reflected in both a reduced rate of [^{3}H]-ouabain binding and a reduced amount of [^{3}H]-ouabain bound under equilibrium conditions at 37°C. When this data is considered in conjunction with the values for activation energies for the poikilotherm and homeotherm enzymes it is apparent that while the characteristics for activation of the enzymes by Na^+ and K^+ are reasonably similar, the affinities of the enzymes for ouabain interaction are strikingly different.

It should be recalled that whereas activation of ATP hydrolysis by the cations Na^+ and K^+ occurs from opposing surfaces of the membrane, inhibition of the enzyme system by ouabain is thought to occur only from the outer surface of the membrane (Whittam, 1962; Skou, 1965).

In seeking to pursue the source of this difference in sensitivity to ouabain, we also compared the temperature dependence of a partial reaction of ($Na^+ + K^+$)-ATPase, K^+ activated, ouabain-sensitive acyl-phosphatase (PNPP-ase) as this reaction is believed to be oriented towards the outer surface of the membrane (Askari & Rao, 1971; Schwartz, Lindenmayer & Allen, 1975). In general the temperature dependence for K^+ activated PNPP-ase was very similar for both beef brain and crab nerve membrane preparations, although considerably lower activation energies were calculated for both systems than was the case for the corresponding overall reaction for ATP hydrolysis. Apparently this "terminal" reaction is not a rate limiting step in the hydrolysis of ATP. However in marked contrast to the recent report

by Walker and Wheeler (1975) that K^+ activated PNPP-ase of rabbit kidney yields a linear temperature dependence between 8°-40°C, while giving a non-linear response in the presence of 0.2 mM ouabain, we found that both crab nerve and beef brain preparations gave non-linear Arrhenius plots whether the system was assayed by K^+ activation or by inhibition by 2 mM ouabain (data for ouabain inhibition not shown). Analysis of these Arrhenius plots yields values for the thermal transition (T_c°) between 20-25°C. Whereas the calculated value for T_c° for PNPP-ase and $(Na^+ + K^+)$-ATPase were identical in beef brain membrane preparations, this was not the case for crab nerve preparations where the difference in these calculated values was about 10°C.

Whether this represents a true biological variation between species or merely reflects the deficiencies in the calculation of a value for T_c° from Arrhenius plots of enzyme rate data cannot be determined from the present experiments. However it is interesting to speculate that this difference arises from the more assymmetric distribution of lipids between the "inner" and the "outer" surfaces of $(Na^+ + K^+)$-ATPase containing membranes of the crab nerve compared to the $(Na^+ + K^+)$-ATPase containing membranes obtained from the brains of beef. Examination of these two enzyme systems (PNPP-ase and $(Na^+ + K^+)$-ATPase) in a wider range of species might confirm our suggestion although such an approach must remain indirect at best.

A more direct measure of lipid involvement in membrane $(Na^+ + K^+)$-ATPase function was sought by labelling both crab nerve and beef brain preparations with the fluorescent probe 12-AS, and measuring the effect of temperature on

the fluorescence polarization. These studies give a measure of the lipid mobility of the membranes, at least in areas adjacent to the probe molecules (Radda, 1971; Radda & Vanderkooi, 1972; Bashford, Morgan & Radda, 1976; Vanderkooi, Landesberg, Selick and McDonald, 1977).

Considerable differences were now found between the poikilotherm and homeotherm enzyme preparations. Whilst $(Na^+ + K^+)$-ATPase preparations from beef brain were non-linear and showed a thermal transition with a mid-point at 25°C just as we have previously reported for sheep and pig kidney (Charnock & Bashford, 1975), the $(Na^+ + K^+)$-ATPase containing membrane from crab nerve displayed a linear change in fluorescence polarization from 8°C to 32°C, where a thermal transition could be observed. This linearity of fluorescence polarization in the 8-32°C temperature range has been observed by us previously in other poikilotherm preparations including the crab Cancer pagurus (Charnock, Bashford & Ellory, 1976) and some lobsters (Homarus valgaris) obtained from Scotland (Charnock, Simonson & Almeida, 1976). However the temperature range studied on both these previous occasions did not permit the detailed study of fluorescence polarization much above 30°C and thus perhaps failed to reveal the thermal transition we have observed with $(Na^+ + K^+)$-ATPase from Cancer magister. Thus the strong positive correlation that can be found for the temperature dependence of $(Na^+ + K^+)$-ATPase, PNPP-ase and fluorescence polarization in membrane preparations from homeotherms which also show a high sensitivity to ouabain, cannot be so easily observed in membrane preparations from poikilotherms which have a much reduced sensitivity to ouabain.

Although the precise localisation of the fluorescent probe 12-AS in

the lipids of biomembranes is not known, it seems likely from our results with beef brain preparations that there is some specificity for this probe which is apparently reporting from a domain associated with the hydrolytic function of the $(Na^+ + K^+)$-ATPase enzyme protein. Conversely, with the crab preparations the probe appears to be more likely to be reporting from a domain more closely associated with a partial reaction of the enzyme which displays a marked reduction in sensitivity to ouabain and is probably located at the "outer" surface of the membrane. Again this would suggest an increased lipid assymmetry in the membranes of crab nerves compared to the beef brain preparations.

However all of our enzyme data can be explained on the basis of increased mobility of membrane lipids in preparations from poikilotherms compared to homeotherms. It is of course well known that crustacea have a relatively high cholesterol content and some preliminary lipid analyses of our enzyme preparations has shown a two-fold increase in the cholesterol: phospholipid ratios obtained with crab compared to enzyme preparations from sheep kidney (Charnock, J.S. & Miller, N.G., unpublished data). It is clear that very detailed analyses of the membrane lipids of these systems is required to answer this question.

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TABLE 1. Apparent activation energies of $(Na^+ + K^+)$-ATPase preparations obtained from homeotherms, awake hibernator and poikilotherms

Tissue	n	Ea_I*	Ea_{II}*	T_c°	Ea_I:Ea_{II}
Beef brain	9	18.1 ± 1.1	37.0 ± 3.4	20.0 ± 1.0	0.49
Ground squirrel brain	6	14.2 ± 1.2	32.4 ± 1.4	19.8 ± 0.4	0.44
Ground squirrel kidney	5	21.4 ± 1.4	45.1 ± 6.2	16.7 ± 2.2	0.47
Rabbit kidney	10	20.4 ± 1.0	44.5 ± 3.4	18.2 ± 1.7	0.46
Sheep kidney	3	16.8 ± 0.2	41.1 ± 7.1	18.2 ± 3.2	0.41
+ Lobster axon	5	13.2 ± 0.9	23.7 ± 3.4	22.0 ± 3.3	0.56
+ Crab axon	3	12.5 ± 1.5	28.5 ± 1.9	16.2 ± 2.3	0.44

* Values for Ea_I and Ea_{II} were given as kcals/mole.

+ 0.4 mM ouabain was used to inhibit $(Na^+ + K^+)$-ATPase from homeotherm and awake hibernator preparations, 1 mM ouabain was used for the preparations from poikilotherms.

TABLE 2. Relationship of $[^3H]$-ouabain binding and specific activity of membrane preparations of $(Na^+ + K^+)$-ATPase from a homeotherm and a poikilotherm

Preparation	n	*Specific Activity (1) (Mean ± SE)	#Rate of binding $[^3H]$-ouabain (2) (Mean ± SE)	+Equilibrium level $[^3H]$-ouabain binding (3) (Mean ± SE)	3/1
Beef brain	4	31.3 ± 1.5	2.42 ± 0.15	113 ± 8.6	3.62
Crab nerve	3	12.7 ± 4.24	0.041 ± 0.01	6.00 ± 1.15	0.48

All measurements were made at 37°C.

* Specific activity given as µmoles Pi liberated from substrate/mg protein/hour.

\# Rate given as pico moles $[^3H]$-ouabain bound/mg protein/sec.

\+ Equilibrium level given as pico moles $[^3H]$-ouabain bound/mg protein after 10 min incubation.

Final concentration $[^3H]$-ouabain was 5×10^{-7}M. (ref. Charnock, Simonson & Almeida, 1977).

FIGURE LEGENDS

Fig. 1 Dose-response curve of ouabain-inhibition of $(Na^+ + K^+)$-ATPase from homeotherms, awake hibernators and a poikilotherm. Enzyme activity determined at 37°C in the presence of 80 mM Na^+ and 20 mM K^+ plus variable ouabain as indicated. (o - o), beef brain; (Δ - Δ), ground squirrel brain; (▣ - ▣), crab nerve. The respective values for 50% inhibition (K_i) are 1.3×10^{-6}M, 2.5×10^{-6}M and 1.3×10^{-4}M.

Fig. 2 Temperature dependence of PNPPase activity of beef brain (o - o) and crab nerve (□ - □) enzyme preparations. Rates are expressed as a percentage of the rate at 37°C. Results are the means of four experiments. The insert uses the same symbols to show the experimental data given as Arrhenius plots. The values for T_c° were 20.8 and 24.3°C for beef and crab respectively. There is little difference in the values for Ea_I and Ea_{II} between preparations, about 8.2 and 14.9 kcals/mole respectively.

Fig. 3 Temperature dependence of fluorescence polarization of membrane preparations of $(Na^+ + K^+)$-ATPase labelled with 12-AS. (□ - □), crab nerve; (o - o), beef brain. Excitation wavelength 385 nm, emission 435 nm. Membrane enzyme preparations labelled at 37°C with 20 pico moles 12-AS/μg protein. Decending temperature gradients were employed for both preparations. Results expressed are the means of four polarization determinations at each temperature.

FIG. 1

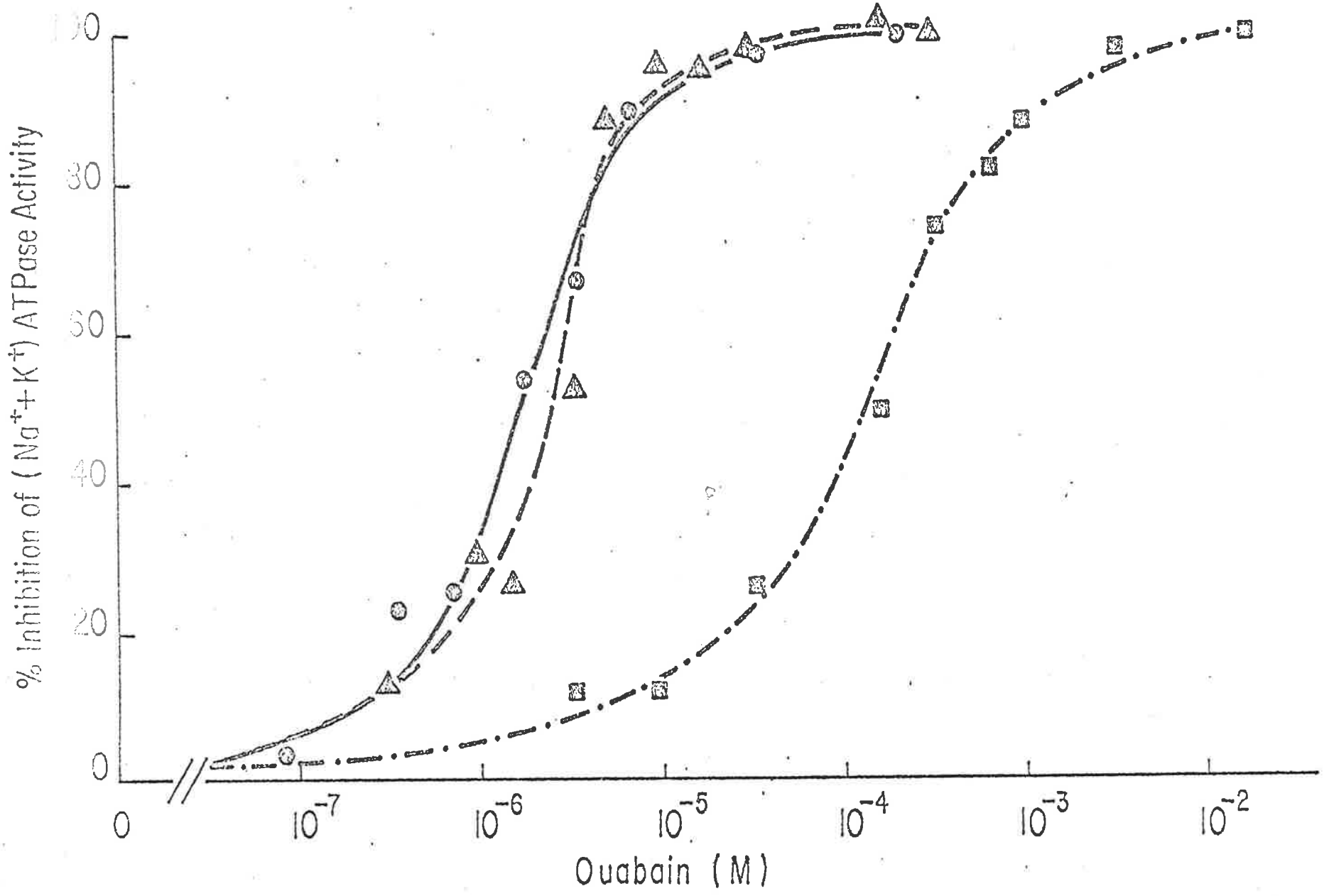
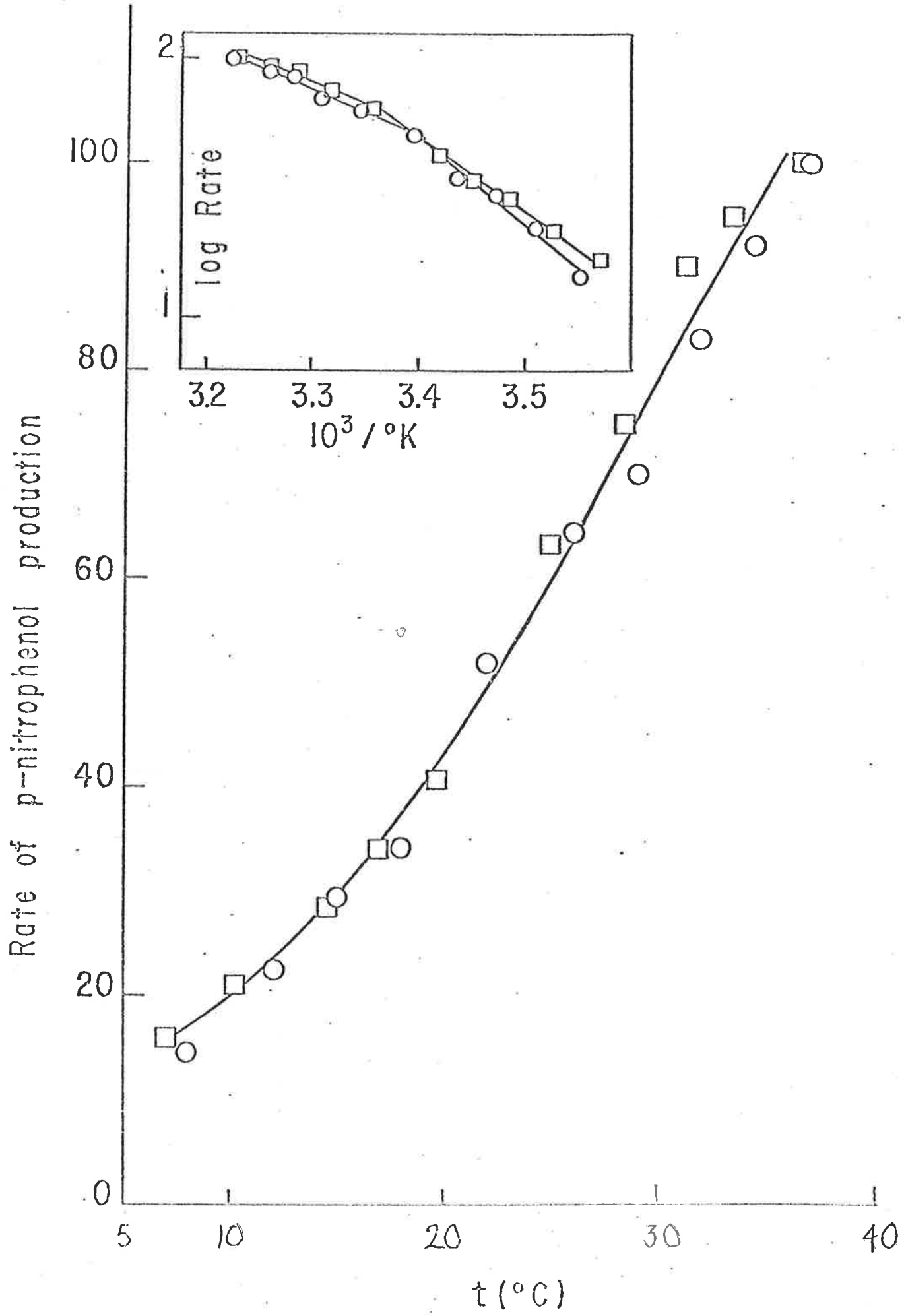

% Inhibition of ($Na^+ + K^+$) ATPase Activity
100
80
60
40
20
0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
Ouabain (M)

FIG. 2


Rate of p-nitrophenol production
100
80
60
40
20
0
5
10
20
30
40
t (°C)
2
1
log Rate
3.2
3.3
3.4
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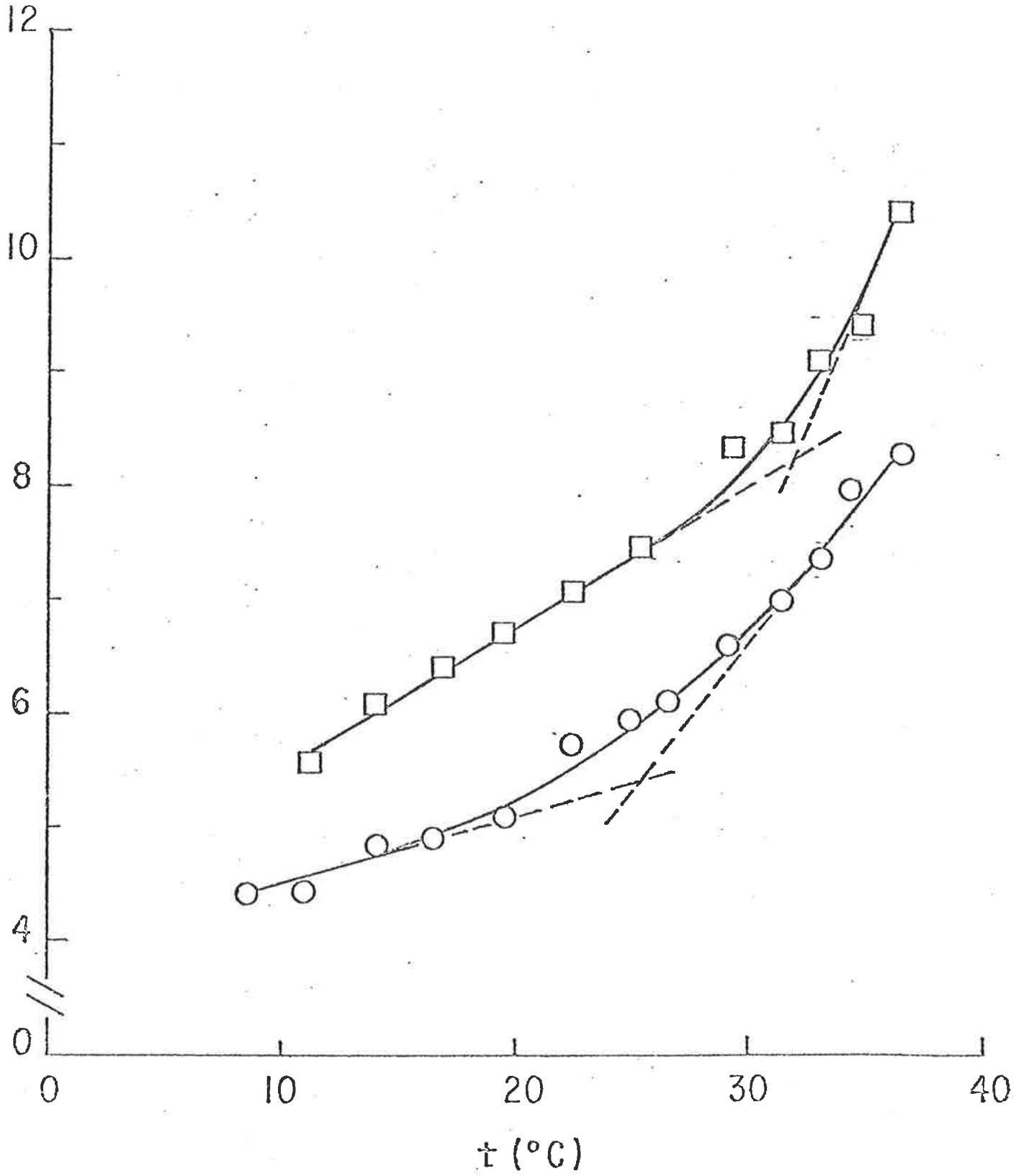
10³ / °K

FIG. 3



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AN ELECTRON SPIN PROBE STUDY OF ($Na^+ + K^+$)-ATPase-CONTAINING MEMBRANES

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Summary

The modulating effect of membrane lipids on enzyme function has been described by several investigators. We have used the spin probe *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto methyl stearate (M 12-NSE) to study this interaction in ox brain membranes enriched with ($Na^+ + K^+$)-ATPase. This methyl ester of stearic acid is practically insoluble in aqueous media, and consequently spectra of M 12-NSE-labelled preparations are free of "liquid lines".

At least two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE, indicating the presence of two distinct binding sites. At one site the spin label is relatively unrestricted and gives rise to an isotropic spectrum. A second spectrum, which is obtained from spin label at another site, is similar to that which is observed after incorporation of M 12-NSE into phospholipid bilayers. This suggests that this latter site is within the core of the microsomal membrane.

The two binding sites differ in their affinity for the spin probe. The low affinity site is both more abundant in crude preparations and is more easily removed by detergent treatment; spin labels at this site produce isotropic spectra. The high affinity sites are fewer in number and produce broad spectra. In addition these high affinity sites increase in concentration as the enzyme undergoes purification.

The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

This study also demonstrates that the delipidation effects of sodium dodecyl sulfate and sodium deoxycholate on ($Na^+ + K^+$)-ATPase-enriched microsomes from ox brain are not identical.

It is suggested that the two spin probe binding sites represent two different lipid domains, one of which is very closely associated with the ($Na^+ + K^+$)-

Abbreviations: ESR, electron spin resonance spectroscopy; M 12-NSE, *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto methyl stearate; 5-NS, *N*-oxyl-4′,4′-dimethyloxazolidine-5-keto stearic acid.

ATPase enzyme and may reflect a protein-directed phospholipid specificity for this enzyme.

Introduction

The preparation of $(Na^+ + K^+)$-ATPase containing microsomes by differential centrifugation with or without sodium deoxycholate extraction, can result in products that differ significantly in the specific activity of the enzyme. However, the temperature sensitivity of the enzyme is not altered [1]. In contrast, the binding of ouabain to this enzyme, which is also temperature sensitive, is markedly different after extraction of the microsomal preparations with sodium deoxycholate or other detergents [2]. This temperature sensitivity is best described by the non-linearity found in Arrhenius plots which are believed to arise from phase transitions or phase separations of the membrane lipids [3]. Whether these transitions pertain to a specific lipid or a number of lipids is not clear. The use of biophysical techniques such as fluorescence spectroscopy and ESR to investigate the physical behaviour of lipids is extensively used, and we are currently engaged in an electron spin probe study of microsomal membranes rich in $(Na^+ + K^+)$-ATPase. A number of review articles of this object are now available [4—6]. It is widely believed that both steroid and fatty acid spin probes intercalate in the membrane bilayer [6] and are consequently used to detect physical changes in the characteristics of the lipid matrix. However, it is not yet possible to precisely locate the spin label which may report from specific domains, from the bulk lipid, or from both [7,8]. In addition, the spin probes may also dissolve in the aqueous phase and give rise to "liquid lines" [9, 10]. Spin probes in aqueous solution are rapidly reduced by ascorbic acid and a study of the rate of this reaction can provide information as to the location of the spin probe in the bilayer [11—13]. In the present study we present evidence to show that the spin probe, the *N*-oxyl-4′,4′-dimethyloxazolidine-12-keto derivative of methyl stearate (M 12-NSE) which is essentially insoluble in aqueous media, will bind non-covalently at more than one site to ox brain microsomes rich in $(Na^+ + K^+)$-ATPase. Preliminary communications of this work have been made at recent symposia [27,28].

Materials and Methods

Microsomes and liposomes. The preparation, delipidation procedures, and biochemical assay of the $(Na^+ + K^+)$-ATPase of the ox brain microsomes have been described recently by Charnock et al. [2].

Liposomes were made from dimyristoyl phosphatidylcholine (Serdary Research Laboratories, Ontario) or from the lipids extracted from ox brain microsomes. Weighed amounts of lipid were added to a buffered sucrose solution (0.25 M sucrose, 5 mM Na_2 EDTA, pH 7.6, with Tris) and the mixtures sonicated in a Cole-Parmer Ultrasonic Cleaner, Model 884S-4, until a clear solution was obtained.

Electron spin resonance. The spin label, a methyl ester of stearic acid with the nitroxyl on the 12-C position M (12-NSE), was a grift from Dr. J.K. Raison,

Macquarie University, Sydney, Australia. A methanol solution of M 12-NSE was evaporated in a stream of nitrogen and the suspension of microsomes or liposomes was added to the label. After incubation for 10 min at 37°C, the sample was stored at 0°C until it was used for spectral investigation. There was no difference in the spectra that were obtained whether the labelling procedure was carried out directly in the glass capillary sample tube (1.1 mm internal diameter) or in a separate vessel and subsequently transferred. No sample was stored for longer than 24 h.

The ESR spectra were obtained using a Varian V-4502 EST spectrometer. This instrument is equipped with an Alpha Model 3039 digital NMR gaussmeter for magnetic field calibration and a Varian-4557 temperature controller. The temperature of the sample chamber was examined by a copper-constantin thermocouple, to 0.1°C. Microwave frequencies were monitored with a HPX 532B frequency meter.

A number of methods are currently available for the quantitation and semiquantitation of ESR spectra. Using some approximations, McConnell [14], Kivelson [15] and Freed and Fraenkel [16] have developed mathematical methods for the calculation of rotational correlation times of spin probes incorporated into rigid matrices. These approaches are only applicable to rapid motion, such as that seen as isotropic spectra from spin probes in solution. The ESR spectra of fatty acid spin probes in lipid bilayers are often indicative of anisotropic motion [17,18] and a measure of the membrane fluidity, the order parameter, can be obtained for spectra such as these [17,18]. Other methods that have also been used for broad spectra, make use of one or more of the peak heights and although they are semiquantitative, these simple approaches can be very useful in the interpretation of ESR spectra obtained from spin-labelled biomembranes. In this report we have generally confined our results to a qualitative description of the different spectra; where quantitative data is presented, peak heights are used as described in the legends.

The reduction of M 12-NSE by ascorbic acid was examined by exposing the labelled material to 2 mM ascorbic acid, freshly prepared, (pH 7.5 with Tris) and monitoring the changes in peak heights with time.

Results

In the application of electron spin resonance spectroscopy to the examination of biomembranes, two complications are commonly encountered. One of these is the appearance of "liquid lines" due to spin probe that has dissolved in the aqueous phase [9,10], and a second problem is the possibility of line broadening due to interactions between the electrons of the spin probe molecules when pooling or clustering occurs [19,20]. Both these effects can be avoided however, by reducing the amount of spin probe that is employed, but some limitations must first be defined. Preliminary experiments [27,28] demonstrated that any spectral contribution from the empty capillary tube, the residual spin label on the walls of the sample tube, or from label dissolved in the buffered sucrose medium were all negligible and could be contained by limiting the gain settings to a narrow range. In particular we established that at the probe concentration used in the experiments reported here, even after 24 h exposure

to the aqueous buffer, a negligible amount of dissolved label could be detected.

In these preliminary experiments we also examined the spectra of M 12-NSE in liposomes (smectic mesophases). Liposomes made from extracted membrane lipids or from dimyristoyl phosphatidylcholine, when spin labelled by the method we have described, showed a rapid incorporation of spin probe as is commonly reported [21,22]. The spectrum of labelled liposomes displayed the characteristic line shape of a spin probe oriented perpendicular to the plane of the bilayer [17,18]. Moreover, varying the probe : phospholipid ratio from 1 : 14 to 1 : 110 did not cause any major change in the line shape of the spectrum.

Spectral variations of spin-labelled microsomes

Recent work in our laboratory has shown that three characteristics of (Na^+ + K^+)-ATPase from ox brain microsomes, namely the specific activity, the rate of ouabain binding, and the effects of temperature on these enzyme characteristics are not equivalent in untreated and detergent-extracted preparations [1, 2]. Our present experiments also indicate that when untreated or detergent-extracted microsomal preparations of (Na^+ + K^+)-ATPase are spin labelled with M 12-NSE, the differences in the spectra which are obtained reflect the variations in the pretreatment of the membranes. Fig. 1 shows a representative sample of the different types of spectra obtained from a variety of enzyme preparations.

Similar to our findings with dimyristoyl phosphatidylcholine liposomes, in the presence of a suspension of ox brain microsomes, M 12-NSE will diffuse rapidly from the glass surface into the microsomal membrane. The shortest

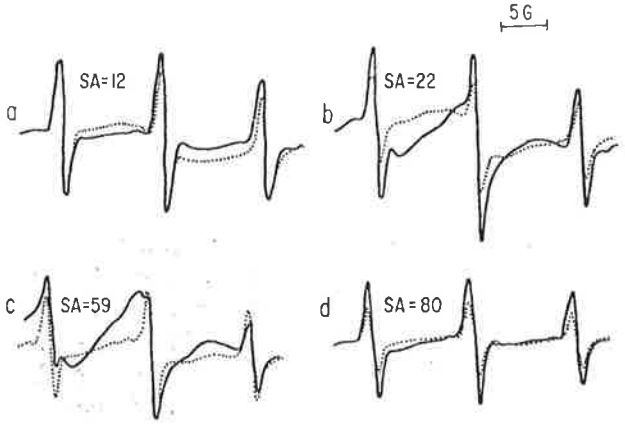

Fig. 1. M 12-NSE in ox brain microsomes. Untreated ox brain microsomes (a,b,c) and after 2 min extraction with 0.1% sodium deoxycholate in the presence of 2 mM ATP and 80 mM NaCl (d) were labelled in sample tubes containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed for at least 1 h prior to spectral examination. Two membrane concentrations from each of the four enzyme preparations were examined. The specific activities (S.A.) of the preparations were, respectively 12, 22, 59 and 80 μmol P_i/mg protein per h as shown on the figure. The membrane concentrations in μg protein/sample were as follows, in each set of data the lower concentration is given as the broken line: a, 1.25 (. . . . . .) and 2.5 (———); b, 4.0 (· · · · · ·) and 16.0 (———); c, 6.6 (· · · · · ·) and 21.0 (———); d, 2.6 (· · · · · ·) and 25.5 (———).

exposure time that was examined was 2 min at 37°C, after which time no further diffusion occurred. In Fig. 1a, a 2-fold difference in membrane concentration is compared. In these experiments both spectra have three sharp peaks equally spaced and of similar intensity, not unlike the isotropic spectrum of a rapidly tumbling nitroxide label in a solvent of low viscosity [6]. This type of spectrum, for which rotational correlation times can readily be determined [14—16], is characteristic of relatively unrestricted labels in the membrane. The lines in the two spectra have very similar intensities, but the low membrane concentration has some distortion of the base line due to residual M 12-NSE on the surface of the sample tube. In Fig. 1b, a 4-fold difference in concentration of membrane protein is compared. Here again the two spectra are similar in appearance and resemble the isotropic spectrum described above. There is, however, a difference in the two spectra. A broad absorption, particularly between the low-field and mid-field peaks, is apparent in the spectrum at the higher membrane concentration. This phenomenon is only observed at the lower probe : membrane ratio ($1.2 \cdot 10^{-4}$ μmol M 12-NSE : 1 μg protein) which suggests that the increase in membrane concentration (16 μg protein/sample) is responsible for the alterations in spectral line shape.

Fig. 1c shows a pair of spectra obtained with a 3-fold difference in membrane concentration. Although this concentration difference is intermediate to the two previous pairs of spectra, the divergence in spectral line shape is by far the greatest. At the lower membrane concentration (6.6 μg protein) the spectrum is typically isotropic, but when the membrane concentration in the sample is raised to 21.3 μg protein the spectrum is no longer isotropic. The form of this spectrum suggests the presence of two probe-binding sites, one of which is highly mobile (isotropic spectrum) and one where the labels are restricted (broad spectrum). Finally, in Fig. 1d we show a pair of spectra that were obtained by spin-labelling microsomes that had been extracted with sodium deoxycholate but in the presence of sodium and ATP [2]. The line shapes of both spectra are typically isotropic despite the fact that the membrane concentration of the two samples differs by an order of magnitude.

It is clear that the observed spectral variations are markedly influenced by membrane concentration. However, if the first three pairs of spectra in Fig. 1 are examined collectively, it is apparent that in addition to the membrane concentration, the specific activity of the enzyme preparations also influences the spectrum. In addition, the spectra shown in Fig. 1d indicate that treatment of the microsomes with detergents also produces changes in the spectral shape.

The effect of membrane (protein) concentration on the spectrum

To further characterize the effects of membrane concentration on spectral type we have examined the spectra of samples containing a fixed amount of probe (2 nmol) and varying amounts of membrane from a single enzyme preparation. A selection of these spectra is shown in Fig. 2. Although the spectra again vary with the membrane concentration, they can be grouped into three categories. At the low membrane concentrations the spectrum is a typical isotropic spectrum characteristic of unrestricted labels. At the very high membrane concentrations, the spectrum is considerably broadened and now resembles the line shape of the spin-labelled dimyristoyl phosphatidylcholine lipo-

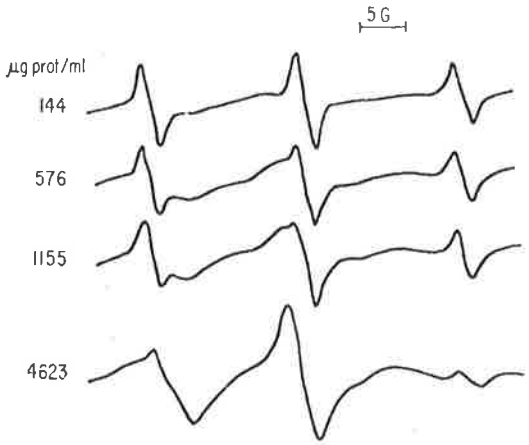

Fig. 2. The effects of membrane concentrations (μg protein) on the spectrum of microsomes labelled with M 12-NSE. Increasing concentrations of untreated ox brain microsomes were added to sample tubes each containing 2 nmol of M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. Note the change in spectrum from sharp to broad as the concentration of the membrane increases.

somes. At intermediate membrane concentrations, the shape of the spectrum is a superposition of the spectra at the extremes, with the relative intensities being determined by the membrane concentration. Thus at low membrane concentrations, the probe binds to sites that permit greater motional freedom, whereas at high membrane concentrations a site that restricts the motion of the nitroxyl is preferred. We define these two spectra as "unrestricted" and "restricted", respectively. The exact ratio of probe : membrane at which a restricted spectrum is obtained is constant for any one microsomal preparation, but may vary from one preparation to another. This latter variation bears some correlation to the degree of purification and specific activity of the enzyme and further evidence for this claim is provided later.

Spectra which are intermediate to the two limiting types described above, are a separate group constituting a mixture of two spectral types. They show the three peaks which are characteristic of the three sharp line spectrum and also display evidence of intermediate peaks. At present these mixed spectra cannot be characterized further. Presumably they arise if both restricted and unrestricted labels contribute to the spectrum.

The effect of 2 mM ascorbic acid on the two sites was also examined. For the unrestricted labels, 50% reduction in peak height intensity had occurred within the first 10 min. This is twice as long as the rate observed for the reduction of M 12-NSE when present as a solution in methanol. In contrast, after 90 min the peak height intensity for restricted spectra had decreased by less than 10%. This differential reduction by ascorbic acid has been reported by others and is believed to reflect the availability of the nitroxyl group for reduction [11—13].

The effect of enzyme purification on the spectrum

The ESR data described above strongly suggest a correlation between enzyme purity and the ESR spectrum. Furthermore, we have previously shown

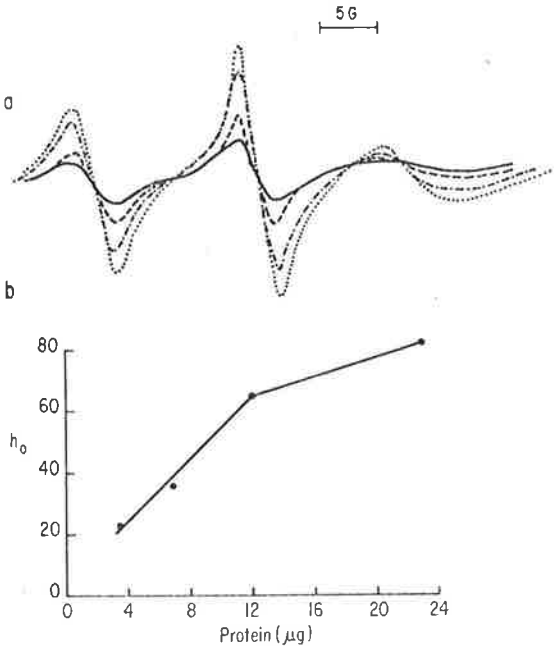

Fig. 3. The effects of membrane concentration after enzyme purification. Increasing concentrations of sodium dodecyl sulfate-extracted ox brain microsomes were added to sample tubes each containing 2 nmol M 12-NSE. Diffusion of the probe was allowed to proceed as described for Fig. 1. (a) Spectral comparison of samples containing 22.8 (· · · · · ·), 11.9 (· — · — ·), 6.9 (- - - - - -) and 3.4 (———) μg protein per sample tube. (b) Plot of mid-field peak height (h_0) vs. protein concentration.

that ox brain microsomes when exposed to low concentrations of sodium dodecyl sulfate in the presence of ATP will undergo dramatic increases in (Na^+ + K^+)-ATPase activity [2]. Thus we have examined the spectra of sodium dodecyl sulfate-treated enzyme preparations. Fig. 3a shows the results obtained from a more active preparation (158 μmol P_i/mg protein per h) than those found after treatment with sodium deoxycholate. Four different membrane concentrations were labelled with a constant amount of 2 nmol of M 12-NSE. Although the amount of membrane added was varied from 3.4 to 23 μg protein, it is quite clear that the spectra are all of the restricted type. This supports the notion that high activity enzyme preparations have a greater proportion of restricted sites than do untreated preparations of lower biochemical activity. If the peak height is plotted against membrane concentration, Fig. 3b, a linear plot is obtained for the first three points. This linearity is good evidence for homogeneous labelling in this system and also for the loss of unrestricted probe-binding sites in sodium dodecyl sulfate-treated microsomes. The highest membrane concentration shows a flattening of the curve, presumably because the amount of M 12-NSE probe is now the limiting factor. Three sodium dodecyl sulfate-treated preparations with elevated specific activities (158, 144 and 142 μmol P_i/mg per h) were spin labelled and examined. In all cases the restricted spectrum was observed with no evidence of mixed or unrestricted spectra.

Discussion

We have described a method which permits the spin labelling of biological membranes by diffusion of the spin probe from the surface of the glass sample tube. By using M 12-NSE, "liquid lines" are avoided because the solubility of this label in an aqueous medium is very low. This method permits the use of minimal amounts of spin probe, and also avoids contamination of the sample with organic solvents.

Liposomes of dimyristoyl phosphatidylcholine or extracted membrane lipids, demonstrate the characteristic behaviour of a typical nitroxyl spin label intercalated into a lipid bilayer. The spectrum was typical of a nitroxide restricted to motion within a cone described by the methylene chain [23]. Saturation of the liposome with spin label did not alter the line shape of the spectrum. Thus the bilayers of liposomes made with dimyristoyl phosphatidylcholine or with lipid extracts of microsomes are homogeneous with respect to binding sites for M 12-NSE.

Conversely, when ox brain microsomes are spin labelled with M 12-NSE a variety of spectra may result. At high probe : membrane ratios the spectrum is isotropic, resembling that of a nitroxide tumbling freely in solution. Presumably such a spectrum must arise from nitroxides residing at unrestricted sites. At the low probe : membrane ratios the spectrum becomes broadened and now bears similarity to that of spin-labelled liposomes. In this situation the nitroxide is in a location that partially restricts its motion. These two limiting cases are quite distinct and are best interpreted as originating from spin probes at two independent sites, an unrestricted motion site, and a restricted motion site. The spin labelling of the two sites with M 12-NSE is clearly influenced by the probe : membrane ratio, and the evidence suggests that although the restricted sites are fewer in number they possess greater affinity for the M 12-NSE.

The effect of membrane concentration on the spectrum varies from one enzyme preparation to another, and this variation can be correlated with the degree of purification of the membrane-bound $(Na^+ + K^+)$-ATPase. As the enzyme is purified the activity increases and this results in a loss of unrestricted sites with a corresponding increase of restricted sites. However, it is possible to obtain a restricted spectrum from a preparation with low specific activity, if the probe : membrane ratio is greatly reduced. Presumably an unrestricted spectrum does have a weak underlying component from restricted sites.

The spectra of sodium deoxycholate-extracted microsomes are influenced by membrane concentration, and in this respect are qualitatively similar to those of untreated microsomes. Brief exposure to sodium deoxycholate in the presence of ATP and Na^+ resulted in an elevation of the specific activity of the enzyme, apparently without a marked reduction in the number of unrestricted sites. By contrast, extraction with sodium dodecyl sulfate resulted in an increase in specific activity which was accompanied by a decrease in unrestricted sites. Presumably, under the conditions employed by us, this detergent interacts with proteins and also with lipids, as has been previously reported [24]. Preliminary examination shows a marked increase in lipid : protein ratios after extraction with sodium dodecyl sulfate compared to either untreated or sodium deoxycholate-extracted preparations.

The following summary characterizes the two sites that we have defined as unrestricted and restricted for the non-covalent binding of M 12-NSE to ox brain microsomes. (1) Two types of spectra may be obtained when ox brain microsomes are spin labelled with M 12-NSE; (2) These two spectral types are due to two different binding sites; (3) These two binding sites differ in their affinity for the spin probe; (4) The low affinity site is more abundant in crude preparations and is easily removed. The high affinity sites increase in concentration as the enzyme undergoes purification; (5) The two sites are quite distinct in their sensitivity to ascorbic acid, the low affinity site showing a considerably greater rate of reduction by this agent.

Since the reporter molecule M 12-NSE is a lipid probe, it is a reasonable assumption that the two sites reflect two different lipid domains. Although specific localization is not possible at present some comment is warranted. The domain of the unrestricted site, if it is in the core of the bilayer is a region of very great fluidity. Using the method of Kivelson [15], we have calculated tumbling times which range from $1.4 \cdot 10^{-10}$ s for a typical isotropic spectrum to $1.9 \cdot 10^{-10}$ s for an isotropic spectrum with distinct evidence of restricted labelling (i.e. presence of secondary peaks). These values are similar to the tumbling times we determined for the water-soluble stearic acid probe 5-NS in 0.25 M sucrose buffer; $1.2 \cdot 10^{-10}$ s. A lipid domain in the core of a membrane with similar viscosity to 0.25 M sucrose does not seem likely. However, typical isotropic spectra such as those described in this work, have been described for the lipid core of a smectic bilayer in the liquid crystalline state [16]. An alternative location would be the membrane-water interface. Such a position would comply with the rapid reduction of the spin label by ascorbic acid, but would require a bending of the polymethylene chain as described by Cadenhead et al. [25].

An intramembrane domain for the restricted sites is a definite possibility because of their similarity to the probe binding sites of liposomes. The correlation between restricted sites and specific activity of $(Na^+ + K^+)$-ATPase implies a lipid specificity for the enzyme protein, similar to the "liquid clustering" reported by Lee et al. [26]. Presumably the $(Na^+ + K^+)$-ATPase macromolecule, when present in ox brain microsomes is capable of selective aggregation of a phospholipid cluster of its own preference, not unlike the lipid selectivity displayed by the $(Mg^{2+} + Ca^{2+})$-ATPase macromolecule from sarcoplasmic reticulum [29].

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VARIATION IN SENSITIVITY OF THE CARDIAC GLYCOSIDE RECEPTOR CHARACTERISTICS OF ($Na^+ + K^+$)-ATPase TO LIPOLYSIS AND TEMPERATURE

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Summary

1. The rate of binding of [^{3}H]ouabain to untreated membrane preparations of ($Na^+ + K^+$)-ATPase is a temperature-dependent process displaying a thermal transition close to 25°C. The apparent energies of activation which can be calculated above and below this transition are similar to, but not identical with, those previously reported for activation of the enzyme by cations.

2. Treatment of the enzyme preparation with detergents or lipolysis with phospholipase A eliminates the thermal transition resulting in linear Arrhenius plots.

3. The number of sites available for [^{3}H]ouabain binding is not temperature dependent as the amount of [^{3}H]ouabain bound at equilibrium is not changed between 10 and 37°C.

4. Treatment of the enzyme with phospholipase A results in time-dependent changes in the number of binding sites for [^{3}H]ouabain at equilibrium.

5. Treatment of the membrane enzyme preparations with detergents reveals additional [^{3}H]ouabain binding sites which are extremely sensitive to lipolysis with phospholipase A.

6. There are a number of [^{3}H]ouabain binding sites which remain resistant to lipolysis by phospholipase A in either untreated or detergent-treated membrane preparations.

7. It is suggested that [^{3}H]ouabain binding sites exist in the membrane in at least two different environments, one of which is resistant the other sensitive to attack by phospholipase A.

Introduction

There is a continuing interest in the mechanism of action of the cardiac glycosides as this potent group of pharmacologic agents has been in continuous

therapeutic use since the pioneering work of Withering in 1785 [1]. For example, since it was first suggested a decade ago that ouabain blocks cation transport by inhibiting the turnover of a phosphorylated intermediate of $(Na^+ + K^+)$-ATPase [2—4], there have been numerous studies of the interaction of that cardiac glycoside with particulate preparations of $(Na^+ + K^+)$-ATPase obtained from a wide variety of tissues and species [5—7]. In confirmation of the earlier reports of Schwartz and his colleagues [8,9], Erdmann and Schoner [10,11] have recently produced strong evidence that the characteristics of [^{3}H]ouabain binding to membrane preparations of $(Na^+ + K^+)$-ATPase reflect the properties of a cardiac glycoside receptor.

Studies of the effect of temperature upon both cation activation and ouabain inhibition of $(Na^+ + K^+)$-ATPase in our laboratory suggest that the lipid membranes containing this enzyme exert a differential effect at the sites of cation activation and cardiac glycoside inhibition [12,13]. This paper describes a series of experiments in which this possibility is explored more directly by examining the effect of temperature upon the binding of [^{3}H]ouabain to preparations of $(Na^+ + K^+)$-ATPase following a variety of treatments known to influence membrane lipids.

Materials and Methods

Enzyme preparation. Ouabain-sensitive $(Na^+ + K^+)$-activated ATPase (EC 3.6.1.3) was prepared from both fresh or frozen beef brain by the general procedure of Charnock and Post [14]. Homogenization was carried out at 4°C in the medium described previously [12] using either (a) four strokes of a teflon-glass homogenizer, or (b) one 10 s pulse in a Polytron Homogenizer fitted with a PT-20 generator and operated at setting 8. After removal of the cellular debris by centrifugation at 1000 × *g* for 15 min in a refrigerated Sorvall RC2-B centrifuge fitted with a SS34 rotor, mitochondrial particles were removed by centrifuging at 9000 × *g* for 20 min. A "heavy microsomal" pellet was then isolated by centrifugation at 46000 × *g* for 30 min. This pellet was washed twice by resuspension and sedimentation in buffer (20 mM Tris · HCl/1 mM EDTA at pH 7.6). The washed pellets were resuspended in this buffer and stored at —20°C after rapid freezing in liquid N_2. The protein content of these microsomal suspensions, which was determined by the method of Lowry et al. [15], ranged from 2 to 8 mg per ml.

Membrane enzyme preparations obtained by both method a and b were treated with 0.1% deoxycholic acid for 10 min at 4°C; with 0.1% deoxycholic acid in the presence of 3 mM ATP for 30 min at 30°C; with 0.05% deoxycholic acid and 2 mM ATP in the presence of 5 mM $MgSO_4$ and 80 mM NaCl for 5 min at 30°C; and with 0.1% sodium dodecyl sulfate plus 4 mM ATP for 30—60 min at 30°C as indicated in the text. After each of these treatments the detergent-extracted membranes were sedimented at 46000 × *g* for 60—120 min and the pellets washed twice by resuspension in buffer.

The procedures for treatment of the enzyme preparations with phospholipase A and subsequent reconstitution was phosphatidylserine were adopted from Imai and Sato [16] and have been described previously [12]. The ratio of phospholipase A to enzyme preparation were determined by preliminary

experiments which monitored the extent of lipolysis by continuous titration of free fatty acid release. Maximum lipolysis usually occurred within 10 min of addition of phospholipase A. Further addition of phospholipase A did not result in further liberation of free fatty acid. Usually this procedure yielded a product having a specific ouabain-sensitive ATPase activity from 20 to 30% of the untreated control preparation. Variations from this time of lipolysis are described in the text.

[^{3}H]Ouabain binding studies. Binding studies were performed in an incubation medium of 100 mM glycylglycine, 2 mM $MgSO_4$, 80 mM NaCl, 2 mM ATP and 0.2 mM H_4EDTA adjusted to pH 7.6 with 1 M Tris/base. Except for some preliminary experiments which are discussed later in the text, the final concentration of [^{3}H]ouabain was $5 \cdot 10^{-7}$ M. The specific radioactivity of the [^{3}H]-ouabain was maintained between 300 and 400 dpm per pmol ouabain.

The bound [^{3}H]ouabain was determined by a rapid Millipore filtration method similar to that employed by others [17,18]. At rapid intervals (as short as 5 s at higher temperatures) 1-ml aliquots were removed from the incubation vessel and filtered on 0.8 μm Millipore filters. Protein retention was virtually complete under all experimental conditions employed, as analysis of filtrates revealed no detectable protein. Assay for ATPase activity in the filtrates also failed to reveal any enzyme activity.

To negate non-specific drug binding to the filters, they were prewashed with 2 ml of a wash solution whose composition was identical to the binding medium but without ATP or radioactivity. The temperature of the binding medium and its respective wash solution was identical. After the initial filtration step, two washes of 2 ml each ensured removal of unbound drug. Increasing the number of washes did not reduce the levels of [^{3}H]ouabain bound.

1-ml aliquots of the incubation mixture were removed for binding analysis at various times after the addition of the enzyme preparations. Binding times were taken to the time the aliquot was applied to the filter. At 37° C the aliquots were removed at 5-s intervals; at lower temperatures the intervals were increased until at 9° C the whole operation took about 250 s. However, at all temperatures samples were removed at much longer intervals (up to 60 min), so that equilibrium levels of [^{3}H]ouabain could be obtained.

Rates of [^{3}H]ouabain binding were determined from the slopes of regression analyses of the levels of bound drug, using the Olivetti program No. 681009. Assays were always in duplicate or more. Individual assays varied less than ±5%, and the mean values were reproducible upon repeated assay of samples stored at −20° C.

The filters were dried in air, disintegrated in 1 ml of methanol and dissolved in 10 ml of 13.5% (v/v) toluene-dioxane fluor and counted to 3% error in a Beckmann LS-100 liquid scintillator.

ATPase activity measurement. All enzyme preparations used in the [^{3}H]-ouabain binding study were routinely assayed for both ouabain-sensitive and ouabain-insensitive ATPase activity under similar conditions to the binding studies.

Enzyme activity was measured by a coupled optical assay system using a Gilford 2400 recording spectrophotometer equipped with a jacketed ethylene glycol/water constant temperature bath [9,19,20]. The temperature of the

spectrophotometer cell was controlled to ±0.2°C. Reactions were performed in 100 mM glycylglycine (pH 7.6) containing 2 mM $MgSO_4$, 80 mM NaCl, 20 mM KCl with 250 mM sucrose added to prevent protein settling. The assay ingredients included 3.14 mM phosphoenolpyruvate (sodium salt) and 64 units of pyruvate kinase, 19 units lactate dehydrogenase and 0.2 mg NADH in a final volume of 3 ml. The enzyme preparation was added to the reaction cuvette and allowed to thermally equilibrate before the reaction was initiated by the addition of ATP to a final concentration of 0.4 mM. Monitoring of NADH oxidation at 340 nm commenced immediately after mixing of the cuvette contents. Mg^{2+}-ATPase activity was followed in the presence of 0.4 mM ouabain, and was subtracted from the total ATPase activity obtained in the presence of Mg^{2+}, Na^+ and K^+ to give the ouabain-sensitive ($Na^+ + K^+$)-ATPase activity.

Determination of activation energies. The effect of temperature on ouabain-sensitive ($Na^+ + K^+$)-dependent ATPase activity, and on the rate of [^{3}H]ouabain binding to these enzyme preparations was determined by a procedure described previously [21]. The data which could be displayed as Arrhenius plots, were further analyzed by the technique of Bogartz [22] for fitting either a single or two intersecting lines, utilizing an APL/360 computer program developed in this laboratory [21]. This analysis yields values for both the apparent activation energies and the critical temperature of the system.

Materials. ATP (disodium salt), glycylglycine, L-histidine (free base), bee venom phospholipase A, phosphoenolpyruvate (sodium salt), pyruvate kinase, lactate dehydrogenase and NADH were all obtained from the Sigma Chemical Co.; [^{3}H]ouabain from New England Nuclear, sucrose (ANALAR grade) from British Drug House Ltd., (Chemical Division); and phosphatidylserine (bovine brain) from Serdary Research Laboratories (Montreal, Quebec).

Results

Preliminary experiments

Although it is well known that two different sets of experimental conditions lead to optimal binding of [^{3}H]ouabain to ($Na^+ + K^+$)-ATPase [20,23—27], we chose to examine [^{3}H]ouabain binding under those conditions which had been previously shown to lead to optimal enzyme phosphorylation, i.e. the presence of ATP, Mg^{2+} and Na^+ [3,4,7,28—30]. In addition, Erdmann and Schoner [10, 11,31] have reported that both maximum drug receptor occupancy and inhibition of ($Na^+ + K^+$)-ATPase activity occur at a ouabain concentration very near $5 \cdot 10^{-7}$ M; although many other workers have shown that the K_i for ouabain inhibition of ($Na^+ + K^+$)-ATPase activity is $5 \cdot 10^{-6}$ M [2,29,30].

We therefore commenced our study by examining [^{3}H]ouabain binding under these reported "optimising" conditions. From preliminary experiments at 37° C we found that in agreement with the reports of Taniguchi and Iida [32, 33], the rate of [^{3}H]ouabain binding only remains linear for about the first 30 s of the experiment; that at this temperature an equilibrium level of bound ouabain is established after about 2 min of incubation; and that this level remains constant thereafter. In addition the binding of [^{3}H]ouabain in the absence of ATP and other activating ligands is negligible compared to that in the presence of both ATP and Na^+. However, in contrast to the findings of Erd-

mann and Schoner [11], we found that the maximum rate of binding was not achieved at $5 \cdot 10^{-7}$ M [^{3}H]ouabain. With ouabain concentrations higher than $1 \cdot 10^{-6}$ M, the rate of drug binding was too fast to be measured experimentally, even by our rapid filtration method, since at 37° C equilibrium levels of binding were reached within 5 s of protein addition. This effect of drug concentration was apparent over the range of enzyme protein concentrations examined (90—240 μg protein/ml). We therefore continued to examine the rate of ouabain binding at a concentration of $5 \cdot 10^{-7}$ M which, although not maximal was technically convenient.

Effect of temperature and lipolysis on the rate of ouabain binding

The effect of temperature upon the rate of [^{3}H]ouabain binding was examined in experiments described in Fig. 1 where it can be seen that the rate of drug binding decreases with decreasing temperature. It is also evident that the linearity of the rate of binding was maintained under the conditions of our experiments. The mean rates of [^{3}H]ouabain binding that can be obtained from these experiments are typical of the values which were used to construct the Arrhenius plots described later in this paper.

All studies of the rate of [^{3}H]ouabain binding were conducted within the linear time period of drug uptake.

In Fig. 2 we give the results of more than 40 experiments using ($Na^+ + K^+$)-

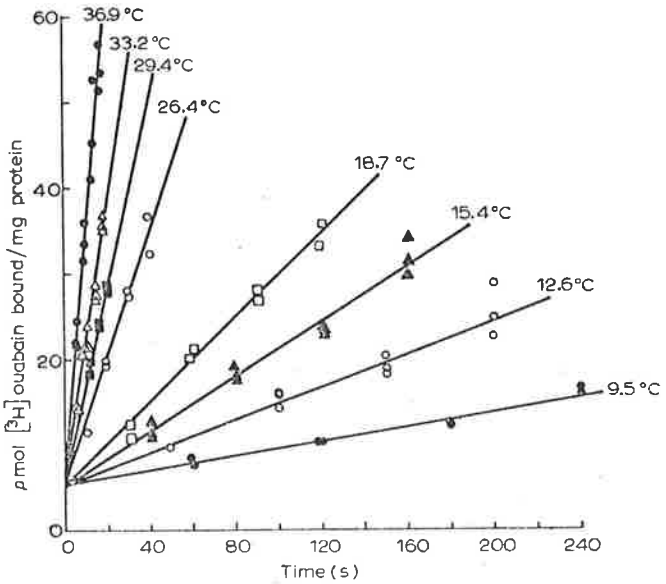

Fig. 1. The effect of temperature on the binding of [^{3}H]ouabain to an untreated membrane preparation of ($Na^+ + K^+$)-ATPase. The enzyme preparation had a specific activity of 16.5 μmol P_i/mg protein per h; 82% of the total ATPase activity of the preparation was inhibited by 0.4 mM ouabain. The protein concentration was 107 μg/ml. The rate of [^{3}H]ouabain binding was determined from the slope of the line obtained at each experimental temperature, using the Olivetti programme No. 681009. The mean initial rates determined in this experiment were from 9.5 to 36.9° C, respectively: 0.05, 0.08, 0.13, 0.20, 0.59, 0.89, 1.24 and 1.95 pmol [^{3}H]ouabain/mg protein per s. Standard error of the means of each temperature were always <10%. Assays were in duplicate or triplicate.

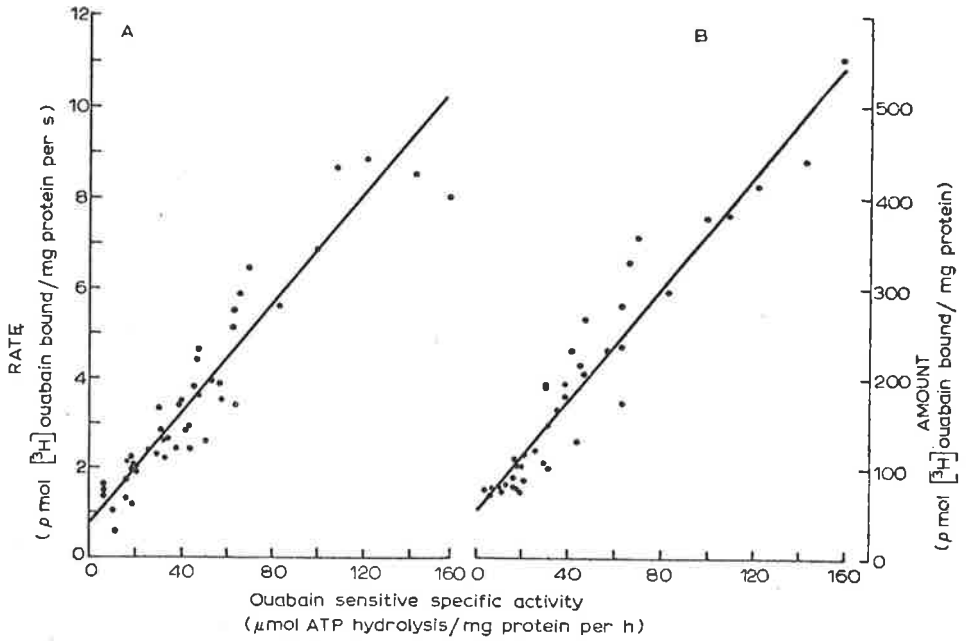

Fig. 2. Relationship of the specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase with the rate of [^{3}H]-ouabain binding (Panel A) and the amount of [^{3}H]ouabain bound at equilibrium (Panel B). All experiments were at 37°C. Enzyme preparations of widely different specific activity were obtained from untreated preparations and following the various detergent extractions with deoxycholic acid and sodium dodecyl sulfate described under Materials and Methods. Rates were determined from aliquots taken during the first 30 s after the addition of the enzyme. Each point represents a single enzyme preparation, assays were in duplicate or triplicate. Amounts of [^{3}H]ouabain binding at equilibrium were determined after 10 min incubation at 37°C and are the means ± S.E. of duplicate assays.

ATPase preparations of widely variable specific activity obtained from both untreated and detergent-treated preparations. The relationship between the rate of [^{3}H]ouabain bound at 37°C and the specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase activity at this temperature is shown in Panel A. It is clear that the rate of [^{3}H]ouabain binding increases with increasing specific activity of these preparations. The correlation shown is significant at the 0.05 level or better. Panel B gives the relationship between the amount of [^{3}H]ouabain bound at equilibrium and the specific activity of ($Na^+ + K^+$)-ATPase at 37°C. It is again evident that a good correlation is obtained between these latter parameters with significance at the 0.05 level or better. Because our data was obtained from both control and detergent-treated preparations, our findings both confirm and extend the earlier reports of Erdmann and Schoner [10,11].

From the information obtained in these experiments we were able to examine the effect of temperature upon [^{3}H]ouabain binding under conditions of ligand, drug and enzyme protein concentration shown to give rates of binding which were as close to the initial rates as we were able to determine by our procedure.

The experimental data obtained from a series of experiments with untreated enzyme preparations and after detergent extractions with deoxycholic acid under various conditions was first displayed as Arrhenius plots and then values

for the apparent energies of activation above and below the transition temperature were calculated [21,22]. The numerical values for these parameters are given in Table I which shows that in four experiments using untreated beef brain enzymes prepared by limited teflon-glass homogenization (Method a), it is possible to construct a non-linear Arrhenius plot for [^{3}H]ouabain binding which can be described by two straight lines intersecting at a transition temperature (T) of 25.4 ± 2.0°C. Calculation of the mean apparent energies of activation above ($E_{a,I}$) and below ($E_{a,II}$) the critical temperature yields values of 18.5 ± 2.1 and 29.1 ± 1.3 kcal/mol, respectively. Comparison of these mean values by the paired "t" test indicates statistical significance at the 0.01 level.

The mean data from another group of six experiments using untreated beef brain enzymes but prepared by Polytron disintegration (Method b) also yields a non-linear Arrhenius plot with an identical value for the transition temperature of 24.6 ± 1.40°C. Although the mean values for $E_{a,I}$ and $E_{a,II}$ which are derived from this plot (20.9 ± 0.33 and 25.7 ± 0.86 kcal/mol, respectively) are not as widely different from each other as are those seen in the previous group, comparison demonstrates statistical significance at the 0.001 level.

Mild detergent extraction of these two apparently similar enzyme preparations with 0.1% deoxycholic acid at 4°C for 10 min reveals some differences in these materials. With enzyme preparations prepared by method a, there are some obvious quantitative changes following extraction, as the mean values for $E_{a,I}$ and $E_{a,II}$ are now 11.7 ± 1.36 and 21.8 ± 2.2 kcal/mol, respectively. The thermal transition in the Arrhenius plot of [^{3}H]ouabain binding is still apparent, although the mean transition temperature is now somewhat higher than before. (T, 29.0 ± 1.3°C). The non-linearity of the temperature dependence of [^{3}H]ouabain binding has remained unchanged.

On the other hand, when enzyme preparations prepared by method b were extracted with deoxycholic acid it was found that the mean value for $E_{a,I}$ (20.1 ± 1.35 kcal/mol) was no longer significantly different ($P > 0.40$) from the mean value for $E_{a,II}$ (22.1 ± 1.59 kcal/mol). That is this mild detergent extraction of these beef brain preparations has given a product which no longer clearly displays a marked thermal transition for [^{3}H]ouabain binding. Preparations obtained by the presumably less disruptive method a are more resistent to alteration by this form of detergent treatment than are preparations obtained by method b.

In addition, Table I also shows the results obtained by treatment of beef brain enzymes (prepared by method b) with deoxycholic acid at higher temperatures as well as treatment with sodium dodecyl sulfate or phospholipase A. All these more vigorous procedures yield data which can best be described by linear Arrhenius plots, as the Bogartz [22] analysis does not reveal either statistically significant differences in the activation energies which could be calculated for $E_{a,I}$ and $E_{a,II}$, or meaningful values for the transition temperatures. It is of considerable interest that incubation of the enzyme preparation with phosphatidylserine after treatment with phospholipase A did not produce a non-linear temperature dependence for [^{3}H]ouabain binding. Clearly both more vigorous extraction of (Na^+ + K^+)-ATPase with detergents and treatment with phospholipase A gives ratios of $E_{a,I}$: $E_{a,II}$ which are close to unity, that is, plots which are best described as linear.

TABLE I

APPARENT ACTIVATION ENERGIES CALCULATED FROM THE RATE OF [^{3}H]OUABAIN BINDING TO VARIOUS ($Na^+ + K^+$)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I}$ ± S.E. (kcal/mol)	$E_{a,II}$ ± S.E. (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	T ± S.E. (°C)	$E_{a,I}$ vs. $E_{a,II}$ P
Untreated	a	4	18.5 ± 2.10	29.1 ± 1.30	0.64	25.4 ± 2.00	<0.01
Deoxycholic acid (4°C)	a	7	11.7 ± 1.36	21.8 ± 2.20	0.54	29.0 ± 1.30	<0.01
Untreated	b	6	20.9 ± 0.33	25.7 ± 0.86	0.81	24.6 ± 1.40	< 0.001
Deoxycholic acid (4°C)	b	3	20.1 ± 1.35	22.1 ± 1.59	0.91	27.2 ± 8.26	>0.40
Deoxycholic acid + ATP (30°C)	b	6	20.4 ± 0.56	21.9 ± 0.37	0.93	43.7 ± 3.06	>0.05
Deoxycholic acid + ATP + Mg^{2+} + Na^+ (30°C)	b	5	22.8 ± 0.37	21.5 ± 1.79	1.06	9.5 ± 2.25	>0.50
Sodium dodecyl sulfate + ATP (30°C)	b	3	21.9 ± 1.84	21.4 ± 1.06	1.02	3.15 ± 3.41	>0.80
Deoxycholic acid (4°C) + phospholipase A *	b	3	26.5 ± 2.43	25.8 ± 2.31	1.03	−27.0 ± 12.4	>0.80
Deoxycholic acid (4°C) + phospholipase A * + phosphatidylserine	b	2	22.2 ± 0.80	24.3 ± 6.25	0.91	40.2 ± 36.7	>0.70

* 5 units phospholipase A for 5 min at 37°C.

TABLE II

APPARENT ENERGIES OF ACTIVATION CALCULATED FROM THE RATE OF ATP HYDROLYSIS BY OUABAIN-SENSITIVE ($Na^+ + K^+$)-ATPase PREPARATIONS AT DIFFERENT TEMPERATURES

	Method	n	$E_{a,I}$ ± S.E. (kcal/mol)	$E_{a,II}$ ± S.E. (kcal/mol)	$\frac{E_{a,I}}{E_{a,II}}$	T ± S.E. (°C)	$E_{a,I}$ vs. $E_{a,II}$ P
Untreated	a	9	18.1 ± 1.10	37.0 ± 3.40	0.49	20.0 ± 1.00	<0.001
Deoxycholic acid (4°C)	a	6	21.8 ± 0.51	49.2 ± 3.90	0.44	17.8 ± 0.80	<0.001
Untreated	b	4	14.6 ± 1.95	29.8 ± 0.30	0.49	20.9 ± 1.31	<0.001
Deoxylcholic acid (4°C)	b	4	19.7 ± 1.89	41.8 ± 3.08	0.47	17.9 ± 0.93	<0.001
Deoxycholic acid + ATP (30°C)	b	5	16.4 ± 0.80	45.4 ± 5.40	0.36	16.6 ± 1.83	<0.001
Deoxycholic acid + ATP + Mg^{2+} + Na^+ (30°C)	b	3	15.1 ± 0.93	32.8 ± 5.30	0.46	19.2 ± 1.55	<0.05
Sodium dodecyl sulfate + ATP (30°C)	b	3	13.6 ± 1.68	30.2 ± 1.20	0.45	20.0 ± 1.27	<0.01
Deoxycholic acid (4°C) + phospholipase A *	b	3	26.9 ± 1.30	20.3 ± 4.20	1.32	30.8 ± 5.00	<0.20
Deoxycholic acid (4°C) + phospholipase A * + phosphatidylserine	b	2	17.6 ± 2.10	35.9 ± 3.45	0.49	22.7 ± 2.40	<0.05

* 5 units phospholipase A for 5 min at 37°C.

By contrast, Table II gives the data for the temperature dependence of ATP hydrolysis by these enzyme preparations which were obtained in paired experiments. As we have demonstrated previously [12,13], there is a marked non-linear response to temperature with all untreated and treated preparations of (Na^+ + K^+)-ATPase except following lipolysis with phospholipase A. Again we have been able to demonstrate that this effect of phospholipase A on the rate of ATP hydrolysis can be overcome by reconstitution of the treated enzyme preparations with phosphatidylserine [12,13]. However, under conditions of our experiments (3 mg phosphatidylserine/mg protein at 37°C for 10 min) reconstitution of phospholipase A-treated enzyme preparations with phosphatidylserine only restored the non-linearity to the Arrhenius plots for ATP hydrolysis and not to those for [^{3}H]ouabain binding (cf. Tables I and II). Nevertheless, it should be noted that the ratio of $E_{a,I}$: $E_{a,II}$ which is obtained for [^{3}H]ouabain binding after treatment with phosphatidylserine shows a tendency towards non-linearity.

We can conclude from these studies of the effect of temperature on the rate of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase that this characteristic of the drug-receptor interaction is lipid dependent, and is more susceptible to change by detergent extraction than is the hydrolysis of the substrate by the enzyme. Apparently membrane lipids are involved in both processes but our preliminary attempts at reconstitution with phosphatidylserine imply that different membrane lipids are associated with these different characteristics of the enzyme receptor system.

Effect of temperature and lipolysis on the amount of ouabain binding

The effect of temperature on the amount of [^{3}H]ouabain which was bound to the enzyme preparation at equilibrium was also examined. The results of a study using untreated enzyme preparations is given in Table III. At true equilibrium temperature does not affect the amount of drug which can be bound; that is, temperature does not alter the number of ouabain receptors available for binding.

TABLE III

EQUILIBRIUM LEVELS OF OUABAIN BOUND TO BEEF BRAIN (Na^+ + K^+)-ATPase AT VARIOUS TEMPERATURES

Binding reactions were started by the addition of protein to a final concentration of 225 μg/ml and terminated by filtration after 30 min, except for the lowest temperature * where equilibrium was not reached until 60 min after protein addition. The concentration of ouabain was $5 \cdot 10^{-7}$ M in all experiments. The values given are the means ± S.D. of four experiments.

Temperature (°C)	[^{3}H]ouabain bound pmol/mg protein ± S.D.
36.8	87 ± 5.7
33	90.6 ± 2.3
29.3	85 ± 2.5
26.5	94.6 ± 5.2
18.5	94.3 ± 5.2
15.3	89 ± 3.6
12	85 ± 4.8
9.3	93.2 ± 4.2 *

However, it is possible to alter the number of ouabain binding sites under certain experimental conditions. For example, although mild treatment of the enzyme preparations with deoxycholic acid at 4°C only leads to a relatively small increase in the specific activity of the enzyme and in the amount of [^{3}H]-ouabain bound at equilibrium, more pronounced detergent treatment with either deoxycholic acid or sodium dodecyl sulfate at 30°C in the presence of ATP produces a marked increase in both enzyme specific activity and the amount of [^{3}H]ouabain bound at equilibrium (Table IV). Apparently, additional binding sites are exposed after detergent extraction, indicating that lipid-lipid or lipid-protein interactions previously prevented ready access of [^{3}H]-ouabain to a potential binding site. It is important to note from the data in Fig. 2 that the increase in [^{3}H]ouabain binding which follows treatment with detergents is proportional to the increase in ouabain-sensitive ($Na^+ + K^+$)-ATPase which also occurs after this treatment.

In contrast to the increase in the amount of [^{3}H]ouabain bound after detergent extraction, Taniguchi and Iida [32,33] have reported that treatment of the enzyme with phospholipase A resulted in a loss of activity and a reduction in the initial rate of [^{3}H]ouabain binding. These workers also reported that the "binding capacities" of the ouabain binding site showed no remarkable change as a consequence of treatment with phospholipase A.

In our experiments we found that the effects of phospholipase A varied with both the time of lipolysis and with pretreatment of the enzyme with detergents. For example, if untreated enzyme preparations are exposed to phospholipase A for only brief periods there is a biphasic effect. This is shown by an initial increase in specific activity and drug binding which is followed by a reduction in both these parameters to near control levels after about 10 min of exposure. This can be seen in Fig. 3 where treatment with 20 units of phospholipase A was followed for 40 min. Under these conditions it is clear that the action of phospholipase A does not result in a marked reduction in the amount of [^{3}H]ouabain bound to ($Na^+ + K^+$)-ATPase.

On the other hand, if the enzyme preparations were first extracted with deoxycholic acid at 4°C, the biphasic effect of phospholipase A was lost. This is shown in Fig. 4 where it can be seen that while the initial levels of both ouabain-sensitive ($Na^+ + K^+$)-ATPase activity and [^{3}H]ouabain binding are

TABLE IV

COMPARISON OF THE SPECIFIC ACTIVITY AND EQUILIBRIUM LEVELS OF [^{3}H]OUABAIN BOUND TO ($Na^+ + K^+$)-ATPase AFTER VARIOUS TREATMENTS WITH DETERGENTS

Specific activity given as μmol ATP hydrolysis/mg protein per h at 37°C. Equilibrium level given as pmol [^{3}H]ouabain bound/mg protein.

Treatment	*n*	Specific activity (mean ± S.E.)	Equilibrium level (mean ± S.E.)
None	14	15.2 ± 2.5	90.5 ± 6.1
Deoxycholic acid at 4°C	16	31.6 ± 3.2	146.5 ± 12.7
Deoxycholic acid + ATP at 30°C	3	96.9 ± 18.0	342 ± 55
Deoxycholic acid + ATP + Mg^{2+} + Na^+ at 30°C	7	60.0 ± 4.8	295 ± 26
Sodium dodecyl sulfate + ATP at 30°C	3	153 ± 17	457 ± 51

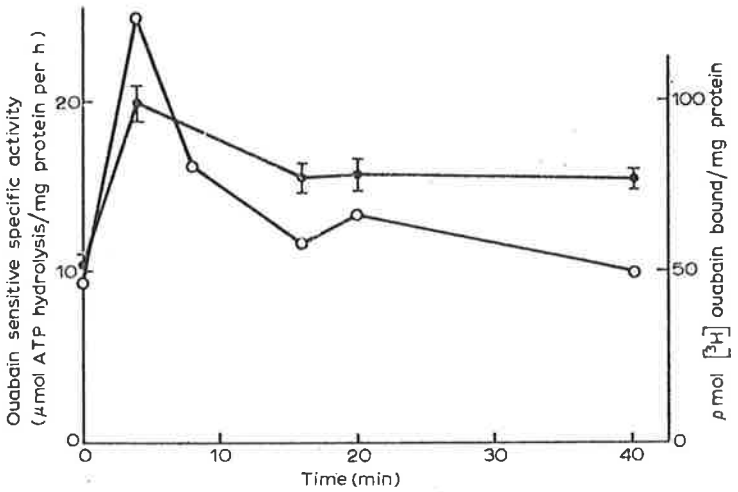

Fig. 3. Effect of progressive treatment at 37°C with 20 units of bee venom phospholipase A/mg protein on the specific activity and binding of [^{3}H]ouabain at equilibrium to otherwise untreated membrane preparations of (Na^+ + K^+)-ATPase. •, [^{3}H]ouabain binding given as pmol/mg protein, values are the means ± S.E. of triplicate determinations from 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P_i/mg protein per h at 37°C; values are means of duplicate assays. 1 unit of phospholipase A hydrolyses 1 μmol of L-α-lecithin to lysolecithin and fatty acid per min at pH 8.5 at 37°C.

much higher than those of untreated enzyme preparations, the action of phospholipase A is to produce an immediate and progressive decline in the specific activity of the preparations which was accompanied by a fall in the amount of [^{3}H]ouabain bound to these preparations under equilibrium conditions. However, under these conditions the amount of [^{3}H]ouabain bound to the prepa-

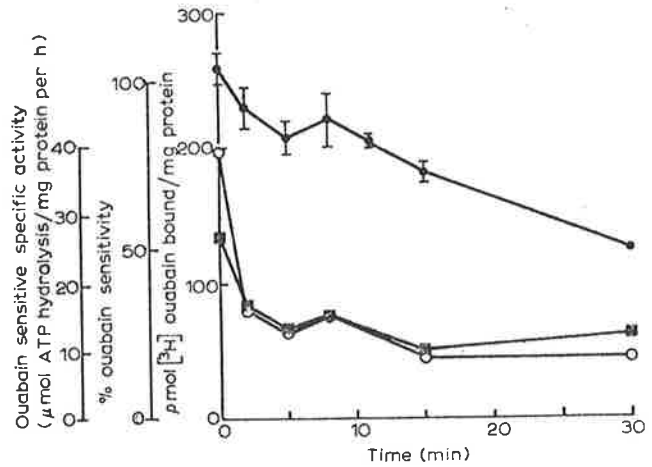

Fig. 4. Effect of progressive treatment at 37°C with 5 units of bee venom phospholipase A/mg enzyme protein on the specific activity and binding of [^{3}H]ouabain at equilibrium to membrane preparations of (Na^+ + K^+)-ATPase after 10 min extraction with 0.1% deoxycholic acid at 4°C. •, [^{3}H]ouabain binding gives as pmol/mg protein, values are means ± S.E. of four times 1-ml aliquots taken after 5 min incubation at 37°C; ○, specific activity given as μmol P_i/mg protein per h at 37°C; values are means of duplicate assays; ▪, ouabain sensitivity expressed as percent of the total ATPase activity of the preparation assayed in duplicate ± 0.4 mM ouabain in the medium as described under Materials and Methods. Note the 4-fold reduction in concentration of phospholipase A from the experiments described in Fig. 3.

TABLE V

EFFECT OF PHOSPHOLIPASE A ON SPECIFIC ACTIVITY, RATE AND EQUILIBRIUM BINDING OF [^{3}H] OUABAIN TO ($Na^+ + K^+$)-ATPase

Specific activity given as μmol ATP hydrolysis/mg protein per h at 37°C. Rate [^{3}H]ouabain binding given as pmol [^{3}H]ouabain bound/mg protein per s at 37°C. Equilibrium level [^{3}H]ouabain binding given as pmol [^{3}H]ouabain bound/mg protein.

Experiment	Control			Phospholipase A		
	Specific Activity	Rate [^{3}H]ouabain binding	Equilibrium level [^{3}H]ouabain binding	Specific activity	Rate [^{3}H]ouabain binding	Equilibrium level [^{3}H]ouabain binding
a	38.7	3.40	194	5.4	2.57	157
b	44.4	3.80	216	8.3	2.28	155
c	29.4	3.34	192	4.6	1.97	133
d	19.6	1.92	102	2.4	0.80	70.7
e	17.6	2.25	102	2.09	0.92	77.4
f	15.8	2.13	79.1	2.05	0.83	57.3
g	18.9	2.07	74.0	2.12	0.59	50.6
Mean ± S.E.	26.3 ± 4.3	2.70 ± 0.29	137 ± 23	3.85 ± 0.90	1.42 ± 0.31	100 ± 17.6
Inhibition (%)	Nil	Nil	Nil	86.2 ± 1.0	50.8 ± 6.1	27.3 ± 1.7

ration does not fall in parallel with the reduction of enzyme activity. This is shown by the data from seven experiments given in Table V, where the reduction in specific activity which occurs after 5 min incubation with 5 units of phospholipase A is compared to the rate and amount of [^{3}H]ouabain binding. Under these conditions the activity of the enzyme has been reduced to 14% of the mean control values, the mean rate of [^{3}H]ouabain binding is 49% of the controls while the mean amount of [^{3}H]ouabain which can be bound at equilibrium is only reduced to 73% of the control level. That is both the biochemical activity of the enzyme and the function of the binding sites are more susceptible to lipolysis with phospholipase A than are the number of sites available.

Discussion

Although there are many reports in the literature concerning the binding of cardiac glycosides to particulate preparations of ($Na^+ + K^+$)-ATPase, much of the data has been conflicting [24—27,31—39]. One contributing factor to this conflict has been the variable methodology employed by the numerous investigators. The studies of Erdmann and Schoner [10,11] have confirmed that under conditions of optimal phosphorylation of the enzyme viz. the presence of ATP, Mg^{2+} and Na^+, binding experiments conducted at equilibrium reflect the number of available cardiac glycoside binding sites per unit mass of enzyme, while very short term rate studies reflect the affinity of these sites rather than the number of sites available. When these distinctions are borne in mind some of the apparent differences reported in the literature can be resolved.

In recent years several laboratories have demonstrated that treatment of membrane preparations of ($Na^+ + K^+$)-ATPase with lipases destroys the thermal transitions which can be observed in the hydrolysis of ATP by this enzyme [12, 13,33,40—42], as well as reduces the rate of [^{3}H]ouabain binding to the preparations in the presence of various combinations of ligands [32,36].

In the present work we have shown that the temperature dependence of the rate of [^{3}H]ouabain binding to untreated enzyme preparations also displays a non-linear relationship with a break in Arrhenius plots at about 25° C. However, the differences in activation energy for this process which can be calculated above and below the critical temperature are somewhat less than the differences in the activation energies for ATP hydrolysis which were determined in paired experiments.

Our conclusions concerning the non-linear effect of temperature upon the rate of [^{3}H]ouabain binding do not agree with those of Siegel and Josephson [23] or Schwartz and his colleagues [35,43]. The experiments by Siegel and Josephson [23] were conducted after 15 min of incubation which under our conditions, would neither reflect the initial rates nor the equilibrium levels of drug binding, particularly in the lower temperature range examined.

Conversely, the experiments reported by Schwartz and his colleagues [35,43] should provide data reflecting the affinity of cardiac glycoside binding sites similar to that observed in our experiments. Although in the experiments of Wallick and Schwartz [43] there are an insufficient number of data points for the construction of Arrhenius plots suitable for the Bogartz [22] analysis

we employ, inspection of their data reveals that their results might also be described as non-linear. Recalculation of their data suggests that like our own findings, the differences in activation energies for [^{3}H]ouabain binding above and below the critical temperature are not as marked as is the case with ATP hydrolysis by these membrane enzyme preparations. Presumably, both our findings and those of Wallick and Schwartz [43] indicate that the binding of [^{3}H]ouabain to (Na^+ + K^+)-ATPase is less influenced by the physical state of the membrane lipids than is activation of the system by cations [12,13,44].

However, it is apparent that lipids do play at least some part in the temperature dependence of [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase as detergent treatment of the preparations resulted in the loss of the discontinuity in temperature dependence and the observation of linear Arrhenius plots. The comparative ease with which detergents remove this effect suggests that the lipids which influence [^{3}H]ouabain binding are not as closely associated with the membrane protein as are those which are responsible for modulation of the temperature dependence of ATP hydrolysis [12,13]. Perhaps the former is a so-called "bulk" lipid phenomenon while the latter is more likely to be the immobilized boundary lipid type recently described by Metcalfe and his colleagues [45,46] for Ca^{2+}-ATPase. In the limited number of experiments we attempted here, it is of considerable interest that although reconstitution of phospholipase A-treated preparations with phosphatidylserine is able to regain the characteristic non-linear temperature dependence for ATP hydrolysis that we have reported before [12,13], that is not necessarily the case for [^{3}H]-ouabain binding. As only one set of experimental conditions for reconstitution with phosphatidylserine was employed here (3 mg phosphatidylserine/mg protein at 37° C for 10 min) it is clear that these attempts at reconstitution will have to be extended before the role of phosphatidylserine in [^{3}H]ouabain binding to (Na^+ + K^+)-ATPase can be decided.

Nevertheless, the partial dependence of [^{3}H]ouabain binding upon lipids is demonstrated by the effects of phospholipase A treatment upon the amount of [^{3}H]ouabain bound to the enzyme preparation at equilibrium at 37° C. With untreated enzyme preparations there is an initial increase in the amount of drug binding which falls off after about 5 min of incubation, but does not fall below control levels during further treatment with phospholipase A. In these experiments the pattern of specific activity of ouabain-sensitive (Na^+ + K^+)-ATPase activity parallels the changes in [^{3}H]ouabain binding which were observed.

On the other hand, if the membrane enzyme preparations are first treated with the detergents deoxycholic acid or sodium dodecyl sulfate at 30° C (in the presence of ATP) there is a very marked increase in both the specific activity of ouabain-sensitive (Na^+ + K^+)-ATPase and the amount of [^{3}H]ouabain bound to the enzyme. This increase in [^{3}H]ouabain binding is now very sensitive to treatment with phospholipase A and is reduced by about 50% in 15 min incubation with a much reduced concentration of lipase.

It should be noted that the final levels of ouabain-sensitive ATPase activity and [^{3}H]ouabain binding which are reached after treatment with phospholipase A are very similar whether detergent-treated or untreated control enzyme was used; apparently this residual enzyme activity and its associated [^{3}H]ouabain binding sites are very resistant to attack by this lipase. In agreement with the

earlier work of Taniguchi and Iida [36], and the recent conclusions of Hansen [47], which were published while this manuscript was in preparation, we also conclude that there are two different types of [^{3}H]ouabain binding sites available in membrane preparations of ($Na^+ + K^+$)-ATPase. Because of the marked sensitivity of only one of these sites to lipolysis with phospholipase A following detergent extraction, it seems very likely that not only are these sites located in different regions of the membrane but that only one site is closely associated with a phospholipid component of the membrane. Whether these sites are equally accessible for cardiac glycoside binding in situ, or whether either of these sites represents a more pharmacologically active "receptor" cannot be determined from the present work, but it is possible that variations in membrane lipids will exert differential effects on the drug-receptor interactions at these different sites. Whether either of these sites resemble the clinically important cardiac glycoside receptor of cardiac muscle remains uncertain [48—50].

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EFFECTS OF ATP AND MAGNESIUM IONS ON THE FLUORESCENCE OF HARMALA ALKALOIDS. RESTRICTIONS FOR THE USE OF HARMALA ALKALOIDS AS FLUORESCENT PROBES FOR $(Na^+ + K^+)$-ATPase

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SUMMARY

1. Harmine and harmaline were investigated as potentially useful fluorescent inhibitors of $(Na^+ + K^+)$activated ATPase.

2. From spectroscopic measurements both compounds were shown to form 1 : 1 complexes with ATP, the dissociation constants being 0.65 mM and 1.83 mM for harmine and harmaline respectively. Addition of Mg^{2+} and enzyme further affected these equilibria.

3. Although it was possible to demonstrate a competitive effect of harmine at the sodium-loading site of the enzyme, other inhibitory effects, including inhibitions of ouabain binding and the ouabain-insensitive ATPase were found.

4. It was concluded that the harmala alkaloids can inhibit $(Na^+ + K^+)$-activated ATPase in a complex way involving both Na- and ATP-binding sites. This severely limits their usefulness as spectroscopic probes.

INTRODUCTION

The hallucinogenic drugs harmine and harmaline are members of the linear tricyclic group of harmala alkaloids which induce disturbances in behaviour and perception [1, 2]. Recently these compounds have been shown to inhibit the $(Na^+ + K^+)$-ATPase systems of squid retinal axon, rat brain and human erythrocyte membranes, where their mechanism of action is reported to be competitive inhibition at the Na^+-activation site of the system [3, 24]. A similar effect of harmaline as a competitive inhibitor has been reported for Na-dependent amino acid transport in kidney and intestine [4], Na : Ca systems in skeletal muscle* and Na transport in frog skin and crayfish gill [5].

These interesting psychomimetic compounds are spectroscopically active with strong blue fluorescence between 420 and 485 nm. This combination of properties makes these agents potentially useful site-specific fluorescent probes to investigate the

* Lea, T. J., Ashley, C. C. and Ellory, J. C. (1976) submitted for publication.

molecular mechanism of Na-dependent membrane transport processes, and ($Na^+ + K^+$)-ATPase activity in particular.

We have therefore examined the properties of harmine and harmaline both in free solution and when bound to membranes containing ($Na^+ + K^+$)-ATPase activity. Our results suggest that the interaction of these drugs with membrane ATPase systems and their various ligands, is much more complex than has been reported [3, 4, 24] making the interpretation of the fluorescence data more difficult and limiting the usefulness of these agents as membrane probes.

MATERIALS AND METHODS

Membranes rich in ($Na^+ + K^+$)-ATPase activity were prepared from sheep and pig kidney cortex and outer medulla by the procedure recently described by Charnock and Bashford [6]. A membrane preparation of ($Na^+ + K^+$)-ATPase was obtained from crab leg nerve (*Cancer pagurus*) following 30-s disruption in the cold in a Willems Ultra-Turrax mincer (Model TP 18/2 Manufactured by Janke and Kunkel KG) in a medium of 0.25 M sucrose/30 mM histidine/20 mM Tris base/2 mM Na_2 EDTA, adjusted to pH 7.6 by the addition of 1 M HCl. The crude mince was centrifuged at $1500 \times g$ for 10 min to sediment the coarse debris, followed by centrifugation at $12\,000 \times g$ for 30 min to obtain a post-mitochondrial fraction. The supernatant was then centrifuged at $38\,000 \times g$ for 120 min to yield a microsomal fraction containing ($Na^+ + K^+$)-ATPase activity. The kidney enzyme had a specific activity of 1–6 μmol phosphate released/mg protein per h, and was 65–90 % ouabain inhibitable. The crab material gave activities from 2–7 μmol phosphate released/mg protein per h and was 40–70 % ouabain-sensitive Human red cell membranes were obtained by the method of Fortes et al. [7], whose procedure was also used for all determinations of enzyme activity. Protein was determined by the method of Lowry et al. [8], and ($Na^+ + K^+$)-ATPase activity was calculated as the difference in activity in the presence and absence of 0.1 mM ouabain (kidney) or 1 mM (crab), following incubation for 15–30 min in 100 mM NaCl/10 mM KCl/1 mM Na_3ATP/0.5 mM $MgCl_2$/15 mM Tris · HCl pH 7.6 at 37 °C.

Changes in the fluorescence intensity of harmine and harmaline both in solution and after binding to ($Na^+ + K^+$)-ATPase-containing membranes were determined at room temperature using a Hitachi-Perkin Elmer MPF-2A spectrofluorometer. The excitation and emission spectra of both agents were not affected by the catalytic amounts of enzyme protein that were employed in this study.

The binding of [^{3}H]ouabain to human erythrocyte membranes and the determination of *p*-nitrophenylphosphatase activity of sheep kidney ($Na^+ + K^+$)-ATPase-containing preparations were determined by methods described previously [7].

Both harmine and harmaline were obtained from The Sigma Chemical Co. St. Louis, Mo. and were purified by recrystallisation from ethanol.

RESULTS AND DISCUSSION

Harmine fluorescence

The fluorescence excitation and emission spectra of harmine were recorded at room temperature in 10 mM Tris buffer pH 7.4. The excitation spectrum showed a

TABLE I

EFFECT OF ATP ADDITION ON HARMINE FLUORESCENCE

Fluorescence intensity measured at 320 nm excitation and 420 nm emission. Values given have been corrected for ATP addition and volume dilution. At 320 nm the absorbance of 5 μM harmine was 0.086 in the absence of ATP.

ATP (mM)	Corrected fluorescence intensity (%)	% Quenching
Control	78.7	nil
0.025	78.0	1.0
0.049	77.1	2.1
0.074	76.1	3.3
0.098	75.5	4.1
0.147	74.2	5.7
0.195	73.2	7.0
0.243	72.1	8.4
0.290	71.3	9.4
0.336	70.5	10.4
0.383	69.3	12.0
0.429	68.7	12.8
0.474*	68.2	13.4

* The addition of 0.45 mM $MgCl_2$ to this system enhanced the fluorescence intensity (E) by 3.5 %.

maximum of 324 nm and a shoulder at 357 nm. The emission spectrum gave a single maximum at 413 nm. These values were taken from uncorrected machine spectra. None of the peaks were shifted by the addition of the catalytic amounts of enzyme protein used in this study (less than 10 μg protein/ml final concentration).

The fluorescence emission of harmine was quenched 10–20 % by the addition of ATP in normal substrate concentrations (< 2.5 mM). When 350 nm exciting light was employed there was a measurable contribution to the fluorescence emission from the ATP solution. This troublesome artefact was markedly reduced by shifting to the lower 324 nm excitation peak and in all subsequent experiments excitation was with 320 nm light and the emission was measured at 420 nm.

Table I gives the degree of fluorescence quenching measured in a 5 μM solution of harmine upon the serial addition of ATP. In this experiment the degree of quenching reaches a maximum of 13.4 %. The quenching was partially reversed (3.5 %) by the addition of equimolar $Mg\,Cl_2$ to ATP.

The ATP-induced quenching of harmine fluorescence (5 μM) was measured at a number of different concentrations of $Mg\,Cl_2$. Harmine fluorescence was reduced in the presence of 0.05 mM $Mg\,Cl_2$ and abolished in the presence of 0.3 mM $Mg\,Cl_2$. These results obtained with harmine in free solution suggest that a complex interaction between the enzyme inhibitor, the enzyme substrate and the enzyme ligand is possible as well as the interaction of the drug with the Na^+ activation site which has been reported [3, 4, 24].

The mechanism of the fluorescence quenching can be either "static" or "dynamic" [9, 10]. Dynamic quenching is a diffusion controlled process described by the classical Stern-Volmer relation [9–11].

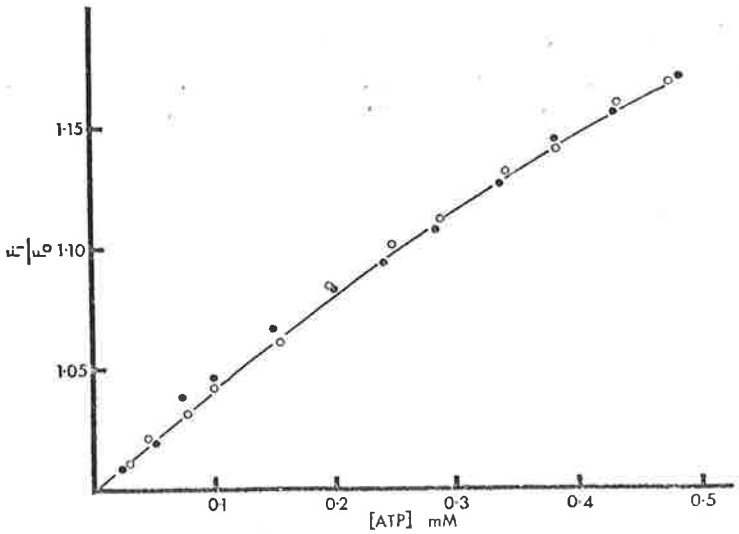

Fig. 1. Stern and Volmer plot for the quenching of harmine fluorescence by ATP. F_1/F_o is the ratio of the fluorescence intensity of free harmine with the intensity in the presence of ATP. Harmine concentration 5 μM in 10 mM Tris pH 7.4 at 25 °C. ATP was added serially as the sodium salt adjusted to pH 7.5 with NaOH. Intensities were all corrected for changes in cuvette volume. ○–○, control in the absence of $MgCl_2$; ●–●, in the presence of crab nerve ($Na^+ + K^+$)-ATPase. The curve approaches a limiting value for F_1/F_o of 1.52.

$F_o/F_1 = 1/1 + K[Q]$ where F_o is the observed fluorescence in the presence of quencher, F_1 the initial fluorescence in the absence of quencher and K the quenching constant. Quenching of this type is characterised by linear plots of F_1/F_o versus $[Q]$ of slope K. In the experiments reported here such plots are non-linear and the value of F_1/F_o tends to a maximum at high concentration of ATP (Fig. 1). This implies that quenching occurs by a static mechanism possibly by the formation of a ground state complex with reduced fluorescence. Support for this type of mechanism is afforded by the observation that the optical density at 320 nm of a 5 μM harmine solution was reduced by the addition of ATP. Under such circumstances the dissociation constant for the harmine/ATP interaction can be calculated from the fluorescence data alone.

The fluorescence enhancement can be defined as $E = F_0/F_1$ where F_o is the observed fluorescence and F_1 the initial fluorescence in the absence of interacting ligand. The limiting enhancement (E_b) is the value of E when all the fluorophore is complexed, and C is the fraction of fluorophore complexed. Now

$$F_o/F_1 = E = 1 - C + C \cdot E_b$$

$$C - E_b \cdot C = 1 - E$$

$$C = \frac{1-E}{1-E_b}$$

A double reciprocal plot of $1/1-E$ versus 1/ATP will yield a value for $1/1-E_b$ at the ordinate intercept. This value can then be used to calculate C, the fraction of fluorophore complexed, at all points on the titration curve. The dissociation constant (K_D)

TABLE II

DISSOCIATION CONSTANT (K_D) OF HARMINE/ATP MEASURED UNDER A VARIETY OF EXPERIMENTAL CONDITIONS

n = number of binding sites per mol of harmine.

Experiment	Conditions	K_D	n
a	Control	0.64±.05	1.09±.06
b	Control	0.69±.05	0.98±.05
c	+Pig $(Na^+ + K^+)$-ATPase	0.79±.10	1.03±.10
d	+Crab $(Na^+ + K^+)$-ATPase	0.75±.13	0.72±.09

for the ATP/harmine interaction and a value for n, the number of binding sites per mol can then be obtained from a graphical solution of the Scatchard relationship [12].

The results of duplicate experiments yielded values for K_D (ATP/harmine) of 0.64 mM±0.05 and 0.69 mM±0.05 with values for n of 1.09±0.06 and 0.98±0.05 respectively. Clearly there is a 1 : 1 harmine/ATP complex formed under these conditions with a dissociation constant near 0.7 mM.

When harmine was bound to ATP in the absence of Mg^{2+} but in the presence of either pig or crab membranes containing $(Na^+ + K^+)$-ATPase there was no significant difference in K_D from the controls although there was apparently some reduction in the value of n for the crab preparation (Table II). However in the presence of Mg^{2+} the interaction becomes complex yielding more than one value for K_D and n (Fig. 2). Such a result could arise if there were more than one site for harmine interaction under these conditions; apparently the presence of enzyme is incidental to this result.

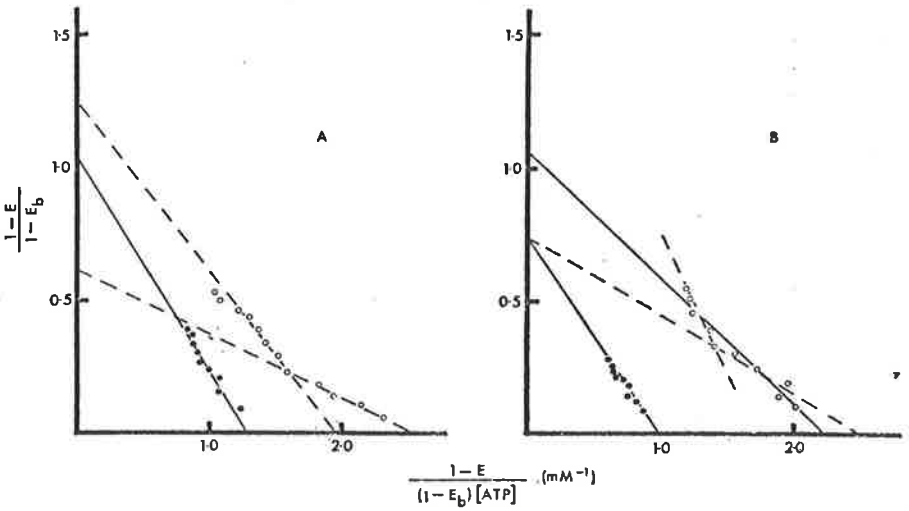

Fig. 2. The effect of Mg^{2+} on the ATP-induced quenching of harmine fluorescence in the presence of $(Na^+ + K^+)$-ATPase-containing membranes. Excitation and emission wavelengths as in Fig. 1. Harmine was 5 μM. 10 mM ATP added as the Tris salt adjusted to pH 7.5 with 0.01 M HCl. Part A: Pig kidney $(Na^+ + K^+)$-ATPase, ●–● control, no Mg^{2+} added; ○–○, 10 mM $MgCl_2$. Part B: Crab nerve $(Na^+ + K^+)$-ATPase, ●–● control, no Mg^{2+} added; ○–○, 10 mM $MgCl_2$.

TABLE III

EFFECT OF ATP ON HARMALINE FLUORESCENCE

Fluorescence intensity measured at 380 nm excitation and 480 nm emission. Values given here have been corrected for ATP addition and volume dilution.

ATP (mM)	Corrected fluorescence intensity (%)	% Quenching
Control	83.4	nil
0.62	76.7	8.0
1.25	73.1	12.4
1.87	70.9	15.0
2.50	68.7	17.6
3.12	67.2	19.4
3.75	66.2	20.6
4.37	65.0	22.1
5.00*	64.2	23.0

* The addition of 5 mM $MgCl_2$ to this system enhanced the fluorescence intensity (E) by 11.2 %.

Similar experiments with 1 μM harmaline, the more saturated analogue of harmine also indicate a marked quenching of fluorescence on the addition of ATP, which could again be partially reversed by the subsequent addition of Mg^{2+} (Table III).

Calculation of the harmaline/ATP dissociation constant in the absence of membrane enzyme gave a value of $K_D = 1.83$ mM with $n = 0.97$. In the presence of

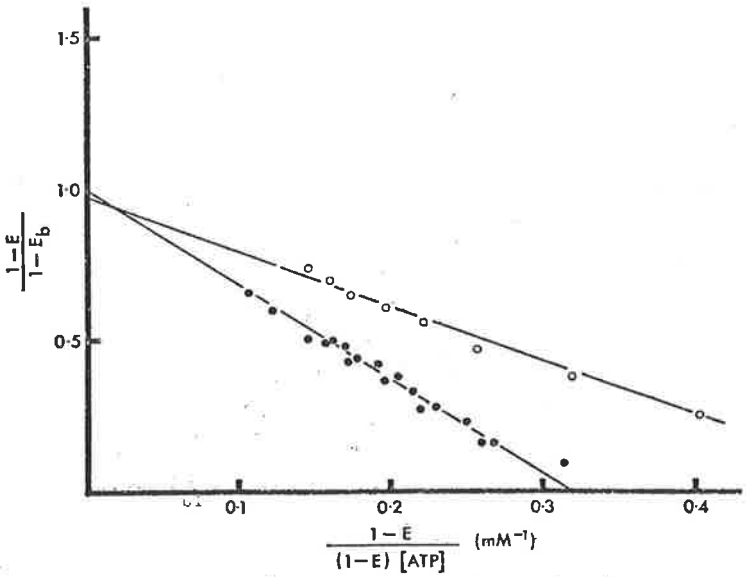

Fig. 3. The effect of $(Na^+ + K^+)$-ATPase membranes on the ATP-induced quenching of harmaline fluorescence. Excitation wavelength 380 nm; emission wavelength 480 nm. Harmaline concentration 1 μM; ATP added as the Tris salt adjusted to pH 7.5 with 0.01 M HCl. ●–●, in the presence of sheep kidney $(Na^+ + K^+)$-ATPase containing membranes; ○–○, control, no enzyme present.

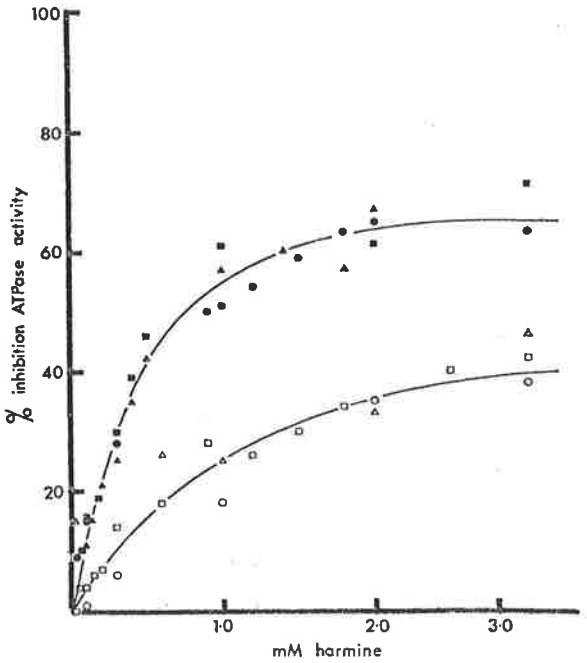

Fig. 4. Concentration dependence of the harmine inhibition of the ATPase activity of sheep kidney membranes. Assay system contained 100 mM Na^+/10 mM K^+/1 mM disodium ATP/0.5 mM $MgCl_2$. 0.1 mM ouabain added where required. Ouabain-sensitive ($Na^+ + K^+$)-ATPase (solid symbols); ouabain-insensitive Mg-ATPase (open symbols). Data are the mean of duplicate assays on three different membrane preparations.

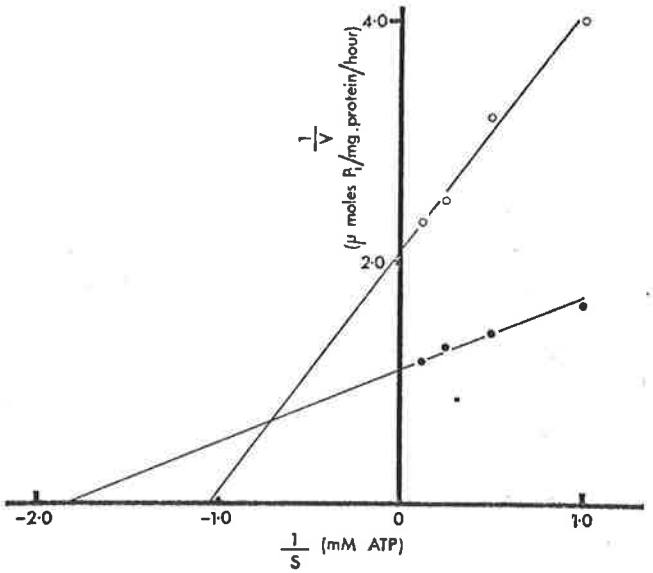

Fig. 5. The effect of harmine on the reaction velocity of sheep kidney ($Na^+ + K^+$)-ATPase. A double reciprocal plot of $1/v$ vs $1/s$. ●–●, control, no harmine added. ○–○, 3 mM harmine. The calculated apparent K_m for ATP in the absence of harmine was 0.54 mM. K_I for harmine was 0.94 mM. Data are the mean of duplicate determinations on a single enzyme preparation.

sheep kidney enzyme the harmaline/ATP dissociation constant was $K_D = 3.1$ mM with $n = 0.99$ (Fig. 3). Thus, although there is still only one binding site per mol the interaction of harmaline with ATP is weaker than that previously found for harmine and ATP.

It is important to note that this decrease in dissociation constant is in agreement with the decrease in inhibitor potency for these agents reported by Canessa et al. [3].

As all these results strongly suggest a direct interaction of both harmine and harmaline with ATP as a potential cause of the inhibition of $(Na^+ + K^+)$-ATPase, or for that matter any other membrane ATPase, a further examination of the mechanism of inhibition of harmine seemed warranted.

A plot of the effect of increasing harmine concentration versus the degree of inhibition of both ouabain-sensitive $(Na^+ + K^+)$-ATPase and ouabain-insensitive Mg^{2+}-ATPase is given in Fig. 4. It is clear that both membrane ATPase systems are inhibited by harmine which in these experiments was able to produce only 65 % inhibition of $(Na^+ + K^+)$-ATPase and 40 % inhibition of Mg^{2+}-ATPase activity at 3 mM drug concentration.

When the effect of harmine on $(Na^+ + K^+)$-ATPase was examined at variable concentrations of ATP, it can be seen from the double reciprocal plots shown in Fig 5 that the intercept is neither on the x nor on the y axis, consistent with some form of mixed inhibition [13].

As Canessa et al. [3] have reported competitive inhibition with Na^+ and harmaline on $(Na^+ + K^+)$-ATPase we therefore re-examined the effect of Na^+ at

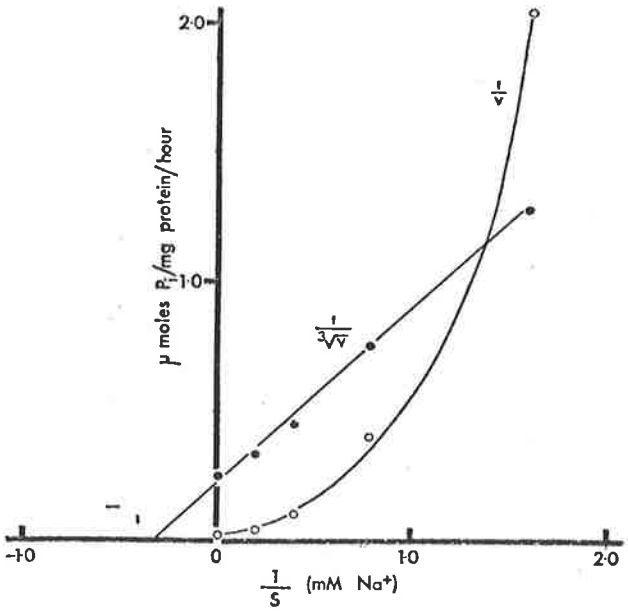

Fig. 6. Comparison of a cubic double reciprocal plot ($1/3\sqrt{v}$ vs. $1/s$) and a double reciprocal plot ($1/v$ vs. $1/s$) of the effect of $[Na^+]$ on the reaction velocity of sheep kidney $(Na^+ + K^+)$-ATPase. Assay system was described under Methods and in Fig. 4. Data are the mean of triplicate determinations on a single enzyme preparation.

fixed concentrations of substrate (Mg^{2+}-ATP) and inhibitor (harmine). Since (Na^+-K^+)-activated ATPase is an asymmetric membrane-bound multisite enzyme, with both external and internal affinities for Na^+ and K^+, kinetic experiments are difficult to interpret unequivocally. Several authors have analysed the Na^+/enzyme interaction in terms of multisite kinetics [14–17]. It now seems generally accepted, however, that results can be well described in terms of three internal Na-loading sites with an affinity for both Na (0.19 mM) and K (9 mM) [18]. Making the cubic reciprocal plot for ouabain-sensitive ATPase activity versus reciprocal Na concentration (Fig. 6) yields a good straight line (in contrast to the simple reciprocal plot which is markedly parabolic). Using this approach harmine can be shown to be behaving as a competitive inhibitor for Na, in confirmation of the results of Canessa et al. [3] (Fig. 7). From five inhibition experiments, the mean value for the x-intercept, representing the apparent K_m for Na but containing a term for K inhibition at the Na site, was 3.26± 0.11 mM for each of three sites. From inhibitor experiments at harmine concentrations from 0.15–1 mM (ATP remaining constant at 2.6 mM), the derived apparent K_i for harmine was 0.28±0.03 mM. Although these values do not represent true kinetic constants they enable us to say that harmine is an effective competitive inhibitor at the Na-loading site.

In order to examine the specificity of harmine inhibition further we examined several of the partial reactions of (Na^++K^+)-ATPase which are believed to separate to some extent the requirements for various ligands [19].

For example, we found that 1 mM harmine was without significant effect upon the K-dependent *p*-nitrophenylphosphatase activity of the kidney enzyme preparation, while at relatively low concentrations it inhibited the binding of [^{3}H]ouabain to

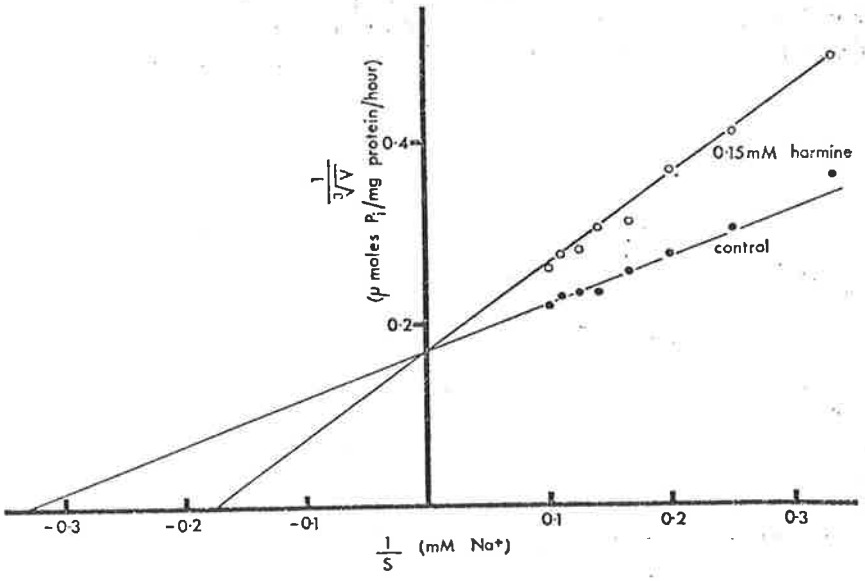

Fig. 7. Cubic reciprocal plot of the effect of [Na^+] on the reaction velocity of pig kidney (Na^++K^+)-ATPase in the presence and absence of harmine. Assay system and results as described under Methods and in Fig. 4 except that [Mg ATP] was 2.6 mM. ●–●, control no harmine added. ○–○, 0.15 mM harmine.

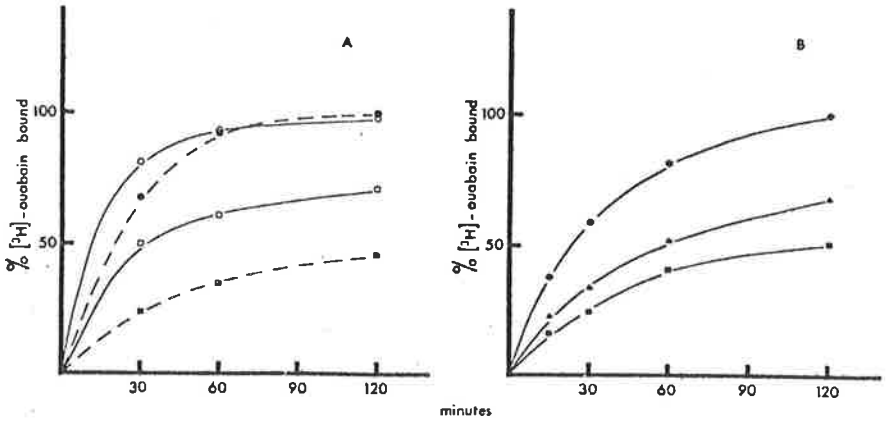

Fig. 8. The effect of harmine on the rate of [^{3}H]ouabain binding to human red cell ghosts. Experimental conditions: 8 nM [^{3}H]ouabain, 100 mM NaCl, 3 mM $MgCl_2$. 10 mM Tris (pH 7.5 at 37 °C), 3 mM Na_3 ATP or 5 mM phosphate where required. Part A: ○–○, ATP, Na^+ and Mg^{2+}; □–□, ATP, Na^+ and Mg^{2+}, plus 1 mM harmine; ●–●, Mg^{2+} and P_i; ■–■, Mg^{2+} and P_i plus 1 mM harmine. Part B: ●–●, Mg^{2+} and P_i control no harmine added; ▲–▲, Mg^{2+} and P_i plus 0.3 mM harmine; ■–■, Mg^{2+} and P_i plus 0.6 mM harmine.

human red cell membranes whether the binding of [^{3}H]ouabain was supported by either ATP and Na^+, or by Mg^{2+} and P_i [20–23]. The results of the [^{3}H]ouabain binding experiments are given in Fig. 8. Thus it is clear that reactions of $(Na^+ + K^+)$-ATPase which superficially do not involve either Na^+ or ATP can also be inhibited by harmine. In other experiments directed towards the specificity of harmine inhibition we re-examined the effect of harmine upon the ouabain-insensitive Mg^{2+}-dependent ATPase of our membrane enzyme preparations, which in the experiments reported

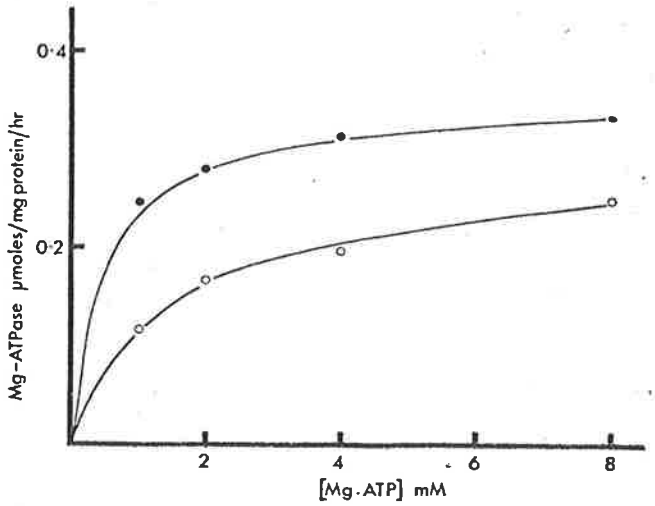

Fig. 9. Effect of harmine on Mg ATPase activity of sheep kidney membranes. Assay conditions as described under Methods and in Fig. 4. 0.1 mM ouabain present in all tubes. ●–●, control, no harmine added; ○–○, 3 mM harmine.

above had already shown considerable inhibition in the presence of 3 mM harmine (cf. Fig. 4).

