PYRUVATE CARBOXYLASE:
PHYSICO-CHEMICAL ASPECTS

A thesis submitted

by

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to

The University of Adelaide

South Australia

for the degree of

Doctor of Philosophy

DEPARTMENT OF BIOCHEMISTRY

UNIVERSITY OF ADELAIDE

SOUTH AUSTRALIA

February, 1978

CONTENTS

SUMMARY

1. Two methods are described for the purification of sheep liver pyruvate carboxylase to homogeneity. The first of these employs molecular sieve chromatography in the presence of SDS to obtain large quantities of homogeneous enzyme in a form suitable for primary sequence determinations. The second, using NAD^+-Agarose affinity chromatography, provides a method for obtaining the enzyme in a highly active, homogeneous form.

2. Some of the physical properties of sheep liver enzyme have been examined. The enzyme has a molecular weight of 4.8×10^5 daltons and is composed of subunits of 1.1×10^5 daltons. This finding is consistent with similar studies conducted on pyruvate carboxylases obtained from a variety of other sources.

3. Electron micrographs of highly purified sheep liver and chicken liver pyruvate carboxylase have revealed that both of these enzymes have a rhombic arrangement of subunits similar to the enzyme isolated from yeast. The square planar tetramer structure previously reported to be chicken liver pyruvate carboxylase (Valentine, R.C., Wrigley, N.G., Scrutton, M.C., Irias, J.J. and Utter, M.F.,

Biochemistry 5, 3111, 1966) has been shown to be a contaminant in the enzyme preparation. Evidence is presented which suggests that this contaminant may be an octomer.

4. An earlier report from this laboratory (Hudson, P.J., Keech, D.B. and Wallace, J.C., Biochemical and Biophysical Research Communications 65, 213, 1975) provided evidence to suggest that 3-bromopyruvate functioned as an affinity label for the keto acid binding site of sheep liver pyruvate carboxylase. To investigate the evolutionary relationship between the keto acid binding sites of pyruvate carboxylase and transcarboxylase from P. shermanii, the effect of this reagent on the catalytic activity of transcarboxylase was investigated. Inhibition of the catalytic activity of this enzyme was observed only at high concentrations of 3-bromopyruvate and the rate of inactivation was increased with either oxaloacetate or pyruvate. Furthermore, using 3-bromo[^{14}C]pyruvate it was found that both the 5S ear and 6S head subunits of this enzyme were modified. These results suggested that, unlike sheep liver pyruvate carboxylase, transcarboxylase does not contain a highly reactive cysteine residue in or near the keto acid binding site. No evolutionary implications

could be drawn from this data.

5. The location and function of the reactive cysteine residues in sheep liver pyruvate carboxylase have been investigated by chemical modification of the enzyme using n-alkylmaleimides and 5,5 -dithio-bis-(2-nitrobenzoic acid). The former reagents inhibited enzymic activity in a biphasic manner with respect to time. The second phase of inactivation occurred at a faster rate when N-ethylmaleimide was replaced by N-butylmaleimide, suggesting that the exxential residue modified during this phase of inactivation was in an hydrophobic environment. The modification of a lysine residue in the Mg^{2+} binding site of the enzyme was suggested to occur during this phase of inactivation of the enzyme. The rate of the inactivation of the enzyme during the first phase was too high to enable detailed analysis of the events occuring during this phase. 5,5 -dithio-bis-(2-nitrobenzoic acid) inactivation of the enzyme was retarded by acetyl CoA and oxaloacetate, suggesting that the cysteine residues modified by this reagent were located in or near the keto acid binding site of the enzyme. Replacement of the 5-thio-2-nitrobenzoic acid group on the modified enzyme with cyanide was accompanied by a recovery of enzymic activity, indicating that the reactive cysteine residues on the enzyme were

not required to function as either nucleophiles or proton-donors in the enzyme catalysed reaction. A scheme has been proposed to account for the events occurring during the inactivation of the enzyme by chemical modifiers.

6. The homology of the subunits of both chicken liver and sheep liver pyruvate carboxylase has been investigated.

A tryptic digest of the chicken liver enzyme, modified with [^{14}C]-iodo acetic acid, resulted in a large number of radioactively labelled peptides. Thirteen of these peptides were isolated and characterized by amino acid analyses and identification of the N-terminal amino acid residue. Since only ten [^{14}C]SCM-peptides can be generated by this method if the polypeptide chains were homologous then this result tentatively suggested that the subunits of chicken liver pyruvate carboxylase may be non-homologous.

Sheep liver pyruvate carboxylase was modified with 3-bromo[^{14}C]pyruvate. Four approximately equivalently labelled peptides were isolated from a tryptic digest of this material. The total number of moles of 3-bromo[^{14}C]pyruvate incorporated

into these four peptides was approximately equivalent to the total number of moles of active sites modified on the enzyme, as determined from the loss of enzymic activity. This result suggested that the sheep liver enzyme may be composed of four non-identical subunits.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material that has been previously published, or written by another person, except where due reference is made in the text.

NEIL H. GOSS

ACKNOWLEDGEMENTS

I wish to thank Professor W.H. Elliott for permission to undertake this project in the Department of Biochemistry, University of Adelaide.

I am grateful to my supervisors, Dr. D.B. Keech and Dr. J.C. Wallace for their advice and constructive criticisms throughout the course of this work and in the preparation of this thesis.

In addition, I wish to thank Professor H.G. Wood for his collaboration in the affinity labelling studies of transcarboxylase, described in Chapter Six of this thesis, and Miss P.Y. Dyer for her collaboration during the study of the electron microscopy of pyruvate carboxylase.

I would also like to thank Miss J.A. Duc, Mr. P.R. Clements and Mr. S.B. Easterbrook-Smith for allowing me to discuss their unpublished results, Mr. M.S. Calder and Miss J. Anderson for skilled laboratory assistance, and Mrs. L. Coates for typing the thesis.

I wish to acknowledge the financial support of a Commonwealth Postgraduate Award for the duration of the project.

ABBREVIATIONS

In addition to those accepted for use in the Journal of Biological Chemistry, the following abbreviations are used in this thesis.

ANS	1-analinonaphthalene-8-sulphonate
CHE-	carboxyhydroxyethyl-
CKM-	carboxyketomethyl-
CoA	coenzyme A
dansyl	5-dimethylaminonaphthalene-1-sulfonyl
DTE	5,5'-dithio-bis-(2-nitrobenzoic acid)
FDNB	1-fluoro-2,4-dinitrobenzene
IAA	iodoacetic acid
NBM	N-butylmaleimide
NEM	N-ethylmaleimide
NMR	nuclear magnetic resonance
OAA	oxaloacetate
P_i	orthophosphate
PMSF	phenylmethane sulphonyl fluoride
POPOP	1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene
PPO	2,5-diphenyloxazole
S.A.	specific activity
SCM-	S-carboxymethyl-
SCMC	S-carboxymethyl cysteine
SDS	sodium dodecyl sulphate
S.E.M.	standard error of the mean
TNBS	2,4,6-trinitrobenzene sulphonic acid
TNB	5-thio-2-nitrobenzoic acid

CHAPTER 1

INTRODUCTION

1.1. The biotin enzymes

Biotin-containing enzymes catalyse the fixation or transfer of bicarbonate in both prokaryotes and eukaroytes. Nine of these enzymes have been characterized to date and these can be sub-divided into three classes, (Table 1.1); the carboxylases, the transcarboxylases and the decarboxylases and are the subject of comprehensive reviews by Moss and Lane (1971) and Wood and Barden (1977).

Examination of the individual members of this group of enzymes has established that they have similar reaction mechanisms, complex quaternary structures and high molecular weights. Furthermore recent primary sequence data obtained from a number of different enzymes has revealed a significant degree of homology around the biotin prosthetic group (Rylatt et al., 1977; Wood and Zwolinsky, 1976).

These similarities encourage closer examination of the general features of biotin containing enzymes when considering pyruvate carboxylase. The most well characterised area of similarity of these enzymes is in the reaction mechanism.

1.2. Reaction mechanism

In all cases where the reaction mechanism of the biotin-containing enzymes have been examined it has been found that the overall reaction (1.1)

$$ATP + HCO_3^- + RH \rightleftharpoons ADP + P_i + RCO_2^- \quad \ldots 1.1$$

can be divided into two partial reactions:

First Partial Reaction:

$$ATP + HCO_3^- + E^{biotin} \rightleftharpoons ADP + P_i + E^{biotin\ CO_2} \quad \ldots 1.2$$

Second Partial Reaction:

$$E^{biotin\ CO_2} + RH \rightleftharpoons E^{biotin} + RCO_2^- \quad \ldots 1.3$$

where R is either acetyl CoA, pyruvate or urea. With transcarboxylase, methylmalonyl CoA replaces ATP and HCO_3^- as the carboxylating species.

This "minimal mechanism" has been deduced from initial velocity and product inhibition data and is supported by observations that isotopic exchange between the components of one partial reaction can occur in the absence of the components of the other partial reaction. Furthermore the biotin$\sim CO_2$ intermediate has to be isolated in a number of cases and

the site of carboxylation identified as the 1'N-nitrogen atom on the biotin molecule. (Knappe et al., 1962; Lane and Lynen, 1963; Wood et al., 1963; Waite and Wakil, 1966; Rylatt, 1976).

This data formed the basis for the concept of the general mechanism of the biotin enzymes where the partial reactions catalysed by these enzymes are envisaged to occur on spatially distinct subsites and the covalently bound biotin prosthetic group serves as a "shuttle" to convey the CO_2 moiety from one site to the other.

In recent years other evidence has become available to support this general concept. Firstly, acetyl CoA carboxylase from E. coli was dissociated into three different subunits, viz., the biotin carboxylase, the carboxyl carrier protein (containing the covalently bound biotin) and the carboxyl transferase subunit (Alberts and Vagelos, 1972; Polakis et al., 1974). The biotin carboxylase subunit was shown to carboxylate free biotin in the absence of the other two subunits thereby locating the binding sites of ATP and bicarbonate on this subunit. Similarly the carboxyl transferase subunit could carboxylate biotin using malonyl CoA, thereby locating the CoA-ester binding site on this subunit. These studies provided strong

evidence for the spatial separation of the two subsites of this biotin enzyme. Studies by Wood and his group also were able to locate the binding sites of the keto acid and the CoA esters on the different subunits of transcarboxylase (Chuang et al., 1975; Chapter 6). In addition, NMR studies with chicken liver pyruvate carboxylase using Cr-ATP^{2-} have shown that the two subsites on this enzyme are greater than 10Å apart. (Scrutton et al., 1973)

Therefore it would appear that a strong body of information is available to support the concept of spatially distinct binding sites for the two partial reactions catalysed by the biotin-containing enzymes.

1.3. Pyruvate carboxylase

Since a comprehensive review of pyruvate carboxylase (E.C.6.4.1.1.) has been published recently (Utter et al., 1975), this section will emphasize the more recent developments in the understanding of the reaction catalysed by pyruvate carboxylase, as some of these findings have a direct bearing on the work to be presented in later chapters of this thesis.

1.3.1. The reaction pathway

The reaction catalysed by pyruvate carboxylase

is depicted in equation 1.4.

$$MgATP + HCO_3 + \text{pyruvate} \xrightleftharpoons[\text{Acetyl CoA}]{Mg^{2+},\ Me^{1+}} MgADP + P_i + \text{oxaloacetate} \quad \ldots 1.4$$

Studies aimed at elucidating the reaction mechanism have been carried out on the enzymes isolated from A. niger (Feir and Suzuki, 1969), rat liver (McClure et al., 1971 a,b,c), chicken liver (Barden et al., 1972) and sheep kidney (Ashman et al., 1972). The information obtained in these studies from initial velocity and product inhibition data were interpreted to suggest that the reaction catalysed by pyruvate carboxylase preceded by a non-classical Bi-Bi-Uni-Uni-Ping-Pong mechanism (fig 1.1a). This interpretation was consistent with the minimal mechanism proposed for other biotin containing enzymes.

Recently however the interpretation of the kinetic data which supports this mechanism has been challenged. Warren and Tipton (1974 a,b), working on the enzyme isolated from pig liver, have proposed that the reaction proceeds via a sequential-type mechanism in which no products of the reaction are released until all of the substrates are bound (fig 1.b). Such a mechanism provides an explanation for the kinetic data, in particular the "mixed" interactions between pyruvate

and bicarbonate, but fails to explain the results obtained from the isotopic exchange experiments. If the reaction preceded by a sequential mechanism then it would be expected that the components of one partial reaction would affect the rate of isotopic exchange occurring between the components at the adjacent site. No effect such as this has been observed for any pyruvate carboxylase studied.

A solution to these conflicting interpretations of the data has been proposed by Easterbrook-Smith et al. (1978) working with pyruvate carboxylase isolated from sheep liver. These authors found that at high concentrations of pyruvate, the reaction did indeed proceed by a sequential mechanism. However, a mathematical analysis of the data suggested that the reaction may proceed via a non-sequential pathway at low concentrations of pyruvate. It was reasoned that at low concentrations of pyruvate both products of the first partial reaction (ADP and P_i) may be released before pyruvate binds, but at high concentrations of pyruvate this does not occur (Easterbrook-Smith et al., 1976b). This data therefore predicts a change in the reaction mechanism depending upon the concentration of pyruvate and thereby accounts for all of the available experimental data.

1.3.2. Activation of the reaction by acetyl CoA.

The rates of the reaction of pyruvate carboxylases isolated from chicken liver, rat liver and sheep kidney are increased by a factor of at least four-fold in the presence of acetyl CoA (Ashman et al., 1972; McClure et al., 1971a). Furthermore these enzymes exhibit a sigmoidal reaction velocity response to increasing concentrations of acetyl CoA (Barritt et al., 1966; Scrutton and Utter, 1967).

In the chicken liver enzyme 3-4 molecules of acetyl CoA bind to the tetrameric form of the enzyme (Frey and Utter, 1977), suggesting that one molecule of acetyl CoA binds for each enzyme active centre. The binding of this ligand to the enzyme has been characterised by the determination of the Hill coefficient (n_H) which represents a function of the number of molecules bound to the enzyme and the extent of their interaction. A Hill coefficient of greater than unity is usually interpreted to indicate homotropic cooperativity in ligand binding. The Hill coefficient for acetyl CoA binding to avian pyruvate carboxylase has been calculated to have a value of approximately 3.0, whereas a value of approximately 2.0 has been obtained for all mammalian sources of the enzyme studied. Thus the interpretation from this data has

to date been that acetyl CoA binds to the enzyme in a homotropic cooperative fashion.

Recent work in this laboratory by Easterbrook-Smith (1977) has indicated that this may not be correct: acetyl CoA may bind to the enzyme in non-cooperative fashion. This author proposed that the observed sigmoidal response of the enzyme reaction velocity with increasing concentrations of acetyl CoA was largely due to two factors:

(1) the influence of acetyl CoA on the K_m value for bicarbonate and pyruvate.

Ashman et al. (1972) had shown that acetyl CoA significantly decreased the K_m value for bicarbonate and pyruvate. It was reasoned therefore that when acetyl CoA was the variable ligand, the fixed concentration of either pyruvate or bicarbonate, although saturating at high concentrations of acetyl CoA, would become non-saturating as the concentration of acetyl CoA was decreased. To test this hypothesis the Hill coefficients for acetyl CoA at different fixed concentrations of either pyruvate or bicarbonate were determined. It was found that as the concentration of either of these ligands was increased the Hill coefficient for acetyl CoA decreased. This indicated that at least

one factor responsible for the higher than unity values for Hill coefficient of acetyl CoA was that, at low concentrations of this ligand the enzyme was not saturated with pyruvate and bicarbonate.

(2) the phenomenon of irreversible inactivation of the enzyme upon dilution.

Under the normal conditions of the assay for pyruvate carboxylase activity, but in the absence of acetyl CoA, the enzyme undergoes an irreversible loss of activity due to dilution (Ashman et al., 1972). This inactivation would give rise to a sigmoidal velocity response curve as the concentration of acetyl CoA increased. At higher concentrations of enzyme (above 4 units per ml) this effect should not be observed however.

To test this, an experiment was performed where all components were saturating, regardless of the acetyl CoA concentration, and the enzyme concentration was raised to a level where inactivation did not occur, even in the absence of acetyl CoA. It was found that the reciprocal of the velocity plotted as a function of the reciprocal of the acetyl CoA concentration gave a curve that was concave downward. This was in contrast to the normal conditions of the assay where a concave upward double reciprocal plot was obtained. Thus the major factors contributing to

the sigmoid velocity profile have been isolated and therefore there appears to be no need to postulate homotropic cooperative binding of acetyl CoA to the enzyme.

In addition to this study, the interaction of the enzyme with acetyl CoA was examined by making use of the observation that pyruvate carboxylase catalyses a slow deacylation of acetyl CoA (Scrutton and Utter, 1967). The deacylase activity has been assumed to be associated with the acetyl CoA binding site on the enzyme since Frey and Utter (1977) have detected only 3-4 such binding sites on the chicken liver enzyme and also the presence of substrates of the pyruvate carboxylase reaction have been shown to enhance the rate of this reaction (Scrutton and Utter, 1967; Ashman, 1973). In an experiment designed to measure the rate of deacylation of acetyl CoA catalysed by pyruvate carboxylase as a function of varying acetyl CoA concentration, Easterbrook-Smith (1977) obtained an hyperbolic response, suggesting that the binding of acetyl CoA to the enzyme under these conditions occurred in a classical Michaelis-Menten type manner.

1.3.3. The molecular basis for the activation of the enzyme by acetyl CoA

In a recent study in this laboratory by

Clements et al. (1978), the molecular basis for the activation of pyruvate carboxylase by acetyl CoA was investigated. Three approaches were employed:

(1) The effect of various components of the acetyl CoA molecule on the isotopic exchange reactions catalysed by pyruvate carboxylase were examined. This approach resulted in the conclusion that the adenosine moiety of acetyl CoA exerted its effect at the ATP/HCO_3 binding site while the acetyl pantetheine moiety of the ligand exerted its effect at the keto acid binding site.

(2) The inactivation of the enzyme upon dilution was investigated. It was found that upon dilution of the enzyme the rate of the first partial reaction exchange decreased in parallel with the loss of overall enzymic activity. The second partial reaction exchange however remained unaffected. Furthermore dilution of the enzyme into solutions containing either acetyl CoA, CoASH protected the enzyme against the inactivation process but similar experiments using solutions containing acetyl pantetheine failed to afford any protection against the inactivation process. In addition to this it was also noted that

dilution of the enzyme into apolar or non-ionic solutes retarded the rate of inactivation of the enzyme. These findings suggested that the inactivation of the enzyme upon dilution was due to hydration of the first partial reaction site and that the adenosine moiety of acetyl CoA prevented inactivation of the enzyme by excluding water from this site.

(3) The nature of the inactivation process was further investigated using circular dichroism. This approach revealed that major changes in the structure of the polypeptide chain occurred following dilution of the enzyme. Dilution of the enzyme into solutions containing either acetyl CoA or apolar solutes prevented these alterations to the structure of the enzyme however.

The results obtained from these three approaches were unified into an hypothesis which suggested that acetyl CoA was oriented with the adenosine moiety near the first partial reaction site and the acetyl pantetheine moiety near to second partial reaction site. The adenosine moiety of acetyl CoA was proposed to protect against dilution inactivation by preventing an unfavourable conformational alteration which resulted

in hydration of the first partial reaction site.

1.3.4. The requirement for divalent metal ions

Pyruvate carboxylases isolated from all species so far examined have an essential requirement for a divalent metal ion. Mg^{2+} has the most marked effect although Mn^{2+} (Bais and Keech, 1972) and Ca^{2+} (Dugal, 1973) can replace the function of this ion. These cations appear to have a dual function.

Firstly pyruvate carboxylase has been shown to utilize $MgATP^{2+}$ rather than ATP^{4-} as the substrate (Keech and Barritt, 1967; Cazzulo and Stoppani, 1967, 1969; Feir and Suzuki, 1969; Cazzulo *et al.*, 1970; McClure *et al.*, 1971a; Bais and Keech, 1972; Warren and Tipton, 1974a).

Secondly, Mg^{2+} appears to activate the enzyme since the optimal concentration of this ion is several fold higher than the nucleotide required for maximal activity. This additional effect of Mg^{2+} has been investigated in this laboratory by Duc (1978). This investigator found that Mg^{2+} significantly reduced the apparent K_a value for acetyl CoA and also reduced the Hill coefficient for the binding of this ligand. Determinations of the dissociation constant for the

Mg^{2+}-acetyl CoA complex revealed that in the standard assay mix for pyruvate carboxylase, approximately 94% of the acetyl CoA would exist as a complex with Mg^{2+}. Furthermore by monitoring the protection by acetyl CoA against TNBS inactivation of the enzyme, in the presence and absence of Mg^{2+}, it was found that only in the presence of Mg^{2+} were kinetic constants for acetyl CoA obtained which agreed with those obtained from initial rate studies. When the inactivation of the enzyme upon dilution was studied under similar conditions as described above the same result was obtained. The locus of interaction of this ion with acetyl CoA was found to be the 3'-phosphate on the ribose ring of the adenosine moiety of this molecule by using [^{31}P] NMR (see Chapter 5).

On the basis of these quite compelling results Duc (1978) postulated that the true allosteric activator of pyruvate carboxylase was the Mg^{2+}-acetyl CoA complex.

1.3.5. The physical properties of pyruvate carboxylase

The physical properties of pyruvate carboxylase isolated from a variety of eukaryote sources are remarkably similar. Table 1.2 illustrates that all of these enzymes have very similar molecular weights (ca. 5.0×10^5 daltons) and can be dissociated into subunits

of approximately 1.1 - 1.3 x 10^5 daltons. Furthermore there are four moles of biotin per mole of enzyme, suggesting that there may be one biotin prosthetic group per subunit of the enzyme. In the case of chicken liver pyruvate carboxylase it has been demonstrated that this is the case. This was achieved by dissociating the enzyme in cold 0.4M urea into the individual polypeptide chains and passing the enzyme through a Sepharose-column containing covalently linked avidin. Approximately 90% of the material applied to the column was retarded whereas in a control experiment, in which the column was pretreated with a ten-fold excess of biotin, almost no protein was bound. Clearly, if the four biotin prosthetic groups were bound to only two of the four polypeptide chains then only 50% of the material applied to the avidin-Sepharose would have been retarded. (Barden et al., 1975)

Electron microscopy of the enzymes isolated from chicken liver, calf liver, turkey liver and yeast support the proposition that these enzymes are tetramers. However, whereas the vertebrate forms of this enzyme exhibit a cyclic arrangement of the subunits, the yeast enzyme appears in the electron micrographs with the subunits arranged at the corners of a rhombus (Utter et al., 1975). The implications of this structure, and its relationship to the enzymes isolated from sheep liver and chicken liver is discussed in detail in

Chapter 4 of this thesis.