By varying the concentration of Mg-ATP we could demonstrate that the level of inhibition of Mg-ATPase activity was decreased with increasing substrate concentration (Fig. 9). In view of our earlier findings of the effect of Mg^{2+} on harmine/ATP fluorescence quenching it seems likely that the Mg-ATP interaction is stronger than the harmine/ATP interaction. However, both reactions are probably important in determining the degree of inhibition of these systems. In general it would seem that the very complex interactions of harmine with both the substrate for these reactions (Mg-ATP) and as a competitive inhibitor with the activating ligand (Na^+) would suggest that the inhibitory action of these agents is not confined to competition with Na^+ at the sites of Na^+ activation [3, 4, 24]. This lack of specificity will greatly limit the usefulness of these agents as fluorescent probes in an examination of the molecular mechanism of membrane transport reactions.

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A Fluorescent Probe Study of the Lipid Mobility of Membranes Containing Sodium- and Potassium-Dependent Adenosine Triphosphatase

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SUMMARY

CHARNOCK, JOHN S. & BASHFORD, C. LINDSAY (1975) A fluorescent probe study of the lipid mobility of membranes containing sodium- and potassium-dependent adenosine triphosphatase. *Mol. Pharmacol.*, **11**, 766–774.

The temperature-activity relationship of a membrane ($Na^+ + K^+$)-ATPase preparation [Mg^{2+}-dependent, ouabain-sensitive, ($Na^+ + K^+$)-activated ATP phosphohydrolase, EC 3.6.1.3] obtained from sheep kidney cortex and medulla was determined and found to be very similar to that previously reported for preparations of this enzyme from either rabbit kidney or ox brain. These temperature-activity relationships can be shown as Arrhenius plots which characteristically are nonlinear and have transition temperatures near 22°. Two noncovalently bound fluorescent probes, 12-(9-anthroyl)stearic acid (12-AS) and *N*-phenyl-1-naphthylamine (NPN), were used to label the hydrophobic core of the partially purified membranes rich in ($Na^+ + K^+$)-ATPase. The fluorescence polarization of these probes was determined between 10° and 40°. The rotational relaxation times (ρ) for each probe were then calculated, and secondary plots of reciprocal relaxation time vs. reciprocal temperature were constructed. The plots for membranes labeled with 12-AS and NPN were nonlinear and showed transition temperatures near 22°, in good agreement with the transition temperature of the hydrolytic activity of the enzyme. A similar transition temperature was detected by right-angle light scattering of an unlabeled microsomal preparation of ($Na^+ + K^+$)-ATPase and of an aqueous suspension of liposomes made from a total lipid extract of the enzyme-containing membranes, thus excluding any direct effect of addition of the fluorescent probes to the membranes. The transition temperatures observed under all experimental conditions were very similar. We conclude that the nonlinear temperature-activity relationship of ($Na^+ + K^+$)-ATPase and the nonlinear fluorescence polarization–temperature profile both arise from a temperature-dependent change in the molecular mobility of the membrane lipids in the immediate environment of the probes and the "active center" of the ($Na^+ + K^+$)-ATPase subunits. These changes illustrate the strong cooperative effect between the physical state of the membrane lipids and the functional state of the enzyme protein in this particulate membrane enzyme system, and suggest a powerful modulating effect of membrane lipids in regulating enzyme activity, or drug-receptor interactions more generally.

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[2] Recipient of scholarships from the Medical Research Council (London) and Hertford College, Oxford University.

INTRODUCTION

The mechanism of energy transduction by biological membranes has been of inherent interest for many years, both mitochondrial and plasma membrane systems having been studied extensively (1). In the plasma membrane the majority of mechanisms which have been proposed to describe the transduction process at the molecular level involve conformational changes of membrane components. These changes are usually associated with the activity of membrane ATPases (2–5). Recent work in other systems has focused attention upon the cooperative effect of membrane lipids in modulating membrane protein activities (5, 6) and suggested that changes in the action of membrane-bound enzymes might well be accompanied by changes in the properties of the associated membrane lipids. The use of fluorescent probe techniques to detect changes in membrane lipids may have advantages over such other methods as electron spin resonance labeling, as they are frequently more sensitive (7–9). Vanderkooi (10) and Radda (11) have discussed in detail the use of fluorescent probes to examine changes in fluidity of membrane lipids, and very recently Bashford, Harrison, Radda, and Mehdi (12) used fluorescence polarization measurements to correlate the mobility of lipids in human thyroid membranes with other biological activities of their system. The latter technique is especially useful for detecting changes in the orientation of fluorescent molecules in both rigid and fluid environments (8, 9, 11, 13–15). When coupled with the use of fluorophors which are known to interact with specific areas of the membrane (15, 16) these techniques can be very suitable for detecting changes in fluidity in defined regions of biological membranes.

In the present study we utilized the fluorescent properties of two such compounds, 12-(9-anthroyl)stearic acid and *N*-phenyl-1-naphthylamine, to examine the lipid mobility of membrane preparations of $(Na^+ + K^+)$-ATPase [Mg^{2+}-dependent, ouabain-sensitive, $(Na^+ + K^+)$-activated ATP phosphohydrolase, EC 3.6.1.3] prepared from the cortex and outer medulla of sheep kidney. These probes were used previously to examine lipid behavior in artificial and natural membrane systems, where they are noncovalently bound (10–12, 17). Binding to membranes results in a blue shift and enhancement of their fluorescence emission spectra (7, 10–12, 17). The location of the bound probes is thought to be deep within the hydrocarbon core of the membranes (16); hence they are potentially very suitable agents for detecting changes in the fluidity of the lipids within this region.

Membrane preparations of $(Na^+ + K^+)$-ATPase have been shown to display nonlinear temperature-activity relationships, which might be explained by two different conformational forms of the enzyme above and below the transition temperature of the system. Both these forms of the enzyme would be capable of hydrolyzing substrate ATP in the presence of activating ligands but would have very different energies of activation (18–20). However, it is also well known that modification of the membrane lipids of this system will markedly alter the temperature-activity relationship of the $(Na^+ + K^+)$-ATPase (21, 22). In particular we have described how mild treatment with phospholipase A can abolish the transition temperature previously observed in the system, and the restoration of this effect by subsequent treatment with phosphatidylserine (23, 24). Thus a direct effect of membrane lipids upon $(Na^+ + K^+)$-ATPase may be responsible for the large change in activation energy which occurs in this system, without the primary cause lying in a major conformational change in the enzyme protein. This paper describes experiments designed to study more directly than previously the lipid-protein interactions of $(Na^+ + K^+)$-ATPase by correlating the motional characteristics of the membrane lipids of the system with the temperature-activity relationship of ouabain-sensitive ATP hydrolysis by the enzyme.

METHODS

Preparation of enzyme. The enzyme was prepared from the cortex and outer medulla of sheep kidney by a simple modification of the general procedure of homogeni-

zation and differential centrifugation first described by Charnock and Post (25). Following the separation of both a nuclear and then a mitochondrial fraction, the crude "heavy microsomal" fraction was collected as a pellet by centrifuging the post-mitochondrial supernatant at 35,000 × *g* for 1 h. It was resuspended in 10 mM Tris–1 mM EDTA (pH 7.4) and then diluted with a solution containing 3 mM Na_2ATP, 2 mM EDTA, 50 mM imidazole, and 0.2% sodium deoxycholate (pH 7.4) until the protein to detergent ratio was 1:1. After standing overnight at 4° the enzyme preparation was collected as a pellet by centrifugation at 35,000 × *g* for 2 hr. It was washed by centrifugation through a medium free of both ATP and deoxycholate, collected as a pellet at 35,000 × *g* for 2 hr, and then resuspended in 10 mM Tris–1 mM EDTA (pH 7.4) as before. In some later experiments incubation with 0.1% sodium dodecyl sulfate (60 min at 37°) replaced the overnight treatment with sodium deoxycholate (26). Small aliquots were rapidly frozen in an acetone–Dry Ice mixture for storage at −20° before use. These preparations can not be regarded as quite as highly purified as others recently reported (26–28), but they had a specific activity of 3–10 units/mg of protein (i.e., micromoles of ATP hydrolyzed per minute at 37°). They were more than 80% sensitive to inhibition by 0.1 mM ouabain and could be stored frozen without loss of activity for at least 4 weeks. As such they proved suitable for the type of study reported here.

Temperature-activity relationship of $(Na^+ + K^+)$*-ATPase.* This was determined by the general procedure described previously (19, 20). The assay for phosphate release from ATP was carried out in a buffered medium containing 50 mM glycylglycine–30 mM imidazole, 0.5 mM EDTA, 100 mM NaCl, 10 mM KCl, 2.5 mM Na_2ATP, and 2.5 mM $MgCl_2$ at pH 7.4. When necessary 0.1 mM ouabain was added to the system. Enzyme activity was determined with variable aliquots of enzyme suspension (25–100 μl) for 5-min incubation periods at the required temperature between 5° and 40°. The linear rate of inorganic phosphate release was determined by the method of Hurst (29) over a 4-min period, using a Technicon AutoAnalyzer (30).

Fluorescence polarization. When polarized light falls onto a group of molecules, those molecules with their axes parallel to the plane of polarized light will be preferentially excited. If the excited molecules do not move out of the plane of polarization, the maximum fluorescence will pass through a second polarizer oriented in the same plane. Conversely, if all the excited molecules move out of the plane of polarization, no fluorescence will reach the detector. Hence changes in the intensity of polarized fluorescence can reflect motion of the molecules during their fluorescence lifetime (13). Thus fluorescence polarization is a convenient parameter for following changes in the fluidity of the environment of a fluorophor within a lipid medium. This principle, first proposed by Weber (14, 31), was employed in the construction of a suitable instrument to measure fluorescence polarization by Barratt, Badley, Leslie, Morgan, and Radda (15). Their instrument uses vertically polarized exciting light and dual photomultipliers mounted at right angles to the sample chamber. This arrangement allows the simultaneous measurement of fluorescence at right angles to the direction of excitation, and through a pair of polarizers oriented both vertically (I_{11}) and horizontally (I_1) with respect to the plane of excitation. Continuous recording of the components of the fluorescence emission is thus possible, permitting the automatic computation of the fluorescence polarization p, which is defined (8, 13, 32) as

$$p = \frac{I_{11} - I_1}{I_{11} + I_1}$$

The instrument is also fitted with a temperature-controlled sample compartment, which permits measurement of the fluorescence polarization between 10° and 40°. For each enzyme preparation the measurements were determined in at least quadruplicate. Values for the rotational relaxation time ρ were calculated using the Perrin equation (33) previously described by Weber (14, 31) and more recently used by Bashfold *et al.* (12):

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_0} - \frac{1}{3}\right) \times \left(1 + \frac{3\tau}{\rho}\right)$$

where p is the measured polarization, p_0 is the limiting polarization of a rigidly held, randomly oriented array of fluorophors, and τ is the fluorescence lifetime. The values for p_0 were obtained from the literature (34, 35) while those for τ were measured on an ORTEC nanosecond pulsed fluorometer (12). The latter parameter was assumed to have the same temperature dependence as the fluorescence intensity (I), which was measured directly (32, 36). Preliminary fluorescence intensity and fluorescence spectra measurements were determined on a Hitachi Perkin-Elmer MPF-2A spectrofluorometer. The fluorescent probes 12-AS and NPN[3] were stored as 1 mM stock solutions in methanol in the cold. Sufficient probe to give a workable signal was added to about 100 μg of enzyme protein suspension at room temperature and allowed to equilibrate until the maximum fluorescence enhancement was observed. The final concentration of added probe was usually less than 1 μmol/50 μg of protein (37). The concentration of methanol in the experimental system did not exceed 0.3% (v/v).

As had been found before, the absolute value of ρ can vary slightly between experiments, but the internal consistency of any set of experimental values obtained with a given enzyme preparation is better than 98% (15).

Light scattering. Right-angle light scattering observations were made with an Aminco-Bowman spectrophotofluorometer by setting both the excitation and emission controls at 300 nm, and recording the effect of variable temperature (T) upon relative intensity according to the method described by Bangham, Hill, and Miller (38).

A total lipid extract of our enzyme-enriched membranes was obtained by the solvent extraction method of Nelson (39), and an aqueous preparation of liposomes was prepared according to the procedures of Bangham and his associates (38, 40).

[3] The abbreviations used are: 12-AS, 12-(9-anthroyl)stearic acid; NPN, *N*-phenyl-1-naphthylamine.

RESULTS

The temperature-activity relationship of ouabain-sensitive $(Na^+ + K^+)$-ATPase of sheep kidney is shown as an Arrhenius plot in Fig. 1. There is a sharp discontinuity in the rate of hydrolysis of ATP by this system between 21° and 22°. The apparent energy of activation above this transition temperature (T) is 15.7 kcal/mole, and is significantly less than that determined below this temperature (34.4 kcal/mole). Although these values for the apparent energies of activation above and below the tran-

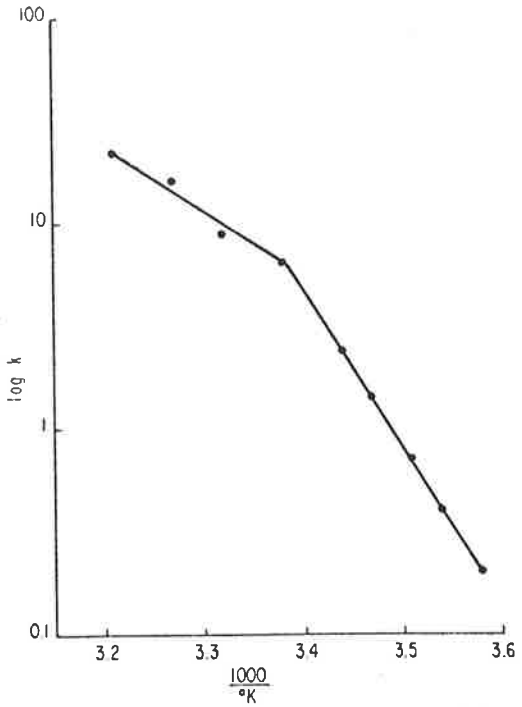

FIG. 1. *Arrhenius plot of ouabain-sensitive $(Na^+ + K^+)$-ATPase activity*

Enzyme preparation (25–100 μl; 0.91 mg of protein per milliliter) was incubated for 5 min at various temperatures in a buffered medium containing 100 mM NaCl, 10 mM KCl, and 2.5 mM MgATP with and without 0.1 mM ouabain. The rate of ATPase activity was obtained by continuously monitoring inorganic phosphate release from ATP at each temperature and then determining the slope obtained between the first and the fifth minute of reaction (30). The difference between the slopes obtained with and without 0.1 mM ouabain was calculated to give ouabain-sensitive $(Na^+ + K^+)$-ATPase activity. The transition temperature (T) is near 22°; the value for the apparent activation energy above this transition temperature is 15.7 kcal/mole, while the value below the transition temperature is 34.4 kcal/mole.

sition temperature are very similar to those reported previously for rabbit kidney and ox brain preparations (20, 23, 24), the transition temperature of 21–22° is slightly higher than that seen previously. Whether this reflects a significant species difference or is due to such environmental factors as season or diet of the experimental animals is not known at present.

The effect of temperature on the fluorescence polarization of (Na^+ + K^+)-ATPase-enriched membranes labeled with 12-AS is shown as mean reciprocal plots of rotational relaxation time ($1000/\rho$) vs. reciprocal temperature ($1000/T$) in Fig. 2. It is clear that there is a marked change in the value of ρ with temperature over the whole range examined. In addition there is a departure from linearity near 22° for all three enzyme preparations. Although the values for ρ vary slightly from preparation to preparation, the data are the means of at least quadruplicate observations at a given temperature and the standard errors are sufficiently small to lie within the points shown. The internal consistency of any set of experiments is very high (15), as is evident from the slopes of the lines, which are very similar for all three enzyme preparations.

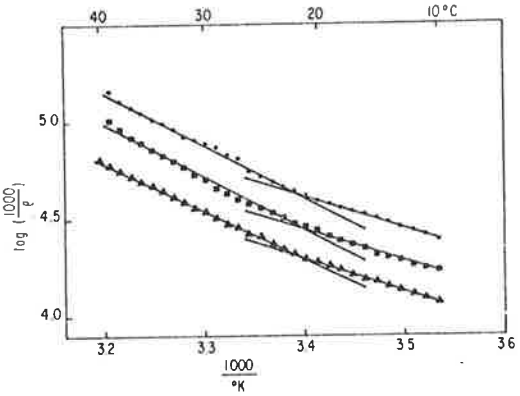

FIG. 2. *Effect of temperature on fluorescence polarization of (Na^+ + K^+)-ATPase labeled with 12-AS*

Either 3 or 10 μl of 1 mM 12-AS in methanol were incubated with varying amounts of enzyme protein suspension for 30 min at room temperature in 3 ml of buffer (10 mM Tris–1 mM EDTA, pH 7.4). The temperature dependence of fluorescence polarization was determined by the method of Bashford and Radda (12, 16) by alternately heating and cooling the sample between 10° and 40°. The fluorescence excitation was at 385 nm; emission, 437 nm (12). ●——●, enzyme treated with sodium deoxycholate, 137 μg of enzyme protein, 3 μl of probe; ■——■, enzyme treated with sodium deoxycholate, 117 μg of enzyme protein, 10 μl of probe; ▲——▲, enzyme treated with sodium dodecyl sulfate, 3.3 μg of enzyme protein, 10 μl of probe. Each point is the mean of at least four observations; the standard errors are small and lie within the experimental points. The slopes for all three experiments are very similar, and the transition temperatures are between 21° and 22°.

A similar result was found when NPN was used as the fluorescent probe. Although some hysteretic effect was found between heating and cooling with this agent, both temperature plots again showed a departure from linearity near 22°, with the value obtained on heating being about 2° higher than that seen on cooling. A double-reciprocal plot of the mean rotational relaxation time ($1000/\rho$) vs. temperature ($1000/T$) is shown for two enzyme preparations in Fig. 3. The data are the means of at least quadruplicate observations, and the standard errors again lie within the points shown. The mean transition temperature is very similar to that found in the hydrolysis of substrate by these preparations and in the fluorescence polarization–temperature profile when the membranes were labeled with 12-AS.

It is significant that these effects of temperature are similar to those previously observed in both human and bacterial membranes labeled with fluorescent probes (12, 41) and in some plant and animal systems labeled with paramagnetic probes (5, 6, 42). However, as the introduction of extrinsic probe molecules into the (Na^+ + K^+)-ATPase system must inevitably perturb it to some extent, we sought to confirm our findings by the use of the less sensitive but nonperturbing light scattering method recently described by Bangham *et al.* (38).

Figure 4 shows the results of an experiment again using a membrane preparation of (Na^+ + K^+)-ATPase obtained from sheep kidney. When a double-reciprocal plot of relative intensity (RI) vs. absolute temperature (T) is constructed from the mean data of three experiments it is clear that the resultant plot is nonlinear and

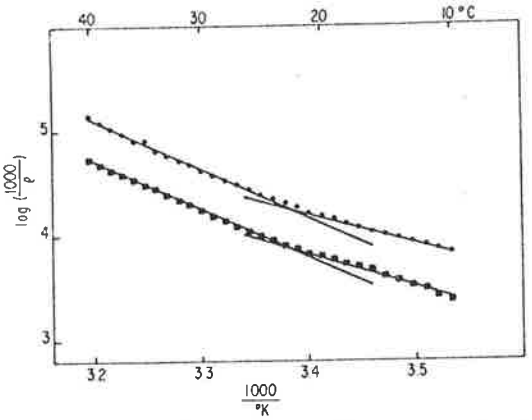

FIG. 3. *Effect of temperature on fluorescence polarization of $(Na^+ + K^+)$-ATPase labeled with NPN*

Either 6 or 10 μl of 1 mM NPN in methanol were incubated with varying amounts of enzyme protein suspension at room temperature for 5 min in 3 ml of buffer (10 mM Tris–1 mM EDTA, pH 7.4). The temperature dependence of fluorescence polarization was determined by the method of Bashford and Radda (12, 16) by alternately heating and cooling the sample between 10° and 40°. The fluorescence excitation was at 340 nm; emission, 407 nm (12). ●——●, enzyme treated with sodium deoxycholate, 137 μg of enzyme protein, 6 μl of probe; ■——■, enzyme treated with sodium deoxycholate, 117 μg of enzyme protein, 10 μl of probe. Each point is the mean of at least four observations; the standard errors are small and lie within the experimental points. The slopes for both experiments are very similar, and the transition temperatures are between 21° and 22°.

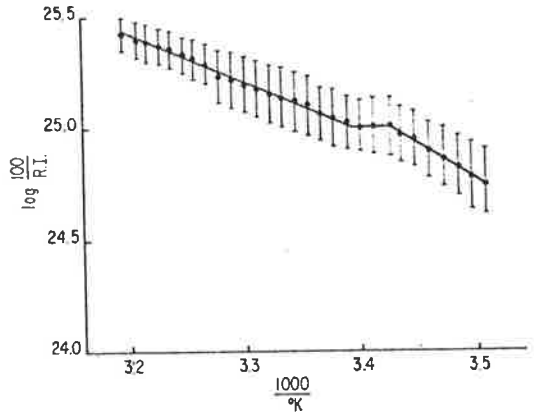

FIG. 4. *Effect of temperature on right-angle light scattering of $(Na^+ + K^+)$-ATPase-enriched membranes*

A sodium deoxycholate-treated membrane suspension (400 μl; 380 μg of protein per milliliter) was diluted to 2 ml in 10 mM Tris–1 mM EDTA buffer at pH 7.4. The percentage relative intensity (R.I.) was measured at 300 nm through an ascending temperature gradient from 10° to 38°. The points shown are the means of three sets of observations. A transition occurs between 18° and 22°. The rates of change in relative intensity are similar above and below the transition.

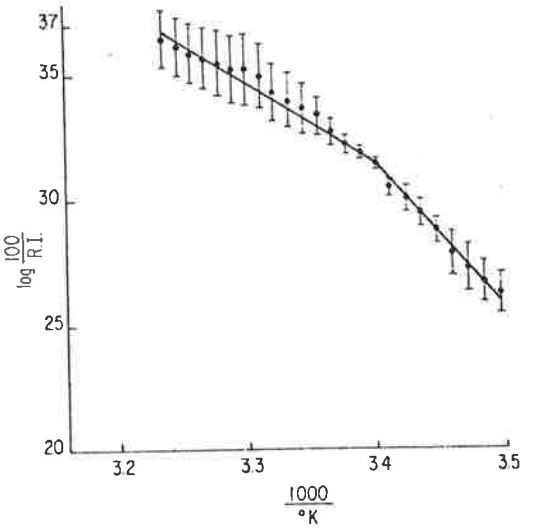

FIG. 5. *Effect of temperature on right-angle light scattering of a liposome preparation from $(Na^+ + K^+)$-ATPase-enriched membranes*

Membrane lipids (2.75 mg) were resuspended in 6 ml of water distilled from alkaline $KMnO_4$. The percentage relative intensity (R.I.) was measured at 300 nm through an ascending temperature gradient from 12° to 36°. The points shown are the means of five observations. The transition temperature is near 21°.

that a temperature-dependent transition occurs between 18° and 22°. The position of this transition is in good agreement with the finding of a similar transition temperature by means of the fluorescent probes and the hydrolytic activity of the enzyme. We believe that this excludes a direct effect of probe addition to membrane lipids as the cause of the temperature transitions observed. If the transition in fluorescence polarization and in light scattering is a reflection of a change in the properties of the membrane lipids per se, it should also be possible to demonstrate this phenomenon in preparations of the isolated lipids. We therefore examined the effect of temperature on the right-angle light scattering of a liposome preparation made from a total lipid extract of our $(Na^+ + K^+)$-ATPase-enriched membrane system (38, 40). The mean data from five experiments are

given in Fig. 5 as a double-reciprocal plot of relative intensity (RI) vs. absolute temperature *(T)*. The data obtained are in excellent agreement with our previous experiments (Figs. 1–4), as there is again a nonlinear response to temperature with a transition point near 21°.

Thus the nonlinear temperature dependence of the hydrolytic activity of (Na^+ + K^+)-ATPase can also be seen in the rotational relaxation time of two fluorescent probes incorporated into the lipids of the supporting membranes, as well as in the light scattering measurements made both in the intact membrane and in liposome preparations derived therefrom.

DISCUSSION

Several previous studies reported from this and other laboratories have demonstrated that untreated membrane preparations of (Na^+ + K^+)-ATPase, prepared from both rabbit kidney and ox brain, possess nonlinear temperature-activity relationships which are characteristically reflected in discontinuous Arrhenius plots. These plots demonstrate transition temperatures close to 20°, and values for the apparent energy of activation for the hydrolysis of substrate ATP are about 3 times greater below the transition temperature than above it (19–22, 43).

Very similar results for a preparation of (Na^+ + K^+)-ATPase from sheep kidney were obtained in the present study, although the transition temperature found here was consistently 1–2° higher than that seen with enzyme preparations examined previously. This small difference may be due to a different source of the enzyme, or perhaps to such factors as the dietary intake or environmental temperature of the experimental animals.

It has been suggested that the very significant increase in the apparent energy of activation of (Na^+ + K^+)-ATPase which occurs below the transition temperature could be brought about by a temperature-dependent conformation of the system which is much less favorably oriented toward the hydrolysis of substrate ATP than that which occurs above the transition temperature (18, 19). More recent work has strongly implicated the nature of the membrane lipids in influencing the rate of substrate utilization by this enzyme (21–24). However, little direct evidence of the physical state of the membrane lipids is presently available (44, 45). The recent use of fluorescent probes to examine the molecular mobility of membrane lipids in other systems (7–12, 16, 17, 46) suggested that this technique is suitable for a direct study of the role of lipids in influencing the temperature-activity relationship of (Na^+ + K^+)-ATPase.

12-AS and NPN are fluorescent probes which bind noncovalently to biological membranes and penetrate the hydrocarbon core of the membrane (11, 12, 16, 47). Therefore they are capable of reflecting changes in the mobility of the membrane lipids in this region. Because fluorescence polarization techniques are suitable for following the motion of fluorescent molecules in a variety of media (8, 9, 11, 13–15), we examined the fluorescence polarization vs. temperature profiles of (Na^+ + K^+)-ATPase-containing membranes labeled with either 12-AS or NPN. The temperature range was that previously used to examine the hydrolytic activity of the enzyme. The good correlation in transition temperature observed with fluorescence labeling and by measuring hydrolytic activity indicates that changes in the molecular mobility of the membrane lipids coincide with changes in the ability of the enzyme to hydrolyze its substrate. The changes in probe rotation which are seen here probably reflect fluidity changes in the hydrophobic region of the membrane, as these probes are unlikely to reveal alterations in the physical properties at the polar-apolar interface regions of the membrane. It therefore seems prudent to refer to the observed changes as *thermal transitions* rather than phase changes or phase separations in the membrane lipids.

In addition, simple right-angle light scattering observations demonstrated thermal transitions in both untreated membrane and liposome preparations. These thermal transitions probably reflected a temperature-dependent change in the refractive index, volume, and particularly in

the case of the microsomal membrane studies, the integrity of the systems.[4]

In all these studies the use of temperature perturbations reveals the correlation between the physical state of the membrane lipids and the hydrolytic activity of $(Na^+ + K^+)$-ATPase, which is a property of the enzyme dependent on the protein components of the system (48–51). Our experiments cannot preclude a direct effect of temperature upon the conformation of the "active center" of $(Na^+ + K^+)$-ATPase. Nevertheless, the observation that all the transition temperatures occurred near 22°, and under widely different experimental conditions, strongly suggests that the effects are due to a fundamental property of the membranes. Although these temperature-dependent transitions must reflect a cooperative interaction between the protein and the lipids of the system, we conclude that the primary effect is a marked increase in the fluidity of the lipids in the membrane adjacent to the active centers of the $(Na^+ + K^+)$-ATPase protein.

This conclusion strongly supports the concept that the lipids of biological membranes have a powerful modulating effect upon membrane enzyme activity in general (5, 6, 12), and upon the transport and hydrolytic function of $(Na^+ + K^+)$-ATPase in particular (23, 24, 52, 53). Finally, it seems reasonable to suggest that both drug-receptor and hormone-receptor interactions in biological membranes might also be modulated by similar changes in the properties of membrane lipids adjacent to macromolecular binding sites. Indeed, the recent work of Kenakin, Krueger, and Cook with H_1 and H_2 histamine receptors (54) is in direct accord with this hypothesis.

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[4] A. D. Bangham, personal communication.

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Temperature–Activity Relationships of Cation Activation and Ouabain Inhibition of (Na $^+$ + K$^+$)-ATPase

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The nonlinear temperature-activity relationship of membrane preparations of (Na$^+$ + K$^+$)-ATPase gives rise to discontinuities in Arrhenius plots of this enzyme. The different apparent energies of activation of (Na$^+$ + K$^+$)-ATPase which are observed above and below the critical temperature of the system have been considered to result from different conformational forms of the enzyme protein. Because both activation of (Na$^+$ + K$^+$)-ATPase by cations, and its specific inhibition by cardiac glycosides may be influenced by the conformational form of the enzyme protein, we have reexamined the effect of temperature upon the activation energy of the system under the different experimental conditions of cation activation and ouabain inhibition.

Our results indicate that the activation of (Na$^+$ + K$^+$)-ATPase by cations, is less influenced by change in temperature than is inhibition of the enzyme by ouabain. In addition, mild lipolysis by phospholipase-A had a marked effect upon the ouabain-dependent response of the enzyme to temperature, but not upon the cation-dependent response. The effect of phospholipase-A can be overcome by reincubation of the treated preparation with phosphatidyl serine.

We conclude that the ouabain-dependent temperature effects of (Na$^+$ + K$^+$)-ATPase are more dependent upon the integrity and nature of the membrane lipids than are the cation-dependent responses. It is possible that phosphatidyl serine plays a unique role in this regard.

It has been known for many years that the activity of membrane-bound (Na$^+$ + K$^+$)-ATPase is dependent upon the amount and composition of the membrane lipids (1–4). Procedures such as treatment with phospholipases, or extraction with detergents or lipid solvents, frequently lead to either partial or total inactivation of the enzyme system (5–9). Very recently the work of several investigators has demonstrated that the temperature–activity relationship of this enzyme is also dependent upon the nature and physical state of the membrane lipids (10–16). Phosphatidyl serine has been shown to overcome the effect of partial removal of a lipid moiety by either phospholipase-A (11, 12) or vigorous treatment with deoxycholate (14, 17, 18).

Discontinuities in Arrhenius plots of (Na$^+$ + K$^+$)-ATPase activity are also considered to arise from different conformational forms of the enzyme protein above and below the critical temperature of the system (19, 20). However, it is now clear that the membrane lipids, and particularly phosphatidyl serine, play a major role in determining the conformational form and activation energy of (Na$^+$ + K$^+$)-ATPase (10–12). Because both the activation of (Na$^+$ + K$^+$)-ATPase by cations, and its specific inhibition by ouabain, are thought to be influenced by the conformational form of the enzyme protein (20) we have re-examined the effects of cation activation and ouabain inhibition on (Na$^+$ + K$^+$)-ATPase activity as a function of temperature, in order to examine this hypothesis further.

MATERIALS AND METHODS

The methods for the preparation of $(Na^+ + K^+)$-ATPase from rabbit kidney cortex (21) and the treatments with lipases or detergents (12) have been described in detail in previous publications. Treatment of the enzyme preparation with 2 M NaI was carried out according to the procedure described by Nakao *et al.* (22). The assay of $(Na^+ + K^+)$-ATPase was again carried out by the general procedure we have used before (12), but additional reaction tubes were included so that activation of the enzyme by 80 mM Na^+ plus 20 mM K^+ could be determined separately from the inhibition of the enzyme obtained with 10^{-4} M ouabain. Blanks for the possible effect of ouabain upon Mg^{2+}-dependent *basal* ATPase (12) were always included in the assay system, and the values shown in the tables and text of this paper were corrected for any hydrolysis of ATP by this source. Arrhenius plots of the temperature–activity relationships of cation activation and ouabain inhibition of $(Na^+ + K^+)$-ATPase were obtained by the procedures we have described previously (12). The values for activation energy (Ea_I and Ea_{II}) above and below the critical temperature (T°c) were determined with the aid of the computer program we have developed in this laboratory (10). The mean values for Ea_I, Ea_{II} and T°c ± their standard errors, were compared by the Student's t test.

RESULTS

The general characteristics of the enzyme preparations from rabbit kidney cortex which were used in this study are shown in Table I. At 37°C, about 80% of the total ATPase activity of the *heavy* microsomal membrane preparation is due to ouabain-sensitive $(Na^+ + K^+)$-ATPase.

TABLE I

CATION ACTIVATION AND OUABAIN INHIBITION OF RABBIT KIDNEY PREPARATIONS OF $(Na^+ + K^+)$-ATPase[a]

Assay conditions	% Activity[b]
Enzyme omitted	<0.5
Mg^{2+}	18
Mg^{2+} + ouabain	15
$Mg^{2+} + Na^+ + K^+$	100
$Mg^{2+} + Na^+ + K^+$ + ouabain	19

[a] Enzyme preparation not treated with detergent or lipase, but stored at −10°C for 5 days before assay.

[b] All assays determined at 37°C for 15 min. Values given are the means of at least five preparations, with determinations performed in duplicate. The mean specific activity of the *enzyme* is 23.7 ± 1.9 μmol Pi/mg protein/h.

Untreated $(Na^+ + K^+)$-ATPase

The effect of temperature upon the apparent activation energy of *untreated* preparations of rabbit kidney $(Na^+ + K^+)$-ATPase is shown as an Arrhenius plot in Fig. 1. The characteristic discontinuity in this plot is again apparent, whether the enzyme activity is determined by activation by cations (80 mM Na^+ + 20 mM K^+) or by inhibition by cardiac glycoside (10^{-4} M ouabain), thus confirming our earlier findings (10, 12) and those of other workers (11, 13). However, it should be noted that the apparent activation energies of the enzyme which can be derived from these plots are not identical under the two experimental situations being compared here. Although the divergence in values of Ea_I above the critical temperature are much smaller than the divergence in values of Ea_{II} below the critical temperature, the values for both Ea_I and Ea_{II} by cation activation are significantly different from those obtained by ouabain inactivation ($p < .05$). Interestingly, the points of inflection in the Arrhenius plots, that is the

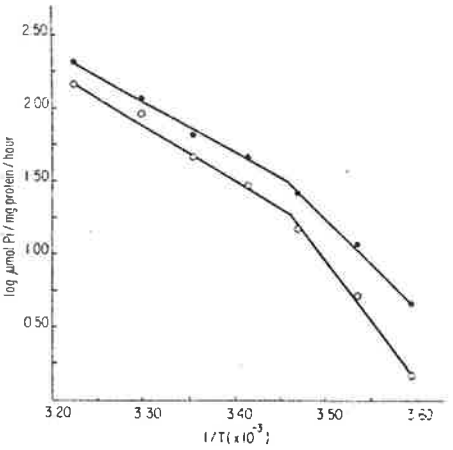

FIG. 1. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (○——○) of untreated membrane preparations of $(Na^+ + K^+)$-ATPase. The mean critical temperature (T°c) for four experiments is 16.7 ± 1.6°C and is identical for all experimental conditions. The computed values for Ea_I above T°c are 15.4 ± 0.7 and 19.1 ± 1.3 kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for Ea_{II} below T°c are 27.5 ± 1.9 and 43.1 ± 3.9 kcal/mol, respectively.

computed critical temperatures (T°c) occur at about 17°C, and are not significantly different under these two experimental conditions.

Detergent Treatment

Previous studies from this laboratory have shown that *mild* treatment with the detergents deoxycholate or Nonidet P40 resulted in significantly increased hydrolytic activity of ($Na^+ + K^+$)-ATPase, but no change in the temperature-activity relationship or the apparent activation energy of the system (12). In this study the Arrhenius plots that were obtained by both cation activation and ouabain inhibition of the system after partial removal of membrane lipids by deoxycholate, or 0.2% Nonidet P40 are shown in Figs. 2 and 3, respectively. The results obtained are very similar to those reported previously (12); again there is a divergence in the apparent activation energies of ($Na^+ + K^+$)-ATPase when determined by either cation activation or by ouabain inhibition. These results are also similar to those shown in Fig. 1 for untreated membrane preparations. The divergence in apparent activation energies is always greater in the temperature range below the critical temperature which, in this group of experiments is about 19°C.

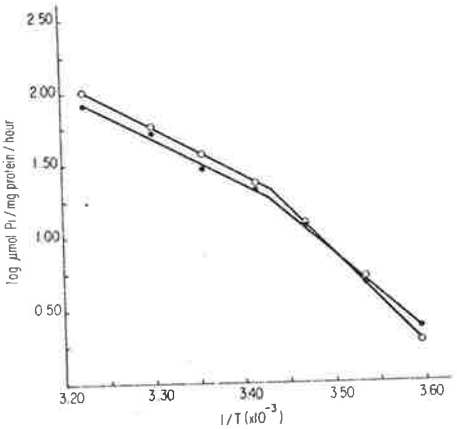

FIG. 2. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (○——○) of membrane preparations of ($Na^+ + K^+$)-ATPase after treatment with 0.1% deoxycholate.

The mean critical temperature (T°c) for four experiments is 18.7 ± 1.4°C, and is identical for all experimental conditions.

The computed values for Ea_I above T°c are 17.5 ± 2.1 and 17.8 ± 1.7 kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for Ea_{II} below T°c are 30.4 ± 5.9 and 36.3 ± 4.2 kcal/mol, respectively.

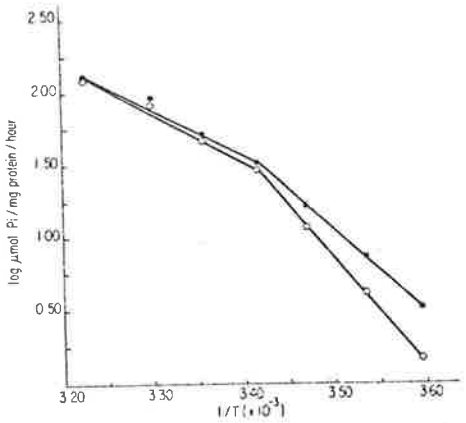

FIG. 3. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (○——○) of membrane preparations of ($Na^+ + K^+$)-ATPase after treatment with 0.2% Nonidet P40.

The mean critical temperature (T°c) for four experiments is 19.5 ± 1.2°C and is identical for all experimental conditions.

The computed values for Ea_I above T°c are 15.6 ± 0.6 and 16.4 ± 0.5 kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for Ea_{II} below T°c are 25.6 ± 1.0 and 37.2 ± 5.1 kcal/mol, respectively.

Treatment with NaI

After treatment with 2 M NaI (22), the temperature-activity relationship of ($Na^+ + K^+$)-ATPase is qualitatively similar to that of the untreated controls when enzyme activity has been determined by inhibition with 10^{-4} M ouabain. There is a sharp discontinuity in the Arrhenius plot at a mean temperature of 14.3 ± 5.9°C. The apparent energy of activation above this critical temperature is 20.6 ± 2.1 kcal/mol and 56.2 ± 24.2 kcal/mol below T°c (Fig. 4). These values are not significantly different from those previously obtained under these experimental conditions (12).

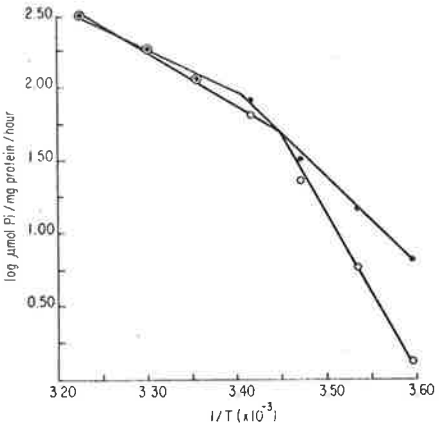

FIG. 4. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (O——O) of membrane preparations of $(Na^+ + K^+)$-ATPase after treatment with 2 M NaI. The mean critical temperature (T°c) for three experiments is 20.6 ± 4.1°C for activation by cations, and is 14.3 ± 5.9°C when the enzyme activity is determined by inhibition with ouabain.

The computed values for Ea_I above T°c are 15.4 ± 1.1 and 20.6 ± 2.1 kcal/mol for activation by cations and inhibition by ouabain, respectively.

The values for Ea_{II} below T°c are 27.0 ± 1.0 and 56.2 ± 24.2 kcal/mol, respectively.

Conversely, when enzyme activity is determined by activation with cations, some change is apparent in the observed temperature-activity relationship. Although a discontinuity in the Arrhenius plot is still apparent, this now occurs at a significantly higher temperature of 20.6 ± 4.1°C than in the untreated controls ($p < .05$). The apparent activation energy above the critical temperature is now 15.4 ± 1.1 kcal/mol, and that below T°c is 27.0 ± 1.0 kcal/mol. All these values are significantly different from those of the untreated control (Fig. 1).

Lipase Treatment

Although it is well-known that prolonged digestion of biological membranes with phospholipases will completely inactive membrane preparations of $(Na^+ + K^+)$-ATPase (1, 13, 23, 24, 25), we have shown previously that under very mild conditions, treatment with phospholipase-C does not significantly change the ATPase activity of these preparations compared to appropriate controls (12). Although phospholipase-C is known to interact with many acidic phospholipids, we have again demonstrated in this study that *mild* treatment with this lipase did not change the temperature-activity relationship of $(Na^+ + K^+)$-ATPase, when determined by either cation activation or by ouabain inhibition (Fig. 5). Under both conditions, $Ea_{II} > Ea_I$. Again the activation energy of $(Na^+ + K^+)$-ATPase when activated by cations was significantly less than the value obtained when the enzyme was inhibited by 10^{-4} M ouabain ($p < .05$). Like the effect seen after treatment with NaI, the critical temperature now found in the presence of cations was higher in these preparations than that seen with untreated controls.

Conversely, mild treatment of $(Na^+ + K^+)$-ATPase with phospholipase-A produced a marked effect on the temperature-activity relationship of the enzyme when determined by ouabain inhibition. There was a complete loss of the

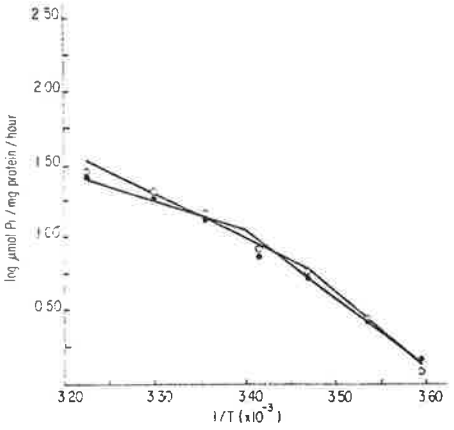

FIG. 5. Arrhenius plots of inorganic phosphate liberation of ATP by cation activation (●——●) and ouabain inhibition (O——O) of membrane preparations of $(Na^+ + K^+)$-ATPase after treatment with phospholipase-C.

The mean critical temperature (T°c) for four experiments with cation activation is 21.2 ± 3.4°C, and 16.4 ± 0.9°C for inhibition by ouabain.

The computed values for Ea_I above T°c are 11.4 ± 0.4 and 14.1 ± 0.9 kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for Ea_{II} below T°c are 20.0 ± 0.9 and 25.9 ± 1.4 kcal/mol, respectively.

inflection point in the Arrhenius plot of this enzyme which had remained apparent after all other treatments examined. A unique value of 24 kcal/mol for the apparent activation energy is now obtained. This effect of treatment with phospholipase-A was not seen with cation activation of the enzyme as a discontinuity in the Arrhenius plot was still apparent under these conditions (Fig. 6). However, the difference in values between Ea_I and Ea_{II} was now smaller than usual, and the point of inflection in the plot could not be determined with as much confidence as before. The computed values for Ea_I, Ea_{II} and T°c were 22.0 ± 0.5 kcal/mol, 36.0 ± 6.7 kcal/mol and 18.3 ± 2.7°C, respectively.

When the phospholipase-A treated preparations of $(Na^+ + K^+)$-ATPase were subsequently reincubated with phosphatidyl serine at a concentration of 3 mg phospholipid/mg of enzyme protein, the temperature-activity relationship which is shown in Fig. 7 was obtained.

The inflection point in the Arrhenius plot previously obtained in the presence of 10^{-4} M ouabain is regained (Figs. 1–5), thus confirming our earlier observation (12) and that of other workers (11, 13). The values for Ea_I, Ea_{II} and T°c which were computed under these experimental conditions, and in the presence of ouabain, were 20.2 ± 1.1 kcal/mol, 43.6 ± 7.2 kcal/mol and 18.7° ± 0.5°C, respectively. Conversely the effect of phosphatidyl serine upon cation activation of phospholipase-A treated $(Na^+ + K^+)$-ATPase is only slight, with very similar values for Ea_I and Ea_{II} being obtained before and after incubation with phosphatidyl serine (Table II). The apparent change in the critical temperature observed may be due to an excess of phosphatidyl serine in the system (24).

Therefore in order to detect any possible *direct* effect of this acidic phospholipid, a previously *untreated* enzyme preparation was also incubated with phosphatidyl serine. The temperature-activity relationship observed was not significantly

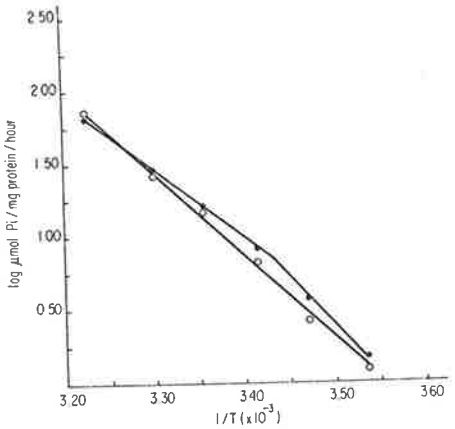

FIG. 6. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (O——O) of membrane preparations of $(Na^+ + K^+)$-ATPase after mild treatment with phospholipase-A. There is no inflection point in the plot determined after ouabain inhibition, whereas the mean critical temperature (T°c) for four experiments after cation activation is 18.3 ± 2.7°C. The computed values for Ea_I and Ea_{II} after cation activation are 22.0 ± 0.5 and 36.0 ± 6.7 kcal/mol, respectively. The unique value for Ea obtained after ouabain inhibition is 24.0 ± 3.9 kcal/mol.

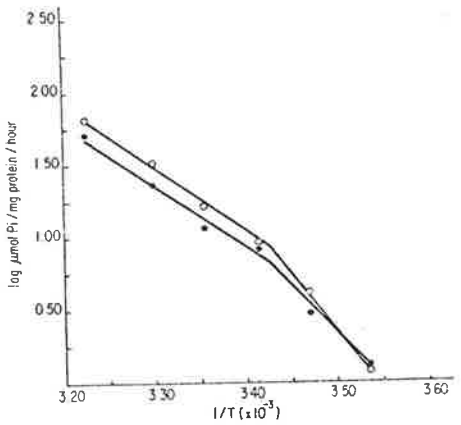

FIG. 7. Arrhenius plots of inorganic phosphate liberation from ATP by cation activation (●——●) and ouabain inhibition (O——O) of membrane preparations of $(Na^+ + K^+)$-ATPase after mild treatment with phospholipase-A, followed by reincubation with phosphatidyl serine. Identical inflection points are determined under both experimental conditions at 18.7 ± 0.5°C (mean of four experiments).

The computed values for Ea_I above T°c are 21.6 ± 0.5 and 20.2 ± 1.1 kcal/mol for activation by cations and inhibition by ouabain, respectively. The values for Ea_{II} below T°c are 28.7 ± 1.2 and 43.6 ± 7.2 kcal/mol, respectively.

TABLE II

APPARENT ACTIVATION ENERGY OF CATION ACTIVATED ($Na^+ + K^+$)-ATPase AFTER VARIOUS TREATMENTS[a]

Conditions	No. of experiments	Ea_I above $T°c$[b]	Ea_{II} below $T°c$[b]	$T°c$
Untreated control	4	15.4 ± 0.7	27.5 ± 1.9	16.7 ± 1.6
Sodium iodide	3	15.4 ± 1.1	27.0 ± 1.0	20.6 ± 4.1
Deoxycholate	4	17.5 ± 2.1	30.4 ± 5.9	18.7 ± 1.4
Nonidet P40	4	15.6 ± 0.6	25.6 ± 1.0	19.5 ± 1.2
Phospholipase-C	4	11.4 ± 0.4	20.0 ± 0.9	21.2 ± 3.4
Phospholipase-A	4	22.0 ± 0.5	36.0 ± 6.7	18.3 ± 2.7
Phospholipase-A and phosphatidyl serine	4	21.6 ± 0.5	28.7 ± 1.2	18.7 ± 0.5
Phosphatidyl serine	3	17.0 ± 0.7	30.7 ± 1.7	16.2 ± 1.3

[a] Temperature-activity relationship determined by cation activation with 80 mM Na^+ and 20 mM K^+, see Charnock and Post (21).

[b] Values for Ea_I and Ea_{II} given as kcal/mol ± standard error of the mean.

different from those seen with untreated controls (Fig. 1) and is therefore not shown.

In general all the experiments reported in this paper indicate that the activation of the enzyme preparations by Na^+ and K^+ ions is less influenced by change in temperature, than is the inhibition of the enzyme by ouabain. This overall effect is illustrated in Fig. 8.

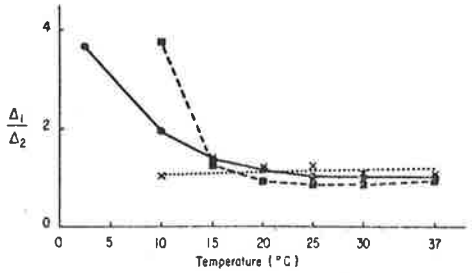

FIG. 8. The differential effect of temperature upon cation activation (Δ_1) and ouabain inhibition (Δ_2) of ($Na^+ + K^+$)-ATPase. (●——●) untreated enzyme preparation (values taken from Fig. 1). (×- - -×) after treatment with phospholipase-A (values taken from Fig. 6). (■- - -■) after treatment with phospholipase-A and reincubation with phosphatidyl serine (values taken from Fig. 7).

The data points are the mean specific activity of four enzyme preparations.

DISCUSSION

The temperature–activity relationships of ($Na^+ + K^+$)-ATPase have been shown to be nonlinear over the range of 5–37°C, with the point of inflection at about 18°C. Above this critical temperature ($T°c$) the apparent activation energy (Ea_I) of the overall hydrolysis of adenosine-triphosphate to adenosine-diphosphate and inorganic phosphate by this enzyme, is not greatly different whether enzyme activity is determined by its activation by the cations (sodium plus potassium) or by its inhibition by the cardiac glycoside ouabain in the presence of $Na^+ + K^+$. Conversely, there is a much greater difference between the apparent activation energies which are determined below the critical temperature (Ea_{II}) under these two experimental conditions; the mean value obtained by cation activation of untreated enzyme preparations being about 60% of that determined by ouabain inhibition. This decrease in activation energy is not influenced by procedures known to partially remove lipids from such preparations (24). Apparently activation of ($Na^+ + K^+$)-ATPase by specific cations is much less influenced by changes in temperature, particularly below 18°C, than is its inhibition by cardiac glycosides. This suggestion is reinforced by the observed effect of treatment with NaI. This potentially chaotropic agent is known to disrupt lipid–protein and protein–protein interactions in biological membranes but does not solubilize their lipid components (26, 27). In these experiments, treatment

of (Na^+ + K^+)-APTase preparations enhanced their specific activity but did not effect the apparent energy of activation of the enzyme in any way when its function was determined by inhibition by ouabain. However, when enzyme activity was determined by activation by cations. the transition temperature ($T°c$) was significantly changed by treatment with NaI, although the apparent activation energies were not altered.

Although further experiments will be required to more clearly establish the point, it is possible that the effect of NaI is more directly related to conformational changes in the protein moiety of the enzyme preparation than to altered phase transitions in the lipid moiety.

In this connection it is of great interest that *mild* treatment of our membrane preparations of (Na^+ + K^+)-ATPase with bee-venom phospholipase-A (12), has a profound effect upon the apparent activation energy of the process of ATP hydrolysis when determined by cardiac glycoside inhibition, but much less effect upon the process of ATP hydrolysis by this enzyme when determined by cation activation. Under conditions of cation activation, the nonlinearity of the temperature-activity relationships persists after phospholipase-A treatment, with an inflection point near 18°C and values of about 22 kcal/mol for Ea_I and 36 kcal/mol for Ea_{II} (Table II). When the apparent activation energy is measured by ouabain inhibition an inflection point cannot be determined under these conditions. The unique value for *Ea* which is now obtained between 5° and 37°C is 24.0 ± 3.9 kcal/mol. In addition, when phospholipase-A treated preparations are incubated with phosphatidyl serine (the ratio of phospholipid to enzyme protein was 3:1), the cardiac glycoside-determined response becomes qualitatively similar to that seen with an untreated control preparation; that is, an inflection point is again apparent, and Ea_{II} is much greater than Ea_I.

Phosphatidyl serine has been reported to be preferentially bound to delipidated membranes (24). Apparently, reconstitution of phospholipase-A treated (Na^+ + K^+)-ATPase by phosphatidyl serine restores both cardiac glycoside sensitivity and temperature dependence to the enzyme (6, 12, 14). *Mild* treatment with phospholipase-C does not produce this effect, although it seems probable that longer incubation of the enzyme with this lipase could reproduce the action of phospholipase-A.

In summary, this study has confirmed that the temperature–activity relationship of membrane-bound (Na^+ + K^+)-ATPase is nonlinear over the range 5°–37°C for the enzyme activity determined either by activation by cations or by inhibition with ouabain. However, the temperature dependence of ouabain inhibition is greater than that of cation activation, particularly below the inflection point of a temperature–activity plot. Mild lipolysis by phospholipase-A has little effect upon the cation activation of the enzyme at any temperature examined, but has a very marked effect in decreasing the apparent activation energy below 18°C when this is determined by inhibition of the enzyme by ouabain. The action of phospholipase-A can be overcome by reconstitution of the enzyme preparation with phosphatidyl serine.

These findings suggest that the lipid components of the membrane undergo a temperature-dependent phase transition at about 18°C, which in turn influences the activity of the (Na^+ + K^+)-ATPase enzyme protein. This cooperative effect is much more apparent when ouabain inhibition of the enzyme is examined rather than cation activation. The discriminating effect of phospholipase-A plus phosphatidyl serine suggest that a unique lipid component of the membrane, perhaps phosphatidyl serine in general but possibly even a phosphatidyl serine moiety at a special location in the membrane (24, 28), is particularly involved in imparting cardiac glycoside sensitivity and temperature dependence to (Na^+ + K^+)-ATPase. It seems much less probable that this suggested lipid moiety is involved in imparting cation activation to the system. Presumably it is this suggested lipid site which is greatly concerned with

the binding of cardiac glycosides, but not the binding of sodium or potassium ions to the membrane system in general.

ACKNOWLEDGMENTS

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Activation Energy and Phospholipid Requirements of Membrane-Bound Adenosine Triphosphatases[1]

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In order to evaluate the role of lipids in the function of membrane ATPase reactions, the apparent activation energies of membrane-bound $(Na^+ + K^+)$-ATPase and membrane-bound Mg^{2+}-ATPase have been measured under conditions frequently supposed to alter the membrane lipids *in vitro*.

In the case of $(Na^+ + K^+)$-ATPase, the untreated enzyme was shown to have two different activation energies as shown by an Arrhenius plot comprising two straight lines which intersect at the "critical temperature." Treatment of the preparation with detergents or with phospholipase C causes some alteration in the specific activity of the enzyme but did not significantly alter the activation energies or the critical temperature. After treatment with phospholipase A, however, the Arrhenius plot appeared linear over the entire temperature range studied. Subsequent treatment of phospholipase A-treated preparations with phosphatidylserine restored the control response.

Conversely, untreated preparations of Mg^{2+}-ATPase give an Arrhenius plot which is neither linear nor composed of two intersecting straight lines. This plot, which we regard as curvilinear, does not permit a unique value of the activation energy to be determined. The shape of this plot is unaltered by detergent or by treatment with phospholipase C. In contrast to $(Na^+ + K^+)$-ATPase, it is also unaffected by treatwith phospholipase A or phospholipase A followed by phosphatidylserine.

We conclude that although $(Na^+ + K^+)$-ATPase and Mg^{2+}-ATPase are frequently closely associated in many membranes, their functions involve the presence of different membrane lipids.

The recent studies of Raison and Lyons (1, 2) have shown that the properties of some membrane-bound enzymes are greatly influenced by the physical state of the membrane lipids. In particular, these workers have demonstrated the close relationship which exists between enzyme activity, the apparent activation energy of the system, and the motional freedom of the membrane lipids (1, 2).

It therefore seems likely that the sudden "breaks" or sharp departures from linearity that have been observed in the Arrhenius plots of membrane-bound $(Na^+ + K^+)$-ATPase (3–6) prepared from a variety of sources might both result from, and be influenced by, the type and state of the phospholipids in the membranes. This enzyme system would seem to be well suited to the study of this phenomenon as it has long been known that its activity can be reduced by a variety of treatments which remove lipids or their components from membranes (7–9), and that activity can frequently be restored by replacement of either phospholipids (10–14) or cholesterol (15, 16) to the system.

This paper describes the effects of a variety of lipid extraction procedures upon the apparent activation energy of $(Na^+ + K^+)$-ATPase both above and below the critical

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temperature for the system, and the effect of phosphatidylserine replacement following treatment of the membrane preparations with phospholipases. The effect of these treatments on the activation energy of Mg^{2+}-ATPase, which was always associated with these preparations, is also described.

MATERIALS AND METHODS

"Heavy" microsomal membrane preparations rich in ouabain-sensitive ($Na^+ + K^+$)-ATPase but also containing ouabain-insensitive Mg^{2+}-ATPase activity were obtained from the renal cortex of mature New Zealand white rabbits by the procedure first described by Charnock and Post (17). The membrane enzyme preparations were suspended and stored at 4°C in a sucrose-buffer mixture of 0.275 M sucrose and 0.2 mM EDTA adjusted to pH 7.6 by the addition of 1 M Tris-base. The protein content of these enzyme suspensions was determined by the method of Lowry *et al.* (18) and was maintained between 1 and 3 mg protein/ml.

Both the ouabain-sensitive and ouabain-insensitive ATPase activity of the freshly prepared microsomal membrane fractions was obtained by the assay procedure of Charnock and Post (17) both prior to and after other experimental procedures described below. The assay conditions were adjusted so that the reaction velocity obtained was constant throughout the incubation periods employed, and closely approaches the $V_{max(t^0)}$ for both ouabain-sensitive ($Na^+ + K^+$)-ATPase and Mg^{2+}-ATPase. Excess substrate (Mg·ATP) was always present, and the concentration of ADP generated by the reaction was never allowed to exceed 20% of that of the initial concentration of ATP. The reaction velocities so obtained were converted to rate constant (μmoles P_i liberated/mg protein/hr) before calculation of the Arrhenius graphs of temperature dependence of these enzyme systems.

Extraction with deoxycholate. Two volumes of freshly prepared membrane suspension (3 mg protein/ml) were mixed with 1 vol of 0.2% Tris-deoxycholate (pH 7.2) and allowed to stand for 16 hr at 4°C. The extracted membranes were recovered by centrifugation at 40,000*g* for 30 min and resuspended in the sucrose-buffer described above.

Extraction with Nonidet P40. One volume of freshly prepared membrane suspension was mixed with 4 vol of 1% Nonidet P40 nonionic detergent (kindly supplied by Dr. J. K. Raison) and allowed to stand for 30 min at 4°C. The extracted membranes were recovered and resuspended as above.

Phospholipase A. When necessary, the membrane suspension was diluted with sucrose buffer to 1 mg protein/ml and was incubated with 2.67 μg/ml of bee venom phospholipase A (Sigma Chemical Co.) in a medium adapted from that described by Imai and Sato (19). The final concentration of reagents in the incubation solution was: bovine serum albumin, 7.35 mg/ml; Tris base, 14.7 mM; 2-mercaptoethanol, 33.7 mM; EDTA, 0.035 mM; $CaCl_2$, 5.6 mM; sucrose, 0.55 M; adjusted to pH 7.4 by the addition of 1 M Tris base. After 5 min incubation at 37°C to insure temperature equilibration of all components of the system, the reaction was started by the addition of phospholipase A. After a further 12 min incubation at 37°C the reaction was stopped by the addition of 2 vol of 57 mM EDTA–321 mM Tris (adjusted to pH 7.4 by addition of 1 M HCl) to 3 vol of reaction mixture and immediately diluted to 200 ml with ice-cold distilled water. The particulate membrane fraction was then recovered by centrifuging at 77,500*g* for 3 hr and resuspending in the sucrose buffer solution employed before to again yield a protein content of 1 mg/ml. Some of these phospholipase A-treated preparations were then incubated for 10 min at 37°C with an equal vol of phosphatidyl serine (3 mg/ml) immediately before the determination of temperature dependence described below.

Phospholipase C. The membrane suspension was diluted to a protein content of 0.8 mg/ml and incubated with 0.2 mg phospholipase C (prepared from *Clostridium welchii* by the Sigma Chemical Co.) in a mixture containing a final concentration of imidazole, 12.5 mM; $CaCl_2$, 3.7 mM; NaCl, 64.0 mM; KCl, 16.0 mM; and $MgSO_4$, 4.8 mM (adjusted to pH 7.1 with 1 M HCl) according to the method of Smith and Kemp (20). After bringing to constant temperature the reaction was started by the addition of phospholipase C and incubated for 1 hr at 37°C. The reaction was stopped by cooling to 4°C, and the particulate membrane fraction recovered by centrifugation at 40,000*g* for 30 min. The pellet was resuspended in sucrose buffer as described above.

Activation energy. The effect of temperature on ouabain-sensitive ($Na^+ + K^+$)-ATPase activity and ouabain-insensitive Mg^{2+}-ATPase activity of both the untreated and treated membrane preparations was determined by the procedure we have described previously (4) and the data so obtained used to construct Arrhenius graphs from 5° to 37°C. These graphs were again analyzed with the assistance of the APL\360 computer program developed in this laboratory (4) to yield values for the apparent activation energies and critical temperatures of both ATPase reactions, and to provide the statistical confidence limits for the number of lines which can be fitted to the plots.

RESULTS

Ouabain-sensitive ($Na^+ + K^+$)-ATPase. In agreement with our earlier findings (17) the specific activity of ouabain-sensitive ($Na^+ +$

K^+)-ATPase was increased about twofold after 16-hr extraction with a low concentration (less than 0.1 %) of deoxycholate (DOC). A similar but quantitatively greater effect was obtained after 30-min extraction with 0.2 % Nonidet P40 when enzyme activity was enhanced about fivefold. The incubation conditions for treatment with either phospholipase A or phospholipase C were adjusted after preliminary experiments, so that any changes in enzyme specific activity were relatively small when compared to the effects obtained with Nonidet P40. These results are shown in Table I.

The apparent activation energies of detergent-treated enzyme preparations were calculated from the Arrhenius graphs shown in Fig. 1 and were not significantly different from those of the untreated control preparations. The calculated mean values for the apparent activation energies for these enzyme preparations above (E_{aI}) and below (E_{aII}) the critical temperature (T_c°) are given in Table II.

Although brief incubation of the membranes with phospholipase A was without significant effect upon the specific activity of the enzyme preparations at 37°C, there was a marked effect upon the temperature dependence of the reaction velocity over the whole experimental range. This is shown by the Arrhenius graph given in Fig. 2 where the sharp "breaks" in the plot which previously characterized these preparations are no longer apparent. Computer-assisted analysis of the data (4) indicated that a single straight line can now be fitted to the data points with a high degree of statistical confidence. This linear relationship yields a unique value of about 24 kcal/mole for the apparent activation energy of treated ($Na^+ + K^+$)-ATPase determined between 5–37°C (Table II).

Reconstitution experiments with phosphatidylserine after phospholipase A treatment resulted in the reappearance of the "break" in these plots and returned the temperature dependence of the enzyme to that indistinguishable from the untreated controls. Addition of phosphatidylserine alone to untreated enzyme preparations was without significant effect upon either enzyme specific activity or temperature dependence; the values for E_{aI} and E_{aII} are given in Table II.

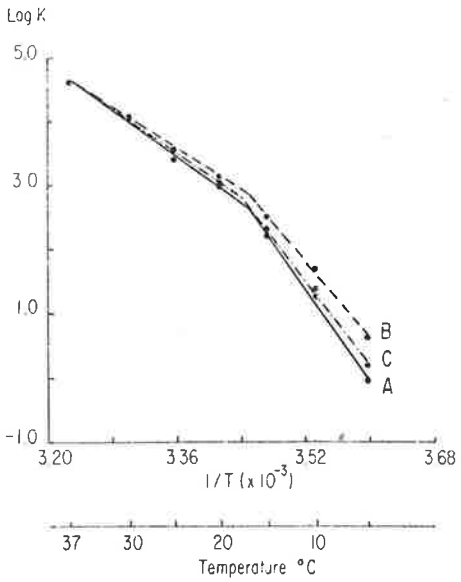

FIG. 1. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-sensitive ($Na^+ + K^+$)-ATPase before and after detergent treatment. The data points were obtained by calculation of the difference in activity of the enzyme preparations assayed with 80 mM Na^+, 20 mM K^+, and 4 mM Mg·ATP ± 1 mM ouabain. Plot A is the control before detergent treatment; Plot B is after extraction with DOC and Plot C is after extraction with Nonidet P40. The values for the apparent activation energies which were derived from these plots are given in Table II.

TABLE I

THE EFFECT OF VARIOUS TREATMENTS ON THE ACTIVITY OF MEMBRANE ATPASE

Conditions[a]	% Ouabain-insensitive Mg^{2+}-ATPase	% Ouabain-sensitive ($Na^+ + K^+$)-ATPase
Untreated control	100	100
Deoxycholate	126	212[b]
Nonidet P40	186[b]	520[b]
Phosphatidylserine	88	150
Phospholipase A	102	130
Phospholipase A plus phosphatidylserine	96	82
Phospholipase C	113	158[b]

[a] Details of treatment are given under *Materials and Methods*. Values given are the means of at least six separate experiments.

[b] Value significantly different from untreated control ($P < 0.05$).

TABLE II

APPARENT ACTIVATION ENERGY OF ($Na^+ + K^+$)-ATPASE AFTER VARIOUS TREATMENTS

Conditions	No. of experiments	E_{aI} above $T_c°$ [a]	E_{aII} below $T_c°$ [a]	$T_c°$
Untreated control	6	19.1 ± 1.3	43.1 ± 3.9	16.5 ± 1.5
Deoxycholate	6	17.8 ± 1.7	36.3 ± 4.2	18.5 ± 2.3
Nonidet P40	13	16.4 ± 0.5	37.2 ± 5.1	19.5 ± 1.8
Phospholipase A	7	23.9 ± 1.5[b]	24.0 ± 3.9[b]	—
Phospholipase A and phosphatidylserine	5	20.2 ± 1.1	43.6 ± 7.2	18.7 ± 0.5
Phosphatidylserine	3	19.5 ± 1.2	39.7 ± 1.7	20.4 ± 1.2
Phospholipase C	5	14.1 ± 0.9	25.9 ± 1.4[b]	16.4 ± 0.9

[a] E_a given as kcal/mole ± standard error.
[b] Values for E_{aI} and E_{aII} are significantly different ($P < 0.05$) from untreated controls.

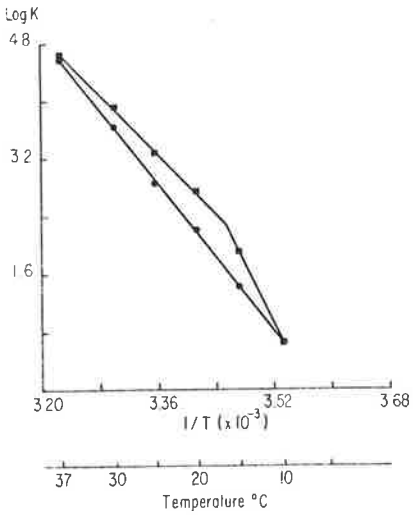

FIG. 2. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-sensitive ($Na^+ + K^+$)-ATPase after treatment with phospholipase A. The lower plot (●) shows the effect after short-term treatment with phospholipase A where the linear plot no longer displays a critical temperature. The upper plot (■) shows the effect of phosphatidylserine addition after treatment with phospholipase A and again displays the nonlinearity of the response similar to that of the untreated control shown in Fig. 1. The values for the apparent activation energies which were derived from these plots are given in Table II.

Treatment with phospholipase C gave results qualitatively similar to those obtained for the controls, but resulted in a decrease in the apparent activation energy of ($Na^+ + K^+$)-ATPase below $T_c°$ as was also seen after treatment with phospholipase A (Table II). Subsequent addition of phosphatidylserine was without effect.

Ouabain-insensitive Mg^{2+}-ATPase. The specific activity of ouabain-insensitive Mg^{2+}-ATPase was significantly increased by treatment with Nonidet P40, but was not markedly changed by treatment with DOC, phosphatidyserine, phospholipase A, or phospholipase C. The activities observed before and after treatment are given in Table I. The effect of temperature on the reaction velocity of Mg^{2+}-ATPase of untreated microsomal membrane preparations was examined in six separate experiments. The mean reaction velocities at temperatures from 5 to 37°C were obtained and used to construct the Arrhenius plot shown in Fig. 3a. A computer-assisted analysis of this data suggested that the plot was not linear over this range, nor could it be fitted by two intersecting straight lines with a high degree of confidence. Seemingly, this data is most reasonably regarded as being curvilinear between 5–37°C, a conclusion which precludes the assignment of a meaningful unique value for the activation energy of this process over this range. However, the data will allow an estimate of the range of values which can be obtained. For example, the values estimated from the control curve shown in Fig. 3a extend from 12.0 kcal/mole at higher temperatures to 25.5 kcal/mole at the lower temperatures. Incubation with phosphatidylserine alone did not significantly alter this observation, again yielding a curvilinear plot shown in Fig. 3b, with a similar estimated

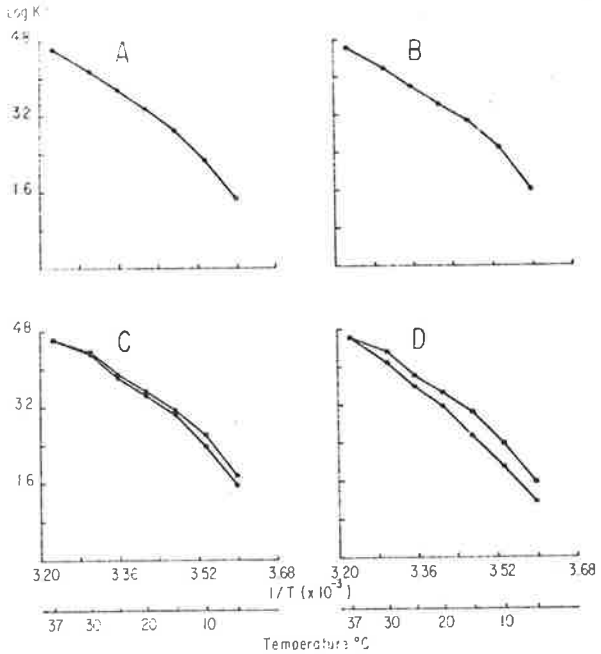

FIG. 3. Arrhenius graphs of inorganic phosphate liberation from ATP by ouabain-insensitive Mg^{2+}-ATPase after various treatments. (A) untreated control; (B) after addition of phosphatidylserine alone; (C) lower curve (●) after Nonidet P40; upper curve (■) after DOC; (D) lower curve (●) after phospholipase A; upper curve (■) after phospholipase A followed by addition of phosphatidylserine. The values for the range of apparent activation energies which were derived from these plots are given in the text.

range of values for activation energy (12.2–23.6 kcal/mole).

Extraction with deoxycholate was also without effect on the temperature dependence of Mg^{2+}-ATPase, as the Arrhenius plot obtained after treatment with this agent also appeared to be curvilinear, again yielding values estimated between 11.3–25.1 kcal/mole. Similarly the data obtained after Nonidet P40 extraction did not show any effect of this agent upon the temperature dependence of ouabain-insensitive ATPase activity; the Arrhenius plots after treatment with DOC and Nonidet P40 being virtually identical (Fig. 3c).

However, unlike the findings with ouabain-sensitive (Na^+ + K^+)-ATPase, there was also no significant change in the temperature dependence of ouabain-insensitive Mg^{2+}-ATPase after treatment with phospholipase A or after treatment with phospholipase A followed by reincubation with phosphatidylserine (Fig. 3d). That is, the Arrhenius plots obtained after treatment with these agents could neither be fitted by a single straight line nor by two intersecting straight lines with any acceptable degree of statistical confidence. Treatment with phospholipase C was also without effect upon the temperature dependence of Mg^{2+}-ATPase and is not shown in Fig. 3.

Thus, all of these experiments failed to yield a meaningful unique value for the apparent activation energy of the reaction, but gave a maximum range of estimated values from 11.3 kcal/mole at the high temperatures to 29.4 kcal/mole at the lower temperatures.

DISCUSSION

In previous reports from this laboratory (3, 4) we have shown that the temperature dependence of ouabain-sensitive (Na^+ + K^+)-ATPase is nonlinear between 5–37°C; resulting in two widely different apparent activation energies above and below a criti-

cal temperature of about 18°C. This finding is confirmed in the present study. We have also shown that these apparent activation energies are unchanged by alterations in the total cation concentration of the incubation medium, or by different periods of storage which produce variable decreases in enzyme specific activity (4).

Our present findings, in which detergent treatment results in large increases in enzyme specific activity without change in apparent activation energies of this enzyme, reinforce our previous conclusion that this thermodynamic parameter is independent of the enzyme specific activity and thus does not reflect the number of *active centers* which are operational. Rather it is a measure of the functional ability of individual sites and, as such, can be influenced by molecular changes within the membrane matrix.

After brief incubation with phospholipase A which was adjusted to maintain the specific activity of $(Na^+ + K^+)$-ATPase at or near the control level, there was a marked change in the temperature dependence of this enzyme which was now linear between 5° and 37°C, thus giving a unique value for the apparent activation energy of this membrane bound enzyme of approximately 24 kcal/mole.

In addition, the effect of treatment with phospholipase A could be reversed by reincubating the lipase-treated membranes with phosphatidylserine. Similar findings have recently been reported by several other workers (6, 21) who have independently confirmed the reactivating action of this phospholipid upon lipase-treated membrane preparations. However, the selectivity of phosphatidylserine for this effect has not been demonstrated as partial reactivation of membrane ATPase has also been reported with phosphatidylethanolamine (11) and lysolecithin (12). More recently the studies of Noguchi and Freed (15) and Jarnefelt (16) have shown that cholesterol may have a similar action following organic solvent extraction of the membranes.

In this regard it is of interest that treatment of the membranes with phospholipase C did not result in the conversion of the temperature dependence of ouabain-sensitive $(Na^+ + K^+)$-ATPase to a linear relationship, suggesting that a selective cleavage of the phospholipids involved in this phenomenon is an important determinant in this effect. It is not surprising, therefore, that addition of phosphatidylserine after treatment with phospholipase C was without effect upon the apparent activation energy of the enzyme.

Conversely, neither treatment with detergents nor lipases resulted in any detectable change in the temperature dependence of the ouabain-insensitive Mg^{2+}-ATPase associated with these membrane preparations. This latter enzyme function could not be described as being either linear or be adequately fitted by two linear subsections separated by a critical temperature. Seemingly our conclusion from the examination of the data from separate experimental situations is valid, and that when all the data from more than 40 experiments is pooled, the resultant Arrhenius plot of ouabain-insensitive Mg^{2+}-ATPase must be regarded as a curve; that is, the temperature response of this enzyme is demonstrably different from that obtained with ouabain-sensitive $(Na^+ + K^+)$-ATPase. This finding suggests that the phospholipids closely associated with the *active centers* of ouabain-sensitive *transport* ATPase are either distinct from those associated with the physiologically less well-defined ouabain-insensitive Mg^{2+}-ATPase enzyme system, or, somewhat less probably, there are no phospholipids associated with Mg^{2+}-ATPase activity.

However, whichever of these suggestions is correct, it seems reasonable to conclude that the now well-established requirement of ouabain-sensitive $(Na^+ + K^+)$-ATPase for phospholipid (8, 10–12) as well as the determining role of phospholipid in the temperature dependence of this system, must now be regarded as characteristics which distinguish this "transport enzyme" from the Mg^{2+}-dependent but ouabain-insensitive *basal* ATPase with which it is closely associated within the membrane matrix.

It is also reasonable to conclude that the influence of phospholipids upon the activation energy of ouabain-sensitive *transport* ATPase must have profound implications

for active sodium transport mechanisms in hibernating mammals and other temperature dependent species (22–24).

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$^{45}Ca^{++}$ UPTAKE AND ATPASE ACTIVITY OF SARCOPLASMIC RETICULUM (SR)

The role of Ca^{++} ions in the control of metabolic and mechanical activity by muscle fibres is well known, but the biochemical mechanism by which these effects are mediated is not yet clear. However, it is now well established that the membranes of the SR have the property of rapidly lowering the concentration of free Ca^{++} from about 10^{-6} M to below the threshold necessary for the activation of the contractile mechanism, *i.e.* $>10^{-7}$ M (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1961, 1963).

In vitro the membranes of the SR can be isolated from muscle homogenates as closed vesicles. In the presence of an energy source such as ATP, and a Ca^{++} precipitating agent like oxalate, large amounts of Ca^{++} may be accumulated within the interior of these vesicles. During this accumulation of Ca^{++} there is a concurrent utilization of energy reflected by the hydrolysis of ATP$\rightarrow$ ADP $+$ Pi which is mediated by a Ca^{++} dependent 'ATPase' reaction located in the vesicular membrane (Hasselbach, 1964*a*,*b*).

Whether this process represents a nucleoside-enhanced form of Ca^{++} binding (Ebashi, 1965) or a true metabolically dependent Ca^{++}-transport system (Hasselbach, 1964*a*,*b*) has been the subject of much investigation. Today many workers favour the latter view.

EFFECT OF CHEMICAL AGENTS AND DRUGS

One approach to this problem has been to determine whether a variety of chemical and pharmacological agents affect both the uptake of Ca^{++} and the hydrolysis of ATP by SR to the same extent (Hasselbach and Makinose, 1963; Martonosi, 1964; Martonosi and Feretos, 1964).

Some of our recent data on this point are shown in Table I. Clearly the findings with the $-$SH binding agents N-ethyl-maleimide (NEM) and mersalylic acid (Salyrgan) show a high degree of similarity and suggest that both the Ca^{++} uptake and Ca-ATPase of SR require functional $-$SH groups for operation.

Furthermore, the data given in Figure 1 show that at 4×10^{-5} M Salyrgan there is no

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TABLE I *Inhibition of Ca^{++} uptake and Ca-ATPase of sarcoplasmic reticulum by various agents**

Additions (M)	Ca^{++} uptake	Ca-ATPase
1×10^{-3} NEM	91	86
5×10^{-5} Salyrgan	61	58
1×10^{-3} quinidine	95	34
2×10^{-2} procaine	70	20

* Values are in % inhibition of untreated control and are the means of duplicate assays performed on 3 separate preparations of rabbit muscle sarcoplasmic reticulum.

inhibition of either Ca^{++} uptake or Ca-ATPase activity. When the concentration is increased to 6×10^{-5} M complete inhibition is observed.

This narrow range of effective drug concentration strongly suggests that the —SH groups necessary for Ca^{++} uptake and Ca-ATPase by SR are identical, and that these phenomena occur at the same site.

In general, experiments of this type have frequently shown that whenever Ca^{++} uptake is reduced, then energy utilization is also reduced to a comparable extent (Hasselbach and Seraydarian, 1966), suggesting that Ca^{++} uptake and energy utilization are tightly coupled processes.

On the other hand, the experiments with quinidine and procaine (*cf.* Table I) do not exhibit this same degree of similarity as both these agents apparently reduce the uptake of Ca^{++} to a much greater extent than they affect Ca-ATPase activity.

However, it is possible that agents such as quinidine (Fuchs *et al.*, 1968) and procaine (Bondani and Karler, 1970) act directly on the membrane to alter the permeability properties

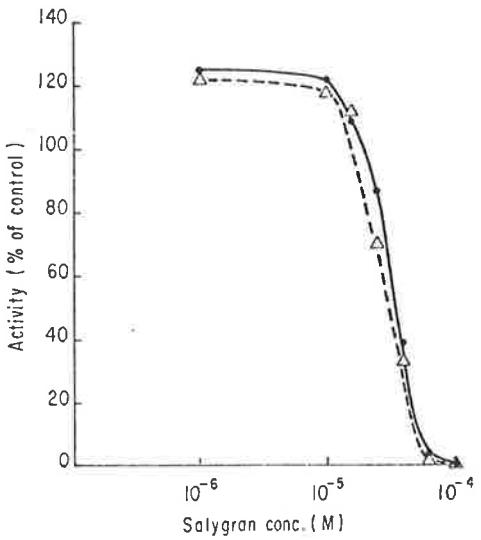

Fig. 1 Effect of varying concentrations of Salyrgan on Ca^{++} uptake (●——●) and Ca-ATPase (△——△). SR preparations were preincubated with Salyrgan for 35 min at 37° C. Control and pretreated SR were then incubated for 1 min for $^{45}Ca^{++}$ uptake determinations and for 5 min for Ca-ATPase activity measurement. All assays were in duplicate.

of SR. If this occurs there could be greater efflux of Ca^{++} from treated vesicles than untreated controls, with an apparently reduced net uptake of Ca^{++}. Both additional Ca^{++} efflux studies and perhaps electron microscope investigations of treated preparations would be required to clarify this point.

EFFECT OF TEMPERATURE

If we examine the effect of temperature upon the uptake of Ca^{++} and Ca-ATPase activity of SR we can avoid the possibility of direct effects of chemical agents on membrane structure and permeability.

An earlier study by Inesi and Watanabe (1967) showed that both the initial rate of Ca^{++} uptake and Ca-ATPase of their SR preparation exhibited very similar temperature dependence. These investigators observed straight line relationships in Arrhenius plots of log product formation versus the reciprocal of absolute temperature (over the range 5° to 20°C and with experimental data points obtained at intervals of 5°C). Their data yielded very similar values for the apparent energy of activation (Ea) for both processes, either in the presence or absence of the Ca^{++} precipitating agent oxalate, again supporting the contention that Ca^{++} uptake and Ca-ATPase are tightly coupled processes. These data are shown in Table II.

TABLE II *Apparent energy of activation (Ea) of Ca^{++} uptake and Ca-ATPase of sarcoplasmic reticulum**

Conditions	Ca^{++} uptake	Ca-ATPase
0.1 mM Ca^{++}	13.0-16.0	13.8-16.0
0.1 mM Ca^{++} + 5 mM oxalate	20.4-30.0	22.6-26.8

* Ea given as kcal/mole. Assay conditions: 5 mM ATP, 5 mM $MgCl_2$, 20 mM Tris-maleate; pH 6.8. (From Inesi and Watanabe, 1967.)

These workers felt that the increase in value for Ea when oxalate was added to the system could be explained by a different rate-limiting step in the two experimental conditions.

Interestingly, at about the same time Yamamoto and Tonomura (1967) published the results of their detailed kinetic study of Ca-ATPase of SR which also included an estimate of the apparent energy of activation measured over the range of 0°-37°C. When the concentration of substrate ATP was 1 mM, which was the highest level employed by these workers, they also observed a straight line relationship which in the absence of oxalate yielded a value for Ea of 26.2 kcal/mole. This is almost double the value of approximately 15 reported by Inesi and Watanabe (1967) under these conditions. Perhaps the difference lies in the different levels of ATP employed in these two studies, with a concentration of only 1 mM ATP in the study by Yamamoto and Tonomura (1967) being rate limiting.

In some recent studies of temperature dependence of a different membrane ATPase system which is also involved in ion transport (*i.e.* Na + K-ATPase) we had observed a non-linear Arrhenius plot over the range 0°-37°C, with a so-called discontinuity or point of inflection in the plot occurring at about 20°C (Charnock *et al.*, 1971*a*, *b*). These data are shown in Figure 2.

The literature contains numerous examples of this type of observation and considerable speculation has occurred as to the physiological significance of such thermal anomalies (Bělehrádek, 1957; Massey *et al.*, 1966; Drost-Hansen, 1970; Kumamoto *et al.*, 1971; Charnock *et al.*, 1971*a*, *b*). For example, Massey *et al.* (1966) contend that the existence of two

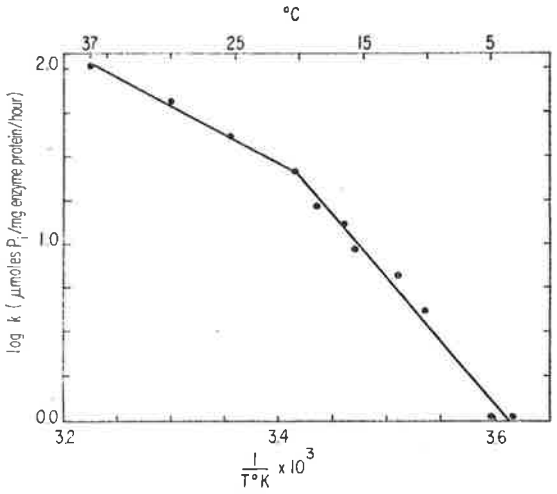

Fig. 2 Arrhenius plot of log rate constant against 1/absolute temperature of ouabain-sensitive ($Na^+ + K^+$)-ATPase of rabbit kidney microsomes. (From Charnock *et al.*, 1971*b*.)

widely divergent energies of activation reflects the existence of two different but stable conformational forms of the same enzyme. We ourselves have speculated for ($Na^+ + K^+$)-ATPase that these forms should display widely different characteristics of ligand binding (Charnock *et al.*, 1971*a*,*b*).

Clearly if an enzyme which displays this phenomenon is involved in ion transport, as is Ca-ATPase, then such a conclusion would have considerable implications in understanding the transport mechanism of the system.

We therefore re-examined the temperature dependence of Ca^{++} uptake and Ca-ATPase of SR over the temperature range of 0°-37° and at a concentration of ATP (5 mM) which was not rate limiting.

In Figure 3 it can be seen that we were able to confirm the linearity of the Arrhenius plot of Ca^{++} uptake by this system, although the mean value for the apparent energy of activation (Ea) was 18.2 kcal/mole. This is somewhat less than the lower limit (20.4-30.0 kcal/mole) reported by Inesi and Watanabe (1967) when oxalate was present in the system.

Conversely, the data shown in Figure 4 do not reveal a linear relationship between temperature and Ca-ATPase activity over this range. There was a marked departure from linearity with the point of inflection occurring at about 10°C, and not at 20°C as was the case with ($Na^+ + K^+$)-ATPase (Charnock *et al.*, 1971*a*,*b*).

The apparent energies of activation obtained from this study are summarized in Table III

TABLE III *Effect of temperature on apparent energy of activation (Ea) of Ca^{++} uptake and Ca-ATPase of sarcoplasmic reticulum**

Temperature range (°C)	Ca^{++} uptake	Ca-ATPase
0-10	18.2	33.4
10-37	18.2	16.3

* Ea given as kcal/mole. Assay conditions are described in Figures 3 and 4.

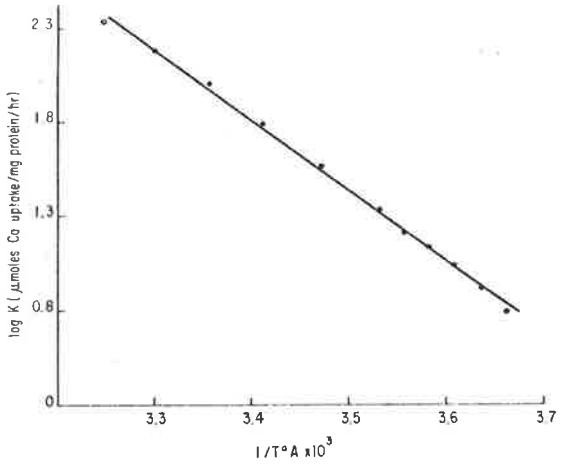

Fig. 3 Arrhenius plot of log rate constant against 1/absolute temperature of $^{45}Ca^{++}$ uptake of rabbit muscle sarcoplasmic reticulum. Assays were for 1 min at temperature shown. Incubation mixture contained 5 mM $MgCl_2$, 5 mM ATP (disodium salt, Sigma) 3 mM potassium oxalate, 0.35 mM $CaCl_2$ plus a tracer amount of $^{45}Ca^{++}$ (specific activity 10 mCi/mg), 0.25 mM EGTA, 50 mM KCl and 5 mM Tris base, pH 7.4.

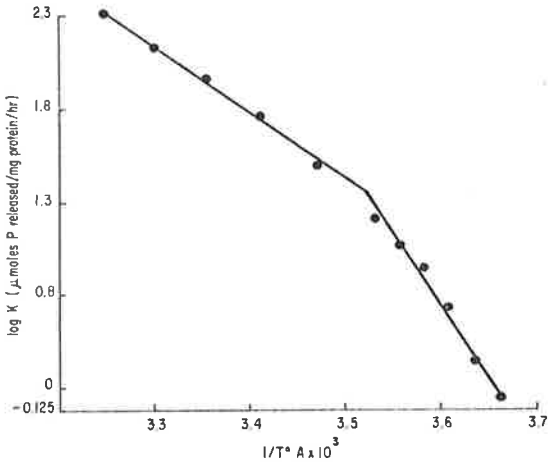

Fig. 4 Arrhenius plot of log rate constant against 1/absolute temperature of Ca-dependent ATPase activity of rabbit muscle sarcoplasmic reticulum. Ca-dependent ATPase activity was calculated as the difference between inorganic phosphate liberation from substrate ATP $\pm$ Ca^{++}. Assay conditions were similar to those given in Figure 3 without addition of $^{45}Ca^{++}$. Incubations were for 5 min at temperatures shown.

where it can be seen that for the higher temperature range (10°-37°C) the value of Ea was 16.3 kcal/mole; this increased to 33.4 kcal/mole below the critical temperature of 10°C.

Whilst a value of Ea of 16.3 kcal/mole for Ca-ATPase is not meaningfully different from that of 18.2 kcal/mole for Ca^{++} uptake above 10°C, there can be little doubt that below the critical temperature of 10°C the value of 18.2 kcal/mole for Ca^{++} uptake is significantly different from that of 33.4 kcal/mole for Ca-ATPase.

Although no explanation can be offered for the difference between our present findings and those of other workers (Inesi and Watanabe, 1967; Yamamoto and Tonomura, 1967), it is important to note that our results are the product of many determinations made at small temperature intervals, and that observer bias has been diminished by fitting linear regression lines by the method of least squares to our experimental data points. The actual calculations were performed by a computer routine developed by Dr. D. Cook of our department for the APL/360 computing system available at the University of Alberta.

Whilst the interpretation of our data must remain conjecture at the present time, these findings suggest that while Ca^{++} uptake and Ca-ATPase of SR membranes may be both morphologically and functionally closely related within the membrane, there is some new evidence to suggest that these sites are neither identical, nor do they utilize the energy of hydrolysis of ATP through identical transformations.

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DEMONSTRATION OF TRANSPORT ADENOSINE TRIPHOSPHATASE IN THE PLASMA MEMBRANES OF ERYTHROCYTE GHOSTS BY QUANTITATIVE ELECTRON MICROSCOPY

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A quantitative electron microscopic method for the demonstration of (Na^+ + K^+)-adenosine triphosphatase (ATPase) in human erythrocyte ghost membranes is described which differs from that employed previously by introducing several modifications which greatly reduce the possibility that variations in section thickness or contamination or sublimation in the electron beam could effect the result. This procedure makes use of the greater electron density of Cs^+ rather than K^+ ions as an activator of (Na^+ + K^+)-ATPase, thus avoiding the lack of specificity of other histochemical or cytochemical procedures. The transient formation of an ion-carrier state during the hydrolysis of ATP by erythrocyte ghost membrane (Na^+ + K^+)-ATPase was shown by a significant increase in membrane density when Cs^+ ions accumulate at the membrane. This accumulation occurs only in the presence of ouabain. The increase in membrane specific gravity was a generalized phenomenon and was not localized at discrete areas of the membrane, suggesting that (Na^+ + K^+)-ATPase "pumping sites" are distributed uniformly throughout the total membrane structure.

Previous work of Skou (41) and Aldridge (3) has shown that Cs^+ ions can substitute for K^+ ions, but not for Na^+ ions, in the activation of transport adenosine triphosphatase (ATPase) (EC 3.6.1.3), that is, that membrane-bound adenosine triphosphatase activity which normally requires both Na^+ and K^+ for full activation and which can be inhibited by low concentrations of cardiac glycosides (12). In a previous study, we took advantage of the greater electron density of Cs^+ ions than potassium ions to demonstrate by quantitative electron microscopy the accumulation of cation by microsomal preparations of transport ATPase under certain experimental conditions (13).

In the experiments reported in this paper, we have extended these observations to the "intact" plasma membranes of human erythrocyte ghost preparations, in which the role of transport ATPase in coupled sodium and potassium transport has been investigated in detail (26–29, 37, 39, 42). The present investigation has been facilitated by modifications in our original techniques of quantitative electron microscopy, which now make it possible to reduce greatly, if not eliminate, the potential effects of contamination and sublimation in the electron beam and variations in section thickness. These modifications, which are relatively simple and have been described in full elsewhere (7, 8), greatly improve the comparison between observations within an experimental series.

We evaluated our procedures further by their quantitative application to the deposition of insoluble Pb salt by this membrane ATPase reaction. Virtually all such methods depend upon the principle that insoluble lead phosphate, which is formed by interaction of the inorganic phosphate end product of the ATPase reaction and lead nitrate in the incubation medium, accumulates at the site of phosphate liberation from the active center of the enzyme (25).

Such methods have had general histochemical use for the localization of membrane phosphatase reactions (20, 21, 31, 32, 47), and many variations have been attempted for the specific identification of transport ATPase (15, 34, 35). However, recently these methods have been extensively criticized for failing to preserve

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enzyme activity in the presence of heavy metals or fixatives and to promote nonenzymatic hydrolysis of ATP and release of inorganic phosphate (6, 36, 38, 45). Because of these and other deficiencies (44), the method is now generally regarded as being of questionable scientific value (1). Nevertheless, some recent modification in fixation techniques (19, 46) and the greatly reduced concentration of Pb^{++} required for our experimental procedure suggested that a reexamination of this problem was warranted.

MATERIALS AND METHODS

Preparation of ghost cells: Human erythrocyte ghost preparations of low hemoglobin content were prepared by hypotonic hemoloysis at fixed pH. The method employed was adapted from that of Dodge, Mitchell and Hanahan (17). Heparinated human blood was obtained from the blood bank and the packed red cells were separated by centrifuging at 1000 × *g* for 20 min at 4°C. The plasma and buffy coat were removed by aspiration and the red cells were washed twice by the addition of 3 volumes of 0.22 *M* sucrose, 12 *mM* glycyl-glycine and 1 *mM* ethylene diaminetetraacetate (EDTA) (sodium salt) adjusted to pH 7.8. The cells were again separated by centrifuging at 1000 × *g* for 20 min at 4°C, and the supernatants were discarded. The packed washed red cells were hemolyzed by the addition of 4 volumes of hypotonic solution (5 *mM* Tris-chloride, 2 *mM* EDTA, pH 7.8) and the hemolysate was separated from the ghost cells by centrifuging at 16,000 × *g* for 10 min at 4°C. This latter step was repeated. Residual hemoglobin of the ghosts was reduced further by washing the preparation three times in 2 volumes of 25 *mM* Tris-Cl (pH 7.8) and separating the pale "hemoglobin-free" red cell ghosts by centrifuging at 16,000 × *g* for 10 min at 4°C. The hemoglobin content of the cells was determined by the spectrophotometric procedure of Crosby, Munn and Furth (16) and was reduced from an original level of 165–185 mg/ml washed red cells to 0.5–1.5 mg/ml washed ghosts by these procedures.

Incubation of cells and enzyme assay: For incubation in the Cs^{+}-containing medium, 5.0 ml packed ghost cells were diluted to a final volume of 10 ml by addition of reagents so that the final composition of the medium (referred to later as medium Cs) was 100 *mM* Tris-glycyl-glycine buffer, pH 7.6, 80 *mM* NaCl, 20 *mM* CsCl, 2 *mM* $MgCl_2$. Two millimolar disodium ATP and 0.1 *mM* ouabain were included where indicated in the tables and text. Incubations were at 37°C and for varying times given in the tables and text. After the desired incubation time, the reaction was stopped by rapid cooling of the flasks and contents to below 4°C and separation of the cells from medium by centrifuging at 16,000 × *g* for 5 min at 2°C. Supernatants were collected by aspiration, acidified and used to determine inorganic phosphate liberation from ATP by methods previously described in full (10).

For incubation in the Pb^{++}-containing medium (referred to later as medium Pb) 20 *mM* KCl was substituted for the cesium salt and 0.1 *mM* Pb $(NO_3)_2$ was added to the medium described above. This concentration of Pb^{++} was shown by preliminary experiments to inhibit partially, but not abolish, ouabain-sensitive transport ATPase activity (46). Incubations were at 37°C for the times indicated in the tables and text. Enzyme activity was again determined by the methods referred to above (10).

Morphologic examination of ghosts: Ghosts cells were harvested after separation by centrifugation and examined either as unstained preparations under phase contrast microscopy or as a stained preparation after treatment with Victoria blue 4R (46). Only preparations revealing large numbers of *intact* ghost membranes were processed for electron microscopic examination and enzyme activity. These preparations were fixed in 6.25% glutaraldehyde in 36 *mM* Na acetate, 36 *mM* Na barbitone buffer, pH 7.6 (9), for 1 hr at 4°C. The cells were then washed overnight in the buffer to remove any remaining glutaraldehyde and postfixed in 2% osmium tetroxide in the acetate-barbitone buffer. Dehydration was achieved by serial extraction with acetone and the preparations were then exposed to acetone-Araldite mixtures (without accelerator) of increasing Araldite concentration over a period of 3 days (46). Finally the ghost preparation was exposed to 100% Araldite plus accelerator for about 7 hr before polymerization at 50–60°C for a further 3 days.

Silver-gray sections were mounted on carbon-collodion films and examined without further staining at 60 kV in a Siemens Elmiskop I, fitted with accessory voltage stabilizers, a 10-μM objective aperture and a Faraday cage exposure meter. The Faraday cage was used to select sections of ~50 nm thickness by comparing their transmission with that of the naked carbon film (8) and to standardize the photographic exposures.

The current passing through a naked portion of the carbon film was measured and adjusted to a constant value so that a constant exposure time could be used. All of the other photographic conditions were kept as constant as possible; Ilford Special Lantern Contrasty plates were used and were developed for 4 min in Ilford PQ-Universal developer (1:9) at 20°C. These conditions gave plates which had optical densities (O.D.) of ~2.5 for the images of the naked film, ~1.5 for the empty resin and ~1.4 to ~1.0 for the membranes.

Plates were exposed at a standard magnification

of 40,000× which was checked with a grating replica. They were taken "blindly" without the observer being aware of the conditions of the experiment and with only enough attention being paid to the membranes to ensure that there were some in the field of view. Approximately two plates were examined from distant regions of each section. Each plate included a portion of naked carbon film, either at the edge of the section or via a hole completely through it. In spite of these new measuring techniques, which eliminate most of the errors due to contamination and sublimation, gross differential variations in these from membrane to membrane and experiment to experiment could still produce some errors. Therefore, an experimental routine was evolved to minimize this possibility. The minimal possible illumination was adopted as a standard, with the double condenser adjusted to give the minimal illuminated area; focusing was performed on a region adjacent to that to be photographed, so that only rapid minor final adjustments needed to be made; and the selected region was only exposed to the beam for a brief period. That the amount of sublimation was indeed minimal was confirmed on a number of occasions by subsequent micrographs taken at low power when the photographed region was compared with unexposed areas (8). An anticontamination device is fitted to our electron microscope (Siemens Elmiskop I), but it was not used in these experiments since this instrument is characterized by a very low contamination rate and because of the danger of removing large amounts of material from the specimen (4).

The plates were measured, again in a blind random sequence, in a Joyce-Loebl IIIC microdensitometer, using a slit width of 30 μm, which was $\sim 1/10$ of the width of the images of the membranes. The calibration of the recordings was done with the aid of external standard neutral density filters (±0.01 O.D.). Random number tables were used to select the portion of membrane to be measured. This was rejected if "unit membrane" structure was not visible, or if there was any technical inadequacy.

One portion of membrane was traced per plate. The difference in optical density (M) was measured between the image of the naked carbon film and the least dense portion of the image of the membrane: the difference (R) was also obtained between the images of the film and the mean of the empty resin. (It was necessary to use the resin outside the ghosts since their interiors usually still contained enough material to make their images ~0.1-0.2 O.D. more transparent.)

It has been shown previously (7, 8) that for the dry specific gravity (S.G.) of the membrane,

$$S.G._{(membrane)} = \frac{(10^M - 1)}{(10^R - 1)} \times S.G._{(resin)}.$$

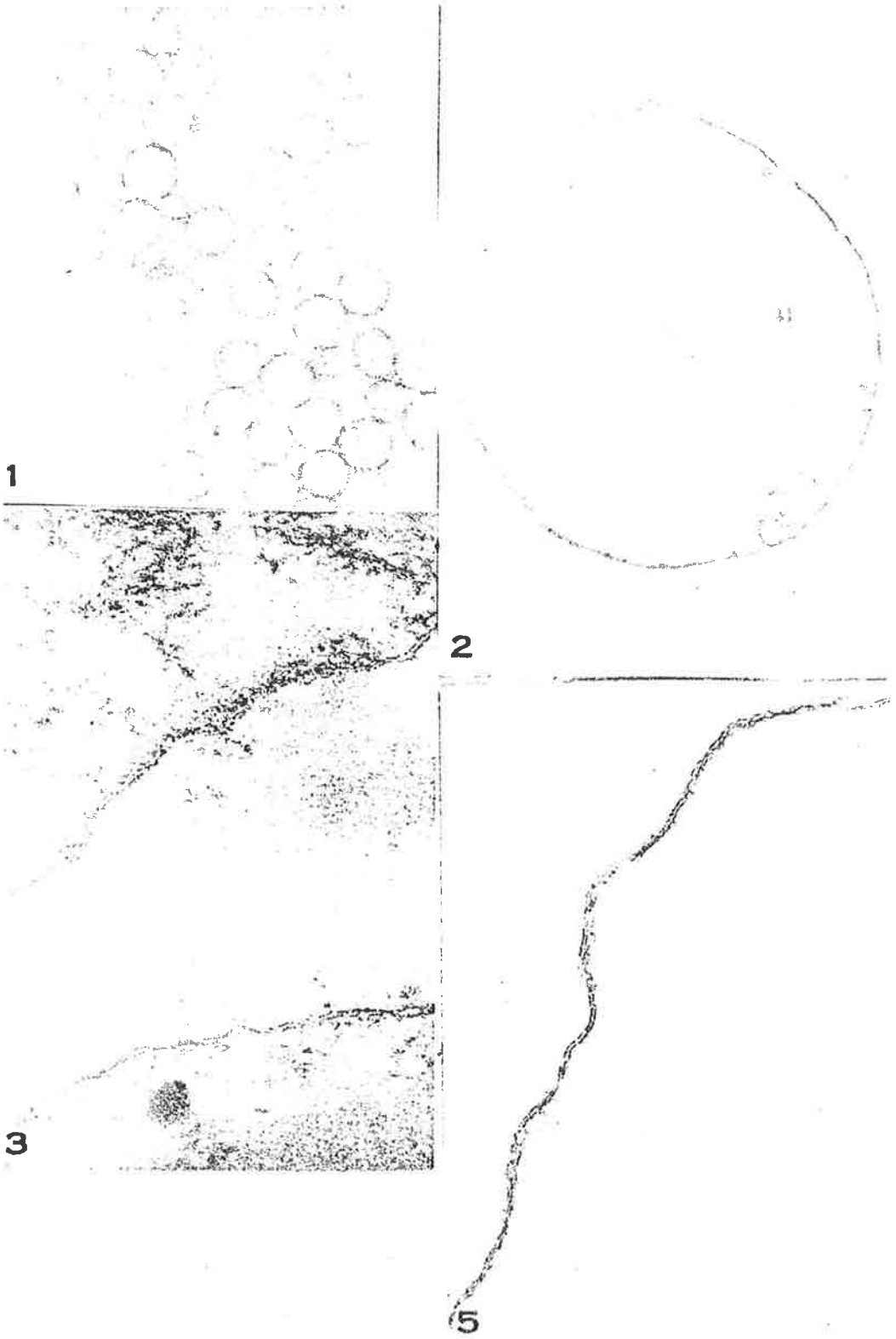
This relationship is independent of section thickness and of the effects of sublimation or contamination, provided that they are uniform for the resin and the membrane. Since we wished only to compare the various membranes with each other, using the S.G. of the resin as a constant, even differential variations of contamination and sublimation between the membrane and the resin would not effect the results, provided that the differential is constant from one experiment to the next. In this paper, the results are expressed as the ratio by which the S.G. of the resin must be multiplied to yield the dry S.G. of the membranes. It should be noted that, while it was theoretically better to compare the integrals of the traces of the membranes, the finite slit width of the microdensitometer made it impossible to determine the actual start of the membrane portion of the trace; hence, the maximal height of the trace was used since this is also a function of the S.G. of the membrane. Statistical analysis of the experimental data was carried out by the unpaired t-test modified for unequal variances (43).

RESULTS

Although we employed a random rather than a selective method for sample selection in this study, the plasma membranes of the red cell ghosts were always intact. This is shown by both phase contrast light microscopy and low power electron microscopy before incubation of the cells (Figs. 1 and 2). At the higher magnification required for membrane examination after incubation, it was often difficult to distinguish between the inside and the outside surface of these structures (Fig. 3) until microdensitometry traces were obtained which revealed the presence of residual electron-dense material near the inner surface of the membrane (Fig. 4).

An important criterion in our study has been that concurrent determinations of transport ATPase activity must be obtained for every experiment subjected to a quantitative examination of membrane density. Table I compares the liberation of inorganic phosphate by erythrocyte ghost ATPase in a sodium plus cesium medium to that obtained in the more usual sodium plus potassium medium. As has been reported by other workers (3, 41), there is a reduction in ATP hydrolysis when K^+ is replaced by equimolar Cs^+, but it is apparent that 0.1 mM ouabain inhibited a very similar proportion of the total ATPase activity obtained whether K^+ or Cs^+ was the co-cation with Na^+ in these experiments.

Incubation with Cs^+: In Table II we give the results of a typical experiment in which enzyme activity was established, and in Table III are the microdensitometry values which were obtained following incubation of these erythrocyte ghosts in medium Cs described above. It is apparent from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2
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Table II that the enzyme was active under these conditions and displayed a constant level of sensitivity to ouabain throughout the incubation period. From Table III it can be seen that the mean ratios of the specific gravity of the membrane to the specific gravity of that of the embedding medium (hereafter referred to as S.G. ratio), which were obtained at all time intervals when membranes were incubated in the presence of ATP and ouabain, were significantly greater ($P < 0.01$) than after incubation under control conditions, that is, in the absence of either ATP or ouabain. This increase in ratio signifies an increase in specific gravity, which in our view (13) corresponds to a deposition of Cs^+ ions under these experimental conditions. Clearly, when both ATP and ouabain were present in the incubation system, there was a significant increase ($P < 0.01$) in specific gravity of the erythrocyte ghost membranes even at "zero time" in the experiment. This observation suggests that the formation of a reaction product by the ATPase enzyme is not an absolute requirement for Cs^+ deposition but that some intermediate step in the reaction is more probably involved (2, 10, 13, 14). In addition the mean S.G. ratio also increased after 30 min incubation in the presence of ouabain. This may reflect the accumulation of an intermediate in the system where over-all hydrolysis of ATP

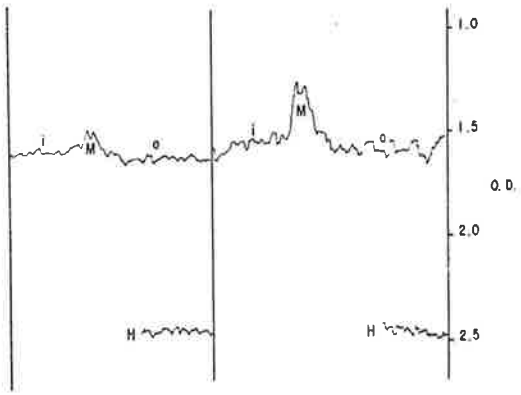

FIG. 4. A typical microdensitometer tracing showing the increase in peak height obtained by scanning the membrane of washed human red cell ghosts. The double peaks are recorded from the inner and outer lamella of the plasma membrane. A control tracing obtained after incubation in medium Cs in the absence of ouabain is shown on the left. The right tracing was obtained after incubation in medium Cs in the presence of ouabain. M is membrane, i is the tracing from inside the cell and o is the tracing from outside the cell. H is the tracing recorded from a hole through the section. The O.D. is given as standard units of optical density.

TABLE I

Effect of Substitution of Cs^+ for K^+ on the Activity of Transport ATPase of Erythrocyte Ghosts

Additions	Specific Activity[a]	Percentage Inhibition by Ouabain
$Na^+ + K^+$	5.88	
$Na^+ + K^+$ + ouabain ...	3.10	47
$Na^+ + Cs^+$	4.93	
$Na^+ + Cs^+$ + ouabain ..	2.64	46

[a] Incubation medium contained 80 *mM* NaCl, 2.5 *mM* $MgSO_4$, 2.5 *mM* Na_2 ATP, 0.12 *mM* EDTA and 62.5 *mM* glycyl-glycine, buffered with Tris base to pH 7.6; 20 *mM* KCl or 20 *mM* CsCl and 0.1 *mM* ouabain were included where shown. Enzyme activity was measured as micromoles inorganic phosphate liberated per milligram red cell ghost protein N per hour at 37°C. Values given are the arithmetic mean of three observations (in duplicate) on 1.0 ml ghost preparation. Final incubation volume was 4 ml.

TABLE II

Enzyme Activity of Erythrocyte Membranes Incubated with Cs^+[a]

Conditions	Enzyme Activity[b]	
	10 min	30 min
$Na^+ + Cs^+$	0.98	2.29
$Na^+ + Cs^+$, ouabain	0.52	1.20
Percentage ouabain inhibition	47	48

[a] Erythrocyte ghost preparation was that also employed in the Cs^+ experiments described in Table III.

[b] Enzyme activity given as micromoles inorganic phosphate liberated by 5 ml ghost suspension/hr at 37°C. The test system was similar to that given in Table I but the final volume was 10 ml.

FIG. 1. Phase contrast micrograph of intact washed human red cell ghosts rendered hemoglobin-"free" by the method described in the text. ×1,330.

FIG. 2. Low power electron micrograph of red cell ghost preparation shown in Figure 1. ×13,000.

FIG. 3. Electron micrograph of the plasma membranes of two adjacent red cell ghosts after 10-min incubation in medium Cs without the addition of ouabain. A small amount of electron-dense material is visible near both membranes, sections of which display trilaminar structure. × 120,000.

FIG. 5. Electron micrograph of plasma membrane of red cell ghost after incubation in medium Cs in the presence of ouabain. ×120,000.

TABLE III

Specific Gravity of Erythrocyte Membranes Incubated with Cs^{+}[a]

Conditions	Specific Gravity[b]		
	0 min	10 min	30 min
Control[c]	1.93 ± 0.12 (14)	2.03 ± 0.18 (10)	1.79 ± 0.06 (12)
+ATP	1.78 ± 0.06 (10)	1.82 ± 0.08 (12)	1.99 ± 0.11 (12)
+Ouabain	2.45 ± 0.29 (10)	2.26 ± 0.16 (10)	1.81 ± 0.08 (10)
+ATP + ouabain[d]	3.15 ± 0.25 (15)	3.02 ± 0.20 (18)	3.62 ± 0.59 (15)

[a] Erythrocyte ghost preparation was that also employed in the Cs^{+} experiments described in Table II.

[b] Specific gravity given as the mean S.G. ratio ± S.E. of the specific gravity of the membrane over that of the Araldite resin (1.3 gm/cc). The number of separate observations is given in parentheses.

[c] Control incubation with Cs^{+} but without either ATP or ouabain.

[d] The values obtained under these experimental conditions are significantly different ($P < 0.01$) from the controls, irrespective of the times of incubation. Probability (P) was determined by the unpaired t-test modified for unequal variances (38).

is blocked by ouabain (2, 10, 11, 13, 14), although the effect is small and not significantly different from that found at shorter incubation times ($P > 0.01$). The mean S.G. ratio under all other incubation conditions did not vary significantly from the controls ($P > 0.01$).

The increase in the standard error of the mean S.G. ratio found in the presence of ATP and ouabain (Table III) implies that by comparison with the control membranes, at least some portions of the membranes are very much more electron-opaque than other areas. Even if one excluded such areas from the evaluation, for example, by excluding all areas having S.G. ratios of more than 3.5, the mean value of the remaining portions of membranes still showed very marked differences from the controls mentioned before.

We attempted to distinguish between an increase in electron opacity at the inner or outer lamella of the unit membranes and found that in these experiments (Table III) the inner lamella was usually somewhat more electron-opaque than was the outer. However, both lamellae were considerably more dense than the controls, and the differential alteration between inner and outer lamellae amounted to only about 10% of the total increase and did not reach statistical significance (*cf.* Fig. 4).

It is important to note that although we found some areas of membranes more electron-opaque than others, we did not obtain clear evidence for discrete localization of Cs^{+} deposits, but rather found that the increase in electron opacity was more generalized over the entire plasma membrane (Fig. 5). These results are in agreement with those reported earlier for microsomal preparations of transport ATPase (13) and suggest that the so-called transport ATPase "pumping sites" are spread over the total erythrocyte surface.

Incubation with Pb^{++}: There can no longer be serious doubt that the concentration of Pb^{++} employed in many histochemical procedures for the localization of phosphatase reactions, including membrane transport-ATPase, is frequently inhibitory to that function of the enzyme system which is necessary to produce the histochemical stain (6, 36, 38, 45). For example, the concentration of Pb^{++} in the procedure of Wachstein and Meisel (47) is 3.6 *mM*, which completely inhibits the enzymatic release of inorganic phosphate from ATP by transport ATPase (46). In addition, it is now known that prolonged incubation of ATP with such concentrations of Pb^{++} leads to the nonenzymatic hydrolysis of ATP (38, 44), which also introduces a serious artifact into the system.

We examined the effect of low concentrations of this heavy metal upon the ouabain-sensitive portion of erythrocyte ATPase activity which was found upon incubation of ghost membranes in a medium containing both Na^{+} and K^{+}. The results are shown in Figure 6 where it can be seen that in this experiment almost total inhibition of (Na^{+} + K^{+})-ATPase activity occurred with a concentration of lead of 0.3 *mM*, and that even 0.1 *mM* Pb^{++} produced 60% inhibition of enzyme activity at the concentration of ghost cells per milliliter of medium employed.

However, this lower concentration of Pb^{++} was selected for the incubation in medium Pb, but the concentration of ghosts per milliliter of system was increased to nearly double that used in the experiments described in Figure 6. The results of the enzyme assay and electron microscope examination are presented below. Table IV shows that control incubations without Pb^{++} revealed ouabain-sensitive ATPase activity after both 5-min and 60-min incubation. When Pb^{++} was included in the medium (Pb), ATP hydrolysis was initially reduced as was

expected, but the enzyme retained some sensitivity to ouabain. As noted previously by Trebilcock (46), the degree of inhibition by Pb^{++} was apparently reduced with increased incubation time, presumably as a result of increased nonenzymatic hydrolysis of ATP and the consequent reduction in free Pb^{++} concentration because of the formation of insoluble Pb phosphate salts. However, some ouabain sensitivity (28%) was retained under these conditions.

No gross change in the electron microscope appearance of these cells was observed during the experiment (Figs. 7 and 8).

The mean S.G. ratio of membranes incubated in the presence of Pb^{++} is given in Table V. There was no change in the mean S.G. ratio after short term incubation in the presence of ouabain with or without Pb^{++}. However, in the presence of Pb^{++} alone, there was a significant increase in the S.G. ratio ($P < 0.01$) after short term incubation, with the mean ratio increasing further after 60-min incubation. Such a result would be predictable if indeed ATPase activity was necessary for the liberation of inorganic phosphate from the enzyme substrate, which then combined with Pb^{++} to form an insoluble Pb salt. Both lamellae of the unit membrane increase in electron opacity in this situation, but in these experiments it was the outer lamella which usually increased slightly more than the inner one (Fig. 9). However, as was the case in the Cs^+ experiments, the differential increase was again less than 10% of the total increase and did not reach statistical significance. Clearly, incubation in this Pb^{++}-containing medium produced an increase in membrane specific gravity which increased with incubation time, in accordance with the view that ATP hydrolysis is required for this effect.

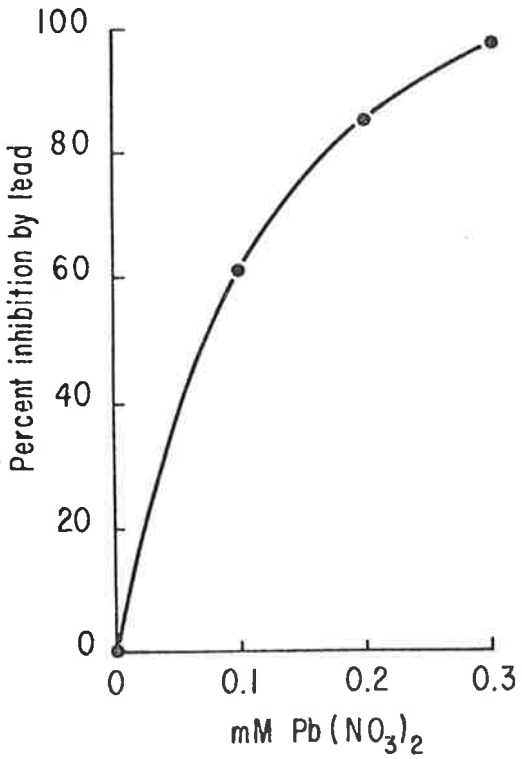

FIG. 6. Effect of lead on ouabain-sensitive transport ATPase, *i.e.*, (Na^+ + K^+)-ATPase, of washed human red cell ghosts.

TABLE IV

Enzyme Activity of Erythrocyte Membranes Incubated with Pb^{++}[a]

Conditions	Enzyme Activity[b]	
	5 min	60 min
Control[c]	0.44	2.22
+ Ouabain	0.28	1.12
+ Pb^{++}	0.35	2.30
+ Ouabain + Pb^{++}	0.28	1.59

[a] Erythrocyte ghost preparation was that employed in the Pb^{++} experiments described in Table V.

[b] Enzyme activity was determined as in Table II.

[c] Control incubation with Na^+, K^+ and ATP but without either Pb^{++} or ouabain.

Thus, in both the Cs^+ and Pb^{++} experiments, a particular set of experimental conditions resulted in a significant increase in the specific gravity of the erythrocyte ghost membranes. Although it was noted in the Cs^+ experiments that some portions of these membranes had much greater than average local increases in specific gravity, inspection of the plates and micrographs obtained after incubation of human red cell ghosts in either medium Cs or medium Pb showed that the increases observed were not due to local large deposits of material; all that was present was a great increase in the electron opacity of the unit membrane.

Because of the demonstration by Gillis and Page (22) that lead phosphate deposits tend to precipitate preferentially at certain proteinaceous structures, and that such clumps of insoluble material have frequently been cited as evidence for the ultrastructural localization of many membrane phosphatase reactions (24) including that of transport ATPase (19, 34, 35), we compared the electron microscope appearance of enzymatically generated changes in electron opacity with that of erythrocyte ghost membranes incubated in an exogenously generated lead phosphate-containing medium.

This was achieved by preloading the erythrocyte

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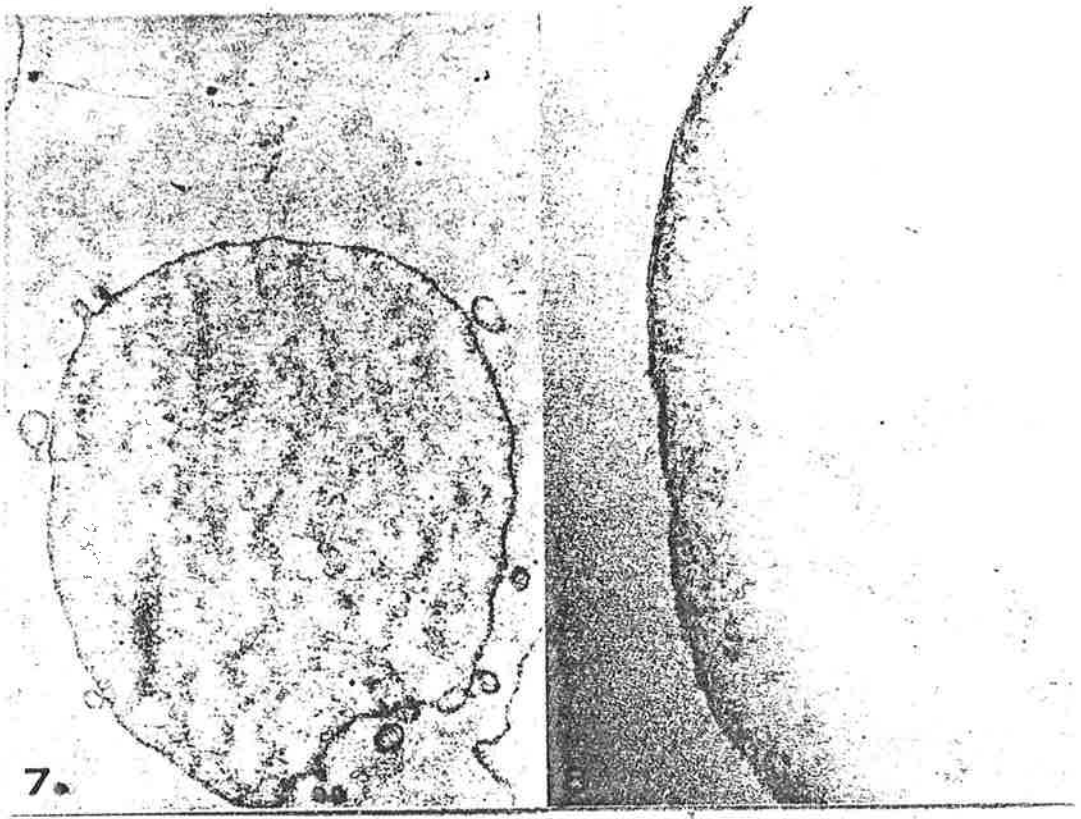

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ghosts with phosphate during the "reversal of hemolysis" stage of their preparation (27). These phosphate-loaded ghosts were then incubated in the absence of ATP, *i.e.*, in the absence of the specific substrate for this membrane phosphatase reaction (10). After 10 min when assay of the medium revealed that the cells had retained the majority (but not all) of the phosphate, lead nitrate was added to the medium. The reactants were then rapidly chilled and the ghost preparation was prepared for an electron microscope study identical to those described above.

The result of this study is given in Figure 10, which shows erythrocyte ghosts after brief incubation in nonenzymatically generated lead phosphate. There are numerous clusters of lead phosphate deposits adjacent to the external surface of the membrane. These deposits bear a striking resemblance to those frequently cited in ultrastructural studies of phosphatase reactions (21, 32) and do not resemble the diffuse but more general increase in membrane electron opacity we observed after incubation in the medium containing a low concentration of Pb^{++} and in which enzymatic generation of inorganic phosphate occurred.

TABLE V

Specific Gravity of Erythrocyte Membranes Incubated with Pb^{++}[a]

Conditions	Specific Gravity[a]	
	5 min	60 min
Control[b]	1.51 ± 0.04 (10)	1.32 ± 0.09 (12)
+Ouabain	1.36 ± 0.03 (12)	1.35 ± 0.03 (12)
$+Pb^{++}$[c]	1.80 ± 0.09 (20)	2.18 ± 0.21 (15)
+Ouabain + Pb^{++}	1.38 ± 0.02 (12)	1.34 ± 0.06 (10)

[a] Specific gravity given as in Table III.

[b] Control incubation with Na^+, K^+ and ATP but without either Pb^{++} or ouabain.

[c] The values obtained in the presence of Pb^{++} alone are significantly different ($P < 0.01$) from those found under all other conditions. Probability (P) was determined by the unpaired t-test modified for unequal variances (38).

DISCUSSION

The integral role of $(Na^+ + K^+)$-ATPase in the coupled transmembrane transport of sodium and potassium ions is now well established (12, 28, 42). Thus, a transient property of cation binding has been postulated for this enzyme system (41), and, indeed, Jarnefelt and von Stedingk (30) and Charnock and Post (10) have demonstrated an ATP-dependent binding of ^{22}Na to microsomal preparations of this enzyme. More recently Lev and Pisareva (33) have described a potassium-stimulated release of Na^+ from preparations of transport ATPase during the hydrolysis of ATP.

In a previous paper (13), we described a semiquantitative electron microscopic procedure for the demonstration of Cs^+ accumulation by microsomal transport-ATPase when ATP was present in the system but its over-all hydrolysis had been blocked by the enzyme inhibitor ouabain. From our earlier work with this enzyme system (10, 11, 14), and that of many subsequent investigators (2), it can be stated that the presence of ouabain under these conditions results in an accumulation of phosphorylated intermediates in the over-all reaction. Our previous results thus indicated an accumulation of Cs^+ ions on the microsomal membrane, concurrent with an increase in the membrane level of phosphorylated components of the transport system. The results of this present study support this interpretation and extend the observation to the intact membranes of erythrocyte ghosts which concurrently demonstrated hydrolysis of ATP via ouabain-sensitive $(Na^+ + K^+)$-ATPase activity.

From the results of the Cs^+ experiments it is apparent that significant ($P < 0.01$) increases in membrane specific gravity occur when both ouabain and ATP are present in the incubation system, but there is no absolute dependence upon incubation time for this effect. In accordance with the commonly accepted view of the biochemical mechanism of ATP hydrolysis by transport ATPase (2), we interpret this result to indicate a significant deposition of Cs^+ on

FIG. 7. Electron micrograph of human red cell ghosts incubated for 5 min at 37°C in Pb^{++}-containing medium in the presence of ouabain. ×12,000.

FIG. 8. Electron micrograph of human red cell ghost membrane after incubation for 60 min at 37°C in Pb^{++}-containing medium in the presence of ouabain. ×120,000.

FIG. 9. Electron micrograph of human red cell ghost membrane after incubation for 60 min at 37°C in Pb^{++}-containing medium in the absence of ouabain. ×120,000.

FIG. 10. Electron micrograph of human red cell ghosts after brief incubation in a medium containing exogenous lead phosphate but without ATP. ×9,000.

the membrane, when the level of phosphorylated membrane components has been elevated and the over-all hydrolysis of ATP to adenosine diphosphate and inorganic phosphate has been blocked by the enzyme inhibitor ouabain. The observed accumulation of this ion on the membrane only when ATP and ouabain are present together suggests that the Cs^+ binding seen here is specifically related to a functional state of $(Na^+ + K^+)$-ATPase and, in contrast to the observations of Hoffman (29), is not influenced by nonspecific binding of Cs^+ ions to the red cell surface.

Furthermore, the data presented here are in accord with the operational involvement of a phosphorylated enzyme "intermediate" in the transient ion carrier function of this enzyme in the transport process (11–14, 30, 33, 41, 42).

From an early study of the effect of cardiac glycosides on the movement of ions in red cells, Glynn (23) was able to calculate that only some 1200–2700 "carrier sites" might be involved per cell. In considering this "remarkably low" estimate he says, "One has, of course, no idea of the size of a carrier site, but on any reasonable estimate it is difficult to avoid the conclusion that only a minute fraction of the cell surface can be employed in ion transport." Since that time it has become fashionable to think of, and, indeed, to seek for, these hypothetically discrete carrier or pumping sites on the red cell surface. Using ^3H-labeled ouabain, Hoffman (29) and Dunham and Hoffman (18) have made even lower estimates of the number of carrier sites per red cell, although a similar study by Baker and Willis (5) results in a very much higher estimate than the values derived by Glynn (23).

Because we are not yet able to provide any evidence on the size of a single so-called carrier site, and that in terms of modern concepts of membrane structure (40) we should consider the possibility of cooperativeness between the various components of biologic membranes (48), it seems reasonable today to think that much larger areas of the cell surface may be involved in ion transport, although the points of attachment to the cell surface of inhibitors such as ouabain could be limited.

Our finding of apparently uniform distribution of accumulated cation across the cell surface is in keeping with this latter view and does not support the belief that discrete pumping sites exist upon the erythrocyte surface.

However, it must be recognized that if such pumping sites were both very small and very numerous, so that the 50-nm thick sections of membranes examined here are large in relation to them, then an averaging effect across the thickness of the electron microscope section could account for our findings.

The significance of the results obtained by the modified Pb staining procedure is much more difficult to evaluate. Although the results suggest that the increase in membrane specific gravity is a direct consequence of inorganic phosphate liberation by $(Na^+ + K^+)$-ATPase and clearly demonstrate an inhibitory effect of ouabain upon this process, the time dependence of the reaction must permit the introduction of major artifacts into the system. Furthermore, although ATP hydrolysis was reduced in the presence of ouabain it was not abolished, and one might have expected some increase in membrane density as a result of phosphate release from this source. No explanation can be offered for the absence of this effect except perhaps that the incubation time was insufficient to demonstrate it.

The concept that insoluble Pb salts preferentially accumulate at the enzymatic site of inorganic phosphate liberation (25) must also be seriously questioned, as "free" ionic Pb^{++} is involved in salt formation, not some form of "bound" phosphate which is involved in the hydrolytic enzyme reaction. Consequently, the absence of discrete deposits of Pb salts in our experiments adds support rather than detracts from the significance of our results. However, in the absence of convincing evidence that Pb^{++} deposition reflects the cytochemical localization of enzyme action, the value of this procedure remains in doubt.

It had been hoped that by examination of the microdensitometry tracings we might have been able to distinguish changes in electron opacity between the inner and outer lamella of the unit membrane structure, but as noted above, the changes observed were not of sufficient magnitude to permit this distinction. On the other hand, the introduction of modifications in our procedures have greatly reduced the possibility of artifacts in our present experiments compared to those reported previously (13). The elimination of most of the effects of

sublimation and contamination by referring the measured optical density to that of the naked film (7) and in the use of the embedding medium as a reference standard which eliminates the effects of varying section thickness (8) has enabled much better comparisons to be made between experiments in the same series.

The possibility of differential sublimation between membranes and resin in the experimental and control groups could still not be totally eliminated. However, if it did exist, and if it were contributing significantly to our measurements, it would still imply a significant interaction between the membranes and the ions in the incubation media under some experimental conditions and not under others. The very minimal amount of sublimation which occurs in our procedure (7, 8) argues strongly that the results are indicative of a true alteration in the specific gravity of the membranes.

It is possible that the sensitivity of the method could be further improved by omitting the postfixation step with osmium. This might necessitate using a very low accelerating voltage and would probably increase the optical density of the plates themselves. The use of image intensifiers both to reduce exposure and improve contrast would also probably improve the method, as might the use of frozen sections or even freeze-dried material. Whether such measures are necessary, in view of the relatively few replications of the observations which are needed to establish the statistical significance of the effects seen in these experiments, becomes a matter of choice for the individual investigator.

In summary, it can be concluded that in both series of experiments it was possible to demonstrate a significant increase in the specific gravity of the membranes under certain experimental conditions which can be readily related to the mechanism of $(Na^+ + K^+)$-ATPase activity. However, from neither series of experiments can the results be interpreted to indicate the cytochemical localization of this enzyme in the sense of discrete or intermittent but recurring sites distributed over the membrane surface.

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Elevated Sodium Plus Potassium Activated Adenosinetriphosphatase in Rats after Chronic Diisopropylfluorophosphate Poisoning

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Abstract—Rats treated with sublethal doses of DFP develop an increase in the specific ($Na^+ + K^+$)-ATPase activity of the heavy microsomal fraction of both brain and kidney homogenates prepared from these animals. The increase in activity was apparent after twelve weeks treatment and after twenty-four weeks the level of activity was about twice that of control animals. The increased enzymatic activity induced by DFP was not apparent two weeks after drug treatment was terminated. Apparently microsomal enzyme induction follows treatment with DFP but rapidly returns to control levels in the absence of this drug.

Introduction

Because a role for acetylcholine and cholinesterase in central neural function is generally accepted, the importance of this system to human and animal behaviour has been extensively examined. Particular emphasis has been given to studies of cholinesterase inhibition and its consequences (1, 2, 3). It is widely held that the toxic effects of acute diisopropylfluorophosphate poisoning are due to the accumulation of acetylcholine at synapses and myoneural junctions, while recovery from a sub-lethal dose of this cholinesterase inhibitor coincides with the recovery of cholinesterase activity. This view must be qualified by the observation that in rats with experimentally induced, chronically low cholinesterase levels, an attenuation of the signs and symptoms of toxicity occurs (4).

As there is a seemingly complete behavioral recovery and an absence of tissue pathology in rats having lowered cholinesterase activity for as long as six months, it might be inferred that other biochemical changes occur to account

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for this adaptation. This paper reports the changes in the specific $(Na^+ - K^-)$-activated ATPase of cerebral and kidney microsomes which occurred in animals during chronic poisoning with diisopropylfluorophosphate.

Experiments were conducted to determine the long term effects of DFP treatment, the time necessary before these changes appeared, and whether or not recovery occurred when drug treatment ceased.

Methods

The tissues for this study were obtained from adult female Wistar rats. Diisopropylfluorophosphate (DFP) was injected into the right gastrocnemius muscle at a dose of 1 mg/kg body weight. This acute dose of DFP was followed by booster doses of 0.5 mg/kg every 72 hours thereafter given alternatively into the right or left gastrocnemius muscle. Peanut oil was used as the biologically inert vehicle for drug administration. The effect of this treatment on cholinesterase activities of a number of different tissues of the rat has been reported elsewhere (4).

At least four rats were used for each treatment; they were killed by decapitation, the brain and kidneys removed and the wet weight of these tissues was obtained. The tissues were then stored for variable periods at $-26°$ and thawed immediately before preparation of the enzyme. For kidney preparations the cortex was dissected from medullary tissue, whereas with cerebral tissue the white matter was cut away. Pooled tissue from at least four animals was then homogenised in a buffered 0.25 M sucrose medium which has been described previously (5). Differential centrifugation was employed for the separate removal of a combined cell debris, erythrocyte and nuclear fraction, at 1,000 g for 20 min; the mitochondrial fraction was then separated at 8,000 g for 20 min; the post-mitochondrial supernatant was then centrifuged at 35,000 g for 30 min and the microsomal pellet collected. All centrifugations were carried out in the S-34 angle rotor of a Servall RC2 refrigerated centrifuge at $+2°$. The microsomal pellet was transferred to a hand-operated homogeniser (volume 50 ml) and gently resuspended in 35 ml of 0.25 M sucrose by a few strokes with a teflon pestle. The particulate material was again separated by centrifugation at 35,000 g for 30 min. The gelatinous pellet so obtained was resuspended once more in a hand-operated homogeniser in a volume of 0.25 m sucrose so that the pellet derived from 2 g wet weight of tissue was dispersed in 1 ml of fluid.

These preparations are similar to the "heavy" microsome fractions used in previous studies of this enzyme (5) and were again stored for variable periods at $-8°$ until assayed for enzyme activity. Because of the effect of storage time on enzyme activity (13, 14, 12) the preparation and assay of enzymes from the tissue of control and experimental animals was always performed simultaneously. The use of an untreated control group of animals with each treatment group was also adopted for this study in order that any effect of variable storage

time would be nullified. Results are thus presented as % of their appropriate controls.

The assay of ouabain-sensitive ($Na^+ + K^+$)-activated ATPase activity was performed at 37° using the procedure described in full elsewhere (5). In this assay system, the hydrolysis of ATP is measured in the presence of ATP (2 mM), Mg^{2+} (2 mM), Na^+ (80 mM) and K^+ (20 mM) both in the presence and absence of 10^{-4} M ouabain. ($Na^+ + K^+$)-ATPase is taken as the difference in activity between these two conditions (8). The protein content of each enzyme suspension was estimated by the method of Lowry *et al.* (6). The quantity of enzyme suspension added to each assay system was adjusted after preliminary assay if this was necessary, to ensure that the velocity of ATP hydrolysis was linear with respect to time for at least 15 minutes. Values of activity were expressed as μmoles inorganic phosphate liberated from substrate ATP/mg enzyme protein nitrogen/hour at 37° C.

Results

Initially, experiments were performed to compare the specific activity of ($Na^+ + K^+$)-activated ATPase in the "heavy" microsomal fraction from the brains of rats which had been treated with DFP for twenty-four weeks, with that of an identical group of untreated rats from the same colony. In all experiments, treatment was terminated 24 hr before the animals were killed in order to reduce the possibility that DFP itself might be carried over into the enzyme preparation and perhaps interfere in the enzyme assay.

TABLE I

The effect of Chronic DFP Treatment on the ($Na^+ + K^+$)-activated ATPase of Rat Brain Microsomes

Treatment	No. of Animals	Mean Wet Weight (g) of Brain	(1) Specific Activity of ($Na^+ + K^+$)-ATPase	% Control
none	40	1.51	165 ± 16	100
24 weeks DFP	8	1.56	290 ± 23	176

(1) μmoles Pi/mg protein N/hour at 37°.

The results are shown in Table I. It can be seen that the mean specific activity of ($Na^+ + K^+$)-ATPase from 40 rat brains pooled from 10 separate experiments was 165 ± 16 μmoles Pi/mg protein nitrogen/hr; whereas, that from chronically

treated rats obtained in two separate experiments was 290 ± 23 μmoles Pi/mg protein nitrogen/hr. This level represents a 76 % increase over that of the control group.

The second group of experiments was performed both to confirm this initial finding and to define some limits on the time of onset of this increased enzyme activity. It is apparent from the data shown in Fig. 1 that again a marked increase in enzyme activity occurred after treatment with DFP. In these experiments the level of enzyme activity was increased by 35 % over the controls after twelve weeks of treatment, continued to rise during the period of treatment, and was increased by more than 100 % after twenty-four weeks of treatment. Although the enzyme activity in brains of animals treated with DFP for less than twelve weeks appears to be somewhat elevated above the control levels, this increase is not statistically significant.

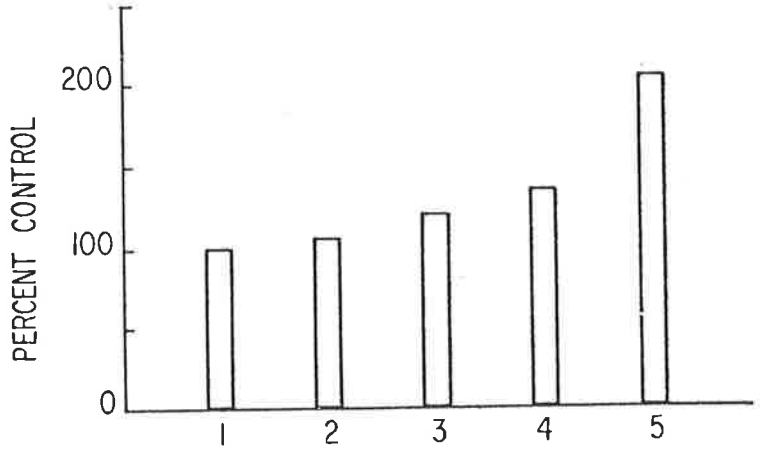

FIG. 1

Effect of duration of treatment with DFP on $(Na^+ + K^+)$-ATPase of rat brain heavy microsomes. Treatment times: 1, untreated control group; 2, four weeks; 3, six weeks; 4, twelve weeks; 5, twenty-four weeks. Values shown are the means obtained from at least 4 animals per group.

In another group of experiments, rats which had been treated with DFP for twelve weeks were killed one, two or three weeks after cessation of this treatment. A group of untreated rats, was again included as controls.

The result of this experiment is summarized in Fig. 2. Again, there is an increase in $(Na^+ + K^+)$-ATPase activity of 35 % over control levels in the brains of the DFP-treated animals. Two weeks after cessation of treatment, the level of enzyme activity had returned to that of the control animals.

To determine whether the increases in enzyme level seen in these experiments were localized to the brain, or represented a more generalized response to DFP treatment, the enzyme activity in the kidney cortex was determined in a number

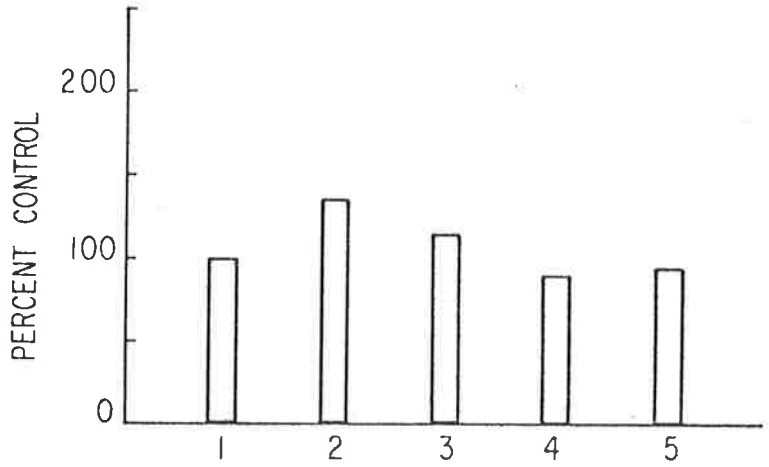

FIG. 2

Effect of withdrawal of treatment with DFP on ($Na^+ + K^+$)-ATPase of rat brain heavy microsomes. Treatment: 1, untreated control group; 2, twelve weeks with DFP; 3, one week after withdrawal following twelve weeks of treatments with DFP; 4, two weeks after withdrawal of treatment; 5, three weeks after withdrawal of treatment. Values shown are the means obtained from at least 8 animals per group.

of these animals. Heavy microsome preparations from the kidney cortex were used to examine this postulate. Unlike Duggan and Noll (7) we found that ($Na^+ + K^+$)-activated ATPase obtained from rat kidneys was always present at a specific activity comparable with that previously reported for guinea-pig

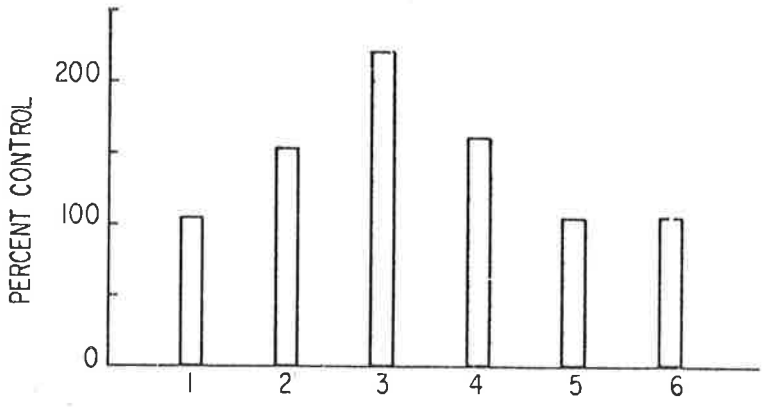

FIG. 3

Effect of duration of treatment with DFP and withdrawal from treatment, on ($Na^+ + K^+$)-ATPase of rat kidney heavy microsomes. Treatment: 1, untreated control group; 2, twelve weeks on DFP; 3, twenty-four weeks on DFP; 4–6, one, two and three weeks after withdrawal after twenty-four weeks of treatment. Values shown are the means obtained from at least 8 animals per group.

preparations (5, 8), and these activities were obtained without pre-treatment of the enzyme preparation with detergent (7).

Following administration of DFP to the rats, a marked similarity in the response of the kidney cortex $(Na^+ + K^+)$-ATPase to that of the brain was found. The level of enzyme activity in untreated controls was 115 ± 18 μmoles Pi/mg protein nitrogen/hr. This was increased by 53 % after treatment for twelve weeks and 120 % after treatment for twenty-four weeks. $(Na^+ + K^+)$-ATPase activity in the kidney was also found to return to control levels two weeks after withdrawal of treatment with DFP (Fig. 3).

Discussion

$(Na^+ + K^+)$-ATPase was selected as an enzyme for study in these animals because of the role of this enzyme in cation transport and the related phenomenon of neuronal excitability (8, 10). There is ample evidence for the presence of the enzyme in considerable amounts in the membrane elements of neuronal tissue (9).

The results presented in this paper demonstrates a marked increase in sodium and potassium stimulated ("transport") ATPase activity in the heavy microsomal fraction of rat brain and kidney after chronic treatment of the animal with DFP. The increase in activity refers specifically to that microsomal enzyme system which requires Mg^{2+}, Na^+ and K^+ for activity and which is inhibited completely by 10^{-4} M ouabain (8, 10).

The increase in activity is shown as specific activity per unit microsomal protein nitrogen and expressed in this way can be interpreted as showing a real increase in enzyme activity rather than simply an increase in the microsomal yield. This increase in activity is apparent in both brain and kidney tissue after treatment of the animals with DFP for twelve weeks, and becomes very marked after treatment for twenty-four weeks. The level of enzyme activity returns to that of control animals two weeks after cessation of treatment.

It is tempting to believe that this increase in "transport" ATPase activity is an adaptive response to the harmful effects of the organophosphorus compound. There is evidence that DFP is a potent inhibitor of the $(Na^+ + K^+)$-activated ATPase of beef kidney membranes (10, 11) and the enzyme appears to be identical to that prepared from rat cerebral or renal cortex. While this adaptive response need not be in any way connected with behavioural recovery noted previously (4), it may constitute an important clue as to the direction for further investigation.

Acknowledgments—We are grateful to Miss P. Sutterby, Miss H. Potter and Mr. D. McKee for their expert technical assistance and care of the animals. The work of one author (P.H.G.) was supported by a grant from the U.S. Public Health Service (NB. 04427) and the Australian Research Grants Commission.

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ETHACRYNIC ACID ACCUMULATION BY RENAL TISSUE

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Abstract—During incubation in isotonic glucose–salt medium, thin slices of rabbit kidney tissue accumulate [2-^{14}C]ethacrynic acid against a concentration gradient. Saturation does not occur in 60 min of incubation, by which time the accumulation is about 10-fold when expressed as a slice : medium ratio. Electron microscopic examination of tissue integrity revealed that there was some swelling and other associated changes after 60 min of incubation also, and the experiments were thus not carried out for longer periods. [2-^{14}C]ethacrynic acid uptake was markedly lowered by reduction of incubation temperature, omission of glucose from the medium or anoxia. Addition of the inhibitors of cellular metabolism, 2,4-dinitrophenol or sodium azide, or addition of either probenecid or ouabain, which are known to block transport processes, also resulted in a striking decrease in drug accumulation. These results strongly suggest that a metabolically dependent active transport process is involved in the accumulation *in vitro* of ethacrynic acid by thin slices of rabbit kidney tissue. When slices of rabbit liver tissue were substituted in these experiments, the degree of [2-^{14}C]ethacrynic acid uptake was greatly reduced and may represent only nonspecific binding to tissue slices. It is suggested that the capacity of renal tissue to accumulate ethacrynic acid *in vitro* may reflect a process which *in vivo* enables significant concentration of this drug at pharmacological receptor sites within renal cells. Thus, observed plasma levels of this drug in whole animals may not represent effective pharmacological concentrations.

ALTHOUGH the diuretic and natriuretic action of ethacrynic acid is well documented, the biochemical mechanism by which these effects are mediated is not fully understood.[1–3] Several recent studies have shown that the distribution of water and the movement of electrolytes in renal tissue are effected by ethacrynic acid,[4,5] but whether these effects follow an inhibition of cellular energy production[4,6] or a more direct action upon membrane transport systems is not clear.[3,7]

In addition, several recent reports[8,9] have confirmed the inhibitory action of ethacrynic acid upon isolated microsomal preparations of the cardiac glycoside sensitive sodium-plus-potassium activated adenosine triphosphatase, ($Na^+ + K^+$)-ATPase, which is thought to be involved in the so-called "sodium pump".[10] However, this effect can only be demonstrated *in vitro* at a drug concentration which greatly exceeds that reported in the plasma of experimental animals during diuretic episodes.[11]

A number of investigators have suggested that this discrepancy might be explained by some form of drug accumulation by renal tissue,[11,12] although the data available do not provide direct evidence for this hypothesis.[3,9]

We have sought further information regarding possible drug accumulation *in vivo* by examining the uptake of [2-^{14}C]ethacrynic acid by thin slices of rabbit kidney tissue under a variety of experimental conditions, which include the effects of inhibitors of cellular metabolism and transport. The greatly increased level of cellular organization

present in these experiments, compared to those with isolated enzyme preparations, has enabled us to show that ethacrynic acid is actively accumulated against a concentration gradient, that this process is dependent upon temperature and substrate, and can be markedly reduced by anoxia, dinitrophenol, azide, ouabain and probenecid.

MATERIALS AND METHODS

Slices of fresh rabbit kidney or liver were cut by hand microtome[13] to a uniform thickness so that when trimmed to 1 cm^2 the slices had a mean initial weight of 50 $\pm$ 5 mg. The first slice was discarded so that all slices used had two cut surfaces. The trimmed weighed slices were kept ice-cold in isotonic glucose–salt medium of the following composition: 143 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 10 mM CH_3-COONa, 5 mM Na_2HPO_4, 1 mM $CaCl_2$ and 10 mM glucose, adjusted to pH 7·4 by the addition of 0·1 N HCl. The osmolarity of this solution was determined by a Standard laboratory osmometer freezing point depression apparatus (Advanced Instruments Inc.) and was 314 mOsmoles/l.

For the incubation of tissue, [2-^{14}C]phenoxy-acetic ethacrynic acid (obtained as a gift from Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A.) was adjusted to pH 7·4 by the addition of solid Tris base (Schwarz/Mann ultrapure) and added to the incubation medium to give a final concentration of ethacrynic acid of $7{\cdot}4 \times 10^{-7}$ M. This concentration corresponds to that plasma level which might reasonably be expected to follow administration of a low clinical dose (10–50 mg) of this drug to man.

Either pure oxygen or nitrogen (for the anoxic experiments) was vigorously bubbled through this solution for at least 1 hr prior to use. When used, inhibitors were added to the medium prior to gassing. The concentrations employed are given in the text. Each tissue slice was incubated in a final volume of 5 ml of solution. The flasks were placed into a Dubnoff metabolic shaking apparatus at the required temperature and continuously flushed with gas throughout the incubation period. After incubation for the required times, the tissue slices were removed from their flasks with flat-nosed forceps and twice dipped into 10 ml of fresh medium without ethacrynic acid; the slices were then drained on absorbant tissue and transferred to vials for radioactivity counting. The tissue was dissolved overnight in 0·5 ml of commercially available NCS tissue solubilizer (Amersham/Searle) and 10 ml of toluene fluor was added (containing 5% BBS2, Beckman Biosolv and 0·5% 2,5-diphenyloxazole). Two drops of 4% $SnCl_2$ in 0·1 N HCl were then added and the tissues dark adapted overnight prior to counting in a Beckman LS-100 liquid scintillator. The samples were counted to a preset error of 3 per cent. The incubation medium was also counted to enable expression of results as slice:medium ratio at all time points during the experiment.[14]

Chromatography. After incubation in [2-^{14}C]ethacrynic acid, some slices were resuspended in 2 ml of ice-cold distilled water for at least 48 hr. The tissue was then separated by centrifuging at 0°, and the protein-free aqueous extracts so obtained were concentrated by evaporation under reduced pressure and spotted on strips of Whatman No. 1 paper. Ascending chromatography in butanol–acetic acid–water solvent (120:3:50) was carried out for 3 hr, according to the procedure of Bourke *et al.*,[15] using authentic ethacrynic acid as a marker. Spots were visualized under ultraviolet light, cut from the paper, and counted for radioactivity as above.

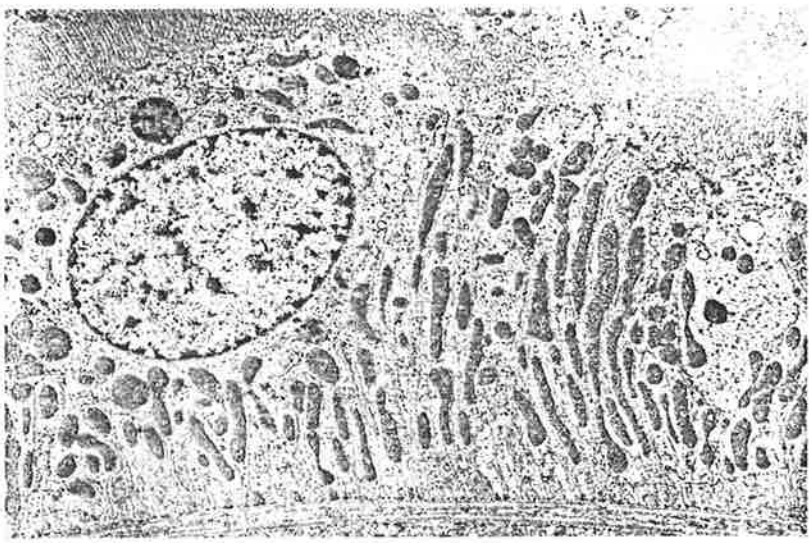

FIG. 1. Electron micrograph of untreated rabbit kidney tissue slice before incubation (magnification 5×10^3).

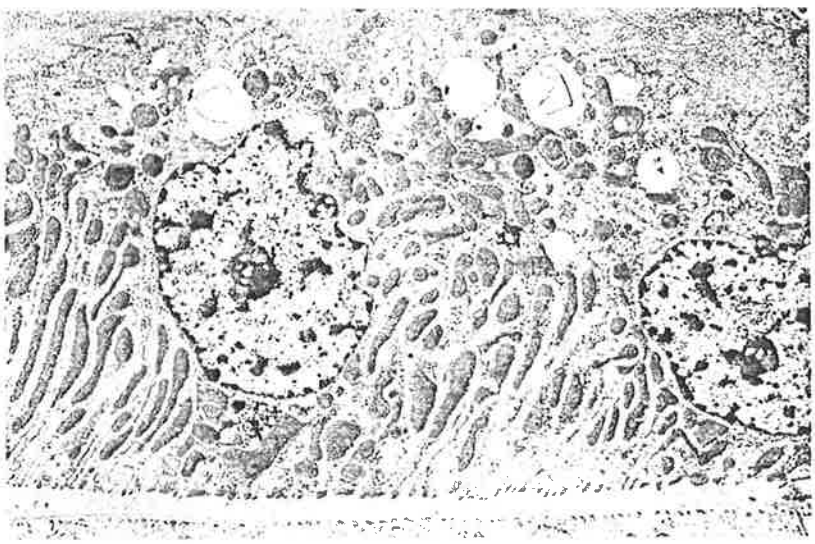

FIG. 2. Electron micrograph of untreated rabbit kidney tissue slice after 10 min of incubation at 37° in an oxygenated isotonic medium containing the salts and glucose as described in the text (magnification 5×10^3).

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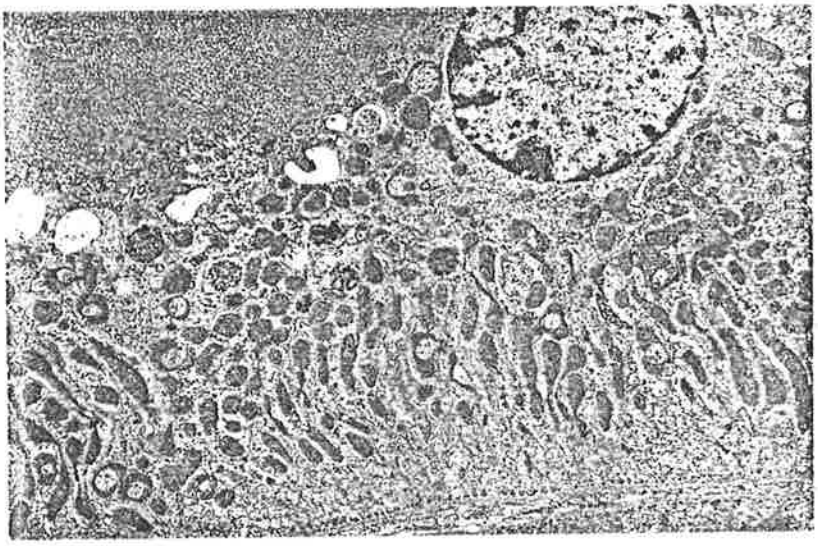

FIG. 3. Electron micrograph of rabbit kidney tissue slice after 10 min of incubation at 37° in an oxygenated isotonic medium containing the salts and glucose as described in the text, and with the addition of $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid. The medium was buffered to pH 7·4 by the addition of Tris base (magnification 5×10^3).

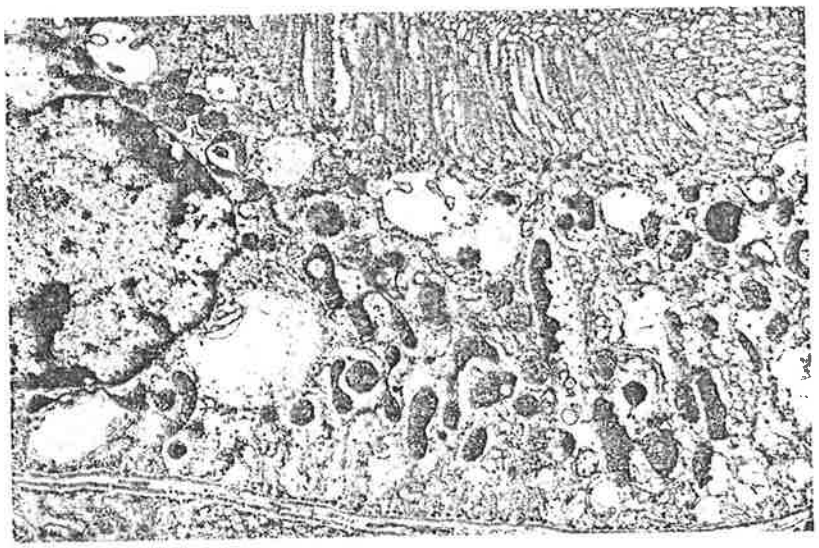

FIG. 4. Electron micrograph of rabbit kidney tissue slice after 60 min of incubation in the medium and under the conditions described in Fig. 3 (magnification 7×10^3).

Extracellular space. A measure of extracellular space was obtained from the distribution of [1-^{3}H]mannitol as described by Rosenberg *et al.*,[14] who made use of the observation that mannitol was not actively transported into cells.[16] The distribution ratio (disintegrations per minute per gram wet weight of tissue: disintegrations per minute per milliliter of incubation medium) was obtained after various incubation times.

Electron microscopy. Tissue slices were fixed in 5% glutaraldehyde in phosphate buffer (990 mOsmoles/l.), post-fixed in OsO_4, and dehydrated by serial treatment with graded ethanol before embedding in Epon. Sections were stained with uranyl acetate and lead citrate before examination in the electron microscope.

RESULTS

Tissue integrity. Electron microscopic examination was carried out on slices of rabbit kidney used in these experiments. Both freshly cut tissue and incubated slices were examined. Figure 1 shows control tissue before incubation. The basal membranes were intact with the nucleus, nuclear envelope, microvilli and mitochondria apparently normal. No signs of swelling or cellular disruption, were evident. After short-term incubation, there were some changes in mitochondrial morphology with a few organelles appearing vacuolated (Fig. 2). No marked differences were seen when ethacrynic acid was present in the medium, other than a slight increase in mitochondrial vacuolation (Fig. 3).

After 60 min of incubation, however, there was evidence of tissue swelling. The basement membrane, plasmalemma and nuclear membrane were intact, but cell organelles and microvilli were swollen. There were many more vacuolated mitochondria, although others appeared unchanged from the control before incubation. Occasional myelin figures were now present. Although no clear differences were seen, the addition of ethacrynic acid to the incubation medium may have enhanced these changes (Fig. 4). Marked cellular disruption or membrane damage was not observed under any experimental conditions, but sufficient changes and swelling had occurred to suggest that incubations should not be prolonged beyond 1 hr.

Some investigators have attributed the experimental effects of ethacrynic acid to direct interaction with anaerobic[6] or oxidative[17] metabolism of the cell. In both studies cited, the concentration of ethacrynic acid was at least 1000-fold greater than that used in the experiments reported here. The pharmacological relevance of experimental effects observed with such high drug concentrations must remain conjecture until a drug-accumulating mechanism is demonstrated. In our experiments, morphological evidence did not suggest significant cell damage by ethacrynic acid at a concentration of $7{\cdot}4 \times 10^{-7}$ M. Furthermore, the extracellular space, as measured by the distribution of trace amounts (8×10^{-8} M) of [1-^{3}H]mannitol, was also determined in the presence and absence of ethacrynic acid. The data given in Fig. 5 show that there was a marked uptake of [1-^{3}H]mannitol over the first 45 min of incubation, but that no significant further increase occurred by 60 min. The uptake of [1-^{3}H]mannitol was identical in the presence and absence of $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid and the mean distribution ratio (given as a percentage) at equilibrium was 48 per cent. Thus, there were no abrupt changes in membrane permeability or water distribution during the period of our experiments. Further confirmation of this conclusion came from

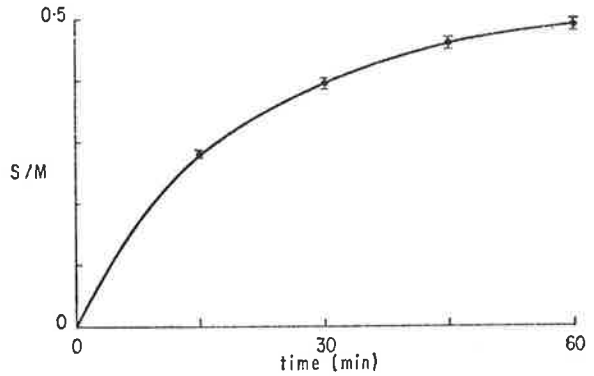

FIG. 5. Distribution (slice:medium) of [1-^{3}H]mannitol by thin slices of rabbit renal tissue. Observations are the mean ± S. E. where N = 8. The data were identical ± $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid.

routine determination of total tissue water before and after incubation, which also indicated no significant change during incubation and was not influenced by the low concentration of ethacrynic acid employed in this investigation.

Uptake of [2-^{14}C]*ethacrynic acid.* The uptake of radioactively labeled ethacrynic acid by thin slices of rabbit kidney cortex was followed in an oxygenated isotonic medium containing salts and glucose. Incubations were for the times and at the temperatures described in the text and figures; the assays were performed in at least duplicate. Tissue was obtained from different rabbits and incubations were carried out on different days. The collected data from 29 experiments with $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid are shown in Fig. 6 which, although by no means the total number of such experiments performed, serves for reference as our bulked control.

Although a concentration of $7{\cdot}4 \times 10^{-7}$ M drug represents that which might reasonably occur after administration of a low dose of this diuretic to man, we also

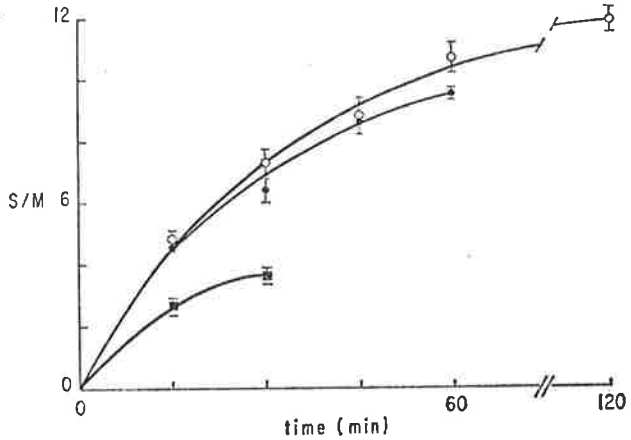

FIG. 6. Time course of uptake of [2-^{14}C]ethacrynic acid by thin slices of rabbit renal tissue under control conditions described in text, with a 100-fold increase in drug concentration, and with glucose omitted from the medium. The points shown are mean ± S. E.: ○, $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid (N = 29); ●, $7{\cdot}4 \times 10^{-5}$ M ethacrynic acid (N = 8); ■, $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid but in the absence of glucose (N = 7).

examined the effect of a 100-fold increase in drug concentration *in vitro* to explore the possibility of saturation. No such effect was observed, although there was a reduction of about 10 per cent in relative concentrating effect after 60 min of incubation at the higher drug concentration ($7{\cdot}4 \times 10^{-5}$ M). These data are also shown in Fig. 6, where the results are presented as slice:medium ratio plotted against time. Chromatographic recovery and identification of radioactively labeled material[15] after incubation of the tissues showed that all the counts were essentially in the form of ethacrynic acid rather than in possible degradation products, since no other ^{14}C- labeled spots were detected and recovery of total counts was greater than 98 per cent. This finding is in agreement with that of Beyer *et al.*,[11] who also reported little $^{14}CO_2$ production after administration of "acetate"-labeled ethacrynic acid to rats, and with a more than 90 per cent recovery of drug in unchanged form.

The slice:medium ratios obtained with $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid were greatly in excess of unity, with a mean value of $10{\cdot}7 \pm 0{\cdot}5$ after 60 min of incubation. When considered with the extracellular space measurement of approximately 0·5 as determined previously (Fig. 5), these values show that this degree of uptake cannot be accounted for by diffusion alone. Some form of accumulation, either binding or transport or both, must have occurred.

In a smaller series (N = 7) of experiments, which are also shown in Fig. 6, the effect of omitting glucose from the medium was observed. After 30 min of incubation, the uptake of [2-^{14}C]ethacrynic acid was markedly reduced, being only 55 per cent of that of untreated controls, thus demonstrating the need for glucose as substrate for this reaction.

Unless otherwise stated, all further experiments were conducted for 1 hr with glucose present in the medium. They were always carried out as paired studies, including untreated controls, but for ready comparison between experiments are usually discussed as percentages of the uptake of the bulked controls.

Effect of temperature. Some earlier experiments had shown a marked temperature dependence of ethacrynic acid uptake by guinea-pig kidney slices, an effect which was not apparent with slices of liver obtained from the same animal (Table 1). The effect of temperature upon [2-^{14}C]ethacrynic acid uptake by rabbit tissue was therefore examined in more detail. The results are shown in Fig. 7, where after only 15 min of incubation a marked effect of temperature was apparent which persisted throughout the entire period of observation. After 60 min of incubation at 0°, the drug uptake

TABLE 1. EFFECT OF INCUBATION TEMPERATURE ON THE UPTAKE OF [2-^{14}C]ETHACRYNIC ACID BY SLICES OF GUINEA PIG KIDNEY CORTEX AND LIVER*

Tissue	Temp.	No. of experiments	Per cent mean dry wt.	Ethacrynic acid ($\mu\mu$moles/ mg dry wt.)†	Per cent control
Kidney	37°	11	$19{\cdot}7 \pm 0{\cdot}8$	$2{\cdot}32 \pm 0{\cdot}62$	100
Kidney	0°	6	$21{\cdot}5 \pm 1{\cdot}6$	$0{\cdot}78 \pm 0{\cdot}18$	34
Liver	37°	7	$22{\cdot}0 \pm 1{\cdot}0$	$1{\cdot}01 \pm 0{\cdot}19$	44
Liver	0°	4	$22{\cdot}5 \pm 0{\cdot}3$	$0{\cdot}67 \pm 0{\cdot}11$	29

* Previously unpublished results of J. S. Charnock, H. A. Potter and D. McKee (1968).
† Means ± S. E.

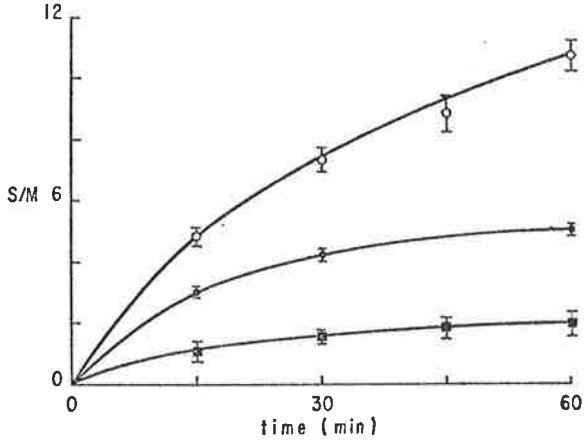

FIG. 7. Effect of temperature on [2-^{14}C]ethacrynic acid uptake. ○, Uptake by rabbit kidney at 37° where N = 29; ▣, uptake by rabbit kidney at 0° where N = 6; ●, uptake by rabbit liver at 37° where N = 5.

represented less than 20 per cent of that seen at 37°. It is likely that this fraction represents the nonspecific drug binding previously reported by Beyer *et al.*[11] When slices of rabbit liver were substituted for kidney, the uptake of ethacrynic acid at 37° was also greatly reduced, reaching only 47 per cent of that found with kidney, thus confirming our earlier observations with guinea-pig tissue (cf. Table 1).

Effect of anoxia and metabolic inhibitors. The experiments in the absence of glucose and the marked effect of temperature had suggested a dependence upon cellular metabolism for ethacrynic acid uptake. This possibility was further examined by substitution of nitrogen for oxygen in the gas phase, both before and during incubation. The results of incubation under nitrogen are given in Table 2. There was some uptake of labeled drug during the first 15 min of incubation, which, however, did not increase appreciably during the remainder of the incubation period. At 60 min, the level under nitrogen was 50 per cent of that found under control conditions with oxygen. The observation that this level of uptake is greater than that found at low temperature presumably indicates only a partial block of metabolism by the substitution of nitrogen

TABLE 2. EFFECT OF INCUBATION CONDITIONS AND INHIBITORS ON THE UPTAKE OF [2-^{14}C]ETHACRYNIC ACID BY SLICES OF RABBIT KIDNEY CORTEX

Incubation conditions	No. of experiments	*Slice/medium ratio*			
		15 min	30 min	45 min	60 min
Oxygen	29	4·85 ± 0·03	7·34 ± 0·04	8·81 ± 0·07	10·69 ± 0·49
Nitrogen	4	3·64 ± 0·22	3·95 ± 0·04	4·92 ± 0·21	5·31 ± 0·17
2,4 Dinitrophenol, 0·1 mM	4	3·64 ± 0·19	3·85 ± 0·28	5·07 ± 0·64	5·03 ± 0·29
Sodium azide, 5 mM	4	2·21 ± 0·17	3·87 ± 0·34	4·36 ± 0·23	4·77 ± 0·19
Ouabain, 1 mM	7	2·95 ± 0·21	3·45 ± 0·13	4·45 ± 0·31	5·11 ± 0·33
Probenecid, 0·1 mM	4	1·80 ± 0·11	2·54 ± 0·13	3·25 ± 0·19	3·74 ± 0·26

* Values given are means ± S. E.

for oxygen. Certainly no attempt was made to remove possible traces of oxygen from the nitrogen supply.

The effects of the metabolic inhibitors, dinitrophenol (0·1 mM) and sodium azide (5 mM), are also shown in Table 2. Both agents markedly reduced ethacrynic acid uptake with the effect of azide being somewhat more pronounced (45 per cent of control at 60 min). Interestingly, at the concentration of inhibitors employed, both agents reduced the uptake to the level seen with oxygen deprivation but not to that found at low temperature (cf. Fig. 7). When taken together, these experiments strongly suggest a major dependence upon oxidative tissue metabolism for the accumulation of ethacrynic acid by slices of rabbit kidney cortex.

Effects of probenecid and ouabain. Finally, the action of two other pharmacological agents was studied, both of which have been known for many years to block cellular transport processes.[18–21] The results are also shown in Table 2, and again clearly indicate the metabolic dependence of ethacrynic acid uptake in this tissue. The action of probenecid (0·1 mM) seemed to occur more rapidly than that of ouabain (1 mM) and to be somewhat more pronounced, in that by 60 min it had reduced the mean level to 35 per cent of the control, whereas with ouabain the mean level was 49 per cent of control.

The action of these inhibitors of active transport and that of the inhibitors of oxidative metabolism reinforce the observations of dependence upon substrate, oxygen and temperature and strongly suggest that in large part the accumulation of [2-^{14}C]-ethacrynic acid observed in these experiments is due to the presence of an active transport system in rabbit renal cortex.

DISCUSSION

These studies *in vitro* were undertaken to determine whether renal tissue is capable of significantly accumulating the diuretic agent ethacrynic acid from an incubation medium, a process which may reflect the capacity of the organ *in situ* to accumulate the drug from the plasma and to raise its effective concentration at some pharmacological receptor site within kidney cells.

Earlier experiments with kidney slices of guinea-pig, a species not thought to be particularly sensitive to ethacrynic acid,[11] had suggested that some accumulation of this drug had occurred against a concentration gradient, and these observations were confirmed and extended using tissue from the more drug-sensitive rabbit species.

The data show that a marked drug accumulation is possible, with concentration factors of 10-fold being commonplace. Even when a concentration of drug was used which exceeded by 50–100 times that which might be expected in man, no evidence was obtained to indicate that the uptake process was saturated. Thus, very large increments may exist between the concentration of drug in the plasma of animals and that at some pharmacological target site or receptor within the renal cells.

In this regard Nechay *et al.*,[3] who seriously questioned the hypothesis that renal ($Na^+ + K^+$)-ATPase was the pharmacological site for ethacrynic acid action, showed that the plasma level of this drug in dogs during maximal diuretic response was only about 1 μg/ml, whereas that in urine collected at the same time was approximately 20-fold greater. This observation seems to support our contention that considerable drug accumulation may be possible within renal tissue. The actual concen-

tration achieved at a particular receptor site could conceivably be greater than that observed here, as our data do not indicate anything about the possible distribution of drug within the tissue slice, merely reflecting the overall concentrating effect achieved on the assumption of homogeneous distribution throughout the whole tissue. It seems not unreasonable to consider that some form of compartmentalization could exist within the kidney which would effectively raise the drug concentration further.

Because Beyer *et al.*[11] had reported that substantial amounts of radioactivity were found in the liver as well as in the kidneys of dogs after intravenous administration of 10 μc of "acetate"-labeled ethacrynic acid, we also examined the liver of both rabbits and guinea-pigs for drug accumulation in our system. The degree of concentration observed did not reach half of that found with the kidney of either of these species, perhaps suggesting a measure of nonspecific drug binding to tissue, but more importantly, some organ specificity for the ethacrynic acid accumulation process in the kidney.

That drug uptake must result in accumulation against a concentration gradient is clearly shown by the magnitude of the slice:medium ratios encountered and by concurrent measurement of "extracellular space". Even if mannitol substantially underestimates this space, and it is generally agreed that some overestimation is more likely, these concentrations of drug cannot be accounted for by diffusion alone. Quite clearly from our results a portion of the uptake observed was not abolished by removal of substrate from the system, substitution of nitrogen for oxygen, reduction of incubation temperature to zero or introduction of metabolic inhibitors. Presumably this fraction reflects the so-called nonspecific uptake reported by Beyer *et al.*[11] and may be related to the binding of drug to the cut surfaces of the tissue slices.

Although subsequent experiments were not corrected for this fraction, the action of the metabolic inhibitors and transport inhibitors is unequivocal in that both types of agents reduce the uptake of the drug profoundly, and argues strongly in favor of a metabolically dependent active uptake process.

Whether this process is specific for ethacrynic acid or reflects a more general renal system for the uptake and perhaps excretion of organic acids of this type cannot be stated at present. Certainly probenecid is widely regarded as the prototype inhibitor of classical organic acid transport systems in the kidney, but its action is also known to lack specificity.[21] Similarly, the action of ouabain is of interest, as this may reflect a more general cardiac glycoside sensitivity of an organic acid transport system, perhaps by dependence of the system upon the maintenance of adequate sodium ions gradients as is the case for sugars and many other substances.[22] On the other hand, a direct involvement of the ouabain-sensitive renal ($Na^+ + K^+$)-ATPase in ethacrynic acid accumulation cannot be excluded,[8,9] and this enzyme system must remain a potential target site for at least part of the pharmacological action of ethacrynic acid as well as the cardiac glycosides.

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The Role of Cations and Other Factors on the Apparent Energy of Activation of $(Na^+ + K^+)$-ATPase

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Data concerning the temperature dependence of ouabain-sensitive $(Na^+ + K^+)$-activated ATPase have enabled estimates of the apparent activation energies of this process to be obtained. Arrhenius plots show a point of inflection at about 20°; at higher temperatures the activation energy is about 13.5 kcal/mole while below this temperature the value increases to 28.5 kcal/mole. Storage at −5° or reduction in total cation concentration without alteration of the Na^+:K^+ ratio causes no significant change in these values, although the specific activity is markedly reduced. Reduction in the sodium concentration alone, however, increases the apparent activation energy at lower temperatures. These results support the hypothesis that two independent processes are involved in ATP hydrolysis, one operating above the critical temperature and one operating below this temperature. Storage, or reduction in the concentrations of both sodium and potassium ions, appears to reduce the number of functional ATPase units, without significantly altering the properties of those which can still hydrolyze ATP. Reduction in the sodium concentration alone, however, may also cause some inhibition of all units. This is more marked at lower temperatures, and may arise from competition by potassium for sodium-binding sites.

In a recent communication, Charnock, Doty, and Russell (1) reported the effect of temperature on H^+ ion release by ouabain-sensitive $(Na^+ + K^+)$-ATPase obtained from rabbit kidney. These investigators used the Arrhenius plot of log rate of H^+ release against the reciprocal of temperature to obtain estimates to the apparent activation energy of this process. That portion of the "total" ATPase activity which was sensitive to ouabain was found to have an apparent activation energy of 10.2 kcal/mole above 14°, but below this temperature no ouabain-sensitive product formation was detectable by the technique employed.

We report here further studies on ouabain-sensitive $(Na^+ + K^+)$-ATPase which were carried out to assess whether assay of ATPase activity by the determination of H^+ release gives results which are similar to those obtainable by the determination of inorganic phosphate production which is more commonly employed. In addition, we have determined whether the activation energy obtained from the Arrhenius plot can be regarded as a general characteristic of $(Na^+ + K^+)$-ATPase or is variable with different preparations of this enzyme; whether it is affected by storage of the preparation or by the concentration of ligands available.

MATERIALS AND METHODS

Ouabain-sensitive, sodium plus potassium-activated adenosine triphosphatase was prepared from fresh rabbit kidney by the method of Charnock and Post (2) and was treated with 0.1% Tris–deoxycholate (pH 7.6) for 12 hr prior to storage at −5°. Care was taken to ensure that adequate substrate (MgATP) was provided during assay so that the observed rate of phosphate liberation was effectively the maximum velocity of the reaction. The reaction was terminated before sufficient product had accumulated for significant inhibition of the enzyme to occur. The reaction was carried out in a final volume of 2 ml

which usually contained 100 mM glycylglycine, 0.2 mM EDTA (free acid), 4 mM $MgSO_4$ and 4 mM ATP (free acid); the system was maintained at pH 7.6 by the addition of Tris base. Where necessary 1 mM ouabain octahydrate (Sigma) was included in the assay to permit estimation of the ouabain-sensitive component of the ATPase. Sodium and potassium ions were added as their chloride salts, to give the final concentrations indicated in the tables and text. The reaction was initiated by the addition of 0.1 ml of enzyme suspension, adjusted to provide 150 μg protein, and incubation times were dependent upon the temperature and the amount of phosphate liberated. For example, the incubation time was increased from 10 min at 37° (in the presence of 80 mM Na^+ and 20 mM K^+) to 2 hr at 5° or under conditions where the cation concentration employed gave rise to low levels of ATPase activity. The reaction was stopped by the addition of 1.5 ml of ice-cold 5% (w/v) TCA, the protein was separated by filtration and the inorganic phosphate content of the filtrate determined by a modification of the method of Fiske and Subba-Row (3) described previously (4). The experimental design was such that control assays were performed on each occasion (± 1 mM ouabain with 80 mM Na^+ and 20 mM K^+ and at 37°). The whole temperature range under investigation was tested with a single enzyme preparation in any one experiment. Incubation temperatures were controlled to ±0.2° in insulated shaker-baths. Reaction rates were expressed as μmoles inorganic phosphate (P_i)/mg protein/hr.

TABLE I

EFFECT OF TEMPERATURE ON P_i LIBERATION BY ($Na^+ + K^+$)-ATPase

°C	μmoles P_i/mg protein/hr		
	$Na^+ + K^+$ (I)[a]	+ Ouabain (II)[b]	Δ (I–II)[c]
37	179.4	42.9	136.5
30	128.5	42.9	85.6
25	80.3	28.0	52.3
20	54.8	25.1	29.7
18	41.3	19.1	22.2
16	32.7	17.7	15.0
14	26.6	14.4	12.2
12	19.7	12.1	7.6
10	14.7	10.3	4.4
5	8.8	7.1	1.7
3.5	5.2	5.2	0
0	3.8	3.8	0

[a] $Na^+ + K^+$; total activity in presence of 80 mM Na^+, 20 mM K^+, and magnesium ions (I).

[b] Ouabain; residual activity in presence of 80 mM Na^+, 20 mM K^+, 1 mM ouabain, and magnesium ions (II).

[c] Δ; Difference between columns I and II above.

RESULTS

When 80 mM Na^+ and 20 mM K^+ were present in the assay system, a portion of the phosphate liberated from ATP was inhibited by 1 mM ouabain at all temperatures above 5°, although the rate of formation of this product was markedly reduced at temperatures below 20°.

Below 5°, however, no ouabain-sensitive phosphate liberation was detectable by the procedure usually employed (Table I). To examine this observation further a series of experiments was carried out utilizing [γ-^{32}P]ATP as substrate, to take advantage of the greater sensitivity of radiochemical assay procedures (5). In these experiments incubations were carried out for as long as 7 hr at 0–1°, when again no significant amount of ouabain-sensitive inorganic phosphate liberation was observed. In addition no ($Na^+ + K^+$) activated ^{32}P liberation was detectable at this temperature (Table II). Arrhenius plots were constructed from experiments where the rate of liberation of phosphate from ATP was plotted as a function of temperature. Such a plot, obtained from a typical enzyme preparation is shown in Fig. 1. At temperatures above about 20° the curve is approximately linear. Near this temperature there is a point of inflection; below about 20° the curve is

TABLE II

LIBERATION OF $^{32}P_i$ FROM γ-ATP^{32} BY ($Na^+ + K^+$)-ATPase AT 0°[a]

Time (hr)	μmoles $^{32}P_i$ liberated/mg protein		
	Mg^{2+}	$Na^+ + K^+$	+ Ouabain
2	0.78	0.69	0.68
4	2.42	2.60	2.81
7	4.44	4.45	4.49

[a] Mg^{2+} is control; $Na^+ + K^+$ shows activation by 80 mM Na^+ plus 20 mM K^+ in presence of Mg^{2+}; ouabain shows inhibition by 1 mM ouabain in presence of 80 mM Na^+, 20 mM K^+, and Mg^{2+}. Values are means of duplicate assays of two separate experiments with two similar enzyme preparations.

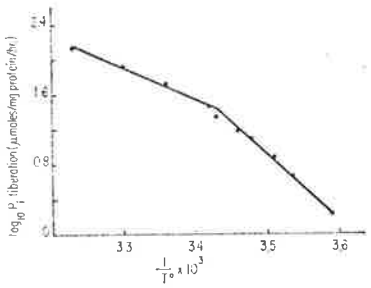

FIG. 1. A typical Arrhenius plot of inorganic phosphate liberation by ouabain-sensitive (Na^+ + K^+)-ATPase. The values were obtained as the difference in activity ± 1 mM ouabain with 80 mM Na^+, 20 mM K^+, and 4 mM MgATP. Assays were in duplicate. Activation energies above and below the transition temperature are 14.0 and 30.6 kcal/mole, respectively.

again approximately linear although the slope, and hence the apparent energy of activation of the process is much increased. Similar points of inflection have been shown to occur in the Arrhenius plots obtained with other enzyme preparations (19–21). The temperatures at which these inflections in Arrhenius plots are observed have been referred to as critical or transition temperatures (14, 19).

From the results of 24 experiments involving six different enzyme preparations we estimate the apparent activation energy for the liberation of phosphate by (Na^+ + K^+)-ATPase to be 13.5 kcal/mole above 20°, and 28.5 kcal/mole below this temperature. These values for the apparent activation energy were obtained by the method of least squares, and, in fact, cannot be determined below a temperature of about 5° where presumably the apparent activation energy is very high indeed. The apparent activation energy obtained at temperatures above 20° by this method is closely similar to that obtained by the previous study of H^+ ion release (1), and the overall Arrhenius plot confirms the marked increase in this parameter at lower temperatures.

In order to confirm the visual impression that the Arrhenius plot between the temperatures of 5 and 37° is composed of two intersecting straight lines, the data were first analyzed by the technique of Bogartz (9) for fitting two such lines to a cloud of points. By this means the point of inflection was found to lie between 18 and 21° (Fig. 2). Application of the F test (22) to the portions of the curve lying above and below the point of inflection showed that neither deviated significantly from linearity (F values 2.067 and 2.26 are not significant). Both analyses were facilitated by use of the APL/360 computing system available at the University of Alberta. Thus, our evidence strongly suggests that the Arrhenius plot for phosphate liberation by (Na^+ + K^+)-ATPase can adequately be described by two intersecting straight lines rather than the curvilinear form suggested by some other workers (6–8).

Comparison of the values computed for some individual enzyme preparations is given in Table III, where it can be seen that

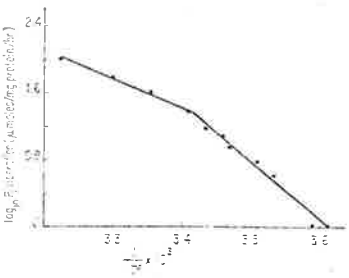

FIG. 2. An Arrhenius plot of inorganic phosphate liberation by ouabain-sensitive (Na^+ + K^+)-ATPase based on 24 experiments involving six different enzyme preparations. The assay conditions were identical to those given in Fig. 1. The mean activation energies obtained above and below the critical temperature (18–20°) are 13.5 kcal/mole and 28.5 kcal/mole, respectively.

TABLE III

APPARENT ENERGY OF ACTIVATION OF (Na^+ + K^+)-ATPase

Enzyme	No. of determinations	Specific activity[a]	Apparent energy of activation[b]	
			Ea_{I} 25–37°	Ea_{II} 5–18°
1	8	118	14.2 ± 0.5	28.3 ± 4.1
2	4	179	13.6 ± 0.7	30.6 ± 2.2
3	4	113	13.6 ± 0.7	21.2 ± 2.2

[a] Specific activity of ouabain-sensitive ATPase measured at 37° with 80 mM Na^+, 20 mM K^+, ±1 mM ouabain.

[b] All values of Ea are mean kcal/mole ± SE.

the variation between preparations is small, and that the values obtained are in close agreement with those reported above.

Since our observations were obtained with a number of different preparations of (Na^+ + K^+)-ATPase enzymes, we examined the possible effects of variable specific activity (obtained as a result of varied storage time at −5°) upon the apparent energy of activation when measured at a fixed concentration of ligands.

The results of seven experiments are shown in Table IV where the value for the apparent activation energy above 25° was compared with the specific activity obtained under control conditions at 37°. Although the specific activity values range from 46 to 179 μmoles P_i/mg protein/hr, the values of apparent activation energy obtained with these preparations range only from 12.6–14.2 kcal/mole.

Because of the influence of both Na^+ and K^+ ions on the rate of hydrolysis of ATP by this ouabain-sensitive enzyme system, we have explored the effects of varying ligand concentrations on the Arrhenius plots. Initially we confirmed the earlier observation of Charnock and Opit (11) that considerable product formation could occur over a wide range of monovalent cation concentration provided the ratio of Na^+:K^+ was maintained. The results obtained with a single enzyme preparation examined at different temperatures are shown in Fig. 3 which shows that a major decline in enzyme activity does not occur until the total monovalent cation concentration falls below about 25 mM, although the rates are clearly very dependent upon temperature. It is of interest to compare these results with those obtained when the concentration of ligands has been varied independently (2, 11–13); and we have thus extended our observations to determine the effect of changing either the total ligand concentration or the ratio of sodium:potassium ions on the apparent energies of activation of ouabain-sensitive (Na^+ + K^+)-ATPase.

Initially we compared the values for apparent energy of activation that were obtained when the ratio of sodium:potassium ions was maintained but their total concentration was reduced 16-fold. The results are given in Table V where it can be seen that in comparison with control values obtained with 80 mM Na^+ and 20 mM K^+, the apparent activation energy above 25° appears to become only marginally smaller with the decrease in cation concentration. No statistically significant alteration in value was obtained over either temperature range examined.

In a further series of experiments the concentration of Na^+ was reduced from 80 mM to 20 mM while that of K^+ was retained

TABLE IV

EFFECT OF STORAGE ON SPECIFIC ACTIVITY AND APPARENT ENERGY OF ACTIVATION OF (Na^+ + K^+)-ATPase

Enzyme	Days of storage[a]	Specific activity[b]	No. of determinations	Ea[c]
a	5	118	4	14.16 ± 0.52
b	8	117	8	13.61 ± 0.66
c	21	179	2	14.00 ± 1.03
d	4	152	4	12.65 ± 1.69
e	12	129	4	13.57 ± 1.25
f	9	113	2	13.61 ± 0.19
g	33	46	2	13.14 ± 1.35
h	27	62	2	13.74 ± 1.46

[a] Enzyme suspension stored in 0.5 M sucrose at −5°.

[b] Specific activity measured at 37° in the presence of 80 mM Na^+ and 20 mM K^+ on day of experiment.

[c] Ea, apparent energy of activation determined from 25 to 37°C; all values of Ea are mean kcal/mole ± SE.

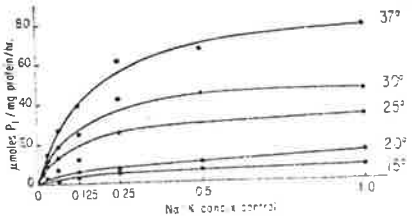

FIG. 3. The effect of temperature on the activity (μmoles P_i liberated/mg protein/hr) of ouabain-sensitive (Na^+ + K^+)-ATPase at different concentrations of Na^+ and K^+ but with the ratio of Na^+:K^+ maintained at 4:1. Assays were in duplicate. Control activity was obtained with 80 mM Na^+, 20 mM K^+, and 4 mM MgATP and is shown on the abscissa as unity.

TABLE V

EFFECT OF CHANGING TOTAL CATION CONCENTRATION ON APPARENT ENERGY OF ACTIVATION OF $(Na^+ + K^+)$-ATPase

Enzyme preparations	No. of observations	Specific activity[a]	Concentrative (mM)		Ea_i[b] (25–37°)	Ea_{ii}[b] (5–18°)
			Na^+	K^+		
a	8	117	80	20	13.61 ± 0.66	28.33 ± 4.1
e	4	129	80	20	13.57 ± 1.25	21.11 ± 2.24
l	4	152	10	2.5	11.90 ± 1.47	—[c]
m	4	130	10	2.5	12.00 ± 0.94	—[c]
n	3	135	5	1.25	11.76 ± 0.11	28.58 ± 1.85
o	3	142	5	1.25	12.39 ± 0.01	29.87 ± 2.38

[a] Measured at 37° with 80 mM Na^+ and 20 mM K^+ on day of experiment.

[b] Ea, apparent energy of activation over temperature range shown; all values of Ea are mean kcal/mole ± SE.

[c] Experiments not done.

TABLE VI

EFFECT OF REDUCED $[Na^+]$ ON APPARENT ENERGY OF ACTIVATION OF $(Na^+ + K^-)$-ATPase

Enzyme preparation	Specific activity[a]	Ea_i[b] (25–37°)	Ea_{ii}[b] (5–18°)
p	76	16.20 ± 1.68	58.60 ± 20.93
v	135	14.46 ± 5.41	22.14 ± 5.33
r	129	13.40 ± 1.13	51.52 ± 6.89

[a] Measured at 37° with 80 mM Na^+ plus 20 mM K^+ on day of experiment.

[b] Ea, apparent energy of activation over temperature range shown; all values are mean kcal/mole ± SE of observations in triplicate.

at 20 mM; thus reducing the Na^+:K^+ ratio to unity. The results of these experiments are given in Table VI where above 25° there was again no statistically significant change in the value of the apparent energy of activation, although the values obtained in this case appear to be marginally increased over control values. The results obtained at the lower temperature range suggest that there may be an increase in apparent energy of activation despite the large experimental variation which exists under these conditions.

DISCUSSION

There is now abundant evidence that the Arrhenius plots of ouabain-sensitive $(Na^+ + K^+)$-ATPase are nonlinear, whether this parameter is measured by liberation of inorganic phosphate (6–8, 10) or by H^+ formation (1) during the hydrolysis of substrate ATP by this enzyme.

Although the Arrhenius plots obtained by these two methods have some features in common; for example, higher activation energies are found at lower temperatures and the point of inflection or critical temperature is at about 15–20°, the data obtained by measurement of H^+ ion formation suggested that all ouabain-sensitive product formation ceased below about 14° (1). The results of the present study, however, suggest that in the 5–20° temperature range, some ouabain-sensitive activity is present and that only at temperatures below 5° does the rate of ouabain-sensitive phosphate liberation become negligible. Thus, it is important to note that all methods employed in these studies, including the release of ^{32}P from [γ-^{32}P]ATP, have failed to detect any ouabain-sensitive product formation when the temperature is less than 5°; a conclusion which is in agreement with a large number of observations in the literature (2, 6–8, 17, 18).

At least two possible explanations can be offered for the change in slope of the Arrhenius plots of ouabain-sensitive $(Na^+ + K^+)$-ATPase. One explanation could be that the plot represents the varying temperature dependence of two different steps of the overall reaction, for example, the Na^+ stimulation of phosphorylation of the enzyme and the K^+ activation of the dephosphorylation of the phosphorylated enzyme complex.

Although such an explanation may seem attractive, it has been argued by both Belehradek (16) and Dixon and Webb (23) that two simultaneously occurring processes will not yield the type of Arrhenius plot observed, but would produce a true curvilinear form.

Furthermore, such a view would necessitate that at least one of the composite reactions of ($Na^+ + K^+$)-ATPase did not occur to any major extent below the critical temperature observed; whereas both Na^+-activated phosphorylation and K^+-stimulated dephosphorylation of the phosphoenzyme intermediate complex have been reported to occur at 0° (24–27). On the other hand, Kumamoto, Raison, and Lyons (14) have reasoned convincingly that two independent processes are required to produce a "break" or abrupt change in slope of an Arrhenius plot derived from a biological system. In addition, they have provided evidence for the existence of mutually exclusive changes in phase from one transition state to another. The magnitude of the change in apparent energy of activation above and below the critical temperature which is seen in our experiments, and which is at least 15 kcal/mole is also sufficient to overcome some of the theoretical objections which have been raised to the acceptance of this interpretation of discontinuities in Arrhenius plots (16).

Thus, in general we are in agreement with the conclusions of Kumamoto, Raison, and Lyons (14). The significance of this interpretation of our data regarding the role of ($Na^+ + K^+$)-ATPase in the function of the sodium pump is discussed elsewhere (15).

The apparent discrepancy between ouabain inhibition or Na^+ plus K^+ activation of P_i liberation at low temperatures which has been reported by Gruener and Avi-Dor (6) was not confirmed by our experiments in which a deliberate excess of ouabain (1 mM) was employed to replace the suboptimal concentration used by others.

Examination of the data obtained with individual enzyme preparations reveals that the apparent energies of activation are independent of the period of storage and the specific activity of the preparations. Thus, it may be supposed that the apparent activation energies obtained are characteristic of ouabain-sensitive ($Na^+ + K^+$)-ATPase and are not related to the number of functional ATPase sites or units which are operative at any one time. Conversely, the changes in specific activity which are known to occur on storage of these enzyme preparations must be related to the number of functional ATPase units which are operating, rather than to any change in the activity of all such units.

Because of the decrease in the total phosphate release that occurs at lower temperatures and at suboptimal cation concentrations or ratios, the reliability of data obtained under such conditions must decrease. Nevertheless, within the limits of interpretation of such experiments the data suggest that decreasing the amount of total cation but maintaining the ratio of Na^+:K^+ can greatly reduce enzyme activity without affecting the apparent energy of activation. This would follow if only a few of the available ATPase units were fully operative under these conditions. On the other hand, decreasing the concentration of Na^+ ions while maintaining the original concentration of K^+, which may be thought of as potential inhibition of sodium activation by potassium ions, perhaps elevates slightly the apparent energy of activation above 25° and even more probably increases the apparent energy of activation at lower temperatures.

Such an effect might follow from more effective competition for Na^+ activation sites by K^+ ions at lower temperatures, thus leading to partial inhibition of all ATPase units, rather than to the number of units which remain functional under these conditions. It would have to be supposed that above about 20°, the affinity of the enzyme for Na^+ is such that equimolar K^+ does not effectively compete for Na^+-activation sites.

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Role of Energized States of $(Na^+ + K^+)$-ATPase in the Sodium Pump

IN an earlier paper[1] we have presented a model for a sodium pump based on the operation of the adenosine triphosphatase component of membranes which is sensitive to ouabain and is activated by sodium and potassium; that is $(Na^+ + K^+)$-ATPase. We attempted to correlate the biochemical properties of this enzyme system as they were then known with the essential properties of Na^+ transport systems. The model suggested further experiments which could clarify the role of $(Na^+ + K^+)$-ATPase in ion transport, and some experimental evidence is now available for the stoichiometry of ouabain binding to isolated enzyme preparations[2,3] although differences in the experimental techniques which have been used make the data equivocal.

More recently several other models for the sodium pump also based upon the operation of $(Na^+ + K^+)$-ATPase, and which incorporate many of our suggestions, have appeared[3,4]. We now wish to discuss our original model in the light of recent experimental evidence related to the temperature dependence of the $(Na^+ + K^+)$-ATPase reaction[6,25] which we believe is consistent with our earlier contentions.

Our concepts can be summarized as follows. The enzyme $(Na^+ + K^+)$-ATPase is structurally and functionally integrated with the cell membrane. Adsorption of Na^+ ions induces activation of the "enzyme centre" so that reaction with substrate ATP can occur. Phosphorylation of the membrane enzyme component or components follows. As a result of the formation of this "intermediate" condition, a structural or conformational change occurs in the protein component of the membrane which effects the conformation and functional properties of the whole lipoprotein membrane structure. The subsequent absorption of sufficient external K^+ ions to this energized conformation in the membrane now allows water to attack the phosphorylated "enzyme centre" permitting hydrolysis of the phospho-intermediate complex; the release of bound Na^+ ions with the restoration of the initial shape of the lipoprotein. This last reaction may possibly be analogous to an elastic recoil mechanism. Each enzyme cycle results in the net outward movement of Na^+ from the cell and the net movement of K^+ into the cell.

A modified version of our original hypothesis which avoids the symbolism of earlier illustrations[1,5,24] and incorporates only a simple notation for different conformational states of the transport enzyme can be stated as:

$$(a)\quad E + ATP \xrightarrow{Mg^{++}} [E_I - P] + ADP$$
$$[E_I - P] \longrightarrow E + P_i$$
$$(b)\quad E + ATP + Na^+ \xrightarrow[K^+]{Mg^{++}} [E_{II} \sim P]Na + ADP$$
$$[E_{II} \sim P]Na^+ \longrightarrow E + Pi + Na^+$$

In this scheme E_I and E_{II} represent different conformational states of the same enzyme with only the formation of the state E_{II} dependent on the adsorption of Na^+ ions. Reaction (*a*) is thought to be relatively insensitive to ouabain and is little affected by temperature or *p*H[6-8], whereas reaction (*b*) is highly sensitive to ouabain and is temperature and *p*H dependent[6-12]. The status of the two postulated conformational forms of $(Na^+ + K^+)$-ATPase, represented in our scheme by $[E_I - P]$ and $[E_{II} \sim P]$, is controversial at present. Some workers have suggested that these "intermediates" are formed by separate pathways and under different ligand conditions[3,16], while more recently others have suggested that these "intermediates" are capable of interconversion[16,21]. There is experimental evidence that the two forms of the "intermediates" are chromatographically identical[20], yet stability studies indicate that they differ both in dissociation characteristics and in inhibitor binding properties[22].

The reaction scheme we propose is consistent with this latter view, and we further postulate that reactions (*a*) and (*b*) will possess different apparent energies of activation and that there is a "critical temperature" above which the conformation E_{II} is predominant, whereas below this temperature conformation E_I predominates.

The existence of such a critical temperature range can be supported by our data[6,25] showing the effect of temperature on H^+ release and inorganic phosphate liberation by $(Na^+ + K^+)$-ATPase, and by a re-examination of the data of other workers[7-12]. All these experiments provide information for the construction of Arrhenius plots which display some general characteristics. These plots are non-linear, that is, the Arrhenius laws are not obeyed over the whole temperature range examined; all show an increase in slope and thus an increase in the apparent energy of activation at lower temperatures (Fig. 1).

Similar findings have been reported for other enzymes[13-15,23] and may be more general than was at first realized. While some investigators have regarded the data obtained with $(Na^+ + K^+)$-ATPase as belonging to a single

population[9-12], and have thus presented their observations in curvilinear form, we have considered the possibility that at least two distinct populations may be present.

We have therefore reprocessed the available data[6,9-12] by the technique of Bogartz[19] for fitting intersecting straight lines to a cloud of points, using the APL/360 Computing System available at the University of Alberta. A value for "critical temperature" was obtained from the graphic presentation of these data and was found to lie between 18° and 20° C in all cases. A value for Ea_{II} was computed for temperatures well above this point (25°–37° C), and similarly a value for Ea_{I} was computed for a temperature range below the critical temperature (5°–15° C) whenever sufficient data were available.

Although information so obtained can only be regarded as semi-quantitative (Table 1) it clearly supports our contention that two energetically distinct reactions are concerned in the hydrolysis of ATP by membrane preparations of $(Na^+ + K^+)$-ATPase. We believe that these data reflect the existence of an energy barrier which in our model[1,5,24] is overcome by the absorption of Na^+ ions to the enzyme, and which is probably associated with a local change in entropy and conformation[9,13,23]. Our contention that the formation of some specific phospho-enzyme complex occurs as a distinct stage having a step-like energy barrier[1,5,24] is thus supported. Furthermore, it seems reasonable to suppose that this energy step is associated with a change in the three dimensional structure of the enzyme itself, although there is no incontrovertible evidence that it is associated with either structural deformation, change in cationic binding characteristics or ouabain binding to the enzyme.

Table 1 Apparent Energies of Activation of $(Na^+ + K^+)$-ATPase Preparations obtained from the Literature

Reference	Enzyme source	*Ea_{II} (above 20° C)	*Ea_{I} (below 20° C)
†Gruener and Avi-Dor[9]	Rat brain	7.8	39.5
Swanson[10]	Guinea-pig brain	18.6±6.8	43.3±6.8
Bowler and Duncan[11]	Rat brain	20.0±3.2	41.7±7.5
†Neufeld and Levy[12]	Calf brain	12.0	51.0
Charnock *et al.*[6,25]	Rabbit kidney	13.8±0.7	28.5±1.8

* Ea given as kcalories/mol.

† Not recalculated; values taken directly from Gruener and Avi-Dor[9] and Neufeld and Levy[12].

Values given were obtained from Fig. 1 and ref. 25.

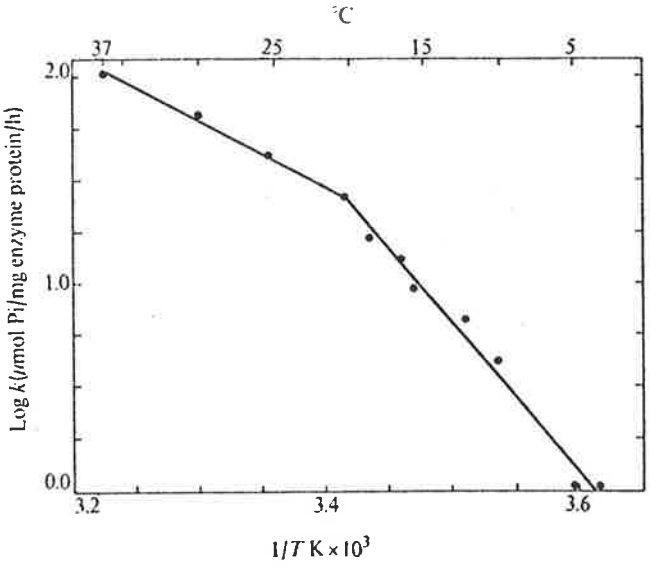

Fig. 1 Arrhenius plot of log rate constant against 1/absolute temperature $\left(\log k \nu \frac{1 \times 10^3}{T}\right)$ derived from the inorganic phosphate liberated by rabbit kidney ($Na^+ + K^+$)-ATPase (μmol Pi/mg enzyme protein/h) during the hydrolysis of ATP. Each point is the mean of eight or more observations from four separate enzyme preparations, with the value obtained at 37° C regarded as the internal control and set at 100%. Incubation conditions were Na^+ 80 mM; K^+ 20 mM; Mg^{2+} 4 mM; ATP (Tris salt) 4 mM; ouabain where required was 1 mM. Incubation times ranged from 15 min at 37° C to 120 min at temperatures below 10° C. The values of k shown were obtained from the difference in activity ± ouabain.

For technical reasons many recent studies with ($Na^+ + K^+$)-ATPase have been carried out at low temperatures[16–18] on the assumption that only the overall velocity of the reaction was reduced. We believe our findings[6,25] suggest that these studies may have been carried out with the enzyme in a conformational state which is not relevant to its function in an ion transport system. We suggest, therefore, that caution is necessary in the interpretation of, for example, ion or ouabain binding studies carried out below the critical temperature for ($Na^+ + K^+$)-ATPase[1,3,16–18].

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The Effect of Temperature on the Activity of $(Na^+ + K^+)$-ATPase

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The marked temperature dependence of product formation by microsomal preparations of ouabain-sensitive $(Na^+ + K^+)$-ATPase prepared from rabbit renal cortex has been confirmed by extremely sensitive pH measurement. It was possible to confine measurements of H^+ release to a change of less than 0.02 pH units. With periods of observation not exceeding 20 sec, the linearity of H^+ release could be maintained. Below 14° there is no detectable H^+ release which is sensitive to inhibition by 0.2 mM ouabain. Above this critical temperature, however, the reaction increases markedly throughout the range examined. The ouabain-insensitive or "residual" ATPase of these preparations also shows a dependence upon temperature, but only above a critical temperature of 24°.

The discontinuities in the Arrhenius plots of these enzyme activities strongly suggest the existence of more than one stable conformational form at different temperatures.

Because of the parallelism which exists between the behavior of sodium-plus-potassium-activated, ouabain-inhibited adenosine triphosphatase i.e., $(Na^+ + K^+)$-ATPase, and the energy-dependent translocation of monovalent cations across biological membranes, there can be little doubt of the intimate role this enzyme system plays in the so-called sodium pump (1–3). The precise mechanisms by which ion translocation occurs is not known. Much attention has been focused on the possible carrier function of the phosphoryl compound which is formed by the transfer of the terminal group of substrate γ-ATP^{32} to microsomal preparations of $(Na^+ + K^+)$-ATPase during the hydrolysis of substrate (4–6). Several workers have suggested that the operation of the carrier depends upon some conformational change induced in its structure, although direct experimental evidence for this is lacking (6–9). Although many previous investigations have demonstrated that the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase is temperature dependent (10–12) little systematic study of this parameter has been performed. Recently Massey and his colleagues have made a detailed examination of the effects of temperature on enzyme activity and concluded that information so gained can best be interpreted in terms of different stable conformational forms of enzyme protein (13).

We have therefore reexamined the effect of temperature on $(Na^+ + K^+)$-ATPase activity using a H^+ measuring procedure to follow enzyme activity (8, 14, 15) in order to utilize the great sensitivity of this method to detect product formation even at low temperatures.

MATERIALS AND METHODS

Particulate membrane preparations were prepared from the renal cortex of adult New Zealand white rabbits by a method essentially similar to that reported previously (10). After treatment with tris-deoxycholate at pH 7.6, assay at 37° in a medium containing 80 mM NaCl and 20 mM KCl resulted in at least 70% of the ATPase activity being capable of inhibition by 0.2 mM ouabain. The hydrolysis of ATP by these preparations was continuously monitored by measurement of the change in pH which was associated with the formation of inorganic phosphate from substrate ATP (14).

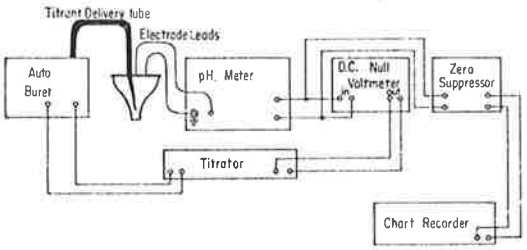

FIG. 1. A schematic diagram of the physical assembly employed to record the changes in pH. All components are readily available commercially except for the zero suppression box which is described in Fig. 2.

The experimental apparatus for following these changes in pH is shown in Fig. 1. With the exception of the zero suppression box all of the units which make up the assembly are available commercially. The components are as follows: Instrument Laboratories Model 245 pH meter; Radiometer Type TTA-31 microtitration assembly with thermostated sample cell; Type TT 111 titrator and Type ABU1b syringe buret (capacity 0.25 ml); Hewlett-Packard Model 7101B 10-in. strip chart recorder with Model 1750A preamplifier and Model 419A dc null voltmeter. Sample temperatures were measured directly with a Yellow Springs thermistor thermometer, and were controlled to $\pm 0.5°$ with a thermostated water circulator. The circuit of the zero suppression box is shown in Fig. 2. Although this may be constructed from readily available parts, the accuracy of the measurements depends upon the linearity of the ten turn potentiometer. It should be noted that the gain of this system is large thus shielding and grounding are critical. The largest source of noise is static electrical fields induced by the stirrer drive belt on the titration assembly, hence it is necessary to maintain a sufficient ionic concentration in the water coolant to provide effective shielding.

The electrical equipment was calibrated to read directly, which allows accurate measurements of pH. The pH meter must be adjusted (buffered) for both the isoelectric point and the slope (i.e., mV/pH unit). Our particular pH meter is especially suitable, as it not only operates in an expanded scale mode of 1 pH unit full scale, but has a "recorder" output of 100 mV for a full scale meter deflection on any range. In addition the recorder output exactly follows the meter movements unlike some other pH meters where this condition is not met. The output from the pH meter is fed to the recorder through the zero suppression box. Depending on the recorder gain (either 100 or 10 mV full scale) and the setting of the zero suppression box, the output is displayed on the recorder at either 1 or 0.1 pH unit full scale. In the latter case any 0.1 pH interval may be selected for display on the chart. The zero suppression box output and the recorder gain are adjusted against the meter reading of the pH meter so that both the recorder chart and the zero suppression control read correctly in pH units. The output of the pH meter is also fed to the dc null voltmeter. This is used as an impedance matching device (adjustable gain) between the pH meter and the titrator. The input impedance of the titrator is too low to permit direct coupling to the Instrument Laboratories pH meter. This, however, permits the titrator to be run in an expanded scale mode allowing the titrator to adjust the pH of the sample very closely. Thus the pH may be both measured and adjusted to ± 0.001 pH.

For small changes in pH the H^+ ion release is directly proportional to the release of inorganic phosphate from the substrate; however, several other workers have observed that this proportionality is not maintained over a range of pH change (8, 15) because the proportionality constant depends not only on the pH but also on the degree of ionization of the phosphate ion released. This factor has not been determined; however, the reactions took place over a very small pH range, and the system was calibrated immediately by back titration over this range.

In addition to 80 mM NaCl and 20 mM KCl, the reaction medium contained a final concentration of 2 mM $MgCl_2$ in 2.5 mM phosphate buffer at pH 7.15. ATP (disodium salt) was added to 8.5 mM to initiate the reaction and where necessary 0.2 mM ouabain (octahydrate) was included. The ATP and ouabain were obtained from Sigma Chemical Co., St. Louis; all other chemicals were analytical reagent grade and water distilled from an all Pyrex still and stored in polyethylene containers was used for all solutions. Measurements of enzyme activity were made by diluting a 0.1-ml aliquot of enzyme in 1 ml of salt buffer solution in a titrator vessel which was incubated at the tem-

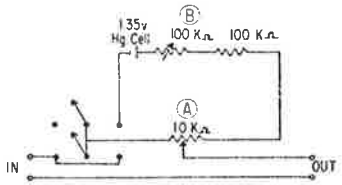

FIG. 2. Electrical circuit of zero suppression box. A is a ten turn potentiometer having 0.25% linearity tolerance and fitted with a calibrated dial (Beckman Duodial) B is also a ten turn wire wound potentiometer.

perature required. The recorder was started and the diluted enzyme sample allowed to equilibrate (30–120 sec) to a stable pH base line. ATP solution (0.1 ml) was then injected into the reaction mixture, with continuous recording of the pH. The chart was run at 0.1 in./sec. After a period of 20 sec repeated aliquots of 0.01–0.05 ml of 0.04 N NaOH were added from the syringe buret to calibrate the enzyme system. The samples were run in pairs, with and without ouabain.

The final calibration of the system was made for each individual sample by reference to the pH changes caused by addition of the aliquots of base. This amounts to a back titration over the pH range covered by the reaction, and yields a figure for equivalents of H^+/pH unit in that range. Over the very short ranges involved this is linear. The slope of the pH recording was then converted, using this calibration factor, to equivalents of H^+/min/g of protein.

When necessary the electrodes were cleaned with pepsin in 0.1 N HCl solution which effectively removed absorbed protein.

RESULTS

A typical trace of the pH change recorded during the hydrolysis of ATP by a rabbit kidney microsomal preparation is shown in Fig. 3. For experimental convenience all rate measurements were taken 15 sec after addition of substrate ATP to the system. Although the release of hydrogen ions has been reported by other investigators to be nonlinear over a 10-min incubation period (8, 15), it can be seen from our data that linearity is achieved when measurements are confined to 20 sec incubation and the pH is measured as described under Methods. This observation is in agreement with that of Wheeler and Whittam (14).

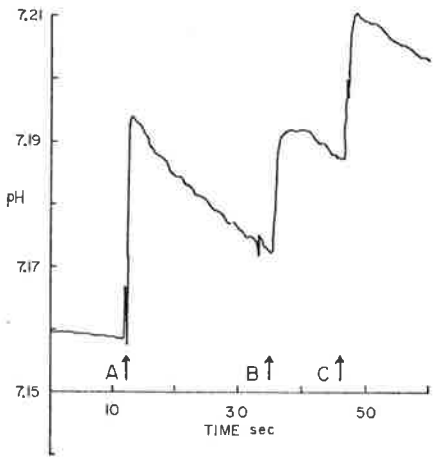

FIG. 3. A typical trace of the change in pH recorded versus time during the hydrolysis of ATP at 37°, by a microsomal preparation of ($Na^+ + K^+$)-ATPase obtained from the cortex of rabbit kidney. Substrate ATP was added at point A; the system was calibrated during the experiment by the addition of 2 µl of 0.04 N NaOH at the times marked B and C. Details of final calibration of the system are given under Methods. This particular trace was obtained under control conditions in the absence of ouabain.

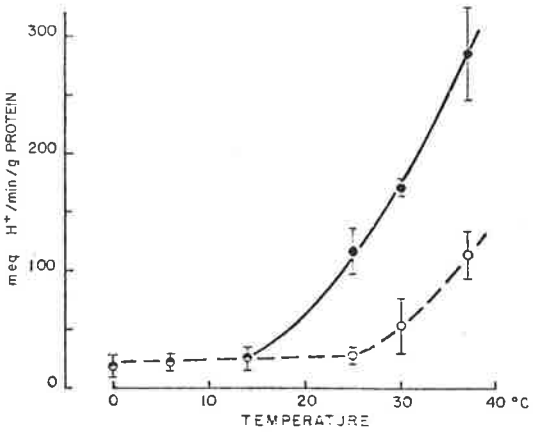

FIG. 4. The effect of temperature on the rate of production of H^+ during the hydrolysis of ATP by a microsomal preparation obtained from the cortex of rabbit kidney. ●, in the absence of ouabain; ○, in the presence of 2×10^{-4} M ouabain. Each point represents the mean of from four to ten individual measurements with the standard deviation shown as vertical bars.

The effect of temperature on H^+ formation during the hydrolysis of ATP, both in the presence and absence of ouabain, is shown in Fig. 4. The data demonstrate the marked reduction in H^+ ion release which occurs when ouabain is added to the reaction mixture. In incubation systems containing sodium and potassium ions in the amounts and ratio employed here, the reduction in activity seen here is generally thought to reflect ouabain inhibition of microsomal ($Na^+ + K^+$)-ATPase (2, 10).

Both the ouabain-sensitive H^+ ion release, and the ouabain-insensitive residual activity are clearly independent of temperature below certain critical temperatures for

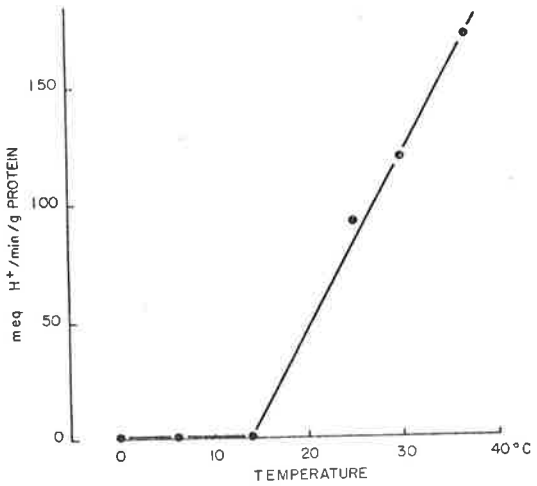

FIG. 5. The rate of production of H^+ by ouabain-sensitive ($Na^+ + K^+$)-ATPase as a function of temperature. The points are calculated from the difference in the mean rates of H^+ production in the absence and in the presence of ouabain given in Fig. 4.

the two systems (14 and 24°, respectively). Above these critical levels both activities increase markedly with increasing temperature throughout the range examined.

The mean difference in H^+ ion release which is sensitive to ouabain, i.e., equivalent to H^+ production by ($Na^+ + K^+$)-ATPase can be calculated from this data and is given in Fig. 5. This also shows a marked temperature dependence with a critical temperature of 14°. It is of interest that even with the sensitive method of pH measurement employed here, no product formation could be detected below 10° in agreement with the earlier findings of Charnock and Post (10).

The mean data shown in Figs. 4 and 5 can be used to construct Arrhenius plots for the calculation of apparent activation energies (E_a) for the "total" ATPase in the presence of sodium and potassium ions, for the "residual" ATPase when ouabain is added to the system, and for "ouabain-sensitive" ($Na^+ + K^+$)-ATPase obtained by difference. These plots are given in Fig. 6 where the apparent activation energies are 13.7 kcal/mole for total ATPase and 21.2 for residual ATPase.

The apparent activation energy calculated for ouabain-sensitive ($Na^+ + K^+$)-ATPase is 10.2 kcal/mole. The value for E_a for all reactions below 14° is 2.2 kcal/mole. All these values are in the normal range for reactions in solution and do not imply unusual properties for any of these reactions.

However, inspection of the Arrhenius plots of these ATPase activities shows that

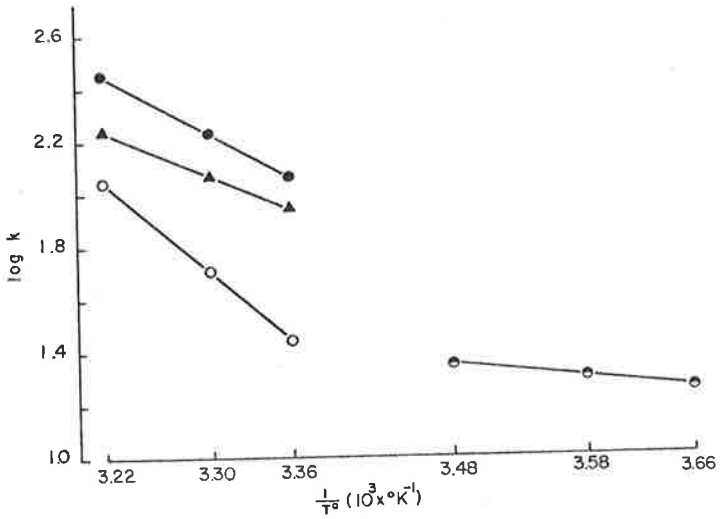

FIG. 6. Arrhenius plots of rate constant vs. absolute temperature (log K vs. $1/T^0$) obtained from the data shown in Figs. 4 and 5. ●——●, total ATPase activity in the absence of ouabain; ○- - -○, residual activity in the presence of ouabain; ▲——▲, calculated difference plus or minus ouabain, i.e., ouabain-sensitive ($Na^+ + K^+$)-ATPase.

the curves are only linear at temperatures above 25° and that the changes in slope which are seen are so abrupt that they resemble the so-called "sharp breaks" or discontinuities in Arrhenius plots which have recently been reported for a number of other enzymes (13, 16, 17).

DISCUSSION

Earlier observations of the effect of temperature on ($Na^+ + K^+$)-ATPase have demonstrated that no ouabain-sensitive product occurred when the assay temperature was less than 10° (10–12). However, all these observations were dependent upon the sensitivity of the procedures used for the determination of inorganic phosphate, and may not have been adequate to detect the extremely small quantities of product which may have been formed at lower temperature.

The data obtained here are not subject to the same limitations, but depends upon the sensitive detection of shifts in pH due to concomitant liberation of H^+ ions with phosphate release from substrate, and approximate the initial rate (8, 14, 15). Clearly these experiments (cf. Fig. 5) reinforce the previously reported observation that the ouabain-sensitive portion of ATP hydrolysis by microsomal preparations of this enzyme is extremely temperature dependent, and demonstrate that below a critical temperature of 14° there is no detectable product formation.

The abrupt change in slope of the Arrhenius plots of our data is similar to those reported for a number of other enzymes (13, 16, 17). The significance of this observation has been extensively studied by Massey *et al.* (13) who concluded that a temperature-dependent transformation from one stable form of an enzyme to another probably explains the type of Arrhenius plot encountered. On the other hand a physical effect on some membrane component such as a lipoprotein may also account for the effect seen here (18).

Nevertheless, in the case of ($Na^+ + K^+$)-ATPase, it is certainly tempting to speculate that several different conformational forms of the enzyme exist, which have different energies of activation, and which may be related to the selective binding properties of the enzyme which form an integral part of several models of ion transport (2, 7–9).

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ETHACRYNIC ACID INHIBITION OF $(Na^+ + K^+)$-ACTIVATED ADENOSINE TRIPHOSPHATASE

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Abstract—Ethacrynic acid was found to inhibit microsomal preparations of sodium+potassium-activated adenosine triphosphatase obtained from guinea pig kidney cortex. The degree of inhibition of ethacrynic acid was influenced by the concentration of potassium ion present and was greatest when potassium ion was least. Examination of the effect of ethacrynic acid on the transfer of ^{32}P from γ-labeled substrate ATP^{32} to an intermediate formed during ATP^{32} hydrolysis demonstrated no inhibition by ethacrynic acid in the presence of 20 mM Na^+, but considerable inhibition in the release of inorganic ^{32}P from this complex upon the subsequent addition of K^+. Again this effect was greatest when the potassium concentration was least. The antagonism between ethacrynic acid and potassium ions may be explained by this mechanism.

THE PHYSIOLOGICAL role of sodium+potassium-activated adenosine triphosphatase (transport ATPase) in the active transport of monovalent cations across biological membranes is now generally accepted.[1–3] The inhibitory action of ethacrynic acid and other diuretic agents on this enzyme system both *in vitro* and after administration to the whole animal[4, 5] has raised the possibility that this interaction may be the basis for the pharmacological effects of diuretic agents, although such a mechanism has been questioned by some investigators.[5, 6]

In accordance with a previous suggestion by Nechay *et al.*,[6] a recent study in this laboratory has shown that isolated slices of guinea pig kidney cortex are capable of accumulating 2-^{14}C-ethacrynic acid from an incubation medium by a temperature-dependent process.† Although the accumulation of drug observed was only about 10-fold, equilibrium was not attained in these experiments and such a mechanism may explain the discrepancy between the plasma levels of this drug which are observed after therapeutic doses of ethacrynic acid to both man and experimental animals[4, 7] and the levels required to demonstrate enzyme inhibition *in vitro*.[4, 6] Thus the pharmacological significance of ethacrynic acid inhibition of transport ATPase remains of considerable interest and a more detailed understanding of the mechanism is desirable.

The experiments described in this paper were conducted to re-examine the inhibitory effect of ethacrynic acid on a sodium+potassium-activated ATPase prepared from guinea pig kidney cortex, by utilizing a sensitive test system which employs γ-ATP^{32} as substrate, and to further examine the competitive effect of K^+ on this reaction, as this ion has recently been observed to influence the ethacrynic acid inhibition of transport

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† J. S. Charnock, H. A. Potter and D. McKee, unpublished results.

ATPase.[6] By measuring the transfer of ^{32}P from substrate to an acid-stable phosphorylated protein intermediate of sodium–potassium ATPase,[8–11] further insight into the mechanism of ethacrynic acid effects was obtained.

MATERIALS AND METHODS

Sodium–potassium-activated ATPase preparations were obtained from guinea pig renal cortex by a method described previously.[12] ATPase activity was measured by the release of inorganic ^{32}P from γ-ATP^{32} at pH 7·5. Inorganic ^{32}P content of the reaction system was determined after charcoal absorption of nucleotide phosphate by the method of Crane and Lipmann.[13] In these experiments, transport ATPase activity was defined as the hydrolysis of γ-ATP^{32} which was inhibited by 0·1 mM ouabain when enzyme preparations were incubated in the presence of 80 mM Na^+ and 20 mM K^+ ions.

The method for isolation of the acid-stable ^{32}P-labeled intermediate complex and the per cent of ^{32}P liberated has been described previously.[10] γ-Labeled ATP^{32} was obtained as the tetra-ammonium salt from the Radiochemical Centre, Amersham, Bucks, and had a specific activity greater than 1400 mc/m-mole. This material was diluted with disodium ATP (Sigma) and adjusted to pH 7·5 by the addition of solid Tris immediately before use.

Ethacrynic acid was obtained as a gift from Merck & Company Inc., (Rahway, N. J.) and was rendered water-soluble by the addition of solid Tris to give solutions of pH 7·5.

^{32}P radioactivity was determined in a Nuclear Chicago gas flow apparatus.

RESULTS

Preliminary experiments demonstrated that under the standard conditions of assay, i.e. in the presence of 80 mM Na^+ and 20 mM K^+, 85 per cent of the total ^{32}P liberated from γ-ATP^{32} could be inhibited by addition of 0·1 mM ouabain to the medium, and that the rate of ^{32}P liberation remained linear throughout the period of incubation.

The effect of varying concentrations of ethacrynic acid on ouabain-sensitive ATPase was then determined under these "standard" conditions of cation activation. The results are shown in Table 1, where it can be seen that inhibition of enzyme activity

TABLE 1. EFFECT OF ETHACRYNIC ACID ON OUABAIN-SENSITIVE ATPASE OF GUINEA PIG KIDNEY CORTEX

Ethacrynic acid (mM)	ATPase activity*		Δ†	% Inhibition
	Na + K	Na + K, ouabain		
Control	2·45	0·37	2·08	0
0·01	2·42	0·37	2·05	1
0·1	2·30	0·37	1·93	7
1·0	1·67	0·35	1·22	41
5	0·82	0·32	0·50	61
10	0·45	0·29	0·16	91

* Results are expressed as μmoles ^{32}P liberated from γ-ATP^{32}/0·1 ml of enzyme suspension (0·24 mg protein)/15 min at 37° and are the mean of five observations; 80 mM Na^+, 20 mM K^+ and 0·1 ml ouabain present where indicated.

† Calculated value for ouabain-sensitive transport ATPase, e.g. 85% of control activity is ouabain sensitive.

increased with the concentration of ethacrynic acid. At 1 mM ethacrynic acid, 41 per cent of the ouabain-sensitive component of the ATPase activity was inhibited, but there was no significant effect upon ouabain-insensitive ATPase activity. However, when 10 mM ethacrynic acid was employed, 91 per cent of the ouabain-sensitive component was inhibited and there was also more than 20 per cent inhibition of the ouabain-insensitive ATPase of these preparations.

Thus, at high concentrations, ethacrynic acid was not completely specific for transport ATPase, confirming the previous observations of Duggan and Noll.[4] Furthermore, under these conditions of assay, ethacrynic acid failed to demonstrate any greater degree of inhibition toward ouabain-sensitive ATPase than that reported by other investigators.[4, 6]

However, greatly increased sensitivity to ethacrynic acid inhibition could be demonstrated when the K^+ concentration of the assay system was decreased. Table 2

TABLE 2. EFFECT OF [K^+] ON ETHACRYNIC ACID INHIBITION OF OUABAIN-SENSITIVE ATPASE OF GUINEA PIG KIDNEY CORTEX

Expt.	[K^+] (mM)	Ethacrynic acid (mM)	ATPase activity* 80 mM Na^+	80 mM Na^+, 0·1 mM ouabain	Δ†	% Inhibition transport ATPase
A	20		2·74	0·38	2·36	
	20	1	2·04	0·38	1·66	29
	2		2·64	0·36	2·28	
	2	1	1·38	0·36	1·02	55
	0·2		1·44	0·20	1·24	
	0·2	1	0·66	0·20	0·46	63
B	20		3·00	0·46	2·54	
	20	5	2·07	0·43	1·64	36
	0·2		0·83	0·23	0·60	
	0·2	5	0·33	0·21	0·12	80

* Results are expressed as μmoles ^{32}P liberated from γ-ATP^{32}/0·1 ml of enzyme suspension/15 min at 37°. Expt. A contained 0·28 mg protein/0·1 ml; expt. B contained 0·32 mg protein/0·1 ml. Results are the mean of duplicate assays.

† Calculated value for ouabain-sensitive transport ATPase.

demonstrates that the inhibition by 1 mM ethacrynic acid could be increased from 29 to 63 per cent by lowering the [K^+] from 20 to 0·2 mM. This effect was also demonstrated when 5 mM ethacrynic acid was employed and here the degree of inhibition was increased from 36 to 80 per cent when the [K^+] was reduced from 20 to 0·2 mM. These results confirm and extend the recent observations of Nechay *et al.*,[6] who noted a 6 per cent increase in sensitivity toward ethacrynic acid when the potassium concentration of their medium was varied over a 10-fold range.

Previous work from this laboratory[14] has demonstrated that the transfer of ^{32}P from γ-ATP^{32} to an intermediate of the transport ATPase reaction[8–11] can be adequately demonstrated by the addition of 20 mM Na^+ to the medium and that dephosphorylation of this complex can be achieved by the subsequent addition of low concentrations of potassium ions. This system was thus ideal for an examination of effects of ethacrynic acid on transport ATPase in the presence of varying concentrations of K^+ ions.

The mean result of five experiments is given in Table 3, where control experiments again confirmed the effect of Na^+ and K^+ ions upon the incorporation into and the release of inorganic ^{32}P from the acid-stable intermediate of transport ATPase.[7–11] The increase in inorganic ^{32}P liberation on the addition of 20 mM Na^+ to the system is attributed to the presence of a trace amount of ammonium ions introduced with the labeled substrate.

When 5 mM ethacrynic acid was added to the system, there was an increase in the transfer of ^{32}P from substrate to intermediate, which in every case was accompanied by a decrease in the amount of inorganic ^{32}P liberated from the enzyme substrate. The increased ^{32}P transfer found in the presence of 20 mM Na^+ indicates that ethacrynic acid does not inhibit the formation of the ^{32}P-labeled complex, whereas the

TABLE 3. EFFECT OF ETHACRYNIC ACID ON THE TRANSFER OF ^{32}P FROM γ-ATP^{32} TO AN INTERMEDIATE OF (Na + K)-ATPASE*

Additions	Control		5 mM Ethacrynic acid	
	Counts ^{32}P incorporated	% ^{32}P liberated	Counts ^{32}P incorporated	% ^{32}P liberated
None	606	18	725	8
20 mM Na^+	3398	28	5647	16
20 mM Na, 5 mM K^+	515	36	805	25
20 mM Na, 0·5 mM K^+	715	38	969	27
20 mM Na, 0·05 mM K^+	1590	41	3260	22

* Results are the mean of five experiments (assays in duplicate). Counts ^{32}P incorporated given as cps/mg protein nitrogen; per cent inorganic ^{32}P liberated is equivalent to the per cent γ-ATP^{32} substrate hydrolyzed in 10 sec.

reduced liberation of inorganic ^{32}P from this complex suggests that ethacrynic acid inhibits the breakdown of this complex, which is necessary for the overall hydrolysis of ATP by transport ATPase.