The subunits of eukaryote pyruvate carboxylases have been inferred to be homologous from a failure to resolve these subunits under dissociating conditions. In addition, the experiment outlined above would favour this interpretation, as would the sequence studies around the biotin prosthetic group reported by Rylatt et al. (1977) (see section 1.4.3.). However these experiments are equivocal in establishing that the entire polypeptide chain of each subunit is identical in amino acid sequence. A more detailed examination of this question is presented in Chapter 7 of this thesis.

In contrast to the eukaryote pyruvate carboxylases, the enzymes isolated from P. citronellolis and A. vinelandii have molecular weights of 2.5×10^5 daltons. In addition, analysis of the constituent polypeptide chains of the P. citronellolis enzyme indicated that it was composed of two different polypeptide chains of molecular weight 6.5×10^4 and 5.4×10^4 (Table 1.2). The biotin prosthetic group was located in the larger of these two subunits (Barden et al., 1975). In view of the fact that the molecular weight of these two subunits together is approximately equivalent to the molecular weight of the protomers of the eukaryote pyruvate carboxylases it is tempting to

suggest that this bacterial pyruvate carboxylase is an evolutionary precursor of the eukaryote enzymes.

The avian liver enzymes are dissociated at $4^{o}C$ into 7S monomers. This dissociation is accompanied by a loss of catalytic activity. However upon rewarming the enzyme solution, the catalytic activity and tetrameric structure of the enzyme can be restored. Furthermore, the monomers obtained from cold inactivation of the enzyme are unable to catalyse either the exchange of [^{32}P]orthophosphate into ATP or the exchange of [^{14}C] pyruvate into oxaloacetate, and yet retain the ability to catalyse the exchange of [^{14}C]ADP into ATP (Irias et al., 1969). Since this latter exchange reaction is catalysed by pyruvate carboxylase and is unaffected by treatment of the enzyme with avidin (Scutton and Utter, 1965b) it would appear that cold inactivation of the chicken liver enzyme may be associated with an inability of the biotin prosthetic group to bind to the two partial reaction subsites.

1.4. Approaches to an understanding of the structure and function of enzymes

The understanding of the catalytic events occurring during the reaction catalysed by pyruvate carboxylase has, in the past relied largely, though

not wholly, upon analysis of the kinetic response of the enzyme to a given set of conditions. While it is clear from the preceding sections of this chapter that a great deal of information about the mechanism of the enzyme has now been accumulated by this approach, no single approach can result in a complete understanding of an enzyme catalysed reaction. Therefore in the following section a number of other complimentary approaches and their prospects of success with pyruvate carboxylase will be discussed.

1.4.1. X-Ray crystallography

Analysis of the x-ray diffraction patterns of protein crystals is the most powerful single technique which can be employed for the investigation of enzyme structure. The results obtained from high resolution x-ray crystallographic data enable the precise location of the individual atoms in the molecule and so provide definitive information on the size, shape and symmetry of the molecule (c.f. Blake, 1975). The active centre of many enzymes has been precisely located and defined from crystals containing enzyme-substrate analogue complexes and this has enabled a deeper understanding of the catalytic processes of these enzymes. In some cases the use of the Fourier difference technique has revealed changes in the electron density maps in the presence and absence of these substrate analogues and

so lent support to the concept of a flexible active site (Koshland, 1960). One striking example of this "induced fit" has been reported by Lipscomb et al. (1968) in a study of carboxypeptidase A. Upon binding of the substrate glycyltyrosine to the enzyme, tyrosine 248 of the enzyme swings down some 12Å so that its hydroxyl group is next to the amide bond of the substrate to be split, and the carbonyl of the amide bond is pressed against the enzyme-bound zinc atom. Clearly, findings such as this greatly facilitate the understanding of the catalytic events occurring at the molecular level.

Unfortunately, there are several problems associated with the general use of x-ray crystallography at the present time. Not the least of these is the availability of suitable crystals of the enzyme. To be useful the crystal is required to be reasonably well ordered and of sufficient size (more than about 0.1mm in each dimension) (Eisenberg, 1970). Furthermore, the size of the crystal lattice (which corresponds to the diffraction pattern obtained) is inversely related to the size of the unit cell of the protein crystal. This means that, at the present time x-ray crystallography is limited to proteins which have unit cells with edges not greater than about 400Å, (Eisenberg, 1970), and therefore this technique cannot be applied to very large proteins. In addition, the full structure

determination of a protein requires a set of heavy atom crystals that are isomorphous with the crystals of the parent enzyme, and these are often quite difficult to obtain.

Even though the problems outlined above have been overcome for some proteins of moderate molecular weight (e.g. lactate dehydrogenase, Adams et al., 1970; glyceraldehyde-3-phosphate dehydrogenase, Biesecker et al., 1977), the complete understanding of the enzyme structure and function has relied on information obtained from the primary sequence of the enzyme and the chemical modification of certain hyperreactive functional amino acid residues. These three methods are in fact complimentary and each provides a unique contribution to the overall understanding of the enzyme catalysed reaction.

With regard to pyruvate carboxylase, x-ray crystallography is not yet developed to the state of sophistication necessary to analyse this large oligomeric protein. Therefore, other techniques which shed light on the structure of the enzyme and the functional groups in the enzyme have been employed.

1.4.2. Chemical modification studies

The contribution made to the understanding of

the structure and function of enzymes through the use of chemical modification studies has long been recognised (c.f. Spande et al., 1970). The success of this approach has relied on the observation that, in a large number of cases, specific modification of one or a few amino acid side chains of the enzyme occurs. The reason for this selective and limited modification is due to the vastly enhanced reactivity of certain amino acid side chains when compared to other side chains of the same type in the enzyme or the isolated amino acid. For example, of the 17 sulphydryl groups in rabbit muscle phosphofructokinase, one reacts with DTNB 2×10^4 times as rapidly as in the denatured enzyme (Kemp and Forest, 1968). This type of information clearly illustrates the importance of the tertiary structure of the enzyme in accelerating the reactivity of certain functional groups in enzymes. In particular, the microenvironment surrounding the amino acid residue determines the velocity of the reaction of the functional group with the reagent.

Cohen (1970) has discussed in detail various factors which could influence the reactivity of an amino acid side chain with a chemical modifier. These factors include the polarity of the microenvironment, hydrogen bonding effects, field or electrostatic effects, steric effects and a variety of other factors which relate directly or indirectly to the local

environment surrounding the functional group. Such a diverse number of influences prevents the prediction of the results obtained from an investigation of an enzyme with chemical modifiers until a significant amount of information has been accumulated. It is therefore desirable to examine the effects of a variety of chemical modifiers of different structure, size and polarity in order to gain a full understanding of the relationship between the hyperreactive amino acid residues and the events occurring at the active centre of the enzyme.

The selection of the reagents used in the chemical modification of an enzyme plays an important part in determining the specificity of the modification. Two basic approaches have been used:

(1) the use of "group-specific" reagents (c.f. Stark, 1970)

(2) the design of active site directed reagents or "affinity labels" (c.f. Baker, 1967).

Affinity labels differ from "group specific" reagents in possessing a structural resemblance to a substrate or other ligand of the enzyme and so, by virtue of this analogy enable, in theory, a more

specific modification of a given ligand binding site. The extent to which this specificity is realized depends largely on the nature of the ligand binding site and the functional group incorporated into the affinity label. The distinction between these two categories of chemical modifiers is further discussed in Chapter 5.

Both group specific reagents and affinity labels have been applied to the investigation of pyruvate carboxylase. However, the enzyme isolated from chicken liver contains a large number of highly reactive cysteine residues (Palacian and Neet, 1972) and this feature has deterred the use of chemical modification of the enzyme from this source as a probe for the elucidation of the catalytic events occurring during the reaction. One successful reagent that has been used on the chicken liver enzyme is TNBS, first reported by Ashman et al. (1973) to specifically modify a lysine residue in the sheep kidney enzyme. Ashman et al. (1973) noted that modification of the sheep kidney enzyme by this reagent inhibited the acetyl CoA-dependent reaction catalysed by this enzyme but stimulated the acetyl-CoA-independent reaction. Similar effects were observed with the enzymes isolated from rat liver (Scrutton and White, 1973) and yeast (Scrutton and White, 1974). Modification of the chicken liver enzyme

with TNBS causes loss of the acetyl CoA-dependent activity and is presumed to have a similar effect on the acetyl CoA-independent activity as it does on the enzymes from other sources (Scrutton et al., 1977). The loss of activity of these enzymes in the presence of TNBS is retarded, although not completely prevented, in the presence of acetyl CoA. From an extensive investigation of this effect, using analogues of acetyl CoA, it has been concluded that the reactive lysyl residue is probably not located in the acetyl CoA binding site however. (Scrutton et al., 1977).

The sulphydryl groups of chicken liver pyruvate carboxylase have been investigated using group specific modifiers (Palacian and Neet, 1970, 1972) and these studies, together with the results obtained from affinity labelling of the sheep liver enzyme are discussed in detail in Chapter 5.

Another area of chemical modification studies of enzymes which is continuing to develop is the use of bifunctional crosslinking reagents. (Wold, 1967). These reagents can be used to introduce both inter- and intra-molecular crosslinks into enzymes. Inter-molecular crosslinking provides information on the spatial arrangement and symmetry of oligomeric proteins (Hucho et al., 1975; Hajdu et al., 1976) and has been used

with considerable success in defining the proximity of the individual proteins which make up the 30S and 50S subunits of the E. coli ribosome (Lutter et al., 1974; Clegg and Hayes, 1974). Intra-molecular crosslinking studies enable an assessment of the distances between reactive amino acid residues within the tertiary structure of an enzyme. (Lomant and Fairbanks, 1976; Reisler et al., 1974; Givol, 1969). By systematic variation of the distance between the functional groups on the chemical crosslinking reagent it may be possible to use this approach to "scan" around a particularly reactive amino acid residue in a protein, as suggested by Henkin (1977).

1.4.3. Primary sequence determination

The determination of the primary sequence of a protein provides information essential to the understanding of the conformation of enzymes and hence to their biological activity. As has been clearly illustrated from x-ray crystallography, the interplay and precise arrangement of essential amino acid residues in the three dimensional structure of the enzyme provides the key to understanding the efficiency with which enzymes catalyse chemical reactions. The primary sequence of an enzyme is the major determinant involved in maintaining this precise arrangement of amino acid side

chains and is therefore an integral part of an overall understanding of enzyme catalysed reactions.

The information gained from primary sequence determination can in turn be used to chemically synthesize proteins and so obtain "synthetic" enzymes. A notable example of this approach is the work of Gutte (1977) who was able to demonstrate that a synthetic 63-residue analogue of ribonuclease possessed full catalytic activity when compared with the native 124-residue enzyme.

This study, and others where large pieces of the polypeptide chain have been removed without affecting the catalytic activity of the enzyme (Hill and Smith, 1956; Nylander and Malenstrom, 1959) illustrate that not all of the polypeptide chain of an enzyme is involved in maintaining the correct positioning of the amino acid side chains involved in the catalysis. The bulk of the protein molecule presumably also contains the binding sites for allosteric effectors of the enzyme catalysed reaction, and regions which are involved in forming oligomeric structures (e.g. multi-enzyme complexes) or locating the enzyme in the correct position in the cell (e.g. membrane attachment).

Another area in which primary sequence data

has been useful is in assessing the evolutionary relationships between proteins isolated from different species. The protein for which the most complete information is presently available is cytochrome c. Of the 104 residues possessed by all species in this protein, 35 residues have been found to be constant. In particular an 11-residue sequence comprising residues 70 through 80 is totally conserved in all sequences, indicating the vital importance of this region to the function of this protein (Nolan and Margoliash, 1968).

This study, and a number of other comparative studies of the sequences around amino acid residues involved in the catalytic events occurring at the active sites of enzymes (see Dayhoff et al., 1972) illustrate that amino acid sequences that are conserved through evolution are generally involved in some important aspect related to the biological function of that protein.

One important contribution to the evolution of the biotin enzymes is the recent work of Rylatt et al. (1977). In this study the tryptic peptides containing the covalently bound biotin prosthetic group of pyruvate carboxylase from sheep, chicken and turkey liver were isolated and sequences. The primary sequence of these three peptides was highly conserved and furthermore showed a strong similarity to the biotin-containing

peptides isolated from P. shermanii transcarboxylase and E. coli acetyl CoA carboxylase (Wood and Barden, 1977) (Table 1.3). In particular the sequence just before and including the biotin prosthetic group had been rigidly conserved. Since it has been estimated that bacteria and eukaryotes diverged approximately 3,000 million years ago (Dayhoff and Eck, 1972) this degree of conservation provided strong evidence that the biotin enzymes evolved from a common ancestral precursor. Work directed towards the further development of this theme is presented in Chapter 6.

The approach used by Rylatt et al. (1977) was to employ the strong affinity of avidin for biotin to facilitate the isolation of the biotin peptides. This method had the decided advantage of not requiring homogeneous enzyme as starting material. Furthermore it is likely that, by employing other methods of cleaving the polypeptide chain (e.g. chymotrypsin, or reducing the number of trypsin cleavable sites by modifying either arginine (e.g. with phenylglyoxal ; Takahashi 1968) or lysine residues(e.g. with maleic anhydride; Butler and Hartley, 1972)) that the known amino acid sequence of the tryptic peptide around the biotin prosthetic group could be expanded.

However it would seem likely that there would

be a limit to how far this procedure could be employed, due to the insolubility of the large peptide obtained. Therefore it would appear that to solve the entire primary sequence of this enzyme (approximately 1100 amino acid residues) more orthodox peptide purification procedures would need to be employed. This would be a formidable task although not beyond the scope of the existing technology. For example, the complete sequence of E. coli β-galactosidase (1021 amino acid residues) has recently been published (Fowler and Zabin, 1977) and the primary sequence determination of NAD^+-specific glutamate dehydrogenase from N. crassa (approximately 1160 amino acid residues) is in progress (Veronese et al., 1974).

1.5. Objectives

From the preceding sections it is clear that the areas of investigation which hold most promise for expanding the understanding of the reaction catalysed by pyruvate carboxylase in the near future are chemical modification studies and the determination of the primary sequence of peptides from in and around the active centre of the enzyme. The characterisation of the functional amino acid residues, via chemical modification studies would provide a basic framework on which to interpret the data obtained from the kinetic

studies of the enzyme at the molecular level. The isolation of peptides containing these functional residues would enable predictions on the region surrounding these functional residues and, in conjunction with crosslinking studies, facilitate the development of a 3-dimensional picture of the active site.

Clearly however, to realize these long term objectives it was necessary to develop a reliable method of obtaining reasonable quantities of fully active homogeneous enzyme and therefore this was undertaken. Following the completion of this objective some of the physical properties of the enzyme from this source, including its appearance in the electron microscope, were investigated.

In addition, as part of a general study of the functional amino acid residues in sheep liver pyruvate carboxylase the role of the cysteine residues in the enzyme catalysed reaction was investigated.

TABLE 1.1

The three classes of biotin utilizing enzymes.

I Carboxylases

- A. Acyl CoA carboxylases
  - 1. Acetyl CoA carboxylase
  - 2. Propionyl CoA carboxylase
  - 3. β-Methylcrotonyl CoA carboxylase
  - 4. Geranyl CoA carboxylase
- B. α-Keto acid carboxylases
  - 1. Pyruvate carboxylase
- C. Amido carboxylases
  - 1. ATP : urea amidolyase

II Transcarboxylases

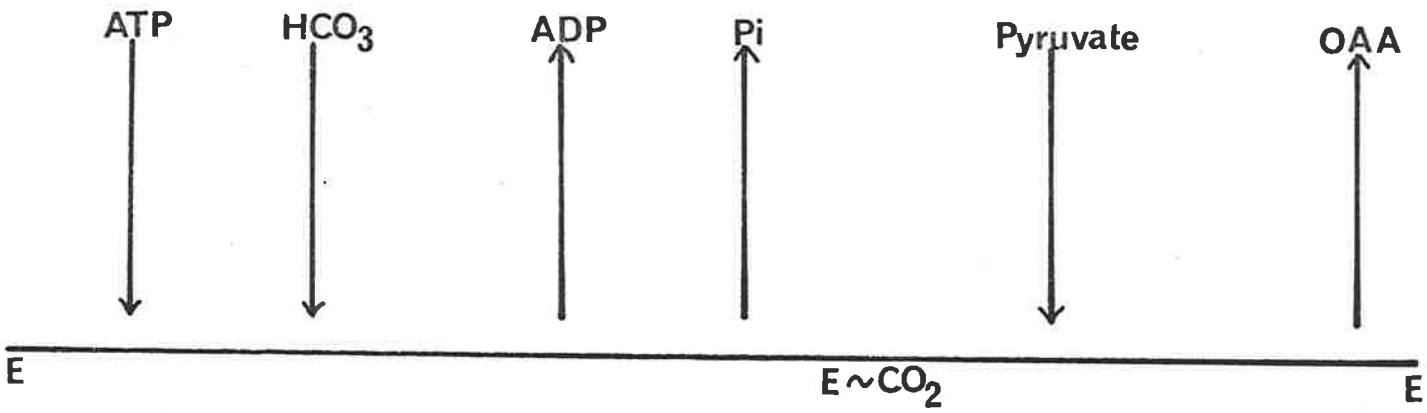
- 1. Methylmalonyl CoA : pyruvate transcarboxylase

III Decarboxylases

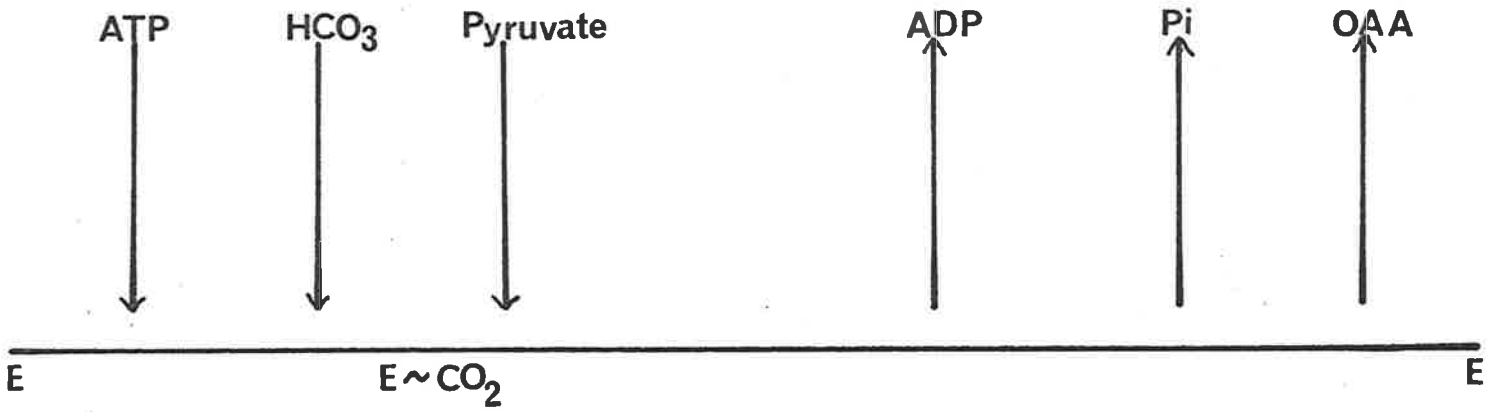
- A. α-Carboxyacyl CoA decarboxylases
  - 1. Methylmalonyl CoA decarboxylase
- B. β-Keto acid decarboxylases
  - 1. Oxaloacetate decarboxylase

FIGURE 1.1

A scheme showing the two alternative mechanisms for pyruvate carboxylase.



a. Ping-Pong Mechanism



b. Sequential Mechanism

TABLE 1.2

Physical properties of pyruvate carboxylase from a variety of sources

1. Bais, (1974).
2. Barden *et al.*, (1975)
3. Warren and Tipton, (1974a).

species	monomer moleculer weight x 10^{-5}	native molecular weight x 10^{-5}	moles biotin / moles enzyme
Sheep kidney[1]	1.1	4.8	4
Chicken liver[2]	1.25	5.0	4
Turkey liver[2]	1.1	5.0	4
Calf liver[2]	1.2	5.0	4
Rat liver[2]	1.3	5.0	4
Pig liver[3]	1.3	5.2	4
Human liver[2]	1.3	-	-
Yeast[2]	1.25	4.75	4
P. citronellolis[2]	0.65	2.56	2
	0.54		

TABLE 1.3

The amino acid sequences adjacent to the biocytins of liver pyruvate carboxylases, *P. shermanii* transcarboxylase and *E. coli* acetyl-CoA carboxylase.