This is confirmed by the increased incorporation of ^{32}P and decreased liberation of inorganic ^{32}P found when ethacrynic acid was added in the presence of K^+ ions. As in the previous demonstration of the effect of $[K^+]$ ion concentration on ethacrynic acid inhibition of transport ATPase activity (cf. Table 2), the effect of ethacrynic acid was most marked when the concentration of added potassium was least (0·05 mM).

DISCUSSION

In this paper we have confirmed that ethacrynic acid inhibits the hydrolysis *in vitro* of ATP by guinea pig kidney preparations of transport ATPase and that meaningful depression of activity cannot be measured under "standard" conditions of cation activation when the concentration of the drug is less than 10^{-4} M.[4, 6]

In addition, we have shown that higher concentrations of the drug do not specifically inhibit transport ATPase, which was defined as the ouabain-sensitive component of our test system, but also exert an inhibitory effect upon the residual ouabain-insensitive component of the total ATPase activity.[6]

Similar to the competition known to exist between K^+ and cardiac glycosides for transport ATPase inhibition,[1, 14–16] Nechay *et al.*[6] had observed a small (6%)

decrease in ethacrynic acid effect when the concentration of [K^+] was increased from 2 to 20 mM in their assay system. Thus it was perhaps possible to demonstrate an increased inhibition of transport ATPase by reducing the K^+ content of the assay system rather than by attempting to increase the concentration of drug, which was reported to be approaching maximum solubility under some experimental conditions[6] and certainly required increasingly high concentrations of Tris buffer (approaching 50 mM when 10 mM ethacrynic acid was employed) to neutralize in the experiments reported here.

This expectation was confirmed when about a 2-fold percentage increase in ethacrynic acid inhibition was observed when the concentration of [K^+] was reduced from 20 to 0·2 mM. Hence, ethacrynic acid could be expected to be a more potent inhibitor of transport ATPase in any biological situation in which the K^+ was low. Furthermore, the lack of specificity of this agent toward ATPase reactions, which is seen only at high concentrations of the drug, would not seem to be a serious problem, since both these experiments and others from our laboratory[14] suggest a factor of about 100 X in preference for the ouabain-sensitive component of the ATPase present.

Finally, our experiments with the sequential addition of cations to the reaction mixture and the isolation of a ^{32}P-labeled intermediate compound of the ATPase reaction, which has recently been demonstrated by Kahlenberg *et al.*[11] to be L-γ-glutamyl-phosphate, indicate that ethacrynic acid inhibits the K^+-dependent dephosphorylation step in the overall mechanism of ATP hydrolysis by this enzyme,[1] and thus offer an explanation for the antagonism between this diuretic agent and potassium ions.

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The Effect of Mg^{2+} and Ouabain on the Incorporation of P^{32} from γ-ATP^{32} into Na^+-and K^+-Activated Adenosine Triphosphatase[1]

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The effects of Mg^{2+} and ouabain were examined on the Na^+-stimulated transfer of P^{32} from γ-ATP^{32} to an acid-stable protein moiety recognized as an intermediate in the hydrolysis of ATP by Na^+- and K^+-stimulated ATPase. Using enzyme preparations from guinea pig renal cortex it was found that transfer of P^{32} was sensitive to the concentration of Mg^{2+} employed with an increase observed up to Mg^{2+}:ATP of 10:1, but transfer of P^{32} was reduced when this ratio was exceeded.

Ouabain did not alter the transfer of P^{32} in the absence of Na^+ at any concentration of Mg^{2+} examined, but reduced the transfer of P^{32} in the presence of either 20 mM Na^+ or 100 mM Na^+. Stimulation of P^{32} transfer by ouabain was not observed with either concentration of Na^+ or at any ratio of Mg:ATP.

The K^+ dependent release of inorganic P^{32} from the acid-stable complex was also examined and found to be ouabain sensitive; like the Na^+-dependent transfer of P^{32} the degree of inhibition by ouabain increased with increasing concentration of cardiac glycoside.

Under the conditions of these experiments both component steps in the formation and breakdown of a P^{32}-labelled intermediate of Na^+- and K^+-stimulated ATPase were found to be sensitive to inhibition by ouabain.

The active transport of Na^+ and K^+ across cellular membranes requires ATP and is blocked by ouabain. Since Skou's original demonstration in the peripheral nerve of the crab of an adenosine triphosphatase (ATPase) which required both Na^+ and K^+ ions for activation, and which could be inhibited by ouabain (1) there has been growing evidence that the transport process is dependent upon the function of this enzyme. However, the means by which the transport of ions is achieved remains unknown (2–5).

Skou's early tracer exchange experiments (6) suggested that a phosphorylated complex (intermediate) may exist during the hydrolysis of ATP and that this complex could possibly function as the ion-carrier. Subsequently, by using γ-ATP^{32} as substrate, many investigators have demonstrated that the terminal phosphate of ATP is transferred to a protein moiety of particulate enzyme preparations when Na^+ ions are present, and that dephosphorylation occurs when K^+ ions are subsequently added to the reaction (7–11). Although this phosphorylated compound has recently been identified as L-glutamyl-γ-phosphate, its role as a true intermediate in ATP hydrolysis by (Na^+ + K^+)-ATPase continues to be debated (12–16).

Because of its specific inhibitory function, the action of ouabain on this system has been widely studied and frequently used to define (Na^+ + K^+)-ATPase activity. Furthermore, the precise site of ouabain inhibition is of considerable importance in the design of model systems which seek to account for the

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mechanism of ion transport by this enzyme system (5, 25, 26). However, because of conflicting experimental evidence there is considerable doubt regarding the mode of inhibition of $(Na^+ + K^+)$-ATPase by cardiac glycosides.

Some workers have not observed significant inhibition of Na^+-stimulated transfer of P^{32} from γ-ATP^{32} to the intermediate compound (7, 17) whereas others have reported an inhibition which was increased with increasing concentration of cardiac glycoside (5, 9, 10). In addition there have been several reports of a stimulation of P^{32} transfer on the addition of ouabain, particularly when the Na^+ concentration of the system was low (9, 11) as well as reports of increased hydrolysis of ATP by $(Na^+ + K^+)$-ATPase in the presence of low concentrations of this agent (19–21).

Examination of these reports indicates that a wide variety of experimental conditions have been used in these studies, with not only variable concentrations of monovalent cations employed but also with considerable variation in the concentration of Mg^{2+} present, such that the Mg^{2+} to ATP ratio in these experiments has ranged from approximately unity to more than 200:1 (8, 10, 22).

This paper describes experiments which demonstrate that the effect of ouabain upon the transfer of P^{32} from substrate γ-ATP^{32} to intermediate compound by $(Na^+ + K^+)$-ATPase is influenced by the concentrations of both monovalent and divalent cations which are employed in the test system.

EXPERMENTAL PROCEDURE

A "heavy" microsome preparation of guinea-pig renal cortex was used as a source of $(Na^+ K^+)$-ATPase (18). After ageing at 0° for at least 5 days, more than 70% of the total ATP hydrolyzed under standard conditions of assay (18) could be shown to be inhibited by 0.1 mM ouabain. The method of incubation of enzyme with 5 μM γ-ATP^{32}, the recovery of acid-stable P^{32}-labelled compound and the percentage hydrolysis of ATP^{32} after short-term incubation has been described elsewhere (14).

γ-ATP^{32} of high specific activity (1644 mC/mM) was obtained from the Radiochemical Centre, Amersham, England. Chromatographically purified ouabain was a gift from Sandoz Ltd., Basle, Switzerland; all other reagents were B.D.H. (A.R.) grade. Incubation solutions containing Na^+ and K^+ were checked for contaminant cations by atomic absorption spectrometry (Si-Ro-Spec, type AA3, Techtron Pty. Ltd., Melbourne, Australia).

RESULTS

In these experiments partially purified preparations of $(Na^+ + K^+)$-ATPase were used after at least 5 days ageing at 0°. The degree of ouabain sensitivity of these preparations is shown in Table I. More than 70% of the total ATPase measured under standard conditions (18) was inhibited by 0.1 mM ouabain.

Effect of sodium and potassium ions. When these preparations are incubated at 37° with γ-ATP^{32} for ten seconds a P^{32}-labelled intermediate complex is formed which can be recovered as an acid-stable P^{32}-labelled compound (7, 14, 17, 27).

The level of incorporation of P^{32} into this complex is stimulated by Na^+ ions. When K^+ ions are subsequently added there is a fall in the level of P^{32}-labelled intermediate which is accompanied by an increase in the amount of inorganic P^{32} which is formed.

The effect of Na^+ and K^+ on P^{32} incorporation from γ-ATP^{32} is shown in Table II where it can be seen that both 20 mM and 100 mM Na^+ stimulated P^{32} incorporation and that this increased incorporation could be discharged by the inclusion of 0.5 mM K^+ into the medium.

Effect of ouabain. The effect of various

TABLE I

OUABAIN SENSITIVITY OF AGED PREPARATIONS OF $(Na^+ + K^+)$-ATPase FROM GUINEA-PIG RENAL CORTEX[a]

Experiment	μmoles P liberated/mg protein N/hr			Ouabain inhibition (%)
	$(Na^+ + K^+)$	$(Na^+ + K^+$, Ouabain)	(Ouabain sensitive)	
66	346	54	292	84
70	464	42	422	91
75	308	54	254	82
81	362	82	280	77
82	394	102	292	73
83	302	77	225	74

[a] The enzyme suspension was incubated at 37° under the conditions of assay described in Ref. 18.

TABLE II

EFFECT OF Na^+ AND K^+ ON THE HYDROLYSIS OF γ-ATP^{32} BY, AND INCORPORATION OF P^{32} INTO GUINEA-PIG RENAL CORTEX PREPARATIONS OF $(Na^+ + K^+)$-ATPase[a]

Additions	% ATP^{32} hydrolyzed	Counts P^{32} incorporated/ 100 sec/mg protein
None	10	3400
20 mM Na^+	24	26,200
20 mM Na^+, 0.5 mM K^+	31	5600
100 mM Na^+	25	22,900
100 mM Na^+, 0.5 mM K^+	27	8300

[a] The conditions of incubation and the procedures for the measurement of % ATP^{32} hydrolyzed and for the recovery of the acid-stable P^{32}-labelled intermediate after 10-sec incubation were those described in Ref. 14.

concentrations of ouabain upon the formation and breakdown of P^{32}-labelled intermediate of $(Na^+ + K^+)$-ATPase is shown in Table III.

Ouabain had no effect upon the incorporation of P^{32} in the absence of Na^+ (i.e., Mg^{2+}-ATPase), but in the presence of 20 mM Na^+ ouabain had an inhibitory action at all concentrations examined (10^{-3}–10^{-5} M). At 0.1 mM ouabain there was a 65% inhibition of the stimulation achieved by 20 mM Na^+. At the same time the hydrolysis of γ-ATP^{32} fell from 22% during the incubation period to only 11% when 0.1 mM ouabain was present.

In addition the release of Na^+-stimulated P^{32} by K^+ was also inhibited by ouabain with the greatest effect again being found with 1.0 mM. At this concentration the hydrolysis of γ-ATP^{32} was also reduced from 31 to 15% over the period of incubation.

Clearly under these conditions ouabain can be shown to inhibit both the phosphorylation and dephosphorylation of the intermediate.

Effect of magnesium ions. The effect of magnesium ions upon the incorporation of P^{32} from γ-ATP^{32} was examined over a range of concentrations so that the Mg:ATP ratio varied from 1:10 to 1000:1.

The effect of varying magnesium concentration in the presence of 20 mM Na^+ with and without 0.1 mM ouabain is shown in Table IV.

In the absence of sodium ions, changing the concentration of magnesium ions by a factor of 10,000 times had no appreciable effect upon the incorporation of P^{32}. In the presence of 20 mM Na^+, however, the maximum incorporation of P^{32} occurred when Mg:ATP was increased to 1:1 and declined as the concentration of magnesium was increased further. At Mg:ATP ratio of 1000:1 the degree of sodium-stimulated incorporation had fallen to 14% of that seen with a Mg:ATP ratio of 1:1. Under these conditions the degree of hydrolysis of ATP^{32} had also fallen from 37 to 13%.

There was a marked effect of 0.1 mM ouabain upon Na^+-stimulated incorporation of P^{32} at all levels of magnesium examined except when the Mg:ATP ratio was 1000:1 where there was only 9% inhibition by ouabain, but Na^+ stimulation of P^{32} incorporation is also very low with this high concentration of magnesium ion.

These experiments were again carried out with the same enzyme suspension but with 100 mM Na^+ replacing the 20 mM Na^+ used before. The incorporation of P^{32} again in-

TABLE III

EFFECT OF OUABAIN ON THE FORMATION AND BREAKDOWN OF A P^{32}-LABELLED INTERMEDIATE OF $(Na^+ + K^+)$-ATPase[a]

Additions	% ATP^{32} hydrolyzed	Counts P^{32} incorporated 100 sec/mg protein
None	11	3800
0.1 mM Ouabain	11	3800
20 mM Na^+	22	24,500
20 mM Na^+, 0.01 mM ouabian	15	20,500
20 mM Na^+, 0.1 mM ouabain	12	13,200
20 mM Na^+, 1.0 mM ouabain	11	10,100
20 mM Na^+, 0.5 mM K^+	32	5900
20 mM Na^+, 0.5 mM K^+, 0.01 mM ouabain	28	5700
20 mM Na^+, 0.5 mM K^+, 0.1 mM ouabain	24	8300
20 mM Na^+, 0.5 mM K^+, 1.0 mM ouabain	15	10,200

[a] The conditions of incubation and procedures for measurement are the same as those of Table II.

TABLE IV

EFFECT OF Mg^{2+} AND 20 mM Na^+ ON OUABAIN INHIBITION OF P^{32} TRANSFER TO AN INTERMEDIATE OF $(Na^+ + K^+)$-ATPase[a]

mM Mg^{2+}	Additions	% ATP^{32} hydrolyzed	Counts P^{32} incorporated/ 100 sec/mg protein
.0005	None	13	530
	20 mM Na^+	33	3700
	20 mM Na^+, 0.1 mM ouabain	16	2060
.005	None	15	490
	20 mM Na^+	37	4120
	20 mM Na^+, 0.1 ouabain	17	1890
.05	None	17	720
	20 mM Na^+	31	3490
	20 mM Na^+, 0.1 mM ouabain	16	1830
.5	None	15	570
	20 mM Na^+	22	2530
	20 mM Na^+, 0.1 mM ouabain	16	1160
5.0	None	11	490
	20 mM Na^+	13	990
	20 mM Na^+, 0.1 mM ouabain	11	890

[a] A 0.4-mg enzyme protein was incubated at 37° with 5 μM [γ-ATP^{32}] for 10 sec with varying concentrations of Mg^{2+}; 20 mM Na^+ and 0.1 mM ouabain were present as shown. The % ATP^{32} hydrolyzed and P^{32}-labeled intermediate were measured as in Table II.

TABLE V

EFFECT OF Mg^{2+} AND 100 mM Na^+ ON OUABAIN INHIBITION OF P^{32} TRANSFER TO AN INTERMEDIATE OF $(Na^+ + K^+)$-ATPase[a]

mM Mg^{2+}	Additions	% ATP^{32} hydrolyzed	Counts P^{32} incorporated/ 100 sec/mg protein
.0005	None	16	490
	100 mM Na^+	30	3370
	100 mM Na^+, 0.1 mM ouabain	16	1560
.005	None	16	420
	100 mM Na^+	37	3520
	100 mM Na^+, 0.1 mM ouabain	18	1590
.05	None	23	560
	100 mM Na^+	44	5600
	100 mM Na^+, 0.1 mM ouabain	28	1450
.5	None	26	840
	100 mM Na^+	42	3150
	100 mM Na^+, 0.1 mM ouabain	23	1480
	None	15	480
5.0	100 mM Na^+	24	2120
	100mM Na^+, 0.1 mM ouabain	15	910

[a] These experiments were carried out with the same preparation of $(Na^+ + K^+)$-ATPase and under identical conditions as those in Table IV.

creased with increasing concentration of magnesium ion, but the maximum effect was now found at Mg:ATP ratio of 10:1. When the concentration of Mg^{2+} was increased further the incorporation of P^{32} again declined as also seen with 20 mM Na^+. At Mg:ATP ratio of 1000:1 the level of Na^+ stimulation had fallen to 33% of the max-

imum with the higher concentration of Na^+ possibly affording some protection against the inhibitory action of high concentrations of magnesium ion (Table V).

Generally the degree of ouabain inhibition was greatest when Na^+ stimulation of P^{32} incorporation was maximal; however, the effect of ouabain could be greatly suppressed by ratios of Mg:ATP which exceed 100:1.

DISCUSSION

Only enzyme preparations having a relatively high proportion of ouabain-sensitive ($Na^+ + K^+$)-ATPase were employed in this study as difficulties are introduced into the interpretation of data if significant amounts of ouabain-insensitive Mg^{2+}-ATPase are present.

Preliminary experiments demonstrated that the addition of Na^+ ions to the system resulted in a stimulation of P^{32} transfer to the acid-stable intermediate, and that the subsequent introduction of K^+ resulted in a fall in the level of this P^{32}-labelled intermediate, at the same time as there was an increase in the level of inorganic P^{32} formed. These results confirm previous reports from this and other laboratories which have been interpreted as a demonstration at a two-step reaction mechanism for the hydrolysis of ATP by ($Na^+ + K^+$)-ATPase (7, 9, 23, 27). The first of these steps is now generally thought to be a Na^+-stimulated phosphorylation of a membrane protein component, recently identified as L-glutamyl-γ-phosphate (12). This reaction is followed by a K^+ requiring dephosphorylation which liberates inorganic phosphate.

Both Post, Sen, and Rosenthal (9) and Rodnight, Hems, and Lavin (11) have reported differing effects of ouabain on the Na^+-stimulated transfer of P^{32}, which range from inhibition to stimulation with differing concentrations of Na^+; and we have drawn attention to the effect of varying Mg:ATP ratios employed by different investigators (5) and to the inhibitory action of Mg^{2+} on ATP hydrolysis by this enzyme (24).

We, therefore, examined the effect of 0.1 mM ouabain upon Na^+-stimulated P^{32} transfer, at two concentrations of Na^+ (20 and 100 mM) and using variable ratios of Mg:ATP from 1:10 to 1000:1. These experiments clearly show that the transfer of P^{32} in the absence of Na^+, which is thought to be insensitive to ouabain, was little affected by a ten-thousand-fold change in Mg^{2+} ion concentration. Conversely, the transfer of P^{32} in the presence of both 20 and 100 mM Na^+ was increased by raising the concentration of Mg^{2+} so that Mg:ATP was either 1:1 or 10:1. When the ratio of Mg:ATP exceeded 10:1 then there was a marked decline in Na^+-stimulated P^{32} transfer which was more pronounced with 20 mM Na^+ present than with 100 mM Na^+. Furthermore, 0.1 mM ouabain reduced Na^+-stimulated P^{32} transfer at both concentrations of Na^+ examined and at all ratios of Mg:ATP.

However, where Na^+ concentration was only 20 mM, and with a Mg:ATP ratio of 1000:1, that is, under conditions in which Na^+ stimulation was least effective, then also was the inhibition by ouabain least noticeable.

However, unlike the findings of Rodnight *et al.* (11) we did not find that ouabain stimulated the Na^+-induced transfer of P^{32} at a "low" concentration of this ion. In addition, examination of the effect of a range of concentrations of ouabain upon Na^+-stimulated transfer of P^{32} shows that when the Na^+ concentration is 20 mM, and the Mg:ATP ratio is 1, then appreciable inhibition occurs at all concentrations examined (10^{-3}–10^{-5} M). Similarly the K^+-induced dephosphorylation was also found to be sensitive to ouabain, and this reaction also showed increasing effect with increasing concentration of cardiac glycoside.

These observations may account for the apparent discrepancy between the findings of workers who have reported differing sensitivity of Na^+-stimulated P^{32} transfer to ouabain, where widely differing concentrations of Na^+ ion and variable Mg:ATP ratios have been employed.

Certainly they serve to demonstrate the interdependence of the reactants in the transfer of P^{32} to a phosphorylated intermediate during the hydrolysis of γ-ATP^{32} by ($Na^+ + K^+$)-ATPase, and reinforce the complex nature of this reaction. It is apparent that several model systems of ion

transport by this enzyme (5, 25) may have to be modified to include the observation that both sites of cation activation by the enzyme are sensitive to ouabain inhibition.

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CHAPTER 2

Membrane Metabolism and Ion Transport

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I. Introduction

The belief that the boundary zone of cells consists of specialized structures is widely accepted, although as yet there is no unequivocal evidence to support this view. These structures are referred to as membranes, and are thought to impart one area of morphological, functional and chemical specialization to cells. As Mitchell (1962) has pointed out, the reactions of the cell are not directionless scalar abstractions but have both magnitude and direction in space. An important function of cellular membranes is that they provide a framework for such cellular organization. A further specialized property of membranes is their ability to select between the vast array of ions and molecules encountered in the heterogeneous environment of cells. These specialized properties of membranes provide a major biological function in their capacity to separate phases both between the cell and its environment as well as within the cell itself. It must be remembered, however, that in addition to the maintenance of the *milieu intérieur*, that numerous other phenomena such as excitability and contractility, the properties of cell movement and adhesion, shape change, including pinocytosis and phagocytosis, and the antigen-antibody reactions of the cell are also generally thought to be associated with the structure and function of cellular membranes.

Despite this general agreement, the properties attributed to these specialized structures often depend upon the viewpoint of the investigator. For example, to the physiologist measuring ion fluxes between the cell and its environment, the membrane is an operational barrier to the free diffusion

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of ions; to the electron microscopist the membranes are structurally defined components with recognizable electron densities under different conditions of staining; to the preparative biochemist membranes may be a sedimentable moiety of tissue mashes having more or less defined sedimentation characteristics and containing specific enzymes, whereas the physical chemist may think of membranes as lipoprotein chemical structures having variable physical and chemical properties.

It is our purpose in this discussion of membrane metabolism to attempt some synthesis of these divergent concepts, whilst examining the membrane from the viewpoint of its major biological function, the separation of phases. We shall direct our attention particularly to the movement of ions across these membranes and to the metabolic properties of the membrane responsible for the control of the ionic composition of the animal cell, long known to differ profoundly from the composition of its fluid environment.

It has been known from the work of Macollum early this century that whereas sodium is the principal cation of the extracellular fluid, potassium has this role inside the cell (Macollum, 1905). In addition, it is known that concentration gradients exist for calcium, magnesium, phosphate and other ions. Similar concentration gradients are also found for other charged and uncharged solutes such as amino acids and sugars.

The manner in which cells achieve the regulation of the composition and concentration of these solutes has been the subject of much investigation and speculation. Because the concentration of many ions and solutes determined experimentally in the cell do not agree with those calculated from the thermodynamic parameters of ionic activity and charge difference across the membrane, it has been argued that some other force is associated with the maintenance of these gradients.

For example, that component of the ion flux which is not in accord with the predictions of a physical theory which visualizes the ion movement occurring due to diffusion, and under the influence of potential differences across the membrane, is usually described as *active transport*. This term was first introduced by Wilbrandt in 1937; it is today often defined as that phenomenon wherein an ion is moved against the existing electrochemical potential gradient (Ussing, 1949).

The inherent weakness of this concept lies both in the assumption of the validity of the physical theory as applied to the cell and to our inability to determine accurately the concentration and activity of *free* ions inside the cell.

Some investigators such as Eisenmann (1961) and Ling (1962) have recognized the oversimplification of the notions implicit in the application of this type of physical theory to ion movement through cells, and have therefore attempted to develop more sophisticated physical models of the membrane region and the cell interior. These modified theories take into account varying densities and arrangement of the fixed charges in membrane structures as well as changes in the order of hydration of the ions themselves.

Most of these more sophisticated theories imply that membranes are not

inert inasmuch as their properties do not remain fixed in either a physical or chemical sense, but include the concept that both *in vitro* and *in vivo* the permeability properties of membranes can be altered by changes in the chemical composition of the environment in which they function. Although examination of the varying theoretical approaches to membrane function is of great value in an understanding of the selective accumulation of ions by cells, the theories themselves offer no solution to the underlying problem of how this change in properties is achieved.

At the experimental level there is a considerable body of evidence that nonspecific injury to cell metabolism can interfere drastically with the distribution of ions and solutes. These injuries affect the capacity of the cell to maintain the apparent thermodynamically "unfavourable" concentration gradients, or to selectively accumulate certain ions. For example, exposure to cold or a wide variety of metabolic poisons such as iodoacetate, 2,4-dinitrophenol, fluoride and cyanide have been repeatedly shown to alter ion gradients or the distribution of other solutes in a wide variety of mammalian cell preparations (Flynn and Maizels, 1949; Green and Parpart, 1953; Robertson, 1960).

It is the particular component of ion or solute distribution across membranes which is affected by metabolic injury that we prefer to think of as an operational definition of *active transport*. This definition requires no *a priori* prediction of any physical model for ion transport, only that in some unspecified way the energy for the process is derived from the metabolic activity of the cell.

II. Active Transport

The dependence of active transport upon cellular metabolism is more complex than appears at first sight. In recent years it has become apparent that not only does the metabolism of the cell control active transport, but that the processes of transport themselves also influence cellular metabolism (Mitchell, 1962; Minakami *et al.*, 1964).

An example of this relationship is found in the action of the cardiac glycosides. It has been shown that these agents inhibit the active transport of sodium and potassium ions both *in vivo* and *in vitro* (Schatzmann, 1953) as well as depress the respiration of whole cells. However, these agents are without effect on the mitochondrial enzyme systems which directly mediate respiration of the cell (Whittam and Ager, 1965).

Clearly, this relationship suggests the existence of the type of feed-back control common to the regulation of physiological processes. The major problem with this concept of the dependence of active transport upon cellular metabolism is how to link the energetics of the cell to the physical movement of solutes across cell membranes. From a study of the effect of metabolic inhibitors on the distribution of ions, research workers have developed two divergent points of view.

Historically, the first of these theories originated in the work of Lund (1928), Lundegårdh (1939), Davies and Ogston (1950) and Conway (1951),

who suggested that the energy for active transport is derived from a redox-potential difference across membrane zones and is associated with the enzymatic dehydrogenation of certain substrates and the subsequent transport of electrons down a chain of electron carriers. Conway (1953, 1955) has termed this hypothesis the "redox-pump" theory of active transport.

Recent developments of this concept have been proposed by Mitchell, whose theories imply that the enzymatic mechanism responsible for the movement of ions is localized at the cellular site of electron transport, namely within the membranes of the mitochondria (Mitchell, 1961, 1962). Supporting evidence for this theory can be found in the experimental observations of Moore and Pressman (1964) and Chappell and Crofts (1965).

However, there is ample experimental evidence to demonstrate that any agent which modifies the metabolism of the cell in such a way as to reduce the concentration of ATP within the cell, whether by direct effect on the synthesis of ATP either by electron transport phosphorylation or glycolysis, or by increased breakdown of ATP will certainly affect the ionic gradients of the cell. This evidence has led to the alternative postulate that ATP itself is the direct energy source for *active transport*, and that whilst the mitochondria remain the major site of ATP production within the cell, the membranes of these organelles are not necessarily the site of *active transport* in the whole cell (Bartley and Davies, 1954).

To illustrate the role of the membranes as it has been conceived by protagonists of these two schools of thought, we intend to consider the present-day viewpoint as it relates to one particular pair of monovalent ions, namely sodium and potassium.

A. *Mitochondrial membranes*

One of the earliest membrane organelles to be distinguished within cells were the ovoid bodies known as mitochondria. They are about 1 μ in length and in electron microscope section can be seen to be surrounded by a single osmiophilic outer layer and a pleated inner layer making up the cristae. Except perhaps in very high resolution studies, the electron microscope appearance of these osmiophilic layers is much the same as that of other membrane structures of the cell. However, it is well known that the membranes of the mitochondria are biochemically unique, and possess functions not located elsewhere within the cell. There can be little doubt that the "inner" mitochondrial membranes contain the assembly of enzymes and electron carriers necessary for electron transport and the subsequent generation of ATP by this process.

There has been speculation about the possible role of mitochondrial membranes as enzymatic mediators of ion transport almost since these organelles were first recognized (Claude, 1946). These speculations were further encouraged by the findings of Bartley and Davies (1954), when these

workers described ion permeability characteristics of mitochondria which seemed to depend upon their metabolic status.

Since that time the examination of the role of mitochondria in ion transport has followed two general directions. On the one hand, the capacity of isolated mitochondria to accumulate a variety of ions has been examined in some detail, whereas an alternative approach has been to study the influence of these ions upon the metabolic activity of the organelles themselves.

Because the functions of the mitochondrial membranes will be dealt with more fully in a subsequent chapter, we shall confine ourselves to a summary of the current state of belief regarding these two fields of investigation.

In any discussion of the mitochondrial membrane it is well to remember that different permeability properties have been ascribed to each of the two osmiophilic layers seen in electron microscope sections. For example, it is known that mitochondria are sensitive to the osmotic pressure of their environment and that under hypotonic conditions mitochondria take up water and swell. Much of the evidence concerning mitochondrial permeability has been obtained indirectly from the measurement of this so-called swelling by turbidometric or light scattering techniques.

Since the work of Chappell and Greville (1958), it has been recognized that another type of swelling takes place in mitochondria, even when an iso-osmotic environment is maintained. This swelling requires the capacity of the membranes to carry out electron transport and thus it is often referred to as "metabolic swelling". It is stimulated by calcium ions and can be inhibited by any agents which prevent the transport of electrons.

It has been postulated that these two types of swelling take place in two different compartments of the mitochondria: osmotic swelling being a function of the outer membrane and metabolic swelling being related to the permeability of the inner membranes (Lehninger, 1962).

There is considerable presumptive evidence that the outer membrane is readily permeable to a wide variety of ions and other solutes, while it is generally believed that the inner membrane is rather selectively permeable. For example, the relatively small anions chloride, bromide and nitrate appear to pass through the outer membrane quite readily but fail to penetrate the inner membrane, whereas the much larger phosphate anions penetrate into the inner matrix of the mitochondrion.

Under the majority of experimental conditions *in vitro* the entry of the cation pair sodium and potassium into mitochondria is not selective. It has been shown, again *in vitro*, that calcium, magnesium, manganese and strontium also enter the mitochondria along with phosphate anion, and precipitates of insoluble phosphates can be witnessed under special circumstances; namely, during active respiration when there is no accepter (ADP) present as a "sink" for the high energy phosphate intermediates which are thought to be generated during electron transport. The meaning of this phenomenon in terms of the *in vivo* accumulation of ions remains obscure.

One explanation that has been given for these observations is that the entry of magnesium and phosphate takes place under metabolic conditions

in which there is a rising pH within the mitochondria (Millard *et al.*, 1964). Under these conditions insoluble magnesium phosphates are formed, thereby leading to reduction of free magnesium ion concentration. It is thought that magnesium ions continue to enter mitochondria by diffusion.

The most important ionic selectivity claimed for mitochondrial membranes is that proposed for the hydrogen ion. This concept has become the basis of an interesting theory for the generation of ATP from the accepter substance ADP by electron transport phosphorylation. Essentially this theory, originally proposed by Davies and Ogston (1950) and later expanded by Mitchell (1961, 1962), implies that the "metabolic" secretion of protons occurs during the synthesis of ATP while the splitting of ATP within the mitochondria is accompanied by a reverse movement of protons.

There is evidence that mitochondrial membranes are normally impermeable to the influx of protons. It is further postulated that the efflux of protons from the mitochondria is balanced electrically by a corresponding influx of another cation. Under certain experimental conditions the movement of protons can be shown to be coupled to the movement of potassium ions in a direction opposite to that of the protons. This exchange of potassium ions for protons across the mitochondrial membrane can lead to an accumulation of potassium ions within the mitochondria. The presence of the antibiotic valinomycin is required to demonstrate this effect (Moore and Pressman, 1964). Another antibiotic, gramicidin A, which will also induce the expulsion of protons from mitochondria, does not show the same selectivity towards cation as does valinomycin and will allow other univalent cations to accumulate in exchange for protons (Chappell and Crofts, 1965).

It has long been known that both magnesium and phosphate are required for electron transport phosphorylation. However, the influence of sodium and potassium ions on both mitochondrial respiration and phosphorylation are not so clearly defined. Some workers have claimed to show a specific stimulation of these metabolic activities by potassium ions, but there is also evidence to the contrary (Opit and Charnock, 1965*b*; Chappell and Crofts, 1965).

In any experimental examination of this problem using isolated mitochondria, it must be remembered that the addition of ions to the supporting medium not only provides specific ionic components, but induces a change in the osmolarity of this medium. Because mitochondrial metabolism, like its morphology, is sensitive to osmotically induced changes in the volume of the organelles, the interpretation of such ionic effects is complicated.

Thus, although it seems reasonable that these mitochondrial ion exchange phenomena may well have significance in the capacity of the mitochondrial membranes to translocate protons, as part of their metabolic function, we do not believe that the accompanying accumulation of other cations has real significance in determining the ionic environment of the whole cell.

In general, this belief is based on the following four arguments which seem to us to preclude the mitochondria from this role. First, in red blood cells which are devoid of mitochondria, the selective accumulation of many ions

is identical to that seen in cells having mitochondria. Second, isolated mitochondria appear to show little discrimination between the accumulation of similar ion pairs, for example between sodium or potassium, or between calcium and magnesium. Third, the accumulation of potassium ions in mitochondria is not influenced by the cardiac glycosides which are known to affect both ion accumulation and respiration of the intact cell. Finally, there is evidence that the respiratory rate of organized cell preparations such as tissue slices is sensitive to change in the sodium:potassium ratio of their environment, whereas the evidence for this effect on isolated mitochondria is equivocal.

A plausible explanation for this paradox can be found in the enzymatic properties of other membrane structures of the cell, which are to be discussed below.

B. *Cytoplasmic membranes*

Let us now turn our attention to another group of membranous structures whose metabolism will be examined with respect to ion transport. These structures are generally referred to as the "plasma" membrane and the endoplasmic reticulum. The "plasma" membrane is the lamellar osmiophilic structure, about 80 Å across, which lies at the outer limit of the cytoplasm and which, after the usual electron microscope staining procedures, appears as a clear area sandwiched between two layers of electron opaque material.

The term endoplasmic reticulum currently refers to those masses of folded and pleated membrane structures seen within the cytoplasm of many cells, and which in electron microscopic appearance closely resembles and has dimensions similar to that of the "plasma" membrane. It is generally believed that the endoplasmic reticulum, which may be either smooth or granular in appearance, is in continuity with the "plasma" membrane at least for some portion of the cells "internal" surface, and that these membrane structures constitute a network or skeleton throughout the cell. This knowledge depends almost exclusively on the findings and interpretations of electron micrograph studies and as such must be both incomplete and subject to considerable preparative artifact.

Several cogent points should be borne in mind when considering this information. There are, for example, sometimes startling differences in the electron microscope appearance of membranes when different staining procedures are employed. Furthermore, because of the nature of the procedures involved in fixation of the specimens, one is invariably examining "dead" tissue, and visualization by electron microscopy is only possible with material having an ordered structure or regularly repeating subunits. In the case of the lamellar membrane structures seen after the usual osmium fixation, the "empty space" detected between the more electron dense layers does not represent the absence of material but indicates that the molecular structures in this region cannot be visualized. With the "plasma" membranes this may be as much as 30% of the total structure.

Probably the greatest difficulty in the interpretation of electron micrograph findings is due to the fact that the material which is bombarded in a 20 mμ thick section in the electron microscope produces only a two-dimensional representation of what is clearly a much more complicated three-dimensional structure.

Notwithstanding these problems, consideration of the evidence derived from electron microscopy leaves one with the firm impression that the cell membrane is a branched continuum. Thus what might be thought of as the "outside environment" penetrates deep into the substance of the cell and is not confined at the peripheral boundary. Clearly this view tends to complicate the classical representation of "intracellular" and "extracellular" space as distinctly separable by a membrane diffusion barrier and replaces it with the concept of a cytoskeleton. We find it easy to equate the microscopically identifiable membrane structures of the endoplasmic reticulum with the "operational" concept of a membrane which represents both the site and barrier to free diffusion of solutes into the cell. Hence this seems to be a logical area to look for a membrane function related to the active transport of ions.

It has been widely speculated that ATP itself is able in some way to alter the "permeability" characteristics of membranes. This implies that the membranes themselves play an active role in the metabolism of ATP. How is this interaction to be linked to the movement of ions or molecules across biological membranes, and against thermodynamic potential gradients? The most popular explanation for this linkage is that a "carrier" is involved. In some unspecified manner it is supposed that the transported substance combines specifically with a molecular group in the membrane. This enables movement of the transported substance to occur across the membrane (Osterhout, 1940; Ussing, 1948; Rosenberg, 1948). This theory has been extended to include the idea that either the attachment of the transported substance to the "carrier", or the detachment of the transported substance on the "other" side of the membrane requires ATP directly, or is brought about by an enzyme system which utilizes ATP.

During the last decade two important membrane-bound ATP hydrolysing enzyme systems (termed ATPases) have been characterized and implicated in the active transport of sodium ions across membranes. Both these systems have been proposed as an enzymatic basis of the so-called "sodium pump" responsible for the metabolically dependent ejection of sodium ions from living cells (Dean, 1941; Krogh, 1946).

The first of these systems to be discussed suggests that transport of sodium ions is effected by an ion-carrier whose synthesis and breakdown involves the hydrolysis of ATP by a series of membrane enzymes.

1. *Phosphatidic acid*

This system was first examined by Hokin and Hokin (1960), who had observed that the isolated salt gland of the albatross, when stimulated by acetylcholine, is capable of secreting hypertonic sodium chloride. The

membranes of this gland synthesize significantly increased amounts of phospholipids under these conditions. Chemical analysis of the phospholipids revealed that much of this increased synthesis was accounted for by the phosphatidic acid of the microsomal or membrane fraction of the gland.

After extensive examination of the enzyme composition of the membrane fraction, and in particular the phosphatidic acid generating system, Hokin and Hokin established the presence of a series of ATP-consuming reactions, which lead to the cyclic reformation of phosphatidic acid.

$$\text{1, 2-diglyceride} + \text{ATP} \xrightarrow[\text{kinase}]{\text{diglyceride}} \text{3-phosphatidic acid} + \text{ADP}$$

$$\text{3-phosphatidic acid} + \text{H}_2\text{O} \xrightarrow[\text{phosphatase}]{\text{phosphatidic acid}} \text{1, 2-diglyceride} + \text{H}_3\text{PO}_4.$$

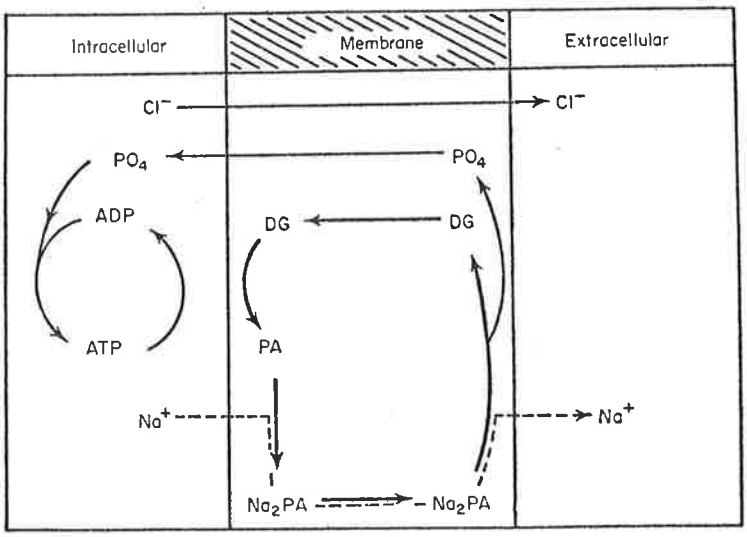

FIG. 1. Postulated phosphatidic-acid sodium pump. This figure is a schematic representation of the membrane region of a cell. The membranes contain diglyceride kinase and phosphatidic acid phosphatase, the two enzymes necessary to perform the cyclic conversion of diglyceride (DG) into phosphatidic-acid (PA), which is thought to act as the carrier for sodium ions (Na_2PA).

Two major features of this "phosphatidic acid cycle" were that phosphatidic acid phosphatase was shown to be stimulated by sodium ions and that the over-all reaction sequence was localized in the membrane fraction and constituted a sodium sensitive ATPase.

Initially it was suggested that the negatively charged polar group of phosphatidic acid would bind Na^+ to form an electrically neutral complex of sufficient solubility to move across the membrane. Thus this cycle of reactions could function as a transducer between chemical energy (ATP) and the lipoprotein component (phosphatidic acid) of the membrane and function as a physical ion (Na^+) carrier system (Hokin and Hokin, 1960). A schematic representation is given in Fig. 1.

Despite the considerable interest aroused by this scheme, and the subsequent discovery that a variety of cell membranes contained the elements of this reaction sequence, the scheme has not gained general acceptance because it has not been possible to meet a number of important requirements.

Kinetic examination demonstrated that there was a maximum ratio of 2 Na^+ transported per mole ATP consumed whereas experimental values, in the whole cell, of the ratios of sodium transported per mole of ATP consumed greatly exceeded this figure. This ratio would represent an energetically expensive load on cellular ATP production. Of more direct significance was the realization that agents like the cardiac glycosides, which inhibit the transport of sodium, were without effect on the synthesis of phosphatidic acid.

Despite some modifications to the original scheme it became necessary to postulate that potential binding sites existed on the lipoprotein membrane other than those of the phosphate groups of the metabolically active phosphatidic acid, and that the reactions of the phosphatidic acid cycle produced conformational changes in the lipoprotein membrane which successively create and destroy specific cation-binding sites (Hokin and Hokin, 1963). Unresolved difficulties related to the maximum possible velocity of the component reactions of the phosphatidic acid cycle and the lack of sensitivity to agents known to inhibit sodium transport remain major objections to the acceptance of this scheme.

An alternative version of this type of carrier hypothesis was first postulated by Goldacre (1952), who suggested that ATP hydrolysis and ion transport may be accomplished by a single enzyme which is specifically adapted to the movement of both sodium and potassium ions by rotation or shape change during the formation of enzyme substrate complexes. An enzyme system which appears to be an attractive candidate for such a role in membrane transport will now be discussed.

2. *($Na^+ + K^+$)-activated ATPase*

The existence of this enzyme system, now sometimes called "transport" ATPase, was first recognized by the Danish physiologist, J. C. Skou, who, in 1957, reported the presence of a membrane-bound ATP hydrolysing enzyme in the leg nerves of the shore crab *Carcinus maenas*.

The most interesting feature of Skou's enzyme preparation was that its ATP hydrolysing property was stimulated by the combined presence of sodium and potassium ions (Skou, 1957, 1960), a feature in accord with the generally accepted view that sodium and potassium ions are transported across membranes by a common mechanism in a linked or coupled reaction (Harris, 1954).

Since this original paper, both interest and experimentation with ($Na^+ + K^+$)-activated ATPase preparations have grown exponentially. Because of the now prevalent belief that this membrane enzyme system plays a central role in the transport of sodium and potassium ions (at least)

across cellular membranes, we now propose to deal at some length with the characteristics of this system.

To present the enzymological features of this enzyme we shall examine in detail the behaviour of this ATPase as it is found in a preparation from the so-called heavy microsomal (membrane) fraction of guinea pig kidney cortical homogenates. Because one source of confusion in reported accounts of this enzyme comes from the wide range of tissue preparations showing ATP hydrolysing activity, we believe it is desirable to commence the discussion with an operational definition of this particular ATPase system.

In our view, this enzyme is best defined as one which hydrolyses the γ-phosphoryl group of adenosinetriphosphate in the presence of magnesium ions. This activity is stimulated by the subsequent addition of sodium and potassium ions together, and this stimulated increment of activity can be abolished completely by the cardiac glycoside ouabain at a concentration of 10^{-4} M. With this purely operational definition in mind, let us consider some of the major biochemical features known about this membrane-bound enzyme.

TABLE I

The effect of Mg^{2+} on guinea pig microsomal ATPase activity

Additions	μmoles P/mg protein N/h*		
	2 mM Mg^{2+}	5 mM Mg^{2+}	10 mM Mg^{2+}
80 mM Na^+, 20 mM K^+	448	371	318
80 mM Na^+, 20 mM K^+, 0·1 mM ouabain	196	183	176
Δ†	252	188	142

* Enzyme preparation incubated with 2 mM ATP in pH 7·6 buffer at 37°.

† Δ is the activity in the presence of 80 mM Na^+ and 20 mM K^+ which was inhibited by 0·1 mM ouabain, i.e. "transport" ATPase (see Charnock and Post, 1963*b*).

a. Biochemical features

(i) *The divalent cations.* Although the presence of magnesium ions is absolutely necessary to produce full activation in combination with sodium and potassium ions, the presence of magnesium ions alone produces only a relatively slow rate of liberation of phosphate from the substrate adenosinetriphosphate. To produce maximal activity the molar concentration of magnesium ions must approximately equal that of the substrate. Increasing the Mg^{2+} concentration beyond this point leads to increasing inhibition of the sodium and potassium stimulated activity. Data which demonstrates this effect is given in Table I.

When magnesium ions are replaced by calcium ions, there is some enhancement of the activity compared to that seen with magnesium alone; but no additional activity is found when sodium and potassium ions are

added. Furthermore, no component of the calcium effect can be inhibited by ouabain and therefore, in terms of our definition, calcium ions can be thought of as inhibiting the enzyme. Neither can barium nor strontium ions replace magnesium ions in this regard.

(ii) *Univalent cations.* If any single alkali metal ion (Li, Na, K, Rb, Cs) is added to the enzyme in the presence of ATP and magnesium, no additional activity is seen beyond that which would occur in the presence of magnesium alone. Obviously, such experiments must be conducted under careful control for contaminant ions in the reaction mixture.

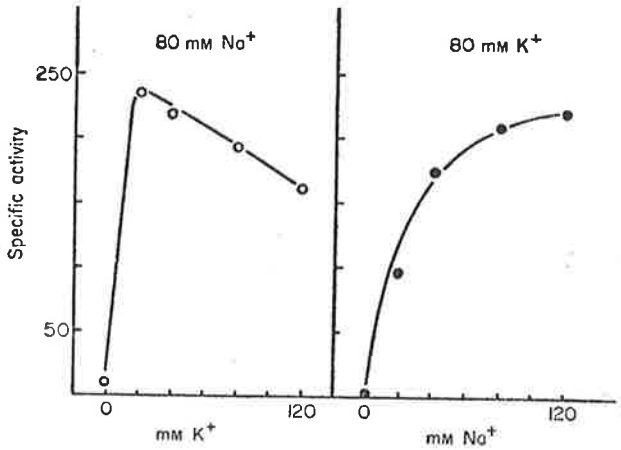

FIG. 2. The effect of Na^+:K^+ ratio on ($Na^+ + K^+$)-activated ATPase. These plots show the effect of varying the concentration of one univalent alkali metal ion in the presence of a fixed concentration of another. Specific activity refers to the release of inorganic phosphate from ATP under standard incubation conditions (Charnock and Post, 1963*b*).

However, when combinations of these ions are employed, several interesting features emerge. To achieve enhancement of ATP hydrolysis, sodium is usually regarded as an absolute requirement and no other ion can replace it. The requirement for the co-cation is not so specific, although it appears from numerous accounts of the enzyme that the effectiveness of the other alkali metal ion varies. In addition, ammonium ions are also effective in combination with sodium ions.

Since 1960 it has been clear that the actions of these monovalent cations are not independent, and a typical effect of one ion upon the other is shown in Fig. 2. From this figure it can be seen that when the sodium concentration is fixed at a relatively high level (e.g. 80 mM) the enzyme activity is extremely sensitive and is proportional to small changes in the concentration of potassium in the range 1–20 mM. On the other hand, when the potassium concentration is fixed at 80 mM, the enzyme activity is similarly sensitive to variations in the sodium concentration over a range of 1–20 mM. This suggests that under certain conditions the rate of ATP hydrolysis by ($Na^+ + K^+$)-activated ATPase is extremely sensitive to relatively small

changes in the concentration of one or other cation required for its activation (Skou, 1960; Charnock and Post, 1963*b*). This interdependence upon ionic concentrations provides the basis for a physiological or "feed-back" control mechanism. This will be referred to again later in connexion with the suggested physiological role of this enzyme.

From this relationship *in vitro* a number of investigators have deduced "optimal ratios" for the conditions necessary to achieve maximum rates of ATP hydrolysis and have attempted to study the stoichiometry of these ionic effects. It must not be overlooked in considering the validity of deductions based on these data that small differences in experimental design have produced "optimal ratios" of Na:K varying from 1:1 to 10:1.

It is of particular interest, however, that whereas high concentrations of potassium ion are inhibitory towards isolated particulate enzyme preparations of kidney cortex, similar concentrations of sodium are not inhibitory.

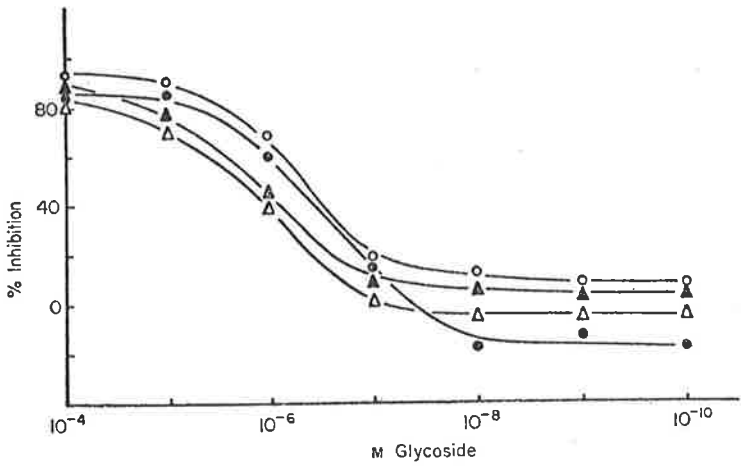

FIG. 3. The inhibition of $(Na^+ + K^+)$-activated ATPase by cardiac glycosides. The figure demonstrates the inhibitory effect of a range of concentrations of ouabain, lanatoside C, digitoxin and cymerol on $(Na^+ + K^+)$-activated ATPase *in vitro*. ▲, Ouabain; △, lanatoside C; ●, digitoxin; O, cymerol.

(iii) *Cardiac glycosides*. The most important and encouraging feature of this membrane-bound enzyme system is its specific and sensitive inhibition by the cardiac glycosides. In the main most workers have used Strophanthin-G (ouabain: 3-0-β-L-rhamnopyranosyl-1β,3β,5,11α,14,19-hexahydroxy-5β-card 20,22,enolide) for their experimental studies solely because it is readily water-soluble. However, almost the whole range of therapeutically effective glycosides has been found to be inhibitory over similar concentration ranges. The effect of ouabain, digitoxin, lanatoside-C and cymerol on a particulate enzyme preparation from kidney cortex is shown in Fig. 3.

Although cardiac glycoside inhibition of the sodium and potassium

stimulated component of the ATPase is virtually complete at concentrations of 10^{-5} M, 50% inhibition is found at 5×10^{-6} M and detectable effects can be seen at concentrations of 10^{-7} M. Such lower concentration ranges are certainly of the same order as the concentration achieved in both man and experimental animals when these agents are used therapeutically.

There are a number of reports indicating that the degree of enzyme inhibition by cardiac glycosides can be influenced by adjustment of the (K^+) concentration. In particular, several authors have reported that as the (K^+) concentration is increased, the inhibitory action of ouabain is reduced (Glynn, 1964; Schatzmann, 1965; Ahmed and Judah, 1965). Various interpretations of this phenomenon have been proposed, including the belief that direct competition exists between potassium ions and ouabain for a particular reaction site on the enzyme surface. This possibility will be discussed on p. 95.

(iv) *Substrate.* Although this enzyme has been defined as an ATP hydrolysing system, it is capable of reaction with the other nucleotide triphosphates. The reactivity towards these nucleotides is much lower than that towards ATP, and only with cytidine triphosphate is there any significantly stimulated activity upon the addition of sodium and potassium ions. From a number of investigations with reasonably purified preparations it appears that other organo-phosphate compounds are not hydrolysed by this enzyme system.

The affinity of the enzyme for ATP has been measured over a range of concentrations, and from these experiments classical plots of the effect of substrate concentration on enzyme velocity have been made. It seems that the K_m for ATP by our preparations of this enzyme is of the order of 2–5 mM and such concentrations of ATP are thought to be those normally found in cells.

This enzyme clearly shows the phenomenon of product inhibition with one of the products (ADP) of ATP hydrolysis. For example, data from our laboratory indicate a 40% reduction in the rate of ATP hydrolysis when equimolar ADP is added to a reaction system containing 2 mM ATP. We have also examined the effect of orthophosphate on this enzyme and this product appears to have no inhibitory effect on ATP hydrolysis in concentrations up to 30 mM, which is generally regarded as being that normally encountered in cells (Charnock, 1963).

These facts not only provide some clue to the possible reaction sequence for this enzyme, inasmuch as they suggest that an ATP⇌ADP exchange reaction may occur whereas an ATP⇌P exchange seems unlikely, but in addition they also suggest that a feed-back control of enzyme velocity may exist which could be related to the existing ATP:ADP ratio in the cell.

A considerable amount of information is available about this enzyme, and for a comprehensive account the reader is referred to detailed reviews by Judah and Ahmed (1964) and Skou (1965). However, there are several additional features of this enzyme which should be mentioned. First there

is the possible effect of anions upon the ability of the enzyme to hydrolyse ATP. Although many attempts have been made to elucidate in great detail the sensitivity of the enzyme to cations, little systematic experimentation has been conducted to study the effect of different anions. We have recently found marked inhibition of the sodium and potassium stimulated ATPase by fluoride, even in the presence of a high chloride·fluoride ratio of 98:2 (Opit *et al.*, 1966). We have also shown that the inhibition by fluoride is not due to interaction with magnesium ion, but is an effect on the enzyme itself. The significance of this finding stems from the well-known effect of fluoride as an inhibitor of potassium influx into red blood cells and its action as a haemolysing agent.

Another group of compounds which inhibit this enzyme and which are important in the field of pharmacology are the lower aliphatic alcohols (Israel *et al.*, 1965, 1966). In our laboratory we have seen definite inhibition of the rate of ATP hydrolysis by ethanol at concentrations as low as 0·5% (w/v). Because of the implications of membrane "permeability" to sodium and potassium in axonal function, these observations may provide the clue to the pharmacological action of ethanol on neural structures.

Before leaving this discussion of the biochemical features of this enzyme we would like to mention the potentiation of activity induced either by ageing or treatment of the membrane preparations with detergents such as deoxycholate (DOC). All these agents appear to have a common effect on membranes in that they produce a selective "denaturation" by extraction of the phospholipid components of the membranes. Indeed it has been reported by Tanaka and Abood (1964) and Tanaka and Strickland (1965) that extensive extraction of phospholipids ultimately destroys the ATPase activity of brain membranes, and this activity could be restored by reconstitution with artificially added lecithin.

Supporting evidence has been obtained by the electron microscope examination of guinea pig kidney cortex microsome enzyme preparations where treatment with DOC has been shown to produce considerable disruption of many membrane vesicles (Charnock *et al.*, 1966).

b. Biological features

(i) *Cellular localization.* The most significant of these features is that the enzyme is membrane-bound and efforts to remove it from the membrane result in its inactivation. Although direct cytochemical localization has been claimed by some workers using ATPase staining of membranes by the usual Gomori "Pb-staining" procedures, the significance of these demonstrations appears to be unconvincing.* Because of the failure of almost all workers to demonstrate any sensitivity of the histochemical stain to cardiac glycosides, we must at present rely on evidence of a different

* The reader is referred to the papers by Novikoff *et al.* (1961), Bonting *et al.* (1962) and Tormey (1966) for critical appraisal of histological staining procedures employing relatively high concentrations of heavy metals such as Pb, Co, Cu and Zu.

nature to demonstrate the structural association of $(Na^{+} + K^{+})$-activated ATPase with cellular membranes.

When a tissue homogenate (in 0·25 M sucrose) is subjected to differential centrifugation a good yield of $(Na^{+} + K^{+})$-activated ATPase is found in a fraction sedimented after the mitochondria (10 000 × *g*) but before forces usually employed to separate the so-called "soluble" enzymes, i.e. (100 000 × *g*). This fraction (the heavy microsomes) can be conveniently sedimented at 35 000 × *g*. From examination of both its morphology and its enzymological behaviour, this fraction is generally regarded as being derived from the endoplasmic reticulum of cells, although the possibility that it contains many fragments of the plasma membrane cannot be discounted (Hokin and Hokin, 1960; Charnock and Post, 1963*b*).

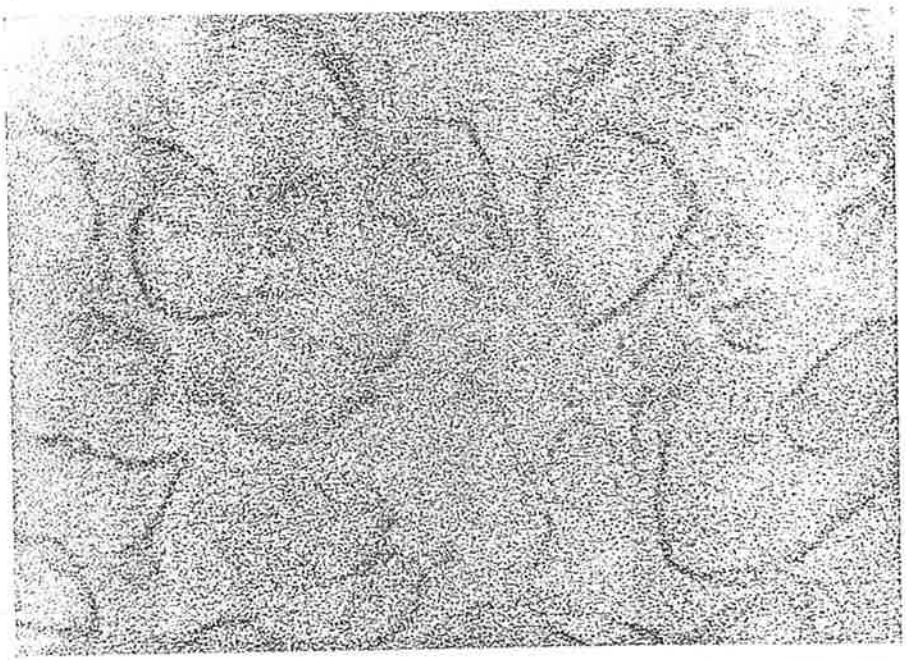

FIG. 4. An electronmicrograph of a "heavy" microsomal preparation of $(Na^{+} + K^{+})$-activated ATPase from guinea pig kidney cortex. Preparation fixed in 6% glutaraldehyde followed by 2% osmic acid, embedded in araldite and examined unstained. × 86 400.

Electron microscope examination of this fraction *in vitro* reveals the presence of vesicles bounded by typical osmiophilic bilaminar membranes. These are probably preparative artifacts formed from fragments of membrane sheets *in situ*, while almost no other structures are visible in electron micrographs of this fraction (Charnock *et al.*, 1966; see Fig. 4). Strong evidence of a similar nature comes from examination of red blood cells from which most of the haemoglobin has been removed. In such cells (ghosts) the plasma membrane is clearly visible, and as judged by both light and electron microscopy there is total absence of other internal structures (see Fig. 5). It has been shown repeatedly that this membrane, which is electron-

microscopically identical with the plasma membrane of cells, contains an ATPase activity which is stimulated by the combined presence of sodium and potassium and which stimulated activity can be inhibited by ouabain (Post *et al.*, 1960).

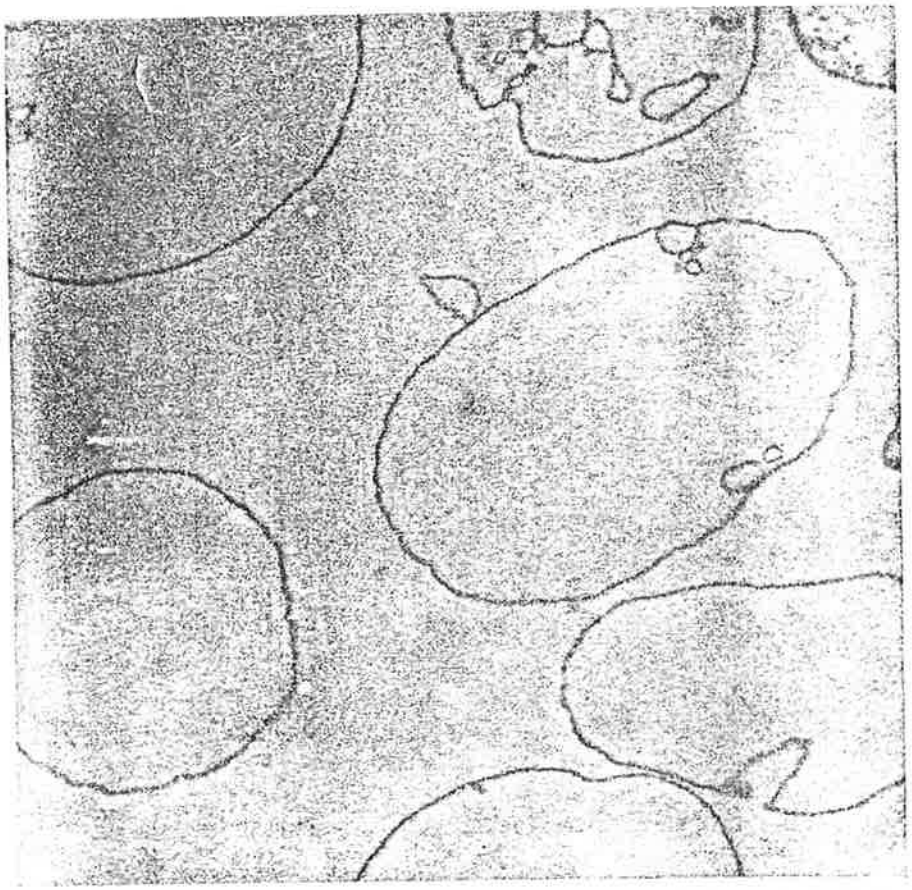

FIG. 5. An electronmicrograph of a preparation of human red cell ghosts obtained after osmotic haemolysis. The preparation has been stained with Pb. Several inclusions of membrane vesicles are visible. ×10 000.

Although this enzyme is also apparently present in the nuclear membrane of cells, the evidence for its presence in mitochondrial membranes is equivocal. Preparations of mitochondria from brain tissue have been reported to show some ($Na^+ + K^+$)-activated ATPase, but it seems entirely possible that this results from the difficulty of obtaining mitochondria free from microsomal contaminants in this tissue.

On the cellular scale an extensive series of examinations has been made with mammalian tissue and the enzyme is found in almost all tissues except in those without cells (serum, lens capsule and vitreous) or those having very low cell density (corneal stroma and adipose tissue) (Bonting *et al.*, 1961).

Such ubiquitous distribution is in accord with the physiological role proposed for this enzyme.

Curiously, the tissue from which some difficulty has been experienced in obtaining effective yields of this enzyme has been cardiac muscle. This difficulty has now been overcome, both by ourselves and other workers, by adopting a preparative technique different from that used to obtain microsomal fractions. The largest yields are found by selective salt extraction of the myosin with lithium bromide (or sodium iodide), and the residual ATPase activity remaining in the morphologically disrupted fibers is principally of the sodium and potassium activated, ouabain inhibited variety (Potter *et al.*, 1966).

Nature has also provided a useful biological experiment by developing strains of sheep having erythrocytes in which the normal Na:K ratio is reversed. Comparison of the red cells from these animals shows that in the sodium-rich cells the levels of $(Na^+ + K^+)$-activated ATPase is considerably lower than that seen in the K^+-rich cells (Tosteson, 1963, 1966). The parotid gland of newborn sheep also shows a sharp increase in the level of this enzyme concomitant with the development of active salivary secretion rich in sodium chloride (Filsell and Jarrett, 1965).

On the phylogenetic scale this enzyme has been identified in the tissues of animals from the order Crustacea upwards.

One further important biological feature of this enzyme has been observed from study of its behaviour in the plasma membrane of red cell ghosts. These important experiments, reported first by Glynn (1961) and later developed by Whittam (1962), suggest that the site of activation by Na^+ is on the inside of the red cell, whereas that for K^+ activation and cardiac glycoside inhibition is on the outside of the cell. These observations parallel the findings of Essig and Leaf (1963) and others, that sodium ion transport through cells from inside to outside, such as in the toad bladder, requires K^+ on the inside (mucosal) surface.

It also appeared necessary in the red cell experiments to have the substrate (ATP) present inside the red cell ghosts. All these experimental findings have led to the belief that this enzyme has a fixed orientation within cell membranes, and that this orientation is asymmetric inasmuch as various activation sites are on opposing surfaces of the membrane (Whittam, 1962).

We are now in a position to consider the possible reaction sequence which may account for the hydrolysis of ATP by this enzyme, when it is provided with Mg^{2+}, Na^+ and K^+ ions.

c. Reaction sequence

Skou's original tracer exchange experiments with ^{14}C-labelled ATP suggested that an intermediate might exist during the hydrolysis of ATP by $(Na^+ + K^+)$-activated ATPase, and this investigator speculated that such an intermediate complex could play a role as an ion-carrier (Skou, 1960). As interest grew in this enzyme, a number of workers began to examine this possibility.

The main experimental approach to the problem of identification of intermediates in this enzyme reaction has been to carry out relatively short-term incubations of the enzyme preparations with low concentrations of [^{32}P] labelled ATP. The rationale for this type of experimentation is based on the belief that because of the great affinity of the enzyme for its substrate, and the probable rapid turnover of such intermediates, these latter complexes will become preferentially labelled during the initial phase of the reaction. Hence, in the early 1960s a number of workers began to examine the reaction between ($Na^+ + K^+$)-activated ATPase and γ-labelled [^{32}P] ATP.

TABLE II

Formation of phosphorylated intermediate of ($Na^+ + K^+$)-activated ATPase

Additions	Counts ^{32}P/mg* protein N/sec	% ^{32}P† released
Control, 2 mM Mg^{2+} only	488	15
2 mM Mg^{2+}, 20 mM Na^+	4128	34
2 mM Mg^{2+}, 20 mM Na^+, 0·5 mM K^+	726	44
2 mM Mg^{2+}, 20 mM Na^+, 0·5 mM K^+, 0·1 mM ouabain	1860	36

* Effect of Na^+, K^+ and ouabain on the incorporation of ^{32}P from γ-labelled [^{32}P]ATP into acid-insoluble protein during the hydrolysis of 0·005 mM [^{32}P]ATP by ($Na^+ + K^+$)-activated ATPase of guinea pig kidney microsomes. Incubation time, 10 sec.

† Per cent ^{32}P released is that portion of the total counts of the reaction system which were not adsorbed by activated charcoal (see Crane and Lipmann, 1953).

Since the first detailed accounts by Rose (1963), Charnock and Post (1963*a*) and Albers *et al.* (1963), there have been many other reports which support the existence of a phosphate-bound protein complex formed during the hydrolysis of ATP by ($Na^+ + K^+$)-activated ATPase. Many workers regard this complex as a phosphorylated membrane enzyme component which functions as an intermediate in the overall hydrolysis of ATP by this system (Whittam *et al.*, 1964; Post *et al.*, 1965; Gibbs *et al.*, 1965; Rodnight *et al.*, 1966; Bader *et al.*, 1966). All these investigators reported the selective sensitivity of this reaction to cations, and it is now generally agreed that Na^+ is required for the formation of the ^{32}P-labelled intermediate complex, whereas K^+ is not. However, in the presence of Na^+, the addition of K^+ to the reaction mixture greatly reduces the number of counts recoverable as an acid-insoluble denatured protein precipitate, although the amount of [^{32}P]ATP hydrolysed under these conditions exceeds that found in the presence of Na^+ alone (cf. Table II). These results have been generally interpreted as evidence for a multistep reaction sequence in which Na^+ is required for the formation (phosphorylation) of the intermediate complex, and K^+ is necessary for the subsequent breakdown (dephosphorylation) of

this intermediate (Charnock and Post, 1963*a*; Charnock *et al.*, 1963). This view has been strengthened by the isolation of a K^+ dependent acyl phosphatase reaction from the enzyme preparations (Rendi, 1966; Bader and Sen, 1966). This reaction is thought to represent the separation of the dephosphorylation step from the overall reaction sequence.

Examination of the implications of such a system has been carried out in great detail and it is now known that a [^{14}C]ADP$\rightleftharpoons$[^{14}C]ATP exchange reaction can be demonstrated, but this reaction does not show sensitivity to either Na^+ or K^+ and cannot be inhibited by ouabain (Swanson and Stahl, 1966; Stahl *et al.*, 1966).

Conversely, the majority of attempts to demonstrate the formation of [^{32}]ATP from inorganic ^{32}P by this enzyme have failed, suggesting that the dephosphorylation of the phosphorylated intermediate complex is virtually an irreversible reaction. However, these experiments have been performed almost exclusively with broken membrane preparations, whereas the very interesting experiments of Garrahan and Glynn (1966) demonstrating the formation of [^{32}P]ATP by the integrated membrane structures of red cell ghosts incubated with ^{32}P indicate the possible importance of morphological integrity in examining the reaction sequence of an enzyme system such as ($Na^+ + K^+$)-activated ATPase, which is so clearly dependent upon structural organization for its proposed biological function.

Despite the apparent gaps in present-day knowledge, there is a general concensus of opinion that ($Na^+ + K^+$)-activated ATPase hydrolyses ATP by a reaction sequence of two or more distinct steps, and that at some stage of the reaction, presumably quite early in the sequence, and in the presence of Na^+, a phosphoprotein intermediate complex is formed.

The following general scheme summarizes the proposed reaction sequence:

$$\text{(1) Enzyme} + [^{32}\text{P}]\text{ATP} \xrightarrow[\text{?Ouabain}]{\text{Na}^+\uparrow} \text{E}\sim{}^{32}\text{P} + \text{ADP}$$

$$\text{(2) E}\sim{}^{32}\text{P} + \text{H}_2\text{O} \xrightarrow[\text{Ouabain}]{\text{K}^+\uparrow} \text{Enzyme} + {}^{32}\text{P}$$

There seems little doubt that the K^+-requiring stage of the reaction is inhibited by concentrations of ouabain sufficient to prevent ATP hydrolysis by the enzyme preparation, but whether this is caused by a direct interaction of the cardiac glycoside at this stage (2) of the reaction sequence or whether the Na^+-stimulated incorporation of ^{32}P into the intermediate is also inhibited by ouabain is not entirely clear. This difficulty arises mainly from the different reaction conditions employed in these studies, and in particular the concentrations of magnesium, ATP and sodium. From the more recent studies of Post *et al.* (1965), Rodnight *et al.* (1966) and data obtained in our laboratory (Charnock and Opit, 1967), it seems that when experimental

conditions are adjusted so that the degree of ^{32}P incorporation is sensitive to the concentration of the sodium ion, then marked reduction of ^{32}P incorporation into the acid-stable phosphate-bound protein precipitate occurs in the presence of 0·1 mM ouabain. The effect of various concentrations of ouabain on the Na^+-stimulated formation of the intermediate, and its breakdown in the presence of Na^+ and K^+ is illustrated in Table III.

TABLE III

Effect of ouabain on phosphorylated intermediate of $(Na^+ + K^+)$-activated ATPase

Additions	Counts ^{32}P/mg* protein N/sec	% ^{32}P† released
Control, 2 mM Mg^{2+} only	238	11
20 mM Na^+	1530	22
20 mM Na^+, 0·01 mM ouabain	1250	15
20 mM Na^+, 0·1 mM ouabain	826	12
20 mM Na^+, 1·0 mM ouabain	634	11
20 mM Na^+, 0·5 mM K^+	350	31
20 mM Na^+, 0·5 mM K^+, 0·01 mM ouabain	356	28
20 mM Na^+, 0·5 mM K^+, 0·1 mM ouabain	517	24
20 mM Na^+, 0·5 mM K^+, 1·0 mM ouabain	633	15

* Effect of different concentrations of ouabain on the incorporation of ^{32}P from γ-labelled [^{32}P]ATP into acid-insoluble protein during the hydrolysis of 0·005 mM [^{32}P]ATP by $(Na^+ + K^+)$-activated ATPase in the presence of 20 mM Na^+ with and without 0·5 mM K^+. Incubation time, 10 sec (see Charnock *et al.*, 1967).

† Per cent ^{32}P released as in Table II.

In attempting an interpretation of such data it is important to keep in mind that the degree of effectiveness of agents under examination will be influenced by both the ATP:ADP ratio, and the Mg:ATP ratio existing during the reaction. Although these factors have received some attention, systematic examination will be required before complete understanding can be achieved. However, one fact emerges clearly from the investigations carried out so far. That is that the reaction between low concentrations of [^{32}P]ATP and $(Na^+ + K^+)$-activated ATPase in the presence of Na^+ ions is extremely rapid and that steady-state levels of the ^{32}P-labelled complex appear to be reached within 1 or 2 sec of initiation of the reaction.

A critical note in the acceptance of this general reaction sequence has been introduced by Schoner *et al.* (1966) who from their findings of the instability of the ^{32}P-labelled complex in the presence of hydroxylamine concluded that the ^{32}P-labelled complex formed in these experiments, could not operate as a true intermediate in the hydrolysis of ATP by this enzyme. However, further examination has revealed that the action of hydroxylamine

in this regard does not preclude the function of this labelled complex as an intermediate (Charnock *et al.*, 1967).

III. The Sodium Pump

We are now in a position to summarize the salient features of the $(Na^+ + K^+)$-activated ATPase, both biological and biochemical, which can be used to make an attractive case for the participation of this enzyme system in the transport of both Na^+ and K^+ across cellular membranes.

(1) This enzyme system is an integral part of cell membranes.
(2) It has widespread occurrence in cell types and species.
(3) The hydrolysis of ATP requires the presence of both sodium and potassium ions.
(4) Potassium appears to be replaceable, but less effectively by those ions which compete with K^+ for transport in the whole cell.
(5) ATP hydrolysis is prevented by calcium, fluoride and other agents known to inhibit active transport in the whole cell.
(6) The highly selective inhibition of the enzyme by low concentrations of cardiac glycosides.
(7) Its activity can influence, as well as be influenced by, the intracellular ATP:ADP ratio; thus the activity of the enzyme can influence the rate of mitochondrial electron transport and hence the respiratory rate of the cell.
(8) In intact structures, such as the red cell "plasma" membrane, the activating ions need to be on opposing surfaces of the membrane, thus suggesting a fixed orientation of the enzyme, a property which could impart the desired vectorial properties to its reactivity.

Although these and other features have been used to conclude an important role for this enzyme in the transport of sodium and potassium ions, the means by which this transport is achieved remains unknown. Almost every worker in this field has put forward some general suggestions, but very few detailed or experimentally verifiable models have been reported. Therefore, we intend to present our own views on this problem, not because we believe they are necessarily correct, but because they might enable the reader to explore the deficiencies in models relating to how an enzyme system acts as a carrier for ions. Although our suggestions are certainly not unique, the ideas to be presented represent an effort to relate what is known about $(Na^+ + K^+)$-activated ATPase, the physiology of ion transport, and what is suspected about the reactivity of protein-containing membranes.

Although the gross chemical composition of animal cell membranes is remarkably constant (protein 50–70%, phospholipid 20–30%, carbohydrate 10–30%), there is no unequivocal evidence concerning the structural arrangement of these components. Since the earliest bimolecular leaflet model proposed by Gorter and Grendel (1925) there have been

many attempts to depict membrane structure. That proposed by Davson and Danielli (1952) is the best known and most useful to date, but its over-simplification is well recognized. More recently Whittam (1964) has proposed a sophisticated model of the red cell membrane which attempts to include some of the known properties of these structures. Yet if one takes into account the fact that in excess of one hundred separate enzyme activities have been isolated from the red cell membranes alone (Pennell, 1964), it is immediately realized that any of the membrane models so far proposed can only represent gross structure, whereas exquisite specialization must occur at the molecular level.

With these considerations in mind, we shall present a model system based upon the important features of $(Na^+ + K^+)$-activated ATPase coupled with the classical Davson-Danielli concept of membrane structure. This particular viewpoint regards cell membranes as two protein lamellae bonded together by a central double layered core of lipid, with the hydrophobic groups of the lipid facing each other and the hydrophilic portions of the lipid molecule effecting the union with the protein.

It follows from this concept of membrane structure that the enzymatic properties of cell membranes are predominately localized in the protein moiety of the structure, that active centres of the enzyme could be expected to be associated with particular amino acid arrangements found along the protein "backbone" and that this "backbone" is both highly polarizable and possesses a large number of extended side-chains with both anionic and cationic groups. For reasons which will become apparent later, we must also assume that the active centre of the $(Na^+ + K^+)$-activated ATPase is situated on the inner surface of the bilaminar membrane structure.

The sequential steps we propose to account for active ion transport, and which would therefore constitute the component reactions of the "sodium pump", are presented in the sequence which corresponds to our view of the reaction sequence of ATP hydrolysis by $(Na^+ + K^+)$-activated ATPase. We suggest that the initial step in the cycle is the formation of associated ion pairs between the monovalent cations of the cell cytoplasm and the anionic side-chains of the inner-facing protein surface of the membrane. It is not necessary in our model that there should be preference for Na-fixed anion ion pairs, for the proportion of these ion pairs which are formed will depend, for example, upon the relative concentration of Na^+ and K^+. (See Fig. 6.)

As we proposed in an earlier account of this model system (Opit and Charnock, 1965*a*), the significance of this assumption is that the hypothesis now includes the features of a feed-back control governed by the relative concentrations of the two major intracellular cations. To initiate operation of the cycle we suggest that a critical number of fixed charge sites adsorb sodium, this number being proportional to the intracellular sodium concentration. Consequently, the activity of the "pumping cycle" will be depressed as the intracellular concentration of sodium falls and the K^+:Na^+ ratio rises.

This sodium ion pair formation is thought to induce, by short-range

interactions, a redistribution of electron density along the polarizable protein "backbone" of the membrane in much the same way as has been proposed for allosteric enzyme effects. In this approach we are employing the concepts independently developed by Koshland (1960), Koshland *et al.* (1962) and Ling (1962).

We suggest, therefore, that the ion pair formation with Na^+ would differ in its effect from that found with K-fixed anion ion pairs; and that when sufficient Na-fixed anionic charged pairs are formed, the proteins of the membrane surface will undergo a change in their electron distribution such that the active centres of the $(Na^+ + K^+)$-activated enzyme become more reactive to their substrate, Mg-ATP. It is this latter interaction which introduces a phosphoryl group into the active centre of the enzyme protein, and which we identify as the phosphoprotein chemical complex (intermediate) referred to earlier.

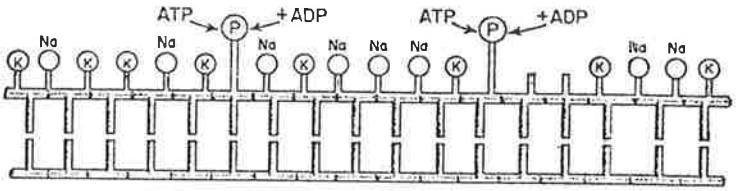

FIG. 6. Diagrammatic representation of a unit segment of membrane in which the horizontal sections depict the inner and outer protein layers, and the vertical broken units between the protein represent the central phospholipid complex described by Davson and Danielli (1952). Projecting from the inner coil are shown the surface orientated anionic groups of the protein with which Na^+ and K^+ have formed associated ion pairs. The longer vertical projections represent the amino acid groups which constitute the active centre of the enzyme. In this figure sufficient Na-fixed anion ion pairs have formed to allow the active centre to react with ATP, and form the phosphorylated intermediate. ATP is shown as the substrate, the Mg^{2+} which is necessary has been omitted for clarity. (Reproduced from Opit and Charnock, 1965, with the permission of the editors of *Nature*.)

The formation of this phosphorylated active centre must now produce a considerable and important change in the charge density of the protein moiety. The outcome of this phenomenon we envisage as a change in spacing or charge distribution along the side-chains accompanied by a change in molecular configuration. We consider that this change in molecular configuration could be sufficient to induce a change in the adsorption energy of the anionic side-chains so that they become strongly K^+ preferring.

In a more detailed account of this model system, we did specify a particular type of deformation which would cause the inward facing protein layer, relatively rich in Na-fixed anion ion pairs, to twist or rotate about the central lipid core, this twist being centred about the active centre of the enzyme protein itself (Opit and Charnock, 1965*a*).

Many variations of such a shape change can be imagined; all that would be necessary for the active outward transport of Na^+ being their translocation to another area of the membrane in which there was a barrier to their back diffusion into the cell interior. However, in the linked transport

of both sodium and potassium, the configurational change must be such that it has the effect of rotating the molecule to face the opposing surface and allow K^+ from the extracellular space to replace the Na^+ originally adsorbed on the protein side-chains (see Fig. 7).

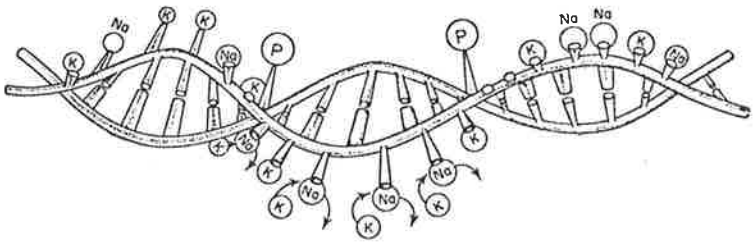

FIG. 7. Representation of the postulated effects of phosphorylation of the active centre of the enzyme. Elongation of the inner protein surface has produced rotation of these protein molecules about the centre of the lipoid core. The site of the phosphorylated active centre is shown as resisting this rotation. The surface orientated anionic groups with their associated ions have rotated outwards following the elongation and twisting of the protein molecule. K^+ for Na^+ exchange is now shown to be taking place at sites of Na^+ adsorption to these groups. (Reproduced from Opit and Charnock, 1965, with the permission of the editors of *Nature*.)

It is thought that this highly deformed transition state of the membrane renders the phosphorylated active centre susceptible to attack by water, with the result that orthophosphate is liberated inside the cell. The loss of these phosphoryl groups once again profoundly affects the electron density of the "backbone" chain of the protein, causing the membrane to revert to its initial configuration, and carrying with it K^+ in the form of K-fixed anion ion pairs (see Fig. 8).

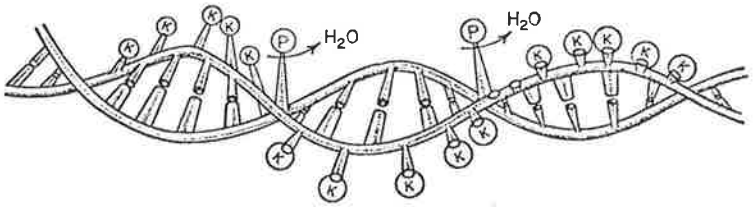

FIG. 8. Diagram showing that, following the K^+ for Na^+ exchange illustrated in Fig. 7, the inductive changes of electron density which take place in the elongated protein molecule lead to changes of field strength in the region of the phosphorylated active centre. As a result of these changes the phosphorylated centre is now readily attacked by water with cleavage of phosphate from the protein. (Reproduced from Opit and Charnock, 1965, with the permission of the editors of *Nature*.)

In this state, some K^+ is again replaced by Na-fixed anion ion pairs as the internal sodium concentration of the cell increases, and hence this sequence of deformation, reformation changes is associated with the net extrusion of Na^+ coupled with the entry of K^+ into the cell. The energy necessary for these changes coming from the hydrolysis of ATP to ADP and orthophosphate.

In this model, although the lipid components of the membrane are not

chemically reactive in the enzymatic sense, they are thought to provide the "recoil" or restoring force necessary for the operation of the cycle. This view provides an explanation for the effects of ageing, or treatment with deoxycholate or other detergents which are known to enhance ($Na^+ + K^+$)-activated ATPase activity initially, but eventually lead to its destruction. Partial removal of lipids may at first reduce the amount of energy necessary to produce the perturbed configurations postulated for the full "pumping cycle", but more extensive removal of lipids could remove the "recoil" mechanism leaving the configuration fixed in a particular form, or eventually lead to the disruption of the integrated structures necessary to perform the overall reaction.

Such a relatively simple scheme as that just proposed also suggests an explanation for a number of the phenomena witnessed, both with the enzyme itself as well as the physiology of active sodium and potassium transport. For example, it is well known that sodium transport falls with falling levels of intracellular Na^+, whereas the rate of transport increases with rising levels of intracellular Na^+; on the other hand, increasing extracellular K^+ also raises the rate of sodium transport. In terms of our model system, these changes would affect the "pumping rate" because increasing the extracellular K^+ concentration would enhance the activity of ($Na^+ + K^+$)-activated ATPase. Lowering the extracellular Na^+ would also activate the enzyme by reducing the possible competition of Na^+ for K^+ activation sites. Conversely, increasing intracellular Na^+ tends to fill the recepter sites and raises the critical level of Na-fixed anion ion pairs necessary for the interaction between the enzyme and ATP. Thus in response to its environment this system can increase the "pumping rate" over that required to maintain normal steady-state conditions. When the increased "pumping rate" has returned the concentrations of cations to normal, the rate will be reduced as the level of ATPase activity will then be less.

With the concept of membrane proteins employed in this model, there must also be side-chains having predominately cationic charges which one could also expect to react with anions to form ion pairs similar to those between Na^+ or K^+ and the anionic side-chains. Thus it might be plausible to believe that ion pair formation between these cationic side-chains and strongly electro-negative anions such as F^- could interfere with the inductive changes in electron density produced by cations. This might explain the action of fluoride in inhibiting ($Na^+ + K^+$)-activated ATPase at chloride: fluoride ratios of 98:2 (Opit *et al.*, 1966).

The inhibitory actions of calcium and magnesium on the enzyme might also be explained by similar effects on charge distribution. However, there is some experimental evidence which suggests that calcium acts, at least in part, by forming the less favourable substrate Ca-ATP (Epstein and Whittam, 1966).

In whole tissues, such as the intestine, the association between active cation transport and the transport of sugars (Ricklis and Quastel, 1958; Csáky, 1963*a*, *b*), amino acids (Nathans *et al.*, 1960; Harrison and Harrison,

1960; Rosenberg *et al.*, 1965), pyrimidines (Csáky, 1961, 1963*a*, *b*), bile acids (Playoust and Isselbacher, 1964), acetate (Gonda and Quastel, 1966), and in the thyroid, iodine (Wolff, 1960), has been well documented. This association may be explained by assuming that all these substances are adsorbed on to the "outer" protein lamellae of the membrane in what we have referred to as the "initial" state of the reaction cycle. If it can be assumed that there is some difference of adsorption of these substances to the membrane material between the phosphorylated and dephosphorylated states of the enzyme, then the deformation-reformation cycle induced by sodium and potassium may account for their active transport into the cell.

As yet we have not attempted to explain the highly specific inhibition of this enzyme model by cardiac glycosides, where detectable falls in ATPase activity can be measured with concentrations as low as 10^{-7} M. Because of the enormous molar concentration differences between the activating ions (in particular K^+) and the inhibitory cardiac glycosides we do not believe on physiological grounds that simple competition alone can explain these effects, although thermodynamically this could be possible. Rather we prefer to speculate that the cardioactive glycosides, like ouabain, all possess a large number of coplanar reactive hydroxyl groups which combine with the enzyme protein. The reactivity of the cardiac glycosides will depend upon the actual configuration state of the enzyme protein itself, and these agents have the net effect of blocking the deformation-reformation cycle by both their molecular size and charge.

To be useful, all models ought to be capable of experimental verification and ideally carry the seeds of their own destruction. The model we have proposed certainly fulfils this criterion because the immediate implications are threefold:

(1) The model suggests that when the enzyme reacts with ATP to produce a high level of the phospho-protein intermediate complex, the whole membrane structure undergoes molecular deformation.
(2) During the deformed transition state the enzyme protein and the adjacent membrane lamella show selective binding for K^+ (or caesium or rubidium).
(3) Ouabain becomes bound to the enzyme; the degree of association being influenced by the conformational state of the protein.

All these implications should be capable of experimental examination and indeed there is evidence to substantiate the view that K^+ (or Cs^+) binding is related to a particular transition state of the enzyme (Charnock *et al.*, 1966).

There are also the reports by Järnefelt (1961), Charnock and Post (1963*a*) and Charnock *et al.* (1963), which describe the ATP-dependent binding of Na^+ to membrane preparations rich in ($Na^+ + K^+$)-activated ATPase, but the technical limitations of these studies make interpretation of the data unreliable. Clearly only a beginning has been made in examining these

postulates, but solutions to the queries raised are vital to further understanding of the role of this membrane-bound enzyme system in active transport.

Almost all models require at some stage the selective binding of ions to membranes, and in general we take the view that any model of enzymatically mediated transport should be based on the theory that the membranes across which substances are transported are dynamic structures whose mechanical, physical and chemical properties can be changed by chemical reactions which occur within the enzyme protein which is itself an integral part of the membrane structure. This is not an original view of membrane properties and some alternative model systems have been proposed to account for a variety of membrane functions. For example, employing just such a dynamic system as that referred to above, Kavanau (1965) has produced a considerable thesis involving the reactivity of the lipid component of membranes, in which he ascribes many of the permeability properties of membranes to this component.

Although we began our model of a Na^+ and K^+ transport system with the premise that the membrane enzyme ($Na^+ + K^+$)-activated ATPase is a highly specific and integral part of the mechanism, and have suggested an example whereby the activity of this enzyme might account for the active transport of glucose, amino acids or anions, we do not wish to suggest that every permeability characteristic of membranes is associated with this particular enzyme. However, we feel that to date, it is the most convincing membrane enzyme system for which a transport function has been proposed.

IV. The Carrier Concept

At the physiological level many experiments have been concerned with the measurement of the movement of ions or molecules across cell membranes and the subsequent deduction of solute distribution between phases separated by biological membranes.

In these types of experiments it has been frequently observed that competition exists for the transport of solutes of similar nature, for example the competition demonstrated between deoxyglucose and glucose or between rubidium and potassium ions. A relatively simple explanation for this, and one which is particularly applicable to uncharged molecules rather than ions, is that competition occurs for a third unknown factor regarded as the carrier vehicle for the transport of these molecules across membranes.

Many physiological models of membrane transport include such terms as *carriers*, *pumps*, *permeases*, *translocation processes* or even *permeability*. Used in this way, these expressions are purely operational terms and as such are often equivalent. Nevertheless, these concepts remain as interesting today as when first proposed by Ussing (1948), Rosenberg (1948) and others, although at the molecular level these terms do little to explain how the membrane achieves the control of solute movement. Furthermore, no

chemical or structural identification of any of the multitude of carriers which have been proposed has ever been achieved.

However, the idea that specific molecular carriers exist to transport specific components across cellular barriers is widespread. A major difficulty with this viewpoint is the need to provide a separate carrier for each separate type of solute transported, on the grounds that the translocation processes involved are highly specific for the various substances (Passow, 1964) and that the competition phenomenon which can be shown to exist functions in the classical enzymatic sense.

More generally it can be considered that the models for solute distribution in the cell fall into two general groups on thermodynamic grounds. First, those which are equilibrium systems and, secondly, those which are based on the steady-state. Historically the first of these systems to be evolved is that known as the Donnan equilibrium. There seems little doubt today that this model does not predict the ion distribution of biological systems, apart from the observation that the living cell appears to behave as a perfect chloride electrode.

Since first introduced, the Donnan model has been developed in varying degrees of complexity so that fixed charges on the membrane as well as the mobile ionic charges are used to predict final ionic distribution. The most detailed of these equilibrium systems is the model evolved by Ling (1962) which treats the cell as a complicated micellar structure containing a network of fixed protein charges. Indeed Ling's work denies the existence of membranes as necessary for the separation of phases.

The second type of model to be mentioned briefly is that based on the idea that the movement of ions across the cell membranes is far from equilibrium and is a steady-state distribution. A comprehensive understanding of the cellular environment seen in this light awaits further development of sophisticated non-equilibrium thermodynamics. Despite a recent upsurge of interest in this field little has yet been done to develop these models with rigour (see Vaidhyanathan, 1966).

Whatever thermodynamic system is used to explain the final distribution of ions and other solutes in the cell, we do know that the cell itself is capable of changes in both ion flux and final ionic distribution and that these changes are themselves often a reflection of the general state of the metabolic activity of the cell. We have already tried to show that the membranes of the cell may have in them the control mechanisms for these changes.

V. Other Ions

Before concluding this chapter on membrane metabolism we shall briefly discuss the transport of some of the remaining electrolytes of the cell across the cellular membranes.

Of the major divalent cations of the cell we know that magnesium ions are relatively plentiful and that the total concentration of both free and bound magnesium inside the cell may well exceed that of the plasma. We

have already mentioned that there is much evidence that isolated mitochondria can accumulate this ion, against a concentration gradient, by a respiration dependent process (Vasington, 1963; Brierley *et al.*, 1963; Chappell *et al.*, 1963). This movement of magnesium into mitochondria is accompanied by an anion, usually phosphate, and may lead to the precipitation of salts of magnesium phosphate within the mitochondria (Chance and Williams, 1955; Chappell *et al.*, 1963; Lehninger *et al.*, 1963; Brierley and Slautterback, 1963).

Whether this process is a mediator of active Mg^{2+} transport by the cells remains speculative, but clearly the presence of intracellular Mg^{2+} is closely associated with the cells' major pathways for the synthesis and breakdown of energy stores. Hence, magnesium ions both influence and are influenced by the over-all state of cell metabolism.

The situation with respect to calcium ions is less clear. Although there are numerous demonstrations of the biological antagonism which exists between calcium and magnesium it is not certain, either from our own work or from the data of others, what the true intracellular concentration of Ca^{2+} normally is, if indeed it ever exists as a free ion inside the cell.

There are plentiful descriptions of the avidity with which calcium ions bind to cell structures (Charnock, 1963) and of the factors which influence this binding (Ebashi and Lipmann, 1962; Hasselbach and Makinose, 1962; Carsten and Mommaerts, 1964). The role of adenosinetriphosphate and other nucleotide triphosphates in this interaction has often been stressed, but the biological significance of these observations remains unclear.

Like the endoplasmic reticulum of soft tissues, isolated microsomal preparations from sarcoplasmic reticulum of muscle contain an ATPase activity which is sensitive to cations. In this case the hydrolytic activity is not as specific as that of the enzyme from endoplasmic reticulum, since any nucleotide triphosphate can serve as substrate (NTPase). However, its action is greatly enhanced by low concentrations of calcium and can be inhibited by the mercurials or the alkylating agent N-ethyl maleimide.

Electron microscope examination of these preparations reveal the presence of vesicular structures bounded by typical double layered membranes. The detailed investigations of Hasselbach and others strongly suggest that these vesicles contain an active NTP dependent calcium "*pump*" which presumably reflects the mechanism by which these membranes transport Ca^{2+} in intact structures *in situ* (Hasselbach and Makinose, 1962; Hasselbach, 1966).

The parallelism between these findings and those reported for $(Na^+ + K^+)$-activated ATPase are striking, although the biochemical features of the calcium stimulated enzyme system are not so well defined at present.

Whether this system is common to all cells or species is not yet known, but further examination is bound to reveal the physiological significance of this membrane enzyme system in the distribution of divalent ions. On the other

hand, the biological significance of the experimentally observed entry of calcium and magnesium ions into isolated animal mitochondria which we have mentioned earlier, and which occurs only under highly artifactual conditions, seems to us to be far less promising.

The role of the phosphate anion in the metabolism of the cell is too well known to be outlined in this chapter, although what regulates the entry and exit of this ion from the cell is not so well understood. There is considerable information available from the study of integrated cell structures *in vitro* showing that the entry of phosphate into cells is linked with the reactions of glycolysis. The operation of the glycolytic cycle tends to increase the level of esterified phosphate within the cell at the expense of non-esterified phosphate. This alone is sufficient to produce an influx of phosphate, but does not necessarily constitute an active transport process. No well-defined enzymatic basis has been demonstrated for this process although glyceraldehyde phosphate dehydrogenase (which links orthophosphate to glyceraldehyde-3-phosphate) has been implicated, at least speculatively, in phosphate translocation.

It has been customary to localize the glycolytic enzyme system with the so-called soluble portion of the cell. However, recent evidence brought forward by Green and his co-workers (Green *et al.*, 1965, 1966) has shown the presence of these glycolytic enzymes in the plasma membranes of red cells. This finding should stimulate a fresh approach to the membrane-bound enzymatic control of phosphate transport, and here the red cell may prove to be an excellent model for a direct examination of the action of hormones and drugs on this process.

In addition, phosphate is also known to move into cells and isolated mitochondria as the anion required to maintain charge neutrality when cation movement has occurred. This latter movement is not specific for phosphate anion, however, and is thought to be a "passive" process that follows energy dependent cation accumulation (Lehninger, 1966).

The uptake of other anions such as acetate and propionate as well as the anions of certain Krebs cycle intermediates (succinate, isocitrate, malate) are also thought to pass across cell membranes by a carrier mediated process. Whether the entry of these substances is secondary to the primary event of cation transport, or whether specific carriers exist for their movement is not yet clear (Chappell, 1966).

VI. Concluding Remarks

In this discussion of membrane metabolism and ion transport we have endeavoured to stress the dynamic state of the cellular membranes and emphasize their role as the cellular locus for control mechanisms not only for ions but for the entry and exit of other solutes.

We have particularly concentrated our discussion on the transport of sodium and potassium ions. Because we believe that their movement is not entirely divorced from the movement of other ions and polar and non-polar

substances, we have used the particular information available to generalize about possible alternative or related mechanisms of transport across membranes.

In addition we have tried to look at the structure and function of "membranes" not only through the eyes of preparative biochemistry, cellular morphology or classical physiology, but to synthesize all these viewpoints into a tangible discussion.

In so doing we have given an account of what, to us, are the major features of membrane behaviour which relate to their physiological function in the separation of phases.

We have attempted to focus attention on the weaknesses of present-day hypotheses and point to gaps in our knowledge that must be filled before further understanding can be achieved.

One of the most significant advances in the practice of medicine in the last twenty-five years has been the awareness of disturbances in the normal distribution of ions in a vast spectrum of disease states in man. Much of the knowledge, and hence the treatment of these conditions, has had, to date, a highly empirical flavour, and it must be clear that our capacity to control these disturbed states will come from our knowledge of the mechanisms controlling the permeability of membranes.

Furthermore, there can be little doubt that an understanding of the target site of hormones such as cortisone, aldosterone, insulin, vasopressin and parathormone is dependent on such fundamental knowledge. The design of such therapeutically active drugs as diuretics must also draw heavily on this basic information.

As a direct outcome of research into the mechanism of one membrane-bound enzyme system, $(Na^+ + K^+)$-activated ATPase, it is at last likely that an enzymatic site of action for the most important group of cardiotonic agents (the cardiac glycosides) has been found by this basic approach, some two hundred years after their introduction to clinical practice.

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Hydroxylamine and a ^{32}P-Labelled Intermediate in Sodium-plus-Potassium Ion-Activated Adenosine Triphosphatase

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An acid-stable ^{32}P-labelled phosphoprotein complex can be recovered after incubation of membrane preparations exhibiting ($Na^+ + K^+$)-activated ATPase* activity with [γ-^{32}P]ATP (Charnock & Post, 1963*a*; Gibbs, Roddy & Titus, 1965; Rodnight, Hems & Lavin, 1966). Formation of this complex is stimulated by Na^+ but not K^+; when both ions are present the steady-state concentration of ^{32}P-labelled phosphoprotein complex is greatly decreased whereas the hydrolysis of [γ-^{32}P]ATP is enhanced. Most investigators have concluded that the ^{32}P-labelled phosphoprotein complex recovered in these experiments is an acyl-phosphate–enzyme derivative that functions as an intermediate (E ~ P) during the hydrolysis of ATP by ($Na^+ + K^+$)-activated ATPase (Charnock, Rosenthal & Post, 1963; Nagano *et al.* 1965; Bader, Sen & Post, 1966).

However, this concept has been challenged by Schoner, Kramer & Seubert (1966), who reported that the Na^+-stimulated formation of ^{32}P-labelled phosphoprotein complex was greatly decreased when the reaction was conducted in the presence of hydroxylamine, but that hydroxylamine did not affect the rate of hydrolysis of ATP by this enzyme in the presence of Na^+ and K^+. From these findings these workers concluded that the ^{32}P-labelled phosphoprotein complex was not an intermediate in the hydrolysis of ATP by ($Na^+ + K^+$)-activated ATPase.

The present experiments are a further examination of the interaction of hydroxylamine with ($Na^+ + K^+$)-activated ATPase.

Materials and methods. Preparation of guinea-pig kidney microsomal ($Na^+ + K^+$)-activated ATPase and the assay conditions for the colorimetric determination of ATP hydrolysis by liberation of inorganic phosphate have been described previously (Charnock & Post, 1963*b*).

The ^{32}P-labelling experiments were carried out

* Abbreviation: ATPase, adenosine triphosphatase.

with 1ml. of enzyme suspension in 1mM-tris–HCl buffer, pH7·5, containing about 0·4mg. of protein N/ml. (Lowry, Rosebrough, Farr & Randall, 1951). The enzyme was added last to a reaction mixture that contained in a final volume of 5ml. carrier-free [γ-^{32}P]ATP (5μM) (an aqueous solution of the tetra-ammonium salt brought to pH7·5 by the addition of solid tris; specific activity 1050mc/m-mole; The Radiochemical Centre, Amersham, Bucks.), $MgCl_2$ (5μM), H_3PO_4–tris buffer, pH7·5 (30mM), and where required Na^+ (20mM) and K^+ (0·5mM) as the chloride salts, ouabain (0·1mM) (Sigma Chemical Co., St Louis, Mo., U.S.A.) and freshly prepared hydroxylamine at the concentrations indicated in the text. After 10min. temperature equilibration, incubation was carried out for 10sec. at 37° and the reaction stopped by the addition of sufficient ice-cold 1% (w/v) trichloroacetic acid solution to bring the pH to 2·5; usually 10–20ml. was required. The precipitated phosphoprotein complex was collected and washed by centrifuging four times with 10ml. portions of ice-cold tris-buffered 1% (w/v) trichloroacetic acid, pH2·5, containing carrier disodium ATP (0·1mM) (Sigma Chemical Co.). All centrifugings were performed at 0°. The inorganic phosphate ^{32}P content of the first acidified reaction mixture was determined after charcoal absorption of nucleotide phosphate ^{32}P by the method of Crane & Lipmann (1953) with an Ecko well-type (N597) crystal scintillator. The washed ^{32}P-labelled phosphoprotein precipitate was dissolved in Lowry's alkaline-copper reagent and samples were taken for protein N determination (Lowry *et al.* 1951). Radioactivity (^{32}P) was determined with an end-window Geiger–Müller tube (Ecko). Sufficient counts were collected to reduce the random error of counting to less than 1%. Hydroxylamine solutions were prepared by passing fresh solutions of hydroxylamine hydrochloride (L.R. grade; May and Baker Ltd., Dagenham, Essex) down a column of Dowex 1 exchange resin (OH^- form). The concentration of chloride-free hydroxylamine (Schales & Schales, 1941) was determined by the bromate procedure described by Vogel (1961).

Table 1. *Effect of hydroxylamine, ouabain and* K^+ *on the incorporation of* ^{32}P *into a phosphoprotein intermediate of* ($Na^+ + K^+$)*-activated ATPase.*

A 1ml. sample of enzyme suspension was incubated in 30mM-phosphate buffer, pH7·5, with 5μM-magnesium [γ-^{32}P]ATP (carrier-free) for 10sec. at 37°, with Na^+ (20mM), K^+ (0·5mM), ouabain (0·1mM) and hydroxylamine (160mM) where shown. Experimental conditions are described in text. ^{32}P-labelled phosphoprotein is given as counts/sec. of ^{32}P/mg. of protein N that remain in the washed acid-stable protein precipitate. [^{32}P]Phosphate formed refers to that portion of the 'total radioactivity' of the acidified reaction system after incubation that is non-adsorbable on activated charcoal.

Expt.	Additions	^{32}P-labelled phosphoprotein	[^{32}P]-Phosphate formed (%)
(i)	None	360	8
	Na^+	2240	15
	Na^+ + ouabain	850	9
	Na^+ + hydroxylamine	510	31
	Na^+ + hydroxylamine + ouabain	860	14
(ii)	None	350	8
	Na^+	1430	13
	Na^+ + ouabain	940	9
	Na^+ + hydroxylamine	470	24
	Na^+ + hydroxylamine + ouabain	800	14
(iii)	None	490	15
	Na^+	4120	38
	Na^+ + ouabain	1890	17
	Na^+ + hydroxylamine	730	61
	Na^+ + hydroxylamine + ouabain	1160	49
	$Na^+ + K^+$	374	44
	$Na^+ + K^+$ + ouabain	980	39

Results and discussion. The results of three labelling experiments are given in Table 1, where all experiments show that, on the addition of Na^+ (20mM), there is a considerable increase (more than fourfold) in the amount of ^{32}P-labelled phosphoprotein complex formed over that in the control situation (5μM-Mg^{2+} only). This increase in ^{32}P labelling is accompanied by a rise in the amount of ^{32}P liberated from [γ-^{32}P]ATP. The addition of ouabain (0·1mM) to the Na^+-containing reaction mixture decreased both the amount of ^{32}P released from [γ-^{32}P]ATP and the amount of ^{32}P-labelled phosphoprotein complex formed. On the other hand, when hydroxylamine (0·16M) was added in the presence of Na^+ there was a marked increase in the amount of ^{32}P released from [γ-^{32}P]ATP whereas the amount of ^{32}P-labelled phosphoprotein complex was decreased to a level similar to that found in the absence of both Na^+ and hydroxylamine. This finding is in agreement with that reported by Schoner *et al.* (1966). However, the addition of ouabain (0·1mM) to a system containing both Na^+ and hydroxylamine inhibited the additional ^{32}P released by the action of hydroxylamine and increased the amount of ^{32}P-labelled phosphoprotein complex to levels clearly greater than that obtained in the controls (twofold). In Expt. (iii) the effect of substituting 0·5mM-K^+ is contrasted with the action of hydroxylamine. It is clear that both hydroxylamine and K^+ decrease the Na^+-stimulated incorporation of ^{32}P into phosphoprotein complex, though increasing the

amount of ^{32}P liberated from [γ-^{32}P]ATP. Hydroxylamine (0·16M) is more effective than 0·5mM-K^+ in this regard. This effect of K^+ has been reported many times previously (e.g. Charnock & Post, 1963*a*; Whittam, Wheeler & Blake, 1964; Post, Sen & Rosenthal, 1965; Gibbs *et al.* 1965) and is generally thought to represent a greatly increased rate of dephosphorylation of the phosphoprotein intermediate under the influence of K^+.

The presence of 0·1mM-ouabain in addition to either K^+ or hydroxylamine again decreases the liberation of ^{32}P from [γ-^{32}P]ATP and increases the amount of ^{32}P-labelled phosphoprotein complex formed. This finding supports the view that hydroxylamine functions as a cation and can replace K^+ in this system.

To examine this further, hydroxylamine (0·36M) was substituted for K^+ (20mM) in three assays of ATPase activity with a standard chemical assay system for the liberation of inorganic phosphate from non-radioactive ATP (2mM) in the presence of 80mM-Na^+ (Charnock & Post, 1963*b*). In these experiments the presence of K^+ increased the phosphate released from 53 to 277μmoles of phosphate/mg. of protein N/hr. whereas addition of hydroxylamine increased the release of phosphate to 93μmoles of phosphate/mg. of protein N/hr. (values are the means of three experiments, assays in duplicate). In every experiment addition of 0·1mM-ouabain decreased the liberation of phosphate to that found with Na^+ alone.

Thus hydroxylamine appears to function as a K^+-like cation in the reaction mechanism of (Na^++K^+)-activated ATPase. The conclusion of Schoner *et al.* (1966) that the ^{32}P-labelled phosphoprotein complex formed (E$\sim$P) from [γ-^{32}P]ATP cannot be an intermediate in the overall reaction mechanism of this enzyme is therefore no longer valid. Whether hydroxylamine acts directly as a cation, or indirectly by the liberation of NH_4^+ ions, is not resolved by these experiments.

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CATION ACCUMULATION BY MICROSOMAL ($Na^+ + K^+$)-ACTIVATED ATPase

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SUMMARY

1. Possible cation accumulation by microsomal ($Na^+ + K^+$)-activated, ouabain-sensitive ATPase was examined by an electron microscopical method.

2. Densitometry measurements of membrane opacity were performed on the electron micrograph plates obtained after incubation of the ATPase preparation under a variety of experimental conditions.

3. To aid visualization of possible cation accumulation, the more electron opaque Cs^+ was substituted for K^+ in the incubation system of many experiments.

4. Increase in membrane density of the microsomal ATPase was observed in the presence of sodium and caesium only when ATP hydrolysis was inhibited by ouabain.

5. This phenomenon was not observed when a ouabain-insensitive mitochondrial ATPase system was examined.

6. This increase in density is interpreted as selective Cs^+ accumulation by the microsomal enzyme membrane fragments.

7. The significance of these results is discussed in terms of a reaction mechanism for ($Na^+ + K^+$)-activated ATPase.

INTRODUCTION

An ATPase which, in addition to magnesium ions, requires the presence of both sodium and potassium ions for activation, and which can be inhibited by low concentrations of cardiac glycosides, has been reported in a wide variety of mammalian tissues[1–4].

The probable relationship of this enzyme system ("transport ATPase") to the movement of Na^+ and K^+ across biological membranes has been reviewed by SKOU[5,6] and JUDAH AND AHMED[7]. Recently OPIT AND CHARNOCK[8] have proposed a model system by which this enzyme may translocate ions across cell membranes.

A number of previous investigations have suggested that during the hydrolysis of ATP by this enzyme, a phosphorylated intermediate complex is formed to which

one or more ions are selectively bound, and that this ion-binding complex constitutes the long-sought "cation carrier" of active transport[3,5–8,10,11].

Although there have been some reports of an ATP-dependent binding of sodium ions to microsomal preparations of this enzyme[9,11,12] and recently an account of a cardiac glycoside-sensitive, ATPase-dependent localization of Na^+ at the plasma membrane of corneal tissue[13], there is as yet little experimental evidence relating the function of this "transport ATPase" system to the selective binding of cations.

We have sought further evidence concerning this function of the enzyme by an electron microscopical examination of a particulate enzyme preparation. Our aim was to study the possible accumulation of cations by the enzyme, and to examine the effect of ouabain upon this situation. Because the work of other investigators had already demonstrated that Cs^+ can replace K^+ (but not Na^+) in the chemical reactions resulting in the hydrolysis of ATP by this enzyme[3,5,14,15], and also because of the greater atomic mass (electron opacity) of Cs^+, this ion was substituted in the majority of experiments where K^+ would normally be used.

METHODS

The enzyme was prepared from the cortex of guinea-pig kidney by a method of tissue homogenisation and differential centrifugation which has been described previously[9]. In this study both a 10000 × *g* sediment (mitochondrial fraction) and a 35000 × *g* sediment (heavy microsomal fraction) were employed. All preparative steps were carried out at 0–2° in a Servall RC-2 refrigerated centrifuge (S-34 angle rotor).

The mitochondrial fraction was collected and washed in 0.25 M sucrose by recentrifuging at 10000 × *g*, collected into a small volume of 0.25 M sucrose and immediately assayed polarographically for respiratory rate and P/O ratio. A Clark oxygen electrode was employed and ADP utilized as phosphate accepter[16]. "Transport ATPase" activity was determined by a standard procedure[4].

After sedimenting the 35000 × *g* "heavy microsome" fraction (from the 10000 × *g* supernatant) the pellet so formed was evenly dispersed in a hand-operated homogeniser into a small volume of either 0.125 M sucrose, or 0.125 M sucrose, 2 mM Tris–deoxycholate mixture (sucrose–deoxycholate) adjusted to pH 7.2. The "enzyme" preparation in 0.125 M sucrose was used immediately either alone or in combination with the mitochondrial fraction. The "enzyme" preparation in sucrose–deoxycholate mixture was aged for 24 h at 2°, as this treatment has been shown to greatly increase transport ATPase activity in these preparations[4]. Respiratory activity and transport ATPase activity were measured as for the mitochondrial fraction.

About 0.5 mg of either "mitochondrial" or "microsomal" protein[17] was added to each incubation flask. Incubations were carried out in a final concentration of 20 mM Tris–glycylglycine buffer (pH 7.6) to which was added 2.5 mM ATP (either disodium or Tris-salt) and 2 mM $MgSO_4$. 80 mM NaCl, 20 mM KCl or 20 mM CsCl and 0.1 mM ouabain were included as required.

The incubations were carried out at 25° for 5–10 min and the enzyme reaction stopped by rapid chilling of the flasks to 4°. The particulate enzyme preparations were separated from the incubation system by centrifuging at either 10000 × *g* or 35000 × *g* for 20 min at 0°. The pellet was then dispersed in 0.25 M sucrose and

washed by centrifuging with or without the addition of 0.2 mM ouabain as required.

The washed mitochondrial or microsomal pellets were mixed, where required, and an equal volume of warm 2 % agar added and the mixture transferred to a polythene planchette and rapidly solidified at 2°.

Blocks of approx. 1 mm^3 were cut and fixed for 6 h in Caulfield's osmium tetroxide solution[18], dehydrated in ethanol and embedded in araldite. "Silver" sections, cut on a Huxley microtome, were mounted on collodion–carbon films and examined in a Siemens Elmiskop I electron microscope using a 15-μ objective aperture.

Ilford "Special Lantern Contrasty" plates were exposed under standard conditions, using a Leitz Microsix-L exposure meter adapted to record the illumination of the fluorescent screen. Constant photographic conditions were employed throughout.

Densitometry was performed on the negatives to provide the numerical data for statistical evaluation. A Lisson microddensitometer was modified for this purpose. The optical magnifications ($\sim 5\times$) were adjusted to give a final magnification of 300000 $\times$. Under these conditions a 2-mm aperture in front of the photomultiplier was covered by a 7-mμ "unit membrane" structure.

Discrete areas of membrane, perpendicular to the plane of the section, were selected for densitometry measurements in order to avoid confusion by adjacent or overlying membranes. Wherever possible background densities were measured adjacent to the object on at least two areas on different sides of the membrane. Since the mitochondrial matrix has appreciable electron opacity, it was often not possible to measure the membranes inside these bodies because adjacent empty resin (for the background densities) could not be measured. With the mitochondria therefore, determinations were made on the outer ends of the cristae membranes or on the limiting membranes continuous with them. For each experiment 10 measurements were made (on a number of negatives) on selected pieces of membrane spaced well apart. Only one measurement was recorded on any one segment of membrane. There were no significant differences between negatives obtained within each set of incubation conditions. Possible observer bias was controlled by measuring plates in random sequence without knowledge of the experimental conditions.

ATPase activity in the presence of Na^+ and K^+, or Na^+ and Cs^+ was determined by the method described previously[4]; inhibition of "transport ATPase" was determined by the reduction in ATPase activity in the presence of 0.1 mM ouabain.

RESULTS

Morphology

After incubation and washing under identical conditions the mitochondrial and microsomal fractions were gently mixed (1:1) with a glass rod in an effort to allow both types of particles to be photographed in the same field. Apparently the mixing did not produce an even disperse as it was found that discrete aggregates occurred (1–10 μ diam.) with clear gaps (10–20 μ) separating the different groups.

Generally the morphology of the mitochondria seen in this study is similar to that reported for these organelles when seen in tissue section, except that the outer limiting membranes are almost always absent, and some cristae appear swollen.

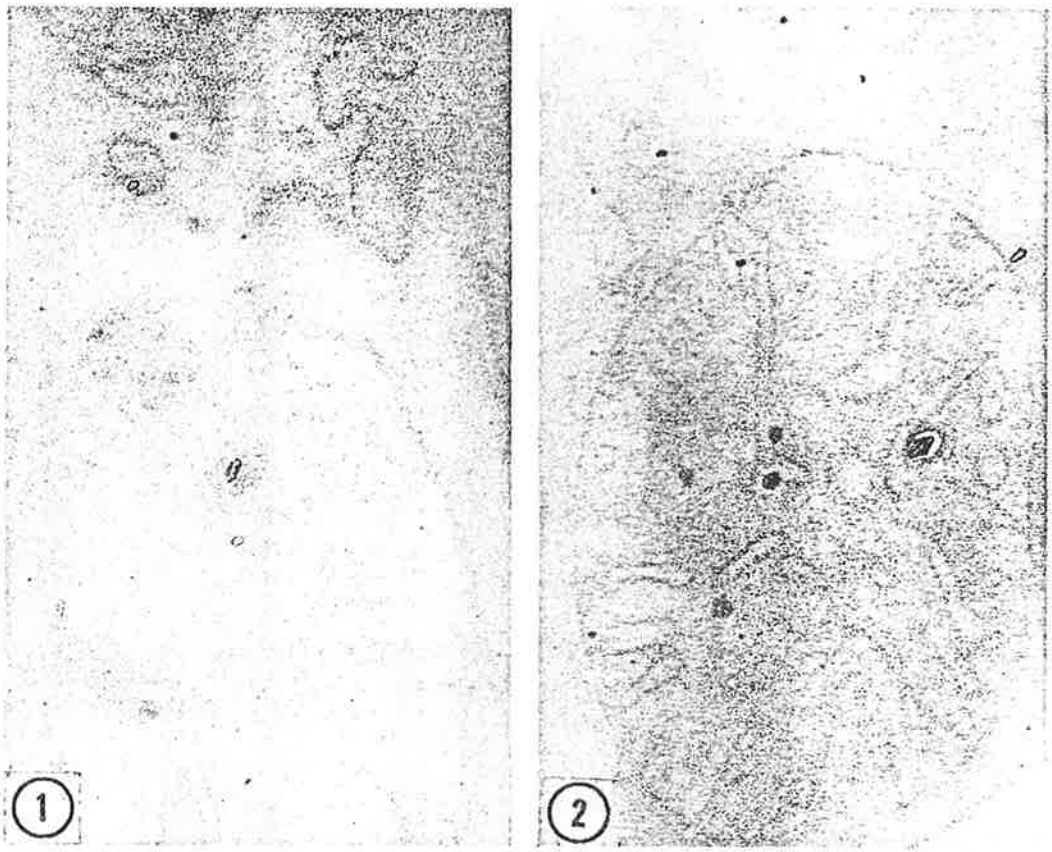

Fig. 1. Mitochondria prepared in 0.25 M sucrose, incubated with ATP, Na^+ and Cs^+. No ouabain present. Embedded in araldite and examined unstained. Magnification 100000 ×.

Fig. 2. Mitochondria prepared in 0.25 M sucrose, incubated with ATP, Na^+ and Cs^+. Ouabain present throughout incubation. Embedded in araldite and examined unstained. Magnification 100000 ×.

Disordered cristae of this type were not used for densitometry measurements. Associated with the "intact" mitochondria were vesicles of varying size; these may be contaminants from the "microsomal" fraction or fragments of broken mitochondria. From the electron micrographs alone it is not possible to determine the origin of these vesicles (Figs. 1 and 2).

The appearance of the microsomal fraction either alone or after mixing with the mitochondria, suggests a preparation consisting of membranes having the "unit" structure described by ROBERTSON[19]. The samples fixed after preparation in sucrose show many large vesicles ($\sim$ 1 μ) in addition to many smaller vesicles ($\sim$ 50 mμ) (Fig. 3). However, after treatment with deoxycholate there is a decrease in the number of large vesicles and there are now many broken segments of membranes (*cf.* Figs. 4 and 5).

Amorphous material, which may be cytoplasmic matrix, is also mixed with the membranes of this microsomal fraction. As was the case with the mitochondrial

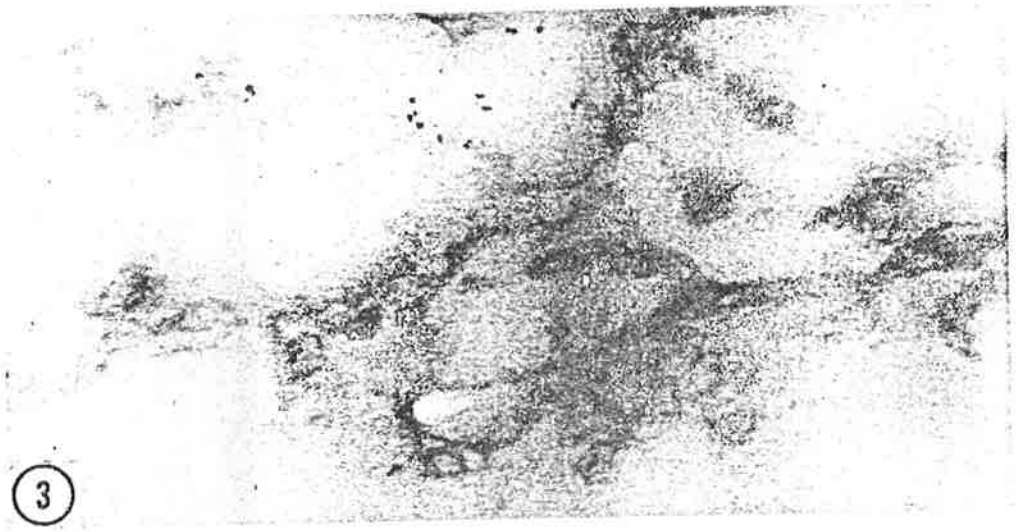

Fig. 3. Microsomal fraction suspended in 0.125 M sucrose without further treatment. Embedded in araldite and stained with lead. Magnification 100000 ×.

fraction it is not possible to determine the cellular origin of either the microsomes or the amorphous material by examination of the electron micrographs alone. However, it should be noted that the method of preparation has been designed to exclude both nuclear and mitochondrial material[1].

Respiratory activity

The result of a polarographic examination of the respiratory activity of both the mitochondrial and microsomal fractions is given in Table I. In these experiments succinate was the substrate, and the oxygen utilization in the presence and absence of ADP was recorded by means of a Clark oxygen electrode[16]. The data obtained show that whilst the mitochondrial fraction utilizes oxygen with good respiratory control and near optimal P/O ratio, the microsomal fraction failed to respire under these conditions. This observation also suggests that the microsomal fraction is virtually free of functional mitochondria.

ATPase activity

The ATPase activity of both the mitochondrial and microsomal fractions of guinea-pig kidney cortex preparation was compared using a standard assay procedure employed in other studies of "transport ATPase" activity[4]. The results are given in Table II which shows that both fractions demonstrate ATPase activity in the presence of Na^+ and K^+, but only the activity of the microsomal fraction was significantly inhibited by 0.1 mM ouabain—a concentration of the cardiac glycoside demonstrated to inhibit "transport ATPase" activity in these preparations*.

The low level of ouabain-sensitive ATPase demonstrated in the mitochondrial preparation suggests that at least the majority of the membrane vesicles observed in

* H. A. Potter, J. S. Charnock and L. J. Opit, unpublished results.

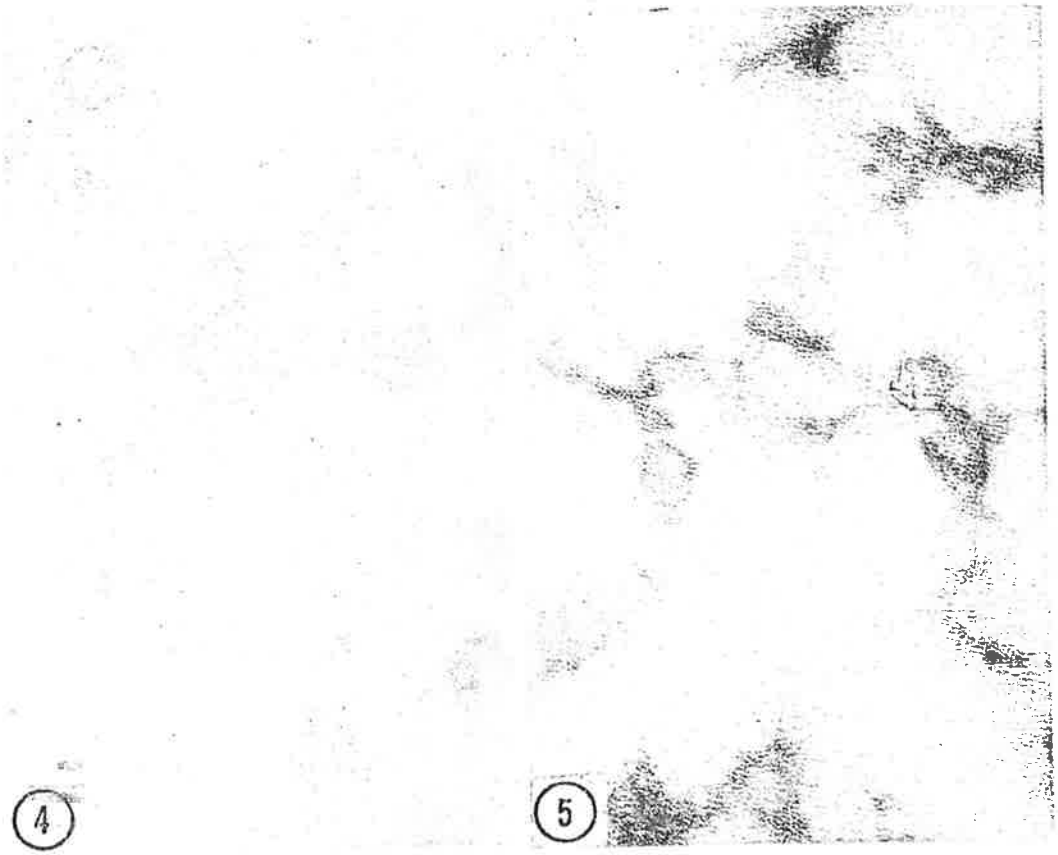

Fig. 4. Microsomal fraction suspended in 0.125 M sucrose, incubated with ATP, Na^+ and Cs^+. No ouabain present. Embedded in araldite and examined unstained. Magnification 100000 ×.

Fig. 5. Microsomal fraction suspended in 0.125 M sucrose, incubated with ATP, Na^+ and Cs^+. Ouabain present throughout incubation. Embedded in araldite and examined unstained. Magnification 100000 ×.

TABLE I

RESPIRATORY ACTIVITY OF MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF GUINEA-PIG KIDNEY

The velocity of O_2 uptake measured polarographically using Clark oxygen electrode, 10 mM succinate as substrate in the system described previously (*cf.* ref. 16). P/O is phosphate:oxygen and R.C. is respiratory control.

Fraction	*μatoms O per 10 mg protein per min*			
	−ADP	*+ADP*	*R.C.*	*P/O*
Mitochondrial	0.30	1.20	4	1.8
Microsomal	—	—	—	—

the aggregates of these organelles (Figs. 1 and 2) are not contaminants from the microsomal fraction and probably are pieces of fragmented mitochondria.

TABLE II

TRANSPORT ATPASE ACTIVITY OF MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF GUINEA-PIG KIDNEY

The standard assay procedure for "transport ATPase" activity has been described previously (see ref. 4). 2 mM ATP and 2 mM $MgSO_4$ were present in all assays. 80 mM NaCl, 20 mM KCl and 0.1 mM ouabain present where indicated. % refers to that percentage of maximum activity ($Na^+ + K^+$) which could be inhibited by 0.1 mM ouabain.

Fraction	*ATPase activity*			
	$Na^+ + K^+$	$Na^+ + K^+$ + *ouabain*	Δ*	%
Mitochondria	81	74	7	9
Microsomes	180	45	135	75

* Specific activity Δ is given as μmoles P_i per mg protein N per h at 37°.

TABLE III

EFFECT OF Cs^+ AND K^+ ON THE TRANSPORT ATPase ACTIVITY OF MICROSOMAL FRACTIONS OF GUINEA-PIG KIDNEY

The conditions of assay were identical with those given in Table II. The specific activity Δ and the % of the activity sensitive to 0.1 mM ouabain are also expressed as in Table II.

Cation added	*ATPase activity*			
	80 mM Na^+	*80 mM* Na^+ *0.1 mM ouabain*	Δ	%
20 mM K^+	424	50	374	88
20 mM Cs^+	400	48	352	88

When equimolar Cs^+ was substituted for K^+ in the usual assay system for "transport ATPase"[4] there was only a slight reduction in the ATPase activity of the microsome preparations (Table III), in general agreement with the finding of others[3,5,14,15].

Membrane density

Other studies have shown that the ability of "transport ATPase" to hydrolyse ATP *in vitro* is not significantly altered when Cs^+ replaces K^+ in the incubation mixture[5,14,15]. Thus in many experiments Cs^+ was substituted for K^+. Table IV contains the results of densitometry measurements on the microsomal membrane enzyme fraction incubated under a variety of experimental conditions. Although different enzyme preparations were used in the experiments reported here, the same enzyme preparation was used under all incubation conditions within each experiment.

Expt. 1 of Table IV shows the effect on membrane density of the incubation conditions necessary for "transport ATPase" activity, contrasted with the same conditions in the presence of the enzyme inhibitor ouabain. It is apparent that considerable increase in membrane density has occurred with ouabain inhibition (*cf.* Figs. 4 and 5).

To determine which of the reactants was responsible for the increase in membrane density Expt. 2 was conducted in which the effect of various combinations of

TABLE IV

THE EFFECT OF OUABAIN ON THE ACCUMULATION OF Cs^+ BY MICROSOMAL $(Na^+ + K^+)$-ACTIVATED ATPase

Preparation in either 0.125 M sucrose or 0.125 M sucrose–2 mM Tris–deoxycholate (pH 7.2). Values are given as the mean absorbance of 10 separate measurements (see text) with the standard error of the mean. Probability (P) represents the comparison of the particular incubation condition with all others within that experiment. 2 mM Mg^{2+} was present in all experiments.

Expt.	*Preparation*	*Reaction components*	*Membrane density*	*P*
1	Sucrose	ATP, Na^+, Cs^+, ouabain	0.186 ± 0.022	<0.001
	Sucrose	ATP, Na^+, Cs^+ —	0.081 ± 0.022	
2	Sucrose	— — — —	0.135 ± 0.009	
	Sucrose	ATP — — —	0.154 ± 0.012	
	Sucrose + deoxycholate	— — — —	0.096 ± 0.012	
	Sucrose + deoxycholate	ATP — — —	0.128 ± 0.009	
	Sucrose + deoxycholate	ATP, Na^+ — —	0.121 ± 0.001	
	Sucrose + deoxycholate	— Na^+ — —	0.117 ± 0.012	
	Sucrose + deoxycholate	ATP — Cs^+ —	0.121 ± 0.014	
	Sucrose + deoxycholate	— — Cs^+ —	0.136 ± 0.015	
	Sucrose + deoxycholate	ATP, Na^+, Cs^+ —	0.109 ± 0.006	
	Sucrose + deoxycholate	— Na^+, Cs^+ —	0.142 ± 0.012	
	Sucrose + deoxycholate	ATP, Na^+, Cs^+, ouabain	0.326 ± 0.024	<0.001
	Sucrose + deoxycholate	— Na^+, Cs^+, ouabain	0.117 ± 0.014	
3	Sucrose + deoxycholate	ATP, Na^+, Cs^+ —	0.106 ± 0.012	
	Sucrose + deoxycholate	ATP, Na^+, Cs^+, ouabain	0.184 ± 0.009	<0.001
	Sucrose + deoxycholate	ATP, Na^+, K^+ —	0.104 ± 0.008	
	Sucrose + deoxycholate	ATP, Na^+, K^+, ouabain	0.118 ± 0.009	

these reactants was examined. The only significant increase in membrane density was again observed in the presence of ATP, Na^+, Cs^+ and ouabain. It was thought that this increase in membrane density could be associated with the adsorption of one of these reactants onto the membrane enzyme. In addition this only occurred when all reactants necessary for "transport ATPase" were present, and the hydrolysis of ATP to ADP and inorganic phosphate was blocked by ouabain.

To examine this possibility further Expt. 3 was performed in which, under conditions of ATPase activity, K^+ was substituted for Cs^+ both in the presence and absence of ouabain. The membrane density was compared with an identical group incubated with Cs^+. Again the only significant increase in membrane density occurred under the conditions seen in Expts. 1 and 2.

Because ATP hydrolysis takes place by "transport ATPase" with either Cs^+ or K^+ we interpret this result as implying that the density increase was due to the interaction of Cs^+ with the membrane enzyme fraction and consider it probable that adsorption of the cation to the membrane itself had occurred under these conditions.

To determine whether this effect was confined to membranes having "transport ATPase" activity, or was common to membranes containing a ouabain-insensitive ATPase, a similar study was made of the mitochondrial membranes. The results of a comparison of membrane density of mitochondrial and microsomal membranes incubated under identical conditions are given in Table V. The experimental conditions were those of Expt. 1 of Table IV.

TABLE V

THE EFFECT OF OUABAIN ON THE ACCUMULATION OF CAESIUM BY MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF GUINEA-PIG KIDNEY

The incubation conditions are described in the text. All the tests contained ATP, Na^+ and Cs^+. Ouabain added where indicated. Membrane density values are expressed as absorbance and are the means of 10 separate measurements ± S.E. of the mean (see Table IV and text).

Fraction	*Membrane density*	
	$Na^+ + Cs^+$	$Na^+ + Cs^+ + ouabain$
Mitochondria	0.079 ± 0.027	0.066 ± 0.055
Microsomes	0.088 ± 0.026	0.221 ± 0.058

It is again apparent that although an increase in membrane density has occurred in the microsomal membranes incubated in the presence of ouabain, no such increase in membrane density was observed with the mitochondrial membranes in the presence of ouabain.

These findings reinforce the conclusion that the observed increase in microsomal membrane electron density which occurred with Cs^+ and ouabain is dependent upon the presence of the selective "transport ATPase" enzyme system and is not a feature of ouabain-insensitive ATPase reactions.

DISCUSSION

Although there is much indirect evidence to support the belief that $(Na^+ + K^+)$-activated, ouabain-inhibited ATPase is the enzymatic basis of active cation transport across cell membranes, there is little experimental evidence supporting the selective cation-binding properties of this system[11–13]. The chemical studies of sodium binding by this enzyme, reported by JARNEFELT[12] and CHARNOCK, ROSENTHAL AND POST[11], are difficult to interpret because the methods employed required sedimenting and extensive washing of the enzyme preparation by time-consuming centrifugal procedures or precipitation of a denatured complex. Because of the instability of the phosphoprotein intermediate involved in the hydrolysis of ATP by "transport ATPase"[20], and the potential role of this intermediate as the ion-binding complex, it was desirable to examine cation binding by a method which did not require either repeated centrifugation or the introduction of denaturants.

For this reason an electron microscopical investigation was undertaken, utilizing Cs^+ as substitute for K^+, to aid the "visualization" of potential ion accumulation. To assess membrane density in the experiments conducted, densitometry was employed instead of relying upon conventional visual evidence of electron micrographs. This was carried out not only to avoid variations in the printing technique itself, and to fulfil the need for a numerical index of comparison, but even more importantly because we considered that cation adsorption to the membrane, if it occurred, would take place as relatively diffuse ion pairs along the enzyme protein chain[8] rather than the formation of massive insoluble precipitates.

The biochemical results presented demonstrate that in terms of respiratory activity and "transport ATPase" activity the mitochondrial and microsomal fractions

of guinea-pig kidney cortex examined here are readily distinguishable. The microsomal fraction contained no respiratory activity, whereas the mitochondrial fraction had an insignificant amount of ouabain-sensitive "transport ATPase" activity. Other studies of mitochondrial ATPase activity suggest that mitochondrial ATPase systems are depressed by the addition of high concentrations of either Na^+ or K^+ and in this respect also are dissimilar to "transport ATPase" activity[21].

Examination of the electron microscopical membrane density data suggests that only when all reaction components necessary for the enzymic hydrolysis of ATP are present, in addition to the enzyme inhibitor ouabain, did the membrane density significantly increase. From previous investigations of the enzyme mechanism[9,11] this situation corresponds to the maximal steady-state level of the phosphorylated enzyme complex.

As this effect was produced by the complete reaction requirements for "transport ATPase" activity and could not be observed either with the substitution of K^+ for Cs^+, nor with the mitochondrial membranes examined under identical conditions, it is reasonable to believe that the increase in membrane density is the result of Cs^+ accumulation during a phase of "transport ATPase" activity.

If the binding characteristics of Cs^+ to "transport ATPase" are similar to those for K^+ (for which Cs^+ was substituted because of its much greater electron opacity than K^+) then the requirement for ouabain suggests that Cs^+ or K^+ binding occurs during the reaction sequence when the enzyme is in the phosphorylated state[9,11]. This finding is in agreement with recent suggestions of OPIT AND CHARNOCK[8] concerning a possible mechanism of action for this membrane enzyme system in active sodium transport.

Although we have interpreted the increase in absorbance of the microsomal membranes as being due to Cs^+ accumulation, other explanations for this phenomenon must be considered. This result might be due to artifacts introduced during the electron microscopy; for example differential changes in the susceptibility of the membrane components to sublimation in the electron beam or induced shrinkage of the membrane could be responsible for increased electron opacity. Even if either of these or other alternatives were found to be valid, the uniqueness of the experimental situation under which the effect occurred would still imply interaction between "transport ATPase" and Cs^+.

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THE SEPARATION OF SODIUM AND POTASSIUM-ACTIVATED ADENOSINE-TRIPHOSPHATASE FROM A SODIUM OR POTASSIUM INHIBITED ADENOSINE-TRIPHOSPHATASE OF CARDIAC MUSCLE

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Summary. Salt extraction of sheep or guinea-pig cardiac muscle was employed to obtain two adenosine-triphosphatase preparations. After extraction of cardiac muscle homogenate with 1 M lithium bromide, an insoluble adenosine-triphosphatase preparation was obtained which was activated by sodium and potassium ions and which could then be inhibited by the cardiac glycoside ouabain. By using 1 M potassium chloride in place of lithium bromide a soluble adenosine-triphosphatase system was extracted which required calcium ions for activation, but which was not activated by sodium and potassium. nor was the activity inhibited by ouabain. Inhibition of the sodium and potassium activated adenosine-triphosphatase of cardiac muscle by low concentrations of ouabain (half maximal 4 x 10^{-6} M) suggest that this cardiac adenosine-triphosphatase displays similar sensitivity to cardiac glycosides as "transport" adenosine-triphosphatase preparations obtained from brain and kidney tissue. It is concluded that the insoluble sodium and potassium activated adenosine-triphosphatase of cardiac muscle may be a major site for the pharmacological effects of cardiac glycosides.

INTRODUCTION.

An adenosine-triphosphatase (ATPase) which is associated with membranes, which requires the presence of both Na^+ and K^+ in a defined ratio for optimal activity and which can be inhibited by low concentrations of the cardiac glycoside ouabain ("transport ATPase"), is now generally believed to be the biochemical basis of active sodium and potassium transport across cellular membranes (Judah and Ahmed, 1964; Skou, 1965; Opit and Charnock, 1965). However, attempts to isolate this enzyme system from cardiac muscle by methods

which have proved satisfactory for the preparation of this enzyme system from kidney cortex or brain yield only small amounts of activity from cardiac tissue (Bonting, Caravaggio and Hawkins, 1962; Schwartz, 1962; Auditore and Murray, 1962 and 1963; Hayashi, Auditore and Uchida, 1963; Kennedy and Nayler, 1965a). The preparation of "transport ATPase" from cardiac muscle is complicated by the presence of large quantities of an "actomyosin-like ATPase" which interferes in most assay systems (Hayashi *et al.*, 1963; Tashima, Nakao, Nagano, Mizuno and Nakao, 1966). In the presence of high levels of this ATPase smaller quantities of the specific membrane "transport ATPase" are difficult to detect.

This paper describes the isolation of two ATPase preparations from cardiac tissue by a method of salt extraction which selectively separates an insoluble $Na^+ + K^+$ activated ATPase from other cardiac ATPase systems. The "transport ATPase" so prepared shows high levels of activation in the presence of Na^+ and K^+ and sensitivity to ouabain inhibition comparable with that previously reported for guinea-pig kidney cortex preparations (Charnock and Post, 1963; Potter, Charnock and Opit, 1966).

Materials and Methods.

Hearts were obtained from exsanguinated sheep, or guinea-pigs killed by decapitation. Five to ten grams of fresh or frozen cardiac muscle were washed in ice-cold 1 mM tris buffer (1 mM tris-HCl, 1 mM ethylene diamine tetra acetic acid (EDTA) adjusted to pH 6·8), to remove blood. The tissue was blotted dry on filter paper and cut into small portions before being homogenised in a chilled Potter-Elvehjem apparatus fitted with a teflon pestle. Sufficient tris buffer was used to give a 10% (w/v) homogenate. The pooled homogenate was strained through gauze to remove connective tissue and other large tissue debris, and the pH of the filtrate readjusted to 6·8 by the addition of 0·2 M tris solution when necessary.

Preparation of ($Na^+ + K^+$)-activated ATPase.

The homogenate was centrifuged at 1,000*g* for 20 min. at 4° (M.S.E. Magnum refrigerated centrifuge). The supernatant was discarded and the sediment resuspended in approximately 100-150 ml. of tris buffer and centrifuged again at 1,000*g* for 20 min. The supernatant was discarded and the pellet resuspended in either (a) tris buffer (pH 6·8); (b) 1 M KCl, or (c) 1 M LiBr as required. Salt extraction was carried out in the cold for times varying from 1-18 hr., using approximately 5 ml. of suspending solution per gram wet weight of original tissue. Some tris buffer solution was then added to facilitate the centrifugation of the particulate matter at 1,000*g* for 20 min. The supernatant was discarded and the pellet resuspended in tris buffer, centrifuged at 1,000*g* for 20 min. to remove excess potassium or lithium salts. Finally, the pellet was dispersed in 1 mM tris-HCl, adjusted to pH 7·6 and assayed for ($Na^+ + K^+$)-activated, ouabain inhibited ATPase activity (Charnock and Post, 1963). Gross morphology of this preparation was observed by phase-contrast microscopy.

Preparation of KCl-soluble ATPase.

A KCl-soluble ATPase was prepared essentially by the methods of Mommaerts and Seraidarian (1946) and Ebashi (1961).

As in the preparation of ($Na^+ + K^+$)-activated, ouabain inhibited ATPase, fresh guinea-pig cardiac muscle was washed with cold tris buffer (pH 6·8) and homogenised

in this medium to give a 10% (w/v) homogenate. After straining through gauze, the homogenate was centrifuged at 1,000g for 20 min. at 4°. The supernatant was discarded and the sedimented pellet resuspended in three volumes of buffered KCl solution (0·6 M KCl, 0·4 M $NaHCO_3$, 0·01 M Na_2CO_3, pH 8·8) and extracted in the cold for 24 hr. The extract was then centrifuged (Servall RC-2 refrigerated centrifuge with S34 rotor) at 35,000g for 30 min. at 4° to sediment particulate matter which was then discarded. The supernatant was diluted by the addition of five volumes of ice-cold glass-distilled water, and then acetic acid added by dropwise addition to bring the pH to 6·5. In the early experiments the precipitated "protein" was sedimented at 7,000g for 10 min. (Servall RC-2) and the precipitate so obtained washed several times by resuspension in glass-distilled water and centrifuging at 7,000g for 10 min. In later experiments the KCl-soluble material was redissolved in KCl and precipitated out several times before being extensively washed with glass-distilled H_2O. The precipitate was finally resuspended in tris-HCl buffer (pH 7·6).

Determination of ATPase activity.

The standard test system (Charnock and Post, 1963) contained 2 ml. of buffer which provided final concentration of 20 mM tris, 100 mM glycylglycine and 0·2 mM sodium EDTA. The substrate was disodium ATP (Sigma) at a final concentration of 2 mM. Sodium, potassium, caesium, manganese and magnesium salts (chlorides or sulphates) were added in small volumes to give the final concentrations indicated in the text. Ouabain was added to give final concentration of 0·1 mM. The final volume of the assay system was 3 ml. and contained 0·1 ml. of "enzyme" suspension usually with 50-150 µg of protein N (Lowry, Rosenbrough, Farr and Randall, 1951). The chilled mixture was incubated in a Gallenkamp metabolic shaking apparatus at 37° for various times. The reaction was stopped by the addition of 1·5 ml. of 5% (w/v) perchloric acid following which all flasks were chilled in ice-water. Precipitated protein was removed by filtration and the inorganic phosphate in the filtrate was measured by the method of Fiske and SubbaRow (1925) as modified by Aldridge (1962), and using amidol as the reducing agent (Skou, 1957).

Protein nitrogen determinations were performed on 0·1 ml. of the "enzyme" suspensions by the method of Lowry *et al.* (1951). The standard reference curve was prepared using a solution of crystalline bovine albumin (Armour) of known protein N content.

Enzyme (ATPase) activity was expressed as µmoles inorganic phosphate liberated from ATP/mg. of "enzyme" protein N/hr. at 37°. That portion of the ATPase activity apparent in the presence of 80 mM Na^+ + 20 mM K^+ which can be inhibited by 0·1 mM ouabain, is referred to as "transport ATPase" activity.

RESULTS.

Morphology.

Phase contrast microscopy of the cardiac muscle homogenate was recorded after various treatments. All photographs were obtained under standard conditions of exposure, development, enlargement and printing. The microscopic magnification through the camera was × 133, and the photographic magnification was a further × 25.

Fig. 1 is a cardiac muscle preparation in tris buffer solution (control) showing the presence of syncytial muscle fragments having the cross-striations typical of cardiac muscle.

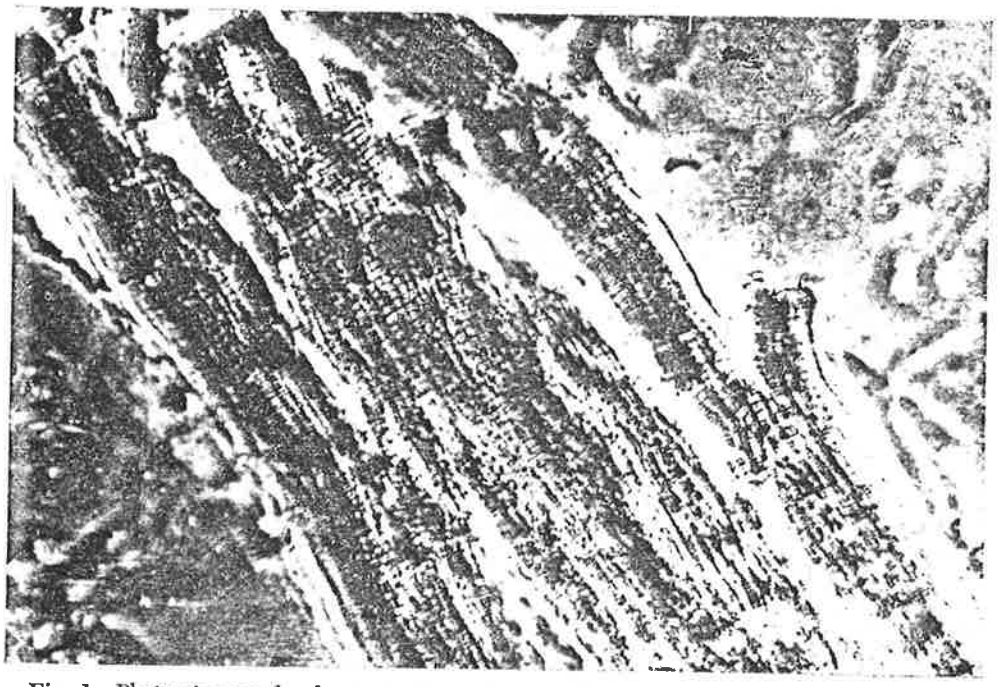

Fig. 1. Photomicrograph of guinea-pig cardiac muscle homogenate examined under phase contrast microscopy (× 133). This preparation has been suspended in 1 mM tris-chloride-EDTA solution (pH 6·8). Photographic enlargement × 25.

Fig. 2 shows cardiac muscle preparation after exposure to 1 M KCl for 6 hr. (Plate A) and 18 hr. (Plate B). Little change is apparent in the gross morphology of the preparation, except for some swelling and the partial disruption of internal structure of the filaments.

Fig. 3 shows cardiac muscle preparation after exposure to 1 M lithium bromide (LiBr). Plate A was taken after 6 hr.' treatment, when considerable swelling had occurred. After 18 hr.' extraction with 1 M LiBr there is marked loss of fine structural detail. Few cross striations are visible while much amorphous material is now apparent (Plate B).

Insoluble ATPase activity.

The ATPase activity of both sheep and guinea-pig cardiac muscle preparations was assayed after extraction in various salt solutions. The results of 18 experiments with sheep tissue are given in Table 1, which shows that extraction of the homogenised muscle with tris buffer (pH 6·8) resulted in an ATPase exhibiting little activation by Na^+ and K^+ which could be inhibited by 0·1 mM ouabain. Only 11% of the "total" ATPase activity assayed under these conditions (in the presence of $Na^+ + K^+$) could be ascribed to the presence of

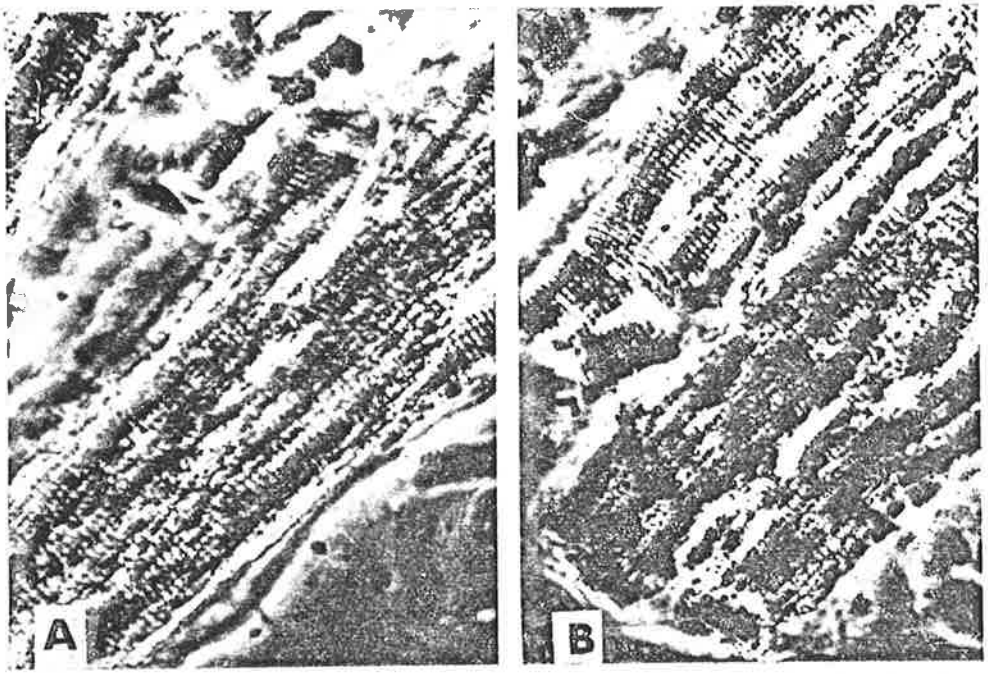

Fig. 2. Photomicrograph of guinea-pig cardiac muscle homogenate examined under phase contrast microscopy (× 133). Plate A: After 6 hr.' extraction in 1 M KCl. Plate B: After 18 hr.' extraction in 1 M KCl.

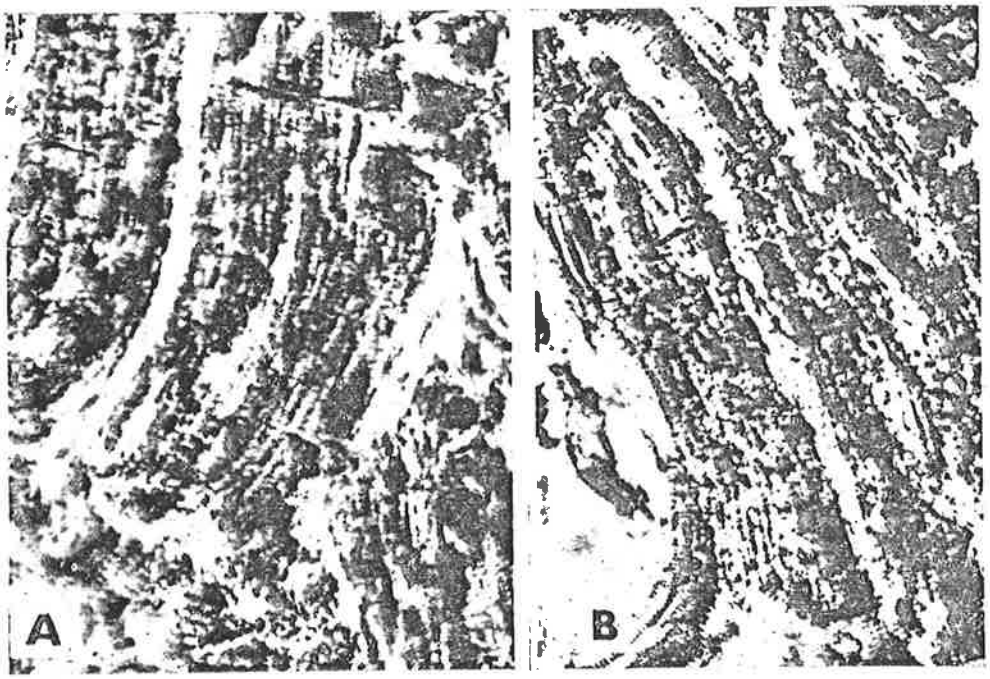

Fig. 3. Photomicrograph of guinea-pig cardiac muscle homogenate examined under phase contrast microscopy (× 133). Plate A: After 6 hr.' extraction in 1 M LiBr. Plate B: After 18 hr.' extraction in 1 M LiBr.

TABLE 1.

The effect of salt extraction on the ATPase activity of sheep heart preparations.

Treatment	Time of extraction	No. of experiments	ATPase activity (μmoles P/mg protein N/hour)†				Ouabain sensitivity
			Control (I)	+Na^+ and K^+(II)	+Na^+ and K^+, ouabain (III)	△‡	
Tris buffer	18 hr	7	43·6±10·7	47·3±10·6	42·1±11·0	5·2	11%
1M KCl	18 hr	6	45·7±13·6	47·7±13·6	31·2±12·5	16·5	34%
1M LiBr*	18 hr	5	15·0± 5·8	73·6±11·6	23·6± 8·7	50·0	68%

Assay conditions are described in the text.

† Values shown are the means for each group (±standard deviation).

* After extraction with LiBr, comparison of control (I) v. Na^+ and K^+ (II) gives probability "p" $<$·01; Na^++K^++ouabain (III) v. Na^++K (II) gives "p" $<$·02.

‡ △ is "transport ATPase" activity, deduced from (II-III).

TABLE 2.

The effect of salt extraction on the ATPase activity of guinea-pig heart preparations.

Treatment	Time of extraction	No. of experiments	ATPase activity (μmoles P/mg protein N/hour)†				Ouabain sensitivity
			Control (I)	+Na^+ and K^+(II)	+Na^+ and K^+, ouabain (III)	△‡	
Tris buffer	18 hr	5	104·3±56·9	95·0±39·2	82·8±39·5	12·2	14%
1M KCl	18 hr	3	50·7±12·0	59·0±19·2	27·7± 6·7	31·3	53%
1M LiBr*	18 hr	5	40·0±18·5	109·0±46·9	43·6±15·3	65·4	60%

The assay conditions are identical with those of Table 1, and are described in the text.

† The values shown are the means for each group (±standard deviation).

* After extraction with LiBr, comparison of control (I) v. Na^+ +K^+ (II) gives "p" $<$·02; Na^+ + K^++ ouabain (III) v. Na^++K^+ (II) gives "p"$<$·02.

‡ △ is "transport ATPase" activity, deduced from (II-III).

TABLE 3.

The effect of time of lithium bromide extraction on the ATPase activity of a guinea-pig heart preparation.

Treatment	Time of extraction	ATPase activity (μmoles P/mg protein N/hour)†				Ouabain sensitivity
		Control (I)	+Na^+ and K^+(II)	+Na^+ and K^+, ouabain (III)	△*	
Tris buffer	0 hr	160	132	118	14	11%
1M LiBr	2 hr	47	80	47	33	41%
1M LiBr	6 hr	24	72	32	40	56%
1M LiBr	12 hr	24	84	32	52	62%
1M LiBr	18 hr	24	88	32	56	64%

† The assay conditions are described in the text, and are identical with those of Tables 1 and 2.
* △ is "transport ATPase" activity deduced from (II-III).

"transport ATPase". The ATPase activity of the preparation obtained by 18 hr.' extraction with 1 M KCl was also similarly little affected by the presence or absence of $Na^+ + K^+$. The ouabain sensitive portion of the $Na^+ + K^+$ stimulated ATPase activity of the preparation was 36%. The ATPase activity of the preparation obtained by 18 hr.' extraction with LiBr was greatly reduced in the absence of both Na^+ and K^+; however, when these cations were present considerable activation of ATPase was seen ($p < \cdot 01$). This increase in activity was largely abolished by 0·1 mM ouabain. The mean proportion of maximal activity of the preparation attributable to the presence of "transport ATPase" was now 68%.

Table 2 shows the results of 13 similar experiments using guinea-pig cardiac muscle extracted with salt solutions. The results obtained with this tissue were similar to those for sheep hearts.

A more detailed examination of the effect of LiBr extraction on guinea pig cardiac muscle preparations is shown in Table 3. This shows a progressive increase in the specific activity of ($Na^+ + K^+$)-activated, ouabain inhibited "transport ATPase" occurring with increasing time of exposure to LiBr.

TABLE 4.

Comparison of the ATPase activity of the KCl-soluble and the KCl-insoluble fractions of guinea-pig heart preparations.

Fraction	Incubation conditions	ATPase activity†
I. KCl-insoluble§	2mM Mg^{2+}	35
	2mM Mg^{2+}, 80mM Na^+, 20mM K^+	71
	2mM Mg^{2+}, 80mM Na^+, 20mM K^+, 0·1mM ouabain	29
	2mM Ca^{2+}	85
	2mM Ca^{2+}, 80mM Na^+, 20mM K^+	67
	2mM Ca^{2+}, 80mM Na^+, 20mM K^+, 0·1mM ouabain	69
II. KCl-soluble*	2mM Mg^{2+}	7
	2mM Mg^{2+}, 80mM Na^+, 20mM K^+	4
	2mM Mg^{2+}, 80mM Na^+, 20mM K^+, 0·1mM ouabain	3
	2mM Ca^{2+}	27
	2mM Ca^{2+}, 80mM Na^+, 20mM K^+	36
	2mM Ca^{2+}, 80mM Na^+, 20mM K^+, 0·1mM ouabain	33

§ The KCl-insoluble fraction was extracted for 24 hr. at 0° and washed twice in tris buffer (pH6·8) to remove excess K^+.

* The KCl-soluble fraction was twice reprecipitated from 1M KCl by the addition of 5 vols glass-distilled water and washed with tris buffer.

† ATPase activity of both fractions is given as μmoles P liberated from ATP/mg. protein N/hour under the standard assay conditions described in the text.

KCl-soluble ATPase activity.

This material was obtained by reducing the ionic strength of a 24-hr. salt extract of cardiac muscle at 0°. The precipitated material was washed with water after reprecipitations from potassium chloride.

The effect of added ions on the ATPase activity of this material was then examined. Unlike the KCl- or LiBr-insoluble enzyme preparations, the KCl-soluble enzyme showed no activation on the addition of Na^+ and K^+ to preparations containing Mg^{2+} ions.

Substitution of equimolar Ca^{2+} for Mg^{2+} (in the absence of Na^+ and K^+) resulted in proportionally greater increase in KCl-soluble ATPase activity than that seen with KCl-insoluble ATPase preparations. The addition of Na^+ and K^+ to either the KCl-soluble or insoluble ATPase preparations in the presence of Ca^{2+} did not increase the activity of either preparation. In addition, the presence of 0·1 mM ouabain under these conditions had no effect. Comparison of the results obtained with KCl-soluble and KCl-insoluble ATPase preparations assayed under identical conditions is given in Table 4.

A further examination of the effect of divalent ions on KCl-soluble ATPase activity of cardiac muscle showed that Mn^{2+}, Mg^{2+} or Ca^{2+} were able to increase the activity of the ATPase; Ca^{2+} was the most effective. There was some dependence upon divalent ion concentration for this effect, and at low concentrations the effects of both Ca^{2+} and Mg^{2+} appeared to be additive. These results are given in Table 5.

TABLE 5.

The effect of Ca^{2+} and Mg^{2+} ions on the ATPase activity of the KCl-soluble fraction of guinea-pig heart preparations.

Experiment	Protein N added	Concentration of ions added†	ATPase activity*
I	0·10 mg	Nil	6
		2mM Mg^{2+}	9
		2mM Mn^{2+}	12
		2mM Ca^{2+}	14
		2 mM Mg^{2+}, 2mM Ca^{2+}	26
		2mM Mg^{2+}, 20mM Ca^{2+}	46
		2mM Mg^{2+}, 200mM Ca^{2+}	31
		20mM Mg^{2+}, 2mM Ca^{2+}	36
		200mM Mg^{2+}, 2mM Ca^{2+}	6

† Incubated for 30 min. at 37° under standard assay conditions described in text.
* ATPase activity is given as μmoles P liberated from ATP/mg. protein N/hour.

The effect of monovalent ions was examined in reaction systems already containing either Mg^{2+} or Ca^{2+} or both divalent ions. The results of this investigation are given in Table 6.

Once again the addition of 80 mM Na^+ plus 20 mM K^+ in the presence of Mg^{2+} failed to elicit the increase in activity characteristic of "transport ATPase". When calcium ions were introduced into the reaction system,

either alone or in combination with Mg^{2+}, the activity increased greatly. However, the subsequent introduction of either Na^+, K^+ or Cs^+ ions at a concentration of 80 mM resulted in a decrease in ATPase activity (Expt. III, Table 6).

TABLE 6.

The effect of ions on the ATPase activity of the KCl-soluble fraction of guinea-pig heart preparation.

Experiment	Protein N added	Concentration of ions added	ATPase activity†
II	0·06 mg	2mM Mg^{2+}	17
		2mM Mg^{2+}, 80mM Na^+, 20mM K^+	13
		2mM Mg^{2+}, 80mM Na^+, 20mM K^+, 0·1mM ouabain	8
		2mM Mg^{2+}, 5mM Ca^{2+}	55
III	0·125 mg	Nil	10
		2mM Mg^{2+}, 80mM Na^+, 20mM K^+	12
		2mM Mg^{2+}, 80mM Na^+, 20mM K^+, 0·1 mM ouabain	8
		28mM Ca^{2+}	87
IV	0·18 mg	2mM Mg^{2+}, 5mM Ca^{2+}	70
		2mM Mg^{2+}, 5mM Ca^{2+}, 0·1mM ouabain	62
		2mM Mg^{2+}, 5mM Ca^{2+}, 20mM K^+	70
		2mM Mg^{2+}, 5mM Ca^{2+}, 80mM K^+	30
		2mM Mg^{2+}, 5mM Ca^{2+}, 80mM Na^+	33
		2mM Mg^{2+}, 5mM Ca^{2+}, 80mM Cs^+	33
V	0·15 mg	5mM Ca^{2+}	45
		5mM Ca^{2+}, 0·1mM ouabain	45
		5mM Ca^{2+}, 80mM Na^+	38
		5mM Ca^{2+}, 80mM Na^+, 0·1mM ouabain	36
		5mM Ca^{2+}, 80mM K^+	36
		5mM Ca^{2+}, 80mM K^+, 0·1mM ouabain	36

† ATPase activity is given as μmoles P liberated from ATP/mg. protein N/hour.

With preparations reprecipitated from KCl this effect was less apparent (Expt. IV, Table 6). These effects were not influenced by ouabain at a concentration known to inhibit "transport ATPase".

Rate of hydrolysis of ATP by insoluble ATPase.

The rate of hydrolysis of ATP by the (Na^+ + K^+)-activated, ouabain inhibited ATPase of these preparations was examined by incubation at 37° for periods up to 90 min. Fig. 4 shows the results of a typical experiment using a guinea-pig cardiac muscle preparation obtained by extraction with 1 M LiBr for 18 hr. The rate of hydrolysis of ATP by this preparation in the presence of 80 mM Na^+ + 20 mM K^+ was linear for the first 60 min.' incubation, but showed departure from linearity by 90 min. The rate of ATP hydrolysis obtained in the presence of 0·1 mM ouabain was less and remained linear throughout 90 min. Similar results were obtained with all other preparations

examined, including several preparations obtained from sheep heart. All experiments were therefore carried out with 30 min. incubation periods whenever possible to ensure linear reaction conditions.

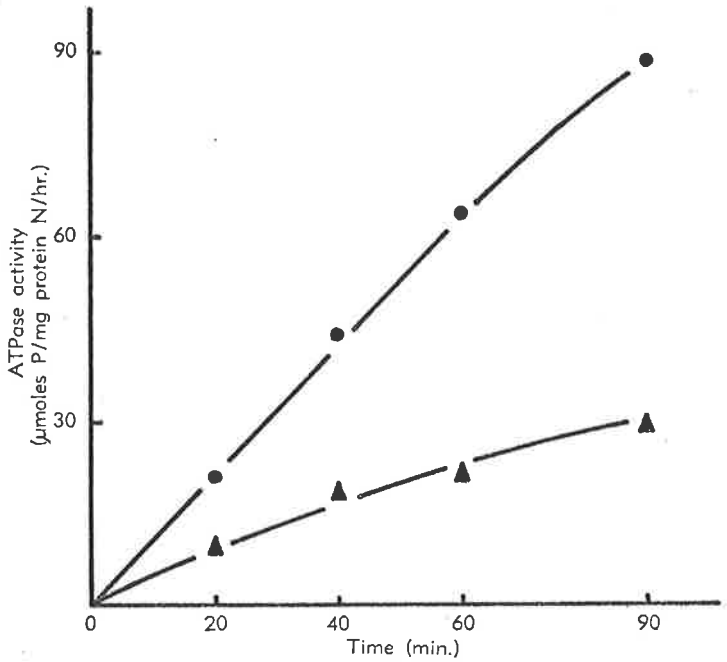

Fig. 4. Effect of time on the rate of hydrolysis of ATP by a lithium bromide extracted (18 hr.) preparation of guinea-pig heart. Assay conditions were 0·1 ml. "enzyme" suspension, incubated at 37° for the times shown; ●——● with 80 mM Na^+ and 20 mM K^+; ▲——▲, with 80 mM Na^+, 20 mM K^+ and 0·1 mM ouabain.

Effect of ouabain on the hydrolysis of ATP by insoluble ATPase.

The effect of various concentrations of ouabain (Strophanthin-G) on the hydrolysis of ATP by (Na^+ + K^+)-activated ATPase obtained from guinea-pig cardiac muscle preparations by 12-hr. LiBr extraction is shown in Fig. 5. No inhibition of (Na^+ + K^+)-activated ATPase was observed at an ouabain concentration of 10^{-8} M, but complete inhibition was obtained at 10^{-4} M. Half maximal inhibition was consistently observed at 4 x 10^{-6} M with enzyme preparations obtained from either guinea-pig or sheep tissue. Comparison with guinea-pig kidney and brain enzyme preparations described previously showed that 4 x 10^{-6} M ouabain also produced half maximal inhibition of "transport ATPase" activity in these preparations (Potter *et al.*, 1966).

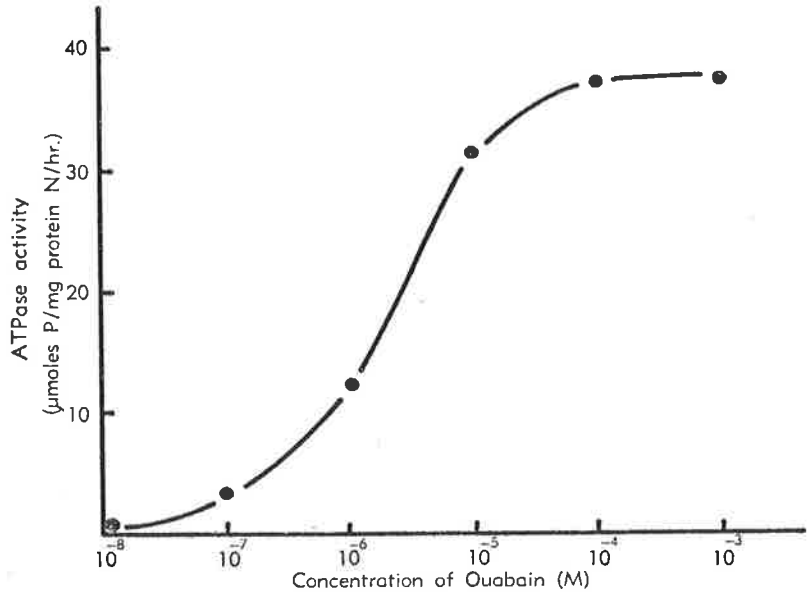

Fig. 5. Effect of ouabain concentration on the hydrolysis of ATP by a lithium bromide extracted (12 hr.) preparation of guinea-pig heart. Assay conditions were 0·1 ml. "enzyme" suspension. incubated for 20 min. at 37°. The values shown were obtained by calculation of the difference $\triangle = (Na^+ + K^+) - (Na^+ + K^+$, ouabain) and represent only that portion of the total ATPase activity which is induced by Na^+ and K^+ and is sensitive to ouabain inhibition, i.e. "transport ATPase" activity. Half maximal inhibition occurs at 4×10^{-6} M ouabain.

DISCUSSION.

Most earlier attempts to isolate a ($Na^+ + K^+$)-activated, ouabain inhibited ATPase from cardiac muscle have centred around the isolation of a "microsomal" fraction similar to that yielding enzyme preparations from kidney and cerebral tissue (Schwartz, 1962; Auditore and Murray, 1962 and 1963; Kennedy and Nayler, 1965a). Bonting *et al.* (1962), Auditore and Murray (1962, 1963), and Lee and Yu (1963) have all shown that this enzyme was detectable in cardiac microsome preparations, and Schwartz (1962) had reported the apparent rise in enzyme activity which followed prolonged ageing of microsomal preparations. However, levels of enzyme activity were usually low in comparison with those reported in other tissues (Schwartz, 1962) or the enzyme displayed greatly reduced sensitivity to either Na^+ and K^- (Lee and Yu, 1963) or ouabain (Kennedy and Nayler, 1965a, 1965b).

From our previous studies with this enzyme isolated from other tissues we were impressed by its association with cellular membranes (Charnock and Post, 1963; Charnock, Opit and Casley-Smith, 1966) and hence sought a method

of preparation which may yield a greater proportion of "membrane structures" than the conventional microsomal isolation procedures. We utilized the findings of Kono and Colowick (1961) who had successfully employed a salt extraction procedure for the isolation of skeletal muscle cell membranes and found that extraction of cardiac muscle homogenate with 1 M LiBr yielded a preparation having clearly demonstrable levels of "transport ATPase". When KCl was substituted for LiBr, this salt extraction procedure yielded a soluble ATPase fraction possessing different properties in respect to cation activation, namely, the absence of activation by Na^+ and K^+ in the presence of Mg^{2+}.

In this respect the soluble ATPase resembles that extracted from red cell membranes (Nakao, Nagano, Adachi and Nakao, 1963) and may be analogous to "erythroaction" described by Ohnishi (1962) and by Ohnishi and Kawamura (1963). The activation of this KCl-soluble ATPase preparation by low concentrations of Ca^{2+} and the subsequent inhibition by high concentrations of K^+ has been a feature of actomyosin ATPase activity known for many years (Mommaerts and Seraidarian, 1946). Repke, Est and Portius (1965) have also reported their use of a mixed salt extraction procedure in an attempt to isolate a cardiac glycoside sensitive ATPase from heart muscle. However, these workers were not able to isolate a "transport ATPase" preparation which was characterised by both activation by Na^+ and K^+, as well as inhibition by cardiac glycosides.

Recently, Tashima *et al.* (1966) have demonstrated the partial separation by extraction with sodium iodide of a cardiac glycoside sensitive "transport ATPase" preparation from rat cardiac muscle microsomes. These workers observed the presence of a Na^+ inhibited ATPase "contaminant" during their separation procedure, similar in behaviour to Na^+ as the KCl-soluble "actomyosin-like" ATPase described here.

Undoubtedly, the presence of this monovalent cation inhibited cardiac ATPase system has obscured the determination of "transport ATPase" activity in many previous studies of this enzyme in heart muscle. We feel that examination of these enzyme systems together (cation activated and cation inhibited ATPase), without prior salt extraction of cardiac tissue, has led to the many reports of low activation by Na^+ and K^+ and the uncertain results of cardiac glycoside sensitivity reported in the literature.

Certainly the findings of Schwartz (1962) and Tashima *et al.* (1966) that the proportion of Na^+ and K^+ activated, ouabain inhibited ATPase of their preparations increased with ageing may be explained on the basis of the two ATPase systems described here, having differing stabilities with respect to time.

While conducting the enzyme studies on heart muscle homogenates, we believed that some information about the localization of the enzyme might be obtained from studies of the gross morphology of these preparations.

Our findings indicated that, as the internal structure of the heart syncitium was destroyed, there was a progressive and parallel increase in the activity of ($Na^+ + K^+$)-activated ATPase, and a concomitant reduction in the Na^+ or K+ inhibited ATPase.

This observation is consistent with the belief that ($Na^+ + K^+$)-activated ATPase is localized in the membrane component of heart muscle cells (Lee and Yu, 1963).

The association of "transport ATPase" systems with membrane components of cardiac muscle, and the sensitivity of this enzyme to low concentrations of cardiac glycosides, must focus attention on the probability of the interaction between cardiac glycosides and "transport ATPase" (Repke, 1965) being the basis of the long-sought pharmacological effects of these agents.

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The effect of anions on ($Na^+ + K^+$)-activated ATPase

The properties of ($Na^+ + K^+$)-activated ATPase, derived from a wide variety of animal tissues have been extensively studied by a number of investigators[1–8]. Recently SKOU[9,10] and JUDAH AND AHMED[11] have reviewed the evidence for the possible role of this enzyme in the active transport of Na^+ and K^+ ions across cellular membranes.

OPIT AND CHARNOCK[12] have proposed a molecular model by which this ouabain-sensitive enzyme system could physically transport Na^+ and K^+ ions, which depends upon the short-range interactions between charged ions and groups within the protein enzyme structure (*cf.* LING[13]). It was considered that such a model reaction system would be influenced not only by cations, but might also demonstrate some influence by anions. We believe therefore that the ATPase activity of this enzyme may reflect the nature of the anion associated with Na^+ and K^+. Our previous investigations and the experiments of other workers have been limited in the main to the use of the chloride salts of selected cations. In this paper we describe the effects of substituting Br^-, I^-, F^-, SO_4^{2-}, NO_3^- and acetate for chloride, whilst retaining sodium (80 mM Na^+) and potassium (20 mM K^+) as the cations.

The methods of preparation of cardiac[14], brain[15] and kidney[16] ($Na^+ + K^+$)-activated, ouabain-sensitive ATPase, and the standard conditions for the determination of "transport ATPase" activity have been described in full elsewhere. "Transport ATPase" activity is defined as that portion of the microsomal or membrane-bound ATPase activity which is abolished by 0.1 mM ouabain in the presence of Na^+, K^+ and Mg^{2+}.

The cardiac and kidney preparations exhibited at least 50% inhibition by 0.1 mM ouabain, whilst the brain preparations used here were 25–50% sensitive to this concentration of ouabain.

Table I demonstrates that the substitution of Br^-, I^-, SO_4^{2-}, NO_3^- or acetate ion for Cl^-, under the standard conditions of assay, was without significant effect upon "transport ATPase" activity prepared from guinea-pig heart, kidney or brain.

TABLE I

THE EFFECT OF ANIONS ON ATPase ACTIVITY

The specific activity is given as μmoles P_i/mg protein N per h at 37°. It represents only that portion of ATPase activity which is stimulated by 80 mM Na^+ + 20 mM K^+, and is inhibited by 0.1 mM ouabain *i.e.* "transport ATPase". % ouabain sensitive represents the % of total activity of the preparation which is inhibited by ouabain.

80 mM Na^+ + 20 mM K^+	*Cardiac ATPase*		*Kidney ATPase*		*Brain ATPase*	
	Specific activity	*% ouabain sensitive*	*Specific activity*	*% ouabain sensitive*	*Specific activity*	*% ouabain sensitive*
Cl^-	44	64	131	70	100	40
Br^-	41	59	139	71	80	37
I^-	35	58	141	72	100	40
F^-	0	0	0	0	0	0
SO_4^{2-}	37	60	118	66	80	29
NO_3^-	46	67	122	72	110	44
Acetate	41	62	126	70	90	39

However, replacement of Cl^- by the F^- anion completely abolished "transport ATPase" activity in all the preparations examined. In these experiments the total F^- concentration was 100 mM.

Hence the minimal concentration of F^- anion required for this effect was examined in experiments with guinea-pig kidney enzyme preparations, in which only a portion of the Cl^- content was replaced with F^-. The result of a typical experiment

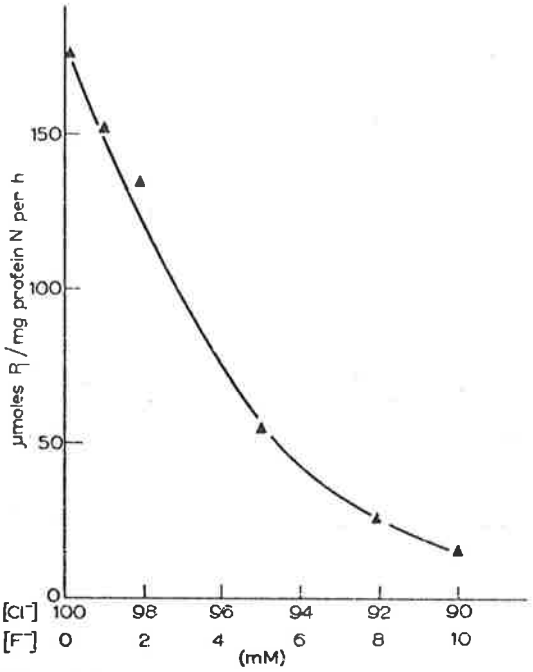

Fig. 1. Effect of varying concentrations of Cl^- and F^- on "transport ATPase" activity. The result shown is that of a typical experiment in which a guinea-pig kidney enzyme preparation was incubated with 80 mM Na^+ + 20 mM K^+, in the presence and absence of 0.1 mM ouabain, and at the chloride/fluoride concentrations given. "Transport ATPase" activity is computed as the difference between these experimental situations (± ouabain), and these derived values are those plotted here.

TABLE II

THE EFFECT OF Mg^{2+} CONCENTRATION ON F^- INHIBITION OF THE ($Na^+ + K^+$)-ACTIVATED ATPase OF GUINEA-PIG KIDNEY MICROSOMES*

The specific activity is given as µmoles P_i/mg protein N per h at 37° and is that portion of the activity stimulated by 80 mM Na^+ + 20 mM K^+ and inhibited by 0.1 mM ouabain. Each value is the mean of three separate experiments.

$[Mg^{2+}]$ (mM)	$[F^-]$ (mM)	*Specific activity*	*% activity*
2	—	252	100
5	—	188	75
10	—	141	56
2	5	104	41
5	5	35	14
10	5	13	5

* Mg^{2+} added as $MgCl_2$.

is shown in Fig. 1 where it can be seen that a concentration of 1 mM F^- in the presence of 99 mM Cl^- has a detectable effect, and that complete inhibition of ATPase activity has occurred at 10 mM F^- in the presence of 90 mM Cl^-.

The standard ATPase assay[6,14,15] always contained 2 mM Mg^{2+}. Because of the possibility that F^- inhibition could be caused through inactivation of the magnesium ion by formation of insoluble MgF_2, experiments were performed with kidney microsome preparations using additional magnesium ions. Table II shows that the inhibition produced by 5 mM F^- was not reduced by an increased concentration of Mg^{2+}, hence inactivation of the Mg^{2+} by complexing with F^- seems unlikely to be the cause of F^- inhibition. Progressive inhibition of ATPase activity occurred with increasing concentrations of Mg^{2+} in the absence of F^-, and this inhibition appeared to be additive with respect to that seen with F^-, thus suggesting that these effects are independent.

The effect of fluoride poisoning of cation transport in the red blood cell is well known[16], where it has generally been assumed to act *via* an inhibition of glycolysis similar to that produced by iodoacetate poisoning[17], or by glucose deprivation[18]. However, it is also recognized that this explanation alone does not provide a complete understanding for the effects of this ion on the red blood cell, although most additional explanations have centered around the undoubted osmotic effects of higher concentrations of F^- (ref. 19).

The appreciable inhibition of $(Na^+ + K^+)$-activated ATPase by low concentrations of F^- described here, and the probable role of this enzyme system in cation transport in the red cell[12,20,21] may offer an alternative hypothesis for this effect.

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Alkali Ion-Sensitive Adenosine Triphosphatase

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From the work of Skou, Post, Whittam, and Glynn during 1957–61 it has been suggested that the enzyme system known as alkali ion-sensitive adenosine triphosphatase is closely associated with the active transport of sodium and potassium ions across cellular membranes.

The salient characteristics which relate this system to the movement of these ions are as follows:

1. The enzyme system is membrane-bound, and virtually ubiquitous in animal cells.

2. It requires the presence of both Na^+ and K^+ ions for activation—a well known requirement for ion transport in whole cells or tissue systems *in vitro*.

3. In the erythrocyte, enzyme activation occurs only when Na^+ is on the inside and K^+ is on the outside of the cell. Thus, the enzyme system possesses vectorial properties and hence directional movement of reactants may be possible.

4. Alkali-activated ATPase is specifically inhibited by the cardiac glycoside ouabain, known to be a potent inhibitor of cation transport both *in vivo* and *in vitro*.

5. Kinetic examination of the reaction *in vitro* shows activation by substrate and cofactors, and inhibition by ouabain at concentrations compatible with physiological conditions similar to those encountered *in vivo*.

An examination of the reaction mechanism of this membrane-bound system by using radioactively labelled reactants (ATP^{32}, $C^{14}ADP$, Na^{24}) has suggested the following sequence of events:

$$1.\quad ATP^{32} + \text{Enzyme} \underset{}{\overset{Na}{\rightleftharpoons}} [E\sim P^{32}] + ADP$$

$$2.\quad [E\sim P^{32}] + H_2O \xrightarrow[\text{ouabain}]{K\uparrow} \text{Enzyme} + P_i{}^{32} + Na + K$$

That is, initially there is a rapid interaction between enzyme and substrate in the presence of sodium ions which leads to the formation of a phosphorylated enzyme complex. K^+ ions cannot replace N^+ in this reaction. The nature of this complex is not completely certain, but it is known that it is phosphoprotein and not phospholipid in nature; the phosphoryl group is probably bound to a histidine residue, although the evidence for this is not unequivocal. The nature of the adjacent amino acid groups is not yet certain.

The second step in the reaction requires K^+ (not Na^+) and leads to the hydrolysis of E∼P (the phosphoenzyme complex). It is this second step which is the site of ouabain inhibition and, from Whittam's data on the erythrocyte system, suggests that this is at the "external" surface of the membrane.

Kinetic examination

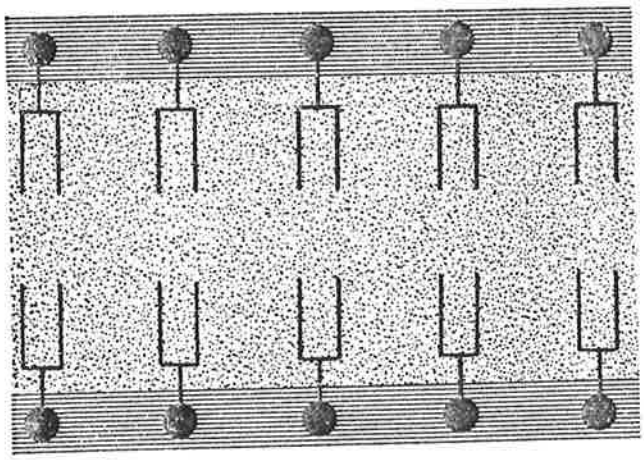
By construction of Lineweaver-Burk plots we have been able to show that ADP can considerably inhibit the rate of ATP breakdown, as would be expected from the reaction mechanism shown above. Introduction of C^{14}-ADP and recovery of C^{14}-ATP confirm the existence of an ADP⇌ ATP exchange reaction. We are at present examining the effect of alkali ions on this reaction.

In addition, we have been only able to demonstrate some inhibition of ATP hydrolysis in the presence of relatively high concentrations of inorganic phosphate (20–30 mM). Whilst this concentration bears no relationship to that formed by ATP breakdown in our *in vitro* system, it nevertheless represents a reasonable approximation of intracellular PO_4 concentration *in vivo*. The fact that negligible inhibition can be detected with 2 mM PO_4 probably explains the lack of experimental evidence for a $P^{32} \rightleftharpoons ATP^{32}$ exchange reaction and is evidence for an almost irreversible decomposition of E~P complex.

Molecular model for Na^+–K^+ transport

How can this information be utilized to provide a molecular model for directional ion transport? How can this model be used to obtain experimental evidence in support of the hypothesis?

With acknowledgement to Professor Ling's basic concepts of enzyme-protein structure and function I would like to present now a molecular model for ion transport recently proposed by my colleague Dr. Louis J. Opit. We believe that this model takes into account the known experimental observations without introducing ill-defined characteristics of carrier substances. First let us consider the Danielli model for bilaminar membrane structure:

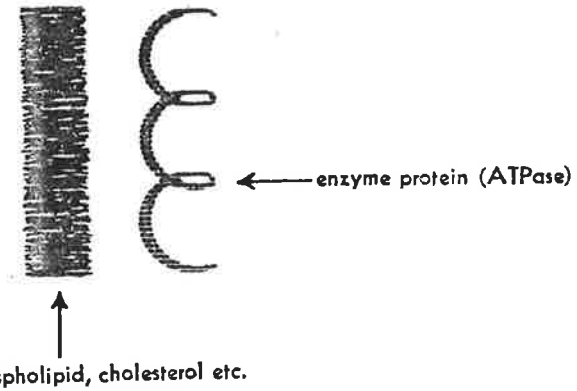


A unit membrane is depicted as a double layer of protein and carbohydrate into which the polar groups of phospholipids are embedded. The tuning fork prongs represent nonpolar groups of phospholipids, and the shaded area represents cholesterol and cholesterol esters.

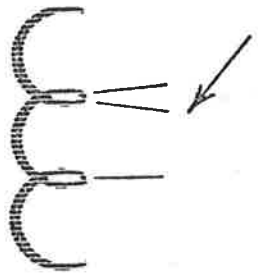
This classical model is oversimplified, as the actual structure is more complicated than this, at least at a secondary level, particularly in respect to the enzyme systems reported in the membrane and the probability that the inner and outer membrane surfaces differ in structure and enzyme content.

For example, we feel that alkali sensitive ATPase is more probably located on the inner surface of the membrane although the component reactions may be situated at different surfaces.

For the sake of this model let us consider the inner surface of this membrane which we choose to depict as follows:



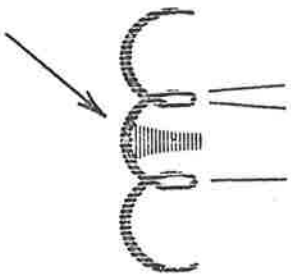
We must first regard the protein coil as a highly resonant and polarizable structure, such as Professor Ling has proposed, and along this coil are many surface-oriented cationic and anionic binding sites (e.g., carboxyl groups) which point towards the inside of the cell. For convenience these are shown as:



Thus the protein-containing enzyme is analogous to an unsaturated resin bed. It is necessary to this model to assume that this structure possesses much greater affinity for Na^+ and K^+ in the unreacted state.

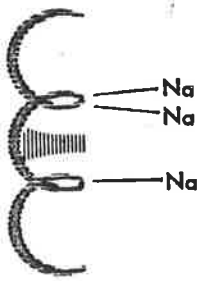
Using Dr. Ling's terminology, there are also other groups interspersed along the protein backbone which he refers to as cardinal sites, and which in our model correspond to the active center of the ATPase enzyme.

These are shown as:



This, then, represents the hypothetical resting state of the enzyme in the absence of substrate and alkali metal ions. Dr. Opit then suggests that the first reaction is a specific binding of intracellular Na^+ to the acceptor sites of the resinlike membrane protein. This process may not require the expenditure of cellular energy in the conventional sense of requiring high energy ~P.

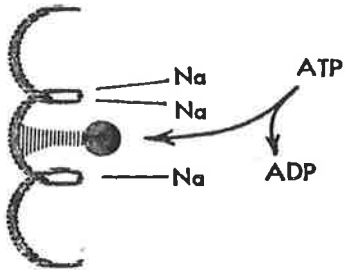
This interaction is shown as:



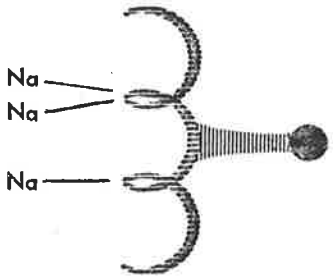
By electrostatic induction dipole-dipole interaction and other common physical forces, this binding is followed by a redistribution of charge along the protein chain, which has been referred to by Professor Ling most descriptively as a zipper effect.

The cardinal sites become activated and now react with ATP to form an energy-rich complex between ~P and enzyme, i.e. $[E\sim P] \ldots_n Na$.

This is represented as:

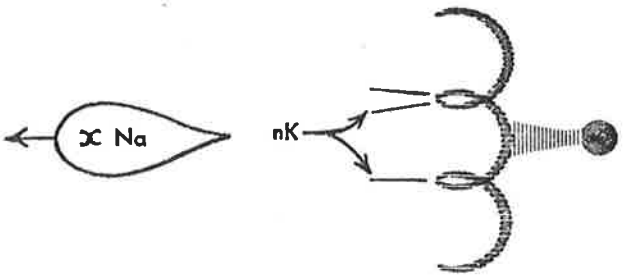


It is now suggested that the introduction of high energy phosphate from ATP into the molecule again induces electron shift throughout the protein chain, resulting in formational change in its tertiary structure. A good example for this type of change is in the contractile protein myosin, which itself is a potent ATPase. This change can be represented thus:



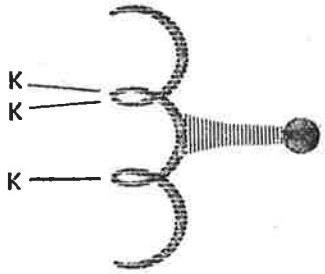
Here it is suggested that the Na-binding sites undergo rotation through 180°. That is, Na^+ groups are now directed away from the surface to which they were originally oriented.

The protein portion of the membrane is now in a state of electron strain, corresponding to an unstable chemical state, which is now susceptible to some form of K^+ for Na^+ exchange. In fact, an unknown number of K^+ atoms now displace the Na^+ previously bound. This can be shown as:

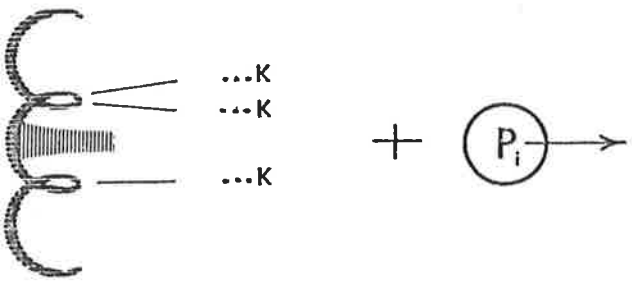


The Na^+ is now released from the membrane on the surface opposite that to which it was originally bound and corresponding now to the outside of the cell.

This step has resulted in the formation of



which must now be susceptible to hydrolysis by H_2O inasmuch as we know that E~P breaks down with the appearance of inorganic phosphate on the inside of the membrane. The rotational force is thus removed from the protein chain, which now reverts to its original form:

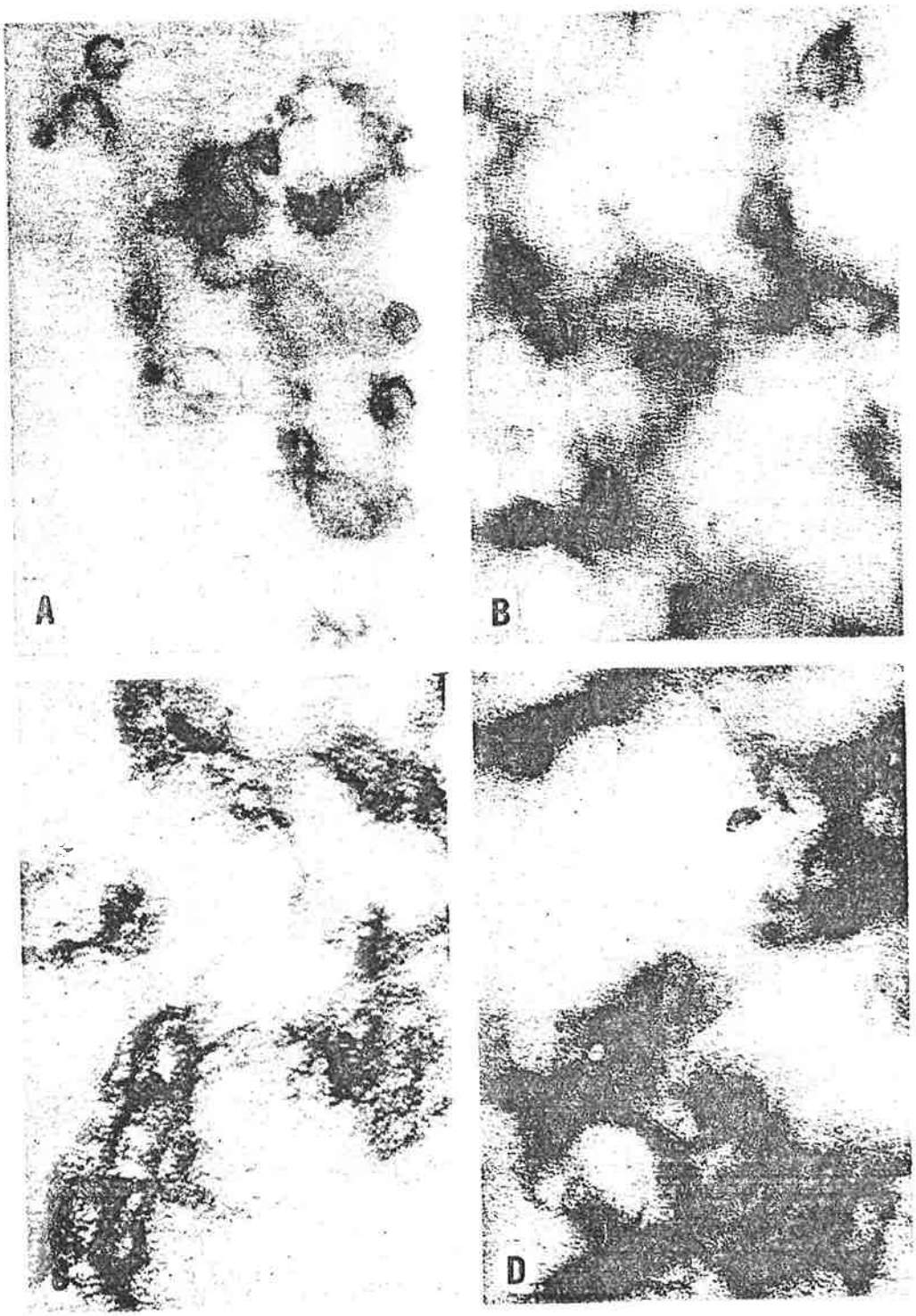


This reversion about the protein axis carries the K^+ ions from the outside to the inside of the cell membrane. In this situation the ion-binding sites will now again bind Na^+ in preference to K^+ and the sequence of reactions will begin again. The consequence of these reactions will be the directional transfer of both Na^+ and K^+ to opposing sides of the membrane.

This, then, constitutes our molecular model for coupled Na^+ and K^+ transport by alkali-sensitive membrane-bound ATPase.

I wish briefly to show you our experimental evidence in support of this model. The data have been obtained in collaboration with Dr. John Casey-Smith of the University of Adelaide's electron microscope unit.

Figure 11 shows a series of electron microscope pictures (at 150,000 magnification) of our usual enzyme preparation obtained from guinea pig renal cortex. This particular preparation is a microsomal fraction free of mitochondrial or nuclear membranes and which contains a little or no ribosomal material. Clearly the essential structures are vesicular bilaminar membranes.



These preparations contain highly active ATPases, of which over 80 percent require alkali ions for activity after treatment with deoxycholate. This highly active preparation represents one major difference between our work and much of that published by other investigators, many of whom have attempted to study this enzyme in preparations having less than 15 percent of their total activity dependent on alkali ions.

By introducing the various reactants of our reaction mechanism into an incubation system, either singly or in combination, and by subsequently removing the particulate enzyme preparation by centrifugation, we were able to examine the electron microscope appearance of these membranes under a variety of conditions. The enzyme preparation was first embedded in agar and fixed with osmium tetroxide, then dehydrated with alcohols, and finally embedded in Araldite. Plates were obtained under standard conditions of exposure, intensity, and developing. These plates were then examined by a method of densitometry which permitted comparison of membrane segments from one plate to another and hence under different experimental conditions.

The data are summarized in table 1, where the unit of measurement is optical density. Each value is the mean obtained from ten randomly selected membranes incubated under each set of experimental conditions. Only one measurement was made on any one segment of membrane.

In one of the experiments shown here we used the technique of substituting Cs^+ for potassium ions in the incubation medium because, although in the biochemical sense these two ions are completely interchangeable, Cs^+ has much greater electron density than potassium and is thus more suitable for electron miscroscope examination than K^+.

This experiment shows that only when the inhibitor ouabain is present in addition to all the other components of the reaction mechanism is there any significant increase in electron density of the membranes. (Compare pl. B with pl. A in fig. 11).

TABLE 1.—Membrane electron density

Experiment 1 Sucrose medium	Mean O.D.	S.E.	p
Control	.096	±.012	
ATP	.128	±.009	n.s.
ATP, Na	.121	±.011	n.s.
Na	.117	±.012	n.s.
ATP, Cs	.121	±.014	n.s.
Cs	.136	±.015	n.s.
ATP, Na, Cs	.109	±.006	n.s.
Na, Cs	.142	±.012	n.s.
Na, Cs, ouabain	.117	±.014	n.s.
ATP, Na, Cs, ouabain	.326	±.024	* * *
Experiment 2 Lead sucrose medium	**Mean O.D.**	**S.E.**	**p**
Na, K	.162	±.009	
ATP, Na, K	.286	±.021	* * *
Na, K, ouabain	.144	±.009	n.s.
ATP, Na, K, ouabain	.146	±.013	n.s.

The other experiment shown in table 1 utilized the well-known histological technique of incubating a phosphate-releasing reaction in a lead-containing medium. Under these conditions the introduction of ouabain now prevents an increase in electron density, and thus acts as a control for the electron density effect of ouabain itself. (Compare pl. C with pl. D in fig. 11).

Returning now to a consideration of plate B (fig. 11): from the sequence of steps in our proposed reaction mechanism, we believe this plate represents the biochemical situation in which we know from repeated experimentation that the level of E~P is maximal. The increase in electron density here we interpret as a deposit of Cs^+ ions on the membrane at the stage represented in our model by figure 9: i.e., when the sodium pump is interrupted at one stage of its molecular reaction sequence.

FIGURE 11

PLATE A.—Vesicular bilaminar membranes incubated in sucrose media containing ATP, Na^+ and Cs^+. These conditions serve as control for plate B.

PLATE B.—Ouabain added to media used for plate A. The hydrolysis of ATP is inhibited, and the membranes are significantly more electron dense.

PLATE C.—Membranes incubated in lead nitrate-sucrose medium containing ATP, Na^+, and K^+. Ouabain also is present. The hydrolysis of ATP is inhibited and no phosphate is released to react with lead.

PLATE D.—Ouabain omitted from medium of plate C. ATP hydrolysis proceeds and lead phosphate deposits significantly, increasing electron density of membranes. The lead deposits in both plates C and D are unrelated to the reaction, as they occur in all controls as well as in test systems in which lead-sucrose medium is used.

All plates obtained at 150,000 X.

Material fixed with osmium tetroxide and embedded in Araldite.

Hence we believe that membrane-bound alkali ion-sensitive ATPase lies at the very heart of the sodium pump. Although no biochemical reaction mechanism can, in its own right, fully explain a vectorial property of membranes such as sodium transport, I trust that this paper has been of interest, as it has been presented with the intention of marrying the ion transport data of the physiologists with the ion reaction mechanism of the biochemists.

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A MOLECULAR MODEL FOR A SODIUM PUMP

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ALTHOUGH there are considerable experimental data relating to the exclusion of Na^+ and accumulation of K^+ in living cells, our knowledge of the mechanism is rudimentary and the molecular basis for this phenomenon remains unknown. Excellent reviews[1,2] stressing these deficiencies continue to be published. From the work of Skou, Post, Glynn and Whittam during 1957–61 there is strong evidence that the enzyme system known as 'Na^+ and K^+ dependent adenosine triphosphatase' is closely associated with the active transport of sodium and potassium ions across cellular membranes.

In summary, the salient characteristics which relate this enzyme system to the movement of Na^+ and K^+ are:

(1) The enzyme system is membrane bound, and virtually ubiquitous in animal cells[3,7].

(2) It requires the presence of both Na^+ and K^+ ions for activation—a well-known requirement for ion transport in whole cell or tissue systems *in vitro*[6–8].

(3) In the erythrocyte, enzyme activation occurs only when Na^+ is on the 'inside' and K^+ is on the 'outside' of the cell[9,10].

(4) Na^+ and K^+ activated ATPase is specifically inhibited by the cardiac glycoside ouabain, known to be a potent inhibitor of cation transport both *in vivo* and *in vitro*[6,7,11].

(5) Kinetic examination of the reaction *in vitro* shows activation by substrate and ions, and inhibition by ouabain at concentrations compatible with 'physiological conditions'[7,12].

Studies by Charnock and Post[13,14], Rose[15] and others[1,16] have suggested the following reaction mechanism for the hydrolysis of ATP by this enzyme in the presence of Mg^{++}, Na^+ and K^+:

$$\text{ATP} + \text{Enzyme} \overset{Na^+}{\rightleftharpoons} \text{E} \sim \text{P} + \text{ADP} \qquad (1)$$

$$\text{E} \sim \text{P} + \text{H}_2\text{O} \xrightarrow[\text{Ouabain}]{K^+} \text{E} + \text{P}_i \qquad (2)$$

The initial reaction step consists of the rapid reversible formation of a phosphorylated complex. This step is

stimulated by Na^+ ions, and no other ion appears able to replace Na^+. The formation of this complex is followed by an almost irreversible decomposition to P_i and free enzyme. This latter reaction is stimulated by K^+ but not Na^+ ions; K^+ can be replaced with either NH_4^+, Li^+, Rb^+ or Cs^+. It is this decomposition which is inhibited by the cardiac glycoside ouabain. The chemical nature of the phosphorylated complex is not yet known, although it seems certain that it is phosphoprotein and not phospholipid[13-17]. Kinetic data from this laboratory also support this general reaction mechanism. The K_m for ATP is 2·8 mM; ADP acts as an inhibitor. Little consistent inhibition is apparent with concentrations of phosphate as high as 30 mM (ref. 12).

Although the details of the reaction mechanism of this membrane-bound ATPase have been extensively studied, the means by which this enzyme effects a vectorial transport of Na^+ and K^+ is unknown. Further advances in understanding the mechanism of Na^+ and K^+ transport therefore require information about how such an ATPase might transport Na^+ against the concentration gradient from the inside of the cell, coupled with the movement of K^+ in the opposite direction. The purpose of this article is to present a molecular model for the directional transport of these ions based on what is known of the ATPase reaction mechanism, and which is consistent with knowledge about Na^+ and K^+ transport characteristics in the whole cell or tissue.

It has been generally accepted that the ATPase enzyme itself is membrane bound. We have recently obtained evidence that the enzyme preparation obtained from guinea-pig kidney cortex consists entirely of bilaminar membrane structures, many of which are vesicular, and which are thought to be derived from the endoplasmic reticulum of the kidney cells[18].

We propose, therefore, to develop the model on the basis of these bilaminar membrane fragments and take as our starting point the configuration of the unit membrane proposed by Danielli. This unit contains a double protein layer, with lipid interposed. We will consider the inner facing protein structure to contain the ATPase enzyme. This belief is supported by the observations of Whittam that hydrolysis of ATP only occurs in the red cell ghost when the ATP is intracellular[10].

If, in the intact cell, these enzyme membrane fragments were associated with cell surface or its prolongations into the cell and the endoplasmic reticulum[19-21], then it is reasonable to suppose that the outer protein surface is directed toward the extracellular compartment.

The inner protein structure containing the ATPase must now be visualized as a highly ordered, polarizable chain, with many surface orientated cationic and anionic groups. Among the groups are situated a much smaller

number of amino-acid groups that constitute the active centre of the ATPase enzyme itself (Fig. 1). The justification for this type of approach to biologically active protein structure has been developed in some detail by Ling[22], who refers to the active centre as the cardinal site.

In the first state of the protein enzyme which we will consider, the model presupposes that the fixed anionic sites will show no strong preference for either Na^+ ions or K^+ ions, but will form associated ion pairs with either ion. The proportion of ion pairs formed by Na^+ will depend on the Na^+ concentration relative to the K^+ concentration. To initiate operation of the 'sodium pump' a critical number of the protein anionic sites must be occupied by sodium ion pairs. Thus we propose to start with the concept that the operation of the 'pumping cycle' has an inherent feed-back control. The enzyme cycle is stimulated by raising Na^+ concentration and depressed as this concentration falls and the K^+ concentration rises. As a result of sufficient sodium ion pair formation, there is a redistribution of electron density throughout the macro-molecule, and this occurs as a result of both direct and indirect interaction between Na^+ and the protein.

As in the protein fixed-charge model developed by Ling[22], it is considered that the short-range interactions predominate in importance, and contribute in particular to the difference between the sodium and potassium protein ion pairs. The inductive effect of the cation on the fixed-charge system will be additive and hence the effect of the sodium ion will bear a relation to the number of fixed anionic sites occupied by sodium ions.

Because the protein will also contain surface orientated cationic groups it will also form associated ion pairs with anions at these sites. The interaction between anion and the protein will also effect the induction of charge by cation–anionic site ion pairs. Thus when sodium and potassium are present as fluoride salts *in vitro* the ATPase activity is virtually abolished[12]. In this model we imply that the highly electro-negative fluoride anion is capable of preventing the inductive changes produced by Na^+ adsorption and which are necessary to initiate operation of the enzyme.

The redistribution of electron density induced by sodium ion pair formation is focused in the neighbourhood of the cardinal sites, and, as a result, this centre now reacts more actively with the substrate (ATP) to form the phosphorylated intermediate (Fig. 1). This step corresponds to the biochemical reaction sequence:

$$\underset{\cdot\cdot}{\overset{Na}{E}} + ATP \rightleftharpoons \underset{\cdot\cdot}{\overset{Na}{E\sim P}} + ADP$$

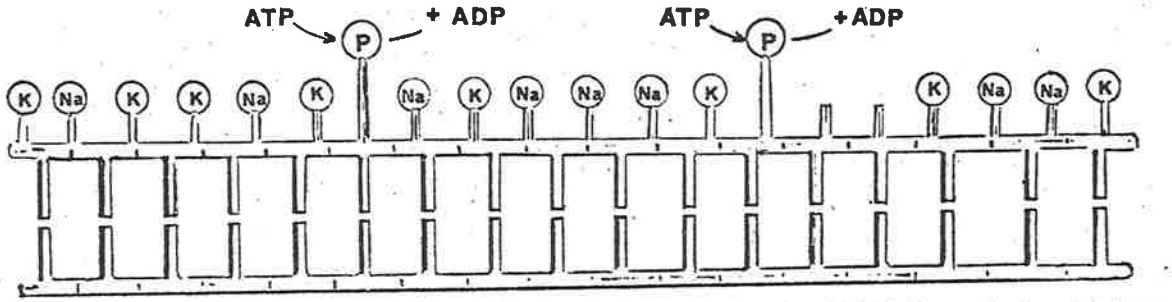


Fig. 1. Diagrammatic representation of a unit segment of membrane in which the horizontal sections depict the inner and outer protein layers, and the vertical broken units between the protein represent the central phospholipid complex described by Danielli. Projecting from the inner coil are shown the surface orientated anionic groups of the protein with which Na^+ and K^+ have formed associated ion pairs. The longer vertical projections represent the amino-acid groups which constitute the active centre of the enzyme. In this figure sufficient sodium ion pairs are considered to have formed to allow the active centre to react with ATP, and form the phosphorylated intermediate. Although the figure shows ATP reacting directly at the cardinal site it has long been established that magnesium ions are required for this reaction. Mg^{++} has been omitted from the figure for clarity

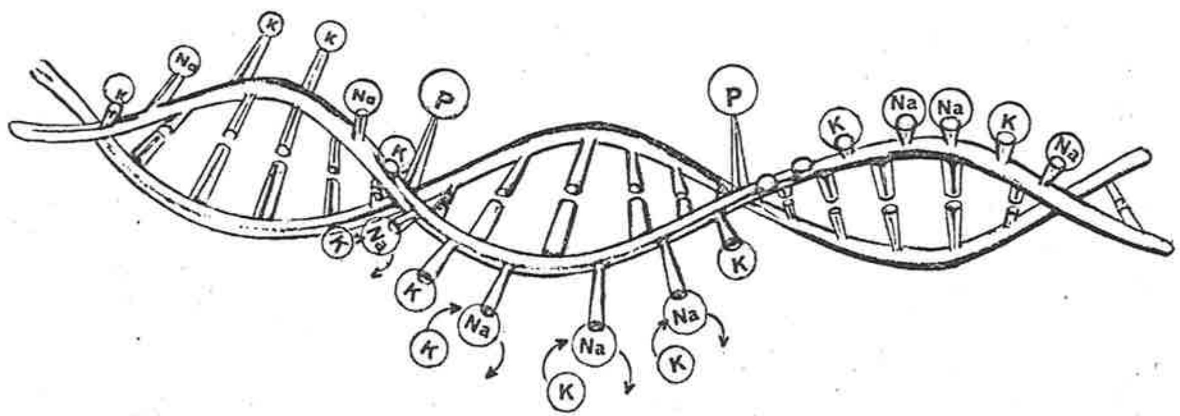


Fig. 2. Representation of the postulated effects of phosphorylation of the active centre of the enzyme. Elongation of the inner protein surface has produced rotation of these protein molecules about the centre of the lipoid core. The site of the phosphorylated active centre is shown as resisting this rotation. The surface orientated anionic groups with their associated ions have rotated outwards following the elongation and twisting of the protein molecule. K^+ for Na^+ exchange is now shown to be taking place at sites of Na^+ adsorption to these groups

As a result of the phosphorylation of the cardinal site, there is a further inductive effect, and a redistribution of electron density along the polarizable chain of the protein. This produces a folding or deformation of the protein as well as a change in the adsorption energy of the fixed anionic site.

The deformation of the enzyme results, in the model, in a rotation outwards of many of the fixed anionic sites, but leaves the phosphorylated site 'anchored' and still inward-facing (Fig. 2). There are many ways in which this deformation might produce such a change in orientation of the molecule. We consider there is evidence for believing that the whole membrane structure is concerned in the transport process, although not directly part of the enzyme.

It is interesting to note that a recent paper by Tanaka and Abood has suggested that lecithin is required to restore activity to this enzyme after treatment with deoxycholate[23]. We would interpret this finding as suggesting that the lipid is a necessary part of the enzyme system, although not involved in the chemical reaction itself.

The deformation suggested, therefore, implies unfolding or lengthening the inner enzyme protein chain with subsequent rotation of the membrane fragment about the central lipoid core. For a membrane unit of 1000 Å long and 60 Å wide, and assuming that the axis of rotation is in the centre of the lipoid core, a change in length of about 50 Å would be required to produce a single 2π spiral.

Because of the adsorption energy changes associated with this phosphorylation and subsequent enzyme deformation, a marked preference for K^+ is now exhibited by the cation binding groups, and the Na^+ at these sites is exchanged for K^+ (Fig. 2). The previous rotation of the membrane now implies that this exchange occurs extracellularly, hence Na^+ has been transported from the intracellular to the extracellular environment. The adsorption of K^+ on to these sites while the macromolecule is in this state of 'electron strain' again redistributes the electron density. In the region of the cardinal sites this alteration in electron distribution renders the phosphate groups more vulnerable to attack by water and cleavage of the phosphate bond occurs with the liberation of orthophosphate.

The liberation of phosphate from the phosphoprotein allows the molecule to return to its initial shape, and as a result the anionic binding sites with their adsorbed K^+ rotate inwards toward the cell centre. Within the cell some of these adsorbed K^+ ions are now displaced by Na^+ and the cycle of reaction begins again.

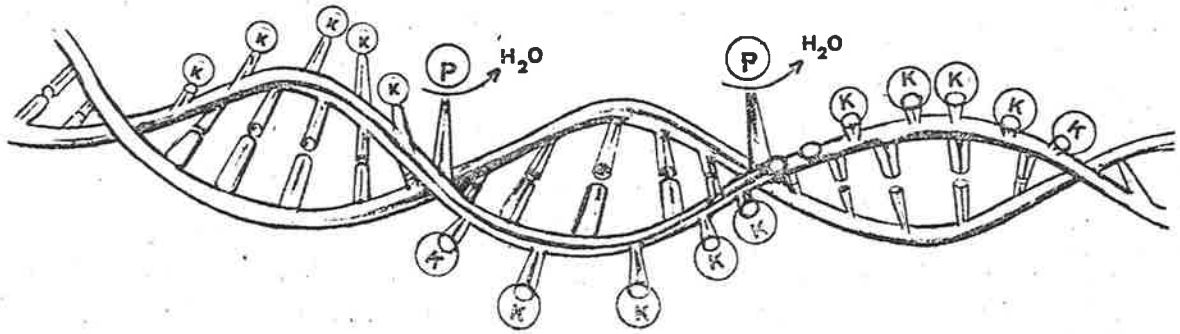


Fig. 3. Diagram showing that, following the K^+ for Na^+ exchange illustrated in Fig. 2, the inductive changes of electron density which take place in the elongated protein molecule lead to changes of field strength in the region of the phosphorylated active centre. As a result of these changes the phosphorylated centre is now readily attacked by water with cleavage of phosphate from the protein

These last hypothetical steps correspond with the second step of enzyme reaction sequence:

$$E \sim P + H_2O \xrightarrow{K^+} E + P_i \qquad (2)$$

Thus the cycle results in net transfer of Na^+ from inside the cell to the outside, coupled with the transport of K^+ in the opposite direction, during which process ATP is hydrolysed to ADP and orthophosphate.

Because there is evidence that the site of ouabain inhibition is at reaction (2) in the sequence given above[13], it seems unlikely that ouabain acts directly at the active centre of the ATPase enzyme. Ouabain does not prevent the formation of the phosphoprotein intermediate but appears to prevent its decomposition[13,14].

For the toxicity and action of cardiac glycosides on ion transport certain features of cardiac glycoside molecule appear essential[24,25]. These features include a *cis*-configuration of the *C–D* ring function, the presence of a β-hydroxy group at C_{14} and an unsaturated lactone ring at C_{17}. A conformational representation of ouabain is shown in Fig. 4. Although the orientation of the molecule will depend on the positions adopted by the lactone ring at C_{17} and the sugar moiety at C_3, as both groups are free to rotate, a conformation could exist where the ketone group and the β-hydroxy at C_{14} face away from the same surface of the ouabain molecule. When oriented in this position, these groups might form by hydrogen-bonding

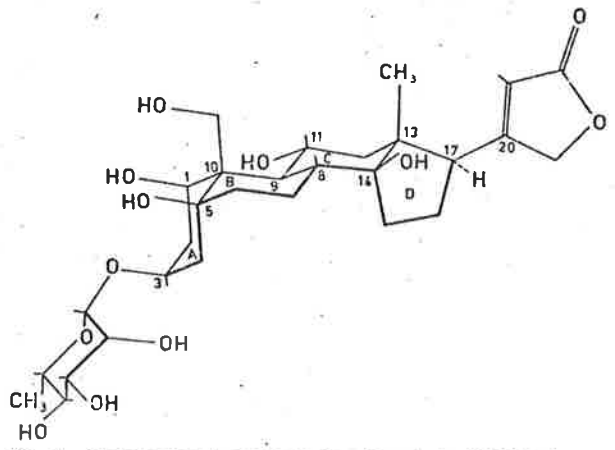


Fig. 4. Conformational representation of ouabain (3-O-β-L-rhamnopyranosyl-1β,3β,5,11α,14,19-hexahydroxy-5β-card 20 (22)-enolide). We are indebted to Dr. J. Mills, C.S.I.R.O. Division of Biochemistry and General Nutrition, Adelaide, for this interpretation of the structure. Rotation of the rhamnose molecule will produce a configuration in which the undersurface of the molecule is hydrophobic, and the upper surface contains projecting OH and =O groups. We envisage that these groups form hydrogen bonds with the extended surface of the inner membrane protein containing the ATPase

the primary attachment of the ouabain molecule to the surface of the ATPase enzyme.

In addition, a cluster of OH groups exist both on the *AB* rings of the aglycone and on the sugar moiety of ouabain. These groups could also form hydrogen-bonds with the protein enzyme surface. Attachment of ouabain to the protein enzyme by the separated clusters of OH-groups would act, in the model presented in this paper, by maintaining the deformed state of the membrane shown in Figs. 2 and 3. This interaction would prevent both release of phosphate from the active centre, and operation of the deformation–restoration cycle required to move Na^+ and K^+ ions in the directions required.

In the absence of direct experimental evidence, such an explanation for the mechanism of ouabain inhibition must remain entirely speculative.

The model presented in this article is an attempt to combine data about the '$Na^+ + K^+$ activated ATPase' into a consistent model which seeks to explain the means by which such an enzyme could produce vectorial Na^+ and K^+ transport.

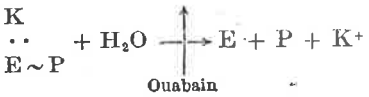
A useful model should be fragile, and ought to suggest the appropriate tests as to its own validity. We believe this model suggests at least two general questions about '$Na^+ + K^+$-activated ATPase' with respect to its capacity to transport these ions.

First, Do transition states of the enzyme exist with selective Na^+ or K^+ binding capacity ? The model, in fact, suggests that selective K^+ binding occurs when the enzyme is in the phosphorylated state (Fig. 3). Recently an answer to this question has been sought using a histochemical electron microscope technique. With this we have been able to show what is believed to be evidence of markedly increased Cs^+ binding by the enzyme membrane during the existence of the phosphoprotein transition state[18]. Because Cs^+ can replace K^+ completely in the enzyme reaction[2,6] we believe that this implies that K^+ binding will occur under the experimental conditions that demonstrated Cs^+ binding. As Cs^+ has a greater electron density than K^+ it is consequently easier to visualize using the electron microscope.

This evidence has necessitated the inclusion of a further step in the reaction sequence to account for a potassium for sodium exchange.

$$\begin{matrix} Na \\ \cdot\cdot \\ E \end{matrix} + ATP \rightleftharpoons \begin{matrix} Na \\ \cdot\cdot \\ E\sim P \end{matrix} + H_2O$$

$$\begin{matrix} Na \\ \cdot\cdot \\ E\sim P \end{matrix} + K^+ \rightleftharpoons \begin{matrix} K \\ \cdot\cdot \\ E\sim P \end{matrix} + Na^+$$



A second question which the model poses is: Does a measurable or detectable degree of molecular deformation occur during or following the formation of the phosphoprotein? One approach to the solution of this question requires direct biophysical examination by such techniques as X-ray diffraction, flow birefringence or light scattering. We have recently been able to show that the particulate enzyme gives a clear orientated X-ray diffraction pattern although as yet we have obtained insufficient resolution to determine the presence of significant deformation[26].

A more indirect approach would be to attempt an estimate of entropy change associated with the hydrolysis of ATP by this enzyme. The model predicts that the entropy values should be high for the ouabain-sensitive Na^+ and K^+ ATPase, and much less for the ouabain-insensitive ATPase which is always associated with our preparations.

Other workers interested in '$Na^+ + K^+$ activated ATPase' have speculated about the mechanism of ion transport by this enzyme. Some authors have considered that either ATP itself, or the phosphate group of the labile phosphoprotein, acts directly as the ion carrier[27]. However, most investigators have treated the enzyme as a polyanionic macromolecule the ion selectivity of which is determined by the turnover of labile phosphate groups[1,2]. Eisenmann[28] has evolved characteristics for such a selective ion binding system which is based on the field strength of the anionic site, and Ling[22] has developed this type of approach with considerable rigour as the basis of specialized protein function in general.

Working with the '$Na^+ + K^+$ activated ATPase' from the electric organ of the electrophoresis, Albers, Fahn and Koval[29] have suggested a model with spatial separation of three enzyme steps. Their model, however, suggests that Na^+ and K^+ movement is coupled with the translocation of phosphate from inside the cell, but fails to account, however, for the return of the phosphate to the cell.

All these models have sought to explain the changing selectivity of binding sites associated with the enzyme 'ion carriers', but none of them has attempted to include an explanation of the vectorial nature of the ion movement, or the highly selective inhibition of both the enzyme and ion transport by the cardiac glycosides. Although the model presented in this article will undoubtedly be proved to be inconsistent as new facts emerge, we believe that it will help to suggest the experiments necessary to uncover these new facts.

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THE EFFECT OF POTASSIUM AND SODIUM IONS ON RAT-LIVER MITOCHONDRIAL OXIDATIVE PHOSPHORYLATION

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(Received March 23rd, 1965)

SUMMARY

1. Measurement of oxidative phosphorylation in rat-liver mitochondria was performed using a platinum oxygen electrode and ADP as acceptor substance in the absence of added alkali metal ions.

2. Under these conditions both active respiration and high P/O ratios were observed with succinate and oxoglutarate as substrates (1.9 and 2.9 respectively).

3. The addition of Na^+ and K^+ either singly or in combination up to a concentration of 75 mM, had little effect on mitochondrial respiration or phosphorylation.

4. Intramitochondrial K^+ was also measured during active respiration and phosphorylation, and showed a considerable fall to about 25% of the initial value during 30 min incubation. Respiration and phosphate esterification were estimated during this period, and were found to be linear. Intramitochondrial Na^+ showed a similar but much less pronounced fall under these conditions.

5. It is concluded from these experiments that Na^+ or K^+ do not stimulate respiration or phosphorylation in rat-liver mitochondria.

INTRODUCTION

Although the stimulating effects of K^+ on the respiration of tissue slices and homogenates is well documented[1-3], the reason for this effect remains uncertain. An effect of the alkali metal ions directly on the mitochondrial process of oxidative phosphorylation was suggested by PRESSMAN AND LARDY[4,5] who claimed that K^+ were required for maximum stimulation of mitochondrial respiration. The discovery of an ATPase (ATP phosphohydrolase, EC 3.6.1.3) in membrane fragments which requires Na^+ and K^+ for activation[6-9] has led to further examination of the effect of alkali metal ions on mitochondrial respiration and phosphorylation.

A preliminary report from this laboratory did not show evidence of such a requirement[10] even when the intramitochondrial K^+ content had been significantly reduced by pretreatment with salicylate *in vitro*[11]. These findings have been extended in this paper.

METHODS

Mitochondria were freshly prepared from the livers of inbred laboratory rats by the procedure of CHARNOCK *et al.*[12] with the omission of KOH from the original homogenising solution. The medium used for measurement of oxidative phosphorylation was a Tris–phosphate buffer solution containing crystalline bovine serum albumin. The final concentration of reactants was 20 mM phosphate, 3 mM Mg^{2+} and 12 mM glucose. The albumin concentration was 1%. The substrates were added as Tris salts (α-oxo-glutaric acid and succinic acid) to give a final concentration of 10 mM. The final pH of the medium was between 6.8 and 6.9. ADP (Sigma) was used as phosphate acceptor in the experiments designed to measure oxidative phosphorylation, and was added in a small volume to provide either 1.0 or 1.5 μmoles ADP in the reaction cuvette per experiment.

Oxygen utilization was determined with a Clark platinum electrode immersed into a perspex cuvette of 4-ml capacity. The cathode was polarized at −0.7 V. The output from the electrode was amplified to drive a 10–100 mV potentiometric recorder and direct tracings were obtained over a 5-min period. The contents of the cuvette were mixed by rotation of a teflon coated bar driven by an external magnetic stirrer. The temperature of reaction was 25°. The reaction was initiated by the addition of 0.3 ml mitochondrial suspension in 0.25 M sucrose, which contained 10–15 mg mitochondrial protein. The solubility of oxygen in the medium was taken to be 0.48 μg-atoms/ml at 25° (ref. 13).

Determination of Na^+ and K^+ content of mitochondrial pellet

Measurement of mitochondrial Na^+ and K^+ content was made under conditions shown to support active metabolism. 0.4 ml of hexokinase (EC 2.7.1.1)–glucose mixture containing 240 K.M. units of hexokinase in 1% glucose, was added to 5 ml of the Tris–phosphate–protein buffer "alkali free" medium, in 20-ml flasks. Tris–ATP (Sigma) was added to give a final concentration of 2 mM. The reaction was started by the addition of 1 ml of mitochondrial suspension in 0.25 M sucrose and the flasks gently shaken in a water bath at 30°. The zero time values for electrolyte assay were obtained by immediately decanting the flask contents after addition of the mitochondria and separating the pellet by centrifuging for 3 min at 20 000 $\times$ g at 0°. Reaction time values were obtained by incubating for either 15 or 30 min after the addition of mitochondria and then separating the pellet as for the zero time assay.

After sedimenting the mitochondrial pellet the supernatant was removed and the tubes drained by inversion. The mitochondrial pellet was then dispersed in 5 ml of distilled water, and the Na^+ and K^+ content of the aqueous suspension of the pellet determined by flame photometry (Gallenkamp apparatus).

The water content of the separated pellet was determined gravimetrically on duplicate pellets after drying at 110° for 24 h. The matebolic activity of the mitochondria was checked by incubating under conditions identical to those used for the ion content assay, and measuring respiration manometrically and phosphate esterification chemically as previously described[12]. Mitochondrial protein was determined by the method of ROBINSON AND HOGDEN[14] as modified by ALDRIDGE[15]. All reagents were of the highest analytical grade available unless otherwise specified.

RESULTS

Fig. 1 shows the record of the respiration of rat-liver mitochondria during a typical experimental run. In the presence of substrate but with the absence of phosphate acceptor the respiration is slow, and on addition of ADP there is an abrupt onset of rapid respiration (State 3 of CHANCE AND WILLIAMS[13]). Following utilization of ADP there is sharp decline in respiratory rate (State 4), thus enabling a satisfactory calculation of P/O ratios as suggested by CHANCE AND WILLIAMS[13].

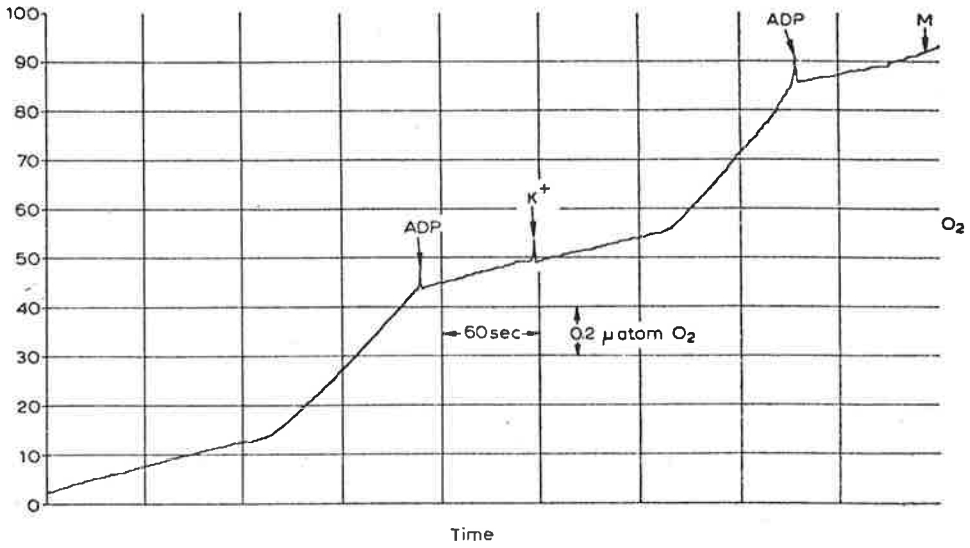

Fig. 1. A typical oxygen electrode record obtained when ADP was used to stimulate respiration in absence of added K^+ or Na^+. The medium contained 20 mM phosphate, 3 mM Mg^{2+}, 10 mM succinate. The temperature of reaction was 25°. Mitochondria added at M and 1 μmole ADP added where indicated. At K^+, KCl was added in a small volume (50 μl) to give a concentration of 25 mM K^+. The effect of further addition of ADP is shown.

Table I shows the results of a group of 5 experiments using Tris–succinate as the substrate. In the absence of added Na^+ and K^+, mitochondria exhibited good respiratory control and near theoretical P/O ratios were obtained. When Na^+ and K^+ were added either singly or together, there was no significant change in respiratory rates, respiratory control or P/O ratios. In Table II are set out the results of another group of experiments done separately to examine the effects of low concentrations of these ions on the respiration of mitochondria utilizing succinate as substrate. These concentrations were selected following the experiments reported by KRALL *et al.*[16] in which it was claimed that the stimulating effect of K^+ on brain mitochondrial respiration could only be demonstrated during the State 3 respiration and when the K^+ concentration was between 2 mM and 13 mM. Again the experiments reported in this paper could not demonstrate any significant effect of alkali metal ions, either singly or added together.

When Tris–α-oxoglutarate was used as substrate, good respiratory control and

TABLE I

THE EFFECT OF Na^+ AND K^+ ON OXIDATIVE PHOSPHORYLATION OF RAT-LIVER MITOCHONDRIA UTILIZING TRIS–SUCCINATE AS SUBSTRATE

The velocity of O_2 uptake measured polarographically using Tris–phosphate–albumin buffer system described in text, with 1.0 μmole ADP added as phosphate acceptor. Temperature of reaction was 25°. The rate of O_2 uptake given as μatoms/10 mg mitochondrial protein/min. Each value shown is mean of 5 experiments.

Concentration of added cation	*Respiration rate*			*P/O ratio*
	Before ADP	*During ADP utilization*	*Following ADP*	
None	0.08	0.41	0.13	1.8
25 mM K^+	0.08	0.42	0.13	1.7
75 mM K^+	0.08	0.40	0.12	1.7
25 mM Na^+	0.08			
75 mM Na^+	0.08	0.43	0.14	1.7
50 mM Na^+ + 25 mM K^+	0.08	0.42	0.13	1.8

P/O ratios near the theoretical maximum were again obtained from the liver mitochondria in the absence of added Na^+ or K^+ (see Table II and III). Concentrations of Na^+ or K^+ up to 25 mM were without significant effect on respiration, respiratory control or P/O ratios (see Fig. 1). With the addition of 75 mM Na^+ or K^+ there was a small reduction in the respiratory rate which occurred during utilization of ADP.

TABLE II

THE EFFECT OF Na^+ AND K^+ AT LOW CONCENTRATION ON OXIDATIVE PHOSPHORYLATION OF RAT-LIVER MITOCHONDRIA

The velocity of O_2 uptake measured polarographically using Tris–phosphate–albumin buffer system described in text. Tris-succinate or Tris–oxoglutarate used as substrate where indicated with ADP added as phosphate acceptor. Temperature of reaction was 25°. The rate of O_2 uptake given as μatoms/10 mg mitochondrial protein. Each value shown is mean of 4 experiments.

Concentration of added cation	*Substrate*	*Respiration rate*			*P/O ratio*
		Before ADP	*During ADP utilization*	*Following ADP*	
None		0.06	0.33	0.07	2.9
6 mM K^+		0.07	0.31	0.07	2.9
5 mM Na^+	Tris–oxo-glutarate	0.06	0.30	0.06	2.9
20 mM Na^+ + 5 mM K^+		0.07	0.31	0.07	2.8
None		0.09	0.41	0.09	1.9
6 mM K^+		0.09	0.40	0.08	1.9
5 mM Na^+	Tris–succinate	0.09	0.41	0.09	1.9
20 mM Na^+ + 5 mM K^+		0.09	0.39	0.09	1.9

TABLE III

THE EFFECT OF Na^+ AND K^+ ON OXIDATIVE PHOSPHORYLATION OF RAT-LIVER MITOCHONDRIA UTILIZING TRIS–OXO-GLUTARATE AS SUBSTRATE

The velocity of O_2 uptake measured polarographically using Tris–phosphate–albumin buffer system described in text, with 1.5 μmole ADP added as phosphate acceptor. Temperature of reaction was 25°. The rate of O_2 uptake given as μatoms/10 mg mitochondrial protein/min. Each value shown is mean of 5 experiments.