Bct = Biocytin

Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Pyruvate carboxylase: sheep	Gly	Glu	Pro	Leu	Val	Leu	Ser	Ala	Met	Bct	Met	Glu	Thr	Val	Val	Thr	Ser	Pro	Val
Pyruvate carboxylase: chicken	Gly	Ala	Pro	Leu	Val	Leu	Ser	Ala	Met	Bct	Met	Glu	Thr	Val	Val	Thr	Ala	Pro	Arg
Pyruvate carboxylase: turkey	Gly	Ala	Pro	Leu	Val	Leu	Ser	Ala	Met	Bct	Met	Glu	Thr	Val	Val	Thr	Ala	Pro	Arg
Transcarboxylase: *P. shermanii*	Gly	?	Val	Leu	Val	Leu	Glu	Ala	Met	Bct	Met	Glu	Thr	Glu	Ile	Asn	Ala	Pro	Thr
Acetyl-CoA carboxylase: *E. coli*	Asn	Thr	Leu	Cys	Ile	Val	Glu	Ala	Met	Bct	Met	Met	Asn	Gln	Ile	Glu	Ala	Asn	Lys

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Enzymes and proteins

Albumin, bovine serum; catalase, bovine liver (E.C.1.11.1.6); cytochrome C, type III, horse heart; glutamate dehydrogenase, type II, bovine liver (E.C.1.4.1.3); glutamic-oxaloacetic transaminase, type I, porcine heart (E.C.2.6.1.1); glutamic-pyruvic transaminase, porcine heart (E.C.2.6.1.2); glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (E.C.1.2.1.12); malate dehydrogenase, porcine heart (E.C.1.1.1.37); ovalbumin; RNA polymerase, *E. coli* (E.C.2.7.7.6) and ribonuclease, type 1-A, bovine pancreas (E.C.2.7.7.16), were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Gamma globulin, human, was obtained from Commonwealth Serum Laboratories, Melb., Aust. Chymotrypsinogen, beef pancreas, was obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Trypsinogen; beef pancreas, was obtained from Nutritional Biochem. Corp., Cleveland, Oh. Pronase, (B grade) was obtained from Calbiochem (Aust.), Pty. Ltd., Sydney, Aust. Avidin and trypsin (L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK) treated, bovine pancrease; E.C.3.4.4.4) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Transcarboxylase; *P. shermanii* (E.C.2.1.3.1) was the generous gift of Professor H.G. Wood.

2.1.2. Radioactive chemicals

Sodium [2-^{14}C] pyruvate, [8-^{14}C]ADP, iodo [2-^{14}C] acetic acid, N-ethyl [2,3-^{14}C] maleimide, and d[carbonyl ^{14}C] biotin were obtained from The Radiochemical Centre, Amersham, England. [^{32}P]-orthophosphate was obtained from the Australian Atomic Energy Commission, Lucas Heights, Australia.

2.1.3. General chemicals

ATP (disodium salt, grade I), ADP (disodium salt, grade I), CoA (grade I), dithioerythritol, DTNB, NADH, oxaloacetic acid, 2-oxoglutarate, sodium pyruvate (type II, dimer free), ninhydrin, dansyl chloride, d-biotin, N-N-dicyclohexyl-carbodiimide, guanidine-HCl (practical grade), SDS, and Trisma base were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. N-ethyl maleimide was obtained from Calbiochem (Aust.) Pty. Ltd., Sydney, Aust. N-N -butyl maleimide was supplied by ICN Pharamaceuticals, Inc., Plainview, N.Y. Acrylamide, N,N -methylenebisacrylamide, N,N,N ,N -tetramethylethylenediamine, thin layer cellulose and silica gel plates (plastic backed) and 1-anilino-napthalene-8-sulphonic acid were obtained from Eastman Kodak, Rochester, New York, U.S.A.

Special enzyme grade ammonium sulphate was obtained from Mann Research Laboratories, New York, U.S.A., and polyethylene glycol, molecular weight 20,000, from Union Carbide Corporation. Sucrose, analytical reagent grade was obtained from Colonial Sugar Refininc Co., Sydney, Aust.

POPOP (1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene), PPO (2,5-diphenyloxazole), and iodoacetic acid were supplied by Koch-Light Laboratories Ltd., Bucks, England. Iodoacetic acid was recrystallized from petroleum ether (b. pt. 60-80^{o}C). Polyamide plates were obtained from Chen-Ching Trading Co., Taipei, Taiwan. 4-Phenyl spiro [furan-2(3), 1 - phithalan] 3,3-dione (fluroescamine) was obtained from Roche Products, Dee Why, N.S.W., Aust.

Blue Dextran, Phenyl Sepharose and all grades of Sephadex and Sepharose were obtained from Pharmacia, Uppsala, Sweden. NAD-Agarose, type 1, was obtained from P-L Biochemicals, Inc., Milwaukee, Wis., U.S.A. DEAE-cellulose (DE-23) was obtained from Whatman Chromedia. (W. and R. Balston, Ltd., England). All solvents were distilled before use. Pyridine was distilled twice from ninhydrin.

2.2. GENERAL METHODS

2.2.1. Measurement of pyruvate carboxylase activity

The enzyme was assayed by a procedure based on that described by Utter and Keech (1963). This involved reduction of the oxaloacetate produced by pyruvate carboxylase, using malate dehydrogenase, with the concommitant oxidation of NADH to NAD^+, and following the decrease in absorbance at 340nm. Assay solutions contained, (in µmoles) in a final volume of 1.0ml; tris-Cl, pH 8.4, (100); ATP, (2.5); $MgCl_2$, (5); HCO_3^-, sodium salt, (20); pyruvate, sodium salt, (10); acetyl CoA, (0.25); NADH, (0.125); malate dehydrogenase, (5 units); and pyruvate carboxylase (0.025 - 0.1 units). The decrease in absorbance at 340nm was followed with either a Unicam SP800 spectrophotometer, or a Varian-Techtron 635-0 spectrophotometer. The cell block was thermostated at 30^oC. The rate of oxaloacetate synthesis was calculated assuming an extinction coefficient at 340nm for NADH of 6.22 $mM^{-1}cm^{-1}$ (Dawson et al., 1969).

2.2.2. Protein estimation

Protein concentrations were determined by the method of Layne (1957) using the equation,

protein concentration (mgs/ml) = $1.55A^{1cm}_{280nm} - 0.76A^{1cm}_{260nm}$

2.2.3. Protein and peptide detection following column chromatography

Protein elution from columns was generally detected by determination of the absorbance at 280nm of the individual fractions. Alternatively, peptides and proteins were detected by a fluorometric procedure slightly modified from the method of Udenfriend et al. (1972). To 0.1ml of sample was added 0.5ml of 0.2M sodium borate buffer, pH 9.0, and then 0.1ml of a 25mg/100ml solution of fluorescamine in acetone was added with mixing. The fluorescence intensity at 480nm was measured in a Perkin Elmer Fluorescence Spectrophotometer Model 203 with the excitation wavelength at 390nm.

2.2.4. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Fairbanks et al. (1971) on 5.6% acrylamide gels.

2.2.5. Determination of biotin concentration

Biotin concentration was determined using the radiochemical assay developed by Rylatt et al., (1977). The assay solution contained in a final volume of 0.5ml; potassium phosphate, pH 7.2 (50 µmole), avidin (0.028 units) and biotin sample (10-100 pmole

biotin). After incubating for ten minutes at room temperature, 0.05ml [^{14}C] biotin (2.2 nmole, 10^5 cpm/nmole) was added, and ten minutes later the avidin-biotin complex was precipitated by adding 0.2ml of 0.1M zinc sulphate, followed by 0.2ml of 0.1M sodium hydroxide. The precipitate was collected by centrifuging, and samples of the supernatant taken and their radioactivity determined using the Triton-based scintillant (Section 2.2.6.). The correlation between the radioactivity of the supernatant and the biotin content of the sample was established by constructing a standard curve using known amounts of unlabelled biotin as reference samples. There was a linear relationship between biotin content and radioactivity for the range 10-70 pmoles of biotin.

2.2.6. Determination of radioactivity

Samples were either placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid containing Triton X-100, 7:3, v/v) or dried onto solid supports (1" x 1" squares of Whatman 3MM paper) and then placed into vials containing 2ml of toluene scintillation fluid (0.3%(^{w}/v) 2,5-diphenyloxazole, 0.03% (^{w}/v) 1,4-bis-2 (4 methyl-5-phenoxazolyl)-benzene, in sulphur-free toluene; Bosquet and Christian, 1960) and counted in a Packard Scintillation Spectrometer.

2.2.7. Preparation of acetyl CoA

Acetyl CoA was prepared by a method similar to that of Simon and Shemin (1953) and purified as described by Ashman (1973).

2.2.8. S-carboxymethylation of pyruvate carboxylase

The protein was dissolved in 0.1M Tris-Cl pH 8.4 containing 6M recrystallized guanidine-HCl to a concentration of 10 mg per ml. A ten-fold molar excess of DTE over total protein thiol was added and the solution incubated for 1 h at 37°C to ensure complete reduction. Recrystallized iodoacetic acid, dissolved in 3.0M tris-Cl, pH 8.0, was then added in equimolar amount to DTE and the solution incubated in the dark at 37°C. The reaction was followed by using the nitroprusside test and was completed within 20 mins. The procedure was repeated to ensure complete S-carboxymethylation and the solution was then dialysed extensively against water containing 0.02% sodium azide and 1.0mM EDTA and freeze-dried.

2.2.9. Amino acid analysis

Samples were hydrolysed under nitrogen in sealed tubes with 1.0ml of 6M HCl containing one drop

of aqueous phenol. Hydrolysates were dried by rotary evaporation and analysed by the procedure of Piez and Morris (1960) using a Beckman 120C analyser modified as described by Harding and Rogers (1971).

2.2.10. Isotopic exchange reactions

The rates of exchange of [^{14}C]ADP or [^{32}P] orthophosphate into ATP and of [^{14}C] pyruvate into oxaloacetate were determined as described by Ashman and Keech (1975).

CHAPTER 3

PURIFICATION OF PYRUVATE CARBOXYLASE

3.1. INTRODUCTION

The purification of pyruvate carboxylase from sheep liver mitochondria can be achieved by the same method as that described for the purification of the chicken liver enzyme by Scrutton and Fung (1972). In this laboratory however, a polyethylene glycol precipitation step is preferred to the Sephadex G-25 gel filtration desalting step described by Scrutton and Fung since this step removes some unwanted protein as well as deionizing the solution prior to ion-exchange chromatography. This purification is rapid and can, on occasions, result in high specific activity preparations of the enzyme from both sources. In general however, glutamate dehydrogenase co-purifies with pyruvate carboxylase during this preparation and, particularly in the case of the sheep liver enzyme, remains as the only significant contaminant following DEAE-Sephadex chromatography. To overcome this problem a 1.07M ammonium sulphate "back extraction" has been introduced into the sheep liver enzyme preparation. At this concentration of ammonium sulphate, glutamate dehydrogenase is largely soluble whereas pyruvate carboxylase is not. This step improves the purity of the preparation but still fails to consistently provide homogeneous enzyme. An outline of this modified preparation is shown in fig. 3.1.

The enzyme prepared by this method is suitable for kinetic studies and consequently the kinetic mechanism of pyruvate carboxylase is known in considerable detail. A complete understanding of the mechanism of CO_2 fixation by pyruvate carboxylase requires however, an understanding of the involvement of the amino acid residues both in the binding of ligands and in the catalytic process, and also primary sequence data, particularly around the active site of the enzyme. Both of these studies require homogeneous pyruvate carboxylase. An investigation was therefore initiated with the aim of removing this final contaminant. Various approaches were adopted in this study and these are discussed in detail below. In addition, some of the physical properties of highly purified sheep liver pyruvate carboxylase were investigated and compared with the properties of pyruvate carboxylase isolated from sheep kidney by Bais (1974).

3.2. METHODS

3.2.1. Preparation of sheep liver mitochondria

All procedures for the isolation of mitochondria were carried out at $4^{o}C$. Sheep livers were placed on ice immediately after removal from the animal, and diced in small segments. These segments were homogenized in a Waring blender with 4 volumes (w/v) of 0.25M sucrose containing $10^{-4}M$ EDTA. The homogenate was centrifuged

at 600xg for 20 min. to remove cell nuclei and whole cells and the supernatant fraction centrifuged at 23,000xg for 15 min. The precipitated material was resuspended in 10^{-4}M EDTA to give a final volume half that of the original 600xg supernatant. The suspended material was centrifuged at 23,000xg for 15 min., resuspended in a minimal volume of 10^{-4}M EDTA and freeze-dried. The dried mitochondria were stored at -15°C over silica gel.

Mitochondria were isolated from chicken liver by the method of Scrutton et al. (1969).

3.2.2. Preparation of sheep liver pyruvate carboxylase

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria by suspending the mitochondria (120g.) in 1750 ml of extraction buffer which contained 25mM tris-acetate, pH 6.7, 3.5mM $MgCl_2$, and 1.7mM ATP. The pH of the solution was maintained between 6.5 and 6.7 during addition of the mitochondria. The suspension was stirred for 20 min., after which time undissolved material was removed by centrifuging at 23,000xg for 10 min. at 4°C.

Ammonium sulphate was added to the supernatant solution to give a final concentration of 1.35M while maintaining the pH between 6.9 and 7.1 by addition of

1M KOH. The suspension was stirred for 20 min. and the precipitated protein collected by centrifuging at 23,000xg for 20 min. at 4°C. The precipitate was suspended in 1.07M ammonium sulphate to a final volume of 5 ml per 10g of mitochondria.

The precipitated protein was collected by centrifuging at 23,000xg for 10 min. at 4°C and suspended in Buffer A (25mM potassium phosphate, pH 7.2, containing 1mM EDTA, 0.1mM DTE and 20mM ammonium sulphate, to give a final protein concentration of 10mg/ml. This suspension was desalted by adding 14.5g of polyethylene glycol (Carbowax 20M) per 100 ml of solution. The suspension was stirred for 30 min. and the precipitated protein was then collected by centrifuging at 23,000xg for 20 min. at 4°C. The protein was then resuspended in 5-6ml of Buffer A per 10g of mitochondria and undissolved protein removed by centrifuging at 23,000xg for 10 min. at 4°C. The supernatant was applied to a DEAE-Sephadex A-50 column (5 x 14cm) previously equilibrated with Buffer A and eluted with a linear gradient from 20mM ammonium sulphate in Buffer A (pH 7.2) to 200mM ammonium sulphate in 25mM tris-Cl, pH 8.0 containing 1mM EDTA and 0.3mM DTE. Pyruvate carboxylase of specific activity 10-20 units/mg protein was routinely obtained using this procedure. The major contaminant in this enzyme preparation was glutamate dehydrogenase and the removal of this contaminant is the subject of this chapter.

Variations in the nature of the gradient (e.g. hyperbolic gradients) or the buffers used in the preparation (e.g. N-ethylmorpholine cations) did not significantly alter the degree of contamination.

3.2.3. Preparation of chicken liver pyruvate carboxylase

Chicken liver pyruvate carboxylase was isolated by the method of Scrutton and Fung (1972) except that the polyethylene glycol precipitation described above was substituted for the Sephadex G-25 chromatography described by those authors.

3.2.4. Storage of the enzymes

The enzymes could be stored at -80°C in storage buffer (0.1M phosphate (K^+, pH 7.0) containing 1.6M sucrose and 1% (v/v saturated $(NH_4)_2\ SO_4$) for several months with negligible loss of activity. During the course of this study it was found that the enzymes could also be stored in either 0.1M tris-Cl, pH 8.4, or Buffer A at -80°C for several weeks without significant loss of activity.

3.2.5. Preparation of Blue Dextran Sepharose

Blue Dextran Sepharose was prepared by the method of Ryan and Vestling (1974) except that the

activation of the Sepharose 4B was performed using cyanogen bromide dissolved in acetonitrile as described by March et al. (1974).

3.2.6. Buffer terminology

The variety of techniques described below required the use of buffers of different composition. To simplify nomenclature, these buffers are described by the numbers of the figures in which they were employed, and their compositions are given in the legends to those figures. The only exception to this is Buffer A, the composition of which is given in section 3.2.2.

3.2.7. Measurement of glutamate dehydrogenase activity

Glutamate dehydrogenase activity was determined by the method described in "biochemica information II" (1975).

3.2.8. Other methods

All other methods employed have been described in the relevant sections of chapter 2.

3.3. RESULTS

3.3.1. Purification of inactive enzyme

The determination of the primary sequence of a protein by conventional methods requires large quantities of highly purified starting material. There is no requirement however that this material be in its native configuration. Hence denaturation of the protein to achieve homogeneity is permissible. Since the molecular weight of eukaryote pyruvate carboxylase monomers is approximately 1.1 - 1.2 x 10^5 daltons (Utter et al., 1975), while that of glutamate dehydrogenase is only 5.3 x 10^4 daltons (Moon et al., 1973), gel filtration under denaturing conditions should readily effect a suitable resolution of these two polypeptides.

Application of this approach by Rylatt (1976) using 8M urea as the denaturant was unsuccessful. This was attributed to the poor solubility of pyruvate carboxylase in this solvent, as observed in this laboratory and reported by Scrutton and Utter (1965a). Contrary to the results of Scrutton and Utter however, guanidine - HCl readily solubilizes pyruvate carboxylase isolated from both chicken and sheep, but the commercially available product is impure, having a high absorbance at 280nm and containing an oxidant. This reagent was therefore recrystallized by the method of Nozaki (1972). The

yields of purified guanidine-HCl obtained were, however, poor (ca. 20%). Considering that this reagent is generally used at a concentration of 6M, it seemed impractical to prepare bulk quantities on the scale envisaged. A more significant factor however is the high viscosity of these concentrated solutions. This can give rise to gel compression, and hence loss of resolution, on the gel types (e.g. Sephadex G-200) required to fractionate monomers of pyruvate carboxylase and glutamate dehydrogenase.

While these objections are not so serious as to exclude the use of guanidine-HCl as the denaturant, an equally effective denaturant was available which did not suffer from the disadvantages discussed above. This denaturant was SDS.

3.3.1.1. Chromatography in the presence of SDS

Sodium dodecyl sulphate is an anionic detergent widely used in the determination of subunit molecular weights of proteins by polyacrylamide gel electrophoresis (Weber and Osborn, 1975). Binding studies on a variety of different proteins indicate that at SDS-monomer concentrations above 8×10^{-4}M, 1.4 g of SDS are bound per gram of protein (Pitt-Rivers and Impiombato, 1968; Reynolds and Tanford, 1970 a, b). Although this low concentration of denaturant reduces the possibility of

gel compression due to viscosity, due to the strength of binding, SDS can be difficult to remove from the protein after a suitable resolution has been achieved. Since this residual strongly bound SDS can interfere with any envisaged study of the protein (e.g. reactivation of enzyme activity, enzymic digestion of the protein) it is important to totally remove all SDS. A number of methods have been reported in the literature to achieve this aim, including ion exchange chromatography in 6M urea (Weber and Kuter, 1971) prolonged dialysis, electrodialysis (Tuszynski and Warren, 1975) or chromatography on Sephadex LH-20 in 70% formic acid (Griffith, 1975). While the peculiarities of the protein being studied and the purpose to which the purified protein is to be put will affect the selection of a method, it appears that total removal of SDS is now not a problem associated with its use as a denaturant.

Pagé and Godin (1969) have reported the isolation of monomers of glutamate dehydrogenase using Sephadex G-200 equilibrated with 0.05M SDS and 10^{-4}M p-hydroxymercuribenzoate. The calibration curve of their column indicated that pyruvate carboxylase should be well resolved from glutamate dehydrogenase and so this system was adopted.

Fig. 3.2 shows the calibration curve of a Sephadex G-200 column (2.5cm x 85cm) prepared as described by Pagé

and Godin. The molecular weight markers used were; ribonuclease (13,700), chymotrypsinogen (25,700), ovalbumin (43,000), glutamate dehydrogenase (53,000) and bovine serum albumin (68,000). Each sample was incubated in 0.05M SDS, 10^{-4}M p-hydroxymercuribenzoate 0.1M sodium borate, pH 8.5 for 24 h at 37°C and then applied individually to the column. The flow rate of the column was 0.16 ml/min and 3.5 ml fractions were collected. The presence of protein in the various fractions was determined using fluorescamine. The molecular weight of the pyruvate carboxylase monomer under these conditions was $1.13 \pm 0.03 \times 10^5$ daltons, in good agreement with the value of $1.1 \pm 0.05 \times 10^5$ daltons obtained by Bais (1974) for the sheep kidney enzyme. The exclusion volume of this column was 1.25×10^5 daltons, considerably lower than the value of 4.5×10^5 daltons reported by Pagé and Godin. However, extensive investigation using columns of various dimensions, upward or downward flow, recrystallized SDS, different batches and swelling conditions of the gel, extremely slow flow rates or replacement of the mercurial with DTE all resulted in the highly reproducible pattern shown in fig. 3.2. It is clear from fig. 3.3 that Sephadex G-200 chromatography in sodium dodecyl sulphate resulted in a satisfactory resolution of the monomers of pyruvate carboxylase and glutamate dehydrogenase.

3.3.1.2. Removal of SDS

A number of methods for the removal of SDS from protein containing solutions have already been mentioned. Of these, two were tested for their ability to separate pyruvate carboxylase from the denaturant. Chromatography using Dowex-1 (Cl^-) as the support and 0.05M tris-acetate pH 7.8 containing 0.01 M 2-mercaptoethanol and 6M urea as the solvent has been reported by Weber and Kuter (1971) to remove SDS from a variety of proteins. However, attempts to utilize this procedure resulted in extensive losses of pyruvate carboxylase. The poor solubility of pyruvate carboxylase in urea solutions was undoubtedly a significant factor contributing to these poor yields.

Chromatography of the purified protein on Sephadex LH-20 using 70% formic acid as solvent (Griffith, 1975) proved to be much more satisfactory. The protein eluted from the Sephadex G-200 column corresponding to pyruvate carboxylase was pooled, dialysed against water until the protein precipitated, and then freeze-dried. The dry powder was redissolved in 10% of its original volume and applied to a column of Sephadex LH-20 (1 x 30cm) previously equilibrated with 70% formic acid at 4^oC. The eluted protein, detected by absorbance at 280nm was pooled, diluted 2-fold with water and freeze-dried. SDS was found to be absent in the protein, using

the method described by Hayashi (1975). Examination of this material using SDS-polyacrylamide gel electrophoresis revealed only one band, indicating that brief exposure of pyruvate carboxylase to 70% formic acid did not result in cleavage of the polypeptide chain (plate 3.1).

The approach described above provides an efficient method of obtaining homogeneous pyruvate carboxylase, free of SDS, which is suitable for the determination of the primary sequence of this large polypeptide.

3.3.2. Purification of active enzyme

This section deals with attempts to isolate pyruvate carboxylase free of glutamate dehydrogenase and in a highly active form. This high specific activity enzyme is necessary for any meaningful affinity labelling or chemical modification studies since contaminating proteins or inactive pyruvate carboxylase may lead to artifactual results.