Concentration of added cation	*Respiration rate*			*P/O ratio*
	Before ADP	*During ADP utilization*	*Following ADP*	
None	0.07	0.36	0.13	2.7
25 mM K^+	0.08	0.36	0.14	2.6
75 mM K^+	0.07	0.28	0.13	2.5
25 mM Na^+	0.08	0.38	0.13	2.7
75 mM Na^+	0.08	0.28	0.13	2.5
50 mM Na^+ + 25 mM K^+	0.08	0.30	0.13	2.5

This inhibition of respiration was accompanied by a small reduction in P/O ratio which also occurred in the presence of both ions when the total added ionic concentration was 75 mM.

Intramitochondrial sodium and potassium content

Although the alkali metal free medium was used as a basis for examining the effect of added Na^+ and K^+, it was recognised that the mitochondria as prepared contain appreciable amounts of these ions[17,18]. It was therefore necessary to determine

TABLE IV

CHANGES IN MITOCHONDRIAL POTASSIUM, SODIUM AND WATER CONTENT IN ALKALI METAL FREE MEDIUM

Mitochondrial pellet incubated at 30° in Tris–phosphate–albumin system and recovered by centrifuging at 20 000 × *g* for 3 min. Cation assay by flame photometry following prolonged water leaching of the resuspended pellet (see text). 5 mM Na^+ concentration occurred when di-sodium ATP replaced Tris–ATP. Control refers to cation content of mitochondrial suspension in 0.25 M sucrose prior to addition to the incubation medium. Ion content, respiration rate and phosphate uptake are given as μg atoms/mg mitochondrial protein. Respiration rate in these experiments was measured manometrically and phosphate uptake by chemical assay of inorganic phosphate esterified.

Added cation	*Mitochondrial ion content*					*Phosphate uptake/min*
	Incubation time	*K^+*	*Na^+*	*% Water content*	*Respiration rate/min*	
None	Control	0.167	0.054	75		
None	Zero	0.061	0.058			
	15 min	0.032	0.035	78	0.060	0.105
	30 min	0.016	0.033	79	0.060	0.105
5 mM Na^+	Zero	0.075	0.061			
	15 min	0.039	0.049	77		
	31 min	0.017	0.049	81		

whether the mitochondrial Na^+ or K^+ content could be lowered without adversely effecting the efficiency of oxidative phosphorylation. This was studied by examining the Na^+ and K^+ content of the mitochondrial pellet at various times during incubation in the medium used for the metabolic studies.

The results are given in Table IV which demonstrates that there was a rapid efflux of K^+ from the mitochondria and imbibition of water following their addition to the alkali metal free Tris–phosphate–protein medium. During the next 30-min incubation there was a continuous efflux of K^+ throughout which time nearly linear respiration and phosphorylation occurred.

After 30-min incubation, the potassium loss was such that the concentration of this ion in the recovered pellet was only 25% of the control level determined at zero time. A similar but less pronounced loss of sodium occurred during 30-min incubation. The pellet water content also rose slightly during the incubation period. When a low concentration of sodium ion was present (5 mM as di-sodium ATP) similar losses of K^+ occurred with a decrease in the loss of Na^+. Measurement of oxidative phosphorylation during the period in which this K^+ efflux occurred showed active respiration and phosphorylation rates which were both linear with respect to the time of incubation.

DISCUSSION

When measurements of mitochondrial oxidative phosphorylation were made using a platinum electrode and utilizing ADP as phosphate acceptor, no increase in respiration rate, respiratory control of P/O ratio could be observed upon the addition of K^+ in any concentration to the incubation system. This finding confirms an earlier report from this laboratory of data obtained by conventional Warburg manometry[10]. However, the previously observed inhibition of mitochondrial respiration and phosphorylation by high concentrations of Na^+ was no longer apparent when a platinum electrode system was employed. Both our earlier manometric data and the results of this study do not support the observations of PRESSMAN AND LARDY[5] that K^+ are required for maximal respiration of rat-liver mitochondria.

UTIDA AND SUGAWARA[19] and KRALL and co-workers[16] have also concluded that K^+ stimulated respiration of brain mitochondria. However, it is generally accepted that the preparation of cerebral mitochondria completely free from nuclear, membrane or microsomal contaminants, is difficult to achieve with brain tissue. It is now known that all these contaminants contain active ATP hydrolysing enzyme systems which are greatly stimulated by Na^+ and K^+ (refs. 8,20), and which could influence the respiration of mitochondria by increasing greatly the rate of ADP regenerated from ATP. The brain is a particularly rich source of this enzyme system[21–13].

Recently a paper by BLOND AND WHITTAM[24] has reported stimulation of kidney-cortex mitochondria by concentrations of K^+ up to 30 mM. These authors also claimed that Na^+ was without effect on the respiration. A study of the data presented in the paper of BLOND AND WHITTAM[24] however, strongly suggest the opposite conclusion. The data clearly shows that maximum respiratory rate of the mitochondria occurred in the absence of added potassium ions, and in the presence of 152.5 mM choline chloride. When the choline was replaced with an equimolar concentration of sodium there was a 40% inhibition of the maximal respiratory rate. These measurements were

made in the presence of ATP but without a glucose–hexokinase–phosphate acceptor system, and are similar to the findings reported in our previous paper[10].

The earlier data of PRESSMAN AND LARDY[4] also demonstrated inhibition of mitochondrial respiration with increasing Na^+ concentration. The discrepancy between these results and the data reported in this paper suggests that some cation effects seen under the experimental conditions of conventional manometry are an artefact due to interaction of cations and the ATP–hexokinase–glucose reaction. MELCHIOR AND MELCHIOR[25] have shown that yeast hexokinase is inhibited by Na^+. In addition SIEKEWITZ[26] has concluded from a study of the site of action of hexokinase when used in this mitochondrial system, that the hexokinase–ATP interaction takes place at the mitochondrial surface. It is tempting to speculate that the inhibitory action of Na^+ might also be at this surface, rather than on intramitochondrial reactions.

In the experiments using the oxygen electrode reported in this paper, the only significant effect of either Na^+ or K^+ on oxidative phosphorylation was 20% inhibition of the maximal respiration (State 3 of CHANCE AND WILLIAMS[13]) and a small fall in P/O ratio when α-oxoglutaric acid was substrate. This inhibition occurred with the highest concentration of ions used and was independant of the nature of the ion. The addition of a solution of ions to a medium however, not only provides specific ionic components, but may produce separate effects resulting from alteration of the tonicity of the solution. Mitochondrial respiration and phosphorylation are known to be susceptible to changes in tonicity[27,28]. SLATER AND CLELAND[29] have also shown that both α-oxoglutaric oxidase and the succinoxidase systems are sensitive to such changes.

In a detailed study of the action of alkali metal ions on mitochondrial ATPase, ULRICH[30] concluded that both sodium and potassium ions were, in increasing concentration, inhibitory to this "enzyme". It is believed that the inhibition of "State 3" respiration seen in this study is compatible with such a non-specific effect, and implies that the substrate-linked phosphorylation occurring with oxidation of α-oxoglutaric acid, is the most sensitive to this inhibition.

Although the medium used in this study is substantially free of Na^+ and K^+ (ref. 10), it is well known that mitochondria contain appreciable amounts of K^+ (refs. 17,18). As it has been claimed that these organelles can accumulate or selectively retain this ion[31,32] it was necessary to study the effect of a reduction of intra-mitochondrial K^+ on oxidative phosphorylation. A direct method for studying this showed that throughout a 30-min period of linear respiration and phosphate esterification in an alkali metal free medium, there was a continuous loss of both potassium and sodium from the mitochondrial pellet. After 30-min incubation the mitochondrial K^+ content had fallen to about 25% of the initial level. Previously we have reported that the 60% loss of mitochondrial K^+ which followed exposure to salicylate[10,11] was without significant effect on the efficiency of rat liver mitochondrial oxidative phosphorylation. These findings suggest that oxidative phosphorylation in these organelles is insensitive to the concentration of K^+.

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STUDIES OF THE MECHANISM OF CATION TRANSPORT

II. A PHOSPHORYLATED INTERMEDIATE IN THE CATION STIMULATED ENZYMIC HYDROLYSIS OF ADENOSINE TRIPHOSPHATE

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SUMMARY.

Membrane-bound cation stimulated adenosine-triphosphatase is thought to be closely associated with active ion transport phenomena. As this enzyme system requires the combination of both sodium and potassium ions for full activation its mechanism of action has been investigated under these conditions. Using adenosine triphosphate labelled with P^{32} only in the terminal position as substrate for the reaction we have been able to show that ATP^{32} is hydrolysed to adenosine diphosphate and radioactive orthophosphate via a phosphorylated intermediate. Sodium but not potassium ions are required for the formation of this intermediate and adenosine diphosphate; potassium ions are subsequently required for dephosphorylation of the intermediate complex with the release of P^{32} orthophosphate — that is, both sodium and potassium ions are required for the turnover of the intermediate in a two step reaction.

The cardiac glycoside ouabain (Strophanthin-G) is without effect upon the formation of this intermediate complex, but can completely inhibit the K^+ — requiring dephosphorylation reaction. Preliminary experiments indicate that sodium ions are bound to the enzyme system during the phosphorylation reaction and are released when this complex is broken down. Solvent extraction procedures designed to remove phospholipid components did not decrease the specific activity of the phosphorylated intermediate which suggests that it is phosphoprotein in nature.

These results support the belief that $Na^+ + K^+$ activated ATPase participates in active cation transport in a physical as well as chemical sense, and a possible biochemical mechanism of active cation transport is suggested.

INTRODUCTION.

The probable participation of membrane adenosine-triphosphatase activity in active cation transport has received widespread support from the recent investigations of Skou (1960), Post and Kinsolving *et al.* (1960), Whittam (1962), Bonting, Caravaggio and Hawkins (1962) and Charnock and Post (1963a, b). However, in a recent review of the function of cell membranes Burgen (1962) has pointed out that the evidence to date does not distinguish

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between the obvious rôle of this enzyme in the supply of energy for a metabolically dependent process and its possible physical participation in the transport of cations.

Skou (1960) has proposed a mechanism in which a phosphorylated enzyme complex is postulated to act as the cation carrier but little definitive evidence is available. In a previous report from this laboratory (Charnock and Post, 1963b) we have described the preparation and properties of an adenosine-triphosphatase obtained by differential centrifugation of a guinea pig kidney cortex homogenate.

Enzyme activity was associated with a "heavy" microsome fraction (35,000 x g) thought to arise from membranous fragments of the endoplasmic reticulum (Siekevitz and Palade, 1958; Hanzon and Toschi, 1959; deRobertis *et al.*, 1962; Landon and Norris, 1963).

The cation stimulated hydrolysis of adenosine triphosphate (ATP) is completely inhibited by low concentrations of the cardiac glycoside ouabain (Strophanthin-G) which has been repeatedly shown by others to inhibit active cation transport in a variety of systems (Schatzmann, 1953; Yoshida, Nukada and Fujisawa, 1961; Taylor, 1962; Whittam, 1962; Wheeler and Whittam, 1962).

In this paper we describe experiments designed to provide further evidence for the existence of a phosphorylated intermediate in the mechanism of enzyme action and its possible rôle as an ion-carrier. By observing the effect of cation environment on the enzymatic hydrolysis of P^{32} labelled adenosine triphosphate (ATP^{32}) in short-term experiments, we have been able to demonstrate the rapid formation of a phosphorylated intermediate complex labelled with P^{32}. This complex requires the presence of sodium ions for its formation and probably binds these ions whilst in the phosphorylated state. Potassium ions are not required in the phosphorylation reaction leading to the formation of the intermediate complex, but as recently suggested by Judah and Ahmed (1963), are required for the dephosphorylation and turnover of the intermediate. On the basis of these experiments a biochemical mechanism has been proposed which suggests a physical rôle for the phosphorylated intermediate compound as the carrier of sodium ions in active cation transport.

Materials and Methods.

The heavy microsome subfraction of guinea pig kidney cortex was prepared from tissue homogenates by the method described previously (Charnock and Post, 1963b). After treatment with 0·1 p.c. sodium deoxycholate the preparation possessed a high specific activity of adenosine-triphosphatase which required 80mM Na+ combined with 20mM K+ for over 90 p.c. of its total activity, i.e. high cation sensitivity also. The activity of individual enzyme preparations was determined as the μmoles inorganic phosphorus released from ATP per mg. protein nitrogen per hour under standard conditions (Charnock and Post, 1963b).

Adenosine triphosphate labelled with P^{32} only in the terminal position was prepared from orthophosphate P^{32} (Oak Ridge National Laboratory, U.S.A.) and ATP (Sigma) by

the exchange reaction described by Pfleiderer (1961), except that the final separation of the labelled adenine-nucleotide (ATP^{32}) was carried out by passage down a column of Dowex-1 in the Tris form.

One ml. of enzyme suspension (which usually contained about 0·5 mg. protein N/ml.) was incubated in the following system: 1 ml. of Tris-phosphate buffer at pH 7·6 (35mM Tris-15mM H_3PO_4) and sufficient substrate (Mg. ATP^{32}) so that no more than 50 p.c. substrate utilization occurred during the incubation period. Obviously the quantity of substrate varied with the specific activity of each individual enzyme preparation and with the duration of each experiment. Usually from 0·2-0·5 μmoles Mg. ATP^{32} was employed. To this system were added various combinations (indicated in the text) of sodium and potassium ions and ouabain. These were added in small volume so that their final concentrations would be 80mM, 20mM and 0·1mM respectively. The final volume of the incubation system was adjusted to 2·5 mls. (without enzyme) and the reaction initiated by the rapid addition of 1 ml. of enzyme suspension. Previous temperature equilibration of both the incubation system and the enzyme suspension was essential. Incubation was conducted at 37° C. for from five to forty seconds as indicated in the text.

Tris-phosphate buffer was used to provide a pool of non-labelled phosphate ions sufficiently large to dilute the specific activity of any inorganic phosphorus (liberated by enzymatic hydrolysis of ATP^{32}) to such an extent that "indirect" incorporation of P^{32} from this source would be negligible. In preliminary experiments the use of this buffer system was found to decrease the rate of hydrolysis of ATP by these enzyme preparations to 70 p.c. of that observed in imidazole-glycyglycine buffer of the same pH. The reaction was stopped by the addition of 10 ml. of ice-cold 10 p.c. trichloracetic acid (TCA), and the protein precipitated by centrifuging for 20 mins. in the cold at 1,000 x g.

The precipitated protein was completely resuspended in 10 mls. of ice-cold 2 p.c. TCA by stirring with a glass rod and washed by resedimenting in the original centrifuge tube at 1,000 x g. The washing procedure was repeated for a total of five times, after which negligible radioactivity could be detected in the supernatant wash fluid.

The original incubation medium which contained much radioactivity was assayed for total counts and orthophosphate P^{32} following the charcoal absorption of nucleotide phosphates by the method of Crane (1958). This procedure provided a determination of the p.c. hydrolysis of ATP^{32} during incubation.

The final washed protein precipitate was redissolved in 2 ml. of Lowry reagent (2 p.c. Na_2CO_3 in 0·1 N NaOH). A small portion (0·1 ml.) was taken for protein assay by the method of Lowry, Rosenbrough, Farr and Randall (1951) using the albumin reference standard referred to previously (Charnock and Post, 1963b) and a suitably larger aliquot (usually 1 ml.) was plated for direct P^{32} counting employing a Nuclear Chicago Gas Flow apparatus. The incorporation of P^{32} into enzyme protein was expressed as cpm P^{32}/mg. protein nitrogen. Total phospholipid extraction of the TCA precipitated protein was performed by the ethanol-chloroform procedure described by Hokin and Hokin (1958). The chloroform phase (phospholipids) could be completely separated from the particulate residue and the distribution of total radioactivity determined (see text). The separated residual material was then heated with 5·7 N-hydrochloric acid for 50 min. at 100° C. and examined by electrophoresis and autoradiography by a modification of the method of Naughton, Sanger, Hartley and Shaw (1960).

RESULTS.

Initially experiments were performed to determine whether "direct" incorporation of P^{32} from ATP^{32} into the adenosine-triphosphatase system occurred during the enzymatic hydrolysis of ATP^{32}; and whether this could be related to

enzyme activity. The degree of "non-enzymatic" P^{32} incorporation was controlled by simultaneous use of similar incubation and assay systems which contain heat denatured enzyme suspensions (105° for 30 mins.) shown to possess no ATPase activity.

The results of five separate experiments using different enzyme preparations (incubated for 5-20 seconds) are given in Table 1. It can be seen that with each preparation examined there was a considerable increase in P^{32} incorporation into the microsomal material associated with enzyme activity. These systems uniformly contained no added cation other than magnesium ions (added as Mg-ATP^{32}).

TABLE 1.

The effect of enzyme activity on the incorporation of P^{32} into a microsomal adenosine-triphosphatase preparation from guinea-pig kidney cortex.

Experiment	cpm P^{32}/mg. protein N		Increase (%)	%ATP^{32} hydrolysis
	Denatured	Active		
i	600	1,300	216	1
ii	600	1,600	267	3
iii	1,000	5,600	560	6
iv	1,400	2,300	164	7
v	3,900	14,300	367	32

Incubation conditions described in text. No sodium or potassium ions added; Mg-ATP^{32} = 0·02mM

The effect of cations on P^{32} incorporation was examined by the separate addition of either sodium or potassium ions to the incubation system, in concentrations previously shown to produce maximal rates of ATPase activity when added in combination (Charnock and Post, 1963b).

The effect of 80M Na^+ and 0·1M ouabain.

In nine experiments the mean incorporation of P^{32} into the adenosine-triphosphatase enzyme system was found to be significantly increased ($P < 0·05$) in the presence of 80mM Na^+ (cf. Table 2). This increase was associated with a mean increase of 12 p.c. in the enzymic hydrolysis of ATP^{32} when sodium ions were present.

The effect of ouabain (0·1mM) on this action of sodium ions was examined in four further experiments using other enzyme suspensions and with the "control" conditions now containing 80mM Na^+. No significant differences could be seen between the systems with and without ouabain. These results are given in Table 2.

The effect of 20mM K^+ and 0·1mM ouabain.

In seven experiments the mean incorporation of P^{32} into the adenosine-triphosphatase enzyme system was found to be not significantly changed by the

TABLE 2.

The effect of 80mM Na^+ and 0·1mM ouabain on the incorporation of P^{32} into a microsomal ATPase system.

Experiment	cpm P^{32}/mg. protein N		% ATP^{32} hydrolysis	Experiment	cpm P^{32}/mg. protein N		% ATP^{32} hydrolysis
	control	Na^+			Na^+	80mM Na^+ + ouabain	
a	4,800	6,900	3	—			
b	8,400	26,800	24				
c	14,300	74,000	21				
d	6,600	21,200	18				
e	8,700	35,000	24				
f	12,200	28,400	16	j	7,000	5,400	−6
g	3,100	6,300	3	k	53,000	38,000	−2
h	2,600	8,500	1	l	15,400	13,600	−23
i	26,600	52,300	1	m	4,800	4,000	−3
Mean	9,700	28,820	12	Mean	20,050	15,250	−8
P*	<0·05			P	n.s.		

Incubation conditions described in the text. P=probability. n.s.=not significant. *Using "t" test.

presence of 20mM K ions in the incubation system although there was a mean decrease of 8 p.c. in the enzymic hydrolysis of ATP^{32} when this ion was added alone. The further addition of ouabain (0·1mM) to this system was also without significant effect. These results are shown in Table 3.

TABLE 3.

The effect of 20mM K^+ and 0·1mM ouabain on the incorporation of P^{32} into a microsomal ATPase system.

Experiment	cpm P^{32}/mg. protein N		% ATP^{32} hydrolysis	Experiment	cpm P^{52}/mg. protein N		% ATP^{32} hydrolysis
	Control	K^+			K^+	K^+ + ouabain	
n	4,100	3,500	0	—			
o	2,000	1,900	0	—			
p	2,600	4,000	0	—			
q	3,100	2,800	+1	u	4,300	3,000	−1
r	7,800	7,100	−4	v	14,500	14,100	−1
s	6,600	7,000	−22	w	12,900	11,700	−5
t	14,300	15,500	−28	x	4,300	4,000	0
Mean	5,780	5,970	−14	Mean	9,000	8,200	−2
P	n.s.			P	n.s.		

Incubation conditions described in text. P=probability; n.s.=not significant.

The effect of 20mM K^+ and 0·1mM ouabain in the presence of 80mM Na^+.

Table 4 gives data derived from six experiments measuring the incorporation of P^{32} into enzymatically active microsomal material (ATPase). Different enzyme preparations and ATP^{32} of widely different specific activity were used in each of these separate experiments. The mean incorporation of P^{32} is given under three different conditions of incubation. It can be seen that in the presence of 80mM Na^+ only, there is much greater incorporation of P^{32} from ATP^{32} than in either of the other groups (mean P^{32} incorporation = 28,550 cpm/mg. protein N).

When 20mM potassium ions were also present, the mean P^{32} incorporation decreased to 8,060 cpm/mg. protein N. (The mean difference between these levels just fails to reach significance at the 5 p.c. level; $P < 0{\cdot}06$ cf. Table 4.) This analysis is influenced by the wide variation introduced into the control group by the use of ATP^{32} preparations of greatly different specific activity. Under these experimental conditions there was a simultaneous increase in the hydrolysis of ATP^{32} from 24 p.c. with only Na^+ present to 42 p.c. when the incubation system contained both sodium and potassium ions.

When 0·1 mM ouabain and 20mM K^+ were added to a system already containing 80mM Na^+ a level of P^{32} incorporation was obtained which was intermediate between that found with either Na^+ alone or $Na^+ + K^+$ (mean P^{32} incorporation = 16,200 cpm/mg. protein N). The presence of ouabain (0·1mM) in the system prevented further hydrolysis of ATP^{32}.

TABLE 4.

The effect of 20mM K^+ and 0·1mM Ouabain on the incorporation of P^{32} into a microsomal ATPase system, in the presence of 80mM Na^+.

Experiment	Na^+		$Na^+ + K^+$		$Na^+ + K^+$ + ouabain	
	cpm P^{32}/mg. protein N	% ATP^{32} hydrolysis	cpm P^{32}/mg. protein N	% ATP^{32} hydrolysis	cpm P^{32}/mg. protein N	% ATP^{32} hydrolysis
vi	15,400	—	10,900	—	14,000	—
vii	14,800	—	3,900	—	14,900	—
viii	74,000	71	7,300	90	24,400	88
ix	6,300	11	3,400	37	5,800	43
x	8,500	5	5,300	29	7,600	28
xi	52,300	8	17,700	12	30,500	15
Mean P^{32} incorporation	28,550	24	8,060	42	16,200	43
P			0·06*		n.s.**	

* Probability compared with Na^+ using paired differences.

** Probability compared with $Na^+ + K^+$ using paired differences.

Sequential addition of cations and ouabain to incubation system.

It should be noted that the data presented in Tables 1-4 were obtained from experiments in which enzyme was added to incubation systems already

containing all other components of the system, and in which all groups were incubated for equal times.

To learn more of the mechanism of the above effects experiments were now conducted in which cation sensitive adenosine-triphosphatase preparations were incubated in systems initially lacking in various components which were later added in sequence, and at timed intervals so that step-wise observations could be made.

The mean result of four experiments is given in Fig. 1 which shows the change in P^{32} incorporation into enzymatically active microsomal material with changing incubation conditions and the progressive hydrolysis of ATP^{32} under these conditions.

At zero time, the enzyme suspension was added and the reaction stopped immediately. This procedure provides the control value, for both P^{32} incorporation and p.c. ATP^{32} hydrolysis.

Within five seconds the subsequent addition of 80mM Na ions had produced a threefold increase in P^{32} incorporation (which was not obtained if 20mM K ions replaced sodium ions). However, when potassium ions were then added to the system already containing sodium ions, the nett P^{32} incorporation decreased with time to an apparently constant level only a little above the initial control values.

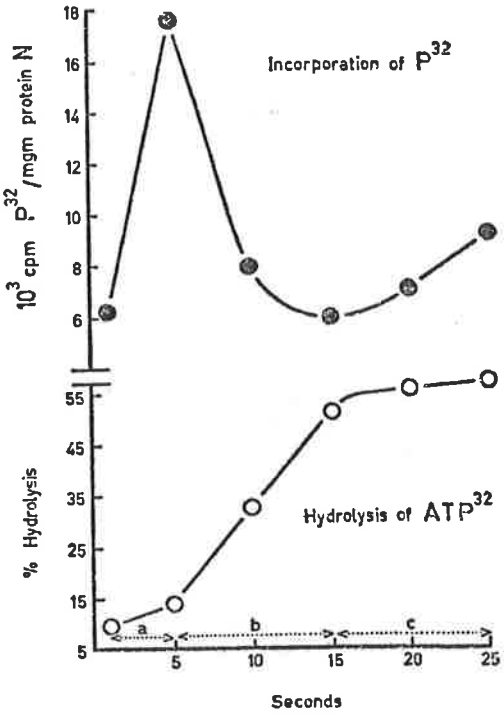

Fig. 1. The incorporation of P^{32} from ATP^{32} into microsomal adenosine-triphosphatase protein and the hydrolysis of ATP^{32} following the sequential addition of 80mM Na^+, 20mM K^+ and 0·1mM ouabain. Period (a) with sodium ions only; (b) sodium and potassium ions; (c) sodium and potassium ions plus ouabain.

●—● Incorporation of P^{32} (cpm $P^{32} \times 10^3$/mg. protein nitrogen).

○—○ P.c. total ATP^{32} hydrolysis measured as appearance of inorganic P^{32}.

Each point represents the mean of four experiments.

At the same time the rate of ATP^{32} hydrolysis increased above that seen in the presence of only added sodium ions. When 0·1mM ouabain was now introduced into the system there was again a sharp increase in P^{32} incorporation and at the same time further ATP^{32} hydrolysis was markedly inhibited.

Binding of Na^{24} to the adenosine-triphosphatase system.

Preliminary experiments using Na^{22} and non-radioactive ATP had indicated that there was an ATP-dependent binding of sodium ions to enzymatically active material, which was not influenced by ouabain but which was reduced in the presence of potassium ions (Charnock and Post, 1963a). This result has been confirmed here in five separate experiments (Table 5) using Na^{24} and agrees with the earlier observation of Jarnefelt (1961) that sodium ions are bound to brain microsomes in an ATP requiring reaction. The addition of ouabain to a system containing potassium ions did not significantly alter the degree of sodium binding.

Preliminary identification of the phosphorylated intermediate complex.

Following incubation with ATP^{32} the reaction is stopped by the addition of cold 10 p.c. TCA and the precipitate extensively washed (5 x 10 mls.) with cold 2 p.c. TCA. This provides radioactively labelled material suitable for the chloroform-ethanol extraction procedure for total phospholipids (Brossard and Quastel, 1963; Hokin and Hokin, 1958). Aliquots of the resultant chloroform phase (total phospholipids) were plated and examined for radioactivity which in every experiment did not exceed 3 p.c. of the total counts introduced into the system. Neither could a significant portion of the total counts be demonstrated in the ethanol phase. Conversely the extracted residue which forms as a distinct layer between the solvent phases could be removed intact and counted directly; this residue always retained more than 85 p.c. of the original total counts. This strongly suggests that the P^{32} is not present in phospholipid (Hokin and Hokin, 1960) but in the fraction usually classified as phosphoprotein (Rose, 1963). Preliminary studies of the phosphorylated product (Atkinson and Charnock, unpublished results) indicate that the binding of the

TABLE 5

The binding of Na^{24} to microsomal ATPase system.

System	Addition	Counts Na^{24}/100 sec/mg. dry weight microsomes
− ATP	none	1,360
+ ATP	none	9,955
+ ATP	ouabain	8,885
+ ATP	K^+	4,785
+ ATP	K^+ + ouabain	4,825

All tubes contained 1 ml. of enzyme suspension and 10mM Na^{24} of varying specific activity. When added, potassium ions were 10mM and ouabain was at a final concentration of 0·1mM.

Incubation was at 23° C. for 20 seconds.

The reaction was stopped and the precipitate washed with 96% ethanol.

Each value is the mean of five experiments.

phosphate differs from that in phosphorylated phosphoglucomutase (Milstein and Sanger, 1961) and phosphorylated alkaline phosphatase (Milstein, 1963) which are very resistant to acid, as much of the radioactivity of these P^{32}-enzymes is present as phosphoserine and phosphoserine-peptides after 30-90 minutes treatment at 100° C. in 5·7N-hydrochloric acid. In contrast to this, in our experiments, orthophosphate was the only radioactive product detected by electrophoresis and autoradiography (cf. Naughton, Sanger, Hartley and Shaw, 1960) after hydrolysis of the P^{32}-ATPase precipitate for 50 mins. at 100° C. in 5·7N-hydrochloric acid. Therefore it is unlikely that the phosphate is present in a phosphoserine residue although steric factors might increase the acid-lability to this extent.

The radioactive product is also unlikely to be a phosphohistidine derivative since these compounds are hydrolysed by the cold trichloracetic acid employed in the washing procedure (Suelter, DeLuca, Peter and Boyer, 1961). If the ^{32}P-phosphoryl group is covalently bound to protein it may be attached to a tyrosyl or cysteyl residue, but bound P^{32}-ATP, P^{32}-polyphosphate or P^{32}-orthophosphate in the precipitate would also be converted into free P^{32}-orthophosphoprotein on heating in acid. Although experiments using 8-C^{14}-ATP as substrate have not yet given any evidence that ATP is bound to the microsomal enzyme, it cannot be concluded at present that the radioactive product is a phosphoprotein in the conventional sense of a covalent phosphorylated protein.

DISCUSSION.

The experiments described above have shown that the terminal phosphorus of ATP^{32} can be directly incorporated into the protein moiety of an adenosine-triphosphatase preparation obtained from the heavy microsome fraction of guinea pig kidney cortex. This fraction has been well documented by others as arising from the endoplasmic reticulum of cells and being membranous in nature (Siekevitz and Palade, 1958; Hanzen and Toschi, 1959; deRobertis *et al.*, 1962; Landon and Norris, 1963; Schwartz, 1963).

During the enzymic hydrolysis of ATP^{32} there is a large increase in direct P^{32} incorporation into this material. This process is greatly stimulated by the presence of sodium but not potassium ions, and is not significantly altered by the addition of ouabain to the system. If the experimental conditions are so arranged that the sodium stimulated incorporation of P^{32} has already occurred, then the subsequent addition of potassium ions leads to a decrease in P^{32} incorporation. At the same time, under the combined influence of both Na and K ions, enzymic hydrolysis of ATP^{32} is increased in accordance with the previous findings of the combined cation requirement of this enzyme system for full activation (Charnock and Post, 1963b). If in studies employing the sequential addition of agents to an enzymically active system, ouabain is added subsequent

to the addition of cations, increased P^{32} incorporation occurs concurrently with inhibition of ATPase activity.

A simple explanation of these findings can be presented in the following hypothesis. Initially Na ions are required for the introduction of P^{32} directly into a component of the enzyme system, i.e. Na^+ promotes the phosphorylation of an intermediate complex during the enzymic hydrolysis of ATP^{32}. Little inorganic P^{32} appears at this stage and this amount is probably induced by a small quantity of K^+ in the incubation system as a contaminant. When K^+ is available, the turnover the phosphorylated intermediate is possible, leading to the cation stimulated hydrolysis of ATP^{32} with the liberation of orthophosphate P^{32}, resulting in a reduction in the nett P^{32} incorporation into the system. That is Na^+ ions induce the phosphorylation of an intermediate and K^+ ions subsequently allow its phosphorolysis. Such a mechanism has already been suggested by Ahmed and Judah (1962) and Judah and Ahmed (1963). Only the K^+ dependent phosphorolysis reaction is inhibited by ouabain, and this block results in the accumulation of the phosphorylated intermediate.

Preliminary experiments suggest that sodium ions are bound to some site on the enzyme at the same time as it becomes phosphorylated, and that in the presence of K^+ ions the sodium is released from these sites (possibly simultaneously with the degree of phosphorolysis). The present experiments do not indicate whether K^+ actually replaces Na^+ on the enzyme and is in turn bound to site or sites on the enzyme complex but such a possibility seems probable.

The following scheme represents a working hypothesis based on the above explanation:

$$(1)\ \text{Enzyme} + ATP^{32} \xrightarrow{{}^{22}Na^+} (\text{Enzyme} \sim P^{32})^{22}Na + ADP$$

$$(2)\ (\text{Enzyme} \sim P^{32})^{22}Na \xrightarrow{K^+} \text{Enzyme} + P_i^{32} + {}^{22}Na + K$$

Site of ouabain inhibition

This mechanism of action involves a phosphorylated-intermediate as first suggested by the tracer exchange experiments of Skou (1960). Although Hokin and Hokin (1960, 1963a, b) have produced evidence for phosphatidic acid as the carrier substance to which sodium ions are bound during cation transport, this suggestion has not been supported by observations in many laboratories (Jarnefelt, 1961; Kanfer and Titus, 1961; Ahmed and Judah, 1962; Heald, 1962; Post and Rosenthal, 1962; Judah and Ahmed, 1963), and is not in accord with the findings reported here or in the recent work of Rose (1963) who also worked with a cation-sensitive ATPase system (obtained from cerebral cortex).

Following the mechano-enzyme concept of Koshland (1960) and the evidence of Whittam (1962) that in the erythrocyte membrane this ATPase system is oriented in an asymmetric manner so that the sites activated by potas-

sium are external to the cell, whilst those activated by sodium are located inside the cell, it can be readily seen that the protein structure of the adenosine-triphosphatase enzyme whilst an integral part of a membrane structure, could itself be regarded as a spatially oriented physical carrier of sodium and potassium ions.

If this is the case, then the phosphorylated intermediate compound described above could now be considered to be the long-sought-after ion carrier.

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Evidence of the Mechanism of Ouabain Inhibition of Cation Activated Adenosine Triphosphatase

OUABAIN has been shown to inhibit electrolyte transport in a number of tissues, including erythrocytes[1,2], brain and kidney[3,4]. Ouabain also inhibits that adenosine triphosphatase obtained from membrane and sub-cellular preparations which is stimulated by sodium plus potassium ions[5–7]. Recent work has shown that this enzyme must play an important part in the mechanism of active ion transport[7–10].

We have been able to demonstrate[11] that a heavy microsome sub-fraction of guinea pig kidney cortex sedimented at 40,000*g* from a tissue homogenate in 0·25 M sucrose and afterwards treated with deoxycholate by a procedure modified from Skou[8] contains in high yield a sodium plus potassium-activated adenosine triphosphatase reaction

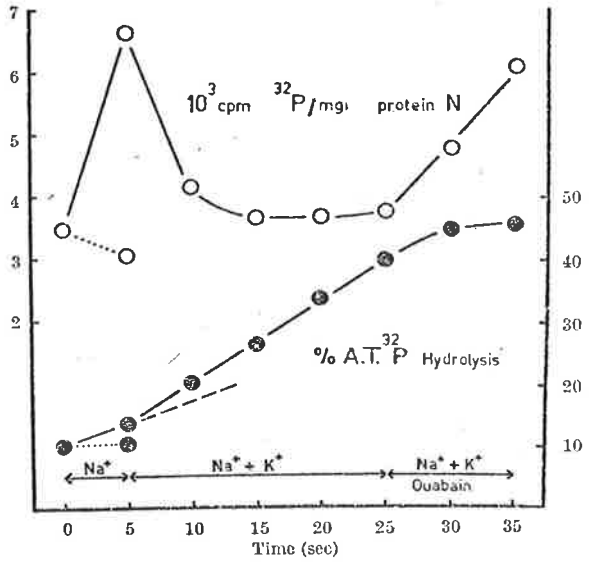

Fig. 1. Incorporation of phosphorus-32 from AT^{32}P into a microsomal adenosinetriphosphatase preparation incubated at 37° in a system to which the sequential addition of 80 mM sodium ions, 20 mM potassium ions and 0·1 mM ouabain was made. O—O, ^{32}P incorporation with Na^+, then Na^+ and K^+, then Na^+ and K^+ and ouabain; O - - - O, ^{32}P incorporation with K^+; ●—●, percentage hydrolysis of AT^{32}P with Na^+, then Na^+ and K^+, then Na^+ and K^+ and ouabain; ● - - - ●, percentage hydrolysis of AT^{32}P with K^+. The results are derived from a typical experiment

Table 1. Incorporation of Radioactive Phosphorus from $AT^{32}P$ into a Na^+- + K^+-dependent Adenosinetriphosphatase Preparation from Guinea Pig Kidney Cortex

Additions*	c.p.m. ^{32}P per mg protein N	Percentage ^{32}P released from $AT^{32}P$
None	26,000	8
80 mM Na^+	53,000	8
80 mM Na^+ + 0·1 mM ouabain	48,000	7
20 mM K^+	15,000	7
20 mM K^+ + 0·1 mM ouabain	14,000	6
80 mM Na^+ + 20 mM K^+	18,000	15
80 mM Na^+ + 20 mM K^+ + 0·1 mM ouabain	31,000	10

* Microsomal enzyme incubated with 0·02 mM $AT^{32}P$ for 10 sec at 37° in 0·05 M imidazole-glycylglycine buffer *p*H 7·6.

which is inhibited by ouabain and has other characteristics associated with active ion transport systems[6,7,12].

In experiments using this microsomal ATPase preparation and $AT^{32}P$ as substrate we have been able to demonstrate the incorporation of phosphorus-32 into a phosphorylated compound which appears to be an intermediate in the overall hydrolysis of $AT^{32}P$ to ADP and orthophosphate labelled with phosphorus-32 (ref. 13). Table 1 shows that the lovel of incorporation of labelled phosphorus into this microsomal material is greatly increased in the presence of sodium but not potassium ions, and is insensitive to ouabain. When both sodium and potassium ions are present together the hydrolysis of ATP is increased, but the level of phosphorus incorporation is reduced to near control values. On the other hand, addition of 0·1 mM ouabain to the sodium and potassium containing system results in inhibition of ATP hydrolysis and increased incorporation of phosphorus into the microsomal enzyme protein.

On the basis of these and other experiments in which sodium and potassium ions and ouabain were added in sequence to the incubation system (Fig. 1) we propose that the overall hydrolysis of ATP by this sodium plus potassium activated ATPase system proceeds by at least two stages. The first step is the formation of an intermediate compound which is dependent on the presence of sodium ions. The second step of the reaction is the subsequent phosphorolysis of this intermediate compound with the release of orthophosphate; this latter reaction is both

Table 2. Binding of ^{22}Na to a Na^+- + K^+-dependent Adenosine-Triphosphatase Preparation from Guinea Pig Kidney Cortex

Additions*	Specific activity c.p.m. ^{22}Na/mg protein N
None	14,000
ATP	131,000
ATP + 0·1 mM ouabain	150,000
ATP + 2 mM K^+	70,000
ATP + 2 mM K^+ + 0·1 mM ouabain	87,000

* Incubated for 5 sec at 37° with carrier-free ^{22}Na at *p*H 7·6 in 0·05 M imidazole-glycylglycine buffer.

dependent on potassium ions and is completely inhibited by low concentrations of ouabain.

The suggested mechanism is as follows (cf. ref. 13):

$$\text{Enzyme} + \text{AT}^{32}\text{P} \xrightarrow{x\text{Na}^+} [\text{E} \sim {}^{32}\text{P}]\text{Na}_x + \text{ADP} \qquad (1)$$

$$[\text{E} \sim {}^{32}\text{P}]\text{Na}_x \xrightarrow{y\text{K}^+} \text{E} + {}^{32}\text{P}_i + x\text{Na}^+ + y\text{K}^+ \qquad (2)$$

$$\text{Enzyme} + \text{AT}^{32}\text{P} \xrightarrow{x\text{Na}^+ + y\text{K}^+} \text{Enzyme} + \text{ADP} + {}^{32}\text{P}_i$$

Ouabain inhibits the hydrolysis of ATP by blocking the K^+-dependent phosphorolysis and hence turnover of the intermediate complex (reaction 2).

In an examination of the sodium plus potassium activation of an 'intact' membrane ATPase system Whittam has reported an asymmetry of this system which orients the site of sodium activation internal to, and potassium activation external to, the cell surface[14]. Dunham and Glynn[5] have previously shown an antagonism between ouabain and K^+ in this reaction, and these findings imply that the site of this antagonism is at a potassium-sensitive activation site, which is probably external to the cell surface, as also suggested by Post and Albright[15].

Preliminary experiments in which sodium-22 was incubated with this enzyme preparation have shown that there is an ATP dependent binding of sodium ions to this microsomal enzyme system, similar to that described by Järnefelt with brain microsomes[16].

The addition of K^+ to the incubation system resulted in a marked reduction in the binding of sodium-22. Ouabain did not reduce this initial sodium binding but at low concentrations could, in these experiments of only 5-sec duration, partially overcome the effect of potassium ions (Table 2). In other experiments of longer duration the effect of ouabain became more pronounced.

These findings also suggest that the action of ouabain is not on the formation of the phosphorylated complex, which is an intermediate in this reaction, or on the binding of sodium ions to it; but rather ouabain specifically inhibits the K^+-dependent phosphorolysis reaction which normally leads to the release of orthophosphate. In the presence of ouabain this intermediate tends to accumulate as its turnover is blocked.

The precise nature of the phosphorylated complex is as yet unknown, but total phospholipid extraction revealed 85 per cent of the total radioactivity (phosphorus-32) in a residual fraction containing all the original protein. This argues strongly against the complex being phospholipid in nature, for example, phosphatidic acid as suggested by Hokin and Hokin[17], and is in accord with the more recent findings of others[3,18–20].

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STUDIES OF THE MECHANISM OF CATION TRANSPORT

I. THE PREPARATION AND PROPERTIES OF A CATION STIMULATED ADENOSINE-TRIPHOSPHATASE FROM GUINEA PIG KIDNEY CORTEX

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SUMMARY.

The distribution and activity of adenosine-triphosphatase in homogenates and subfractions of guinea pig kidney cortex has been examined. A portion of the activity associated with particulate fractions could be further stimulated, in the presence of magnesium ions, by the combined addition of sodium and potassium ions but not by the addition of either cation alone or in combination with choline.

Treatment of the heavy microsome subfraction with deoxycholate resulted in a large increase in the specific activity of this enzyme, which now required sodium and potassium ions in a ratio of 4 : 1 for over 85 p.c. of its total activity. Under such conditions of cation stimulation, optimum pH was found to be between 7·5 and 8·0, and the enzyme also exhibited considerable temperature dependence and was stable up to 55° C.

The cardiac glycoside Ouabain completely inhibited that portion of enzyme activity stimulated by monovalent cations but was without effect on the activity observed in the presence of magnesium ions alone.

The effect of other reagents and hormones on the cation stimulated adenosine-triphosphatase activity was also examined, with a discussion of the possible significance of this enzyme in active cation transport.

INTRODUCTION.

The concept that membrane adenosine-triphosphatase activity is a participant in the active transport of sodium and potassium ions has gained considerable support from many recent studies (Post *et al.*, 1960; Skou, 1960, 1962; Järnefelt, 1961a, b). Although the most definitive results have been obtained using intact human erythrocytes (Glynn, 1961; Whittam, 1962) where it has been shown that enzyme activity can be stimulated only by K^+ external to the cell and Na^+ internal to the cell, considerable evidence has been obtained from an examination

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of the ATPase of broken cell preparations (Järnefelt, 1962a; Skou, 1962; Aldridge, 1962; Landon, 1962; Landon and Norris, 1963).

The widespread nature of this enzyme has been established by Bonting *et al.* (1961, 1962) who point out that highest activities are found either in nervous tissue or tissue concerned with secretory function. Perhaps some of the best evidence for a rôle of this enzyme in cation transport has come from the numerous demonstrations that that part of the hydrolysis of adenosine-triphosphate which is stimulated by sodium and potassium ions can be inhibited by concentrations of the cardiac glycoside Ouabain. Ouabain is known to inhibit cation transport in a variety of tissues including erythrocytes, brain and kidney preparations (Schatzmann, 1953; Whittam, 1962; Wheeler and Whittam, 1962; Schwartz, Bachelard and McIlwain, 1962).

This paper describes the distribution and properties of an adenosine-triphosphatase preparation in homogenates of guinea pig kidney cortex. The enzyme associated with a microsomal subfraction has a high specific activity, over 85 p.c. of which requires both sodium and potassium ions in addition to magnesium ions for full activation. That portion of the total activity stimulated by sodium plus potassium ions can be inhibited by Ouabain. The effect of some agents known to affect mitochondrial adenosine-triphosphatase or to influence electrolyte balance of the whole organism has also been investigated.

Materials and Methods.

Homogenisation.

Kidneys were obtained from adult guinea pigs killed by decapitation. The capsules were removed from the kidneys which were then chilled over crushed ice. The kidneys were either used immediately or stored at 2° C. for up to three days or −25° C. for much longer periods. For the preparation of the enzyme material the cortex was dissected from the medullary tissue and disrupted in the cold with a Potter-Elvehjem homogeniser fitted with a Teflon pestle, so that 1 gm. of wet tissue was dispersed in 10 ml. of medium. The medium was 0·25M sucrose buffered to pH 6·2 with 0·2 mM EDTA-0·2mM Histidine. This pH has been reported to be favourable for the preservation of the nuclear membrane (Dounce, 1950).

Differential centrifugation.

The whole homogenate was first centrifuged at 1,000 *g.* for 20 mins. to sediment the nuclear material, erythrocytes and larger cell debris. The supernatant was further centrifuged at 10,000 *g.* for 30 mins. to sediment the mitochondria. The supernatant obtained from this procedure was again centrifuged at 35,000 *g.* for 30 mins. to separate the "heavy" microsome fraction. The sediments were washed once by resuspension in 0·25M sucrose and recentrifuged at the original speed. The various sediments were gently resuspended in 0·25M sucrose in the homogeniser now operated by hand and were stored at 2° C. for assay. This procedure provided the following fractions (a) whole homogenate, (b) "nuclear" fraction, (c) mitochondrial fraction, (d) "heavy" microsome fraction, (e) "cytoplasm" fraction. In some experiments the "cytoplasm" fraction was further centrifuged at 100,000 *g.* for 60 mins. whereupon a small sediment ("light" microsomes) and a nearly clear supernatant ("soluble" fraction) were obtained.

In the majority of preparations the "heavy" microsome fraction (d) was diluted with an equal volume of an aqueous solution of 0·2 p.c. sodium deoxycholate and stored at 2° C.

Determination of enzyme activity.

After preliminary experiments the standard test system used for the assay of enzyme activity contained 5 μmoles of Tris-ATP (Sigma), 5 μmoles $MgCl_2$, 100 μmoles Imidazolo, 100 μmoles Glycylglycine, 0·5 μmoles H_4EDTA in a final volume of 3·5 ml. (pH 7·6). Deionised distilled water was used for all reagents. To some reaction vessels sufficient sodium chloride and potassium chloride were added to give final concentrations of 80 mM Na^+ and 20 mM K^+ whereas in other vessels Ouabain (Strophanthin-G, Nutritional Biochem. Corp.) was added to a final concentration of 0·1 mM. 0·1 ml. of "enzyme" preparation (usually about 0·05 mg. protein nitrogen) was added last to the chilled mixtures and quickly transferred to a Dubnoff metabolic shaking apparatus for incubation at 44° C. for from 15 to 30 min.

The reaction was stopped by the addition of 1·5 ml. of 8 p.c. $HClO_4$ and the reactants chilled by immersion in an ice bath. Protein was filtered off and the ice cold filtrate assayed for inorganic phosphate by the method of Fiske and SubbaRow (1925) using the modification of Aldridge (1962), and Amidol reagent (2,4 diaminophenol hydrochloride) as the reducing agent (Skou, 1957).

Protein was determined on 0·1 ml. or less of the various fractions in 0·25M sucrose by the method of Lowry, Rosenbrough, Farr and Randall (1951), except that a standard curve was prepared using a solution of crystalline bovine albumin (Armour) of known protein nitrogen content and all unknown values were compared with this.

Expression of results.

Unless stated in the text all tests for enzyme activity were carried out in the presence of Mg^{2+} and the activation by sodium plus potassium ions was the difference above this "base-line" level.

To avoid enhanced activity by small amounts of cations in the reagents, e.g. treatment of the enzyme preparation by sodium-deoxycholate which finally introduced between 1-2 mM Na^+ to the test solution, control values were determined in the presence of 10^{-4}M Ouabain. The specific activity was simultaneously measured under each set of conditions and expressed as μmoles inorganic phosphate hydrolysed from ATP per mg. protein nitrogen per hour. The difference due to sodium plus potassium activation was then expressed as a percentage of the total activity found in the presence of Mg^{2+} + Na^+ + K^+. This value was termed the p.c. cation activation.

RESULTS.

An adenosine-triphosphatase which required both sodium and potassium ions in combination, in addition to magnesium ions for full activation, was detected in every sub-fraction prepared by differential centrifugation of an homogenate of guinea pig kidney cortex. The highest enzyme activities were found in the sediment obtained at 35,000 *g*. ("heavy" microsomes) and in the "nuclear" fraction obtained at 1,000 *g*. The least activity was detected in the "cytoplasmic" fraction, i.e. the supernatant obtained after centrifuging at 35,000 *g*. In a few experiments this cytoplasmic fraction was further centrifuged at 100,000 *g*. for one hour, whereupon all the enzyme activity was now associated

with the sediment ("light" microsome fraction) and none was detectable in the supernatant ("soluble") fraction.

These results are given in Table 1 from which it can be seen that the distribution of adenosine-triphosphatase activity is similar to that recently reported for rat brain, but not rat liver (Bonting *et al.*, 1962).

The "enzyme" material could be satisfactorily prepared from guinea pig kidney tissue which had been stored either at 0-2° C. for a few days or at − 25° C. for at least one week, although the best results were usually obtained using tissue immediately after removal from the animal. Typical results of three separate preparations using kidneys following different storage conditions are shown in Table 1. The values given therein were obtained from assays performed 18 hours after the preparation of the cell fractions.

Recently Skou (1962) has reported that the addition of low concentrations of the detergent deoxycholic acid (DOC) to the homogenising medium was beneficial in the preparation of a sodium and potassium activated adenosine-triphosphatase from rabbit brain or kidney. In contrast Järnefelt (1961c and 1962a) has reported that the addition of 10^{-4}M DOC to the medium had some inhibitory effect on the adenosine-triphosphatase activity of rat brain microsomes. When the effect of this agent was examined by exposing the isolated "heavy" microsome fraction to a 0·1 p.c. solution of sodium deoxycholate in 0·125M sucrose at 0-2° C. it was found that this treatment rapidly resulted in a marked increase in enzyme activity. This effect became apparent after several hours' treatment and usually persisted for at least three weeks. The increase in activity ranged from 2-8 times that of control preparations suspended in sucrose alone. Unlike the effect reported by Skou (1962) who observed only a depression of Mg^{2+} activated "base-line" activity, under the conditions of these experiments the activity in the presence of magnesium alone was little changed but the specific activity of the sodium plus potassium activated component was greatly enhanced. The effect of DOC treatment on the other tissue fractions was not as great as that found with "heavy" microsomes; the effect with the nuclear fraction being most and that with mitochondria least. The result of DOC treatment may be likened to that of ageing which was also found to increase activity in the control suspensions of "heavy" microsomes in sucrose. These results are given in Table 2.

The effect of pH.

The effect of pH on adenosinetriphosphatase activity was examined over the range 5·5 - 9·5 using appropriate glycylglycine-imidazole mixtures. The extremes of pH were obtained by addition of dilute HCl or Tris. The pH dependent hydrolysis of ATP by "heavy" microsome fractions was examined in the presence of $Mg^{2+} + Na^{+} + K^{+}$, or with Mg^{2+} plus 10^{-4} Ouabain which was added to control possible effects of contaminating monovalent cations. For

TABLE 1.

Specific activity of various fractions prepared from guinea pig kidney cortex.

Fraction	Test	Experiment H			Experiment O			Experiment P		
		Specific activity		p.c. cation activation	Specific activity		p.c. cation activation	Specific activity		p.c. cation activation
Whole homogenate	Control	64			288			105		
	Na +K	126	62	49	375	67	18	160	55	34
Nuclear fraction	Control	76			186			129		
	Na + K	174	98	56	300	114	38	169	40	24
Mitochondria	Control	87			80			140		
	Na + K	139	52	37	102	22	21	177	37	26
Heavy microsomes	Control	72			158			67		
	Na + K	162	90	55	240	82	34	153	86	56
Supernatant fraction	Control	36			28			36		
	Na + K	40	4	10	36	8	22	38	2	5
Heavy microsomes after 24 hrs. in 0·1 p.c. deoxycholate	Control	75			87			75		
	Na + K	605	530	88	650	563	87	405	330	82

Specific activity given as μmoles P_i/mg. protein nitrogen/hour. For standard conditions see text.
H, fresh tissue. O, stored 3 days at 2° C. P, stored 10 days at —25° C.

TABLE 2.

The effect of storage conditions on the cation sensitive ATPase activity of untreated and deoxycholate treated heavy microsome preparations from guinea pig kidney cortex.

Engyne preparation	Modium	Days of storage							
		1 day		3 days		14 days		23 days	
		Specific activity	p.c. cation activation	Specific activity	p.c. cation activitaion	Specific activity	p.c. cation activitation	Specific activity	p.c. cation activation
D	a)	340	52	237	78	195	77	132	78
	b)	464	61	523	87	302	82	214	87
E	a)	74	50	98	61	89	66	194	88
	b)	415	88	394	86	354	87	172	89
F	a)	42	34	108	62	187	79	165	85
	b)	291	93	259	83	386	90	294	90
J	a)	82	34	105	57	244	95	243	91
	b)	563	87	878	92	503	95	418	93
L	a)	86	56	114	80	194	84	104	80
	b)	330	82	424	91	278	90	229	87

Medium a = 0·25 M sucrose; b = 0·125M sucrose plus 0·1 p.c. sodium deoxycholate. p.c. activation = p.c. total activity sensitive to cation stimulation. Specific activity as in Table 1.

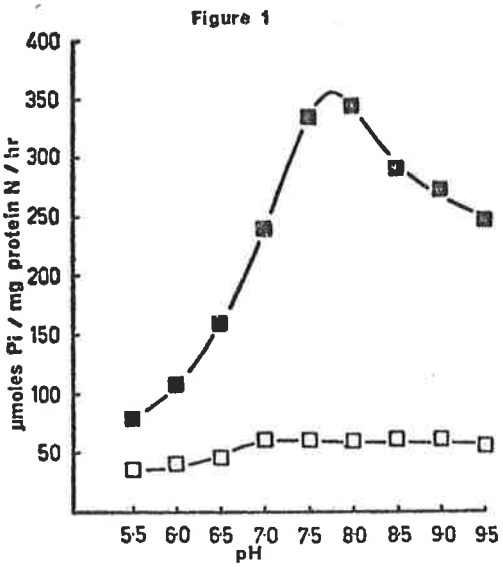

Fig. 1. The effect of pH upon enzyme activity.
■—■ Specific activity in the presence of Mg^{2+}, Na^+ and K^+.
□--□ Mg^{2+} and Ouabain present.
The concentration of ions and other conditions of incubation are given in the text.

example, actual estimation of the sodium content of the complete system used in these experiments showed that 1·3mM Na^+ was present.

All preparations displayed marked monovalent cation stimulated adenosine-triphosphatase activity with a pH optimum between 7·5 and 8·0. This activity being at least fivefold that of the control level measured in the absence of sodium and potassium ions. The activity measured in the presence of only magnesium ions was relatively insensitive to pH change and did not exhibit any sharp peaks in activity although somewhat higher values were found between pH 7·5 and 9·5 (see also Wheeler and Whittam, 1962). The pH of the reaction mixtures was checked after the addition of the enzyme preparation and again after incubation which in these experiments was at 40° C. for 15 minutes. No significant change in pH occurred during incubation. The results are shown in Fig. 1.

The effect of temperature.

The effect of incubation temperature on the hydrolysis of ATP by heavy microsome preparations was examined over the range 0°-85° C. using a twenty-minute incubation period. The results shown in Fig. 2 clearly indicate that the sodium plus potassium activated enzyme has a high temperature coefficient which is not displayed by the microsome preparation in the absence of these cations or in the presence of 0·1mM Ouabain.

Following this finding a standard incubation temperature of 44° C. was therefore adopted as this permitted greater resolution of the magnesium activated ATPase activity from that stimulated by sodium plus potassium than was possible at 37° C.

The effect of ions.

Table 3 shows the mean specific activity of five different enzyme preparations in both the presence and absence, and in different combinations of 2mM Mg^{2+},

2mM Ca^{2+}, 80mM Na^{+} and 20mM K^{+}. The effect of 0·1mM Ouabain and 3mM EDTA is also given.

It is apparent that a small amount of inorganic phosphorus is liberated from ATP in the absence of added ions. This activity is little altered by either the addition of magnesium ions or EDTA; although experiments with preparation (*b*) (ref. Table 3) clearly show that magnesium is essential for further activation by sodium plus potassium ions and cannot be replaced by calcium, the presence of which inhibits further stimulation by the monovalent cations.

Neither sodium nor potassium ions alone are able to produce activation of the enzyme but when added together produce a very great increase in the rate of hydrolysis of ATP. The monovalent cation stimulated increment in activity can be completely inhibited by addition of 0·1mM Ouabain, which reduces enzyme activity to that level found in the presence of magnesium ions only.

That optimal concentrations of sodium plus potassium ions were used to produce activation is demonstrated by Figs. 3 and 4 which show the activity of the enzyme at fixed concentrations of one cation whilst the other is increased

Figure 2

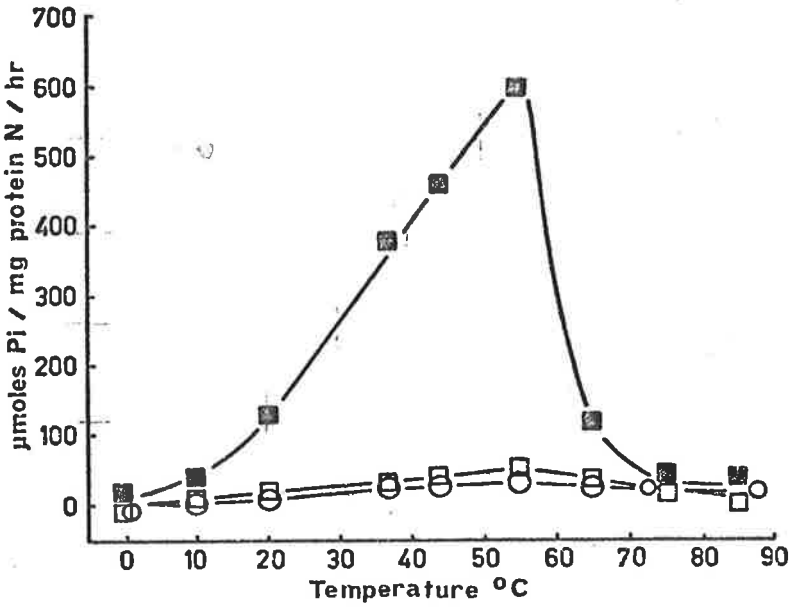

Fig. 2. The effect of temperature on enzyme activity.
■ — ■ Specific activity in the presence of Mg^{2+}, Na^{+} and K^{+}.
□ — □ Mg^{2+} and Ouabain present.
○ — ○ Mg^{2+} only.
The concentration of ions and other conditions of incubation are given in the text.

TABLE 3.

The effect of cations, ouabain and EDTA on the ATPase activity of heavy microsome preparations from guinea pig kidney cortex.

Enzyme preparation	2mM Mg	2mM Ca	80mM Na	20mM K	0·1mM ouabain	3mM EDTA	Specific activity	p.c. activation by Na+K
a	—	—	—	—	—	—	15	—
	+	—	—	—	—	—	19	—
	—	—	—	—	—	+	12	—
	+	—	—	—	—	+	13	—
b	—	—	—	—	—	—	25	—
	+	—	—	—	—	—	31	—
	—	—	+	+	—	—	28	—
	+	—	+	+	—	—	160	640
	—	+	—	—	—	—	41	—
	—	+	+	+	—	—	48	—
	+	+	—	—	—	—	38	—
	+	+	+	+	—	—	51	—
c	+	—	—	—	—	—	56	—
	+	—	+	+	—	—	460	820
	+	—	+	+	+	—	53	—
	+	—	—	—	+	—	51	—
d	+	—	—	—	—	—	48	—
	+	—	+	—	—	—	62	—
	+	—	+	—	+	—	48	—
	+	—	+	+	—	—	317	660
e	+	—	—	—	—	—	42	—
	+	—	—	+	—	—	37	—
	+	—	—	+	+	—	36	—
	+	—	+	+	—	—	350	840

Specific activity is μmoles inorganic P hydrolysed from ATP/mg. protein nitrogen/hour at 44° C.

to at least 120mM. As already shown in Table 3 the addition of either sodium or potassium ions alone was without significant effect upon enzyme activity.

Fig. 3 shows that in the presence of fixed concentrations of potassium ion, the addition of sodium ions leads to greatly enhanced hydrolysis of ATP, e.g. in the presence of 20mM K^+, enzyme activity rapidly increased when sodium ions were also added until maximum activity was reached when sodium ions were 80mM, i.e. a K^+ to Na^+ ratio of 1 : 4 was obtained. No further stimulation was obtained by an increased concentration of either sodium or potassium ions. As previously demonstrated with other ATPase preparations choline could not replace sodium ions (Whittam, 1962; Aldridge, 1962). Ouabain completely abolished the effect of added sodium plus potassium ions but was without effect on the "base-line" enzyme activity. Similarly when sodium ions are present at fixed levels and the concentration of potassium varied between 0-120mM (Fig. 4) it can again be seen that the greatest stimulation was obtained in the presence of 20mM K^+ plus 80mM Na^+. In fact, higher concentrations of

Figure 3

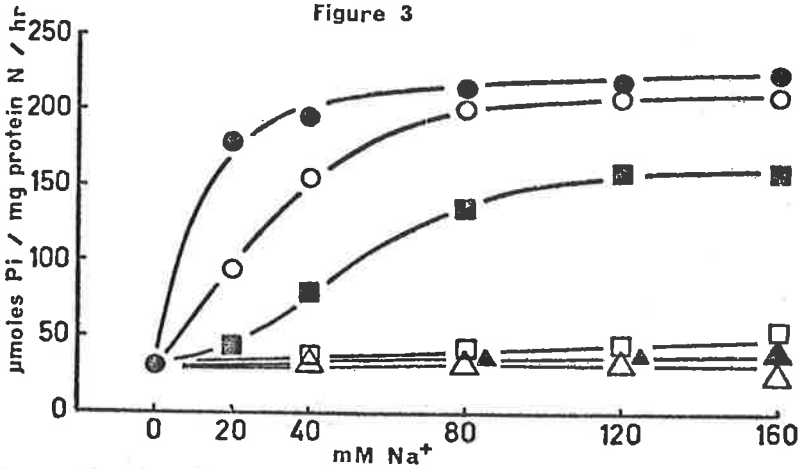

Fig. 3. The effect on enzyme activity of variable concentrations of Na^+ at three constant levels of K^+. The effect of Ouabain and substitution of choline for sodium is also shown.

● — ● Variable Na^+, 20 mM K^+.
○ — ○ Variable Na^+, 80 mM K^+.
□ — □ Variable choline replaces Na^+, 20 mM K^+.
■ — ■ Variable Na^+, 160 mM K^+.
▲ — ▲ Variable Na^+, 0·1 mM Ouabain, no potassium.
△ — △ Variable Na^+, no potassium.

The conditions of incubation are given in the text.

potassium ions were inhibitory to the stimulating effect seen in the presence of sodium ions plus low concentrations of potassium ions. Choline was also unable to replace potassium, and the combined effect of sodium plus potassium was again abolished by Ouabain. Under optimal conditions that component of activity which is induced by sodium plus potassium ions exceeds 85 p.c. of the total activity of the enzyme.

The effect of other agents.

The possible action of a number of agents on this enzyme was examined under standard conditions of cation stimulation and it was found that at the concentrations used 2:4-dinitrophenol, sodium salicylate, aldosterone and hydrocortisone were without direct effect on enzyme activity. In addition to Ouabain, however, it was found that sodium para-chloro-mercuri-benzoate (buffered to pH 7·6 with Tris) was able to completely abolish the activating action of combined sodium and potassium ions, but like Ouabain had no effect upon "baseline" activity measured in the absence of sodium plus potassium ions (cf. Table 4).

Specificity towards substrate.

The following nucleotides were examined as possible substrates for this enzyme; adenosinetriphosphate (ATP), adenosinediphosphate (ADP), adeno-

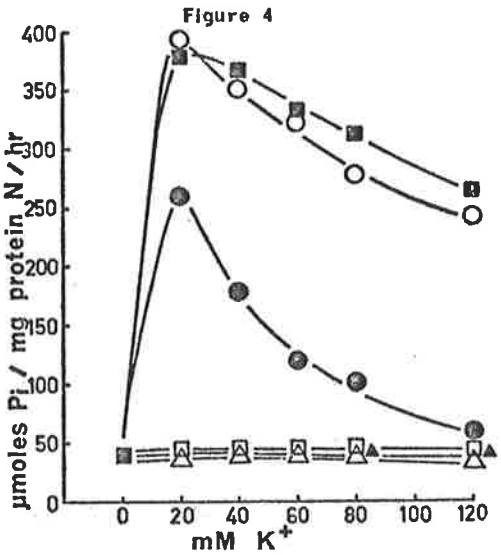

Fig. 4. The effect on enzyme activity of variable concentrations of K+ at three constant levels of Na+. The effect of Ouabain and the substitution of choline for potassium is also shown.

■ — ■ Variable K+, 160 mM Na+.
□ — □ Variable choline replaces K+, 20 mM Na+.
○ — ○ Variable K+, 80 mM Na+.
● — ● Variable K+, 20 mM Na+.
▲ — ▲ Variable K+, 0·1 mM Ouabain, no sodium.
△ — △ Variable K+, no sodium.

The conditions of incubation are given in the text.

sinemonophosphate (AMP), cytidinetriphosphate (CTP), guanosinetriphosphate (GTP), and uridinetriphosphate (UTP). In addition the utilization of glucose - 6 - phosphate, sodium pyrophosphate and sodium β-glycero-phosphate was examined.

The results are given in Table 5 which shows that neither ADP nor AMP was significantly utilized by the sodium plus potassium activated enzyme. On the other hand, CTP was utilized 36 p.c. as much as ATP but utilization of GTP or UTP was negligible as was that of glucose-6-phosphate and sodium pyrophosphate.

This enzyme preparation did not exhibit acid phosphatase activity (sodium β-glycerophosphate was not utilized at pH 5·0) but did exhibit a relatively small amount of alkaline phosphatase activity (sodium β-glycerophosphate at pH 8·5). The alkaline phosphatase activity was inhibited by the subsequent addition of sodium and potassium ions in combination (cf. Table 5).

DISCUSSION.

An adenosine-triphosphatase activity which requires both sodium plus potassium ions for full activation has been prepared from homogenates of guinea pig kidney cortex. This enzyme activity was associated with all particulate fractions prepared by differential centrifugation but the greatest specific activity was associated with the "heavy" microsome fraction. This material is generally thought to arise from fragments of endoplasmic reticulum and be membranous in nature (Hokin and Hokin, 1960; Palade and Siekevitz, 1956; Siekevitz and Palade, 1958; Hanzon and Toschi, 1959; DeRobertis *et al.*, 1961; Landon and Norris, 1963).

Such an enzyme has now been prepared from a wide variety of tissues and species and although there have been large differences in the reported specific activities of these preparations, apparently this enzyme activity can be

TABLE 4.

The effect of agents on the adenosine triphosphatase activity of heavy microsomes.

Enzyme preparation	Addition	Specific activity			p.c. cation activation
		Mg^{2+}	$Mg^{2+}+Na^{+}+K^{+}$	Δ	
n	Control	40	303	263	87
	0·1mM Ouabain	41	39	—2	0
	0·5mM 2,4-DNP	47	320	273	85
	5mM sodium salicylate	43	303	260	86
	Aldosterone (71 μg./ml.)	44	296	252	85
	Hydrocortisone (71 μg./ml.)	44	304	260	85
r	Control	31	160	129	81
	25mM choline chloride	32	160	128	80
	1mM sodium para-chloro-mercuri-benzoate	29	26	—3	0

TABLE 5.

Substrate specificity of ATPase reaction of heavy microsome preparations.

Enzyme preparation	Substrate 5mM	Specific activity			p.c. cation activation
		Control	(Na+K)	Δ	
r	ATP	31	147	116	79
	ADP	11	23	12	50
	AMP	24	19	—5	0
t	ATP	32	310	278	90
	CTP	39	142	101	71
	GTP	53	69	16	23
	UTP	60	69	9	13
p	ATP	46	185	139	75
	Glucose-6-phosphate	0	0	0	0
s	ATP	13	103	90	87
	Sodium pyrophosphate	10	24	14	58
x	sodium β-glycero-phosphate				
	pH 5·5	21	19	—2	0
	pH 7·6	13	13	0	0
	pH 8·5	98	66	—31	—45

All substrates were adjusted to pH 7·6 with tris buffer unless otherwise specified and were at a final concentration of 5mM. Standard incubation conditions (given in text) were used (except that 5mM Mg^{2+} was present).

expected in any tissue containing cells and particularly in tissues having nervous or secretory function (Bonting, Caravaggio and Hawkins, 1962).

Treatment of the microsomal material with a low concentration of sodium deoxycholate (0·1 p.c.) resulted in a marked increase in the specific activity of the sodium plus potassium activated enzymatic hydrolysis of ATP. This effect was similar but not identical with that recently described by Skou (1962) and also observed in this laboratory (Kinsolving, 1962).

There was a marked dependence of activity upon pH and maximum rates of ATP hydrolysis were obtained between pH 7·5-8·0. In this respect this material differs greatly from the adenosinetriphosphatase activity of rat brain microsomes described by Aldridge (1962) but closely resembles that of a rabbit kidney preparation (Wheeler and Whittam, 1962).

The pH dependence was apparent with both freshly prepared preparations and those which had been stored at 2° C. for up to ten days. This phenomenon was not demonstrated by the enzymatic material in the absence of sodium plus potassium ions. Similarly to the observations with pH, the cation activated hydrolysis of ATP was sensitive to temperature (Q_{10} approximately 2 between 15-55° C.) and was stable to a temperature of over 50° C., whereas there was relatively little effect of temperature upon the enzyme activity in the absence of the combination of sodium and potassium ions.

In the absence of sodium and potassium ions only about 20 p.c. of the maximum activity was obtained at pH 7·6 and at 44° C. This "basic" activity was insensitive to both Ouabain and para-chloro-mercuri-benzoate, which completely inhibit that activation obtained in the presence of sodium and potassium. This evidence together with the lack of response to both temperature and pH change suggests that this so-called "basic" activity is a separate process with different sites of enzyme activation. In further support of this suggestion is the finding that calcium ions could replace magnesium for basic activity; in fact, with the same concentration of Ca^{2+} greater activity was obtained than with Mg^{2+}. On the other hand calcium ions could not substitute for Mg^{2+} to produce that enzyme activity which could be further stimulated by the addition of sodium and potassium ions.

Neither sodium nor potassium ions were able to initiate activation alone, nor could choline substitute for either ion in combination with either sodium or potassium. In this regard this preparation is the same as those reported by Aldridge (1962) and Whittam (1962).

A ratio of 4 : 1 sodium to potassium ions gave maximal activity where 80mM Na^+ and 20mM K^+ were employed. Changes in these concentrations, even when the ratio was maintained, produced sub-maximal activity. With fixed concentrations of sodium, high concentrations of potassium were no longer stimulating but produced marked inhibition. This observation suggests that under such conditions potassium ions replace Na^+ from those activation sites normally occupied by sodium ions.

2:4-dinitrophenol and salicylate which are known to effect the magnesium dependent ATPase of isolated mitochondria (Charnock and Opit, 1962) were without effect on this enzyme as were the adrenal hormones aldosterone and hydrocortisone *in vitro*. Although the *in vivo* effect of these hormones on renal ion excretion and reabsorption is well known, both agents have also been found to be inactive *in vitro* when tested with ATPase preparations from cat grey matter and retina (Bonting, Simon and Hawkins, 1961).

Skou (1960) has reported that inosinetriphosphate (ITP) can be utilised as a substrate in place of adenosinetriphosphate (ATP) with crab-nerve microsome preparations, but with ITP additional stimulation in the presence of sodium and potassium ions was not apparent. In these experiments we found that although the microsome preparation from guinea pig kidney cortex would hydrolyse cytidinetriphosphate (CTP) to only 35 p.c. of that found with ATP, a further 70 p.c. activation could be obtained upon the combined addition of sodium plus potassium ions.

ADP was apparently utilized to 10 p.c. the extent of ATP and there was considerable stimulation of ADP hydrolysis when sodium and potassium were added. However, this effect may be due to the possible presence of adenylate-kinase in this preparation. Guanosinetriphosphate (GTP) and uridinetriphosphate (UTP) were utilized to a negligible extent (6 p.c. and 3 p.c. of control ATP level) and demonstrated only very low activation by sodium plus potassium ions. This was also the case with sodium pyrophosphate as substrate, whereas glucose-6-phosphate was not utilized at all.

These findings suggest that this cation stimulated enzyme activity possesses a high degree of substrate specificity towards the terminal phosphate bond of ATP and is not a general nucleotide phosphatase. This preparation was also shown to be nearly free of both acid and alkaline phosphatase activity when tested with β-glycerophosphate at pH 5·5 and 8·5 either in the presence or absence of combined sodium and potassium ions.

At pH 8·5 where hydrolysis was apparently greatest the addition of sodium and potassium ions resulted in a decrease in activity, as was also found to be the result when a commercial preparation of alkaline phosphatase (Worthington Biochemical Corporation, N.J., U.S.A.) was examined.

Although it is probable that this microsomal preparation from guinea pig kidney cortex contains enzymatic activity apart from the "specific" sodium plus potassium activated adenosinetriphosphatase, after treatment with DOC and under appropriate conditions the contribution to phosphate bond hydrolysis from other sources is very low.

Although this enzyme has many of the characteristics of an active cation transport process, Burgen (1962) has pointed out that studies of this type do not resolve the question whether this enzyme is an active physical participant in ion transport as suggested by Skou (1960) and Post *et al.* (1960) or whether it serves as a vital link in the coupling of energy into the transport mechanism.

However, other experiments in this laboratory strongly suggest that this microsomal enzyme system is an actual physical component of a sodium transport process (Charnock and Post, 1963).

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CALCIUM ACCUMULATION AND RESPIRATORY ACTIVITY OF THE SMALL INTESTINE OF THE RAT

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Abstract

The possible dependence of calcium accumulation upon respiratory activity of the rat small intestine has been investigated in vitro. The oxygen consumption of this tissue was relatively insensitive to the concentration of calcium in the medium; and the accumulation of radioactive calcium was greatly dependent upon ambient calcium concentration. At low concentrations of the medium the ratio of tissue calcium to that in the medium greatly exceeded unity, indicating an accumulation of this ion against a concentration gradient. However, this effect was also seen when the tissues were incubated in a gas phase of nitrogen instead of oxygen, demonstrating the lack of dependence of this process on tissue respiration. The rate at which calcium accumulated at 0° also suggests that the accumulation of calcium by the small intestine is not a metabolically controlled process but rather is a binding phenomenon similar to that reported for salivary gland of the rat.

Introduction

Using isolated strips of whole intestine, Schachter, Dowdle, and Schenker (3) found that a restricted segment of the small intestine of the rat can accumulate radioactive calcium to a concentration about five times greater than that of the medium in which it is incubated, and that much of this accumulation was dependent upon the oxidative metabolism of the tissue, although these workers did not directly study tissue respiration.

A recent investigation in this laboratory has shown that slices of rat cerebral cortex can accumulate radioactive calcium from an incubation medium in excess of that accountable by diffusion (1). Although the gradient of calcium formed affected the oxygen consumption of the isolated tissue, the formation of the gradient itself was not dependent upon the oxidative metabolism of the cerebral cortex, but was found to be a binding phenomena similar to that reported for salivary gland (2), kidney, liver, and other tissues of the rat (3). Therefore it was of interest to examine the accumulation of calcium by the small intestine in direct relation to the oxidative metabolism of that tissue.

In an investigation of the active transport of sugars in vitro, Crane and Mandelstam (4) used a sliced preparation of small intestine which permits mixing of tissue from many animals, thus overcoming one of the well-known disadvantages (3, 10) of the older perfusion techniques (5, 6, 7), and which also allows concurrent examination of the respiratory activity of the tissue.

This paper describes the accumulation of calcium by sliced rings of small intestine prepared essentially by the method of Crane and Mandelstam, and the relationship of the accumulation of this ion to the respiratory activity of the tissue in vitro.

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Materials and Methods

Tissue Preparation and Incubation

All tissues were prepared from young hooded rats weighing less than 170 g fed ad libitum on standard laboratory diet without vitamin D supplement. The animals were killed by decapitation. Rings of tissue were prepared from a 5-cm segment of the small intestine taken from the end proximal to the pylorus (see Ref. 3), by the method of Crane and Mandelstam (4). Pooled samples from at least six rats were used in each experiment. Slices of cerebral cortex, liver, and renal cortex were prepared with a chilled Stadie–Riggs microtome (8) and hemidiaphragms obtained after whole diaphragms were excised and the surrounding tissue and bone cut away with scissors. The tissues were chilled by immersion in an ice-cold calcium-free salt solution of 5 m*M* KCl, 120 m*M* NaCl which had previously been gassed for 60 minutes in the cold with either 100% oxygen or oxygen-free nitrogen (9). All tissues were blotted dry on hardened filter paper (Whatman No. 542) and weighed on a torsion balance immediately before transfer to chilled incubation vessels. About 100 mg wet tissue or one hemidiaphragm were added to each Warburg flask which contained, in a final volume of 3 ml, sodium, potassium, magnesium, and strontium ions added as the chloride salts to a Tris–HCl buffer (pH 7) and other additions at concentrations indicated in the text. Glucose (5 m*M*) was also present as substrate except in some experiments with small intestine. Radioactive calcium ions of determined specific activity (Oak Ridge National Laboratory) were added from the side arm of the flasks after temperature equilibration at 0° or 37°. The gas space of the vessels was thoroughly flushed out and filled with either 100% oxygen or purified nitrogen as previously described (1) and respiratory activity of the tissue measured by a standard manometric procedure (9).

Measurement of $^{45}Ca^{++}$ Accumulation

After incubation the muscular tissues were separated from the medium with forceps and blotted free of surface fluid, whereas the slices of brain, liver, and kidney were separated by rapid centrifugal sedimentation and decanting the supernatant fluid, and then washed once by resuspension in 3 ml of the calcium-free salt solution used previously and again harvested by centrifuging and decanting the supernatant fluid. The tissues were thoroughly dried on filter paper and weighed again before transfer to an oven at 105° and drying to constant weight. The dried tissue was quantitatively transferred to digestion flasks and boiled under reflux for 10 minutes with 2 ml concentrated nitric acid. A few drops of 30% hydrogen peroxide were added to the boiling mixture to complete digestion. The cooled digests were made to volume and aliquots plated for determination of radioactive calcium using a thin-window gas flow apparatus (Tracerlab).

Expression of Results

In recent studies of the accumulation of $^{45}Ca^{++}$, Schachter, Dowdle, and

Schenker (3) expressed their data as a ratio of the concentration of the calcium in the tissue to the final concentration of calcium in the medium after incubation (i.e. $^{45}Ca^{++}{}_T : {}^{45}Ca^{++}{}_M$ where $^{45}Ca^{++}{}_T$ was counts per minute/g wet weight of tissue after incubation and $^{45}Ca^{++}{}_M$ was counts per minute/ml medium) whereas Dreisbach (2) made an estimate of "bound" calcium $^{45}Ca^{++}{}_B$ after assuming that at equilibrium the "tissue fluid" contained a concentration of calcium ions equal to that of the medium. In this latter study, $^{45}Ca^{++}{}_B$ was expressed as calculated bound counts per minute/unit dry weight of tissue. Similar methods of expression were used in this study where total tissue calcium $^{45}Ca^{++}{}_T$ is expressed as c.p.m./g original wet weight of tissue before incubation in order to avoid the variation in wet tissue weights which were obtained after different conditions of incubation, e.g. different calcium concentrations leading to different degrees of tissue swelling. "Bound" calcium $^{45}Ca^{++}{}_B$ was calculated as follows:

$$Ca_F \times W_F \times SA = C_T \quad [1]$$

$$C_W - C_T = C_B \quad [2]$$

$$C_B \times \frac{1}{SA} \times \frac{1}{DW} = {}^{45}Ca^{++}{}_B \quad [3]$$

where Ca_F = final concentration of $^{45}Ca^{++}$ in the medium after incubation (c.p.m./ml);
W_F = final weight of tissue fluid (mg) determined as weight loss at 105°;
SA = specific activity of $^{45}Ca^{++}$ (c.p.m./μmoles Ca^{++});
C_T = calculated counts in tissue fluid at equilibrium with medium;
C_W = total counts in wet tissue determined after incubation (c.p.m./mg wet tissue);
C_B = "bound" counts obtained by difference;
DW = determined dry weight of tissue after drying at 105°.

In some later experiments the initial concentration of radioactive calcium in the medium ($^{45}Ca^{++}{}_I$) given as c.p.m./ml, was used in place of the final concentration of radioactive calcium ($^{45}Ca^{++}{}_M$), also given as c.p.m./ml, as these values were always within 10% and usually less than 5% different (see Table I) and substitution of $^{45}Ca^{++}{}_I$ for $^{45}Ca^{++}{}_M$ did not introduce serious error. Duplicate experiments agreed within ±10%.

Results

Accumulation of $^{45}Ca^{++}$ by the Isolated Small Intestine of the Rat

The Effect of Calcium Concentration

When slices of rat small intestine were incubated in media containing from 0.1 m*M* to 100 m*M* $^{45}Ca^{++}$, the accumulation of this ion by the tissue was found to increase with increasing concentration in the medium. After 90 minutes incubation at 37° the ratio of tissue calcium:calcium concentration

TABLE I

Effect of calcium concentration on the respiration and accumulation of $^{45}Ca^{++}$ by the sma intestine of the rat

$^{45}Ca^{++}{}_{I}$	$^{45}Ca^{++}{}_{M}$	Q_{O_2}	$^{45}Ca^{++}{}_{T}$	$^{45}Ca^{++}{}_{T}/^{45}Ca^{++}{}_{M}$	$^{45}Ca^{++}{}_{B}$	$^{45}Ca^{++}{}_{B}/^{45}Ca^{++}{}_{M}$
0.1	0.09	5.7	2.42	25.8	1.2	13.2
0.5	0.46	5.6	1.01	2.2	4.3	9.4
1.0	0.93	6.3	1.86	2.0	6.4	6.8
5.0	4.7	6.3	6.95	1.4	13.2	2.8
10	9.7	7.3	8.30	0.85	14.4	1.5
20	19.5	5.9	16.9	0.87	21	1.1
30	29.0	5.1	21.3	0.74	28	0.97
40	38.8	4.8	31.8	0.82	35	0.91
50	48.8	5.7	32.4	0.68	46	0.96
75	72.5	5.1	54.6	0.76	61	0.81
100	98.0	4.3	60.9	0.62	80	0.82

NOTE: Incubated for 90 minutes at 37° with gas phase 100% O_2. No exogenous substrate or magnesium ion present. Initial concentration calcium in medium $^{45}Ca^{++}{}_{I}$; final concentration $^{45}Ca^{++}{}_{M}$; total tissue calcium $^{45}Ca^{++}{}_{T}$ μg atoms/g original wet weight tissue; calculated bound calcium $^{45}Ca^{++}{}_{B}$ μg atoms/g dry weight tissue taken fo digestion (cf. Dreisbach (2)). Q_{O_2} μl.O_2/mg dry weight per hr.

in the medium ($^{45}Ca^{++}{}_{T}$:$^{45}Ca^{++}{}_{M}$) was greatest where concentration of the medium was least, i.e. 25.8 at 0.1 mM Ca^{++}. This gradient declined sharply to approximately unity at 10 mM Ca^{++} and then remained near this level at all higher concentrations of calcium examined.

The respiratory activity of the sliced intestine (Q_{O_2}) varied from 5.7 at 0.1 mM Ca^{++} to a maximum of 7.3 at 10 mM Ca^{++} and then decreased at higher concentrations of calcium. Evidently the respiration of intestinal tissue is less sensitive to calcium ions than that of isolated rat cerebral cortex (1). No obvious correlation could be found between values of Q_{O_2} and the concentration gradient of calcium established ($Ca^{++}{}_{T}$:$Ca^{++}{}_{M}$). When the amount of "bound" calcium ($Ca^{++}{}_{B}$) was calculated by the method of Dreisbach (2) it was found to increase linearly with concentrations of calcium in the medium greater than 5 mM. These results are given in Table I.

Effect of pH

Changing the pH of the incubation medium from pH 4–11 by using appropriate Tris–HCl buffers resulted in little change in respiratory activity of the sliced small intestine although the Q_{O_2} was reduced slightly below pH 6. Because of the low capacity of some of these buffers the pH of the incubation solutions was always checked after incubation and was found to be less than 0.4 pH unit from their initial values.

With 1 mM Ca^{++} in the medium, the amount of calcium which accumulated in the tissue increased from 1.92 mM at pH 4 to a maximum of 2.81 mM at pH 7 and thereafter declined slightly from this value. The calculated amount of "bound" calcium (Ref. 2) showed a similar but not identical effect which was also maximal at pH 7. These results are given in Table II. All further experiments were conducted at pH 7.

Effect of Temperature on Rate of Accumulation

The rate at which $^{45}Ca^{++}{}_{T}$ accumulated in slices of rat small intestine was

TABLE II

Effect of pH on respiration and accumulation of $^{45}Ca^{++}$

pH	Q_{O_2}	$^{45}Ca^{++}{}_T/^{45}Ca^{++}{}_I$	$^{45}Ca^{++}{}_B/^{45}Ca^{++}{}_I$
4	5.2	1.92	5.0
5	5.9	2.53	8.25
6	6.1	2.73	9.25
7	6.6	2.81	10.65
8	6.6	2.65	8.47
9	6.8	2.56	10.3
10	6.1	2.56	9.0
11	6.6	2.54	9.1

NOTE: Incubated for 60 minutes at 37° with gas phase 100% O_2. No exogenous substrate or magnesium ions present in medium containing 1 m*M* $CaCl_2$, 5 m*M* KCl, 120 m*M* NaCl, 10 m*M* Tris adjusted to pH with HCl/NaOH.

studied at 0° and 37°. Initially the rate at either temperature was extremely rapid although that at 0° was less than that at 37°; after 5 minutes incubation 47% (at 0°) and 63% (at 37°) of the total amount obtained at the steady state level was found. After 60 minutes incubation at either 0° or 37° the amounts of calcium accumulated by the tissue ($^{45}Ca^{++}{}_T$) were identical. Similarly, calculation of the bound calcium at the steady state ($^{45}Ca^{++}{}_{B_{50\ min}}$) after incubation at either temperature gave the same result. However, calculation of bound calcium after 5 minutes incubation at 37° gave only 20% of that found after 60 minutes, and at 0° the calculation resulted in a negative quantity indicating that under these conditions equilibrium between tissue fluid and the medium could not be assumed. Thereafter bound calcium was only calculated after at least 60 minutes incubation. These results are shown in Table III.

TABLE III

Effect of temperature on rate of accumulation of $^{45}Ca^{++}$

	0°			37°		
Incubation time (min)	$^{45}Ca^{++}{}_T/^{45}Ca^{++}{}_I$	% total	$^{45}Ca^{++}{}_B$	$^{45}Ca^{++}{}_T/^{45}Ca^{++}{}_I$	% total	$^{45}Ca^{++}{}_B$
5	0.66	47	<0	0.88	63	0.5
10	0.79	54	0.2	1.10	78	1.5
15	1.00	71	1.3	1.20	86	1.7
30	1.20	86	1.6	1.25	89	2.1
45	1.30	93	2.1	1.30	93	2.2
60	1.40	100	2.5	1.40	100	2.5

NOTE: Incubated in medium containing 1 m*M* $CaCl_2$, 5 m*M* KCl, 120 m*M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* glucose, 10 m*M* Tris–HCl, pH 7. Gas phase 100% O_2. Both Mg^{++} and glucose reduce calcium accumulation below level observed in Tables I, II, and V.

Effect of Additions to Incubation Medium

When exogenous substrate was supplied as either glucose, sodium glutamate, or sodium succinate the respiratory activity of the tissue (Q_{O_2}) increased by over 30% of that found in controls incubated with endogenous substrate

only. However, with each increment in Q_{O_2} there was some reduction in the amount of $^{45}Ca^{++}{}_T$ which accumulated in the tissue, the greatest effect being with succinate where the level of $^{45}Ca^{++}{}_T$ was 84% of that of the control. The addition of magnesium or strontium ions at ten times greater concentration than that of the calcium in the medium was without effect on tissue respiration, but greatly reduced the amount of calcium which accumulated. The effect of magnesium ions (42% decrease) was greater than that of strontium (25% decrease). Incubation with 2×10^{-4} *M* iodoacetic acid (IAA) decreased the oxygen consumption of isolated small intestine to 68% of the control level but had no effect on calcium accumulation.

To examine the effect of acetylcholine the tissues were preincubated for 10 minutes with eserine (which inhibits acetylcholine esterase), and which was shown in control experiments to be without effect on either oxygen consumption or calcium accumulation. Acetylcholine and $^{45}Ca^{++}$ were added together from the side arm and incubation continued for 1 hour. Both respiratory activity and calcium accumulation were decreased by about 10%. Incubation with lysozyme did not alter the respiratory activity of the isolated small intestine but did decrease the accumulation of calcium by 12%, whereas incubation with phospholipase A (heated cobra venom) produced a 23% decrease in Q_{O_2} and a 12% decrease in the amount of calcium accumulated by the tissue. These results are shown in Table IV.

TABLE IV

Effect of incubation conditions on respiration and accumulation of $^{45}Ca^{++}$

Additions	Q_{O_2}	% control	$^{45}Ca^{++}{}_T/^{45}Ca^{++}{}_I$	% $^{45}Ca^{++}{}_T$
Control	6.2±0.8	100	2.4±0.5	100
10^{-2} *M* glucose	8.1±0.2	130	2.3±0.3	96
3×10^{-2} *M* sodium glutamate	8.5±0.9	137	2.2±0.6	92
3×10^{-2} *M* sodium succinate	8.4±0.1	135	2.0±0.7	84
10^{-2} *M* $MgCl_2$	6.4±0.3	103	1.4±0.3	58
10^{-2} *M* $SrCl_2$	6.0±0.5	97	1.8±0.2	75
2×10^{-4} *M* iodoacetic acid	4.2±1.0	68	2.4±0.4	100
10^{-4} eserine	6.6±0.1	106	2.5±0.3	104
10^{-4} acetylcholine $+10^{-4}$ eserine	5.6±1.3	90	2.2±0.6	92
Lysozyme 1 mg	6.2±1.0	100	2.1±0.6	88
Phospholipase A 1 mg	4.8±1.2	77	2.1±0.6	88

NOTE: Incubated for 60 minutes at 37° in medium containing 1 m*M* $CaCl_2$, 5 m*M* KCl, 120 m*M* NaCl, 10 m*M* Tris–HCl, pH 7, with additions shown in table. Gas phase 100% O_2. All values are means ± standard errors.

Effect of Oxygen Tension

The rate of calcium accumulation had previously been examined at various temperatures (cf. Table III). Now it was examined at fixed temperature (37°) but at two widely different levels of oxygen tension. In order to minimize experimental variation these experiments were performed simultaneously with

pooled tissue from at least six rats placed into duplicate vessels and incubated in portions of the same batch of medium which had previously been gassed in the cold with either 100% oxygen or oxygen-free nitrogen (see Methods section). The vessels were now thoroughly swept out with these respective gasses. Table V demonstrates that like previous findings reported in the

TABLE V

The effect of oxygen tension on $^{45}Ca^{++}$ accumulation

Incubation time (min)	Oxygen		Nitrogen	
	$^{45}Ca^{++}_T/^{45}Ca^{++}_I$	% total	$^{45}Ca^{++}_T/^{45}Ca^{++}_I$	% total
5	.73	38	1.60	78
10	.96	50	1.69	83
20	1.23	64	1.82	84
30	1.32	68	1.76	86
45	1.71	89	1.85	91
60	1.92	100	2.04	100

NOTE: Incubated at 37° in medium containing 1 m*M* $CaCl_2$, 5 m*M* KCl, 120 m*M* NaCl, 10 m*M* Tris-HCl, pH 7. No magnesium ions or exogenous substrate added.

literature for liver, salivary gland, and rachitic metaphysis (3), in these experiments segments of rat small intestine accumulate more $^{45}Ca^{++}$ when incubated in an atmosphere of nitrogen than oxygen. Although the initial rate of calcium accumulation is much greater under nitrogen than oxygen the difference has all but disappeared after 60 minutes incubation where the $^{45}Ca^{++}_T$ accumulated in an atmosphere of oxygen nearly equals that found after incubation in nitrogen.

Calculation of bound calcium (Ca^{++}_B) at the end of the incubation period also demonstrated a slightly smaller amount (7.4) formed under oxygen than with nitrogen (8.6). In one other experiment the data of which is not given in Table V it was found that this effect persisted when both substrate (5 m*M*) glucose and iodoacetic acid (2×10^{-4} *M*) were present in the medium of those vessels incubated under nitrogen.

Comparison with Other Tissues of the Rat

Incubation of slices of small intestine, cerebral cortex, hemidiaphragm, liver, and renal cortex of the rat with various concentrations of calcium, sodium, and potassium shows that the respiratory activity ($Q_{O_2\ (60\ min)}$) of these tissues is widely different. Values obtained for renal and cerebral cortex were the highest obtained here but a twofold difference existed in the amount of calcium accumulated by those tissues. Small intestine preparations having a relatively low respiratory activity accumulated considerably more calcium than either brain or kidney (on a wet weight basis) when the ambient concentration of calcium was low (1 m*M*). Again no obvious relationship could be found between respiratory activity and calcium accumulation. These results are given in Table VI.

TABLE VI

Accumulation of $^{45}Ca^{++}$ by various tissues of the rat

Tissue	Additions	Q_{O_2}	$^{45}Ca^{++}_T$	$^{45}Ca^{++}_T/^{45}Ca^{++}_I$
Small intestine	1 m*M* Ca^{++}, 5 m*M* K^+, 120 m*M* Na^-	6.8	2.2	2.2
Small intestine	10 m*M* Ca^{++}, 5 m*M* K^+, 120 m*M* Na^+	6.1	10.7	1.1
Cerebral cortex	2 m*M* Ca^{++}, 5 m*M* K^+, 145 m*M* Na^+	9.3	3.6	1.8
Cerebral cortex	10 m*M* Ca^{++}, 105 m*M* K^+, 40 m*M* Na^+	14.1	14.2	1.4
Diaphragm	10 m*M* Ca^{++}, 5 m*M* K^+, 120 m*M* Na^+	1.3	12.6	1.3
Liver	2 m*M* Ca^{++}, 55 m*M* K^+, 90 m*M* Na^+	6.7	2.4	1.2
Renal cortex	2 m*M* Ca^{++}, 5 m*M* K^+, 120 m*M* Na^+	14.8	1.4	0.7

NOTE: Incubated for 60 minutes at 37° in medium containing 1 m*M* $MgCl_2$, 5 m*M* glucose, 10 m*M* Tris–HCl, pH 7. Gas phase 100% O_2. All ions added as the chloride salts.

Discussion

By using either everted sacs or loops of perfused isolated segments of small intestine many substances have been shown to move against a concentration gradient from the mucosal to the serosal side of these preparations. When this movement is inhibited by metabolic poisons these demonstrations are thought to represent evidence for the active transport of the substance under investigation (11, 12). However, the inherent variability of these techniques is well known as is the complication of non-physiological diffusion through the serosal portion of the intestine (4, 13).

In an effort to overcome some of these difficulties several investigators (4, 3) have measured the accumulation of various substances by isolated strips or rings of tissue. This procedure exposes large areas of cut or damaged tissue not encountered with perfusion or everted sac methods; whether this constitutes an important factor has yet to be determined.

Using a preparation of sliced rings of small intestine (4) these experiments have demonstrated that this tissue can accumulate calcium ions from the medium in excess of that which can be explained by the intracellular tissue fluid coming into equilibrium with the medium. The greatest accumulation was obtained when the external concentration of calcium was low (0.1 m*M*) and at pH 7. Lowering the incubation temperature to 0° reduced the rate at which calcium entered the tissue below that found at 37°, but, as observed by DeLuca and Engstrom with isolated rat kidney mitochondria (14) and Dreisbach with rat salivary gland (2), the rate remained sufficiently high (58% of the total within the first 5 minutes) to suggest the presence of a binding process rather than a metabolically dependent concentration mechanism.

In addition, no obvious relationship could be found between tissue respiratory activity and calcium accumulation. Provision of exogenous substrate raised the oxygen consumption of the tissue and decreased the amount of calcium accumulated whereas the addition of other agents (IAA, acetyl-

choline, lysozyme, phospholipase A) decreased the oxygen consumption and also decreased calcium accumulation. It is of interest to note that in their study with isolated mitochondria DeLuca and Engstrom (14) did not find a direct dependence of calcium uptake upon oxidative phosphorylation although ATP and an oxidizable substrate were beneficial. Both ATP and oxidizable substrate are known to effect water content and hence membrane permeability of mitochondria (15).

The effect of magnesium and strontium ions, which, at the concentrations used, did not affect tissue respiration but greatly reduced calcium accumulation, also suggests competition between these ions and calcium for binding sites in the tissue rather than interference with a metabolic process. Experiments in which the tissue was incubated anaerobically confirmed this suggestion, as not only did $^{45}Ca^{++}$ continue to enter the tissue, but the initial rate of accumulation was greatly increased. Schachter *et al.* have reported a similar increased accumulation of $^{45}Ca^{++}$ by liver, salivary gland, and rachitic metaphysis when incubated under nitrogen rather than oxygen (3). This finding may suggest the presence of an oxidatively dependent expulsion mechanism for calcium ions in these tissues, that is, an outwardly oriented active transport process.

Although these experiments do not provide direct evidence for the nature of the sites of calcium binding, treatment of the tissue with lysozyme or phospholipase A resulted in some decrease in calcium accumulation indicating that groups hydrolyzed by these enzymes are involved.

As the animals used in this study were of intermediate age (12–24 weeks) and of unknown vitamin D status, no direct comparison is possible between these findings and those of Schachter *et al.* (3), who reported an active accumulation of calcium by a similar segment of the small intestine of very young rats, although it does serve to emphasize the restricted physiological significance of such a transport mechanism. Because of the difficulties inherent in any method of examination of tissue "concentration" of ions (2, 3, 16) it is felt that the accumulation of calcium ions against a concentration gradient shown here is less meaningful in terms of an "active transport" mechanism than the absence of direct dependence of this process upon oxidative metabolism.

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THE ACCUMULATION OF CALCIUM BY BRAIN CORTEX SLICES

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THE ANTAGONISTIC action of calcium and potassium ions on the metabolism of isolated brain cortex has been known for a long time (ASHFORD and DIXON, 1935; DICKENS and GREVILLE, 1935) and recently reaffirmed by MCILWAIN (1952), KLEINZELLER and RYBOVA (1957), RYBOVA (1960*a*, *b*), GARDOS (1960). QUASTEL and QUASTEL (1961). Although much is known of the mechanism by which nervous tissue accumulates potassium ions and the dependence of this transport mechanism upon the metabolic and respiratory activity of the tissue (TERNER, EGGLESTON and KREBS, 1950; WHITTAM, 1962) little is known of the mechanism by which calcium ions exert their effect, although these ions are known to be essential for the accumulation of K^+ against a concentration gradient (GARDOS, 1960).

The present study describes an examination of the uptake of radioactive calcium by slices of rat brain cortex under a variety of cationic, and other environmental, conditions shown markedly to change the respiratory activity of these preparations *in vitro*.

MATERIALS AND METHODS

Tissues and Incubation. Young inbred hooded rats were killed by decapitation, and the intact brain was quickly removed and chilled in ice-cold calcium-free solution (5 mM-KCl, 120 mM-NaCl) which had previously been gassed in the cold with either 100 per cent oxygen or nitrogen. The last traces of oxygen were removed from the nitrogen by passing the gas through a train of alkaline pyrogallol and vanadous sulphate (UMBREIT *et al.*, 1957). Slices of cerebral cortex were prepared with a Stadie–Riggs (1944) apparatus and were blotted dry on hardened filter paper. After weighing, about 100 mg. of tissue were added to chilled Warburg vessels containing KCl, NaCl, Tris-HCl buffer pH 7, glucose, and other additions at concentrations indicated in the text. The incubation mixtures had also been gassed in the cold and the vessels were now swept out thoroughly with either oxygen or nitrogen. Radioactive calcium ions (as $^{45}CaCl_2$) of determined specific activity were introduced to the medium from the side-arm of the vessels after temperature equilibration. Incubation was at 37° in a conventional Warburg apparatus.

After incubation, the tissues were separated from the medium by rapid sedimentation in a bench model centrifuge and the supernatant fluid was decanted off. The tubes were drained and dried as far as possible with adsorbent tissue. The slices were washed once by floating in 3 ml of the calcium-free cooling solution previously used, followed by rapid centrifugal sedimentation. The wet tissue was collected on filter paper and blotted dry before weighing again. It was dried to constant weight at 105°, quantitatively transferred to digestion flasks and boiled under reflux for 10 min with 2 ml of concentrated nitric acid. A few drops of 30 per cent hydrogen peroxide were added to complete digestion. The cooled digests were made to volume and portions plated for the determination of radioactivity with a thin window continuous gas flow apparatus.

Expression of results. Concentration gradients of tissue calcium to that in the medium were derived as $^{45}Ca_T^{2+}$: $^{45}Ca_I^{2+}$, where $^{45}Ca_T^{2+}$ is cpm/μg atoms calcium/g original wet weight of tissue (this avoids the variation otherwise introduced by changes in tissue fluid content following different

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conditions of incubation) and $^{45}Ca_I^{2+}$ is cpm/μg atom calcium/ml incubation fluid. As the uptake of ions from the medium was less than 5 per cent of the total radioactivity little error was introduced by using the original rather than final concentration of $^{45}Ca^{2+}$ in the medium. Duplicate experiments agreed within $\pm 10\%$.

RESULTS

By incubating slices of rat brain cortex in a medium containing 2 mM-$^{45}Ca^{2+}$ but with varying concentrations of sodium and potassium ions it was possible to show that varying the respiratory activity of the tissue between Q_{O_2} 10·1–17·3 was without effect on the concentration gradient of calcium established. The addition of 5 mM-sodium glutamate to the medium which already contained glucose as substrate, also elevated the Q_{O_2} but did not effect the calcium gradient, although KREBS *et al.* (1950; 1951) have shown that glutamate increases K^+ accumulation in cortex slices. Incubation in the presence of the cardiac glycoside ouabain (Strophanthin-G) did not change the amount of calcium taken up in these experiments, although at the concentration used (10^{-4} M) it is known to prevent active cation transport in brain and other preparations (WHITTAM, 1962; POST, MERRITT, KINSOLVING and ALBRIGHT, 1960) and decrease respiratory activity (WOLLENBERGER, 1947).

Further evidence for the dissociation of calcium accumulation from the oxidative metabolism of the cortex was obtained from experiments in which slices were incubated with 10^{-4} M-2,4-dinitrophenol. Although the Q_{O_2} of the tissue was reduced to 54 per cent of that found in control experiments the apparent concentration gradient of calcium formed under both conditions was the same. However incubation of the slices with 5 mM-sodium salicylate, which also uncouples oxidative phosphorylation (CHARNOCK, OPIT and HETZEL, 1962), did result in a decrease in the concentration gradient formed. Incubation in the presence of phospholipase A brought about marked inhibition of respiratory activity of the tissue (20 per cent of the control) and would result in eventual disintegration of the slice if incubation were prolonged, (PETRUSHKA, QUASTEL and SCHOLEFIELD, 1959) yet did not appreciably alter the concentration gradient of calcium formed in 60 min.

TABLE 1.—THE EFFECT OF INCUBATION CONDITIONS ON THE RESPIRATION AND ACCUMULATION OF $^{45}Ca^{2+}$ BY RAT CEREBRAL CORTEX

Incubated for 60 mins at 37° in medium containing 2 mM-$CaCl_2$, 5 mM-Glucose, 1 mM-$MgCl_2$, 10 mM-Tris-HCl pH 7. Gas phase 100% O_2. The data were derived as duplicate analyses from two to five separate experiments.

Additions	Ionic concentration (mM) K^+	Na^+	Q_{O_2}	$^{45}Ca_T^{++}$	$^{45}Ca_T^{++}/^{45}Ca_I^{++}$	Per cent control Ca_T^{++}
	5	145	10·1 ± 0·6	4·0 ± 0·4	2·0	100
	25	120	12·2 ± 0·6	3·6 ± 0·5	1·80	90
	55	90	15·9 ± 1·2	3·6 ± 0·3	1·80	90
	105	40	17·3 ± 0·3	3·9 ± 0·3	1·95	97
5 mM-sodium glutamate	5	145	17·6 ± 0·8	3·8 ± 0·1	1·90	95
10^{-4} M-ouabain	55	90	13·6 ± 0·2	3·7 ± 0·2	1·85	92
10^{-4} M-2,4-dinitrophenol	55	90	8·6 ± 0·9	3·8 ± 0·4	1·90	95
5 mM-sodium salicylate	55	90	10·1 ± 1·0	3·3 ± 0·3	1·65	82
phospholipase A*	55	90	3·1 ± 0·1	18·1 ± 0·5	1·81	91

* In phospholipase A experiments 1000 μg lyophylized enzyme per 100 mg wet tissue and 10 mM-$CaCl_2$ were employed.

In all of these experiments (Table 1) there was established an apparent concentration gradient of calcium between tissue and medium ranging between 1·8–2·0 except in the experiments with sodium salicylate where the gradient was 1·65 (82 per cent of control level).

To verify that steady-state conditions were established by the incubation procedure, the rate of accumulation of tissue calcium was examined at two concentrations of

TABLE 2.—THE RATE OF ACCUMULATION OF $^{45}Ca^{2+}$ BY THE CEREBRAL CORTEX OF THE RAT
Incubated at 37° in gas phase, 100% O_2. Medium containing 2 mM-$MgCl_2$, 10 mM-glucose, 55 mM-KCl, 90 mM-NaCl, 10 mM-Tris-HCl pH 7. The data are values from closely-agreeing duplicates.

Time in min	2 mM-Ca_I^{2+}			10 mM-Ca_I^{2+}		
	Ca_T^{2+}	Ca_T^{2+}/Ca_I^{2+}	Per cent Ca_T^{2+} 60 min	Ca_T^{2+}	Ca_T^{2+}/Ca_I^{2+}	Per cent Ca_T^{2+} 60 min
2	0·72	0·36	23	5·4	0·54	33
5	1·32	0·66	43	8·6	0·86	52
10	1·75	0·87	56	10·4	1·04	63
15	1·89	0·94	61	12·6	1·26	77
20	1·93	0·96	62	13·5	1·35	82
30	2·20	1·10	71	14·5	1·45	88
45	2·68	1·34	87	15·4	1·54	94
60	3·08	1·54	100	16·4	1·64	100
90	3·10	1·55	100	16·8	1·68	100
120	3·11	1·55	100	16·6	1·66	100

$^{45}Ca^{2+}$ and incubation was continued until equilibrium was attained. The results of these experiments (Table 2) show that 40–50 per cent of the total calcium taken up by the tissues is accumulated within the first 5 min of incubation and that equilibrium is reached by 60 min.

Since a wide range of respiratory activity had not changed the gradient of calcium accumulated, the effect of aerobic and anaerobic incubation was examined simultaneously on pooled samples of rat cerebral cortex incubated with different concentrations of $^{45}Ca^{2+}$. Under conditions of oxidative metabolism (Table 3), the respiratory

TABLE 3.—THE AEROBIC AND ANAEROBIC ACCUMULATION OF $^{45}Ca^{2+}$ BY THE CEREBRAL CORTEX OF THE RAT
Incubated for 60 min at 37° in medium containing 105 mM-KCl, 40 mM-NaCl, 1 mM-$MgCl_2$, 5 mM-Glucose, 10 mM-Tris-HCl pH 7. Values are the means of three separate experiments.

Ca_I^{2+}	Oxygen			Nitrogen	
	Q_{O_2}	$^{45}Ca_T^{2+}$	$^{45}Ca_T^{2+}/^{45}Ca_I^{2+}$	$^{45}Ca_T^{2+}$	$^{45}Ca_T^{2+}/^{45}Ca_I^{2+}$
1 mM	17·5	2·0	2·0	2·3	2·3
2 mM	16·3	2·6	1·3	2·6	1·3
5 mM	16·7	8·3	1·7	10·9	2·2
10 mM	13·0	13·3	1·3	18·5	1·8
20 mM	10·7	23	1·2	33	1·7
30 mM	9·7	32	1·1	48	1·6
60 mM	8·4	54	0·9	75	1·3
80 mM	8·3	67	0·8	71	0·9

activity of brain slices is decreased more than 50 per cent by high concentrations of calcium ions and the gradients of calcium accumulation decrease from 2·0 at 1 mM

Ca^{2+} to 0·8 with 80 mM Ca^{2+}. However an identical effect on gradient formation was obtained in experiments at very low oxygen tension. Under these 'anaerobic' conditions the gradients equalled or slightly exceeded those obtained with aerobically respiring tissue.

DISCUSSION

The experiments reported here indicate that the isolated rat cerebral cortex can accumulate calcium ions from the medium in excess of the concentration which could be expected if only the tissue fluid came into equilibrium with the medium. However this accumulation could not be attributed to the oxidative metabolism of the tissue as equal gradients were obtained at very low oxygen tension. In addition, conditions of incubation known to effect the active transport of other cations were without effect on calcium accumulation although the converse situation is not so (GARDOS, 1960). Sodium salicylate decreased the $^{45}Ca^{2+}$ concentration gradient, but the absence of effect of 2,4-DNP suggests that this effect of salicylate is more probably related to its ability to complex with ions (WHITEHOUSE, 1962) than its ability to uncouple oxidative phosphorylation.

When taken with the previous findings, the observed rate of accumulation of $^{45}Ca^{2+}$ in these experiments suggests that rat cerebral cortex possesses a high affinity for the binding of external calcium ions similar to that reported for the salivary glands (DREISBACH, 1962). DELUCA and ENGSTROM (1961) found a similar rate of accumulation of calcium by rat kidney mitochondria where a direct dependence upon oxidative phosphorylation was also not apparent.

A non-metabolically dependent binding of calcium ions has also been reported for kidney, liver and the major portion of the rat small intestine by SHACHTER, DOWDLE and SCHENKER (1960) although these authors have described an active transport mechanism for calcium ions at the pyloric end of the small intestine. The nature of the binding material in rat cerebral cortex is not known, but treatment of slices with a preparation of phospholipase A did not decrease the binding of these ions although considerable changes in the integrity of the tissue were apparent.

SUMMARY

The accumulation of $^{45}Ca^{2+}$ by rat brain cortex slices was investigated and found to exceed that accountable to diffusion alone. When the respiratory activity of the tissue was varied over a wide range (Q_{O_2}, 3–17) either by the addition of sodium glutamate, ouabain, 2,4-dinitrophenol, sodium salicylate, phospholipase A, or by alteration of the potassium to sodium ion ratio in the incubation medium, little alteration from control levels was observed in the calcium gradients. In addition the rate of calcium accumulation was rapid; over 40 per cent of the total amount was accumulated within the first 5 min of incubation. Incubation of brain slices in an atmosphere of nitrogen demonstrated concentration gradients of calcium which were equal to those obtained in control experiments where slices were simultaneously incubated in an atmosphere of oxygen. These findings suggest that calcium does not accumulate in rat brain slices by a metabolically dependent process but probably by a binding phenomenon similar to that reported for rat salivary gland.

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PART II

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VARIATIONS IN $(Na^+ + K^+)$-ATPase AND Mg^{++}-ATPase OF THE RICHARDSON GROUND SQUIRREL RENAL CORTEX DURING HIBERNATION

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Short Title: Changes in membrane ATPase during hibernation.

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INTRODUCTION

An important characteristic of hibernating mammals is that much cellular function can persist at body and organ temperatures which are quite inhibitory for non-hibernating species. For example, cold resistant neuromuscular transmission (15), nerve conduction (10), mitochondrial electron transport (3) and K^+ transport (16) have all been reported in a variety of hibernating species.

Because of its integral role in cation transport Willis and Li (17) studied the temperature dependence of renal cortical $(Na^+ + K^+)$-ATPase in several non-hibernators and in the hibernating Syrian hamsters (Mesocricetus auratus) and reported that there was both an increase in the specific activity of the $(Na^+ + K^+)$-ATPase, and that its activity was reduced less by the lowering of the experimental temperature in hibernators than in non-hibernating species; that is, it displayed "cold resistance" compared to its non-hibernating control. In addition Fang and Willis (11) showed that the increase in enzyme specific activity which occurred in hamster renal cortex $(Na^+ + K^+)$-ATPase was demonstrable during a period of cold adaption prior to hibernation and did not alter during the ensuing 50 days of hibernation at 5°C.

Because the increase in enzyme specific activity was not accompanied by an increase in the number of so-called enzyme "pumping sites" either during hibernation (11) or at low temperature (18), it was believed to originate in an increased efficiency of the enzyme system rather than an increase in enzyme concentration in the membrane.

We have examined this interesting hypothesis by determining the ratio

of pumping sites/enzyme activity for $(Na^+ + K^+)$-ATPase as well as some other biochemical characteristics of this system in renal cortex preparations from awake and hibernating Richardson ground squirrels (Spermophilus richardsonii) throughout a full cycle of non-hibernation/hibernation.

MATERIALS AND METHODS

The Richardson ground squirrels (Spermophilus richardsonii) were collected in the field in small traps during May/June 1976. Quarantine periods, laboratory feeding and preparation for hibernation have all been described in another publication (7). The time the animals had been in hibernation was determined by the "sawdust" method described by Lyman (12) and used more recently by Fang and Willis (11).

Except for the awake control animals sacrificed during the summer months, animals were not sacrificed for tissue collection unless they had been in hibernation for at least six days in any hibernation cycle. Microsomal fractions rich in $(Na^+ + K^+)$-ATPase were prepared from the renal cortex of frozen kidneys by the homogenisation procedure employing a Brinkmann Polytron that we have described previously for ox brain preparations (9).

With renal tissue however, the supernatant from the 10,000 x g centrifugation was removed by Pasteur pipette to prevent agitation or decantation of the loosely packed mitochondrial pellet before resuspension and centrifuging the supernatant again at 48,000 x g for 30 mins. The homogenisation medium contained 250 mM sucrose, 30 mM L-histidine, 20 mM Tris-HCl and 2 mM H_4.EDTA, adjusted to pH 6.8 (4). The media for suspension and storage

of the enzyme preparation was similar to this buffered solution but L-histidine was now omitted and the pH was adjusted to 7.6 rather than 6.8.

Enzyme activity was determined by the coupled optical assay procedure we have employed previously (9,14) except that activation of $(Na^+ + K^+)$-ATPase by cations was always measured in addition to inhibition of the system with the cardiac glycoside ouabain (7). The reaction mixture which contained 1.5 mM Tris-ATP and 0.78 µM NADH was temperature equilibrated in the spectrophotometer cuvette before the reaction was started by the addition of 20 µl of enzyme suspension. Reaction velocities were determined over periods of about 10 min.

Cation activation, ouabain inhibition and units of enzyme specific activity have all been described previously (8) as have the methods for determining the initial rates of enzyme activity, its temperature dependence, the rate and amount of binding of [^{3}H]-ouabain to the membrane and the computer assisted analysis of the data (5,9).

Glycylglycine, L-histidine, lactic dehydrogenase, ouabain, pyruvate kinase, Tris-ATP and tricyclohexylamine salts of phospho(enol) pyruvate and NADH were all obtained from the Sigma Chemical Company, [^{3}H]-ouabain was obtained from New England Nuclear Corporation and sucrose (Analar grade) and H_4;EDTA from British Drug House Chemicals Ltd.

RESULTS

Much previous work on the renal cortical $(Na^+ + K^+)$-ATPase of hibernators has been done with relatively crude homogenates of the kidney of Syrian hamster (Mesocricetus auratus) which like the non-hibernating rat

is reported to be remarkably insensitive to inhibition by the cardiac glycoside ouabain (17). We therefore began our study by examining the ouabain sensitivity of our microsomal preparations of $(Na^+ + K^+)$-ATPase from the renal cortex of both awake and hibernating Richardson ground squirrels so that both technical errors and possible seasonal variations in this important characteristic could be eliminated.

From the data presented in Fig. 1 it is clear that 100% inhibition of renal cortical $(Na^+ + K^+)$-ATPase of the Richardson ground squirrel is achieved with concentrations of ouabain near 5×10^{-6}M and that the K_i for 50% inhibition by this agent at 37° occurs near 5×10^{-7}M for both awake and long-term (> 75 days) hibernating animals. Dose response curves obtained with enzyme preparations from animals after short-term hibernation (< 30 days) were not significantly different from the controls (n = 4) as the mean values for K_i were 3.4 ± 0.6 and $5.0 \pm 0.8 \times 10^{-7}$M from two separate experiments respectively. The data for short-term (< 30 days) hibernation is not shown in Fig. 1 for clarity of presentation, but the data for summer controls and long-term (> 75 days) in hibernation is given in full. Thus unlike the Syrian hamster (17) the Richardson ground squirrel renal cortex $(Na^+ + K^+)$-ATPase is just as sensitive to inhibition by ouabain as that previously reported for the non-hibernating New Zealand white rabbit or common laboratory guinea pig (6). The sensitivity of the kidney enzyme is also very similar to that from the brain of these animals which we reported recently (7).

Because our sensitive optical enzyme procedure (9,14) permits the simultaneous determination of both "cation stimulated" $(Na^+ + K^+)$-ATPase

as well as "ouabain-inhibitable" ($Na^+ + K^+$)-ATPase, it was possible to examine both these characteristics of the various enzyme preparations under any experimental conditions. By definition it was also possible to obtain values for the non-sodium stimulated ouabain-insensitive "basal" or Mg^{++}-ATPase which was present in these microsomal enzyme preparations, as the membranes were deliberately not "activated" by further treatment with either detergents or chaotropic agents. Under our assay conditions cation activated ATPase always equalled that determined by inhibition by 0.4 mM ouabain. Both this data and the seasonal variation in the specific activity of these enzymes is shown in Table 1, where it can be seen that there is a significant decrease ($p < .001$) in the activity of ($Na^+ + K^+$)-ATPase during hibernation (both short-term and long-term groups) which is not paralleled by a similar decrease in Mg^{++}-ATPase. On the contrary, after 75 days of hibernation the activity of Mg^{++}-ATPase was more than twice that of either the summer control levels or that found after relatively short periods of hibernation ($p < .05$).

These results are in marked contrast to those reported by Fang and Willis (11) for the cold adapted and hibernating Syrian hamster renal cortex.

Because decreased ($Na^+ + K^+$)-ATPase activity could result from either a generalised reduction in the amount of enzyme protein during hibernation or from a change in the efficiency by which the enzyme carries out its biochemical function, we first studied these alternative possibilities by determining the number of ouabain-inhibitable "pumping sites" by measuring the amount of [^{3}H]-ouabain binding to the enzyme preparations from awake and hibernating animals. Our results are given in Table 2 where it can be

seen that although both the rate and amount of [^{3}H]-ouabain bound to the enzyme preparation falls during hibernation, this fall closely parallels the loss in enzyme specific activity that was reported in Table 1. In addition, the values for [^{3}H]-ouabain binding that were obtained at equilibrium with a concentration of 5×10^{-7}M ouabain under our normal experimental conditions accurately reflects the total number of binding sites available since a separate Scatchard analysis (13) from 25 experiments obtained with various concentrations of ouabain between 5×10^{-8}M and 5×10^{-7}M gave a mean value of 111.6 ± 0.4 pico moles [^{3}H]-ouabain/mg enzyme protein which is not significantly different ($p > .01$) from the 123.1 ± 6.9 pico moles [^{3}H]-ouabain/mg enzyme we reported in Table 2. Because the ratio of ouabain binding sites/enzyme specific activity remains constant whether the animal is hibernating or not, these results strongly suggest that the loss of ($Na^+ + K^+$)-ATPase activity of the renal cortex of the ground squirrel which is seen during hibernation is not due to a change in functional ability of the enzyme, but is probably due to a loss of enzyme protein under these conditions.

However, another approach to the determination of the thermodynamic efficiency of an enzyme process is to measure its energy of activation (Ea) and as this is often achieved by the Arrhenius analysis of temperature dependence (1) it is particularly appropriate in this case.

Thus in a series of thirteen separate experiments, enzyme preparations from summer control, short-term and long-term hibernators were assayed for activity at 3° intervals between 8°-37°C and the resulting data subjected to the Arrhenius analysis and the computer assisted analysis we referred to

before (1,5,7). The results are given in Table 3 which shows that the temperature dependence of all these preparations was "non-linear" under all our experimental conditions. In addition, the calculated values for the apparent energies of activation for ATP hydrolysis by $(Na^+ + K^+)$-ATPase are given for those temperatures above (Ea_I) and those below (Ea_{II}) a single inflection temperature (T_c°) which is obtained from this type of analysis.

It is clear that no changes in any of these parameters occurred during hibernation and thus no changes in the thermodynamic efficiency of this enzyme function were detected by this approach. These results considerably strengthen our conclusion from the $[^3H]$-ouabain binding data given in Table 2 where it seems probable that a loss of enzyme protein occurs during hibernation, while that enzyme protein which remains functions in an unchanged way from that of awake control animals. These results do not support the idea of "cold resistance" developed by Willis and his colleagues using the Syrian hamster as a model for hibernation (11,17).

Furthermore, because the values for the energies of activation above and below the transition temperature are nearly identical to those previously obtained from non-hibernating species (5,9), neither is there evidence that the $(Na^+ + K^+)$-ATPase of the renal cortex of the hibernating ground squirrel functions at any time or temperature in a lipid membrane matrix inherently more fluid than that of comparable non-hibernating species.

In addition to examining the thermal response of ouabain-sensitive $(Na^+ + K^+)$-ATPase, our experiments permitted the concurrent study of ouabain-insensitive Mg^{++}-ATPase which was present in these renal cortex preparations to less than 15% of the total ATPase that could be measured at

37°C. Our results are given in Table 4 which shows that the Arrhenius characteristics of this enzyme are quite different from those of the $(Na^+ + K^+)$-ATPase.

Firstly, the results with awake animals are best described as a linear Arrhenius plot without a thermal transition, but having an apparent energy of activation even less than that seen above the thermal transition in the $(Na^+ + K^+)$-ATPase of the awake controls, i.e., about 12 kcals/mole compared to about 16 kcals/mole for the Ea_I of $(Na^+ + K^+)$-ATPase.

It is also clear that these linear characteristics do not change greatly during hibernation where the values for the apparent energies of activation are not significantly different from the awake controls. However, examination of the values obtained for T_c° are interesting in that the magnitude of the variations from the means that were obtained from either awake or short-term hibernators are such that little significance can be attributed to this calculated value. On the other hand, with enzyme preparations from long-term hibernators the mean value for T_c° was 19.1 ± 0.9°C, suggesting that it may not be merely a function of chance and that under these conditions the "linearity" of the Arrhenius plot may not be unequivocal! However, many more experiments than were possible in this series would be necessary before this could be resolved with confidence.

Finally, our data also permit an examination of any seasonal variation in the effect of temperature upon the ratio of $(Na^+ + K^+)$-ATPase/Mg^{++}-ATPase in the renal cortex membranes of the ground squirrel.

This information is given in Fig. 2 where the percentage of the total ATPase which is sensitive to inhibition by 0.4 mM ouabain (i.e., the ouabain

inhibitable ($Na^+ + K^+$)-ATPase) is plotted against temperature for awake summer control animals as well as after short-term and long-term hibernation.

It is clear from these results that the shapes of the various temperature curves do not change with hibernation, that is, the quality of the temperature effects remains unchanged, but that the amounts of activity are reduced after long-term hibernation. These results are in close agreement with the data presented earlier in this paper.

DISCUSSION

Working with an homogenate of Syrian hamster kidney, Willis and his associates reported that the cation transport enzyme ($Na^+ + K^+$)-ATPase developed a form of "cold resistance" which was manifest in greater activity at low temperatures during hibernation than was found with awake animals during a summer control period. As subsequent work showed that these changes did not involve the number of "phosphorylation sites" on the enzyme, they suggested this occurred through an increase in the efficiency of the enzyme (11,18).

During the course of another investigation we noted that the microsomal ($Na^+ + K^+$)-ATPase of the hibernating ground squirrel (Spermophilus richardsonii) renal cortex decreased sharply during hibernation, while that of its brain rose significantly (7). We therefore sought to re-examine the suggestion of Willis and his associates in some detail as resolution of these differences could have a great influence upon the underlying biochemical concepts of hibernation.

It is clear from the present study that the specific activity of renal cortical $(Na^+ + K^+)$-ATPase of the ground squirrel goes down during hibernation and that this effect can be seen after 30 days of hibernation at 5°C. Conversely, the Mg^{++}-ATPase of these preparations increases although this increase is only seen after more than 75 days of hibernation. Whether this constitutes a general finding amongst hibernating species and is therefore in conflict with the earlier reports of Willis and his associates, or whether this constitutes a major difference between the present ouabain-sensitive hibernating species and the comparatively ouabain-insensitive Syrian hamster employed by Willis et al. (11,17) cannot be determined from the present work.

However, there can be no doubt that in this species of ground squirrels the fall in enzyme activity during hibernation is exactly matched by the fall in the number of ouabain binding sites of the system - a finding which strongly suggests that it is a decrease in the amount of enzyme protein available for cation transport that occurs during hibernation rather than a change in the quality or efficiency of the enzyme.

This suggestion is strongly supported by our finding of unchanged thermal dependence and apparent energy of activation of $(Na^+ + K^+)$-ATPase of renal cortex during hibernation, but does not agree with the concept of development of a form of "cold resistance" which superficially resembles the cold resistance of poikilotherm enzymes at low temperatures (2,8,17).

In comparing our results with ground squirrel and those of Willis and his colleagues with Syrian hamster, one should remember that the hamster enzymes were prepared by a method involving their partial delipidation with

deoxycholate which could have removed a membrane component having a significant modulating action during hibernation. It is for this reason that neither detergents nor chaotropic agents were used in the present study.

It is therefore of great interest that the Mg^{++}-ATPase of these ground squirrel preparations display a form of thermal dependence which is almost identical to that reported by Willis and Li (17) for the Syrian hamster renal cortex, and also to that which we reported for the brain of hibernating ground squirrels in a recent publication (7). Unfortunately, while the role of Mg^{++}-ATPase in membrane function remains unknown, the biological significance of this result must also remain unclear.

However, neither the marked decline in renal cortical $(Na^{+} + K^{+})$-ATPase activity nor the two-fold increase in Mg^{++}-ATPase activity that was found during hibernation provide a ready explanation for the two-fold increase in K^{+} transport activity of kidney slices at 5°C which was observed by Willis (16) in tissues from hibernating hamsters compared to awake controls. Perhaps the major biochemical change in membrane transport function during hibernation lies elsewhere than in the $(Na^{+} + K^{+})$-ATPase activity of these systems?

SUMMARY

In contrast to some earlier reports of effects in Syrian hamsters (11,17) the specific activity of renal cortical ($Na^+ + K^+$)-ATPase is markedly reduced during hibernation in the Richardson ground squirrel, whereas the activity of Mg^{++}-ATPase is markedly increased. In addition, both the 'non-linear' thermal dependence of ($Na^+ + K^+$)-ATPase and the 'linear' thermal dependence of Mg^{++}-ATPase are also unchanged during hibernation indicating no change in the efficiency of the reaction under these conditions. Although the apparent sensitivity of ($Na^+ + K^+$)-ATPase to inhibition by ouabain is unchanged by hibernation (i.e. Ki), we found that the rate and amount of specific [^{3}H]-ouabain binding to these ($Na^+ + K^+$)-ATPase preparations falls significantly during hibernation. This fall closely parallels the fall in enzyme activity, so that the number of ouabain inhibitable "pumping sites"/unit of enzyme activity shows no seasonal variations. These findings strongly suggest that the amount of renal cortex ($Na^+ + K^+$)-ATPase enzyme protein falls during hibernation, but that the enzyme protein which remains functions with identical biochemical characteristics to that of awake summer animals. Hence there is no evidence in the Richardson ground squirrel for the development of the type of "cold resistance" reported by Willis et al. for the tissues of the Syrian hamster.

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FIGURE LEGENDS

Fig. 1: Dose response curves for ouabain inhibition of $(Na^+ + K^+)$-activated ATPase from summer (o) and hibernating (□) ground squirrel renal cortex preparations. Concentrations of ouabain giving 50% inhibition of ATPase activity (K_i) were $5.0 \pm 0.8 \times 10^{-7}$M and $4.8 \pm 0.8 \times 10^{-7}$M for the summer and hibernating kidney preparations and represent the mean ± standard error from four experiments in each group. 100% inhibition of ATPase activity is indicative of complete inhibition of the enzyme activity stimulated by the addition of 80 mM Na^+ to the reaction medium containing 20 mM K^+.

Fig. 2: Seasonal variation in the percentage of ouabain sensitive ATPase in the total ATPase from renal cortex preparations from summer (o), < 30 day hibernating (Δ), and > 75 day hibernating (▨) Richardson ground squirrels. The results are expressed as the means ± S.E. from five, three and five separate enzyme preparations respectively.

TABLE 1: SEASONAL VARIATION IN THE SPECIFIC ACTIVITY OF OUABAIN-SENSITIVE $(Na^+ + K^+)$-ATPase AND OUABAIN-INSENSITIVE Mg^{++}-ATPase OF GROUND SQUIRREL RENAL CORTEX

Condition of Animals	n	Specific Activity*		
		$(Na^+ + K^+)$-ATPase		Mg^{++}-ATPase
		Ouabain-Sensitive	Na^+-Stimulated	Ouabain-Insensitive
Awake controls	7	33.6 ± 1.9	31.5 ± 1.8	5.05 ± 0.61
< 30 days in hibernation	7	12.0 ± 1.6	11.3 ± 1.8	4.0 ± 0.65
> 75 days in hibernation	7	13.3 ± 1.6	10.4 ± 1.5	10.83 ± 2.44

* S.A. given as μmoles Pi liberated from ATP/mg enzyme protein/hour at 37°.

TABLE 2: SEASONAL VARIATION IN THE RATE AND AMOUNT OF [^{3}H]-OUABAIN BINDING TO RENAL CORTEX ($Na^+ + K^+$)-ATPase PREPARATIONS FROM THE GROUND SQUIRREL

Condition of Animals	n	Rate [^{3}H]-ouabain Binding	Amount [^{3}H]-ouabain Binding	Ouabain Binding/Specific Activity*
Awake controls	5	2.53 ± 0.24	123.1 ± 6.9	3.82 ± 0.14
< 30 days hibernation	5	1.25 ± 0.32	46.9 ± 8.4	3.64 ± 0.17
> 75 days hibernation	3	1.31 ± 0.23	53.6 ± 14.4	3.94 ± 0.26

All experiments carried out at 37°C according to the procedures described in detail in reference (9). Values for [^{3}H]-ouabain binding are given in pmoles/mg enzyme protein/sec for the rate and pmoles/mg enzyme at equilibrium for the amount.

* Specific activity is in µmoles Pi liberated from ATP/mg enzyme protein/hour at 37° and is taken from the particular experiments cited in Table 1 which were used for the determination of both enzyme activity and [^{3}H]-ouabain binding.

TABLE 3: SEASONAL VARIATION IN THE APPARENT ENERGIES OF ACTIVATION, TRANSITION TEMPERATURE AND ARRHENIUS ANALYSIS OF OUABAIN-SENSITIVE $(Na^+ + K^+)$-ATPase OF THE GROUND SQUIRREL RENAL CORTEX

Condition of Animals	n	Ea_I	Ea_{II}	T°_c	Arrhenius Analysis
Awake controls	5	15.7 ± 1.3	31.7 ± 2.4	20.4 ± 0.6	non-linear
< 30 days hibernation	3	14.0 ± 1.6	27.4 ± 2.9	20.8 ± 2.5	non-linear
> 75 days hibernation	5	16.2 ± 1.1	30.7 ± 1.8	19.8 ± 0.9	non-linear

Values for the apparent energies of activation are given as kcals/mole and are the means ± standard errors of the individual experiments.

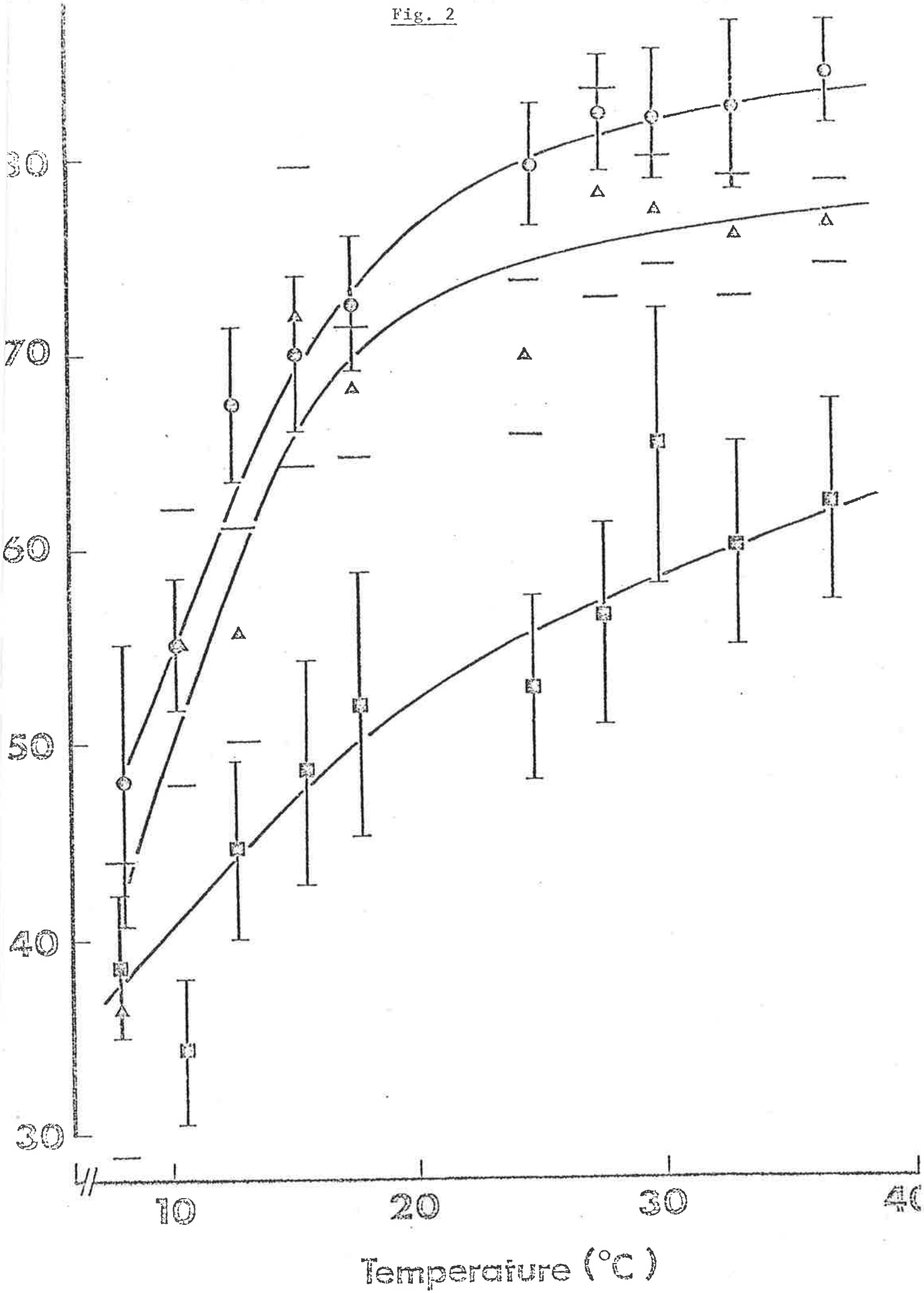
TABLE 4: SEASONAL VARIATION IN THE APPARENT ENERGIES OF ACTIVATION, TRANSITION TEMPERATURE AND ARRHENIUS ANALYSIS OF OUABAIN-INSENSITIVE Mg^{++}-ATPase OF THE GROUND SQUIRREL RENAL CORTEX

Condition of Animals	n	Ea_I	Ea_{II}	T°_c	Arrhenius Analysis
Awake controls	5	11.2 ± 0.9	12.2 ± 1.1	19.8 ± 5.7	linear
< 30 days hibernation	3	13.6 ± 0.6	12.6 ± 1.6	19.6 ± 16.6	linear
> 75 days hibernation	5	10.3 ± 0.7	16.2 ± 1.6	19.1 ± 0.9	non-linear?

Values for the apparent energies of activation are given as kcals/mole and are the means ± standard errors of the individual experiments.

(%) inhibition of (Na^+K^+)-dependent ATPase
0
20
40
60
80
100

Fig. 2



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PROTEIN-LIPID INTERACTIONS IN THE EFFECTS OF TEMPERATURE ON $(Na^+ + K^+)$-ATPase: AN ESR STUDY

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Suggested Running Title:

Temperature sensitivity of spin labelled ($Na^+ + K^+$)-ATPase.

SUMMARY

In a previous study of ox brain membranes rich in $(Na^+ + K^+)$-ATPase, we have described two binding sites for the spin probe M 12-NSE. These sites, designated according to the mobility of the probe as unrestricted or restricted, have now been examined for their thermal sensitivity.

A linear Arrhenius plot is obtained from M 12-NSE at unrestricted sites in untreated and detergent extracted membranes. By contrast label at restricted sites generates a non-linear response with an inflection point at about 22° for both untreated and detergent extracted membranes.

ESR data obtained from detailed studies carried out at 1°C intervals from 2°-37° reveal more than one discontinuity. These multiple discontinuities are progressively reduced by extraction with detergents.

Liposomes made from total lipid extracts of these membrane preparations do not show multiple discontinuities, but give a single inflection point at about 20°C. Hence the membrane protein is implicated in the organisation of the membrane phospholipids.

When examined in similar detail the $(Na^+ + K^+)$-ATPase

activity of these preparations was also non-linear with a strong suggestion of multiple discontinuities at similar temperatures to those described by the spin probe. Extraction with detergents did not change the results.

We conclude that protein-lipid interactions in the membrane can lead to the formation of fluid domains or lipid clusters which influence the thermal behaviour of the membranes and the membrane bound enzyme $(Na^+ + K^+)$-ATPase.

INTRODUCTION

Since the early work of Tanaka and Abood [1] and Tanaka and Strickland [2], the involvement of membrane lipids in the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase has become well documented. Of the many experimental procedures which are used to evaluate this, the use of the Arrhenius analysis [3] of thermal effects upon the rate of the reaction is common. Under many experimental conditions, the Arrhenius plot of $(Na^+ + K^+)$-ATPase is non-linear, yielding a markedly higher activation energy below and a lower activation energy above a critical inflection temperature [4].

This inflection has been associated with a thermal transition in the physical properties of the membrane lipids in which $(Na^+ + K^+)$-ATPase protein is embedded [5,6]. However, it is not clear whether a single lipid species is directly responsible for this phenomenon, or whether the non-linearity is a consequence of a more

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Abbreviations: ESR, electron spin resonance spectroscopy; M 12-NSE, N-oxyl-4',4' dimethyloxazolidine-12-keto methyl stearate.

widespread physical alteration of the bulk lipids of the membrane. While attempts to analyse the lipid constituents of highly purified enzyme preparations have failed to implicate a single phospholipid component [7,8], reconstitution of the enzyme activity has been achieved in several phospholipid liposomes [7,8,9]. Recent work by Palatini et al [10] demonstrates that non-linear Arrhenius plots are obtained when guinea pig kidney $(Na^+ + K^+)$-ATPase was reconstituted into dimyristoyl phosphatidyl glycerol (DMPG) liposomes, but not when reconstitution occurred in dioleoyl phosphatidyl glycerol (DOPG) systems. Although the variations in the thermal inflections which have been reported for $(Na^+ + K^+)$-ATPase enriched membranes are quite wide, for example at 15° by Bowler and Duncan [11] and 31° by Kimelberg and Papahadjopoulous [12], most workers report values near 20°C for their preparations. It seems quite likely that these variable values represent phase transitions or phase separations of the membrane lipids which in turn are greatly influenced by both the source of enzyme material and its method of isolation.

Failure to detect more than one transition in any

one preparation could arise from the limitations of the methods employed to determine thermal effects, with biophysical procedures such as electron spin resonance and fluorescent probes [13,14] being far more sensitive than the less direct measurement of enzyme activity. However, such multiple changes in temperature sensitivity of other membrane enzymes have been reported by Raison and McMurchie [15] and by Wisnieski et al. [16].

We have recently demonstrated [17] that ox brain microsomes enriched with $(Na^+ + K^+)$-ATPase exhibit two distinct populations of binding sites for the lipid spin probe M 12-NSE. When labelled with M 12-NSE, one population of sites produces a sharp spectrum which is characteristic of a highly mobile nitroxyl, which we have described as "unrestricted". The second population has a broader spectrum suggesting that the nitroxyl is not as free, and has been designated as "restricted". Restricted and unrestricted sites are quite distinct in their sensitivities to ascorbic acid, and possess different affinities for the spin label. Furthermore, the proportion of restricted sites increases with the specific activity of the enzyme as it is purified. We have suggested

that this may imply the concentration of a specific lipid species during purification. In this present work we have explored this possibility by examining the effects of temperature on the mobility of the spin probe M 12-NSE in $(Na^+ + K^+)$-ATPase-enriched membranes before and after extraction with sodium deoxycholate and sodium dodecyl sulfate, two detergent treatments which are known to increase the specific activity of the enzyme.

MATERIALS AND METHODS

Microsomes and Liposomes

The preparations, delipidation procedures and biochemical assay of the $(Na^+ + K^+)$-ATPase-enriched ox brain microsomes have been described recently by Charnock et al. [18].

Liposomes were made from dimyristoyl phosphatidyl choline, or from ox brain phosphatidyl serine (Serdary Research Laboratories, Ontario) or from chloroform: methanol total lipid extracts of ox brain microsomes. The lipids, contained in a buffered sucrose solution (0.25 M sucrose, 5 mM Na_2-EDTA, pH 7.6 with Tris) were sonicated in a Cole-Parmer Ultrasonic Cleaner Model

884S-4, until a clear solution was obtained.

The spin label, a methyl ester of stearic acid with the nitroxyl on the 12-C position (M 12-NSE), was a gift from Dr. J.K. Raison, Macquarie University, Sydney, Australia. The spin labelling procedure and the ESR instrumentation have been described in detail previously [17]. In addition, some spectra were obtained using a Bruker B-ER 420 spectrometer coupled to a Varian Magnet VFR-2408. This spectrometer is equipped with a Bruker temperature control unit Model B-ST 100/700, which employs a chrome-alumel thermocouple. The temperature of the sample was maintained to within 0.1° with this accessory which permitted the very detailed temperature studies referred to later in this paper.

A number of methods are currently available for the quantitation of ESR spectra. McConnell [19], Kivelson [20] and Freed and Fraenkel [21] have developed mathematical methods for the calculation of rotational correlation time (T_c) for paramagnetic species. These approaches are only applicable to rapid motion, such as that seen as isotropic spectra from spin probes in solution. By assuming Lorentzian line shapes, and with appropriate

algebra, Reswick and Mortimer [22] have derived the expression which can be used to quantitate data from rapidly rotating spin labels. A convenient form of this expression is the tumbling time (To) which is given below:

$$To = 6.5 \times 10^{-10} Wo[(h_o/h_{-1})^{\frac{1}{2}}-1] \quad s$$

where W is line width, h is line height and the subscripts +1, 0, -1 refer respectively to the low, mid and high field lines. This expression finds application for relative and empirical measurements [23].

On the other hand, the ESR spectra of fatty acid spin probes in lipid bilayers are often indicative of anisotropic motion [24,25]. Other methods that have also been used for broad spectra make use of one or more of the spectral peak heights and although they are only semiquantitative, these latter simple approaches can be very useful in the interpretation of ESR spectra obtained from spin labelled biomembranes. The spectra we have described in this report are essentially isotropic with tumbling times not in excess of 5×10^{-9}M, and consequently can be readily analysed by the method of Raison et al. described above [23]. However, we have observed that the

effects of temperature on the motional freedom of the nitroxyl results in a marked reduction in the intensity of the high field peak (h_{-1}). In some cases the effect of temperature is to reduce this peak to a degree where accurate measurements cannot be obtained. For this reason peak height ratios of the low- and mid-field peaks, h_o/h_{+1}, have also been used.

RESULTS

Quantification of ESR spectra

It is well known that liposomes made from synthetic phospholipids will readily incorporate spin labels with the long axis of the spin probe oriented perpendicular to the plane of the bilayer [24-27]. The spectral line shape which is obtained varies depending on the depth to which the reporter group is embedded into the lipid bilayer.

The spectrum obtained from dimyristoyl phosphatidyl choline liposomes labelled with M 12-NSE is given in Fig. 1. Typical spectra obtained at three different temperatures are presented to demonstrate that as the temperature is lowered all three spectral peaks undergo

a reduction in height and an increase in width which indicates a loss of mobility of the probe due to an increase in rigidity of the methylene chains of the membrane lipids.

However, it is clear from the limited data shown here that in such temperature studies the broadening of the high-field peak (h_{-1}) which occurs will eventually lead to the loss of this peak from the spectrum. On the other hand, changes in the height of the mid-field line were usually much less and accurate measurements could be obtained throughout the entire temperature range. Therefore peak height ratios of h_o/h_{-1}, or h_o/h_{+1} could be obtained with varying degrees of reliability over the whole temperature range to be studied. In addition, values for the tumbling time T_o could be obtained by the method of Raison et al. referred to above [23].

In this experiment with DMPC liposomes there is a gradual change between 37°-23° below which temperature the change becomes much more rapid. This pattern is shown by all the parameters we examined. The data that could be obtained from these various parameters are given in Table 1 as are the calculated values for the

critical temperature (T_c°) which were obtained. It is clear that all methods of quantification which were employed gave very similar values for T_c°, and that the mean value for 23.5°C for the onset of a phase transition in DMPC liposomes is not appreciably different from that reported by Shimshick and McConnell for liposomes of highly purified material [28].

Thus it is apparent from these results that M 12-NSE is a suitable probe for the study of temperature dependent physical changes in lipid membranes, and that the different methods used to quantify the spectral changes yield very similar results which may be regarded as interchangeable for our experimental purposes.

Because biological bilayers contain mixtures of phospholipids, the transition slopes that are obtained are more gradual (and spread over a wider range of temperature) than are those found with single phospholipid systems [28]. Thus although the physical properties of biomembranes might be similarly influenced by temperature, a sudden change like that described in the inset to Fig. 1 seems highly unlikely. Notwithstanding, the Arrhenius analysis is often used to examine the effects of temperature on

membranes, as an inflection point can be derived from non-linear plots, which although not a precise estimate of the onset of a phase transition, it has been frequently used as a measure for comparative studies. As will be seen later, the number of temperature points that are fed into the Arrhenius analysis, and the way that this data is analysed can have very marked effects upon the interpretations which are drawn from these experiments.

M 12-NSE labelling of $(Na^+ + K^+)$-ATPase membranes

Unrestricted sites: When unrestricted sites on ox brain microsomal preparations of $(Na^+ + K^+)$-ATPase are labelled the spectrum obtained is similar to that seen with the nitroxyl spin labels in solution [17]. Although our existing information does not precisely locate these very fluid sites in the membrane, one possible location we have suggested is the surface of the bilayer. Such a situation may imply specificity of the phospholipid head group at these restricted sites which in turn could influence the transition temperature [28].

We have therefore examined the effects of temperature on M 12-NSE labelling of unrestricted sites of ox brain microsomal preparations of $(Na^+ + K^+)$-ATPase both

before and after extraction with detergent as such treatment is known to greatly influence the activity of this enzyme. As the sample is cooled there is a marked change in the intensity of the high-field peak relative to both the low- and mid-field peaks. In a typical experiment this amounts to a change of 30% in the h_o/h_{-1} peak ratios (from 1.5 to 2.0 between 5°-37°). This change corresponds to a three-fold difference in the tumbling time T_o which can be calculated (1.1×10^{-10} to 3.6×10^{-10}s). An Arrhenius plot of the peak height ratio h_o/h_{-1} is given in Fig. 2 which shows that there is no discontinuity in the temperature range examined.

These results are not changed by extraction of the membranes with sodium deoxycholate although this treatment is known to remove membrane lipids and to result in a marked increase in the specific activity of the enzyme. However, the thermal dependence of enzyme activity is <u>not</u> altered by this treatment [6,18].

Thus while the magnitude of the change in temperature dependent spectral characteristics of unrestricted sites of M 12-NSE labelled microsomes is large, it does not appear that the label in this position reports any

thermal transitions which could be attributed to changes in the physical properties of membrane lipids.

Restricted sites: Recently we have demonstrated that M 12-NSE can be bound at sites on ox brain microsomes which greatly restrict the motional freedom of the probe, and which sites increase with increasing purification of the $(Na^+ + K^+)$-ATPase activity of the microsomal enzyme [17]. We have speculated that this correlation might mean selective retention of some specific lipid or lipid array during the purification procedures. To further examine this possibility we have studied the effects of temperature on M 12-NSE labelled restricted sites of ox brain microsomes before and after extraction with detergents.

As can be seen from Fig. 3, the spectrum of the spin probe M 12-NSE bound to the restricted sites of ox brain microsomes is distinct from the spectrum of a nitroxyl in solution or when bound to the more fluid unrestricted sites of the membrane. Although the three peaks of an isotropic spectrum are still present, the peaks tend to be much broader than those seen from unrestricted sites. As the temperature of the sample

is lowered the intensity of the spectrum decreases and the broadening of the peaks is accentuated. In some experiments this becomes so marked in the high-field peak that the measurement of h_{-1} is no longer accurate. For this reason the ratio of h_0/h_{+1} which can be incorporated into a formula for the determination of tumbling time (T_o), was used to evaluate the spectral changes found in this series of experiments.

Fig. 4 gives the Arrhenius plots that were obtained for these ratios from untreated and detergent extracted enzyme preparations. Unlike the plots obtained for M 12-NSE labelled unrestricted sites, it is very clear that none of the present plots are linear. By using the computer assisted analysis we have employed before [4] we can calculate values for the statistically determined single inflection points (the so-called critical temperatures) which occur near 22°C in all experimental conditions. The value for these critical temperatures and the slopes of the plots above and below this single inflection point are very similar before and after detergent treatment. This finding exactly parallels the effects of these detergents upon the thermal

dependence of $(Na^+ + K^+)$-ATPase activity, but does not provide unequivocal evidence in support of the concept of increasing lipid specificity with increasing purification of the enzyme.

However, it is possible that in studies such as these which rely upon a limited number of data points within the temperature range, and a method of computer analyses biased in favour of a single inflection point, that secondary and possibly less marked transitions might become obscured. We have therefore repeated these studies of temperature effects in much greater detail by using many more experimental points and have submitted the resulting data to a variety of methods of analysis.

In order to obtain mean values from many separate experiments the data were normalized to a constant ordinate and plotted as simple ratios of peak height against temperature. Fig. 5 shows our results from four experiments with untreated microsomes. As was seen before, it is clear that a plot with two straight lines intersecting at about 20°C can be drawn; it would however be an inadequate description of these results which reveal three characteristic temperatures at about 30°, 20° and 10°C.

Although there is an interruption which is seen as a step between 28°C and 30°C, there is no change in the slope of the line between 22°C and 37°C. That is, there is no alteration in the rate at which mobility of the probe changes with temperature in this range. On the other hand, at 20°C there is a very marked step in the reaction which is accompanied by a distinct change in the slope of the line between 10-20° compared to that above this point. Clearly this reflects a temperature range in which the mobility of the probe is markedly reduced - and provides the basis for the two-state concept described in our earlier studies with a limited number of data points.

In addition, at about 10°C there is now an even more noticeable inflection point indicating a very marked loss in probe mobility below this temperature. This temperature probably indicates the end of a phase transition and the beginning of a phase with rigid extended fatty acid chains. Shimshick and McConnell have described this phenomenon as the end of the contiguous F phase in which the lipids are still fluid and the beginning of the S phase in which the fatty acid chains are much stiffer [28].

Detergent treated preparations were now examined in

similar detail. Plots of the normalized mean data against temperature are shown in Fig. 6. The data obtained after extraction with sodium deoxycholate is now significantly different from that obtained with untreated enzyme preparations. The changes in the slope of the line are now reduced in magnitude, the critical temperature is a few degrees higher than that seen previously and the step between 27-30° is no longer apparent. The marked discontinuity at 10°C is however still apparent. Collectively, the changes in the plot obtained after extraction with sodium deoxycholate suggest an increased ability of the membrane, when compared to that of untreated microsomes, to accommodate the restricting effects of cooling.

This tendency is even more marked in the data obtained from sodium dodecyl sulfate treated microsomes. Cooling of the labelled microsomes from 37° down to 16°C now causes no interruption in the rate of change of probe mobility either in the form of a step or a change of slope in the plot. Below this temperature there is a small but detectable increase in the slope of the line which remains the same throughout the remainder of the temperature range. That is, there are only two states of

of the membrane probe after treatment with sodium dodecyl sulfate in agreement with our earlier finding with a limited number of data points. However, inspection of the present data suggests an inflection point about 18°C rather than at 22°C as reported in the experiments described in Fig. 4.

M 12-NSE labelling of liposomes

There can be little doubt from this type of study that treatment of the membrane with either sodium deoxycholate or sodium dodecyl sulfate alters the sensitivity of the membrane lipids to changes in temperature. To investigate this further we examined the effects of temperature on spin labelled liposomes made from extracts of the total lipids of the microsomes obtained both before and after extraction with detergents. For comparison we also labelled liposomes made from ox brain phosphatidyl serine as this phospholipid has been strongly implicated in the modulation of $(Na^+ + K^+)$-ATPase activity and temperature dependence [29].

Our results are shown in Fig. 7 where it can be seen that there is a gradual increase in peak height ratio as the temperature is lowered. For both the lipids

of untreated microsomes and for liposomes made from ox brain phosphatidyl serine, this variation with temperature is readily described by two straight lines having significantly different slopes with intersection points at 20° and 16°C respectively.

For liposomes made from total lipid extracts of detergent treated microsomes, there is much less difference in the slopes of the lines above and below an inflection point at 20°C.

Of even more importance is the observation that the multiple discontinuities of untreated microsomes are reduced to a single inflection point in liposomes made from extracts of these preparations. Similarly the liposomes made from lipid extracts of detergent treated microsomes also differ from their parent preparations. The loss of these inflection points strongly suggest a powerful co-operative effect by the enzyme protein on the physical behaviour of the lipid bilayer.

Effects of temperature on $(Na^+ + K^+)$-ATPase

As these detailed electron spin probe studies have revealed hitherto undefined thermal characteristics in the behaviour of $(Na^+ + K^+)$-ATPase containing membranes,

we have re-examined the activity of the enzyme in the same detail by determining changes in ouabain-sensitive enzyme activity at 1°C intervals between 6°-37°C. Both untreated and detergent extracted preparations were studied; the results are given in Fig. 8. In general the data from all three types of enzyme preparations are remarkably similar; in no instance can it be fitted adequately by a single straight line as there is a noticeable discontinuity at about 18°C.

Using the computer analysis for a two line fit [4] we have determined the apparent activation energies for the hydrolytic activity of the enzyme above and below the critical temperature. The values of about 14 kcals/mole above and about 30 kcals/mole below T_c° are very similar to the values we obtained previously in more limited studies [18]. It is possible that other discontinuities which might correspond to the temperature characteristics observed in the electron spin probe studies might also exist in this data but are obscured by the method of analysis.

Using the method employed by Bashford and Radda [30] all three plots can be shown to depart from linearity at

several temperatures. There is a major step at 30°C, a clear discontinuity with a change of slope at about 18°C and a further point of change in the lower temperature ranges which is difficult to describe as our experiments could not be carried out below 6°C because of technical limitations in the instruments.

Thus although in some instances (after extraction with sodium dodecyl sulfate) there are inflection points in the parameters of biochemical function which are not found in the physical properties of the membranes or their lipid extracts, in general the experiments described above continue to reveal good correlation between the physical properties of the membrane lipids and the biochemical activity of an integral enzyme protein.

Because detergent extraction of biological membranes frequently results in significant amounts of residual detergent in the preparations we checked our preparations after extraction with ^{14}C-sodium deoxycholate and ^{35}S-sodium dodecyl sulfate. By using a value of 250,000 for the molecular weight of the $(Na^+ + K^+)$-ATPase trimer [29] we were able to calculate that there were less than 1.5 moles of residual sodium deoxycholate per mole ATPase in

our preparation. It seems unlikely that this could significantly alter the temperature response of the system. Conversely, the residual sodium dodecyl sulfate was much higher; 150 moles/mole ATPase and this amount of detergent may significantly influence membrane behaviour. It is well to remember that after treatment with sodium dodecyl sulfate the thermal response of the enzyme shows a sharp discontinuity near 20°C which is not seen in the ESR data [18]. It is possible that the residual sodium dodecyl sulfate may be acting as a significant fluidizer of the membrane lipids in general. However, an examination of the cholesterol content of the total lipid extract of all three types of membranes (untreated, sodium deoxycholate or sodium dodecyl sulfate extracted) by a modified Liebermann-Burchard reaction [31] also shows that detergent treatment reduced the cholesterol content to 14% and 11% respectively after sodium deoxycholate or sodium dodecyl sulfate, whereas the untreated controls gave values of 16%. Whether this decreased cholesterol content coupled to greater residual detergent we detected accounts for the loss of correlation in results after sodium dodecyl sulfate treatment cannot be determined from the present data.

DISCUSSION

In this study we have investigated further differences in behaviour of the two binding sites for M 12-NSE in ox brain membrane preparations of $(Na^+ + K^+)$-ATPase which we have described previously [17]. The response of the electron spin probe to changes in temperature is quite different at the two sites.

The linear response of M 12-NSE at the so-called unrestricted sites lends further support to our suggestion that this site is inherently very fluid and is probably at or near the membrane interface rather than deep within the membrane core. Wherever it is located, it is not influenced by phase transitions within the membrane lipids.

On the other hand, ox brain microsomes labelled with M 12-NSE at restricted sites reveal multiple temperature dependent inflections in a variety of preparations, particularly when these are examined in studies with maximum data input and minimal bias in the analysis. For example, there is good evidence that at least three such temperature points exist in microsomal preparations which have not been subjected to extraction by detergents.

Similar studies of enzyme activity reveal excellent correlation in this regard and support the general conclusions of Grisham and Barnett [13] and Charnock and Bashford [14] who employed different membrane probes. However, neither of these latter studies was in such detail nor did they reveal the multiple thermal transitions revealed here.

As detergent extraction is applied to these preparations this level of correlation declines as some of the thermal effects upon the enzyme protein cannot be detected in either the detergent extracted microsomes or particularly in liposomes of the total lipid extracts of these preparations.

This is particularly noticeable after treatment with sodium dodecyl sulfate which however leaves about 10 times the amount of residual detergent in the membranes than does treatment with sodium deoxycholate. There can be no doubt that treatment with either detergent results in loss of total lipid and loss in cholesterol. While the result is a somewhat more fluid matrix for enzyme function which corresponds to a marked increase in enzyme specific activity we cannot unequivocally determine

whether there is a retention of a specific lipid(s) essential for enzyme function, but the circumstantial evidence is strong. When it is recalled that brain tissue which is rich in $(Na^+ + K^+)$-ATPase also contains proportionally higher concentrations of fatty acids with 18-C chains [32], it must be considered that the suggested concentration or retention of lipids discussed above may occur by preference for chain length with some unsaturation rather than by phospholipid head groups alone.

The apparent lack of correlation between the effect of temperature on the liposomes made from total lipid extracts of the enzyme preparations and their parent microsomes clearly suggests a very important ordering role for the protein on the lipid. Thus in addition to the selective retention of a lipid species by the protein we suggest that the $(Na^+ + K^+)$-ATPase macromolecule, which is known to span the membrane, also exerts a powerful co-operative influence on the surrounding assembly of phospholipids. After treatment with sodium dodecyl sulfate the spin probe molecules might become bound to areas of lipid which are unrelated to and uninfluenced by the thermal behaviour of the enzyme protein or their

phospholipid neighbors. Such an explanation would require migration of the spin labels from the binding sites in the untreated preparations since the latter clearly do reflect thermal changes in both the enzyme protein and in the phospholipids of the membranes. It seems more likely that in the sodium dodecyl sulfate treated preparations the fluid "pool" containing the spin probe is adjacent to the enzyme, and whereas the fluidity of this region is reduced by cooling, the methylene chains no longer become fully extended within this temperature range. That is, complete conversion from the contiguous F phase to the S phase now does not take place within this limited domain. Presumably the presence of 150 moles sodium dodecyl sulfate per mole $(Na^+ + K^+)$-ATPase could account for this.

Such a model would support our earlier suggestion that the uniqueness of these lipids may be in their chain length rather than their phospholipid head group. Since restricted binding sites for M 12-NSE increase with specific activity [17] it would be expected that in untreated microsomes the fluid "pools" adjacent to the enzyme would not be able to insulate the spin probe from

the rigidifying effects of temperature upon the bulk membrane. After selective retention by detergent treatment they are capable of accommodating this effect. When applied to the hydrolytic action of the enzyme protein, this model suggests the following explanation for the temperature dependence phenomenon.

Firstly, the fluid envelope transmits the rigidity of the bulk membrane to the enzyme protein and at lower temperatures the necessary conformational changes required for function now require more energy due to the loss of an inertia component. Secondly, the change in width of the membrane which would follow the stiffening of the methylene chains, probably necessitates an altered form of the enzyme protein to maintain function.

In conclusion our data suggest a co-operative effect between the $(Na^+ + K^+)$-ATPase protein and the lipid(s) of the membrane which is two-fold. First the protein macromolecule exerts an ordering effect on the phospholipids in the bilayer and secondly, the alterations of the membrane fluidity caused by cooling of the fatty acid chains of the phospholipid in turn impose restraints on the protein macromolecule leading to conformational

readjustment.

Our data do not support or exclude a third possibility, namely a direct effect of temperature on the conformation of the enzyme protein macromolecule. However, linear Arrhenius plots obtained with a variety of delipidated preparations attest to the contrary [6,7,12].

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FIGURE LEGENDS

Fig. 1: The effects of temperature on the spectrum of M 12-NSE in DMPC liposomes. M 12-NSE was allowed to diffuse into DMPC liposomes which were then sampled for spectroscopic analysis. Three representative traces obtained at 37° (·····), at 20° (- - -), and at 16°C (———) are shown. Temperature alteration was achieved by progressively reducing the temperature of the sample chamber. The inset shows an Arrhenius plot of the tumbling time To, which was determined as described in Methods.

Fig. 2: The effects of temperature on unrestricted sites on ox brain membranes labelled with M 12-NSE. The spin probe was allowed to diffuse into the membranes which were then equilibrated overnight at 4°C. About 30 µl of the labelled membranes were introduced into capillary tubes (1.1 mm internal diameter) which were then sealed. Temperature alteration was achieved by cooling the sample in multiple steps of a few degrees between 37°-5°C. The plots represent mean values of 9 determinations for each condition; (+ - + - +) untreated

membranes, and (o - o - o) sodium deoxycholate extracted membranes. A linear thermal response was found under both experimental conditions.

Fig. 3: The effects of temperature on the spectrum of M 12-NSE bound to restricted sites on ox brain membranes. M 12-NSE was allowed to diffuse into ox brain membranes which were then treated as described in Fig. 2. Three representative traces obtained at 37° (·····), at 19° (- - -), and at 7°C (———) are shown.

Fig. 4: A limited study of the effects of temperature and detergent extraction on restricted sites labelled with M 12-NSE. The spin probe was allowed to diffuse into untreated ox brain membranes (· - · - ·), and after extraction with sodium deoxycholate (o - o - o), or with sodium dodecyl sulfate (+ - + - +). All labelled membranes were then treated as described in Fig. 2. A single discontinuity near 20° can be seen under all experimental conditions.

Fig. 5: A detailed study of the effects of temperature on spin labelled ox brain membranes. M 12-NSE was allowed to diffuse into membranes and was equilibrated overnight at 4°. About 30 µl of labelled membrane was treated as described for Fig. 1, except that the multiple temperature steps were at 1°C intervals, between 37°C-2°C. Multiple discontinuities can be seen near 10°, 20° and 30°C.

Fig. 6: A detailed study of the effects of temperature on spin labelled partially delipidated ox brain membranes. M 12-NSE was allowed to diffuse into membranes which had been treated with sodium deoxycholate (· - · - ·), or with sodium dodecyl sulfate (o - o - o). The labelled membranes were then examined as reported in the legend to Fig. 5. Both plots represent mean values with standard error bars; where no bars are given the errors were small and lie within the points shown. Discontinuities near 10° and 26° are seen following extraction with sodium deoxycholate, whereas only a single discontinuity near 18° occurs after extraction with sodium dodecyl sulfate.

Fig. 7: A detailed study of the effects of temperature on spin labelled liposomes. M 12-NSE was allowed to diffuse into liposomes as described in Fig. 1. The results obtained from lipid extracts of membranes treated with sodium deoxycholate (▽) and sodium dodecyl sulfate (o) were not significantly different and showed a single discontinuity near 20°. For comparison the results with a commercial preparation of ox brain phosphotidyl serine (□) and from lipid extracts of untreated ox brain membranes (▣) are given below. Single discontinuities can be seen near 16° and 20° respectively. The results are the mean values of duplicate determinations.

Fig. 8: A detailed study of the effects of temperature on enzyme activity before and after detergent extraction. The $(Na^+ + K^+)$-ATPase activity of untreated ox brain membranes (Δ - Δ - Δ), and after extraction of the membranes with sodium deoxycholate (o - o - o), or with sodium dodecyl sulfate (● - ● - ●) was determined by a continuous assay procedure. For clarity the rates of enzyme activity which are given as μmoles Pi/mg protein/hr have been offset for the different preparations. At

37° the specific activities of the preparations were 44, 125 and 95 μmoles Pi/mg protein/hr for the untreated, the sodium deoxycholate treated and the sodium dodecyl sulfate treated ox brain membranes respectively. Multiple discontinuities are apparent in all preparations.

TABLE 1

THE EFFECT OF TEMPERATURE ON DIFFERENT SPECTRAL PARAMETERS OF SPIN LABELLED DMPC LIPOSOMES

Parameter	37°	23°	21°	T_c*
h_{+1} mm	100	47	42	22.37
h_o mm	100	57	36	28.69
h_{-1} mm	100	36	10	23.37
h_o/h_{+1}	100	121	160	22.39
h_o/h_{-1}	100	159	351	22.69
To x 10^{-10}	100	217	423	23.96

* As determined by computer programme using 10 temperature values from 21° to 37°. The mean value for T_c = 23.5 ± 0.53 S.E.

Fig. 1

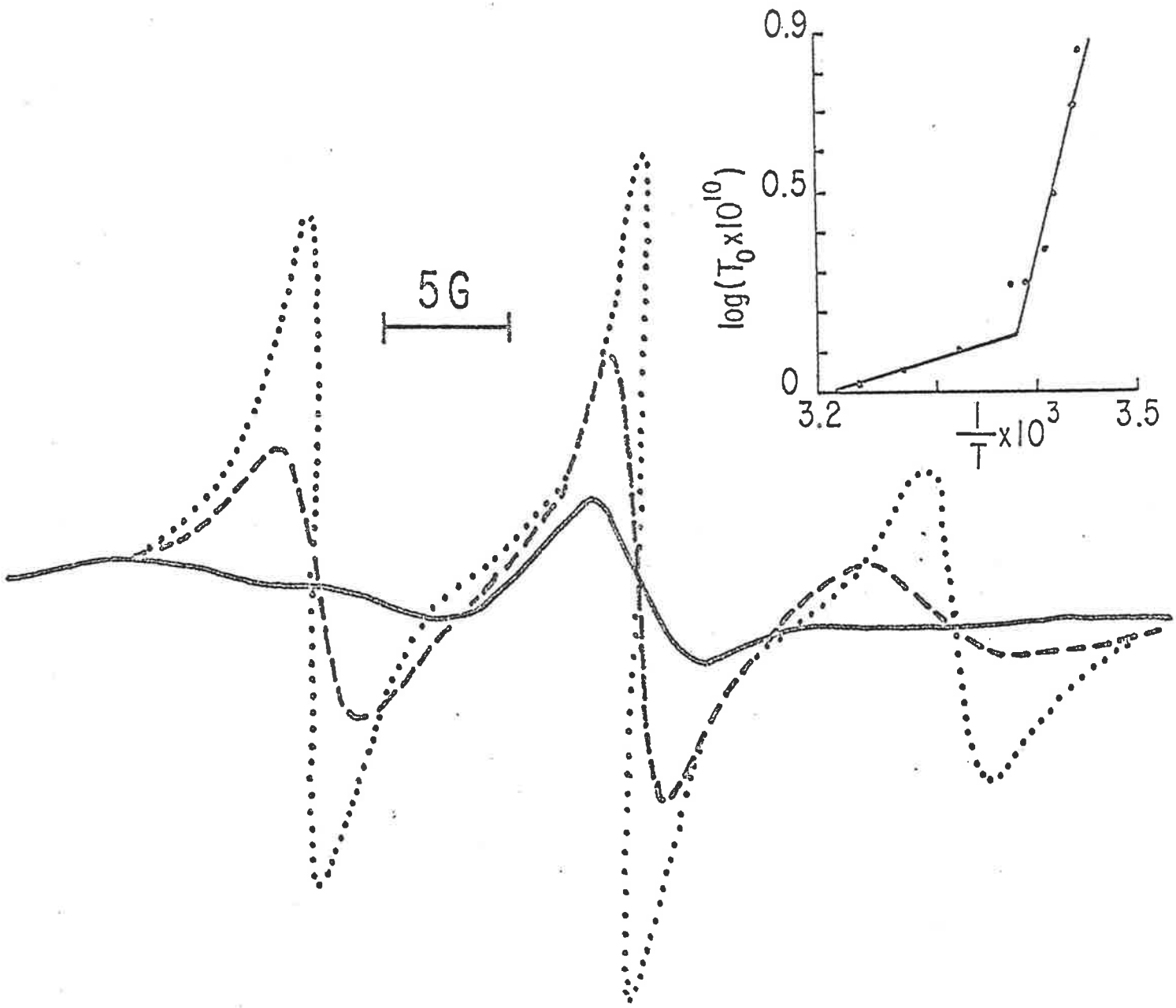
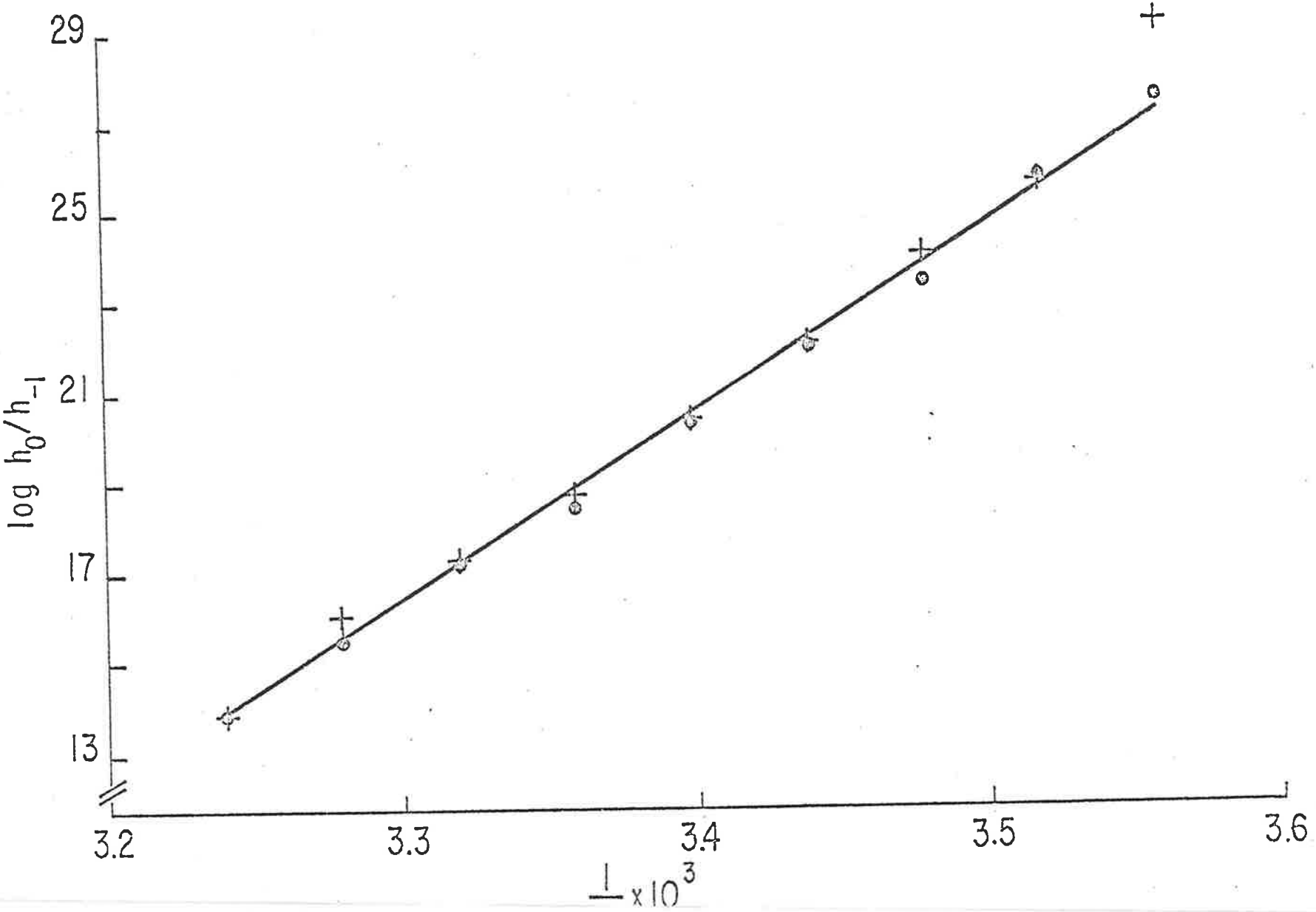


Fig. 2



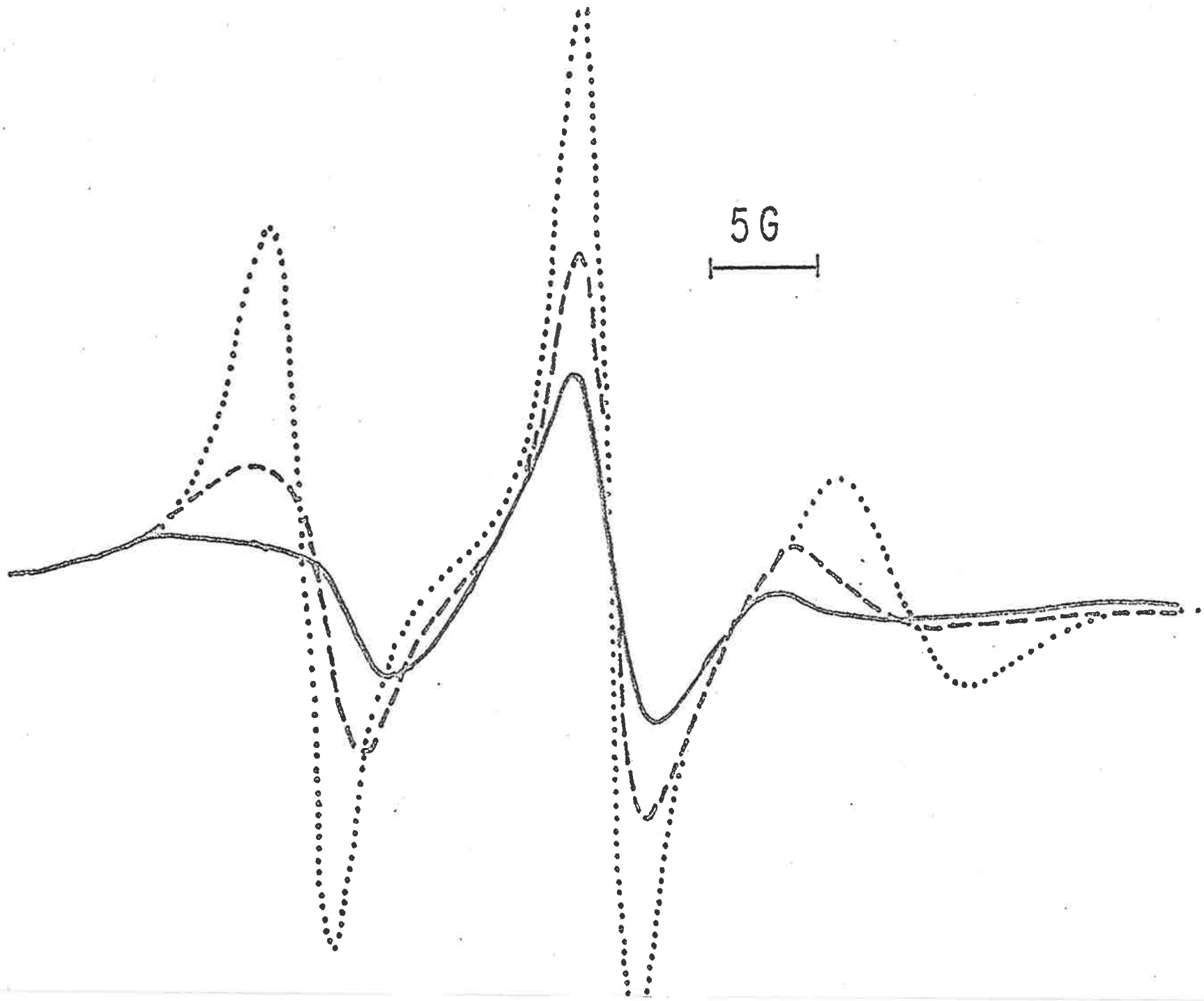
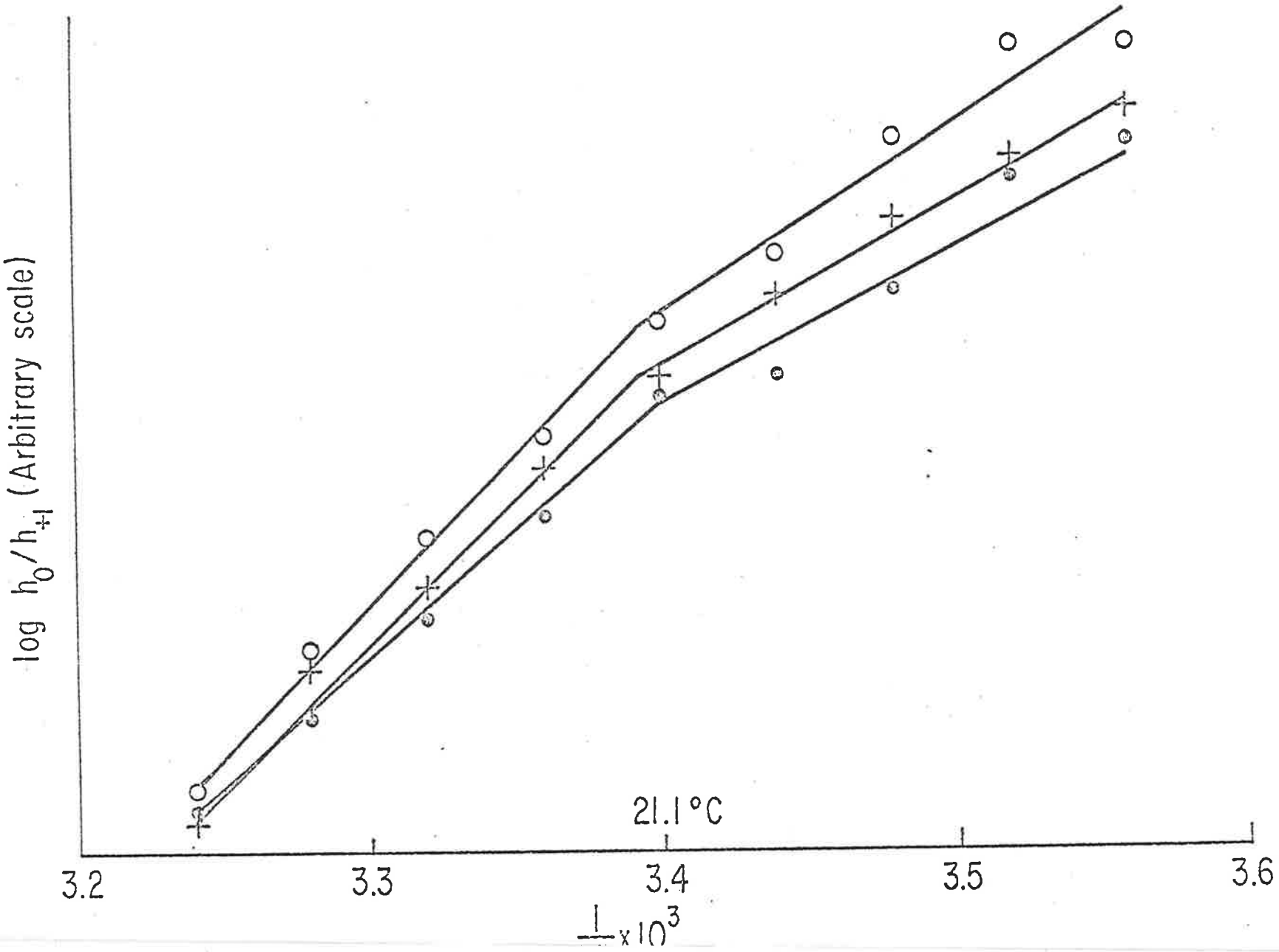
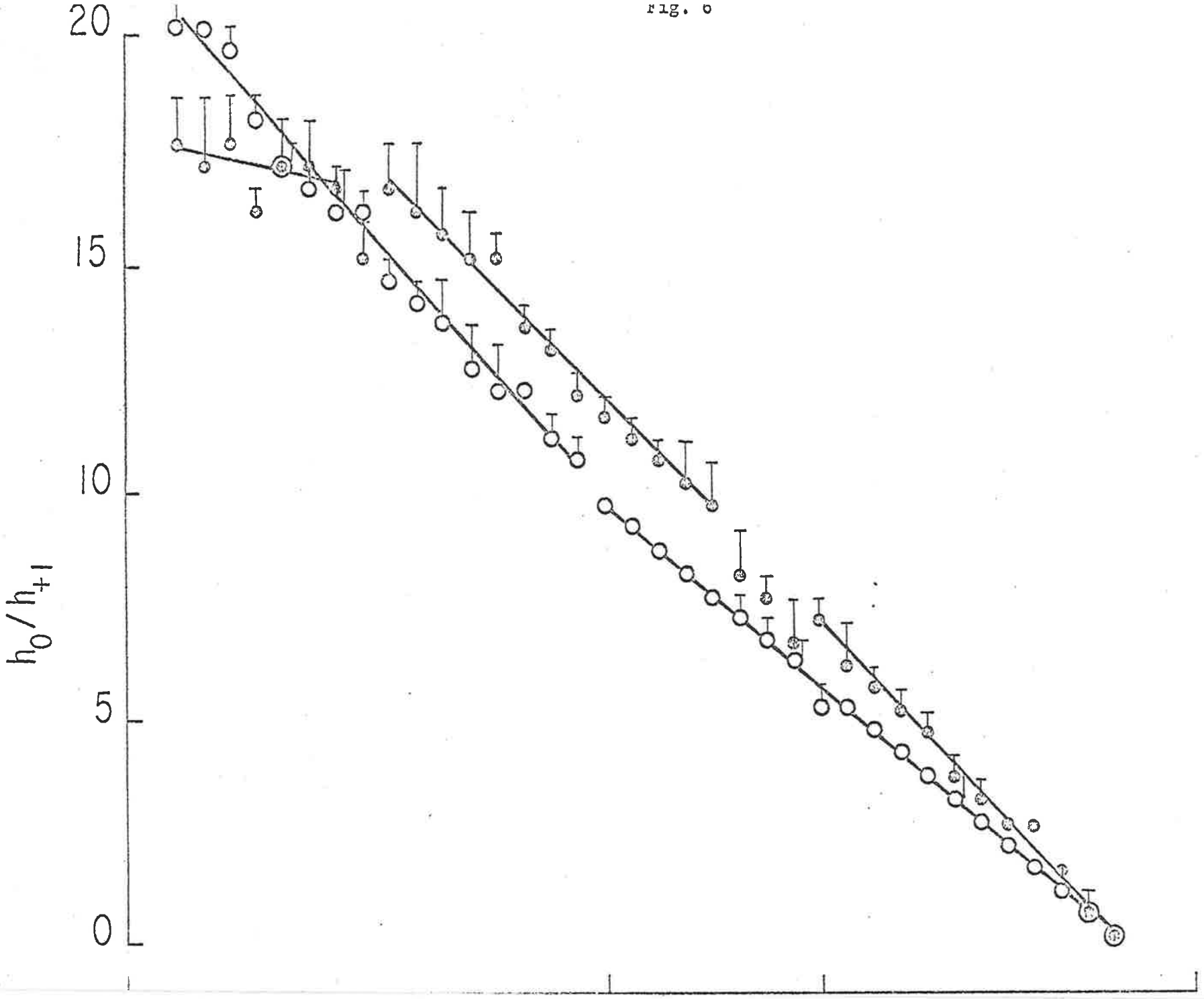

5G

Fig. 4



h_0/h_{+1}
0
5
10
15
20

Fig. 6



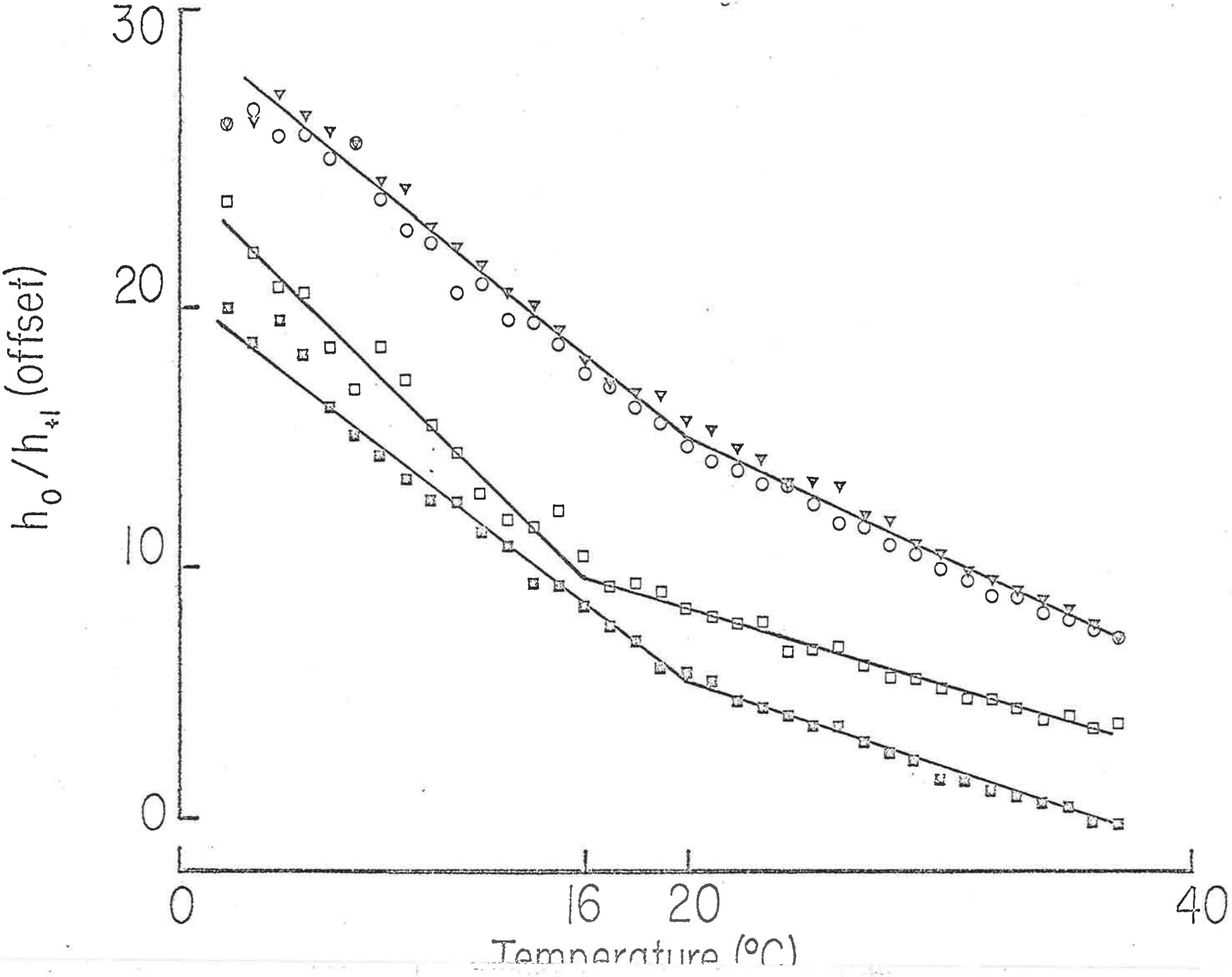
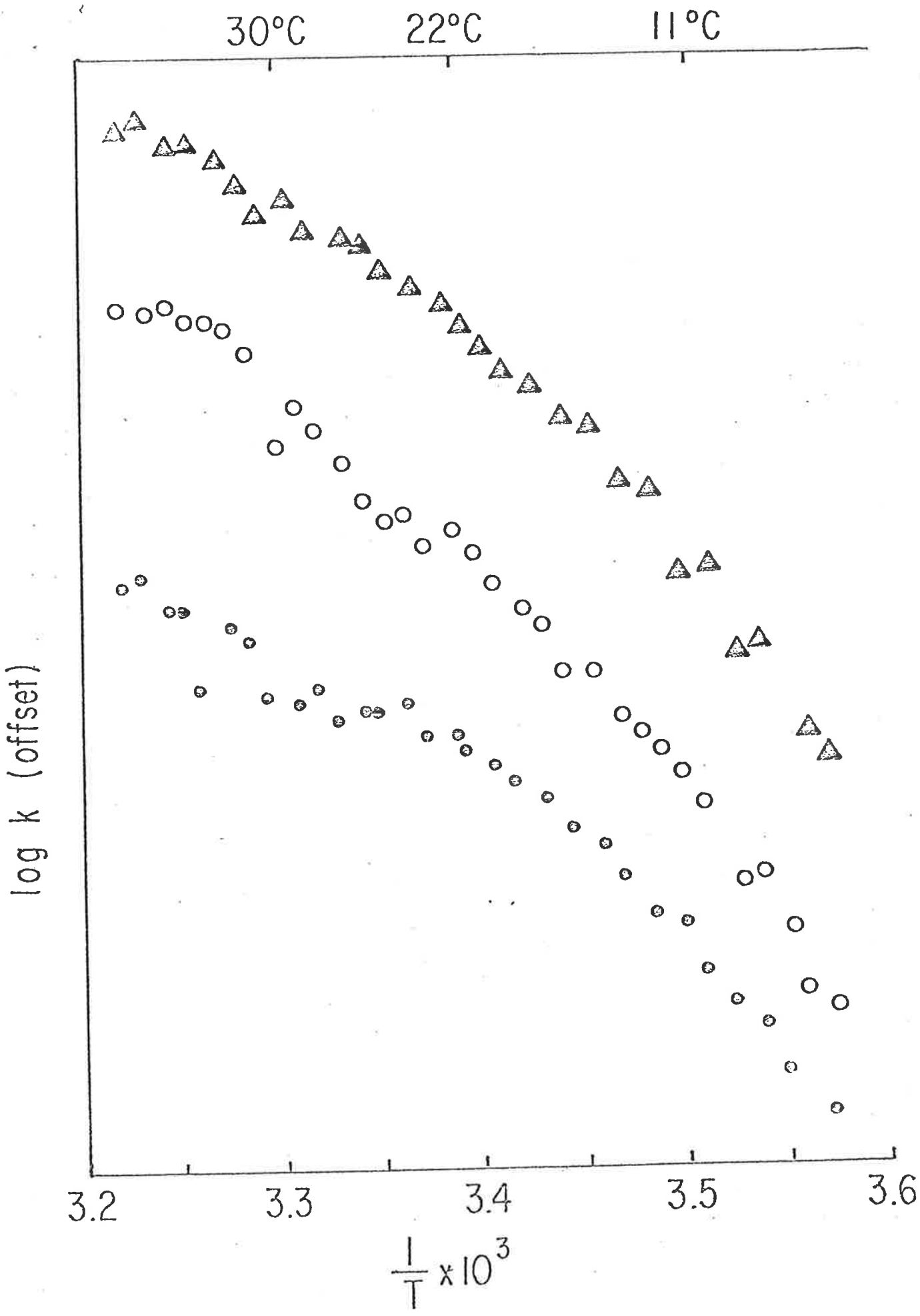

30
20
10
0
h_o / h_{+1} (offset)
0
16
20
40
Temperature (°C)

Fig. 8



Science (Submitted, 1977).

MEMBRANE ELASTICITY AS A COMPONENT OF THE ENERGY TRANSDUCTION MECHANISM OF $(Na^+ + K^+)$-ATPase

A vast body of evidence has accumulated which attests to the temperature sensitivity of the transport enzyme $(Na^+ + K^+)$-ATPase. When presented as an Arrhenius plot this phenomenon can be described by two distinct energies of activation above and below a critical temperature, such that the enzyme function is less efficient at the lower temperatures[1]. This effect has also been examined by physical techniques such as electron spin resonance and fluorescence polarisation and the results parallel the thermal sensitivity of the enzyme[2,3,4].

Fig. 1 shows the effect of temperature on untreated $(Na^+ + K^+)$-ATPase membranes prepared from ox brain, as determined by enzyme activity, and also by spin labelling the membranes with N-oxyl-4',4'-dimethyloxazolidine-12-keto methyl stearate (M 12-NSE). The two methods show good correlation of the biphasic response to temperature. In the case of the spin probe study, this biphasic response represents two distinct rates of change of molecular motion, with the more rapid change occurring at the higher temperatures. This is in direct contrast to the behaviour of the probe when it is intercalated in synthetic bilayers; on cooling these bilayers there is a sudden acceleration of the loss of molecular motion at the onset of the phase transition[4,5]. The transition that occurs in the bilayer is an alteration from a liquid crystalline arrangement of flexible methylene chains to a gel phase with stiff extended chains[5]. We deduce from this that the extended chains result in an increase in membrane width (perpendicular to the plane of the bilayer) which occurs suddenly and is responsible for the steeper of the

two slopes. Thus it is important to inquire why this situation is reversed in biological membranes. Of immediate interest in this regard is whether the protein macromolecule is implicated.

To examine this further we have studied the effects of temperature on spin labelled liposomes made from total lipid extracts of this enzyme preparation and the results are also shown in Fig. 1. Quite clearly these liposomes resemble synthetic phospholipids[4,5], with the more rapid rate of change taking place at the lower temperatures. We must therefore conclude that functional membrane bound enzymes exert an effect on the lipid bilayer to prevent or reduce the increase in width. We may now re-describe the temperature phenomenon as follows:

On cooling a biological membrane, in addition to a decrease in fluidity, there is also an expansion or increase in the width of the membrane, as some of the methylene chains stiffen and become extended. At lower temperatures, membrane bound enzymes, in order to maintain function, re-arrange the phospholipids in their immediate environment so as to contain this expansion. Presumably this occurs by lateral re-arrangement with sequestration of phospholipids containing unsaturated long chain fatty acids.

The sequestration of specific lipids by the enzyme protein implies that purification would lead to concentration of these lipids. In an earlier publication we had observed that purification of $(Na^+ + K^+)$-ATPase by extraction with sodium dodecyl sulfate did suggest concentration of specific lipid species[6]. We have therefore repeated the investigation described above using membranes that were pretreated with sodium dodecyl sulfate. The results are shown in Fig. 2. The parameters that were examined previously,

namely, the enzyme activity, the mobility of the spin labelled microsomes, and the mobility of the spin labelled liposomes are now identical and apparently fail to support the postulated concentration of a lipid species. If, however, the labelled microsome study is conducted with a very gradual cooling process of not more than 1° in 4 minutes, then this is not the case. This is also shown in Fig. 2. Now the two slopes bear greater similarity to the liposome studies with the greater slope being manifested at the lower temperatures. Therefore, as reported by M 12-NSE, this lipid domain undergoes expansion with cooling. Furthermore, this result also supports the concept of sequestration and concentration of specific phospholipids. Thus, the expansion appears to have been contained to a degree that permits continued operation of the enzyme, albeit with a greater energy requirement.

A similar study, using a gradual cooling process with labelled untreated microsomes, did not alter the results shown in Fig. 1.

From the data described above it is apparent that membrane fluidity, which is essential for enzyme function, can be seen to exert its influence in two planes, parallel as well as perpendicular to that of the bilayer. This arrangement implies that the membrane behaves like a sheet of rubber with close integration of the forces in the two planes. Inherent in this concept is the tendency by the membrane to return to its equilibrium state. It is therefore most likely that the $(Na^+ + K^+)$-ATPase incorporates the potential energy of this elastic membrane in the energy transduction process that is necessary for transport.

Weiss has used the term "mechanico-chemical coupling" to describe the influence of the lipid on entropy changes in biological membranes[7]. This

elegant review discusses many aspects as diverse as the α-helical content of proteins and the cholesterol content that could influence the elastic forces of the membrane. Our data provide further experimental evidence in support of mechanico-chemical coupling, without implicating a specific component of the system. Quite clearly this enzyme system, being intimately associated with phospholipids, is an excellent tool for further investigation into such related factors.

At least two predictions can be made from this model. Firstly, in the functional condition the two elastic components of the membrane must exist in some fixed equilibrium to prevent disruption of the membrane. Secondly, selective alteration of one elastic component would lead to a change in the other, in an attempt by the membrane to obtain a functional equilibrium again. In the latter concept, one would expect the membrane to be capable of accommodating some degree of change with very little alteration in function of the enzyme.

Some evidence can be cited in support of these predictions. First, partial delipidation of this membrane bound enzyme with phospholipase A does not result in a loss of one or other of the slopes in an Arrhenius plot as was originally thought from the work of Tanigucha and Iida[8]. Instead, a new activation energy is defined which lies intermediate between the former values above and below the thermal transition[9]. Second, reconstitution of delipidated ($Na^+ + K^+$)-ATPase preparations have failed to implicate a specific phospholipid as critical for enzyme function[10]. Third, a comparison of the two activation energies above and below a thermal transition of a variety of enzymes from an equally variable number of species

results in a ratio of Ea_I:Ea_{II} of about 2. We suggest that this constancy reflects a critical relationship of the two membrane vectors that we have described. Finally, we point out that integration of the two membrane vectors must include both halves of the bilayer which are known to have an asymmetric distribution of phospholipids. In view of this, it is not surprising that transmembrane protein molecules all tend to exhibit a biphasic thermal sensitivity.

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FIGURE LEGENDS

Fig. 1: Arrhenius plots of data obtained with $(Na^+ + K^+)$-ATPase enriched ox brain membranes.
Enzyme activity as μmoles Pi/mg protein/hr (· - · - ·); for methods see Ref. 1. Spectral changes of M 12-NSE labelled microsomes (o - o - o) and liposomes made from total lipid extracts of microsomes (+ - + - +), as ratios of peak height h_o/h_{+1}; for methods see Refs. 4,6.

Fig. 2: Arrhenius plots of data obtained with $(Na^+ + K^+)$-ATPase enriched ox brain membranes after purification with sodium dodecyl sulfate; for methods see Ref. 1.
Enzyme activity as μmoles Pi/mg protein/hr (· - · - ·); for methods see Ref. 1. Spectral changes of M 12-NSE labelled microsomes (o - o - o) and liposomes made from total lipid extracts of microsomes (+ - + - +) as ratios of peak height h_o/h_{+1}; for methods see Refs. 4,6. Also shown are spectral changes in peak height ratios when the spectroscopic study was carried out with very slow cooling, 1° in 4 minutes (Δ - Δ - Δ).

Fig. 1

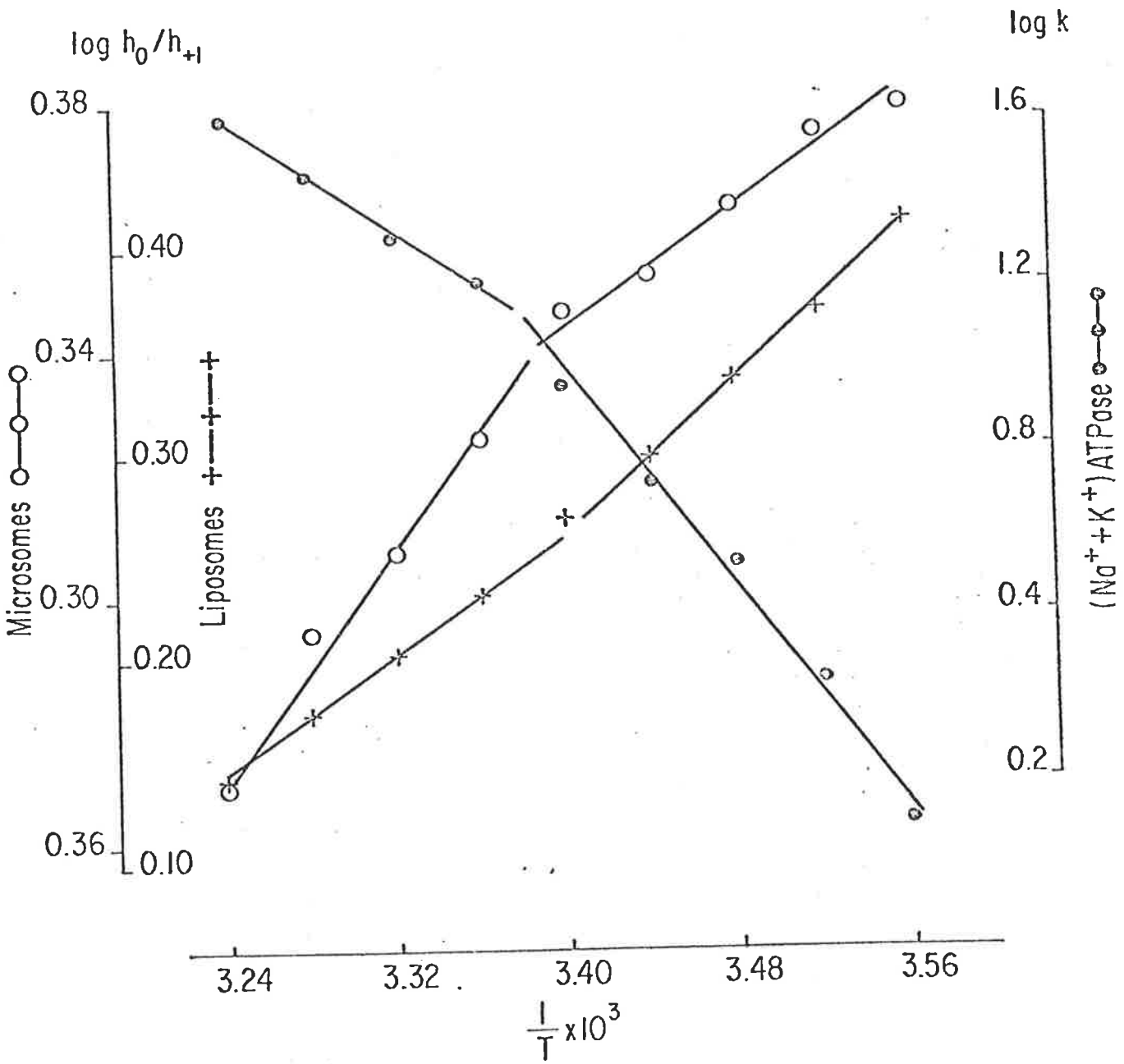
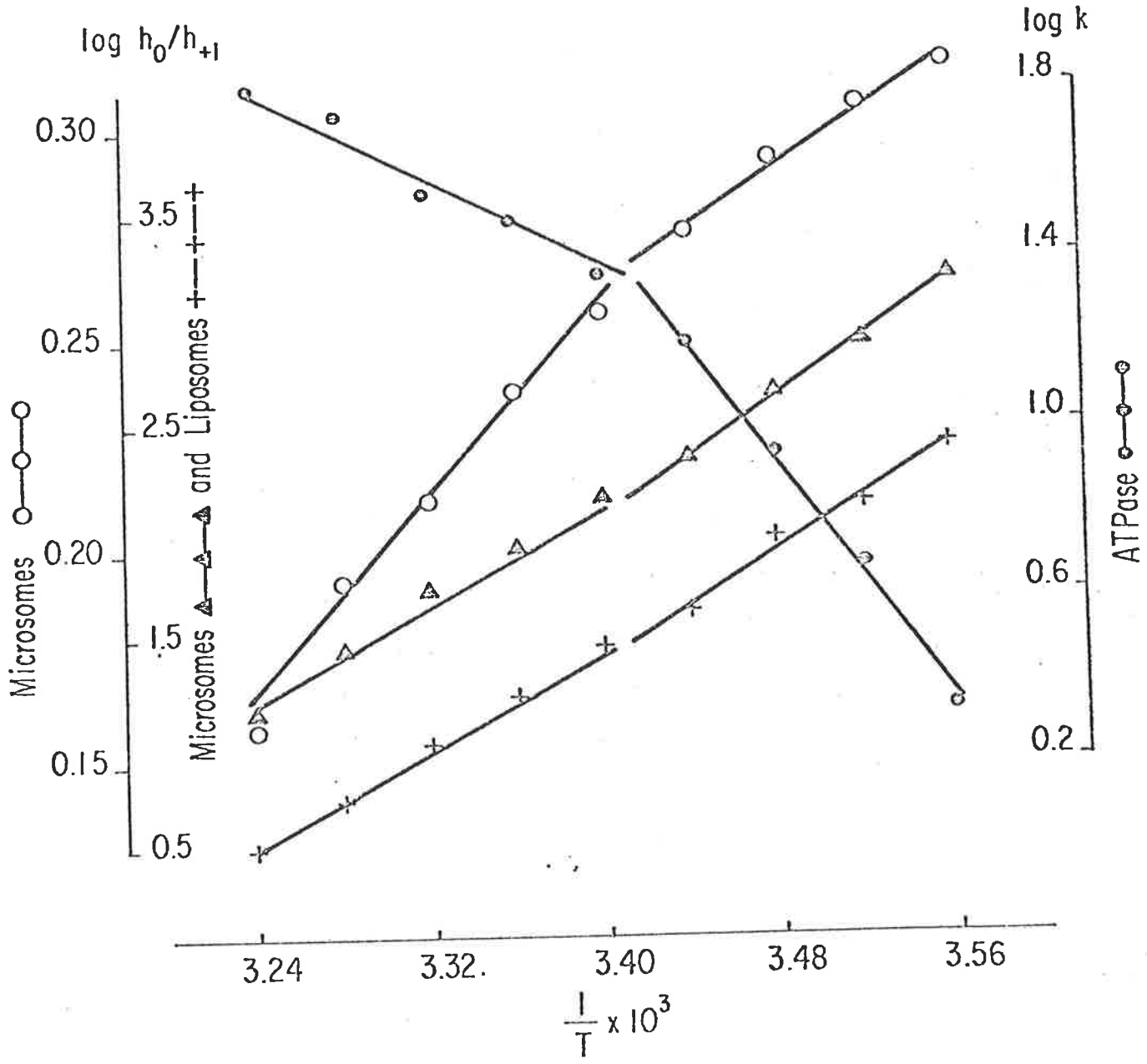

log h_0/h_{+1}
log k
0.38
0.40
0.34
0.30
0.30
0.20
0.36
0.10
1.6
1.2
0.8
0.4
0.2
Microsomes
Liposomes
$(Na^+ + K^+)$ ATPase
3.24
3.32
3.40
3.48
3.56
$\frac{1}{T} \times 10^3$

Fig. 2


log h_0/h_{+1}
log k
0.30
0.25
0.20
0.15
3.5
2.5
1.5
0.5
1.8
1.4
1.0
0.6
0.2
Microsomes
Microsomes and Liposomes
ATPase
3.24
3.32.
3.40
3.48
3.56
$\frac{1}{T} \times 10^3$

PART III

EVIDENCE FOR MEMBRANE-LIPID ALTERATION WITH PURIFICATION OF OX BRAIN $(Na^+ + K^+)$-ATPase

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Banff, Alberta, Canada

ABSTRACT

Membrane bound ($Na^+ + K^+$)-ATPase from ox brain microsomes, when labelled with the ESR spin probe N-oxyl-4'-4'-dimethyloxazolidine derivative of 12-Keto methyl stearate (12-NSE) will generate two types of spectra, which we have suggested arise from two different non-covalent binding sites in the membrane. In order to further examine this hypothesis we have studied the effects of enzyme purification on the ESR spectrum. Our data show that the specific activity of the enzyme can be correlated with the type of spectrum. This evidence suggests that one of the sites may be associated with the enzyme while the other site is a non-specific site in the membrane. This selective incorporation by a fatty acid probe may support the concept of specific aggregation of one or more lipid species by the ($Na^+ + K^+$)-ATPase enzyme. This latter suggestion is supported by an examination of the effects of temperature on the two types of spectra.

The membrane bound enzyme ($Na^+ + K^+$)-ATPase requires lipid for optimal activity, but the mechanism by which this lipid modulation occurs is not known. Attempts to analyze the lipid constituents of highly purified enzyme preparations have also failed to unequivocally establish the identity of a critical phospholipid. Furthermore, reconstitution of the enzyme activity after lipid removal, has been achieved with a number of phospholipids. Thus the identity of the critical phospholipid and its association with the protein continue to be both a mystery and an attractive research goal.

In our laboratory, one area of interest has been the effects of temperature on the membrane lipids associated with this enzyme. It is primarily with this in mind that we chose ESR as an investigative technique. My presentation tonight will describe how ox brain microsomes will bind a spin probe at two different sites, how these sites relate to the specific activity of the enzyme, and finally how this information could mean an aggregation of one or more specific lipid species.

E 1

At the top on the left hand side is shown a formula of the spin probe. It is a methyl ester of stearic acid with the nitroxyl on the 12-C position. 12-Nitroxyl stearic acid or 12-NSE was a gift from Dr. J. Raison from Australia - at the time that we commenced this work lipid spin probes were not commercially available. 12-NSE is relatively unreactive and an advantage of the ester is that it is practically insoluble in aqueous media.

If this spin probe is dissolved in methanol and examined spectroscopically, then we obtain an isotropic spectrum - a spectrum with three sharp peaks of almost equal intensity. A spectrum of this type is characteristic of an unrestricted nitroxyl. So in methanol, the nitroxyl can rotate freely. If however the label is incorporated into liposomes made from dimyristoyl phosphatidyl choline, DMPC, the spectrum is quite different. This is shown superimposed on the methanol spectrum - the continuous line. We still have three peaks but they are considerably broader, and this is because the nitroxyl is undergoing restricted motion. Stearic acid intercalates in the bilayer with its long axis perpendicular to the plane of the bilayer which results in restricted motion of the methylene chain.

The three peaks, defined as the low-field, the mid-field and the high-field, or h_{+1}, h_o and h_{-1}, can be used to quantitate the spectra. A number of methods are available, some very sophisticated and others quite simple, but they all use approximations of varying magnitude. A simple method, often encountered in the literature, is the use of peak height ratios - either h_o/h_{+1} or h_o/h_{-1} and in this work we have used both methods. Although really only semiquantitative this method can be useful in the evaluation of spectral changes.

The labelling procedure is extremely simple. A quantity of 12-NSE in methanol, in this work 2 nmoles, is introduced into a glass tube and the methanol is blown off in a stream of nitrogen. The microsomes or liposomes are added to the tube and warmed to 37°C for 10 minutes. This

labelling procedure can be carried out in the sample tube.

If ox brain microsomes are labelled with 12-NSE a variety of spectra may result. They may be isotropic spectra where the nitroxyl is unrestricted. The line shape is similar to a methanol solution of 12-NSE. Alternatively the spectrum may resemble the broad line shape of the DMPC liposomes - that is, a restricted spectrum. Finally, labelled microsomes might generate a host of assorted spectra that are not in either group. These we call mixed. Using these three groups - unrestricted, restricted and mixed - we can classify the spectra that are obtained with spin labelled ox brain microsomes.

2 2 First the unrestricted spectra. They have three sharp peaks which I emphasize are <u>not</u> due to 12-NSE in solution. We found that these spectra only occurred when untreated and DOC-extracted microsomes were labelled - curiously enough SDS-treated microsomes did not generate sharp spectra. One other requirement for unrestricted spectra was a high probe: membrane ratio.

A second group of spectra are the restricted spectra with the broader peaks. Broad spectra, we discovered, could be obtained with any microsomal preparation providing the membrane concentration was high enough, i.e., we needed a low probe:membrane ratio. Of course as we saw earlier, liposomes also generate broad spectra and the ratios of probe:DMPC do not alter the line shape of the spectrum.

Finally, there are those spectra which do not fall into either group

and must be described as a mixture of the two groups superimposed on one another. These mixed spectra are not seen with liposomes.

From this classification we have drawn the following conclusions:

First: The membrane concentration influences the spectrum.

Second: The two limiting spectral types, the unrestricted and the restricted are due to spin labels at two distinct binding sites.

Third: Treatment of the microsomes with detergents influences the binding sites.

The next slide shows a more detailed examination of the effects of membrane concentration on the spectrum of 12-NSE.

IDE 3

These four spectra have been selected from a larger series. They were all obtained with the same enzyme preparation - an untreated enzyme that had not been extracted with any detergent. The probe concentration was 2 nmoles for all samples.

At the lowest membrane concentration the spectrum is without doubt a sharp three-peak spectrum. As the membrane concentration is increased, an irregularity in the base line appears which becomes a secondary peak. Eventually a broad spectrum is observed. In this broad spectrum the spin labels occupy restricted sites even though the unrestricted sites are still present. This is because the probe concentration is limited and presumably the restricted sites are preferentially labelled. So a restricte spectrum is a consequence of labelling high affinity sites and an unrestricted spectrum portrays low affinity sites.

On examination of the membrane concentrations given here as micrograms of protein, we can conclude further that in this particular preparation the low affinity sites are quite abundant. In constrast, the high affinity sites are relatively rare.

Let me now turn to the observation that detergent treatment influences the binding sites. We routinely use detergents to increase the specific activity of our enzyme. Thus it seemed likely that the detergent effect was really a consequence of enzyme purification.

The next slide demonstrates the relationship between specific activity and spectral shape.

E 4

Each of these three pairs of spectra represents a different untreated enzyme preparation. This is shown by the specific activities, 12 µmoles inorganic phosphate per milligram protein per hour - i.e., 12 µmoles Pi, 22 µmoles and 59 µmoles Pi. Again we have kept the concentration of the spin probe constant at 2 nmoles and have varied the membrane concentration. In all three pairs the lower membrane concentration is shown as a broken line.

The topmost pair compares a two-fold difference in membrane concentration, 1.25 and 2.5 µg membrane. Both spectra are isotropic. There is no evidence of high affinity sites being labelled.

The next pair compares a four-fold difference in membrane concentration. Now we begin to see a difference. The lower membrane concentration has an isotropic spectrum, the higher shows some irregularity. In the

last slide we saw that this irregularity is really due to the high affinity sites beginning to manifest themselves.

If we examine the third pair we note a three-fold difference in membrane concentration, and this is intermediate to the first two pairs. Yet we see a marked difference in the two spectra. An isotropic spectrum at the lower membrane concentration and a mixed spectrum at the higher membrane concentration. In the last slide we showed that a broad or restricted spectrum is a consequence of labelling high affinity sites. In this slide we show that as the tendency for the labelling of high affinity sites increases, so also does the specific activity of the enzyme. Rephrasing this we could claim that the high affinity binding sites for 12-NSE increase as the catalytic sites increase.

With this slide we have attempted to show a correlation between line shape of the spectrum, i.e., pictorial evidence and the specific activity of the enzyme, i.e., numerical evidence. I understand that correlations of this see-and-count type tend to give pure scientists indigestion. To avoid this, or perhaps correct it, we have measured the heights of these secondary peaks and have plotted them against specific activity. The results are shown on the next slide.

On the vertical axis we have the height of the secondary peak measured in mm directly off the chart paper and on the horizontal axis is shown the specific activity of the different ox brain preparations. Three different ox brain preparations are represented, 20 µmoles Pi/mg protein/hr, 35 µmoles

Pi and 56 μmoles Pi. Again the label concentration was maintained at 2 nmoles of 12-NSE per sample tube. With the most impure preparation if 200 μg protein are labelled the height of the secondary peak is about 11 mm. The intermediate one, 35 μmoles Pi the secondary peak increases to about 20 mm again using 200 μg protein/sample tube and the purest enzyme, the 50 μmole preparation the secondary peak is 30 mm high. If a lower membrane concentration is used, 50 μg/sample a similar tendency is observed but less dramatic. This slide then re-affirms the lesson of the last slide: high affinity sites increase as the enzyme is purified.

If this is true then increasing purification should eventually result in a total loss of low affinity sites. We have examined this possibility by spin labelling SDS-treated microsomes. The results are shown on the next slide.

DE 6

Although four SDS-treated preparations were examined the specific activities varied from ≈ 140 to 150 μmoles Pi/mg protein/hr. This slide shows the results from one of these preparations. Here again the probe concentration was maintained at 2 nmoles per sample tube and the membrane concentration was varied. It is quite clear that at all the 4 membrane concentrations that were examined the spectrum was that of the high affinity sites. If the mid-field peak height is plotted against the membrane concentration then the peak heights fall on a single straight line. This again excludes any significant contribution from the unrestricted sites. Failure of the last point to fall on the straight line is because at this point the label does not saturate all the sites of this high activity

preparation. This is because the label was kept constant at 2 nmoles.

From this result it appears that the purification of ox brain microsomes by extraction with SDS results in a selective removal of the low affinity sites, and a concentration of high affinity sites. This selective retention of sites in a membrane, sites which bind a lipid spin probe I emphasize, implies a selective retention of some specific lipid species. However, we realise that we need more evidence before our confidence in this interpretation can be transmitted to our critics. One source of evidence is the chromatographic examination of lipid extracts of high activity preparations. We have only just begun this work and we hope to obtain both qualitative as well as quantitative information.

However, we are aware that information derived from lipid extracts of membranes cannot stand alone because lipid extracts lack the spatial orientation with respect to the protein macromolecule of this pump-cum-enzyme. To correlate data from the extracts with data from the active enzyme, we are making use of this spectroscopic method together with the well-known effects of temperature on this enzyme.

The next slide will remind us what an Arrhenius plot looks like.

LIDE 7

This is an old slide that I have included, to show how we illustrate the effects of temperature on enzyme activity. This is a semi-log plot of enzyme activity in μmoles Pi/mg protein/hr on the X-axis versus the reciprocal of the absolute temperature on the Y-axis. It is a non-linear plot with a bend or discontinuity at about 20°. The plot describes two

activation energies, one above and one below this critical temperature. This is a typical Arrhenius plot, one I am sure you are all familiar with.

We have produced similar semi-log plots of ESR data obtained with 12-NSE and this is shown on the last slide.

E 8

Two graphs are shown on this slide - the unrestricted or low affinity sites and the restricted or high affinity sites. Both plots were obtained with untreated enzyme preparations. Please ignore the arrows - they are there only because I haven't yet made the mental transition from centigrade to reciprocal absolute. But do note these vertical axes.

For the unrestricted sites we have used the h_o/h_{-1} ratio. Now the peak most susceptible to change is generally the high field peak. So the maximum change is best seen with this ratio - h_o/h_{-1}. However, we found that in the case of the restricted sites, the high field peak would sometimes be reduced so excessively as to make measurement at the lower temperatures difficult and somewhat inaccurate. Consequently we have used the mid-field and the low-field peaks - i.e., h_o/h_{+1}.

Although the low affinity sites display a marked sensitivity to temperature they show no inflection or bend - i.e., a linear plot. In contrast the restricted sites or high affinity sites, in addition to the sensitivity to temperature, show a distinct bend at 21°. This inflection point was derived by computer to avoid bias. The similarity of this plot to that of the catalytic activity that you saw in the last slide is striking, but it still does not demonstrate unequivocally the presence of

specific lipids in the vicinity of the enzyme. It does however lend support to this concept. Future ESR studies will compare the temperature effects of different enzyme preparations before and after SDS purification, with temperature studies of lipid extracts of these preparations.

To summarize then, we have demonstrated the following:

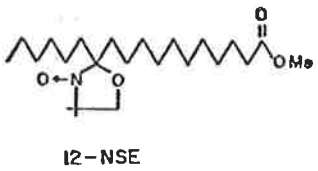
12-NSE will bind to two groups of sites on ox brain microsomes. These two groups differ in their affinity for 12-NSE.

The high affinity sites increase with enzyme purification using SDS extraction, suggesting a correlation between high affinity sites in the lipid with catalytic sites on the protein.

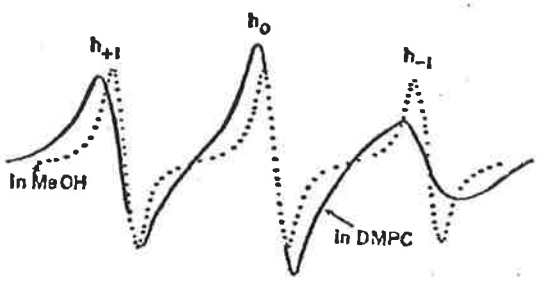
Although both groups of sites are sensitive to temperature only the high affinity sites show a discontinuity at 21°.

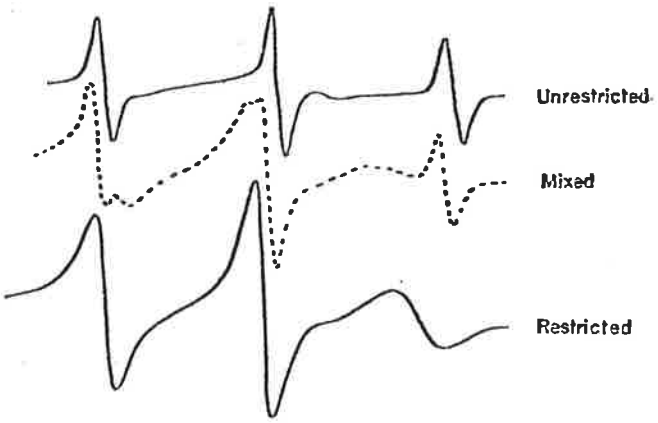
The discontinuity of the high affinity sites in the lipid is similar to the discontinuity displayed by the catalytic activity.

SLIDE 1



12-NSE





SLIDE 2

CLASSIFICATION OF SPECTRA

UNRESTRICTED: Sharp peaks.

Untreated and DOC-extracted microsomes - not SDS.

HIGH probe:membrane

RESTRICTED: Broad peaks.

All microsomal preparations

LOW probe:membrane

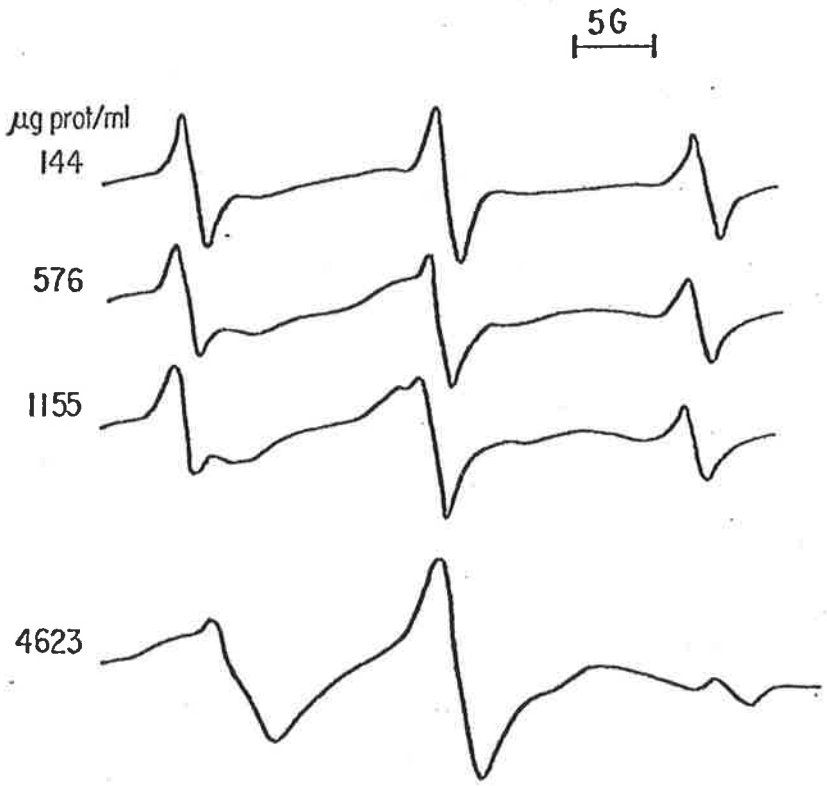
Also with liposomes

MIXED: Sharp and broad superimposed

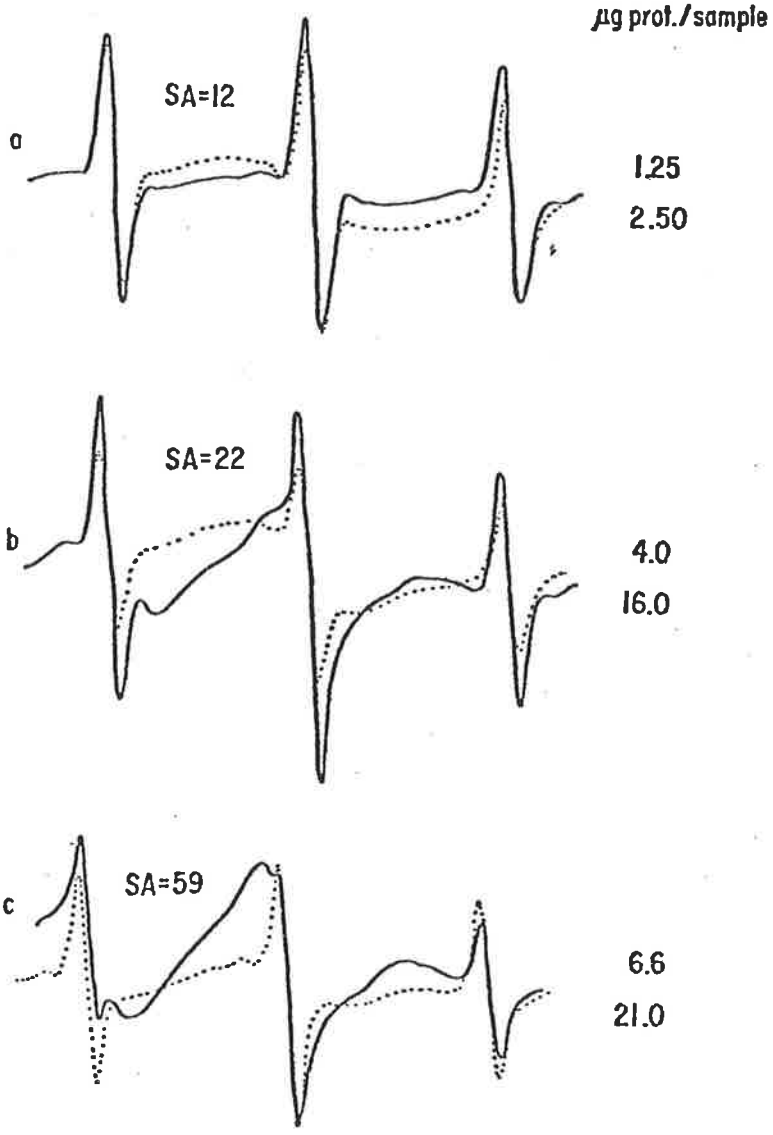
CONCLUSIONS:

1. Membrane concentration influences spectrum.
2. Two binding sites for 12-NSE.
3. Detergent treatment influences sites.

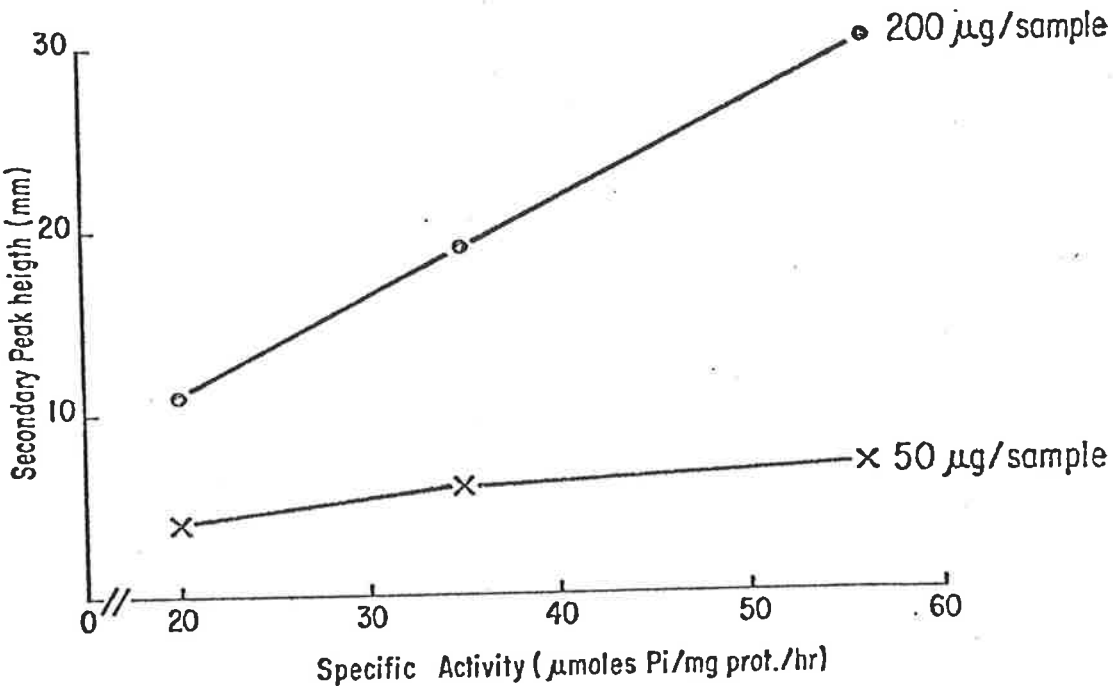
SLIDE 3



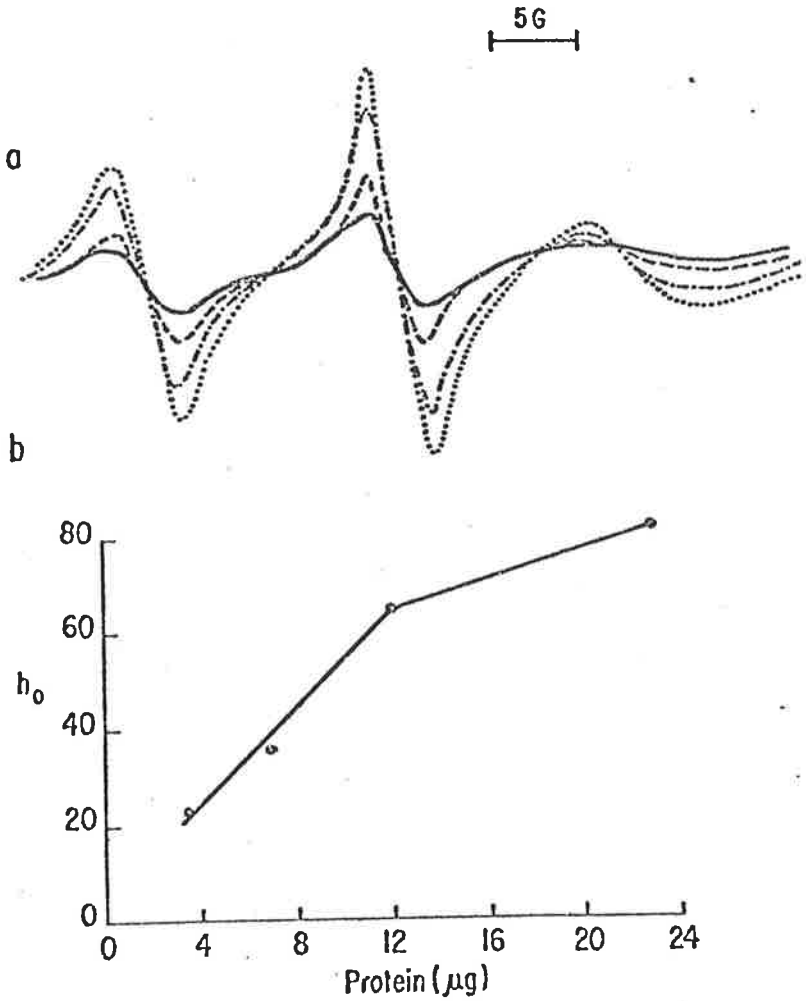
SLIDE 4



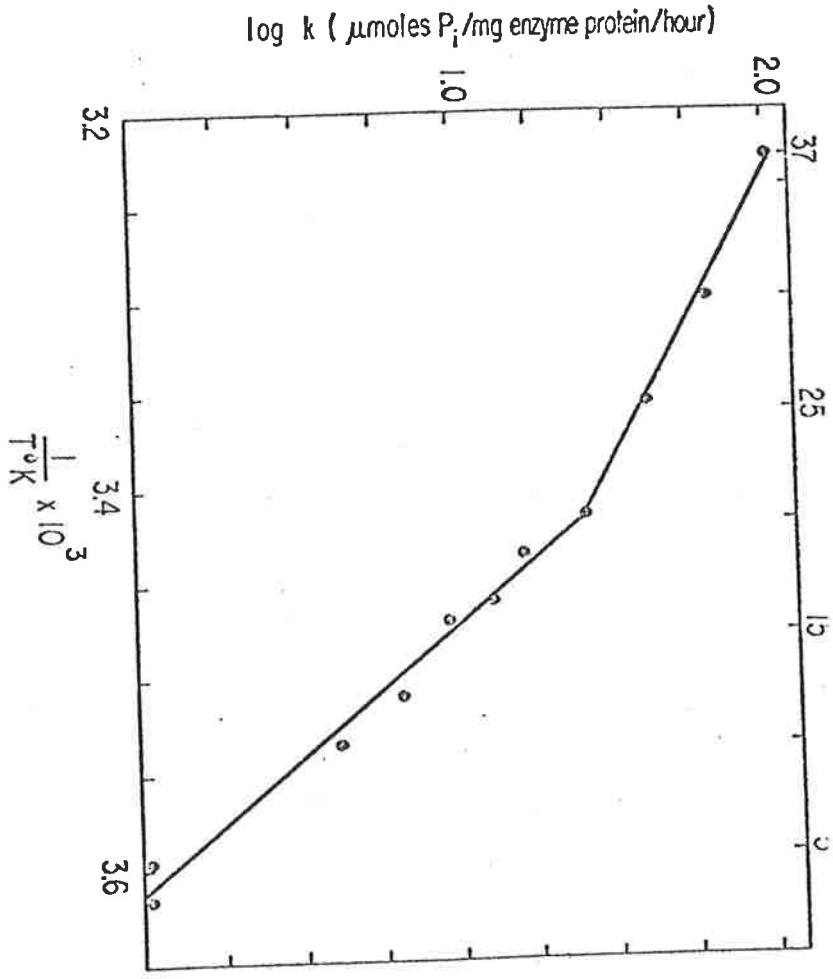
SLIDE 5


30
20
10
0
Secondary Peak heigth (mm)
200 µg/sample
50 µg/sample
20
30
40
50
60
Specific Activity (µmoles Pi/mg prot./hr)

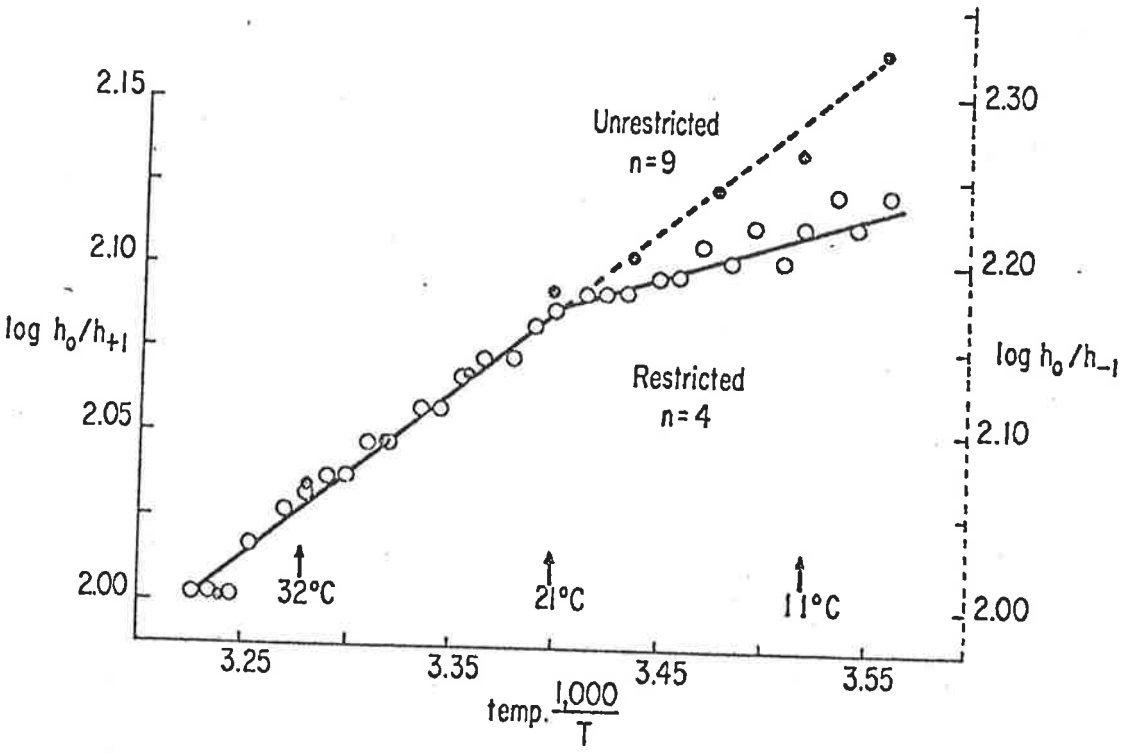
SLIDE 6



SLIDE 7


log k (μmoles P_i/mg enzyme protein/hour)
2.0
1.0
0
3.2
3.4
3.6
$\frac{1}{T°K} \times 10^3$
37
25
15
5

SLIDE 8


2.15
2.10
2.05
2.00
log h_0/h_{+1}
Unrestricted
n=9
Restricted
n=4
32°C
21°C
11°C
3.25
3.35
3.45
3.55
temp. $\frac{1,000}{T}$
2.30
2.20
2.10
2.00
log h_0/h_{-1}

THE SPECIFICITY OF INCORPORATION OF THE FLUORESCENT PROBE N-PHENYL-α-NAPHTHYLAMINE INTO $(Na^+ + K^+)$-ATPase PREPARATIONS

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Banff, Alberta, Canada

ABSTRACT

A very marked enhancement of fluorescence occurs when NPN is incorporated into ox brain membranes rich in $(Na^+ + K^+)$-ATPase. This is accompanied by a blue shift in the maximum wavelength of fluorescence emission.