3.3.2.1. Molecular weight fractionation

Molecular weight fractionation on Sepharose 4B has been used previously by Bais (1974) to improve the purity of sheep kidney pyruvate carboxylase preparations. On occasions, this procedure resulted

in homogeneous enzyme, but due to the variable amounts of glutamate dehydrogenase present in the preparations, it was not satisfactory as a general method. This was due to the fact that glutamate dehydrogenase migrated ahead of pyruvate carboxylase during gel filtration and, at low concentrations, dissociated to lower molecular weight forms (Freiden, 1962). This gave rise to a significant tailing effect and resulted in incomplete separation of the two enzymes. (fig. 3.4).

3.3.2.2. Hydrophobic chromatography

Hydrophobic interaction chromatography has recently been introduced as an alternative method of purifying proteins (Hofstee, 1976). The principle of the technique involves the differing affinities of the hydrophobic regions of proteins for an uncharged hydrophobic group covalently attached to an inert matrix. In theory, any change in conditions which results in weakening of these interactions would allow elution of the bound proteins from the matrix.

The results of a typical attempt to utilize this procedure for the purification of pyruvate carboxylase from the glutamate dehydrogenase remaining after DEAE-Sephadex chromatography is shown in fig. 3.5. The enzyme was applied to a Phenyl-Sepharose column, (1 x 10cm), previously equilibrated with buffer 3.5.I and eluted

with the same buffer. No protein emerged from the column. A gradient of 15 ml of the above buffer and 15 ml of buffer 3.5.II was then established and the elution continued. Protein emerged at the end of this gradient and was completely eluted using a further wash of the second buffer. This protein contained both pyruvate carboxylase and glutamate dehydrogenase activity. The recovery of pyruvate carboxylase activity was approximately 45%. Substantial losses of activity were also observed when N-ethyl morpholine was used as the buffer cation at pH 7.5.

Attempts were made to utilize this procedure in lieu of the polyethylene glycol precipitation in the standard preparation (see fig. 3.1). The protein precipitated from the 1.07M ammonium sulphate "back extraction" in the standard preparation was redissolved in buffer 3.6.I and applied to a Phenyl Sepharose column, (2 x 30cm), previously equilibrated in the same buffer (fig. 3.6). The column was washed with buffer 3.6.I until the bulk of the unbound protein was removed. A linear gradient of 70 ml of buffer 3.6.I and 70 ml of buffer 3.6.II was then applied and once again both enzymes eluted from the column at the very end of this gradient. A marginal resolution of the two enzymes was observed under these conditions, most probably due to the larger column used in these studies. The recovery of pyruvate carboxylase activity was from the Phenyl Sepharose column

was however, very poor (approximately 30%).

It was apparent from these results that both pyruvate carboxylase and glutamate dehydrogenase have regions within their quaternary structure that are strongly hydrophobic. The strength of interaction of these hydrophobic regions with the phenyl-groups on the Sepharose may be great enough to physically distort pyruvate carboxylase, so resulting in the poor yields of active enzyme. These losses of activity preclude the use of hydrophobic chromatography (at least using Phenyl Sepharose) as a means of resolving these two enzymes.

3.3.2.3. Affinity chromatography

The specific interaction of a ligand with a given protein forms the basis of the now well established technique of affinity chromatography. This elegant procedure has facilitated the isolation of a large number of biologically active proteins (Cuatrecasas and Anfinsen, 1971; Lowe and Dean, 1974).

Purification is achieved by passage of a protein solution through an inert matrix containing covalently bound ligand. Proteins possessing affinity for this particular ligand are retarded while all others pass unhindered through the matrix.

Successful application of this technique relies on the choice of ligand to be coupled to the matrix. In the case of pyruvate carboxylase this choice was rather limited. Pyruvate, in the absence of acetyl CoA, binds poorly to the enzyme. Oxaloacetate readily decarboxylates, making it unsuitable for affinity chromatography. Acetyl CoA, while possessing a suitably low K_m value for the enzyme is too expensive to be considered for this type of work. ATP is a logical choice as a ligand to be coupled to a matrix, since the enzyme has a high affinity for this nucleotide (K_m = 0.6mM). Unfortunately, attempts to resolve glutamate dehydrogenase from pyruvate carboxylase using an ATP-affinity column have in the past been unsuccessful. This is presumably because glutamate dehydrogenase has an ADP binding site which interacts with the bound ATP. Direct application of affinity chromatography to pyruvate carboxylase, for the specific purpose of removing glutamate dehydrogenase therefore seems unlikely to be successful.

An alternative approach to this problem was to attempt affinity chromatography directed against glutamate dehydrogenase, thereby leaving pyruvate carboxylase free to elute straight through the column. A number of affinity chromatography systems have been described for glutamate dehydrogenase. The glutamate - affinity system of Blumenthal and Smith (1973) might be expected to lack unique specificity for glutamate

dehydrogenase since α-ketoglutarate can act as an inhibitor of the pyruvate carboxylase reaction (Mildvan et al., 1966). The GTP-affinity system of Godinot et al. (1974) was clearly unsuitable since pyruvate carboxylase can utilize this nucleotide as a substrate (Haywood, unpublished observations). It therefore appeared that the most attractive system for the resolution of these two enzymes was one based on the affinity of glutamate dehydrogenase for NAD^+.

3.3.2.3.1. Blue Dextran - Sepharose affinity chromatography

Through the use of X-ray crystallography it has been observed that many NAD^+ utilizing enzymes have a "dinucleotide fold" (Ohlsson et al., 1974). This is a particular arrangement of polypeptide chains which appears to be heavily conserved and binds NAD^+. Furthermore Thompson et al. (1975) have shown that blue dextran, a sulphonated polyaromatic blue dye convalently attached to dextran, behaves as an analogue of NAD^+ and so, when coupled to Sepharose 4B can be used for affinity chromatography of those enzymes possessing the dinucleotide fold. Since glutamate dehydrogenase utilizes NAD^+ then this blue dextran system was investigated as an economical alternative to an NAD^+-Agarose system.

Blue Dextran Sepharose was prepared by the method of Ryan and Vestling (1974) as described in Section 3.2.4. To establish the viability of the Blue Dextran Sepharose, bovine glutamate dehydrogenase (30 units, 0.5mg) in 20mM potassium phosphate, pH 7.2, 10^{-4}M EDTA, 10^{-4}M DTE (buffer 3.7.I) was applied to a Blue Dextran Sepharose column (0.7 x 1.5cm) equilibrated in the same buffer. The enzyme was retarded and could be eluted with buffer 3.7.I containing 5mM NAD^+ (fig 3.7). Clearly, the system appeared to behave as an affinity chromatography system for glutamate dehydrogenase.

When a mixture of pyruvate carboxylase and glutamate dehydrogenase was applied to this column however, pyruvate carboxylase was also retarded. Upon application of a buffered linear gradient of 0 - 1.0M NaCl both enzymes appeared simultaneously in the eluent. This was accompanied by a significant loss of pyruvate carboxylase activity (>50%). Similar results were obtained when a linear gradient of 0 - 1mM $MgATP^{2-}$ was used as the eluent (fig 3.8). This latter finding was consistent with the previously mentioned interaction of glutamate dehydrogenase with an ATP affinity chromatography column.

From the results presented above it appeared as though pyruvate carboxylase was interacting with the Blue Dextran Sepharose column through the $MgATP^{2-}$

binding site. Since three coenzyme A utilizing enzymes have been reported to bind to Blue Dextran however, (White and Jencks, 1976; Thompson et al., 1975) some interaction with the acetyl CoA binding site on pyruvate carboxylase might also be expected. To test this hypothesis a mixture of pyruvate carboxylase and glutamate dehydrogenase were bound to the Blue Dextran column in buffer 3.7.I. Elution with 0.25mM acetyl CoA in the buffer was then attempted. As can be seen in fig. 3.9, pyruvate carboxylase streaked badly off the column under these conditions. Glutamate dehydrogenase was, surprisingly however, eluted as a single peak of activity. This result implied that glutamate dehydrogenase interacted with acetyl CoA, an observation never previously reported with this well studied enzyme. The fact that pyruvate carboxylase was not effectively eluted with acetyl CoA at a concentration 25-fold higher than its K_a value suggested that this binding site was not the primary locus of interaction of blue dextran with the enzyme.

Similar results to those reported above were obtained using a tris-Cl buffer at pH 8.4. When N-ethyl morpholine was used as the buffer cation however the binding characteristics of the enzyme to Blue Dextran Sepharose was altered. Glycerol (2% v/v) was initially used in these experiments in an attempt to prevent the large losses of pyruvate carboxylase activity which had

previously been observed. Under these conditions pyruvate carboxylase did not bind to the affinity column and was quantitatively recovered. No glutamate dehydrogenase activity was detected in the eluant but the specific activity of the pyruvate carboxylase remained the same as prior to loading. The purity of this material was therefore examined using SDS-gel electrophoresis. These gels revealed the presence of both enzymes in the eluant. It was apparent that glutamate dehydrogenase was inactivated in the N-ethyl morpholine - Cl/glycerol buffer. To confirm this, glutamate dehydrogenase was incubated in this buffer and aliquots removed at various time intervals for enzymic activity determination. Over a period of 2 hr the enzyme lost greater than 80% of its activity (fig. 3.10). Furthermore, when applied to a Blue Dextran Sepharose column this material failed to find. Removal of the glycerol from the buffer resulted in a more complete separation of the two enzymes. Pyruvate carboxylase did not bind and passed through the column. 3% of the glutamate dehydrogenase activity emerged unretarded from the column. The bound glutamate dehydrogenase could be eluted with 1.0M NaCl (fig. 3.11). The recovery of pyruvate carboxylase activity was ca.45% and represented an increase of 3-fold in the specific activity. Once again however, glutamate dehydrogenase underwent inactivation in this buffer, (fig. 3.11, inset) so was not completely eliminated

from the pyruvate carboxylase preparation.

The purification of pyruvate carboxylase via the use of Blue Dextran Sepharose chromatography does not appear to be a viable proposition, since under no conditions studied could complete removal of glutamate dehydrogenase be achieved.

This study did reveal a number of interesting observations, however. Firstly, using the criteria set down by Thompson et al. (1975), it would appear that pyruvate carboxylase contains a structure similar to the dinucleotide fold. This occurs in the ATP binding site and so may more correctly be termed a mononucleotide fold. Of the other CoA binding enzymes listed above, none have yet been reported to be specifically eluted with CoA or its derivatives and so the possibility of a dinucleotide fold in this binding site remains at this stage, speculative. The failure to obtain specific elution with acetyl CoA suggests that this is not the case with pyruvate carboxylase.

Secondly, the elution of glutamate dehydrogenase from these columns with acetyl CoA suggests an interaction between this enzyme and this important metabolite, but rules out the possibility of using the 3'5'ADP affinity system described by Barry et al. (1976) for the isolation of CoA utilizing enzymes for direct affinity

chromatography of pyruvate carboxylase.

3.3.2.3.2. NAD^+ - Agarose affinity chromatography

The lack of specificity observed with the Blue Dextran-Sepharose system led to an investigation of the true substrate, NAD^+, as an affinity ligand for glutamate dehydrogenase. This approach, using a NAD^+ - Agarose affinity column, was found to be entirely satisfactory for the removal of glutamate dehydrogenase from pyruvate carboxylase preparations. The best condition for the resolution of these two enzymes was to apply the enzyme preparation in storage buffer (see section 3.2.4.) directly to a column, 1cm x 5cm, of NAD^+ - Agarose, equilibrated in buffer 3.12.I. Under these conditions both enzymes were retarded on the column although a small amount of inactive protein was eluted with a wash of buffer 3.12.I. By increasing the concentration of the buffer 10-fold while maintaining a constant pH, pyruvate carboxylase could be eluted from the column with recoveries of initial activity ranging from 70 - 100% (fig. 3.12). Glutamate dehydrogenase was either totally absent or present in only trace amounts when the specific activity of the pyruvate carboxylase applied to the column was greater than about 15. When lower specific activity enzyme was used a small amount of the glutamate dehydrogenase originally present eluted with the pyruvate carboxylase. This residual contaminant

could be quantitatively removed by repeating the affinity chromatography procedure. The final specific activity of the enzyme was always in the range 28-30 and was homogeneous on SDS-polyacrylamide gels (plate 3.2).

Fig. 3.13 illustrates several other features of the interaction of pyruvate carboxylase and glutamate dehydrogenase with the NAD^+ - Agarose system.

(1) $MgATP^{2-}$ specifically removed glutamate dehydrogenase from the column. This provided an efficient method of regenerating the NAD^+ - Agarose. Further, it indicated that pyruvate carboxylase was not ligated to the affinity column through the $MgATP^{2-}$ binding site. In fact, since increased ionic strength eluted pyruvate carboxylase, it seems reasonable to suggest that this enzyme binds to the affinity column through ionic interactions. This interaction was clearly an advantage as it enabled the small amount of other contaminating protein to be removed and so accounted for the very high specific activity of the pyruvate carboxylase routinely obtained.

(2) Potassium phosphate buffers at pH 7.2 were an inadequate replacement for the pH 8.4 tris-Cl buffers (i.e. buffer 3.12.I.) described above. This would suggest that NAD^+ - Agarose affinity chromatography may not be applicable to the removal of glutamate dehydrogenase from chicken liver pyruvate carboxylase preparations since this latter enzyme is less stable at pH 8.4 (Keech and Utter, 1963).

The NAD^+ - Agarose affinity chromatography described above enables a routine, facile and highly efficient method of completely eliminating glutamate dehydrogenase from preparations of sheep liver pyruvate carboxylase while maintaining this large enzyme in a highly active form.

3.3.3. Physical properties of sheep liver pyruvate carboxylase

Molecular sieve chromatography on a 1.6 x 65cm column of Sephadex G-200 was used to obtain estimates of both the molecular weight and the Stokes radius of sheep liver pyruvate carboxylase. The standard proteins used to calibrate the column were

individually chromatographed at a flow rate of 0.16 ml/min and their elution volume (Ve) expressed relative to the elution volume of blue dextran (Vo). The molecular weights of the standard proteins were obtained from the literature (see Legend, fig. 3.15). The Stokes radii (a) of the standard proteins were either obtained from the literature (see Legend, fig. 3.14) or, in the case of trypsinogen, calculated from its known diffusion constant ($D_{20,w}$) (Tietze, 1951) using the Stokes-Einstein equation (Gosting, 1956)

$$D = \frac{kT}{6\pi\eta a} \qquad \ldots (1)$$

where k is the Boltzman constant, T the absolute temperature and η the viscosity of the medium.

A plot of the log of the Stokes radii against the relative elution positions of the standard proteins gave a linear relationship which upon extrapolation indicated a Stokes radius for sheep liver pyruvate carboxylase of 74.9 ± 0.6 Å (fig. 3.14). Similar values were also obtained by the method of either Porath (1963) or Laurent and Killander (1964). A similar plot of the molecular weights of the standard proteins against their relative elution positions indicated that sheep liver pyruvate carboxylase had a molecular weight of $5.25 \pm 0.08 \times 10^5$ daltons (fig. 3.15). The molecular weight of the enzyme was also estimated on a Sepharose

4B column (2.5cm x 80cm) using the equation

$$\log \text{mol. wt} = 8.78 - 1.62 \left(\frac{Ve}{Vo}\right)$$

derived by Marrink and Gruber (1969). The relative elution position $\left(\frac{Ve}{Vo}\right)$ of sheep liver pyruvate carboxylase was 1.9, indicating a molecular weight of approximately 5.0×10^5 daltons. Both of the determinations given above are subject to some uncertainty due to two unknown parameters; the degree of hydration and the molecular assymetry of the protein (Ackers, 1975). The molecular weight was therefore also calculated from the Svedberg equation (Svedberg and Petersen, 1940)

$$M = RTs_{20,w} / D_{20,w} (1 - \nu\rho) \qquad \ldots (2)$$

where R is the gas constant, T the absolute temperature, $s_{20,w}$ the sedimentation coefficient at 20°C in water, ν the partial specific volume of the solute, ρ the density of water, $D_{20,w}$ the diffusion coefficient of the solute in water at 20°C and M the anhydrous molecular weight of the protein. The value for the $s_{20,w}$ used was 15.27 ± 0.77, determined by Easterbrook-Smith (1977) for the sheep liver enzyme using the reacting enzyme sedimentation technique (Cohen et al., 1967). The partial specific volume of the enzyme was assumed to be 0.73 cc/gm. The diffusion coefficient,

$D_{20,w}$, was calculated from the Stokes radius of the enzyme using equation (1). The value obtained by this method was 2.67 ± 0.02 x 10^{-7} cm^2 sec^{-1}, which is close to the value of 3.03 ± 0.17 x 10^{-7} cm^2 sec^{-1} obtained by Bais (1974) for the sheep kidney enzyme using low speed centrifugation as described by Kawahara (1969). The molecular weight obtained from combination of the $s_{20,w}$, ν and $D_{20,w}$ values in equation (2) gave an anhydrous molecular weight for sheep liver pyruvate carboxylase of 4.8 ± 0.24 x 10^5 daltons.

The three determinations of the molecular weight of sheep liver pyruvate carboxylase given above are in good agreement with the value of 4.8 ± 0.4 x 10^5 daltons determined for the sheep kidney enzyme by Bais (1974) using ultracentrifugation.

The subunit molecular weight of sheep liver pyruvate carboxylase was 1.13 ± 0.03 x 10^5 daltons as determined by chromatography in SDS (see section 3.3.1.1., fig. 3.2). Polyacrylamide gel electrophoresis in the presence of SDS gave a value of 1.09 ± 0.01 x 10^5 daltons (fig. 3.16). These values were in good agreement with the value of 1.10 ± 0.05 x 10^5 daltons determined by Bais (1974) for the sheep kidney enzyme, in the presence of SDS, using ultracentrifugation.

On the basis of the evidence presented above

it would appear that sheep liver pyruvate carboxylase is a tetramer composed of subunits of equivalent molecular weight. This finding is in accord with similar studies of the enzyme from a variety of other eukaryotic sources (Utter et al., 1975).

The amino acid composition of sheep liver pyruvate carboxylase is presented in Table 3.1. The number of residues were calculated for a tetramer of molecular weight 4.8×10^5 daltons. The results are given as the averages of seven analyses performed on separate hydrolysates of different enzyme preparations.

The half-cystine content of the enzyme was also determined by spectrophotometric titration of a sample of a DTE-free, fully active enzyme with DTNB in 6M guanidine-HCl at pH 8.0. A value of 12.0 ± 0.6 was obtained for the number of moles of free cystine per mole of biotin. Since there are 4.0 moles of biotin per mole of the tetramer (Duc, unpublished observations) this finding supports the amino acid analysis data that there are approximately 48 moles of free cysteine in the active enzyme, and therefore the enzyme contains no cystine residues.

Included in Table 3.1 is the amino acid composition of sheep kidney pyruvate carboxylase obtained by Bais (1974). It is clear from these

analyses that the kidney and liver enzymes, while showing some similarities in the content of a few amino acids, are different. In particular, the liver enzyme has a much higher content of glycine and alanine. These differences suggest an organ specific difference between the ovine pyruvate carboxylases.

3.4. DISCUSSION

The aim of the work described in this chapter was to develop reliable and reproducible methods for the isolation of homogeneous sheep liver pyruvate carboxylase in reasonable yield. This entailed removal of residual glutamate dehydrogenase, the only significant contaminant remaining in the enzyme preparation following DEAE-Sephadex chromatography. The difficulties encountered in achieving a quantitative resolution reflect the remarkable similarity between these two enzymes. In addition, the association - dissociation effects exhibited by glutamate dehydrogenase undoubtedly enhanced the problems involved in achieving such a resolution.

The two methods described in this chapter which successfully overcome these inherent difficulties provided homogeneous pyruvate carboxylase for two different but related approaches to the understanding of the physico-chemical properties of this large regulatory enzyme. The first procedure, in which the enzymes were completely denatured and the resultant polypeptide chains resolved by chromatography under denaturing conditions enables large quantities of material to be purified in a simple fashion. This technique would be most applicable to heavily contaminated material and would provide the necessary quantities of protein required for the complete

determination of the primary sequence of the enzyme. No attempt has been made in this study to renature this material following removal of the SDS. However, since the polypeptide chain remains intact during the entire isolation procedure renaturation studies should be feasible.

The second approach, using the NAD^+-Agarose affinity chromatography system enables highly active pyruvate carboxylase to be obtained in a homogeneous form. Using the system described in this study a maximum of ca. 10 mg of pyruvate carboxylase can be purified per application, though with larger columns higher loads should be possible.

The physical properties of sheep liver pyruvate carboxylase appear to be similar to properties determined by Bais (1974) for the sheep kidney enzyme. Both enzymes have molecular weights of approximately $4.8 - 5.0 \times 10^5$ daltons, and subunits of approximately $1.0 - 1.5 \times 10^5$ daltons. These values are consistent with the reported molecular weights of pyruvate carboxylases obtained from a variety of eukaryotic sources (Utter et al., 1975). From the differences detected in this study between the diffusion constants and the amino acid compositions however, it would appear that there are organ specific differences between the ovine liver and kidney pyruvate carboxylases. From

studies conducted in this laboratory it is clear however that these differences have not, to date, been reflected in the kinetic properties of the enzymes.

FIGURE 3.1

An outline of the steps involved in the purification of sheep liver pyruvate carboxylase.