By various methods of detergent extraction ox brain preparations with a wide range of enzyme activity could be obtained. When these are labelled with NPN the degree of fluorescence enhancement which is observed can be positively correlated with specific activity of the $(Na^+ + K^+)$-ATPase activity.

It is concluded that NPN is incorporated in these preparations in a lipid area of the membrane which is essential for optimal activity of the $(Na^+ + K^+)$-ATPase.

Attempts have been made to correlate the chemical composition and specific activity of ouabain-sensitive ($Na^+ + K^+$)-ATPase preparations at various stages of purification.

Jørgenson (1) showed an increase in phospholipid:protein and in cholesterol:protein ratios as well as an increase in specific activity upon purification of rabbit kidney ($Na^+ + K^+$)-ATPase by treatment with sodium dodecyl sulfate (SDS). More recently, Wheeler, Walker and Barker (2,3) also reported a correlation between specific activity and phospholipid composition of rabbit kidney ($Na^+ + K^+$)-ATPase resulting from detergent extraction with either lubrol WX or deoxycholate (DOC). Apparently specific phospholipids are required for reactivation of partially delipidated systems.

We have already employed the fluorescent probe N-phenyl-α-naphthylamine (NPN) to examine the role of lipid phase transitions in the temperature dependence of ($Na^+ + K^+$)-ATPase (4) and have used this agent here to gain additional information concerning the possible relationship between membrane lipids and enzyme activity.

Although NPN may be a relatively nonspecific lipid probe, both the solvent isotope effects described by Radda and Vanderkooi (5) and the paramagnetic resonance studies of Colley and Metcalfe (6) strongly suggest that this probe inserts deep within the hydrophobic core of the membrane bilayer. The addition of cholesterol to an NPN-labelled phospholipid bilayer results in extrusion of NPN (7) and the fluorescence intensity of N-aryl-naphthylamines is subject to packing constraints (8,9). This agent might therefore be a useful probe of specific regions of the bio-

membrane system.

We report here the changes in fluorescence enhancement of NPN which occurred when this probe interacted with ox brain preparations of $(Na^+ + K^+)$-ATPase subjected to various treatments with detergents. The relationship of this enhancement to the specific activity of the enzyme which we observed suggests that NPN is preferentially inserted into a lipid region of the membrane which is necessary for optimal $(Na^+ + K^+)$-ATPase activity.

METHODS

N-phenyl-α-naphthylamine was obtained from British Drug Houses Ltd. and was recrystallysed twice from 60% ethanol in the dark before use. It was stored at -20°C as a 0.2 mM solution in methanol. All other reagents were analytical grade.

Enzyme Preparations

Ox brain membrane preparations were prepared and assayed as described by Charnock, Simonson and Almeida (10). The delipidation procedures with detergents have also been described previously (11,12). "Untreated" enzyme preparations as well as those obtained after activation by DOC and SDS were employed to provide a wide range of enzyme activities. Specific enzyme activity refers only to that hydrolysis of ATP which was sensitive to 0.1 mM ouabain, and is expressed as μmoles phosphate released from ATP/mg membrane protein/hour at 37°C.

Fluorescence Measurements

Fluorescence measurements were performed on a Perkin-Elmer MPF-4 fluorescence spectrophotometer fitted with a jacketed cell holder which permitted temperature regulation ± 0.1° at 37°C. Experiments were designed such that a constant amount of NPN (1 nanomole per 3 ml reaction mixture) was titrated with increasing amounts of enzyme preparation. The reaction mixture had a final concentration of 4 mM $MgSO_4$, 0.4 mM EDTA, 160 mM NaCl, 40 mM KCl and 200 mM glycylglycine and was adjusted to pH 7.6. Excitation and emission wavelengths were 345 nm and 400 nm respectively unless otherwise specified.

RESULTS AND DISCUSSION

The fluorescence intensity (F_i) of NPN in aqueous solution is negligible (< 1%) compared to that in the presence of our membrane enzyme preparations. This is illustrated in Fig. 1 which also shows the experimental protocol we employed. Note that upon addition of NPN in the presence of membrane the fluorescence intensity increases to a maximum in less than ten seconds which was the minimum time required for our measurements. The level of F_i then decreases erratically by a small amount over a period of a few minutes probably due to a mixing effect. Thereafter there is a slow but linear decrease in F_i for a period of at least one hour which was the limit of our observations. This slow decrease in F_i may be due to the photosensitivity of NPN.

A value for fluorescence intensity was obtained by extrapolation to the time of addition of NPN which was then corrected by subtracting the

contribution to fluorescence intensity by the membrane preparation in the absence of the probe. This value ($F.I_{corr.}$) was later employed in our calculation of maximal values at infinite membrane concentration.

By using a number of membrane enzyme concentrations (μg protein/ml), a rectangular hyperbolic relationship was obtained between fluorescence intensity ($F.I_{corr.}$) and enzyme concentration. This is shown in Fig. 2.

Increasing enzyme concentration resulted not only in an increase in the intensity of fluorescence ($F.I_{corr.}$) but also in a concomitant decrease (a so-called blue shift) in the wavelength of maximum emission. Fig. 2 shows that as the maximum fluorescence intensity of all preparations examined was approached (n = 17), so did the wavelength of maximum emission become constant (405.4 ± S.E. 0.3 nm).

This blue shift of NPN following incorporation into a lipid biomembrane is typical of N-arylnaphthylamine compounds and has been reported previously by others (9). It is thought to arise from an increase in the packing constraint of the probe (8,13) although a decrease in the polarity of the probe environment has also been suggested (14).

The fluorescence intensity:enzyme concentration relationships may be conveniently expressed as double reciprocal plots (9,15) from which we can obtain the value for fluorescence intensity at infinite enzyme concentration (FI_{max}) and thus the concentration of enzyme required to give half maximal fluorescence intensity ($K_{0.5}$). Fig. 3 shows the data obtained from twenty-two experiments using enzymes of widely different $(Na^+ + K^+)$-ATPase activity which had been prepared by three different procedures. As can be seen from the plot, the values for the intercept $\frac{1}{FI_{corr.}}$ are all

quite similar irrespective of the treatment of the enzyme preparation. When considered together (n = 22) the values for FI_{max} that were obtained were one hundred and eighty-six times (186 ± S.E. 10) the fluorescence intensity of the probe in the absence of membrane and demonstrate clearly the magnitude of the fluorescence enhancement which has occurred.

Although the intercepts for $\frac{1}{FI_{corr}}$ were similar, this was not the case for the slopes of the plots ($K_{0.5}/FI_{corr}$) which were widely different depending upon the pretreatment of the membranes and thus indirectly the specific activity of the $(Na^+ + K^+)$-ATPase. In general these experiments suggest that there was a marked decrease in slope as the specific activity of the $(Na^+ + K^+)$-ATPase preparation was increased first by extraction with DOC and even more markedly after extraction with SDS (10,16).

This impression is confirmed by the data given in Fig. 4 which shows the direct relationship between $K_{0.5}$ and the specific activity of all preparations examined. Clearly the value for $K_{0.5}$ decreases as the specific activity of the enzyme preparations increases. There can be little doubt that high specific activity preparations of $(Na^+ + K^+)$-ATPase have much greater capacity to bind or insert NPN than do those of lower activity.

This does not appear to be a result of residual detergent in the preparation. Firstly, some trend was found in the group of untreated preparations which obviously contain no detergent, although the range of specific activities was relatively small (20-32 μmoles Pi/mg protein/hr). However, the range of specific activities after treatment with DOC was from 55-110 μmoles Pi/mg protein/hr and we have no reason to suppose that this arises

from a two-fold increase in residual DOC. The same argument applies to the data obtained after treatment with SDS. The enzyme activities range from 95-150 μmoles Pi/mg protein/hr. The intensity of fluorescence (given here as values for $K_{0.5}$) only change by about 20% but the relationship between specific activity and fluorescence enhancement still appears to hold.

Do these results indicate that the increase in probe binding which is observed, occur because there is an increase in the number of specific probe binding sites which takes place as greater enzyme purification and specific activity is achieved?

At present our data cannot provide a definite answer.

We do know from preliminary gravimetric analysis that the total lipid: protein ratio of our preparations is at least 3 times greater after treatment with SDS than that of the untreated controls. This might indicate that the increase in probe binding only reflects an increase in the total amount of lipid available in the system and does not necessarily reflect a proportional change in enzyme specific activity.

On the other hand, there is only a very small change in the lipid: protein ratio after treatment with DOC. At the same time there is a marked increase in both enzyme specific activity and in fluorescence enhancement.

This seems to suggest that the fluorescent probe NPN is preferentially inserted into a lipid region of the membrane which is necessary for the optimal activity of $(Na^+ + K^+)$-ATPase.

Such a conclusion would be in agreement with our observations on the type of electron spin resonance spectra which are obtained with a nitroxyl stearic acid spin probe (16).

ACKNOWLEDGEMENTS

This work was supported by grant #MT-4945 from the Medical Research Council of Canada.

Fig. 1: Fluorescence enhancement: The change in fluorescence intensity (F_i) following the incorporation of NPN into ox brain membranes rich in ($Na^+ + K^+$)-ATPase. (——) solid line shows the normal experimental protocol where A is the inherent fluorescence of the buffered medium, B is the very small (< 2%) increase which occurs on the addition of the membrane suspension and C the fluorescence enhancement which follows introduction of NPN into the system. (---) dashed line shows the control experiments with NPN in the absence of ox brain membranes. The value for F_i of NPN in aqueous solution is < 1% of the enhancement in the presence of membranes.

Fig. 2: The effect of increasing the concentration of ox brain membrane (µg protein) on the fluorescence intensity (FI_{corr}, o - o) and the wavelength of maximum emission (λ_{max}, x - x). Both parameters show maximum change when more than 1000 µg protein was present in the system described under Methods.

Fig. 3: Double reciprocal plot of fluorescence intensity v. membrane concentration for three different preparations of ox brain $(Na^+ + K^+)$-ATPase. Values for fluorescence intensity at infinite membrane concentration are similar for untreated (o - o), DOC extracted (o - o) and SDS extracted (x - x) membranes. The specific activities of the three preparations examined were respectively 20, 102 and 144 µg Pi/mg protein/hr at 37°C.

Fig. 4: Relationship between values for $K_{0.5}$ and the specific activity of $(Na^+ + K^+)$-ATPase of ox brain membranes. $K_{0.5}$ represents the amount of membrane (µg protein) required to give half maximal fluorescence intensity in the presence of 1 nanomole NPN.
Data from seven different untreated preparations (o); five different preparations after extraction with DOC (x); four different preparations after extraction with SDS (o).

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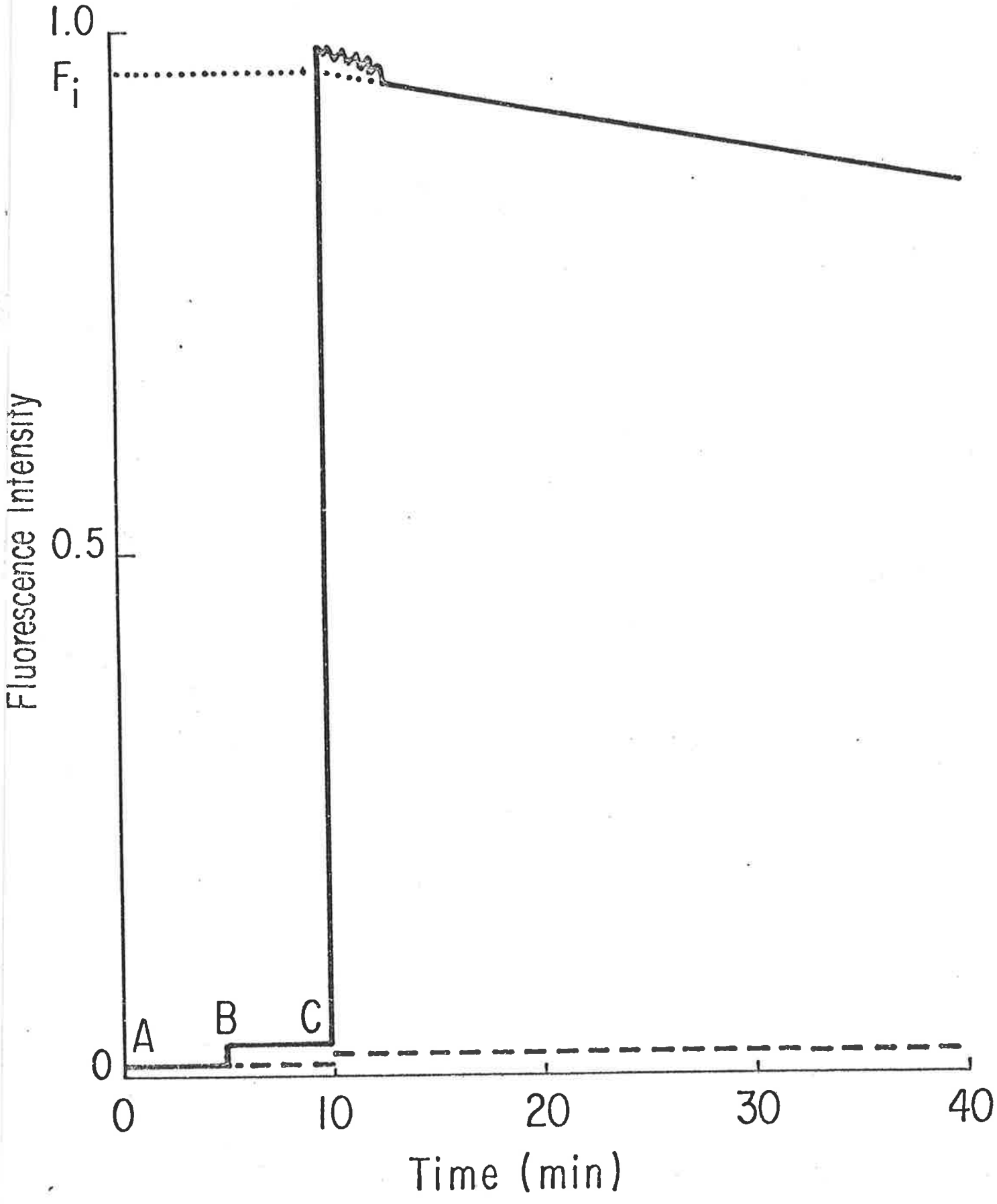

1.0
F_i
0.5
0
Fluorescence Intensity
A
B
C
0
10
20
30
40
Time (min)

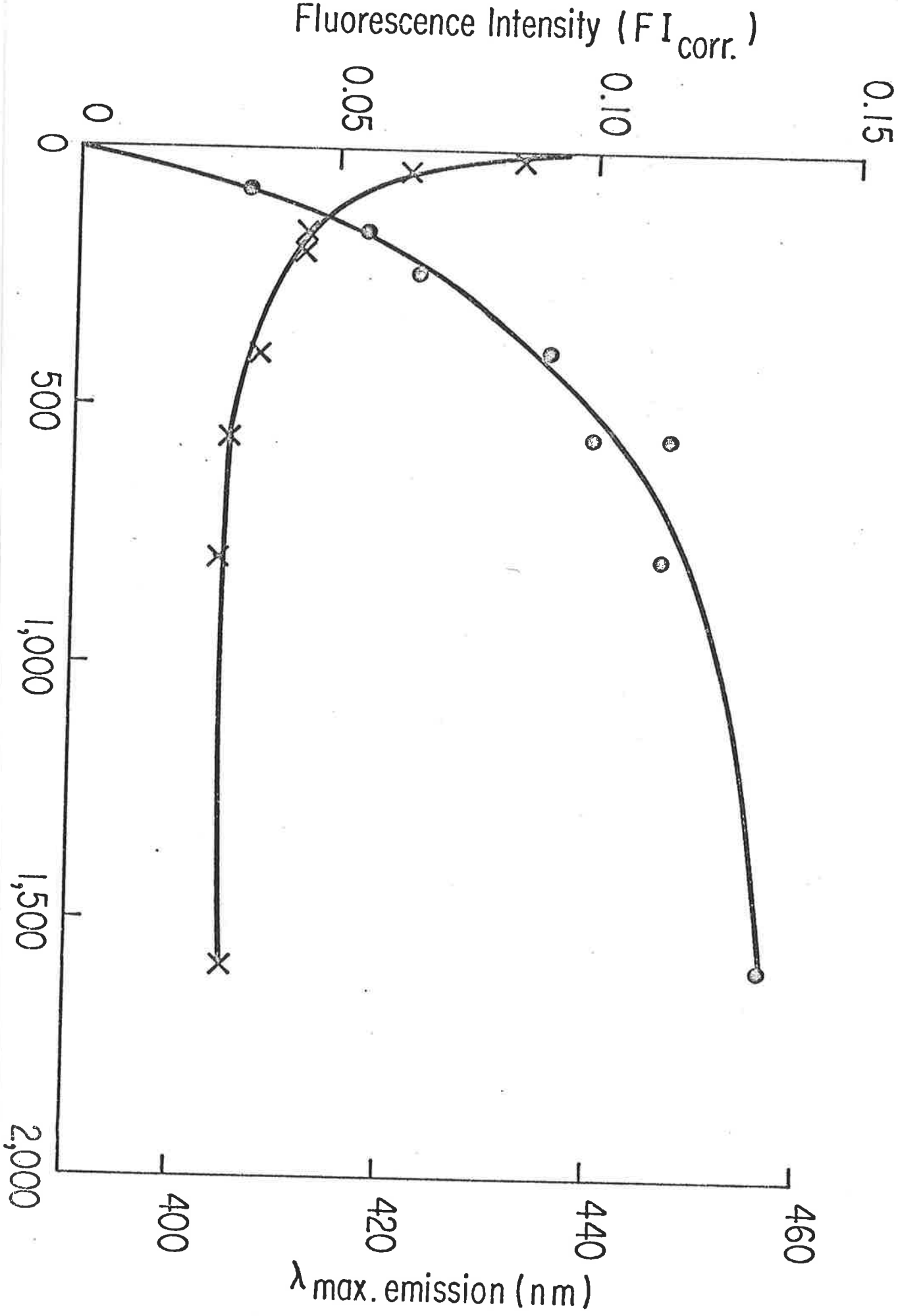
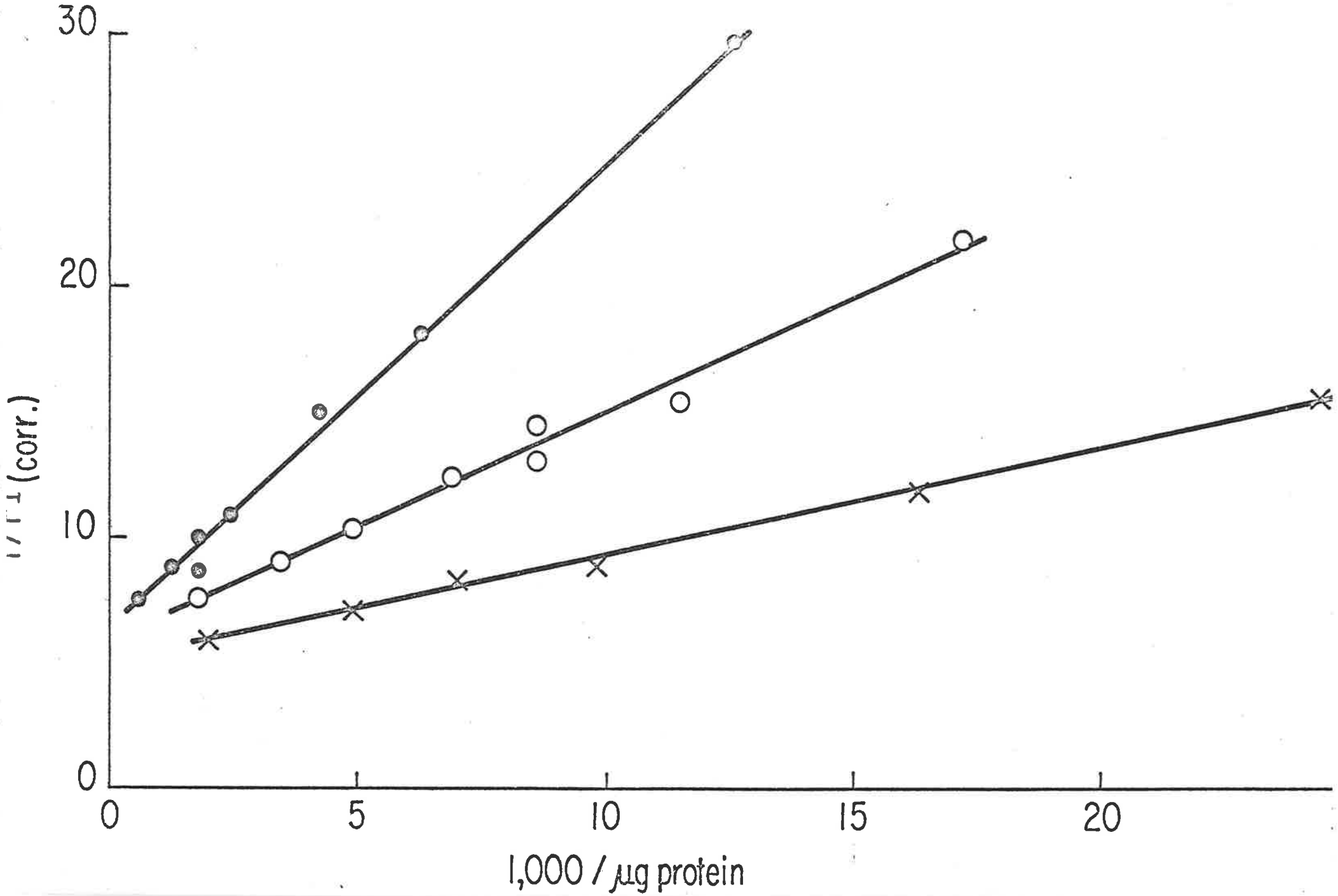
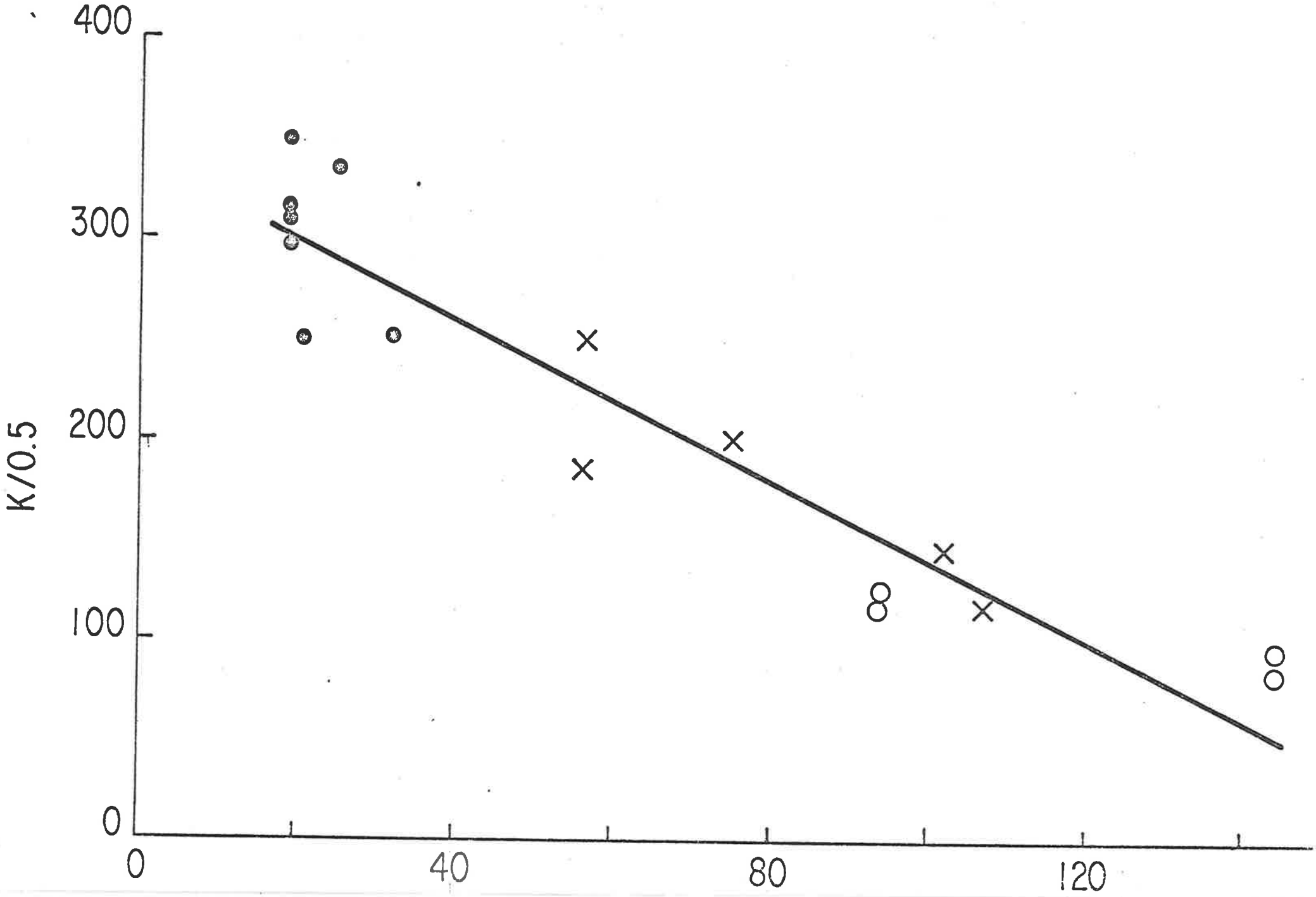

Fluorescence Intensity (F I corr.)
0
0.05
0.10
0.15
0
500
1,000
1,500
2,000
400
420
440
460
λ max. emission (nm)

Fig. 3




400
300
200
100
0
K/0.5
0
40
80
120
8
8

THE BINDING OF CARDIAC GLYCOSIDES TO MEMBRANE RECEPTORS

By

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29TH ANNUAL MEETING OF THE CANADIAN CARDIOVASCULAR SOCIETY

Edmonton, October 18 - 23, 1976

* Supported by the Alberta Heart Foundation

Although it is almost 200 years since Withering introduced digitalis into clinical medicine we still do not know the precise mechanism by which this agent or any other active cardiac glycoside influences the inotropic and arrhythmogenic response of the heart.

Of the many biochemical systems which have been considered as likely targets for these effects, only the inhibitory action of cardiac glycosides on membrane transport reactions has been seriously implicated in these responses. The inhibitory effect of cardiac glycosides on the coupled transport of Na^+ and K^+ ions was first reported by Schatzmann (1) in 1953 and later Skou (2) showed that there was a specific inhibition of the membrane ATPase reaction which is responsible for this process. In 1965 Repke (3) reported that there was a positive correlation between the inhibition of this enzyme system, that is, $(Na^+ + K^+)$-ATPase and the inotropic response of cardiac muscle. Repke (3) found that this correlation was only apparent in the so-called digitalis-sensitive species he examined and on the basis of this evidence he suggested that membrane $(Na^+ + K^+)$-ATPase of cardiac muscle was the long sort "digitalis receptor". More recently Erdmann and Schoner (4) have shown that membrane preparations of $(Na^+ + K^+)$-ATPase will bind radioactively labelled cardiac glycosides - notably [^{3}H]-ouabain - in a way which suggests that there is a direct relationship between the amount of drug bound and the biochemical function of the enzyme system; that is, in vitro there is good experimental evidence to support the concept that membrane $(Na^+ + K^+)$-ATPase has some of the essential properties of a cardiac glycoside receptor system.

We therefore sought to study this process in order to learn more about those factors which influence the binding of these clinically important drugs to their potential receptor site. Although my laboratory was one of the first to isolate $(Na^+ + K^+)$-ATPase from cardiac muscle (5), the preparations that are obtained are extremely impure and are of relatively low activity - for example, only 10-20% of the total ATPase activity is sensitive to ouabain which is the most water soluble of the cardiac glycosides and is thus the most commonly used in in vitro experiments of this nature. These somewhat disappointing results are obtained despite the fact that the muscle has been subjected to extremely vigorous preparative techniques which include both extraction with strong salt solutions and exposure to detergents! At the present time, we do not consider these enzyme preparations suitable for the study of drug receptor interactions as they are neither sufficiently pure nor are they sufficiently active.

We therefore elected to begin our cardiac glycoside binding studies with enzyme preparations obtained from a more amenable source and the work I shall describe today was carried out with a membrane preparation of $(Na^+ + K^+)$-ATPase which was prepared from beef brain. As far as we are able to tell, this enzyme system possesses identical biochemical characteristics to that described in cardiac muscle, but it is at least ten times as active and is over 85% sensitive to inhibition by ouabain (6).

LDE 1 In the first slide you will see that we repeated the experiments of Erdmann and Schoner and were able to demonstrate the strong positive

correlation that exists between [^{3}H]-ouabain binding and the specific activity of the enzyme system. This correlation is apparent whether the results are expressed as the rate of drug binding which is shown in Panel A, or the amount of drug binding which is shown in Panel B. In either case it is clear that there is an increase in drug binding as the activity of the preparations increases. Because of the amount of [^{3}H]-ouabain which binds to the system under equilibrium conditions is a direct reflection of the number of drug receptor sites which are available per unit mass of membrane enzyme, this type of study can be used to examine the factors which affect the number of receptor sites which may be present in the system.

IDE 2

Because detergents are commonly used in the preparation and isolation of ($Na^+ + K^+$)-ATPase from cardiac muscle, we first studied the effect of these agents upon our brain enzyme preparation. In the next slide you will see what happened when we treated our preparations with either deoxycholate or sodium dodecyl sulfate. It is apparent that detergent treatment increases the activity of these preparations and that there is a concomitant increase in the amount of [^{3}H]-ouabain which can be bound after lipid extraction; that is, there is an apparent increase in the number of drug binding sites which are available after detergent extraction! If removing some of the lipids by detergent extraction reveals more binding sites, we wondered what would happen if we treated the enzyme preparation with phospholipase-A, an agent which should split many of the membrane phospholipids into their lyso-compounds

and release free fatty acids from the membrane - a procedure which had been reported to markedly reduce activity of $(Na^+ + K^+)$-ATPase.

LIDE 3 The next slide showed what happened when we treated our enzyme preparation with 20 units of bee-venom phospholipase-A. The open circles (o) are the specific activity of the enzyme, and the closed circles (●) represent the [^{3}H]-ouabain binding which could be measured concurrently. The first point to note is that the effects on enzyme activity and on drug binding parallel each other. Secondly, the effects are biphasic in that there is first a very marked increase in both drug binding and enzyme activity which is rapidly followed by an almost equal decrease in both of these parameters. It is important to note that the final level of drug binding which is reached in these experiments is about 65-70 pmoles [^{3}H]-ouabain per mg enzyme protein and is more than half the maximum level obtained.

However, the experimental result is somewhat different if we first treat the enzyme preparation with detergents and then expose it to

SLIDE 4 subsequent treatment with phospholipase-A. The next slide shows the results of an experiment in which the enzyme was treated for a few minutes with deoxycholate at 30°, the detergent washed away, and the preparation then exposed to only 5 units of phospholipase-A. The open circles (o) again represent specific activity of the enzyme, and the closed circles (●) are the amount of [^{3}H]-ouabain which was bound.

Please note that there is a definite increase in the starting point of these experiments as one would expect after detergent treatment of the enzyme. In these experiments the starting levels are about twice that shown in the previous slide. The action of phospholipase-A is again to effect both these parameters in a parallel fashion although the effect is no longer biphasic. As you can see, there is an immediate and precipitous drop in both enzyme activity and drug binding. The residual level of drug binding is again just over half of the amount that was found with preparations of the enzyme before treatment with phospholipase-A.

We think that these experiments have told us several important things about the binding of [^{3}H]-ouabain to ($Na^+ + K^+$)-ATPase (6). Apparently, there are two different types of ouabain binding sites or drug receptors in these preparations:

1. The first of these sites are apparently readily available for drug binding and are resistant to attack by phospholipase-A. These represent about half of the total number of sites.
2. The remainder of the sites are latent and are only revealed in our experiments when the system is activated by detergent treatment. These latent sites are very sensitive to lipolysis by phospholipase-A and are presumably located in the membrane in a very lipid-rich area.

This interpretation could be very important for our understanding of the drug receptor interaction, as many workers now believe that the lipids of biological membranes exert a powerful modulating or controlling influence over the action of membrane protein receptors. It might therefore be possible to demonstrate some sort of lipid control over the binding of cardiac glycosides to these preparations. We therefore examined this hypothesis by utilizing the effect of temperature to alter the physical properties of membrane lipids which in general are more fluid at the higher temperatures than they are at the lower temperatures of the usual physiological range (0° - 40°C).

DE 5

The next slide shows the results of an experiment in which we examined the effect of temperature upon the rate of [^{3}H]-ouabain binding to preparations of ($Na^+ + K^+$)-ATPase before and after detergent treatment. The results are displayed as an Arrhenius plot which indicates the log of the rate of ouabain binding versus the reciprocal of the absolute temperature. You will see that in the control situation before detergent treatment, which is shown by the closed circles (●), that the effect of temperature is non-linear and displays a sharp discontinuity at about 20°C. This type of result is thought to reflect a dramatic change in the fluidity of membrane lipids in these preparations at about 20° and strongly indicates that drug binding is indeed influenced by the physical state of the membrane lipids. Conversely, detergent treatment of the enzyme which is shown by the open symbols (▭), produces a linear plot which does not necessarily indicate a controlling effect by the remaining lipids. We

believe that this type of experiment supports our previous conclusions that there are two independent sites for cardiac glycoside binding, only one of which is very dependent upon the nature and fluidity of the membrane lipids immediately adjacent to this site.

We are at present trying to introduce certain specific lipids such as phosphatidyl serine or phosphatidyl choline into our system in the hope of learning whether some particular lipid component of the membrane is responsible for the modulation of the ouabain binding site we have observed.

In addition, we feel now that we have learnt enough about the process in brain and how to measure it in the laboratory that we are ready to try again to work with the much more difficult system of cardiac muscle. Whether either of these binding sites can be identified in the muscle, and perhaps more importantly, found to vary in health and disease remains to be seen - but it certainly is of interest to us that we can foresee that a change in a specific membrane lipid could be involved rather than a direct change in the protein of the drug receptor [^{3}H]-ouabain binding site.

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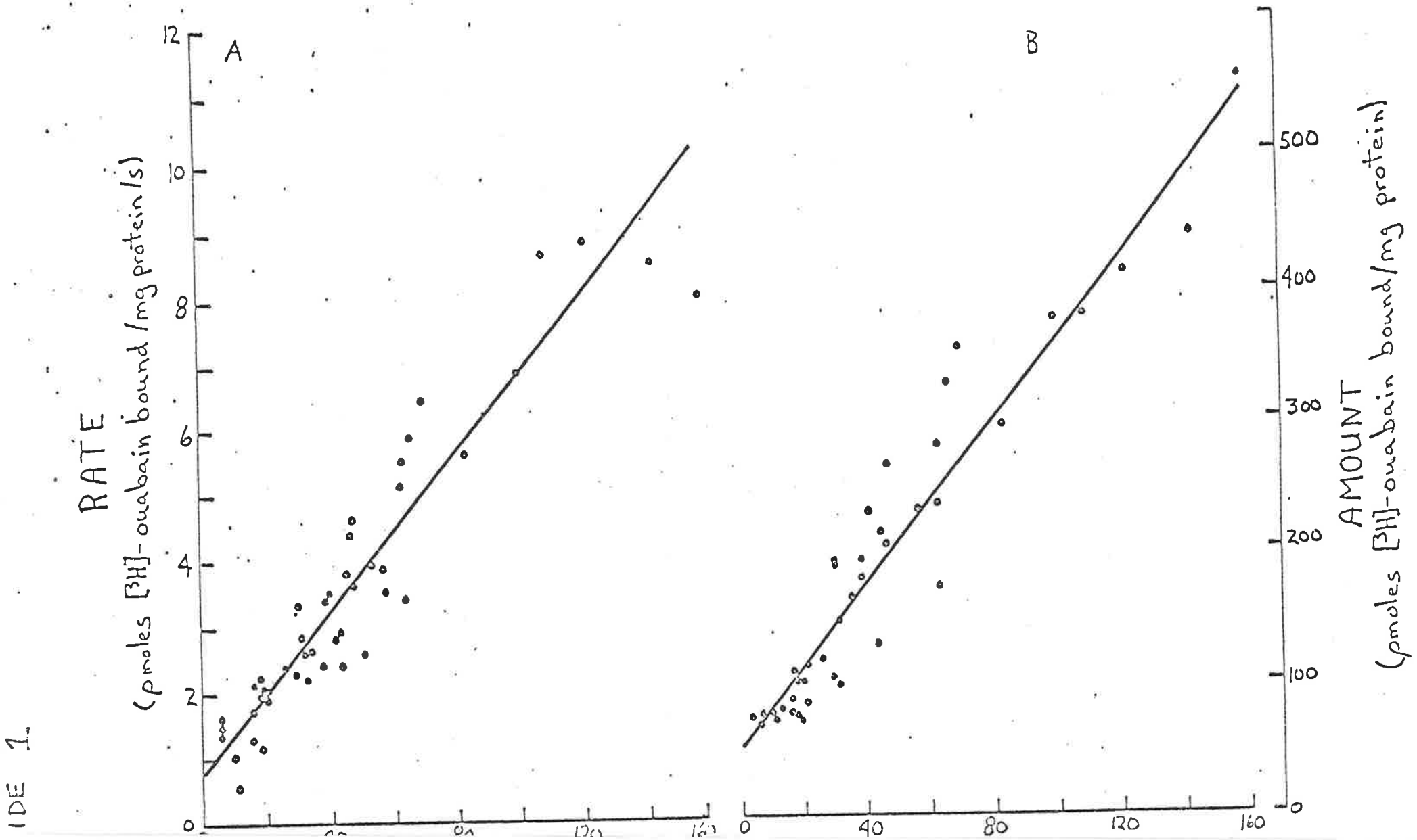
5. Potter, H.A., Charnock, J.S. and Opit, L.J. (1966) Aust. J. Exp. Biol. Med. Sci. 44, 503-518.

6. Charnock, J.S. and Simonson, L.P. (1976) Biochim. Biophys. Acta. In press.

Fig 2A and 2B [illegible]

Variation in Sensitivity of the Cardiac Glycoside Receptor Characteristics of $(Na^+ + K^+)$-ATPase to Lipolysis and Temperature



IDE 1.

DE 2

TABLE IV

COMPARISON OF THE SPECIFIC ACTIVITY AND EQUILIBRIUM LEVELS OF $[^3H]$-OUABAIN BOUND TO $(Na^+ + K^+)$-ATPase AFTER VARIOUS TREATMENTS WITH DETERGENTS

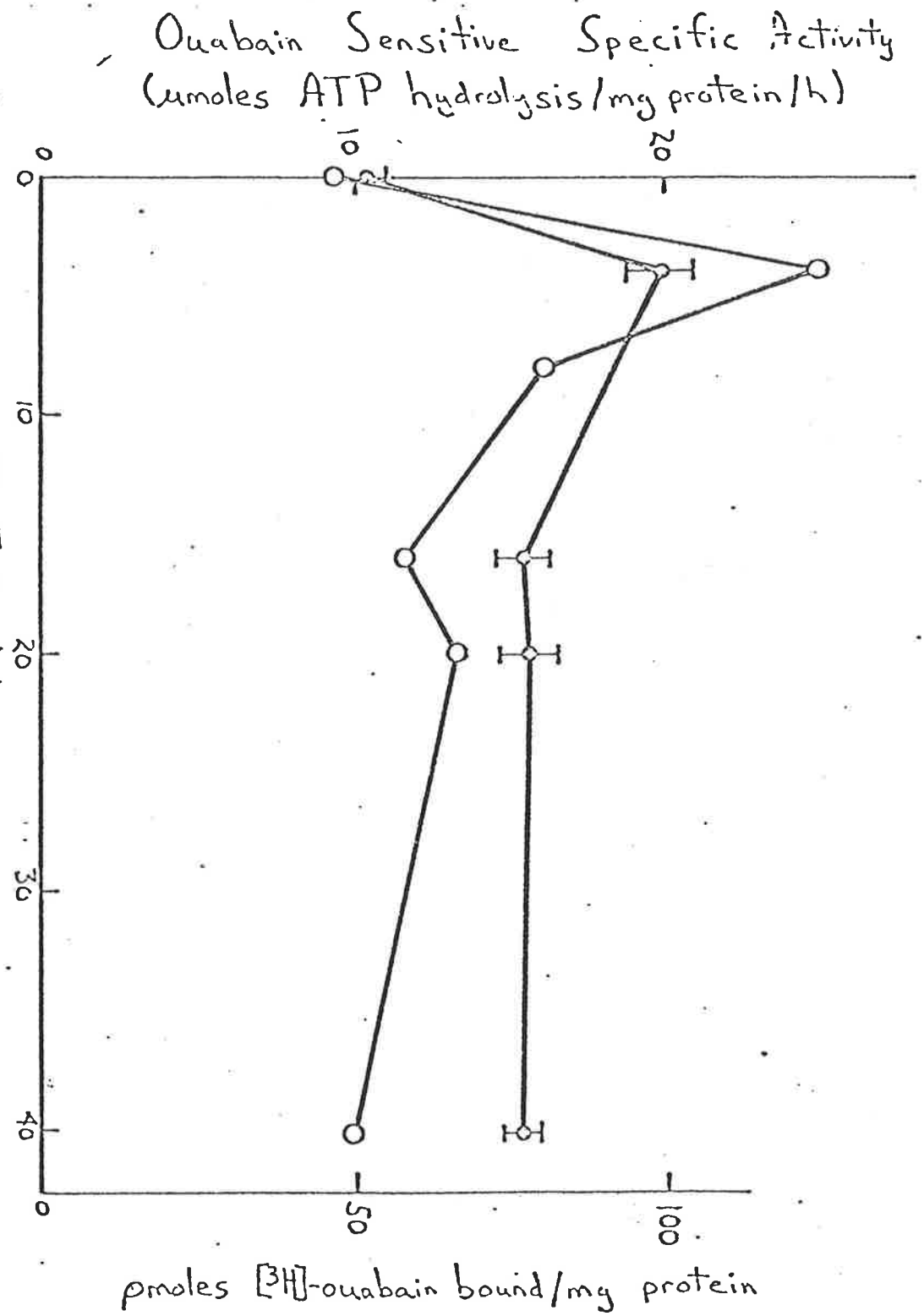
Treatment	n	Mean ± S.E. Specific Activity *	Mean ± S.E. Equilibrium Level **
None	14	15.2 ± 2.5	90.5 ± 6.1
DOC at 4°	16	31.6 ± 3.2	146.5 ± 12.7
DOC + ATP at 30°	3	96.9 ± 18.0	342 ± 55
DOC + ATP + Mg^{2+} + Na^+ at 30°	7	60.9 ± 4.8	295 ± 26
SDS + ATP at 30°	3	133 ± 17	457 ± 51

* Specific activity given as μmoles ATP hydrolysis/mg protein/h at 37°C.

** Equilibrium level given as pmoles $[^3H]$-ouabain bound/mg protein.

SLIDE 3

Fig. 3

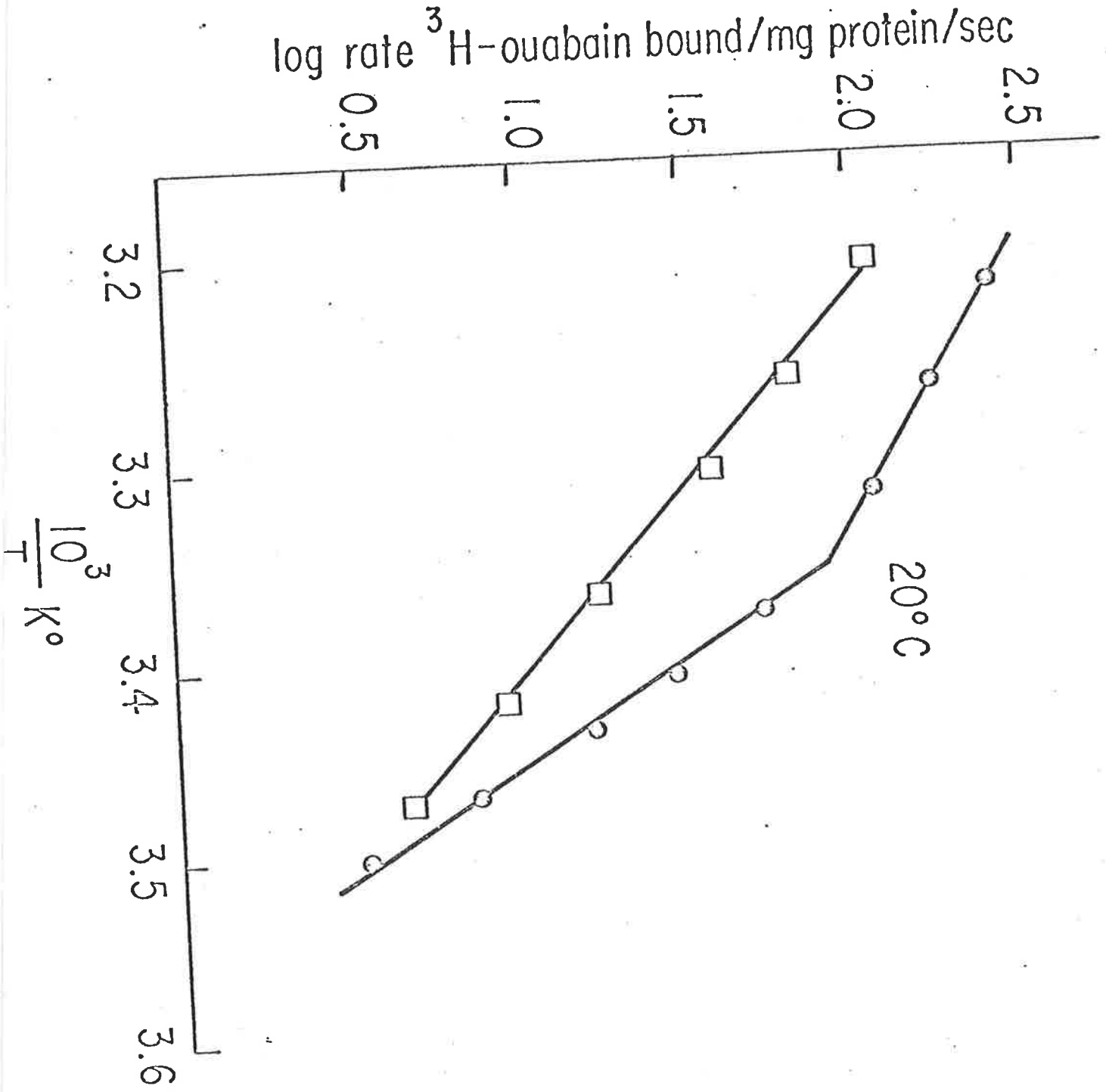


John S. Charnock, Laurits P. Simonson and Anthony F. Almeida
Variation in Sensitivity of the Cardiac Glycoside Receptor
Characteristics of $(Na^+ + K^+)$-ATPase to Lipolysis and Temperature

SLIDE 4

Fig. 4 John S. Charnock, Laurits P. Simonsen and Anthony F. Almeida
Variation in Sensitivity of the Cardiac Glycoside Receptor
Characteristics of $(Na^+ + K^+)$-ATPase to Lipolysis and Temperature

SLIDE 5.


log rate ^{3}H-ouabain bound/mg protein/sec
0.5
1.0
1.5
2.0
2.5
$\frac{10^3}{T}$ K°
3.2
3.3
3.4
3.5
3.6
20°C

SYMPOSIUM ON THE BIOCHEMISTRY OF MEMBRANE TRANSPORT, ZURICH, SWITZERLAND,

JULY 18 - 23, 1976

MEMBRANE LIPIDS AND $(Na^+ + K^+)$-ATPase ACTIVITY.

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Comparison of the effects of temperature on the lipid mobility of membranes containing $(Na^+ + K^+)$-ATPase and the ATPase activity of those systems strongly suggests that membrane lipids play an important role in modulating membrane enzyme activity. This suggestion is further supported by a comparative study of membrane systems obtained from a variety of biological sources. Whereas there is a marked discontinuity at about 18°C in the thermal responses of membrane systems obtained from homeotherms, no such discontinuity is observed in membrane systems from poikilotherms. $(Na^+ + K^+)$-ATPase activity in membranes from hibernators shows both forms of thermal response according to the season. Variation in membrane lipid composition is believed to be the primary modulating cause in these effects.

Membrane lipids and ($Na^+ + K^+$)-ATPase activity.

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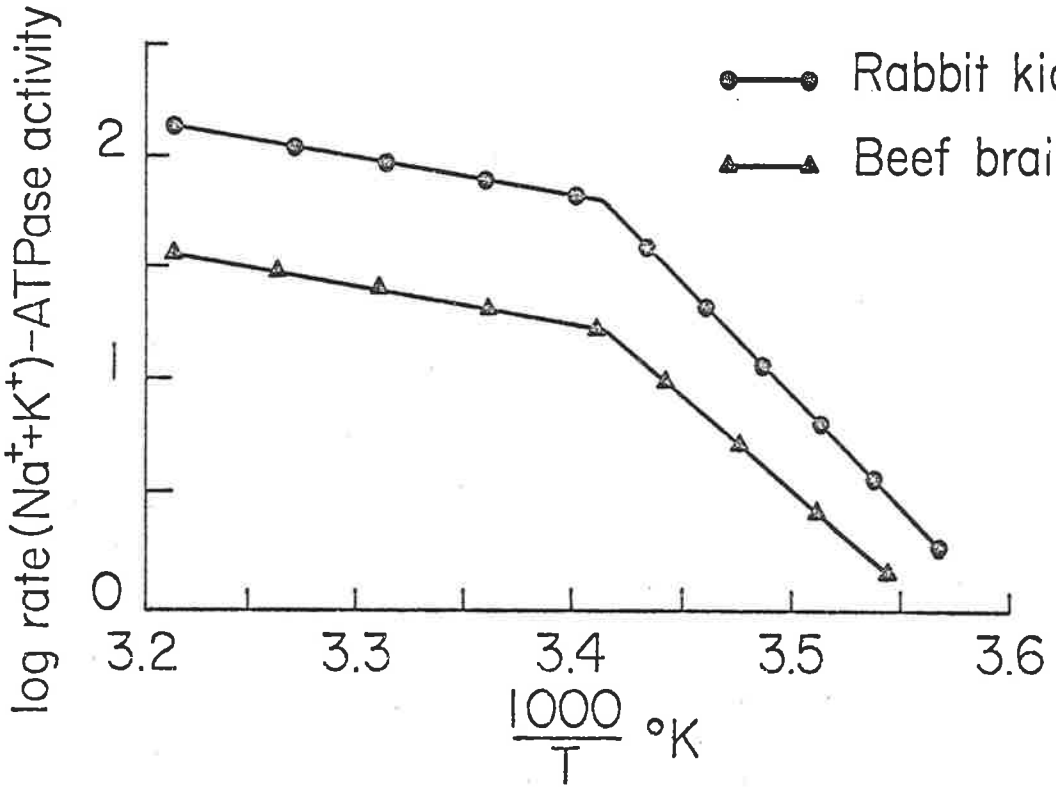
Introduction

For more than a decade it has been known that membrane lipids are required for full activation of ($Na^+ + K^+$)-ATPase. Chemical modification of membrane lipids by detergents or lipases frequently inactivates the enzyme system; activity being restored by reconstitution with such phospholipids as phosphatidyl serine. (PS) Recently by incorporation of the fluorescent probe 12-(9-anthroyl)-stearic acid (12-AS) into membranes rich in ($Na^+ + K^+$)-ATPase we have been able to demonstrate a significant correlation between the mobility of the lipids of the hydrophobic core of the membranes and the ouabain-sensitive activity of the enzyme prepared from a number

of different sources. The temperature dependence of enzyme activity and the lipid fluidity of the membrane matrix were in parallel. (cf. Charnock J.S. and Bashford C.L: Molecular Pharmacology 11, 766, 1975). Apparently temperature dependent changes in membrane lipid mobility play an important role in modulating the activity of this enzyme system. Because there are significant differences in the lipid composition of membranes obtained from homeotherms and poikilotherms a comparative study of the effects of temperature upon the rate of (Na^++K^+)-ATPase derived from a variety of biological sources should confirm this hypothesis.
We have therefore compared the effects of temperature upon (Na^++K^+)-ATPase preparations from beef and rabbits (homeotherms) with those from cold water crabs and lobsters (poikilotherms) as well as examining possible seasonal variations in ground squirrels (hibernators).

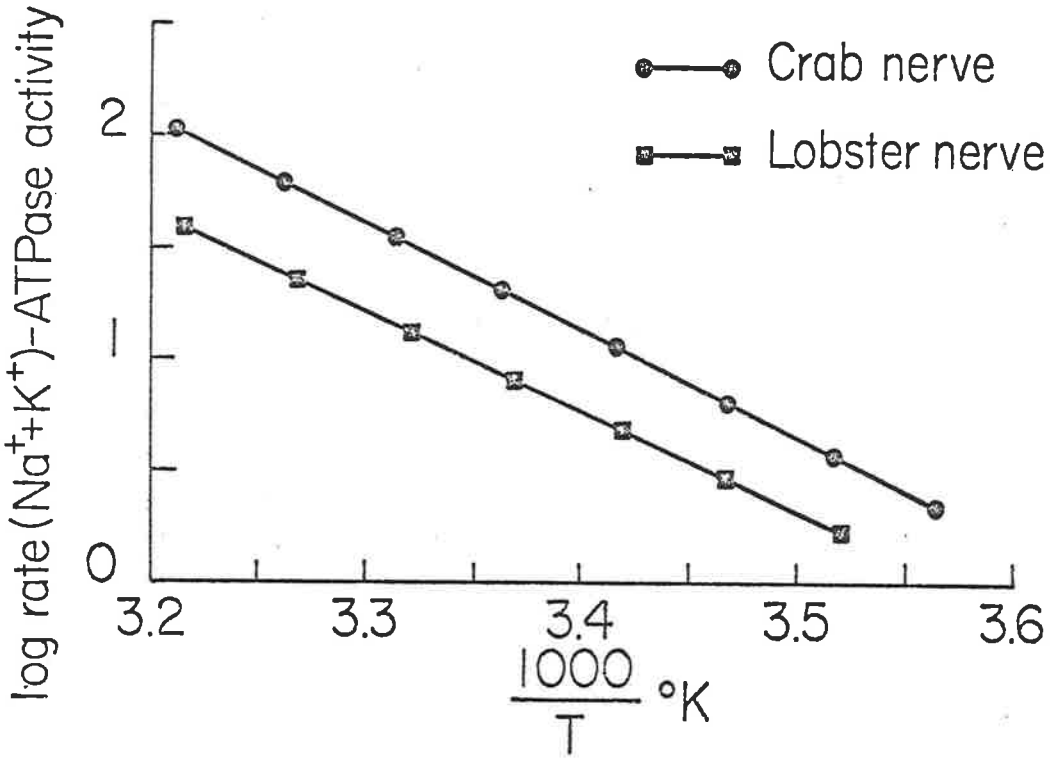
EFFECT OF TEMPERATURE ON $(Na^+ + K^+)$-ATPase FROM HOMEOTHERMS



1) Both rabbit kidney and beef-brain preparations give non-linear Arrhenius plots between 5°-40°C. The marked thermal transition occurs near 20°C in both preparations. Calculation of the apparent energies of activation of $(Na^+ + K^+)$-ATPase activity above and below the point of thermal transition gives values of about 15 kcals/mole and 35 kcals/mole respectively.

2) Identical results have been obtained with enzyme preparations obtained from kidney and brain of sheep, pig and guinea pig.

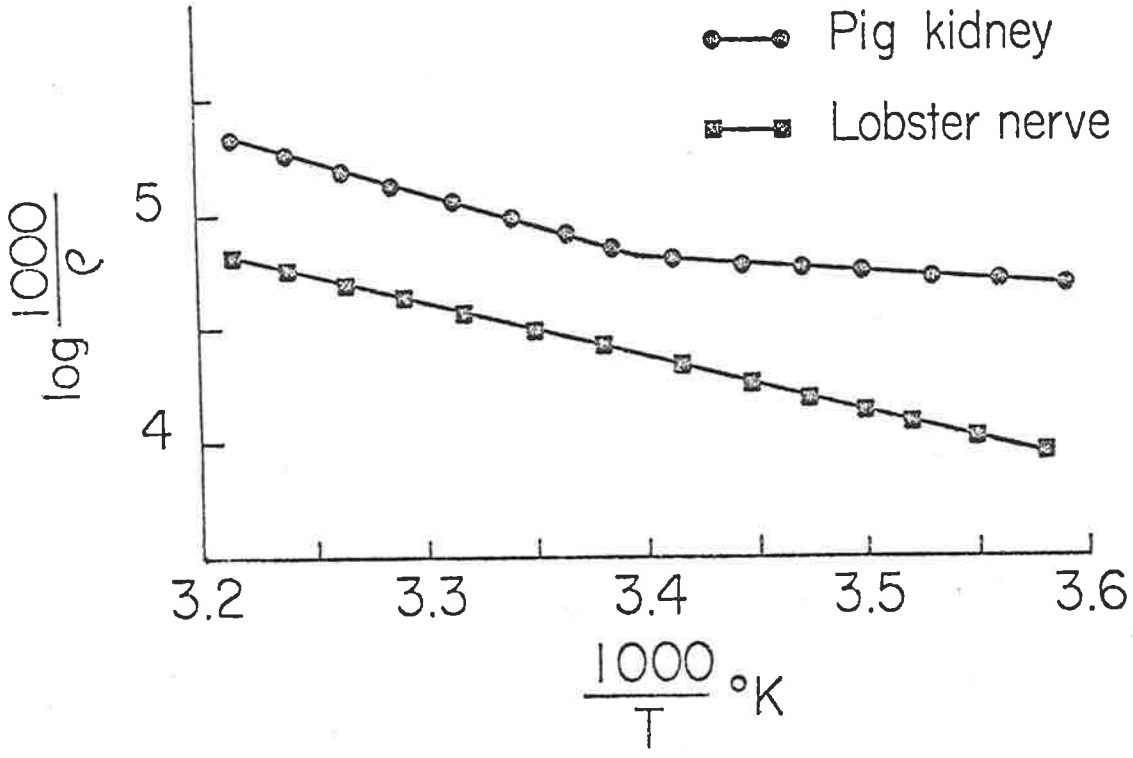
EFFECT OF TEMPERATURE ON (Na^++K^+)-ATPase FROM POIKILOTHERMS



1) Nerve preparations of (Na^++K^+)-ATPase from the walking legs of crab and lobster (ambient temperature 8°C) gave linear Arrhenius plots between 5°-40°C. No thermal transitions were observed in either preparation in contrast to those seen in enzyme preparations from homeotherms.

2) The apparent energy of activation over the whole temperature range was about 20 kcals/mole.

EFFECT OF TEMPERATURE ON THE ROTATIONAL RELAXATION TIME "ρ" OF THE FLUORESCENT PROBE 12-AS INCORPORATED INTO (Na^++K^+)-ATPase MEMBRANES FROM HOMEOTHERMS AND POIKILOTHERMS.



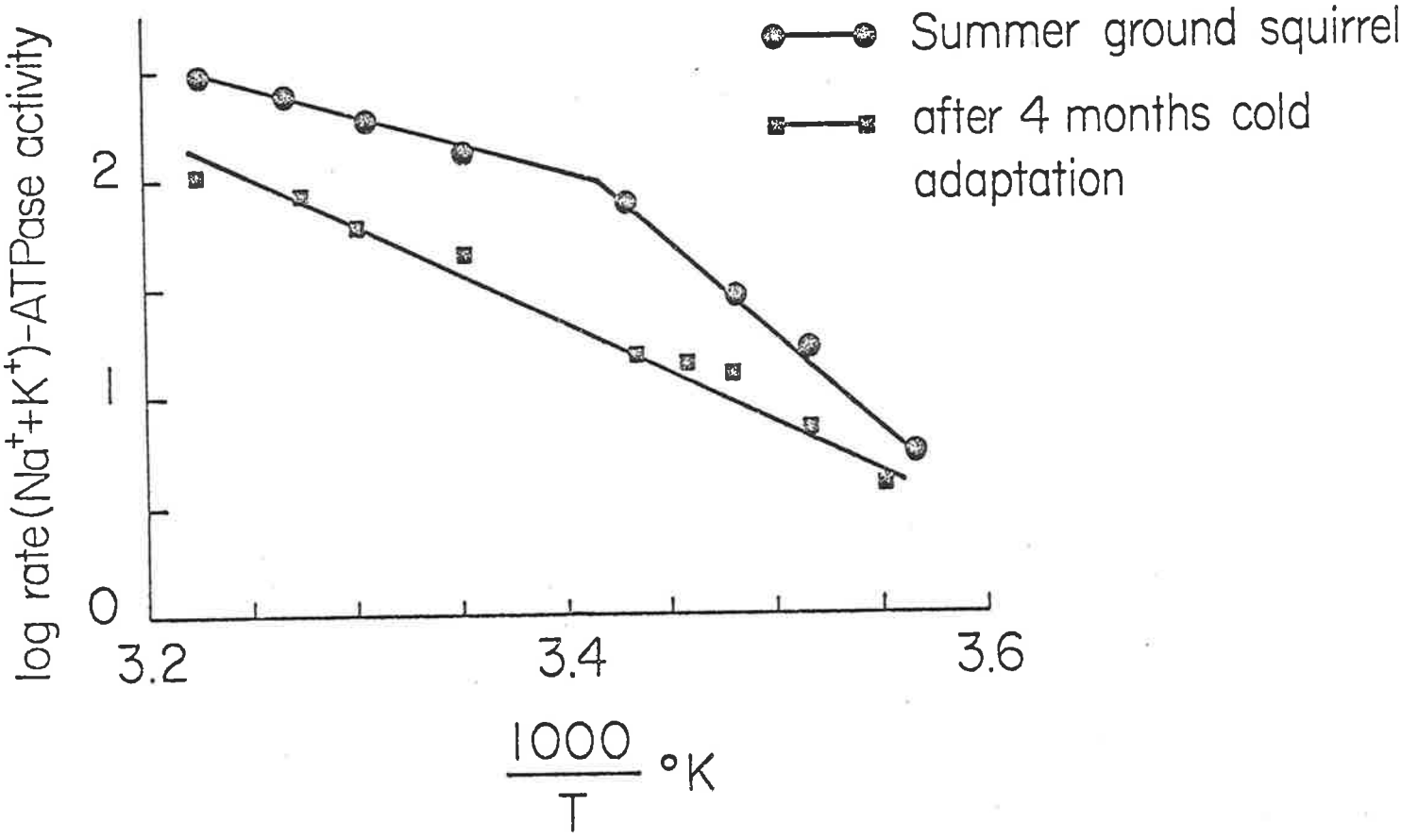
1) Non-linear Arrhenius plot of "ϱ" between 5°-40°C for (Na^++K^+)-ATPase containing membrane from pig kidney (homeotherm, ●—●). Thermal transition is near 20° C similar to that observed in the related ATPase activity experiments.

2) Linear Arrhenius plot of "ϱ" between 5°-40°C for (Na^++K^+)-ATPase containing membrane from the walking leg of lobster (poikilotherm, ■—■). No transition temperature observed similar to the result found in the related ATPase activity experiments.

3) There is a good correlation between (Na^++K^+)-ATPase activity and the molecular mobility of the membrane lipids of enzymes prepared from both homeotherms and poikilotherms.

SEASONAL VARIATION IN THE EFFECT OF TEMPERATURE OF $(Na^+ + K^+)$-ATPase ACTIVITY OF A HIBERNATOR.

(Spermophilus richardsonii)



1) Non-linear Arrhenius plot between 5°-40°C for (Na^++K^+)-ATPase prepared from the brain of Richardson ground-squirrels collected during summer months (June-Sept. 1975). There is a marked thermal transition at 20°C similar to that found in enzyme preparations obtained from homeotherms. Apparent energies of activation above and below the thermal transition are also similar to those calculated for homeotherms (13.9 and 35.3 k cals/mole respectively).

2) Linear Arrhenius plot between 5°-40°C for (Na^++K^+)-ATPase prepared from the brain of Richardson ground-squirrels after 4 months adaption to cold (Jan-Feb 1976). No thermal transition is apparent, similar to the (Na^++K^+)-ATPase results obtained with enzyme preparations derived from poikilotherms.

3) The mean specific activity of ouabain sensitive (Na^++K^+)-ATPase of cold adapted ground squirrels is reduced to 35% of the level of the controls collected and sampled during the summer months.

Conclusions

1) $(Na^+ + K^+)$-ATPase from HOMEOTHERMS display markedly non-linear Arrhenius plots with a thermal transition at about 20° C.

2) $(Na^+ + K^+)$-ATPase from POIKILOTHERMS display linear Arrhenius plots with no thermal transition between 5°–40° C.

3) There is a good correlation between the molecular mobility of the lipids of membranes containing $(Na^+ + K^+)$-ATPase and the ATPase activity of the enzymes of both homeotherms and poikilotherms.

4) A hibernating species (Richardson ground squirrels) displays seasonal characteristics of homeotherms during summer and poikilotherms after four months in the cold.

5) Ouabain-sensitive (Na^++K^+)-ATPase levels in the brain of hibernators are greatly reduced after four months exposure to the cold.

6) These comparative experiments provide strong evidence in favour of lipid modulation of (Na^++K^+)-ATPase activity and suggest that the physical properties of membrane lipids exert biological control over the active transport of cations in many different species.

Proc. Can. Fed. Biol. Soc. 19, #391 (p. 98) 1976.

Mechanism of Harmine Inhibition of $(Na^+ + K^+)$-ATPase

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The HARMALA ALKALOIDS are a group of hallucinogenic compounds which were originally extracted from a native Columbian liana plant for use in a potent concoction employed in ceremonial rituals by the indigenous tribes of Central America.

Today we know that these psychomimetic compounds induce disturbances in behaviour and perception - and at the biochemical level have been shown to interfere with many Na^+ dependent transport processes presumably because of their inhibition of the transport ATPase system of membranes more properly known as $(Na^+ + K^+)$-activated ATPase.

Clinically, at least 3 pharmacologically active components have been identified of which HARMINE and HARMALINE are the more potent. Their closely related structures are shown in Fig. 1. They are linear tricyclic compounds which differ only in the degree of saturation of the N-containing ring.

All of these alkaloids exhibit strong blue fluorescence between 420-485 nm, and it was this property that drew our initial attention as we are seeking to find some chemical tools for a fluorescent probe study of molecular mechanisms of membrane $(Na^+ + K^+)$-ATPase.

What was particularly attractive about these compounds was the report of Cannessa and her colleagues (Cannessa et al., J. Membrane Biol., 13 p. 263-282, 1973) that their inhibitory action was derived from their COMPETITIVE INHIBITION of the Na^+ activation site of the system. Hence we were potentially dealing with a group of fluorescent agents which were thought to be SITE SPECIFIC in ACTION.

We therefore began our study by examining the fluorescent properties of these agents in aqueous solution where we wished to exclude any possible chemical interaction between these compounds and the activating ligands of the $(Na^+ + K^+)$-ATPASE SYSTEM.

Almost immediately it became apparent that both Harmaline and Harmine were capable of forming complexes with the substrate for the enzyme system, that is ATP and that this complex formation was greatly influenced by the availability of Mg^{2+}. For example Fig. 2. shows a simple experiment in which we monitored the fluorescence intensity (E) of a 0.3 mM solution of harmine at different concentrations of ATP in the presence and absence of Mg^{2+}. You will see that in the absence of Mg^{2+} there is a marked fall in Harmine fluorescence which reaches a maximum when the [ATP] = [Harmine]. As Mg^{2+} become available, the quenching effect of ATP is reduced so that at equimolar [Mg^{2+}] = [Harmine] the effect of ATP is virtually abolished. Clearly some form of ATP:Mg:Harmine complex is being formed.

Subsequently, this and many other related experiments were performed which indicated that the interactions of HARMINE with Mg^{2+} and ATP were little changed in the presence of ENZYME PREPARATIONS i.e., the presence of enzyme was incidental to the formation of the Harmine:Mg:ATP complex.

In view of the earlier assertions of Cannessa et al. regarding "Site-Specificity" of the Harmala alkaloids it became important to re-investigate the mechanism of inhibition of (Na^+ + K^+)-ATPase by these agents.

Fig. 3 shows the results of a series of experiments with membrane fractions obtained from pig kidney which contained about 50-60% of their total ATPase activity which was inhibited by 0.1 mM ouabain, i.e., the (Na^+ + K^+)-activated fraction and the remainder insensitive to ouabain, i.e., the Mg^{2+}-ATPase type.

Maximal inhibition of both ATPases could be reached when the Harmine concentration equalled that of the substrate ATP. It is important to

note that in neither case was inhibition complete - and with Mg-ATPase no Na^+ activation sites are involved! Clearly something other than inhibition of Na^+ activation is occurring.

This was confirmed by the next experiments (Fig. 4) in which the effects of Harmine on $(Na^+ + K^+)$-ATPase were examined at variable concentrations of ATP. This sort of plot is typical of "MIXED INHIBITION" as defined by Dixon S. Webb in their classical text on Enzyme Kinetics. We next examined more directly the possible interactions of Harmine with the Na^+-loading site. To do this we examined the effect of Harmine on only ouabain-sensitive $(Na^+ + K^+)$-ATPase at various levels of Na^+.

Because $(Na^+ + K^+)$-ATPase is an assymmetric membrane-bound multi-site enzyme these kinetic experiments are frequently difficult to interpret.

In this case a standard double reciprocal analysis of the effect of $[Na^+]$ leads to a markedly parabolic plot (Fig. 5); whereas plotting the cubic reciprocal of "v" yields a good straight line. We therefore used this cubic reciprocal plot at several concentrations of HARMINE (Fig. 6). The results with 0.15 mM Harmine are shown in this figure where it is clear that Harmine indeed acts as a competitive inhibitor of Na^+ at the Na^+ loading sites. From 5 such experiments with Harmine concentrations from 0.1 mM to 1.0 mM we were able to calculate an apparent K_i for Harmine of approximately 0.3 mM - the [ATP] concentration was 2.5 mM. We therefore felt that we had reasonable evidence that Harmine interacts with both ATP and Na^+ and a strong suggestion that Mg^{2+} could be involved.

To examine the possible "non-specificity" of Harmine inhibition of $(Na^+ + K^+)$-ATPase further, we turned to several of the so-called "partial" reactions of the system which are believed to separate to some extent the requirements for various ligands.

For example it is now known that the inhibitor ouabain can be bound to the enzyme preparations under two separate sets of conditions, one of which - namely in the presence of Mg^{2+} and inorganic phosphate does not involve either ATP or Na^+, i.e., the so-called "BACK REACTION". The results of an examination of the effect of Harmine on ouabain binding are shown in Fig. 7.

In Panel A it can be seen that 1 mM Harmine inhibits ouabain binding whether this is supported by either 1) ATP and Na^+ or 2) Mg + Pi, with the inhibition in the presence of Mg + Pi being quite markedly greater.

In Panel B we show that the effect of Harmine inhibition increases with increasing concentration and reaches about 50% of the control when [Harmine] = 0.3 mM.

Taken in conjunction with our earlier observation of fluorescence changes of Harmine ± Mg^{2+} we feel that there can be little doubt that Mg also interacts directly with Harmine to produce Mg-Harmine complexes. We therefore felt that of the four major activating ligands for $(Na^+ + K^+)$-ATPase, we now had reasonable evidence for direct chemical interaction of Harmine with ATP, Mg^{2+} and Na^+!

All that remained was to look at the possible effects of Harmine at the K^+ activation site and to do this we examined the effect of Harmine on the K^+-dependant phosphatase reaction which is believed to represent the terminal step of the multi-step reaction sequence of $(Na^+ + K^+)$-ATPase.

We employed para-nitro phenyl phosphate as a false substrate for this reaction and were unable to show any effect of 1 mM Harmine in our assay! However in November of 1975, J.D. Robinson reported (Biochem. Pharmacol. 24, 2005, Nov. 1975) that 0.4 mM Harmaline inhibited K^+-phosphatase of rat brain. Robertson employed a fluorescence assay procedure based upon the release of umbelliferone from umbelliferone phosphate as

a substrate. While there can be little doubt that this procedure is more sensitive than the assay procedure of Fortes et al. which we used (Fortes, Ellory and Leu; Biochem. Biophys. Acta 318 p. 262-272, 1973) fluorescence quenching effects between Harmaline and umbelliferone may have occurred.

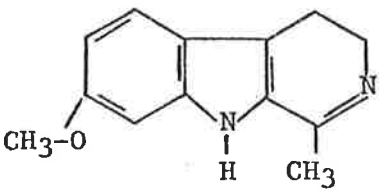
We are currently examining this possible explanation for the discrepancy between our findings and those of Robertson.

In Summary, we have shown that Harmine inhibits $(Na^+ + K^+)$-ATPase by:

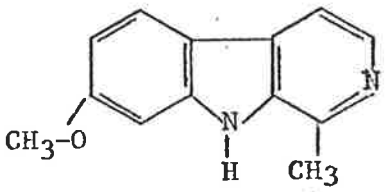
1) interacting with ATP
2) interacting with Mg^{2+}
3) interacting with Na^+

and the possibility exists that it may also interact with K^+. It is therefore clearly not "SITE-SPECIFIC" for Na^+ loading sites as claimed by Cannessa et al., and will be of less value than was originally hoped as a fluorescent probe of the molecular mechanism of $(Na^+ + K^+)$-ATPase.

FIG. 1



Harmaline



Harmine

FIG 2

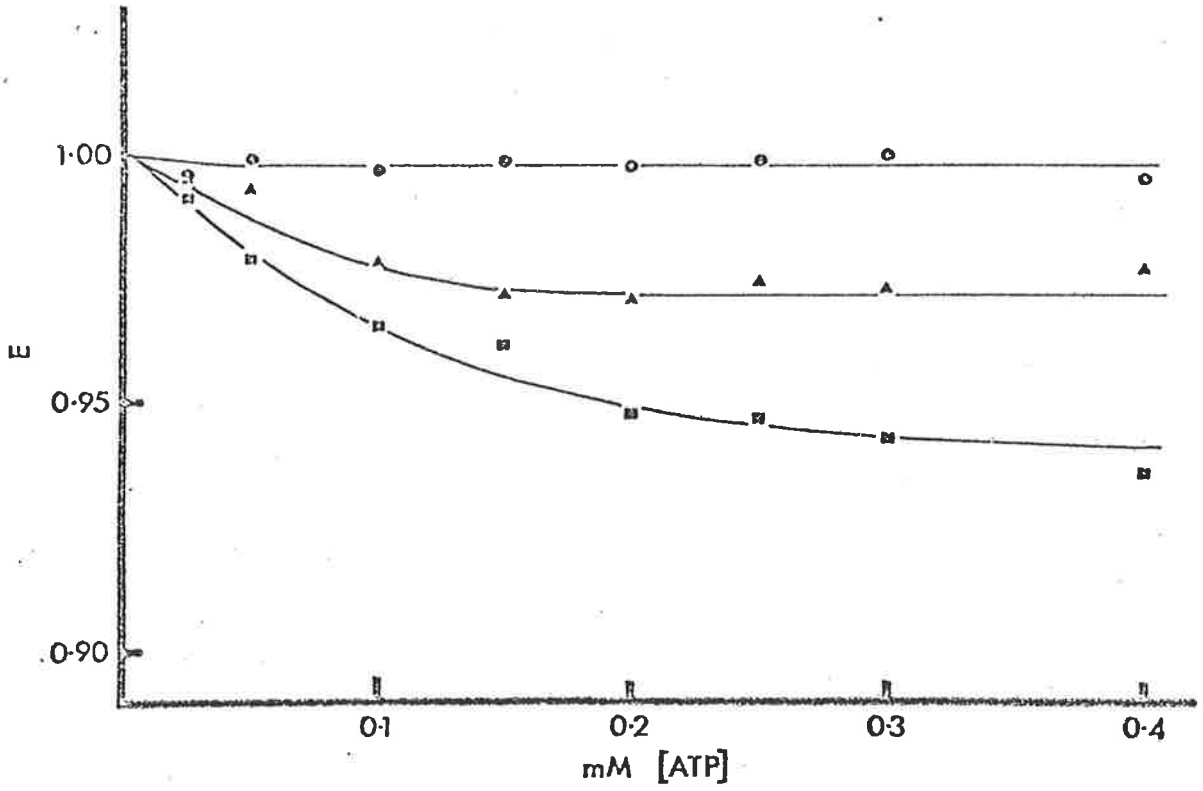

1·00
0·95
0·90
E
0·1
0·2
0·3
0·4
mM [ATP]

FIG 3

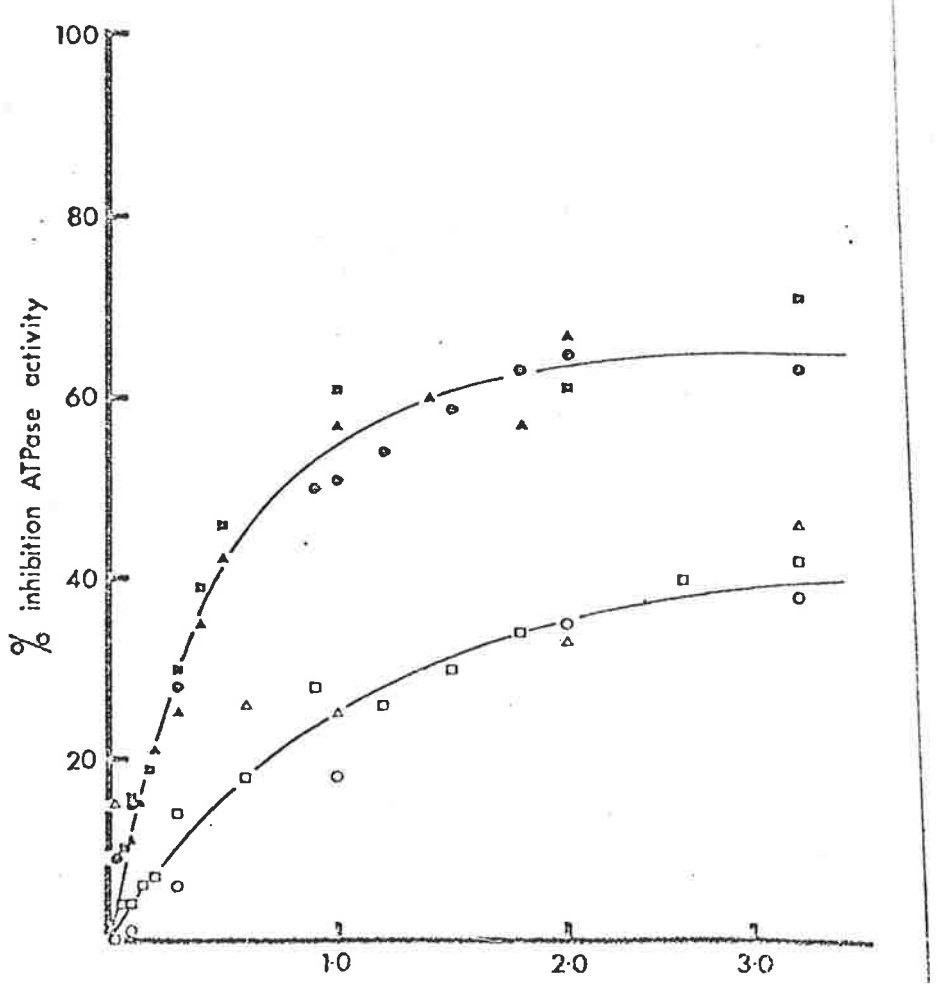

100
80
60
40
20
% inhibition ATPase activity
1·0
2·0
3·0

FIG 4

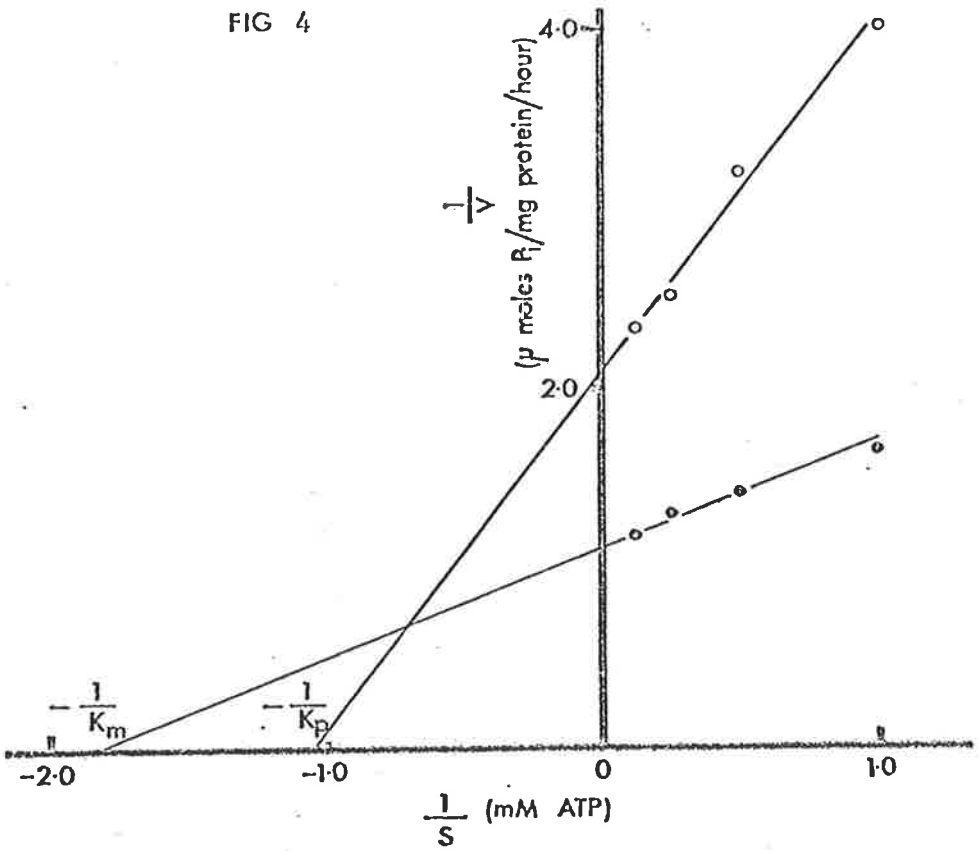

4·0
2·0
$\frac{1}{V}$
(μ moles P_i/mg protein/hour)
$-\frac{1}{K_m}$
$-\frac{1}{K_p}$
-2·0
-1·0
0
1·0
$\frac{1}{S}$ (mM ATP)

FIG 5

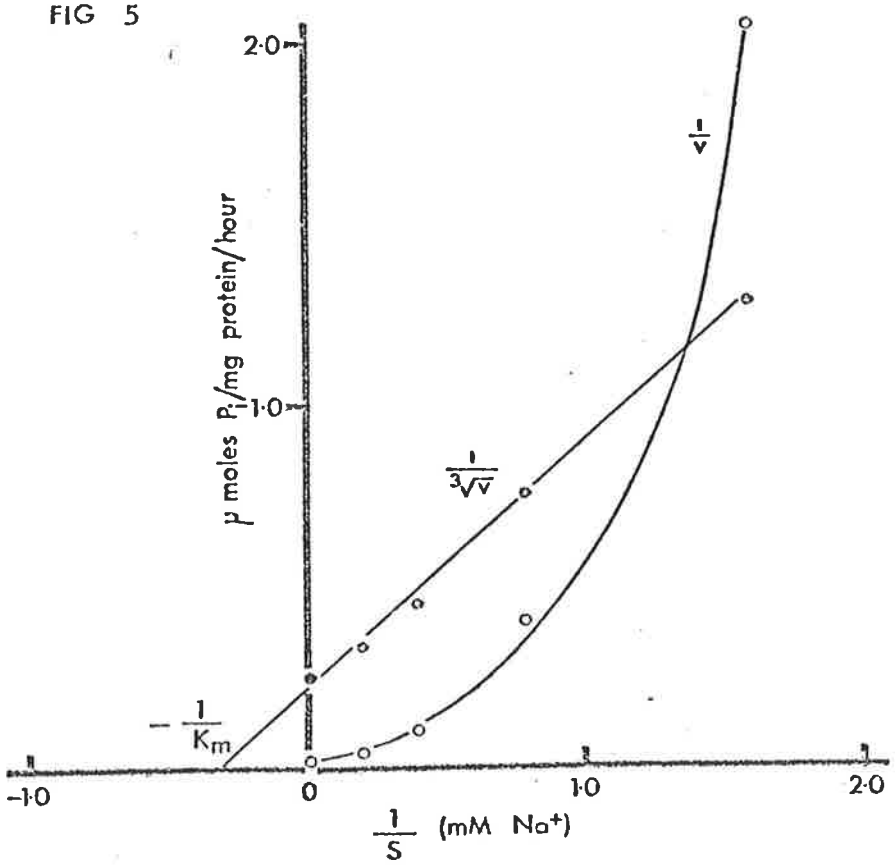

2·0
1·0
μ moles P_i/mg protein/hour
$\frac{1}{v}$
$\frac{1}{\sqrt[3]{v}}$
$-\frac{1}{K_m}$
-1·0
0
1·0
2·0
$\frac{1}{S}$ (mM Na^+)

FIG 6

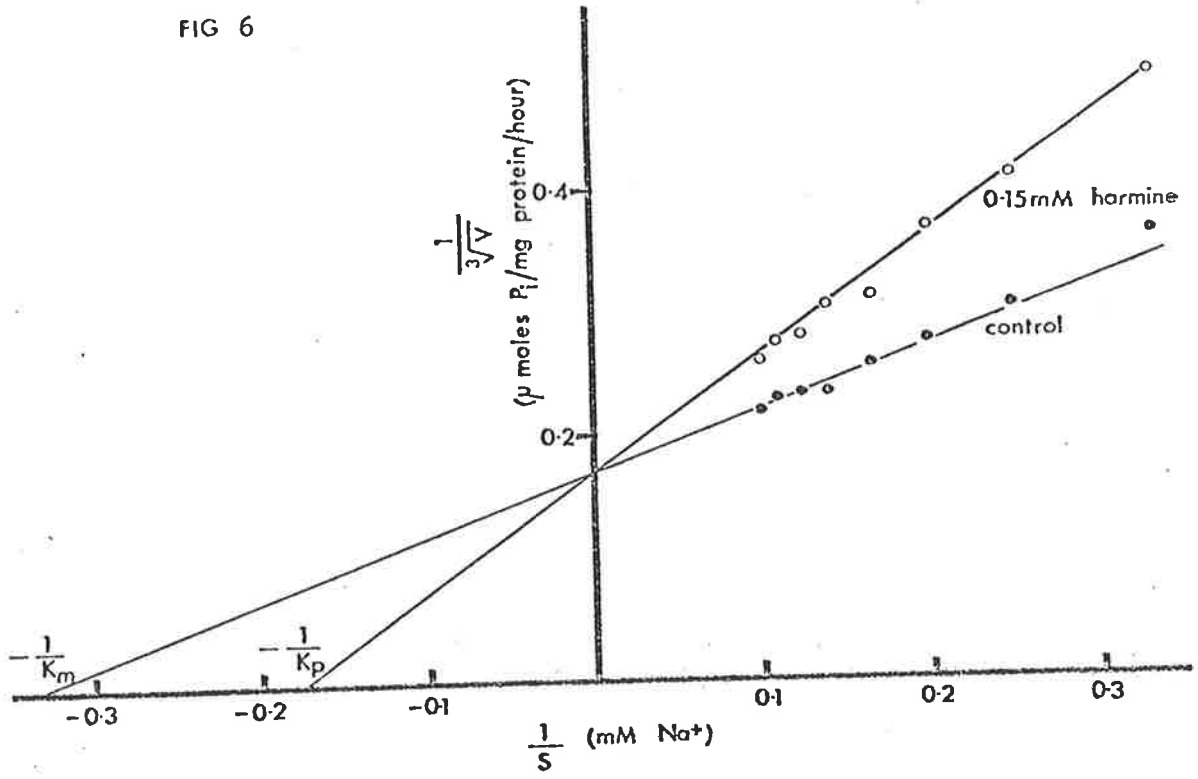
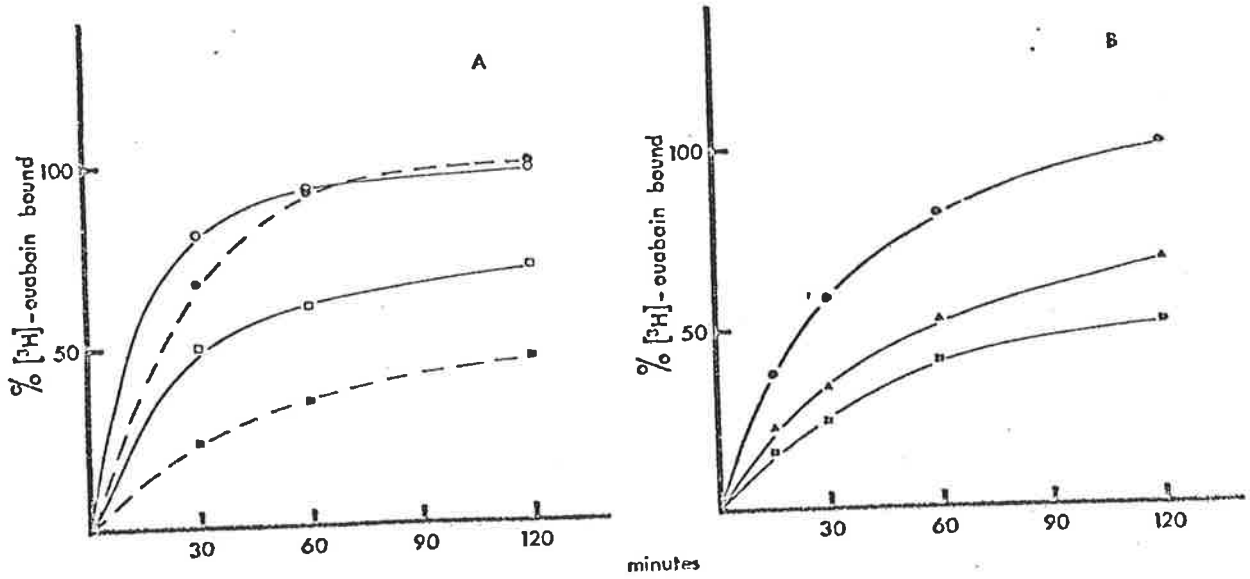

$\frac{1}{\sqrt[3]{V}}$ (μ moles P_i/mg protein/hour)
0·4
0·2
0·15mM harmine
control
$-\frac{1}{K_m}$
$-\frac{1}{K_p}$
-0·3
-0·2
-0·1
0·1
0·2
0·3
$\frac{1}{S}$ (mM Na^+)

FIG 7


A
B
% [^{3}H]-ouabain bound
100
50
30
60
90
120
minutes

EVIDENCE OF HETEROGENEOUS LABELLING OF OX BRAIN MICROSOMES WITH A NITROXIDE LABELLED FATTY ACID PROBE

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CBS and PAABS Symposiun on Structure and Function of Biological Membranes

March 14-19, 1976

Banff, Alberta, Canada

Evidence for heterogeneous labelling of ox brain microsomes with a nitroxide labelled fatty acid probe.

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Dept. of Pharmacology
University of Alberta

Introduction

Membrane phospholipids have been implicated in the hydrolysis of ATP by (Na+K)-ATPase. It is not clear however whether this interference is caused by the "vicinal lipids" or the "bulk lipids" or both. Electron spin resonance spectroscopy (ESR) has been used to study the behaviour of lipids in biological membranes, but precise localization of the spin probe has not been possible. In applying ESR to a study of (Na+K)-ATPase, we have accumulated evidence to suggest the existence of two locations or sites for intercalation of the spin probe, N-oxyl-4',4'-dimethyloxazolidine of 12-keto, methyl stearate (12NSE).

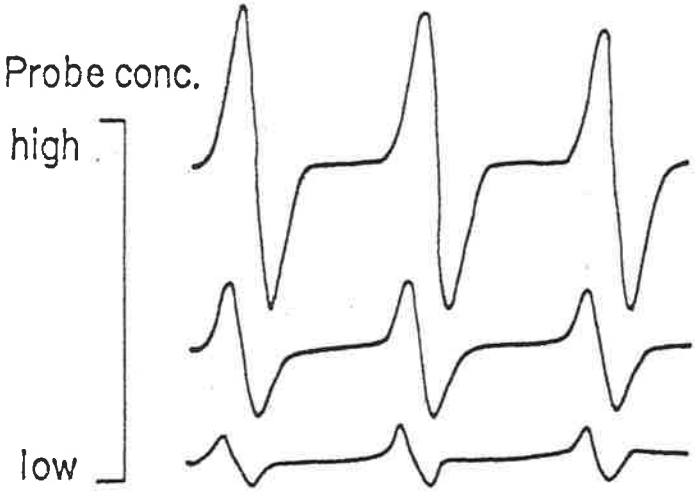
12NSE has a characteristic three line spectrum which can vary in one of two ways:

1) Any simultaneous change in the heights of the three peaks reflects an alteration in the signal intensity, due to changes in concentration of the spin probe.
2) Changes in the spectrum other than signal intensity, (for example line widths or ratio of peak heights) are believed to reflect changes in the immediate environment of the spin probe.

I2NSE in buffered sucrose medium

I2NSE is insoluble in buffered sucrose medium. There is no evidence of any peaks. Increased amounts of spin probe produce a spectrum of an immobilized nitroxyl, due to the I2NSE on the glass.

I2NSE in methanol



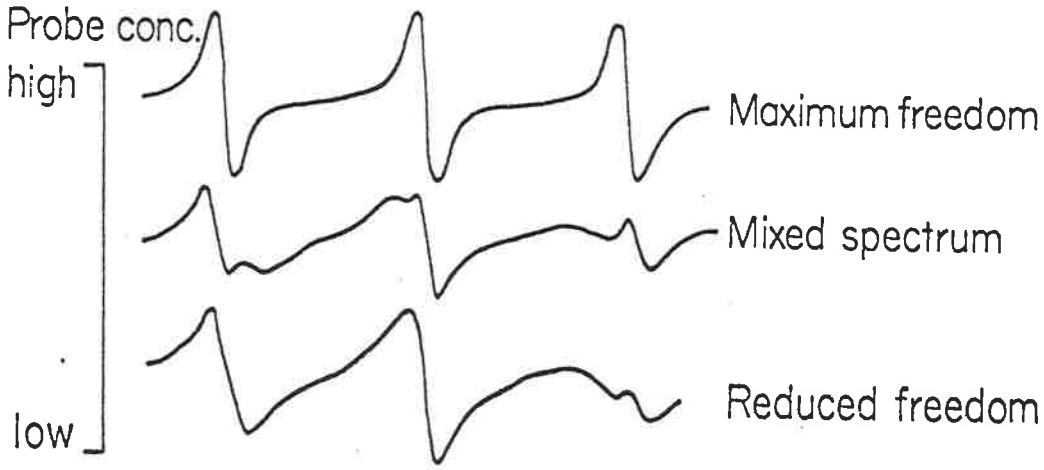
I2NSE in methanol produces a typical sharp three-line spectrum, indicating maximum motional freedom. A change in probe concentration does not influence the shape of the spectrum.

I2NSE in liposomes made from *DMPC or microsomal lipid extracts.

Compared to methanol, I2NSE in liposomes produces a broad spectrum indicating reduced motional freedom. The probe concentration does not influence spectral shape. Spectra from *DMPC or microsomal lipid extracts are identical.

*Di Myristoyl Phosphatidyl Choline

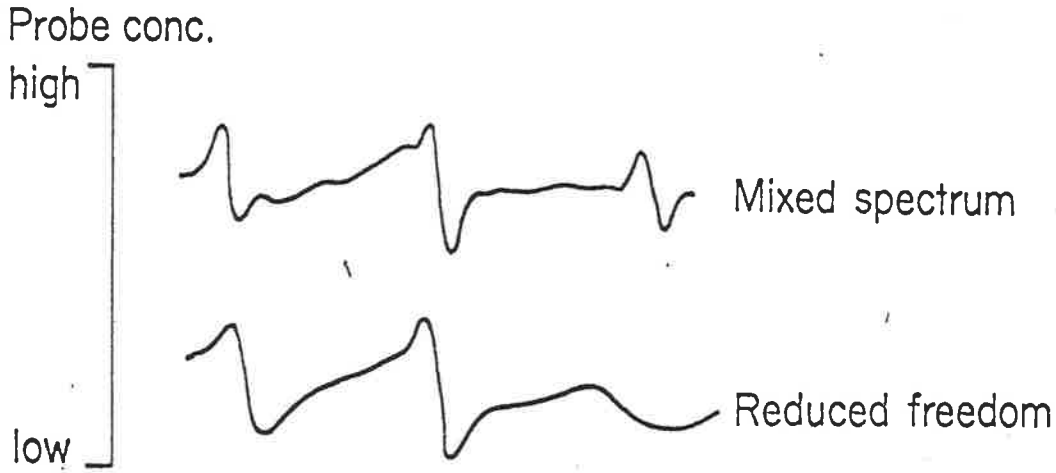
I2NSE in untreated or *DOC-extracted microsomes



I2NSE in untreated microsomes or DOC-extracted microsomes produces varied spectra, which may be sharp, broad, or mixed.

*DeOxyCholic acid

I2NSE in PPL-A or SDS-treated microsomes



I2NSE in PPL-A treated microsomes or in SDS-treated microsomes produces mixed spectra or broad spectra but not sharp three-line spectra.

PPL-A = PhosPhoLipase-A

SDS = Sodium Dodecyl Sulfate

Summary

Spin labelled microsomes may generate varied spectra.

Type I spectrum.

a. Sharp three line spectrum – rapid motion.
b. Observed with untreated and with DOC-extracted microsomes at high probe : membrane ratios.

Type II spectrum.

a. Apparently a mixture of SHARP and BROAD spectra.
b. Observed with microsomes irrespective of treatment, at some critical probe : membrane ratio.
c. NOT OBSERVED with liposomes of DMPC or microsomal lipid extracts.

Type III spectrum.

a. Broad spectrum – restricted motion.
b. Observed with ALL microsomal preparations at low probe : membrane ratios. Also with liposomes made from DMPC and from microsomal lipid extracts.

Conclusions

1. I2NSE will localize itself at two sites in the membrane.
2. One site permits rapid motion, and if these sites predominate, a sharp three line spectrum is observed.
3. A second site restricts the label to some degree, and if it predominates will produce a broad spectrum.
4. If spin probes at both sites contribute to the spectrum then a "mixed" spectrum is observed.
5. In untreated and DOC-extracted microsomes, rapid-motion sites outnumber the restricted-motion sites.
6. Treatment with PPL-A or SDS results in a reduction of rapid-motion sites.
7. Liposomes made with DMPC or microsomal lipid extracts are homogeneously labelled, showing only restricted-motion sites.

II Banff Conference on Biomembranes, Banff, Alberta - November 23rd - 25th, 1973.

J.S. Charnock, Department of Pharmacology, University of Alberta.

ELECTRON PARAMAGNETIC RESONANCE STUDIES OF LIPID MOBILITY IN BIOLOGICAL MEMBRANES

The membrane bound enzyme $(Na^+ + K^+)$-ATPase is now known to be responsible for the biochemical transduction of energy into the active transport of monovalent cations across many biological membranes. Both this enzyme system and the active transport of cations are remarkably temperature sensitive. In previous studies we have shown that the hydrolysis of ATP by $(Na^+ + K^+)$-ATPase is a non-linear function of temperature and can be considered as two separate but intersecting straight lines which on analysis yield two different energies of activation above and below a critical temperature. The experimental data for this observation are reviewed, as is our conclusion that $(Na^+ + K^+)$-ATPase can exist and function in at least two distinct conformational forms.

The role of membrane phospholipids in this phenomenon has been studied and we have demonstrated that phosphatidyl serine plays a critical role in the temperature dependence of this enzyme.

We have commenced an investigation of the effect of temperature on the mobility of membrane lipids in this system, by using the spin-labelled reagent 12NS and examining the resultant electron paramagnetic resonance spectrum.

Our preliminary data indicate that the ESR spectra of 12NS labelled $(Na^+ + K^+)$-ATPase displays significant temperature dependence with lipid mobility, that is the tumbling time 1, increasing with a decrease in temperature. The data obtained to date allow construction of an Arrhenius plot which shows marked similarities to the Arrhenius plot of activation energy referred to earlier. This plot again shows two distinct populations with a transition occurring near to the previously determined critical temperature. These results are not obtained with heat denatured enzyme preparations or a preparation treated with phospholipase-A. We have not yet determined the effect of phosphatidyl serine upon our system but work towards this end is in progress.

As yet, insufficient data are available for any firm conclusion, but we suggest that our findings are in agreement with the hypothesis that the mobility of membrane lipids is all-important in determining the temperature response of $(Na^+ + K^+)$-ATPase. The role that membrane lipids play *in vivo* in influencing the transport function of this enzyme system must await further investigation.

Proc. Canad. Fed. Biol. Soc., 16: 303 (1973)

J.S. Charnock and A.F. Almeida, Department of Pharmacology, University of Alberta, Edmonton, Alberta.

PHOSPHOLIPIDS AND ACTIVATION ENERGY OF MEMBRANE ATPase.

The activation energy of membrane bound ouabain-sensitive ($Na^+ + K^+$)-ATPase varies considerably above and below the critical temperature (T_c°) of about 18°C, with a mean value of 19.2 k cal/mole above T_c° and 43.1 k cal/mole below T_c°. Conversely the activation energy of membrane bound ouabain-insensitive Mg^{2+}-ATPase cannot be described by either a single or by two separate populations of data, and therefore cannot be adequately derived from an Arrhenius plot. We have examined the possibility that this unusual thermal behaviour is associated with direct thermal effects on the physical properties of membrane lipids, by subjecting membrane preparations to a variety of treatments known to act upon membrane lipids. Extraction with 0.1% deoxycholate or 0.2% Nonidet P40 did not alter the thermal response of ($Na^+ + K^+$)-ATPase or Mg^{2+}-ATPase. Treatment with phospholipase A had a marked effect on ($Na^+ + K^+$)-ATPase but not on Mg^{2+}-ATPase. The action of phospholipase A could be overcome by subsequent addition of phosphotidyl-serine to the system. These findings suggest that some specific membrane phospholipids are involved in the thermal response of membrane bound ($Na^+ + K^+$)-ATPase, which is clearly different to that found with the Mg^{2+}-ATPase associated with these preparations.

(Supported by the Alberta Heart Foundation)

Proc. Can. Bio. Fed. Soc. 16, 383 (1973):

A.F. ALMEIDA* and J.S. CHARNOCK, Department of Pharmacology, University of Alberta, Edmonton. The mechanism of ethacrynic acid accumulation.

When incubated under optimal metabolic conditions and in a medium containing ethacrynic acid (EA) at 7.4×10^{-7}M, slices of rabbit kidney cortex will accumulate this drug. Ouabain and probenecid will cause a 50 to 60% inhibition of this process. Phlorizin, the inhibitor of Na^+-dependent sugar transport, inhibits EA uptake by about 70% and a similar effect is seen with a Na^+-free incubation medium. In addition, K^+ will not replace Na^+. It therefore seems probable that a Na^+-dependent co-transport process is involved. Conversely, the transport of EA also bears similarity to a classical organic acid transport process, as it is inhibited by probenecid and is enhanced by acetate in the incubation medium. However, para-aminohippuric acid did not inhibit uptake at 7.4×10^{-5}M. Furthermore, a high concentration of K^+, or the absence of a K^+ gradient did not inhibit the uptake of EA. Thus involvement of the organic acid transport process in the accumulation of EA remains uncertain. In addition, the uninihibited fraction of EA was not saturated by a 1000-fold increase in drug concentration, but increased linearly. Therefore it is unlikely that this uninhibited fraction is accumulated by "nonspecific binding" only.

(Supported by the MRC).

Proc. Can. Fed. Biol. Soc. 15: 460 (1972).

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Quebec, Quebec June 13-16, 1972

Abstract of Paper

Presented

The transport of ethacrynic acid into rabbit renal cortex.

A. ALMEIDA and J.S. CHARNOCK, Department of Pharmacology, University of Alberta, Edmonton, Alberta, Canada.

Some investigators have suggested that ethacrynic acid produces diuresis by inhibiting the sodium pump in the renal tubule. However there is no clear evidence to support membrane involvement or to reject metabolic interference. Inhibition of renal Na-K-ATPase in vitro is possible, but with drug concentrations much in excess of plasma levels found after normal diuretic doses suggesting that some form of concentrating mechanism could exist. A low dose of this drug results in a plasma concentration of about 7.4×10^{-7}M. Using this concentration of drug in an oxygenated buffered phosphate medium, we found that slices of rabbit kidney cortex accumulate ^{14}C-ethacrynic acid about ten-fold when expressed as slice:medium ratio. This value is reduced by half if the slices are deprived of oxygen or if glucose is omitted from the incubation medium. At 0°C the uptake of ^{14}C-labelled drug is markedly reduced from that found at 37°C; the slices attain a two-fold concentration under these conditions. DNP and azide also inhibit this concentrating mechanism by about 60% which suggests the need for oxidative metabolism. The depression of uptake could also be duplicated with probenecid or ouabain. Preliminary experiments show possible sodium dependence for ^{14}C-ethacrynic acid uptake in vitro suggesting involvement of a sodium co-transport system.

Proc. Can. Fed. Biol. Soc. 15: 597 (1972).

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Abstract of Paper

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Anomolous thermal response by membrane bound $(Na^+ + K^+)$-ATPase

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Several investigators have reported that membrane bound preparations of $(Na^+ + K^+)$-ATPase from a variety of sources, do not display a linear response in hydrolytic activity when the temperature is increased over the relatively narrow range of 0°-40°C. This observation is frequently reflected by a discontinuous Arrhenius plot which demonstrates a sharp bend or "thermal anomoly" at about 18-20°C, and results in a greatly increased apparent Energy of Activation over the lower temperature range. At least four different explanations have been offered in the literature to account for such observations. These are a) that the experimental data is artifactual, b) that changes in enzyme activity reflect changes in the order of biological water adjacent to a macromolecular surface, c) direct thermal effects upon the conformation of enzyme proteins, d) thermal effects in the physical properties of membrane lipids associated with membrane bound enzymes. These theoretical possibilities will be discussed briefly, along with some recent experimental findings which suggest that a temperature sensitive lipid-protein interaction in the membrane will account for the thermal anomoly reported.

Proc. Can. Fed. Biol. Soc. 13: 30 (1970).

PROCEEDINGS of the CANADIAN FEDERATION

OF BIOLOGICAL SOCIETIES

Abstract of Paper

Presented

Ethanol inhibition of sodium-plus-potassium activated adenosinetriphosphatase

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The effect of ethanol was determined on ATP hydrolysis by microsomal preparations of sodium-plus-potassium activated adenosinetriphosphatase obtained from guinea-pig cerebral cortex, cardiac muscle and renal cortex.

While little inhibition could be demonstrated with ethanol concentrations of 0.5 percent (v/v) there was marked inhibition with 5.0 percent and almost complete inhibition when the concentration of ethanol was increased to 10 percent.

The mechanism of this ethanol effect was examined by studying the sodium ion sensitive transfer of ^{32}P from γ-ATP^{32} to the phosphoprotein intermediate of the reaction, in addition to the potassium-ion sensitive hydrolysis of this complex. Ethanol did not inhibit the formation, but did inhibit the hydrolysis of the intermediate complex. Furthermore when the concentration of potassium ion was reduced, the effect of ethanol was not significantly changed in these experiments.

Ethanol appears to have a direct effect on the lability of the intermediate complex which is not influenced by the concentration of potassium ion. Furthermore the concentration of ethanol employed in these experiments is that often reached when this agent is used as a solvent for non water-soluble drugs.

Clin. Res., 18: 149 (1970)

J.S. Charnock, Department of Pharmacology, University of Alberta, Edmonton.

ETHACRYNIC ACID INHIBITION OF RENAL (Na + K)-ATPase.

The biochemical mechanism of action of non-thiazide diuretics is largely unknown. However hydrolysis of ATP by (Na + K)-ATPase of renal tubules is believed to be one mechanism for the active reabsorption of Na^+ ions in the loop of Henle. In vitro, this enzyme process can be inhibited by ethacrynic acid (Edecril). We have found that the degree of ethacrynic acid inhibition is influenced by the concentration of K^+ ion and that inhibition is greatest when K^+ ion is least. To examine the mechanism of this competition between K^+ ion and diuretic we have isolated (Na + K)-ATPase containing membranes from guinea pig renal cortex and incubated the preparation with γ-ATP^{32} in order to see the rate of Na^+ dependent formation and K^+ dependent breakdown of a P^{32}-labelled membrane complex which is believed to function as the ion carrier in active membrane transport. 1 mM ethacrynic acid has no effect on the Na^+ dependent formation of the intermediate complex, but greatly inhibited the K^+ dependent breakdown of the intermediate thus reducing enzyme turnover and resulting in enzyme inhibition. Again this effect was influenced by the concentration of K^+ ion and was greatest when K^+ was least, i.e., 0.05 mM K^+ in these experiments. Hence this diuretic agent inhibits renal (Na + K)-ATPase by blocking the K^+ dependent turnover of a labile membrane complex thought to function as an ion carrier. This finding further correlates the proposed biochemical mechanism of action of ethacrynic acid with clinical evidence.

Fed. Proc. 22: 213 (1963)

A PHOSPHORYLATED INTERMEDIATE COMPOUND IN ATP-DEPENDENT SODIUM AND POTASSIUM TRANSPORT

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A simple relationship has been found between sodium ion, potassium ion, the transport inhibitor, ouabain, and the incorporation of the terminal phosphate group of ATP into an insoluble transport ATPase preparation. Phosphorylation was stimulated by sodium ion, dephosphorylation by potassium ion, and the action of potassium ion was blocked by ouabain. In the presence of Na^+ and/or K^+ and/or ouabain a suspension of $(Na^+ + K^+)$-dependent ATPase activity from guinea pig kidney was incubated at 37° for 7 seconds with 0.02 mM Mg ATP labelled with P-32. There was enough activity to hydrolyze all the ATP within a minute. The reaction was stopped with 3 volumes of 10% trichloroacetic acid and the sediment was washed free of soluble radioactive material with 2% TCA. The sediment radioactivity per mg protein was three times greater when incubated with Na^+ than when incubated with K^+. This phosphate compound may be an intermediate in the active transport reaction.

(Supported by U.S.P.H.S., HE-01974-08 (PHY)).