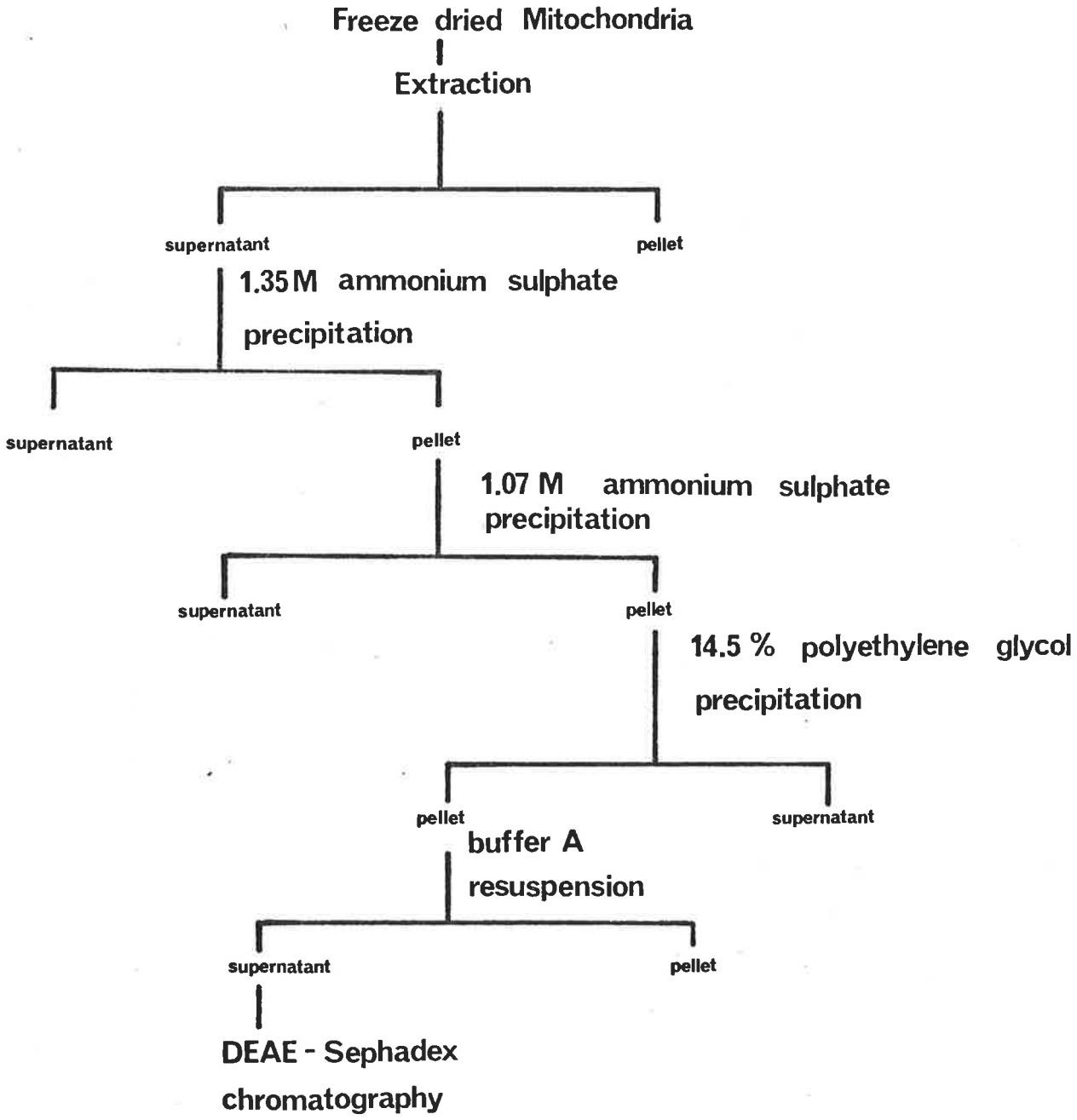

Freeze dried Mitochondria
Extraction
supernatant
pellet
1.35 M ammonium sulphate precipitation
supernatant
pellet
1.07 M ammonium sulphate precipitation
supernatant
pellet
14.5 % polyethylene glycol precipitation
pellet
supernatant
buffer A resuspension
supernatant
pellet
DEAE - Sephadex chromatography

FIGURE 3.2

Calibration curve of Sephadex G-200

Buffer: 0.05M SDS/10^{-4}M p-hydroxymercuribenzoate in 0.1M sodium borate, pH 8.5

Proteins used:

A. sheep liver pyruvate carboxylase
B. bovine serum albumin
C. glutamate dehydrogenase
D. ovalbumin
E. chymotrypsinogen
F. ribonuclase

The molecular weights of the standard proteins were obtained from Weber and Osborne (1975).

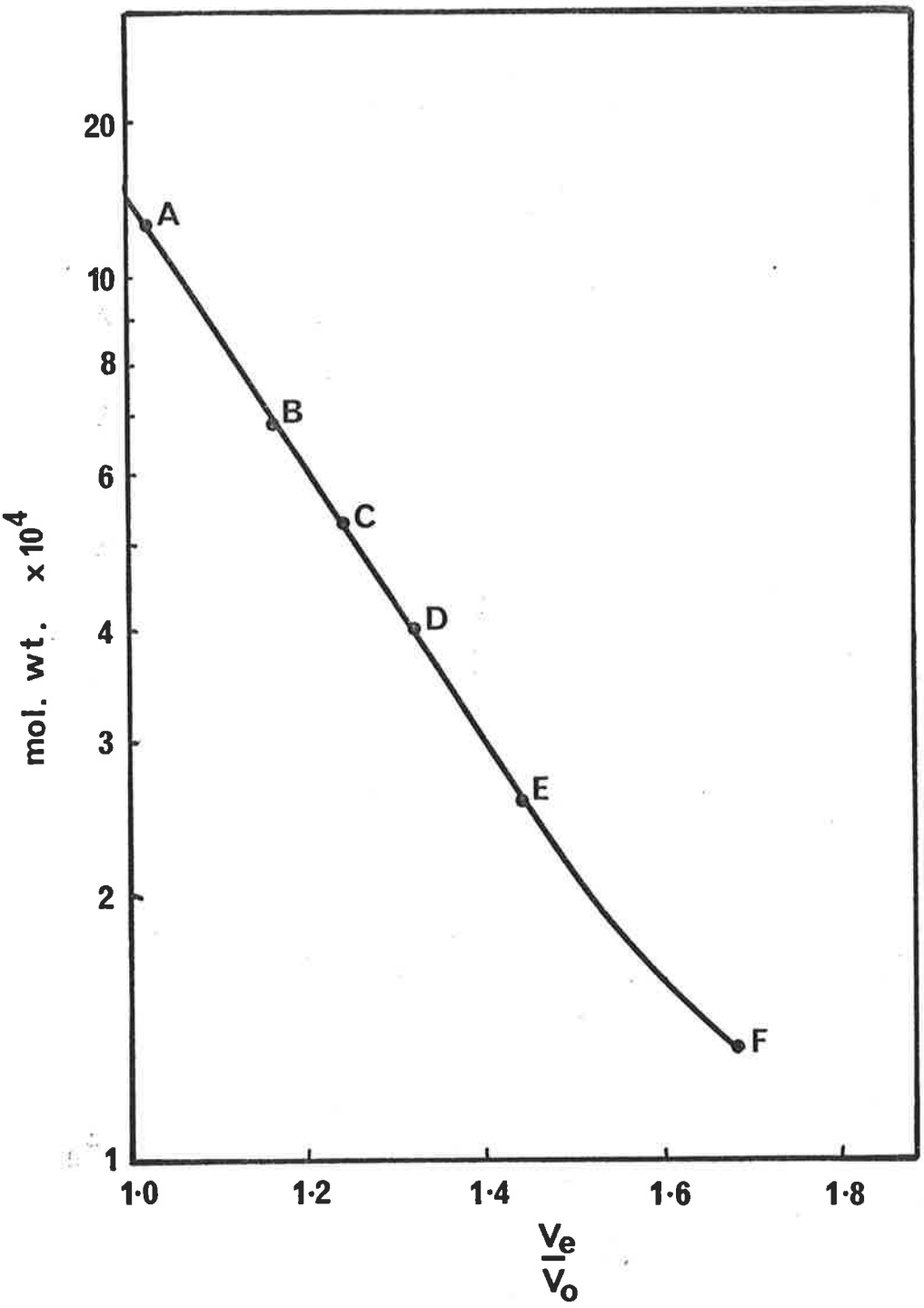

20
10
8
6
4
3
2
1
mol. wt. x10^4
A
B
C
D
E
F
1·0
1·2
1·4
1·6
1·8
Ve/Vo

FIGURE 3.3

Fractionation of pyruvate carboxylase from glutamate dehydrogenase on Sephadex G-200

Column dimensions:	2.5 x 89cm
Buffer:	as for fig 3.2
Flow rate:	0.2ml/min
Fraction size:	3.0ml
Protein applied:	26.7mg
Biotin determination:	50µl aliquots were removed and the biotin content determined by the method of Rylatt *et al.* (1977)

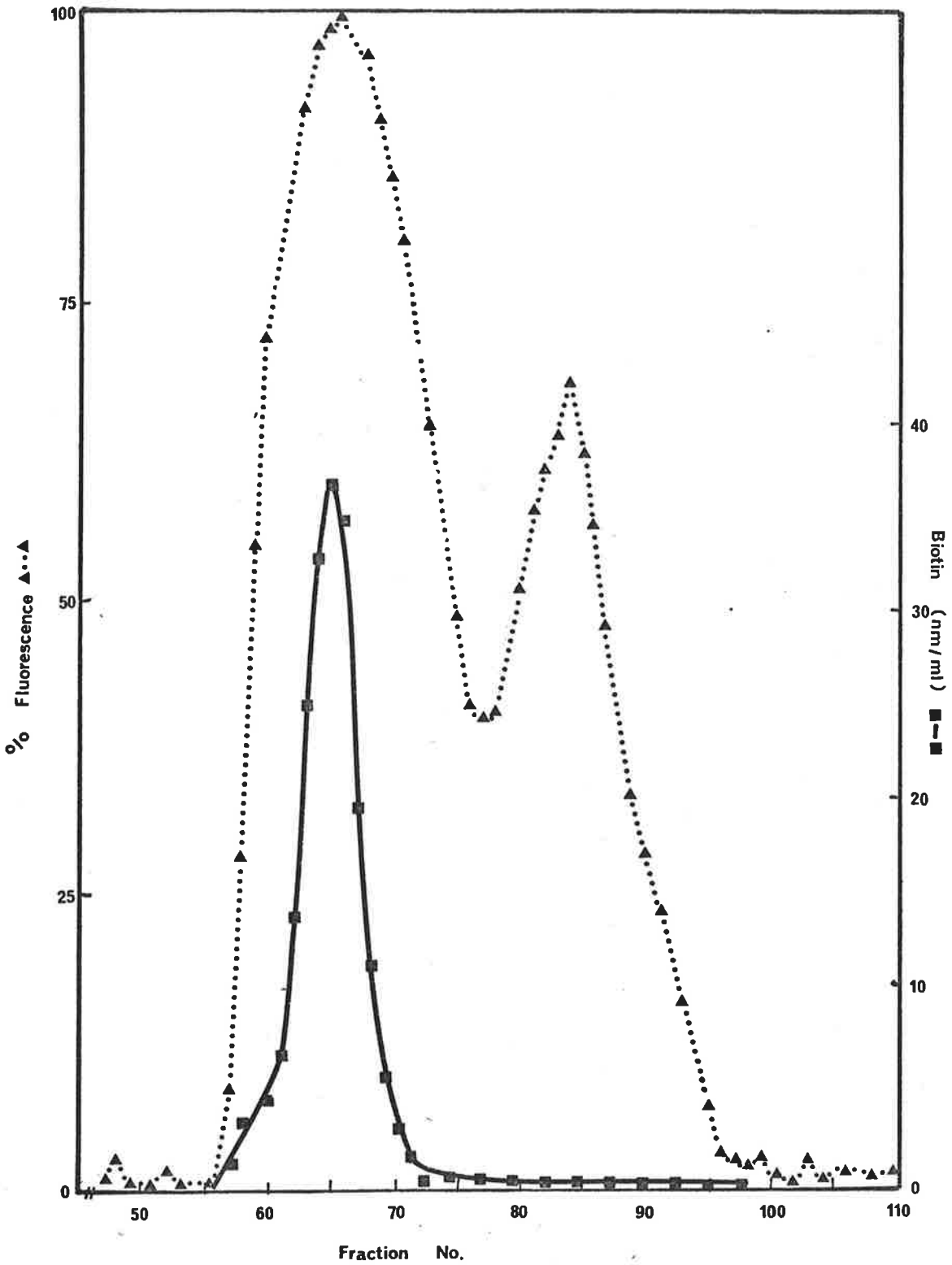

100
75
50
25
0
% Fluorescence ▲···▲
40
30
20
10
0
Biotin (nm/ml) ■—■
50
60
70
80
90
100
110
Fraction No.

PLATE 3.1

SDS polyacrylamide gel electrophoresis of sheep liver pyruvate carboxylase

A: before chromatography on Sephadex G-200 in SDS

B: after chromatography on Sephadex G-200 in SDS

C: pyruvate carboxylase

D: glutamate dehydrogenase

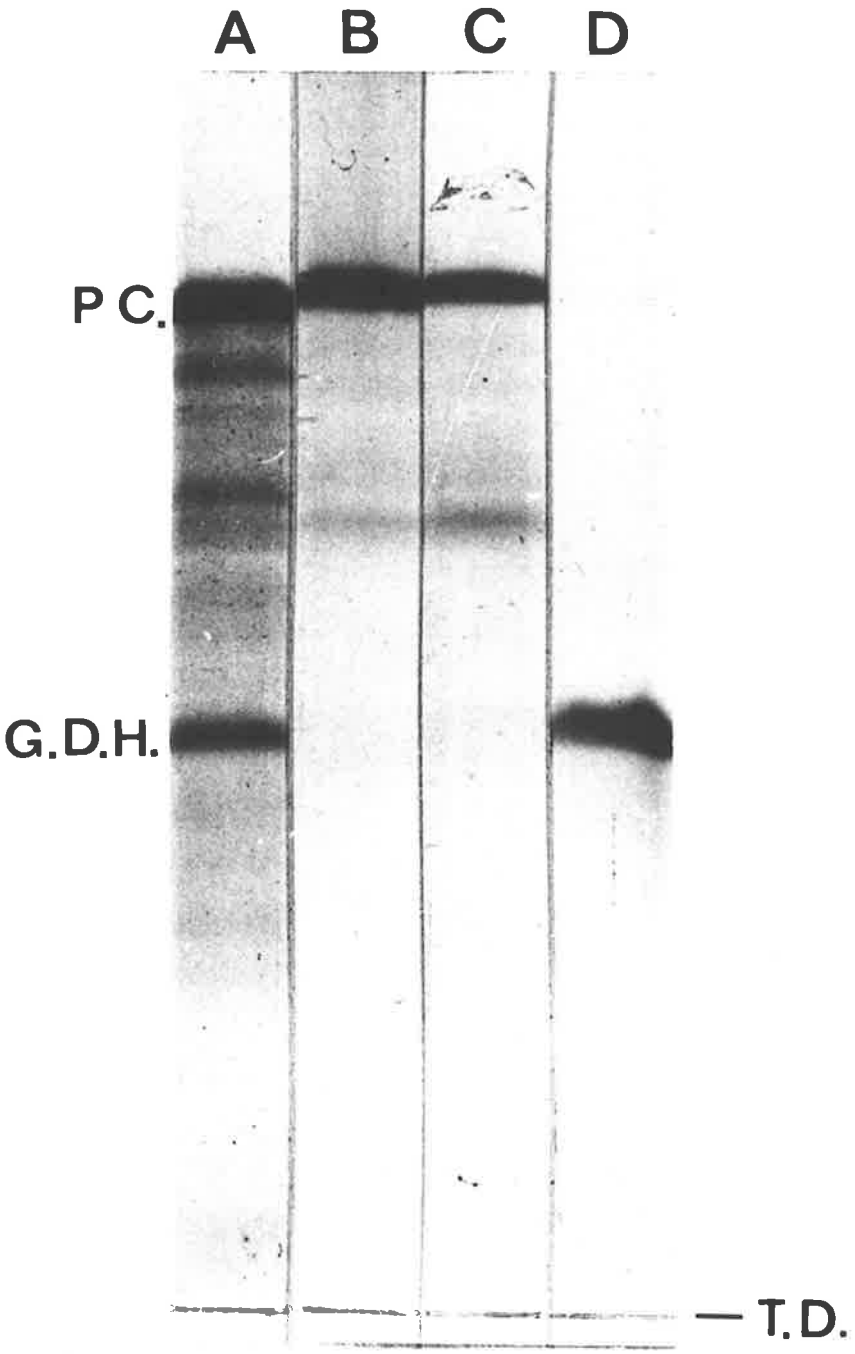

A
B
C
D
P C.
G.D.H.
T.D.

FIGURE 3.4

Chromatography of pyruvate carboxylase on Sepharose 4B

Column dimensions:	2.3 x 55cm
Buffer:	Buffer A
Flow rate:	0.32ml/min
Fraction size:	3.2ml
Enzyme applied:	1447 units; S.A. 7.7

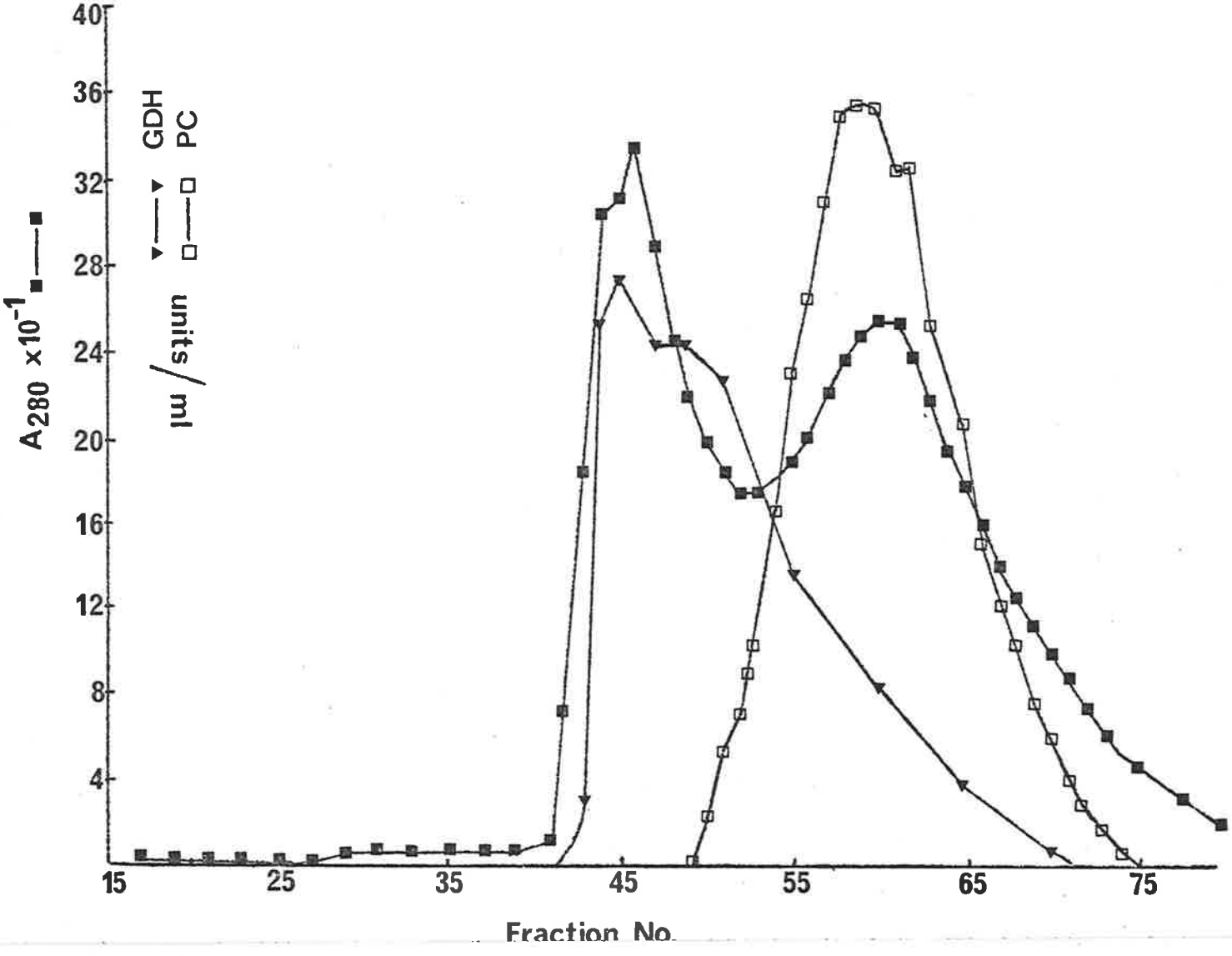

40
36
32
28
24
20
16
12
8
4
$A_{280} \times 10^{-1}$
GDH
PC
units / ml
15
25
35
45
55
65
75
Fraction No.

FIGURE 3.5

Chromatography of pyruvate carboxylase on Phenyl Sepharose

Column dimensions: 1 x 10cm

Buffers: Buffer 3.5.I.

25×10^{-3}M potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, 10% sat'd ammonium sulphate, pH 7.2

Buffer 3.5.II.

25×10^{-3}M potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, 50% $^{w}/_{v}$ ethylene glycol, pH 7.2

Flow rate: 1 ml/min

Fraction size: 1 ml

Enzyme applied: 80 units; S.A. 10.7

Absorbance (280nm): Δ·····Δ

Pyruvate carboxylase activity (units/ml): ●—●

Glutamate dehydrogenase activity (units/ml) O---O

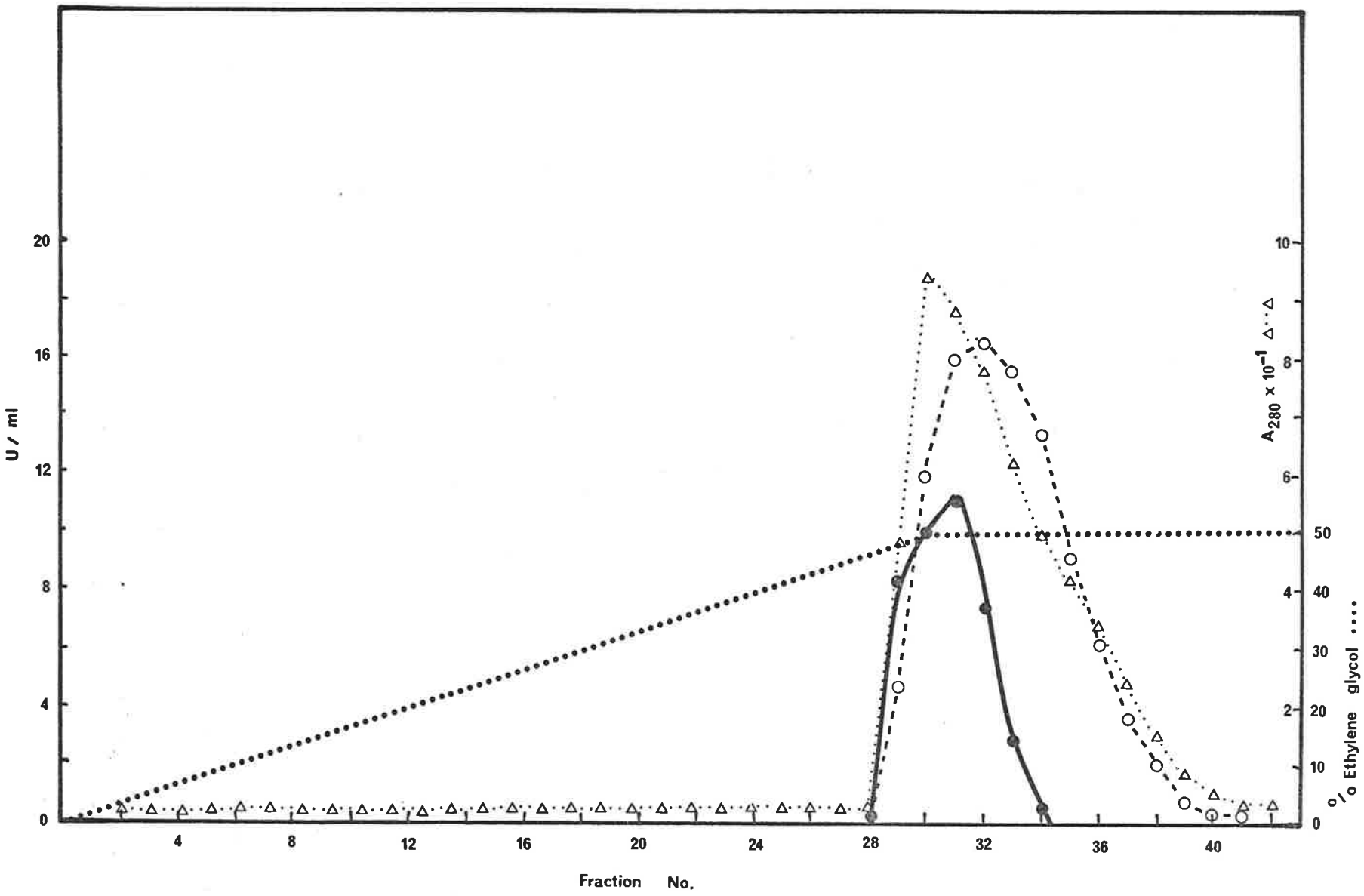

U / ml
20
16
12
8
4
0
4
8
12
16
20
24
28
32
36
40
Fraction No.
$A_{280} \times 10^{-1}$ Δ···Δ
10
8
6
4
2
0
50
40
30
20
10
0
% Ethylene glycol ····

FIGURE 3.6

Chromatography of pyruvate carboxylase on Phenyl Sepharose

Column dimensions: 2 x 30cm

Buffers: Buffer 3.6.I.

0.05M N-ethylmorpholine-Cl, 10^{-4}M EDTA, 10^{-4}M DTE, 10% sat'd ammonium sulphate, pH 7.5

Buffer 3.6.II.

0.05M N-ethylmorpholine-Cl, 10^{-4}M EDTA, 10^{-4}M DTE, 50% $^{w}/_{v}$ ethylene glycol, pH 7.5

Flow rate: 1 ml/min

Fraction size: 2 ml

Enzyme applied: 179 units; S.A. 0.6

Absorbance (280nm): O·····O

Pyruvate carboxylase activity (units/ml): ▲——▲

Glutamate dehydrogenase activity (units/ml): ●---●

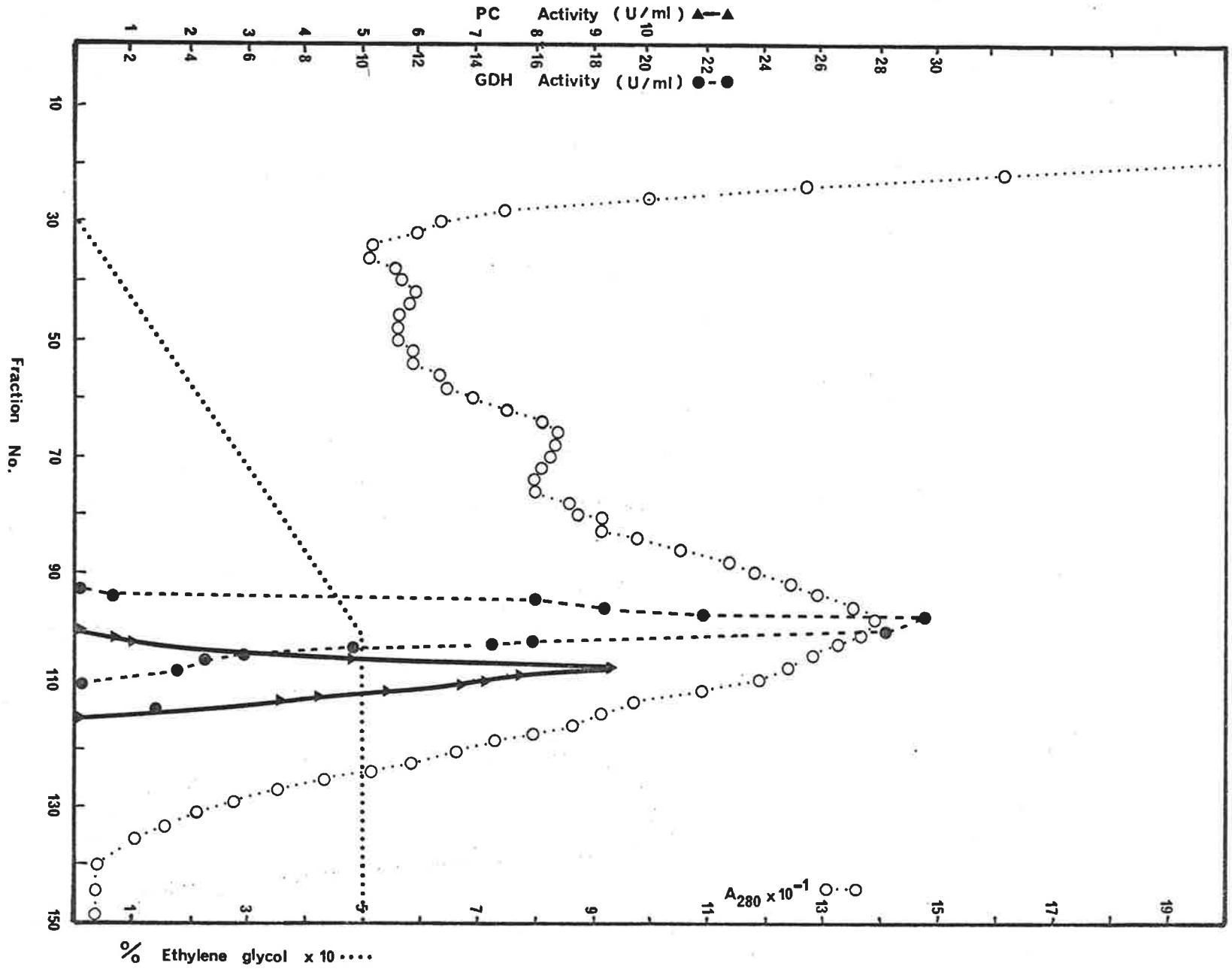

PC Activity (U/ml) ▲—▲
GDH Activity (U/ml) ●-●
Fraction No.
$A_{280} \times 10^{-1}$ o···o
% Ethylene glycol x 10 ····

FIGURE 3.7

Chromatography of glutamate dehydrogenase on Blue Dextran Sepharose

Column dimensions: 0.7 x 1.5cm

Buffer: 20mM potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.2. The arrow indicates the addition of 5mM NAD^{+}.

Flow rate: 0.32ml/min Fraction size: 0.5ml

Enzyme applied: 15 units

FIGURE 3.8

Chromatography of pyruvate carboxylase on Blue Dextran Sepharose

Column dimensions: 0.7 x 1.5cm

Buffers: Buffer 3.8.I.

20mM potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.2

Buffer 3.8.II.

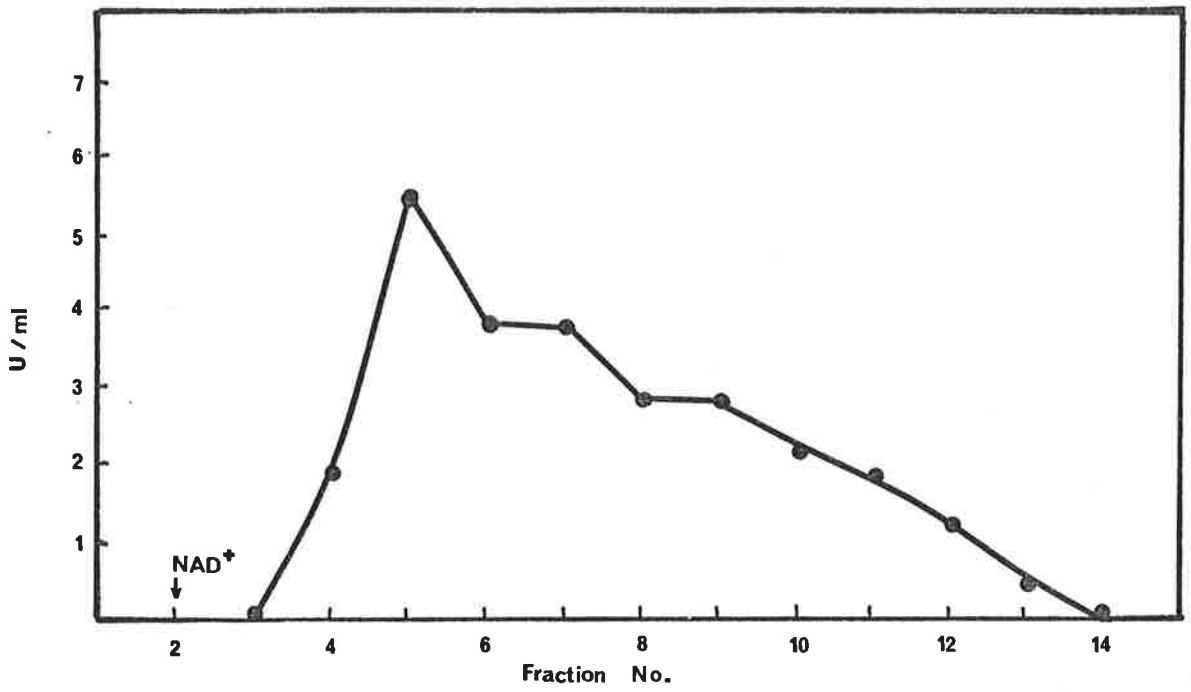
20mM potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.2, 2.5mM ATP, 7mM $MgCl_2$

Flow rate: 0.32ml/min Fraction size: 1.0ml

Enzyme applied: 10 units; S.A. 6.2 units/mg

Pyruvate carboxylase activity (units/ml) ■——■

Glutamate dehydrogenase activity (units/ml) ●·····●


U / ml
7
6
5
4
3
2
1
NAD+
2
4
6
8
10
12
14
Fraction No.

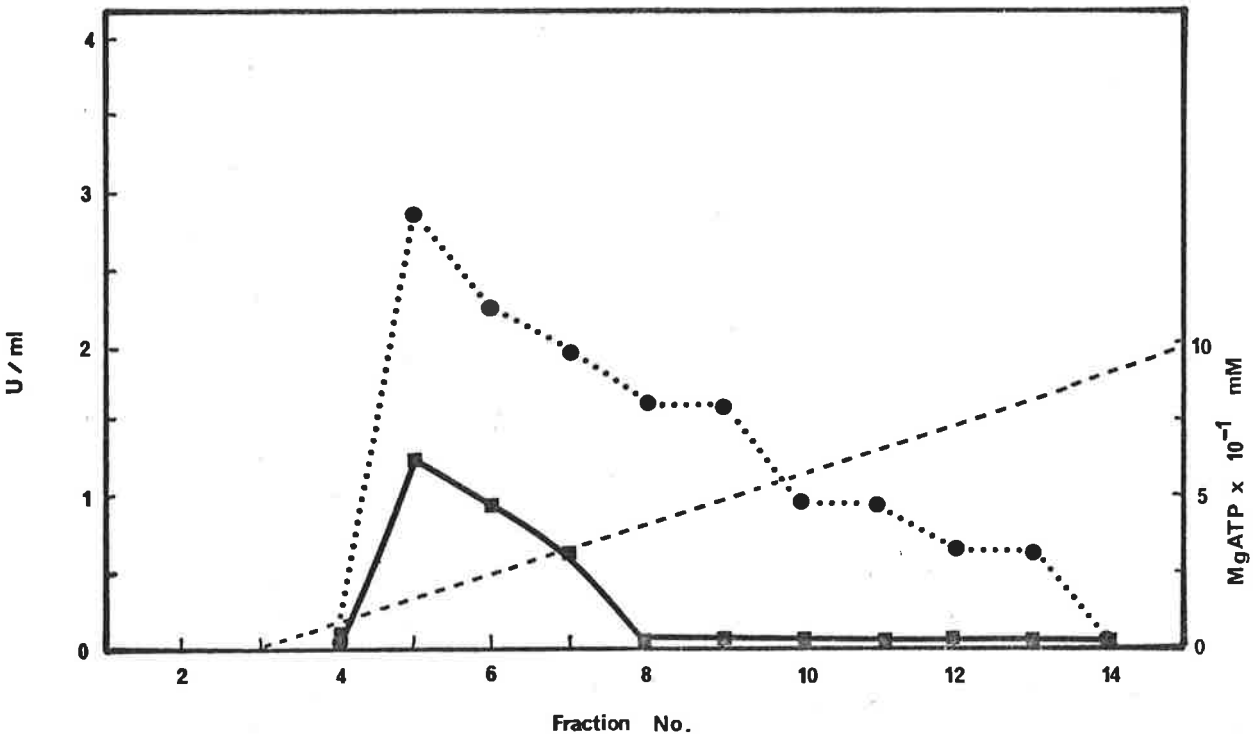

U / ml
4
3
2
1
0
2
4
6
8
10
12
14
Fraction No.
10
5
0
MgATP x 10⁻¹ mM

FIGURE 3.9

Elution of pyruvate carboxylase from Blue Dextran Sepharose with acetyl CoA

Column dimensions: 0.7 x 1.5cm

Buffer: 20mM potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.2.
The arrow indicates addition of 0.25mM acetyl CoA.

Flow rate: 0.32ml/min

Fraction size: 0.5ml

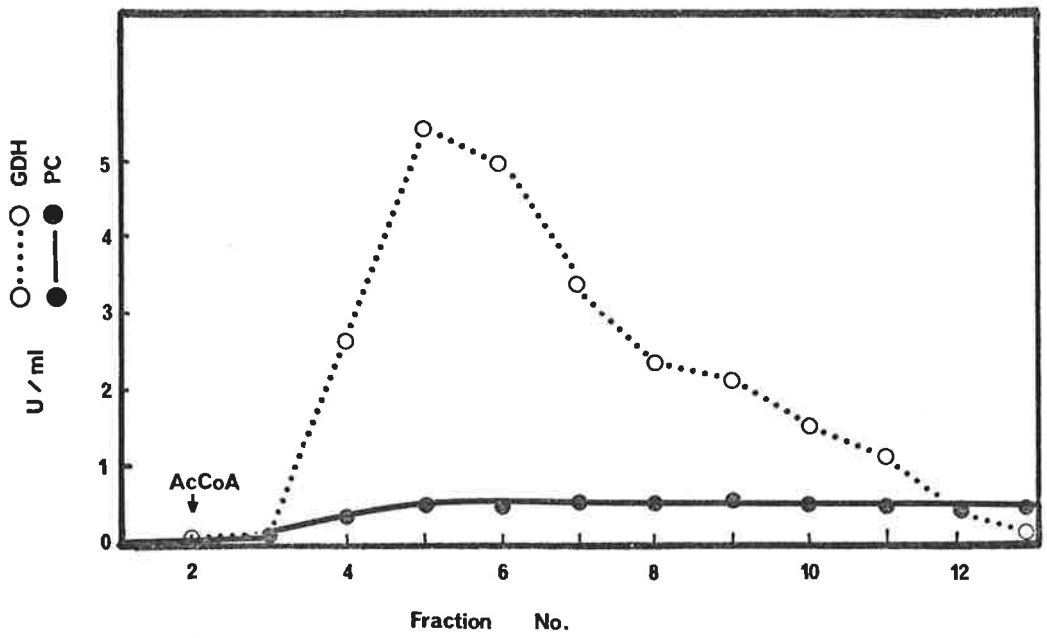
Enzyme applied: 12 units; S.A. 5.8 units/mg

FIGURE 3.10

Inactivation of glutamate dehydrogenase in N-ethyl morpholine-Cl buffer containing 2% glycerol

Buffer 3.10: 20mM N-ethyl morpholine-Cl, 10^{-4}M EDTA, 10^{-4}M DTE, 2% v/v glycerol, pH 7.5.

Glutamate dehydrogenase (30 units) was applied to a Sephadex G-25 column (1 x 10cm) equilibrated in Buffer 3.10 and the enzymic activity of the eluted enzyme was determined at various time intervals.


GDH
PC
U / ml
0
1
2
3
4
5
AcCoA
2
4
6
8
10
12
Fraction No.

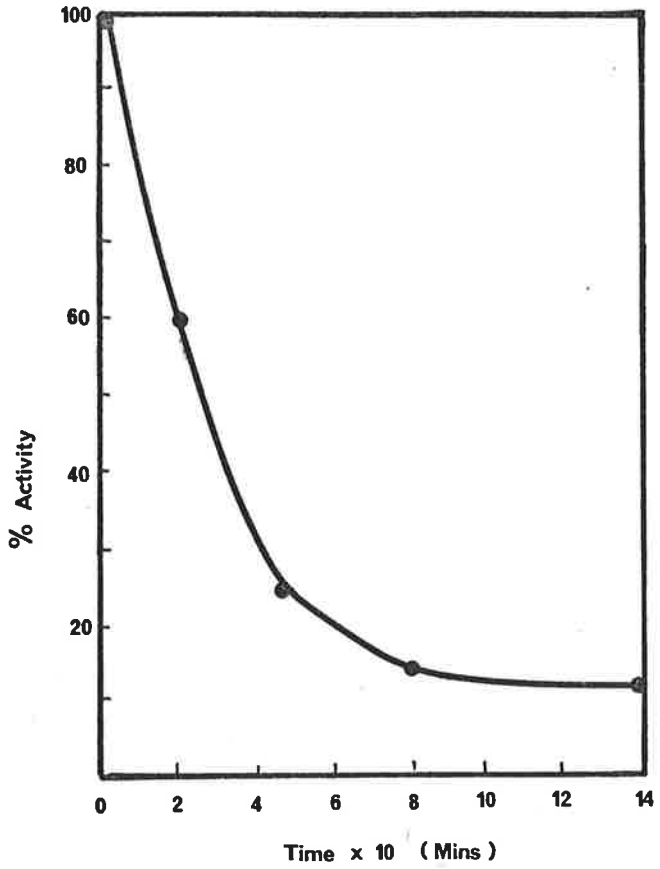

100
80
60
40
20
% Activity
0
2
4
6
8
10
12
14
Time x 10 (Mins)

FIGURE 3.11

Chromatography of pyruvate carboxylase on Blue Dextran Sepharose

Column dimensions:	0.7 x 1.5cm
Buffer:	20mM N-ethylmorpholine-Cl, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.5. The arrow indicates the addition of 1M NaCl.
Flow rate:	0.32ml/min
Fraction size:	1.0ml
Enzyme applied:	10 units; S.A. 8.8 units/mg

FIGURE 3.11, inset.

The inactivation of glutamate dehydrogenase in Buffer 3.11.

The experiment was performed as described in the legend to figure 3.10.

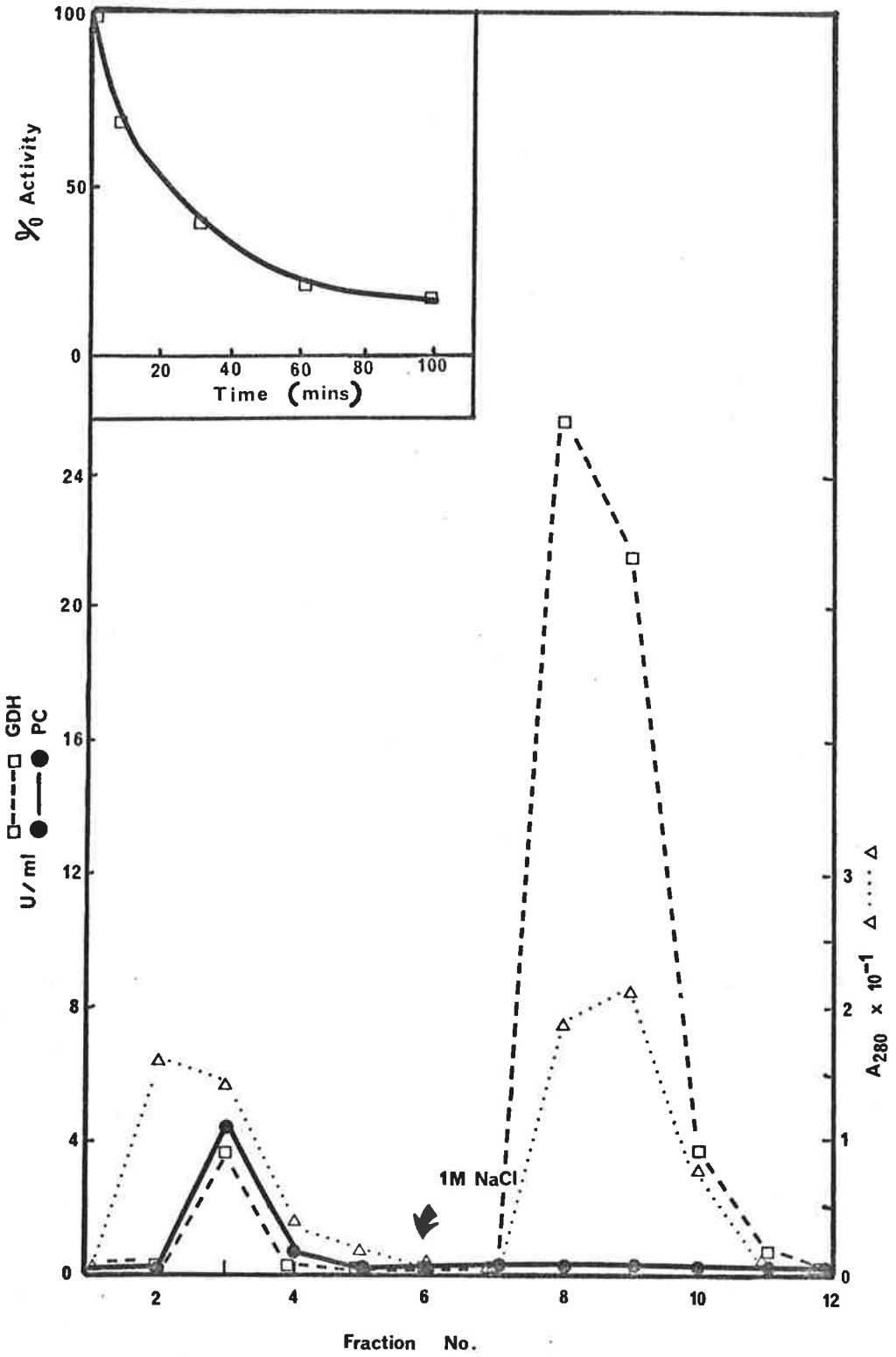

100
50
0
% Activity
20
40
60
80
100
Time (mins)
24
20
16
12
8
4
0
U/ml
GDH
PC
1M NaCl
3
2
1
0
$A_{280} \times 10^{-1}$
2
4
6
8
10
12
Fraction No.

FIGURE 3.12

Chromatography of pyruvate carboxylase on NAD^+-Agarose

Column dimensions:	1 x 5cm
Buffers:	Buffer 3.12.I.
	50mM tris-Cl, 10^{-4}M EDTA,
	10^{-4}M DTE, pH 8.4
	Buffer 3.12.II.
	500mM tris-Cl, 10^{-4}M EDTA,
	10^{-4}M DTE, pH 8.4
Flow rate:	1.0ml/min
Fraction size:	1.0ml
Enzyme applied:	220 units; S.A. 15.0 units/ml

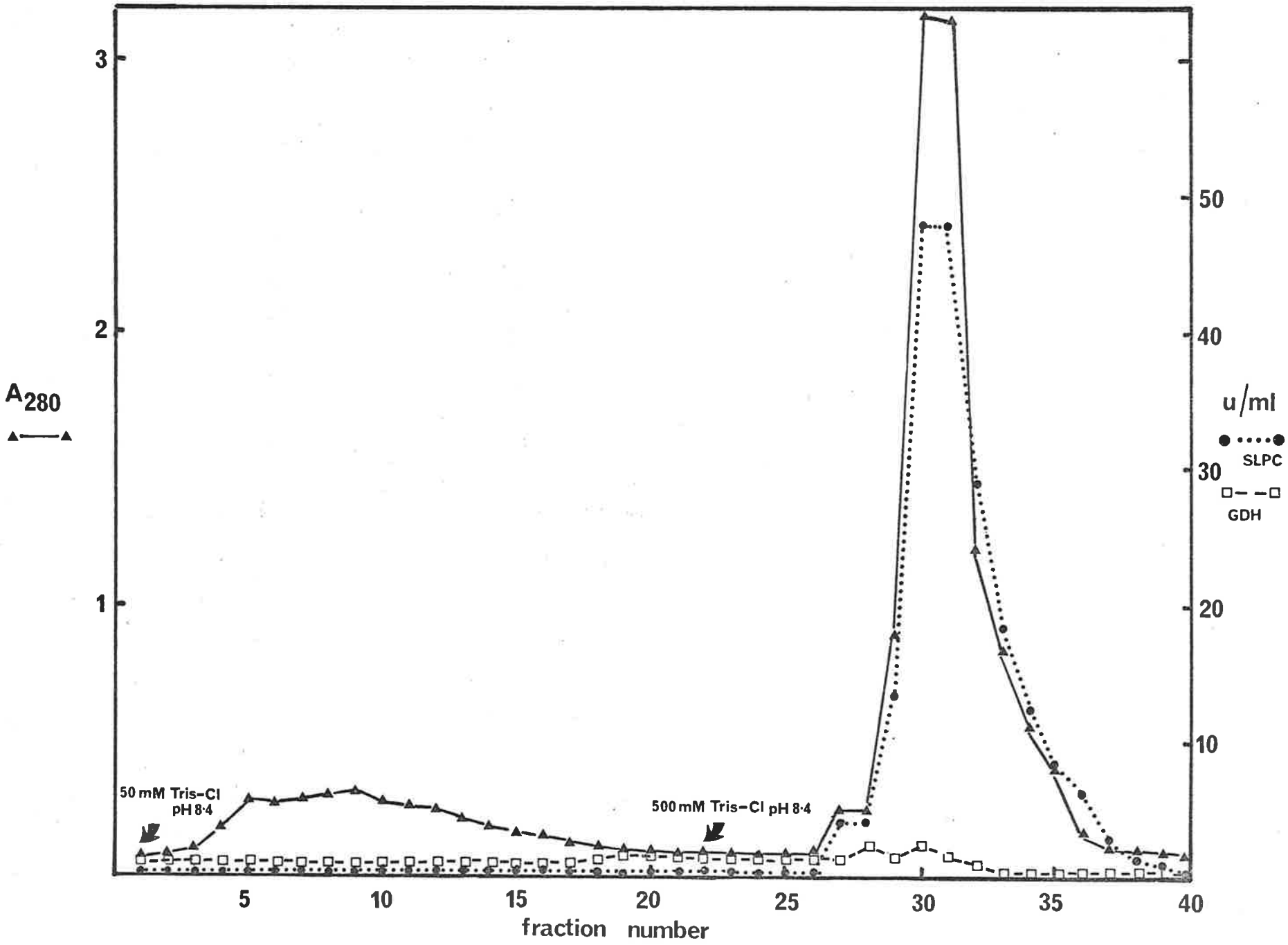

A280
u/ml
SLPC
GDH
50 mM Tris-Cl pH 8·4
500 mM Tris-Cl pH 8·4
fraction number
1
2
3
10
20
30
40
50
5
10
15
20
25
30
35
40

PLATE 3.2

SDS polyacrylamide gel electrophoresis of sheep liver pyruvate carboxylase before (A) and after (B) chromatography on NAD^+-Agarose.


A
B

FIGURE 3.13

Chromatography of pyruvate carboxylase on NAD^+-Agarose

Column dimensions:	1 x 5cm
Buffers:	50mM potassium phosphate, 10^{-4}M EDTA, 10^{-4}M DTE, pH 7.2
	The arrows indicate the addition of: $MgATP^{2-}$ (7mM Mg^{2+} : 2.5mM ATP^{4-}); 100mM potassium phosphate; 500mM potassium phosphate; 500mM Tris-Cl, pH 8.4.
Flow rate:	1.0ml/min
Fraction size:	1.0ml
Enzyme:	75 units: S.A. 10.7

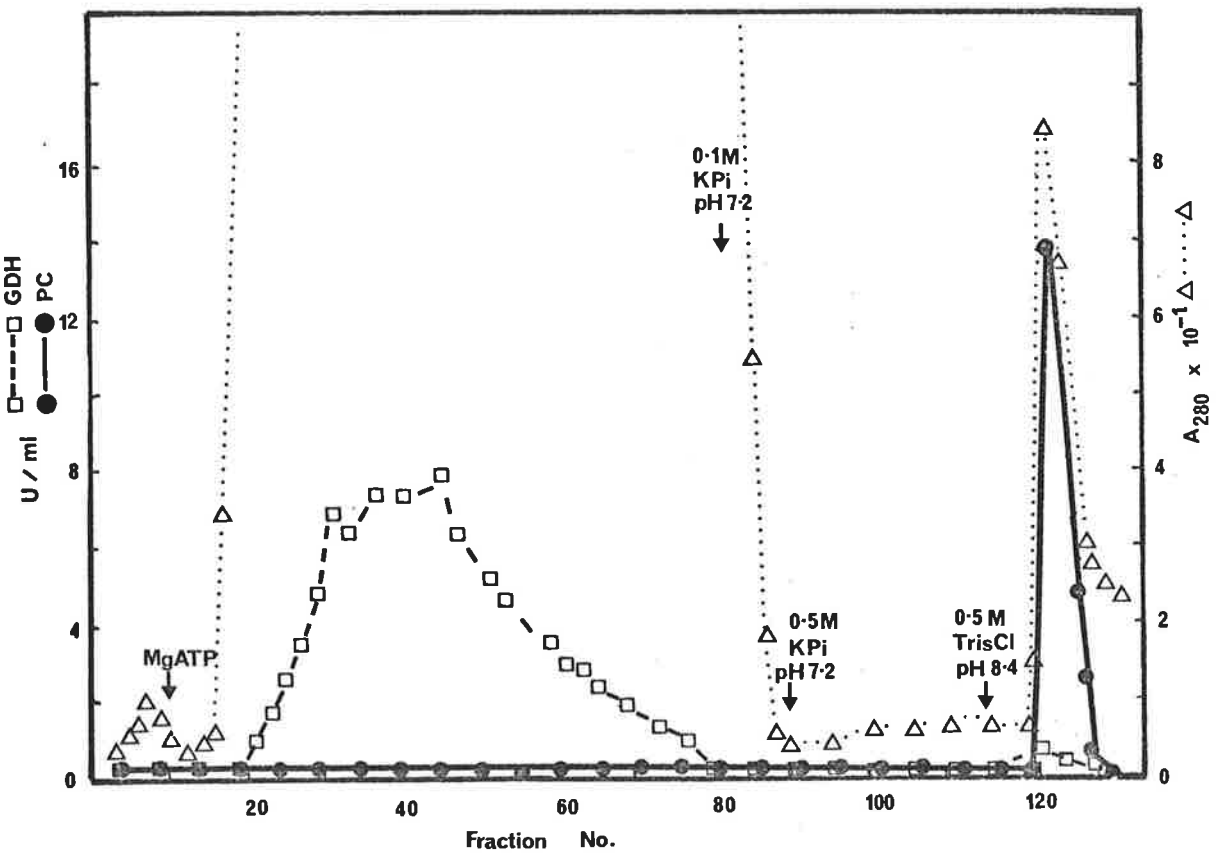

GDH
PC
U / ml
16
12
8
4
0
MgATP
0·1M
KPi
pH 7·2
0·5M
KPi
pH 7·2
0·5 M
TrisCl
pH 8·4
8
6
4
2
0
$A_{280} \times 10^{-1}$
20
40
60
80
100
120
Fraction No.

FIGURE 3.14

The determination of the Stokes radius of sheep liver pyruvate carboxylase.

Column dimensions: 1.6 x 65cm

Buffer: Buffer A

Flow rate: 0.16ml/min

The proteins used were:

(a) Cytochrome C, 16.4Å (Laurent and Kellander, 1964)

(b) Trypsinogen, 20.5Å (Tietze, 1953)

(c) Ovalbumin, 27.3Å (Laurent and Kellander, 1964)

(d) Bovine serum albumin, 35Å (Siegel and Monty, 1966)

(e) Catalase, 52Å (Siegel and Monty, 1966)

(f) Sheep liver pyruvate carboxylase

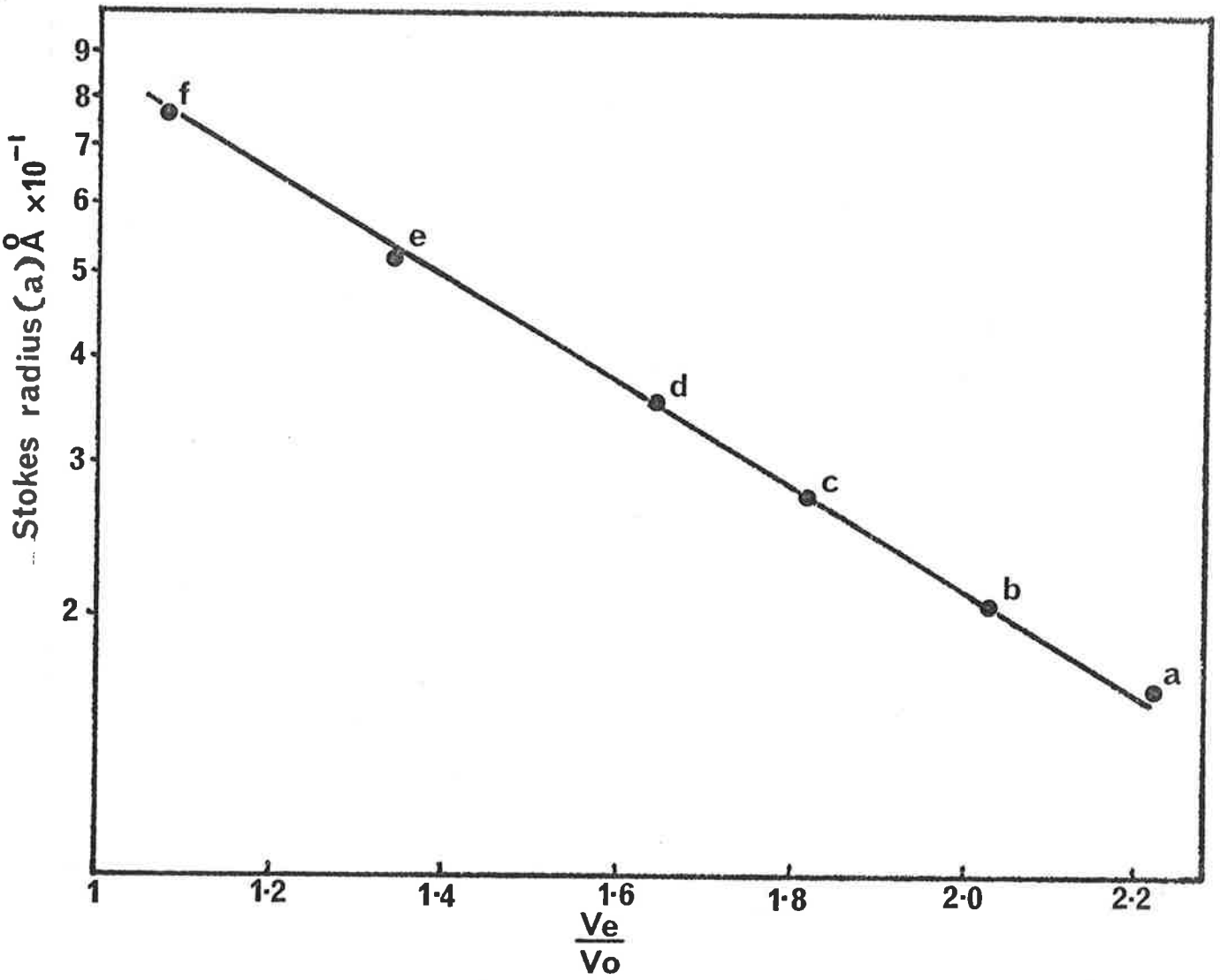

Stokes radius (a) Å ×10⁻¹
9
8
7
6
5
4
3
2
f
e
d
c
b
a
1
1·2
1·4
1·6
1·8
2·0
2·2
Ve
Vo

FIGURE 3.15

The determination of the molecular weight of sheep liver pyruvate carboxylase.

Column dimensions: 1.6 x 65cm

Buffer: Buffer A

Flow rate: 0.16ml/min

The proteins used were:

(a) Cytochrome C (12,400 daltons) (1)

(b) Trypsinogen (23,500 daltons) (2)

(c) Ovalbumin (43,000 daltons) (1)

(d) Bovine serum albumin (68,000 daltons) (1)

(e) Catalase (232,000 daltons) (1)

(f) Sheep liver pyruvate carboxylase

References:

(1) Andrew, 1965

(2) Tietze, 1953

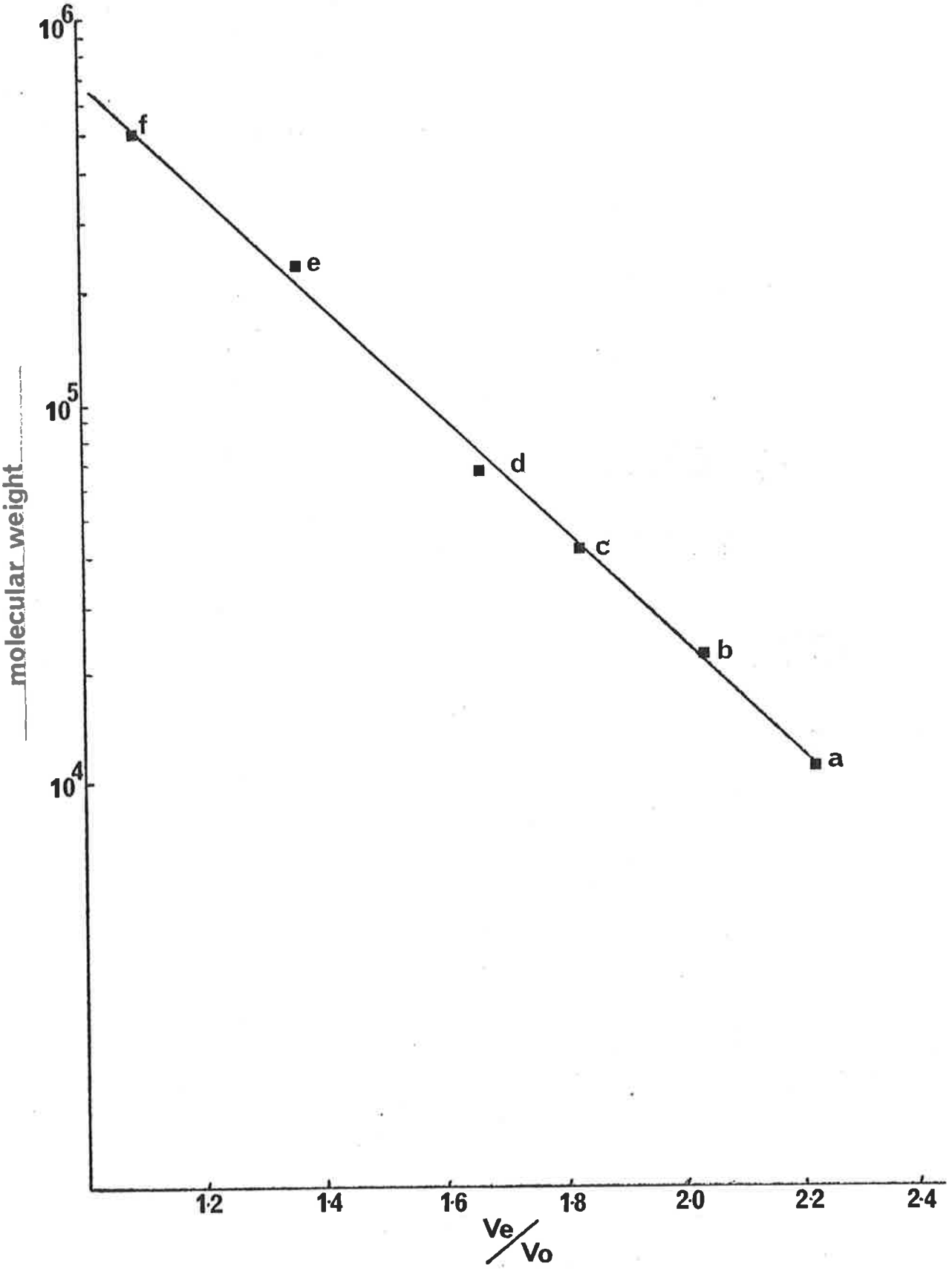

molecular weight
10^6
10^5
10^4
f
e
d
c
b
a
1·2
1·4
1·6
1·8
2·0
2·2
2·4
Ve/Vo

FIGURE 3.16

The determination of the molecular weight of the subunits of sheep liver pyruvate carboxylase

Polyacrylamide gel electrophoresis in the presence of SDS, as described by Fairbanks et al. (1971) was employed.

The proteins used were:

A RNA polymerase (subunit 2)

B RNA polymerase (subunit 1)

C γ-globulin

D Bovine serum albumin (dimer)

E Sheep liver pyruvate carboxylase

F Bovine serum albumin (monomer)

G Catalase

H Glutamate dehydrogenase

I Ovalbumin

J Glyceraldehyde-3-phosphate dehydrogenase

K Chymotrypsinogen

The molecular weights of the standard proteins were obtained from Weber and Osborne (1975).

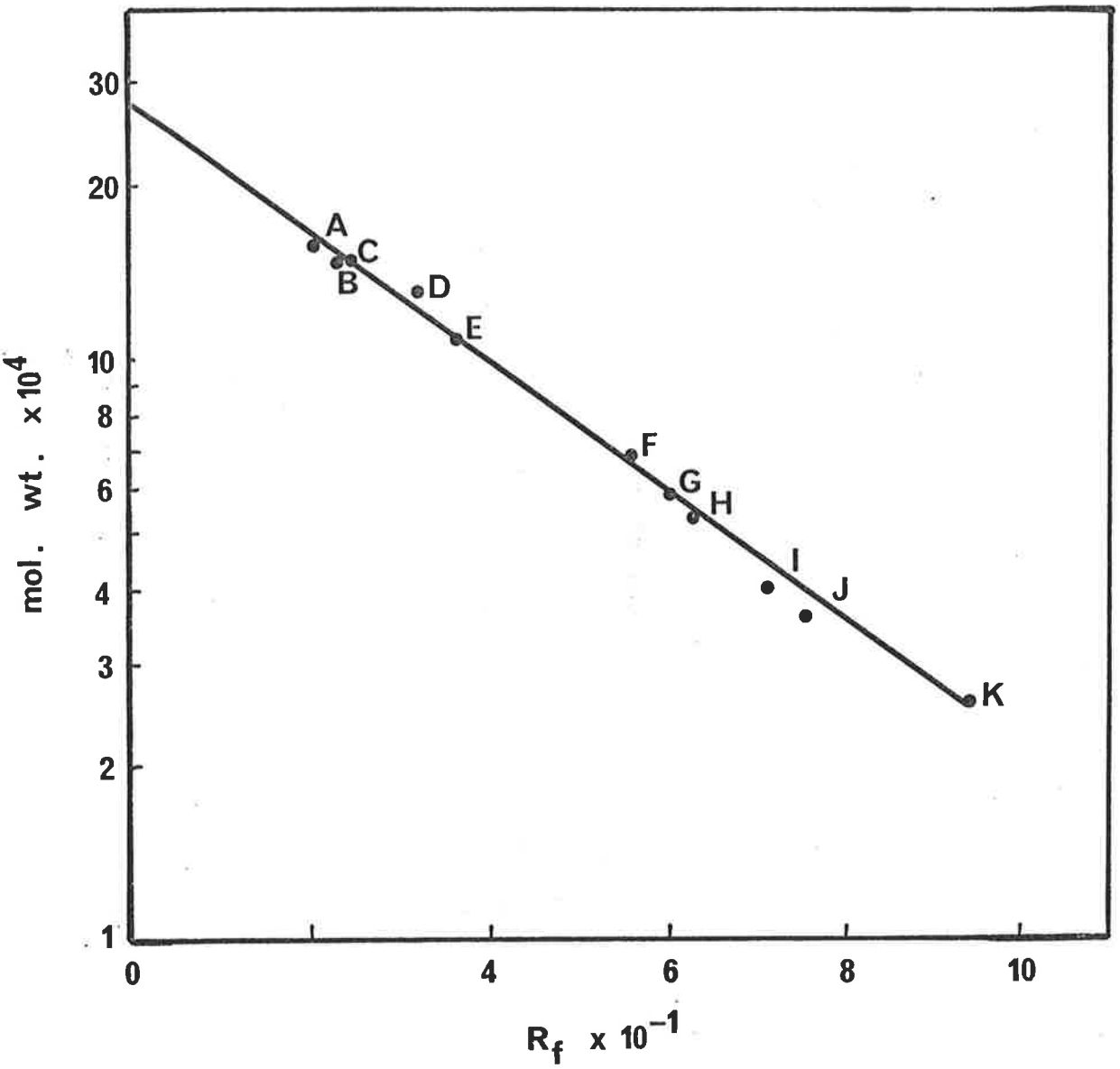

30
20
10
8
6
4
3
2
1
mol. wt. x 10^4
A
B
C
D
E
F
G
H
I
J
K
0
4
6
8
10
R_f x 10^-1

TABLE 3.1

The amino acid analysis of sheep liver pyruvate carboxylase.

Amino Acid	Residues per mole[a] SLPC[b]	SKPC[c]
Aspartic Acid	363 ± 11	332 ± 6
Threonine[d]	256 ± 4	208 ± 11
Serine[d]	288 ± 25	248 ± 9
Glutamic Acid	410 ± 10	468 ± 18
Proline	288 ± 28	222 ± 9
Glycine	744 ± 45	390 ± 21
Alanine	655 ± 36	347 ± 7
Cysteine[e]	49 ± 2	44 ± 4
Valine	356 ± 33	290 ± 23
Methionine	82 ± 16	80 ± 10
Isoleucine	193 ± 23	208 ± 10
Leucine	381 ± 30	352 ± 9
Tyrosine	84 ± 6	106 ± 7
Phenylalanine	142 ± 4	158 ± 8
Lysine	194 ± 20	194 ± 8
Histidine	111 ± 7	113 ± 8
Arginine	177 ± 5	198 ± 3
Tryptophan[f]	29	62

a molecular weight 480,000
b sheep liver pyruvate carboxylase
c sheep kidney pyruvate carboxylase (Bais, 1974)
d corrected for destruction during hydrolysis
e determined as S-carboxymethyl cysteine
f determined spectrophotometrically by the method of Goodwin and Morton (1946)

CHAPTER 4

ELECTRON MICROSCOPY OF PYRUVATE CARBOXYLASE

4.1 INTRODUCTION

The application of electron microscopy in the examination of globular proteins has proved, in many instances, to be particularly rewarding. The technique has most commonly been used to establish the subunit numerology of oligomeric proteins (Haschemeyer, 1970). From this information predictions on the symmetry of the molecule can be made (Valentine, 1968). In some favourable cases it has also been possible to gain information on the shape (e.g. glutamine synthetase, see Haschemeyer, 1970) and the catalytic centres of proteins (Green et al., 1972). The recent work of Boublik et al. (1976) on the 50S subunit of the E. coli ribosome is a notable example of the information that can be gained from the electron microscopy of complex proteins.

Chicken liver pyruvate carboxylase was first examined in the electron microscope by Valentine et al. (1966). The enzyme appeared as a tetramer with the subunits arranged at the corners of a square. The rotational symmetry of the images was either C_4 or D_2, but no distinction could be made between these two point groups. The structures were found to be dissociated by incubating at $4^{o}C$, in agreement with ultracentrifugal studies which indicated that the native enzyme dissociated into inactive monomers

under these conditions (Scrutton and Utter, 1965a).

Similar structures have subsequently been found in turkey and calf liver preparations of the enzyme (Utter et al., 1975). The enzyme isolated from yeast is different however, having subunits arranged at the corners of a rhombus (Valentine, 1968).

An examination of the images produced by highly purified sheep liver pyruvate carboxylase in the electron microscope seemed warranted for two reasons. Firstly, the electron micrographs presented to date would suggest that the basic morphology of the avian and mammalian enzymes has been conserved. Examination of another mammalian source would enable evaluation of this hypothesis. Secondly, it appeared that direct visualization of the catalytic sites on the enzyme may be possible by making use of the specific interaction between avidin and biotin. The reaction of pyruvate carboxylase with avidin would result in avidin binding to the biotin in the active site of the enzyme. Examination of this complex in the electron microscope should therefore reveal the position of the active sites. This approach had previously met with success when applied to trans-carboxylase (Green et al., 1972).

4.2 METHODS

4.2.1. Preparation of negatively stained specimens of pyruvate carboxylase.

Samples of the enzyme were diluted to approximately 0.2 mg/ml in Buffer A. One drop of this solution was laid on a teflon dish and, by touching a carbon coated grid onto the surface of the drop, a bead of the solution was picked up on the grid. Filter paper was touched to the edge of the grid to remove any excess sample without completely drying the grid. About 20 drops of 2% Uranyl Acetate was then applied with a Pasteur pipette without wetting the under surface of the grid. Excess stain was removed with filter paper, and the grid was allowed to dry for 5 min prior to insertion into the microscope.

4.2.2. Electron microscopy

The samples were examined in a Siemens 102 electron microscope, operated at 60 K volts using a 50 μ aperture and photographed on Ilford Electron Microscope film.

4.3 RESULTS

4.3.1. Sheep Liver Pyruvate Carboxylase

Examination of homogeneous preparations of sheep liver pyruvate carboxylase in the electron microscope supported the physical data presented in Chapter 3 that this enzyme was a tetramer (plate 4.1). These micrographs also showed what initially appeared to be a large number of broken molecules. When examined in more detail however it became apparent that many of these "broken" molecules were in fact different views of the tetramer. A model of the enzyme was constructed which accommodated the images observed in the electron microscope. Five different views of individual tetramers are shown in plate 4.2. Below each image is a photograph of the model corresponding to that image. In particular it should be noted that this model explains the trimer-like appearance of the images d and e in plate 4.2. The arrangement of the subunits within the tetramer appears in the form of a rhombus, with one pair of subunits displaced slightly above the plane of the second pair. The appearance of "arms" protruding from the subunits was suggested from the micrographs and more will be mentioned of this unusual feature in latter sections of this chapter.

The approximate dimensions of the molecule

observed in the electron micrographs were 167 ± 4Å (S.E.M., 9 observations) across the longer axis, 143 ± 4Å (S.E.M.) across the shorter axis and 69 ± 2Å (S.E.M.) between the centres of adjacent subunits. Although these values reflect the rhomic appearance of the tetramer they should be considered as only approximate due to the compact nature of the molecule.

By using the equation

$$M = 4/3\pi a^3 N\rho$$

where a is the radius of the molecule, N, Avagadro's number, ρ, the density of the molecule (assumed to 1.36 g/cc) and M, the molecular weight of the molecule, the diameter of a subunit of sheep liver pyruvate carboxylase (molecular weight 1.1×10^5 daltons, see section 3.3.3.) may be calculated to be approximately 64Å. This calculation agrees reasonably well with the size of the observed images, particularly considering that the subunits are unlikely to be perfect spheres. The relationship of the images to the native enzyme was also determined by comparing the effective radius of the observed images to the Stoke's radius of the enzyme in solution. The former value was taken to be approximately 77Å, since this would be the average radius of a particle spinning about the longer and shorter axes of the rhombus. The Stoke's radius was already known to be approximately 75Å from molecular sieve chromatography

(section 3.3.3.) The agreement between these two values is quite good and suggests that the structure observed in the electron microscope is, as expected from the purity of the preparation, sheep liver pyruvate carboxylase.

The most remarkable feature of this study was that the electron micrographs of sheep liver pyruvate carboxylase bore no resemblance to the regular square planar tetramer observed by Valentine et al. (1966) in chicken liver pyruvate carboxylase preparations and later reported by Utter et al. (1975) to be present in the enzyme preparations of both turkey and calf liver. The sheep liver enzyme appeared instead to be similar to the yeast enzyme (Valentine, 1968) where the subunits were also arranged at the corners of a rhombus.

4.3.2. Chicken Liver Pyruvate Carboxylase

The marked contrast between the images presented here for the ovine source of the enzyme and those described by Valentine et al. for the avian enzyme necessitated a reinvestigation of chicken liver pyruvate carboxylase in the electron microscope.

The enzyme used in this study had a specific activity of 29.6 and was homogeneous as determined by electrophoresis on SDS-polyacrylamide gels. (plate 4.3a).

A field view of this material is shown in plate 4.3. No square planar tetramers were seen. Instead the images were very similar to the sheep liver enzyme. The inset (fig. 4.3.b) shows an enlargement of a single molecule and clearly indicated a rhomboid arrangement of the subunits.

Examination of these micrographs enabled a model of the enzyme to be built and a montagé of these images, together with the corresponding view of the model are shown in plate 4.4. The appearance of "arms" protruding from the subunits was noted earlier with the sheep liver enzyme, and these structures were again apparent in the images of the chicken liver enzyme. The arms are most striking in the image Cb. The model (Db) suggests that in this case, an edge-on-view of the molecule is being observed. Furthermore, the photograph of the model Da clearly accounts for the triangular hole observed in the corresponding image Ca. Measurement of these molecules revealed that the longer axis of the rhomboid was 167.7 ± 3.7Å(S.E.M., 51 observations), while the shorter axis was 141.5 ± 1.87Å (S.E.M.). The distance between the centres of adjacent subunits was 69.9 ± 0.81Å (S.E.M.). These values were identical, within the errors of the determination, to the values obtained for the sheep liver enzyme, and were consistent with the size of the chicken liver pyruvate carboxylase molecule.

These structures were however quite obviously different from those described by Valentine et al. as chicken liver pyruvate carboxylase. Since the material used in this present study was established to be homogeneous by SDS polyacrylamide gel electrophoresis while no criteria of purity were given in the earlier study, it became apparent that the square planar tetramers observed by Valentine et al. may have been an impurity in the enzyme preparation used by these authors. This suggested that some consideration be given to the differences between the methods of preparation of the samples of chicken liver enzyme examined.

The enzyme examined by Valentine et al. in which the square planar tetramers were observed was prepared by the method of Scrutton and Utter (1965a). This procedure involved a series of ammonium sulphate "back extractions" with pyruvate carboxylase being solubilized at a concentration of 0.5M ammonium sulphate, pH 6.7. The pyruvate carboxylase examined in this laboratory, in which square planar tetramers were not observed was prepared by the method described in section 3.2.3. The major difference between these two preparations was the use of ion exchange column chromatography. Since this technique functions on different principles to ammonium sulphate precipitation it seemed possible that the ion exchange chromatography

had removed the square planar tetramers from the pyruvate carboxylase examined in this study.

To test this possibility chicken liver pyruvate carboxylase was prepared by the standard procedure described in Chapter 3 and the fractions eluted from the DEAE-Sephadex column (fig. 4.1) were examined in the electron microscope. It was clear from these electron micrographs that square planar tetramers were present in the eluate of this column, being highly aggregated in the early fractions (plate 4.5) and becoming less aggregated and less numerous in latter fractions (plate 4.6). To determine whether these square planar tetramers were an inactive form of pyruvate carboxylase the fractions eluted from the column were assayed for biotin. This cofactor appeared only in those fractions associated with pyruvate carboxylase activity, (fig. 4.1) and so eliminated this possibility.

Measurement of the distance between the centres of adjacent subunits of this molecule gave a value of 64.7 ± 0.86Å (S.E.M., 63 observations). This was smaller than the centre-to-centre distance determined by Valentine et al. for these molecules of 70 - 75Å. However, when the molecules in the plates presented by Valentine et al. were re-measured it was found that, apart from the large super-imposed structure

shown in plate III of these authors' publication, all other general field views gave centre-to-centre distances of 60 - 70 Å. These dimensions were calculated from the magnifications given in each of the plates. Thus it would appear that the square planar tetramers found to be resolved from pyruvate carboxylase activity in this present study and those presented by Valentine et al. as pyruvate carboxylase were the same size.

If the morphologically idential structures found in this present study were the same as those observed by Valentine et al., they should dissociate at 4°C and reassociate upon rewarming to room temperature, since this property was a characteristic of the square planar tetramers previously described. Plate 4.7 shows that this was the case. The square planar tetramers used were obtained from fraction 45 of the DEAE-sephadex frachonation shown previously (fig. 4.1). Incubation at 4°C for 20 mins. resulted in loss of both the aggregated and tetrameric structure of these molecules (plate 4.7.b). Rewarming this material to room temperature caused a reassociation to both the tetrameric and aggregated structure (plate 4.7.c). It therefore became apparent that the square planar tetramers observed by Valentine et al. were an impurity in the preparation of chicken liver pyruvate carboxylase used in the study.

Closer examination of the fractions containing mainly individual square planar tetramers in an unaggregated form revealed what appeared to be a side view of the square planar tetramer (plate 4.8). These images showed no defined subunit structure and appeared as basically two parallel lines. Measurements indicated that they had approximately the same dimensions as the square planar tetramers and so suggested that the square planar "tetramers" may have been octamers composed of subunits arranged at the corners of a cube. If these structures were tetramers, then from the average of the longer and shorter axis of rotation, an effective radius of approximately 74Å could be calculated for these structures. Similar calculations for an octamer gave a value of approximately 92Å. Since the effective radius of pyruvate carboxylase had previously been calculated to be approximately 75Å by the same method then, by using pyruvate carboxylase as a reference point, a distinction between octamers and tetramers could be made using molecular sieve chromatography.

A small amount of unaggregated square planar structures from fraction 45 (fig. 4.1) was therefore combined with chicken liver pyruvate carboxylase and the mixture applied to a Sepharose 4B column (2.5 x 80cm). The elution profile is shown in fig. 4.2. Electron microscopic examination of the fractions revealed that the square planar structures eluted, in an unaggregated

form, well ahead of pyruvate carboxylase, suggesting that they were in fact cubic octamers. This finding would explain the unusually vivid appearance of the square planar "tetramers" since the underlying subunits would reinforce the image.

This final resolution of the two molecules in question provides compelling evidence that pyruvate carboxylase isolated from chicken liver is not a square planar tetramer as described by Valentine *et al.*, and suggests that this error arose through examination of only partially purified enzyme.

4.3.3. Comparison of the Sheep Liver and Chicken Liver Electron Micrographs.

The electron micrographs presented earlier in this chapter indicate that both the avian and ovine pyruvate carboxylases were tetramers with the subunits arranged at the corners of a rhombus. Closer examination of these structures revealed some differences between the images projected by the enzymes from the different sources however. The sheep enzyme appeared to have a fairly compact arrangement of the subunits while the chicken enzyme gave the appearance of a more "open" rhomboid form. Since the dimensions of the enzymes from both sources are very similar, this difference was not likely to be due solely to a distortion of the chicken

liver enzyme during preparation for and examination in the electron microscope. The more open appearance of the chicken enzyme may have been due to a greater penetration of the stain between the binding domains of the individual subunits. This hypothesis appears reasonable given that the chicken enzyme is known to dissociate readily into monomers (Utter et al., 1975). This would suggest that the contact regions between the subunits of the chicken liver enzyme are quite weak. No such dissociation has been observed with the sheep liver enzyme and therefore less penetration of the stain between the subunits might be expected. This would give rise to the more compact appearance of the images of the sheep liver enzyme.

The appearance of the "arms" was also more pronounced in the case of the chicken liver enzyme. This may also be a consequence of a greater penetration of the stain into the chicken enzyme, which would effectively highlight this structural feature. Whether these arms are projections from the main body of the subunit, as shown in the models of the enzymes, or merely stain-impermeable regions or ridges on an oblate subunit cannot as yet be ascertained.

4.3.4. Binding of Avidin to Pyruvate Carboxylase.

The logic involved in locating the active sites of pyruvate carboxylase by treatment with avidin

prior to electron microscopy has been outlined in the introduction to this chapter. Since it was found that the chicken liver enzyme had a more open structure, these experiments were conducted on the enzyme from this source.

The enzyme was reacted with a sixty-fold excess of avidin (based on biotin binding capacity). Under these conditions complete loss of enzyme activity occurred in less than one minute. Excess avidin was removed by gel chromatography on Sephacryl S-200 and the avidin-pyruvate carboxylase complex was then examined in the electron microscope.

A field view of this material is shown in plate 4.9. The rhomboid shape of the molecule appears unaffected by this treatment (see #1, plate 4.9). In some images a small protein can be seen bound to the tetramer (see #2, plate 4.9). The binding was apparently through only one of the subunits and the most common image of this type showed only one small protein bound. Increased magnifications of these structures were however, no more enlightening. (plate 4.10).

The appearance of rhomboids with apparently no avidin bound to them is extremely surprising considering the large excess of avidin used and the tenacity with which this protein binds to biotin ($K_D = 10^{-15}M$, Green, 1975). It appeared more likely

that, in these cases, avidin was bound to the enzyme but could not be visualized because it was either underneath or directly above the pyruvate carboxylase molecule. This would imply that the active sites of the enzyme are at least not on the extremities of the subunits. The inherent flexibility of the biocytin arm may explain the two different forms of the avidin-pyruvate carboxylase complex observed. Since the biotin binding site on avidin is known to be ca.8Å below the surface of this molecule, (Green, 1975) the residual ca.6Å of the 14Å biocytin arm may enable enough variation in the position of the avidin to generate two images in the electron microscope: one in which the avidin is observed by the large enzyme subunit and a second where the avidin has "fallen" to the side of the subunit and so is visible. Alternatively, the potential of avidin to bind four biotin molecules could conceivably give rise to crosslinking between the biotin cofactors of adjacent subunits of the enzyme, if the active sites were close enough together. If this hypothesis is correct, the appearance of avidin molecules bound to only one subunit may be interpreted as a failure of the crosslinking to occur.

The electron micrographs of the enzyme-avidin complex obtained in this study gave no indication as to which of these possibilities was correct and the only conclusion which could be drawn was to suggest that

the catalytic sites of pyruvate carboxylase are not exposed on the outer extremities of the subunits. The absence of large polymers of the avidin-pyruvate carboxylase complex, which could form by inter-molecular crosslinking, is consistent with this interpretation.

4.4. DISCUSSION.

The results of this study indicate that both sheep liver and chicken liver pyruvate carboxylase are tetramers with the subunits arranged in a rhombic configuration. A number of implications arise from this finding.

Firstly, the error made by Valentine *et al.* in assigning the structure of the square planar tetramer to chicken liver pyruvate carboxylase serves to illustrate the problems associated with interpreting electron micrographs of partially purified proteins. In this case the fortuitous correlation between the erroneously high molecular weight originally determined for the native enzyme (Scrutton and Utter, 1965a) and the calculated molecular weight of the square planar tetramer, together with the correspondence between the temperature sensitivity of the observed images and the enzyme, made detection of this error difficult. Such correspondences however clearly indicate the need to establish the identity of the structure being observed. This could be

achieved either by the use of specific reactions (e.g. antibodies), or more reasonably, by ensuring that the material examined was homogeneous (e.g. by polyacrylamide gel electrophoresis).

Secondly, the marked resemblance between the yeast enzyme and the two sources of the enzyme examined in this study would suggest that the rhomboid configuration of subunits has been conserved during evolution. Some discussion in the literature has centred on the appearance of rhomboid tetramers in electron micrographs. For example, the crystals of muscle phosphorylase, when examined in the electron microscope, show a rhombid shape for the tetramers and Valentine and Chignell (1968) described two ways in which this situation could occur:

(1) The subunits of the protein were identical and the rhomboid shape was generated by heterologous binding domains. In this situation the remaining potential links would need to be blocked by some steric hindrance to prevent large oligomers being formed.

(2) The tetramer was composed of two identical dimers containing non-identical subunits (i.e. there are two different types of subunits in the tetramer). The first

possibility was considered unlikely since Monod et al. (1965) have argued that heterologous binding domains would be less stable than homologous ones and that if such a structure did exist it would be modified during evolution to a symmetrical dimer On this basis Valentine and Chignell concluded that the phosphorylase tetramer probably contained two similar, but not identical, subunits. The same conclusion was drawn by Valentine (1968) to explain the rhombic appearance of yeast pyruvate carboxylase.

Haschemeyer (1970) has questioned the validity of such conclusions based on electron micrographic evidence and has pointed out that distorted tetramers of the point group D_2 could give rise to images which approximated a rhombus. However the work presented in this chapter suggests that the sheep liver and chicken liver pyruvate carboxylases are also rhomboids. Thus, Haschemeyer's objection would now require that the pyruvate carboxylases from all three sources undergo a similar distortion. Such a regular distortion of the enzymes from different species would appear unlikely. Furthermore, if the arguments of Monod et al. regarding the instability of heterologous binding domains between idential subunits are

accepted, then it would appear from the conservation of the rhomboid structure in the yeast, chicken and sheep enzymes, that no evolutionary "pressure" has been applied to alter these enzymes to symmetrical dimers.

It was therefore concluded that the most likely reason for the appearance of these enzymes as rhomboids was that they contain non identical subunits.

PLATE 4.1

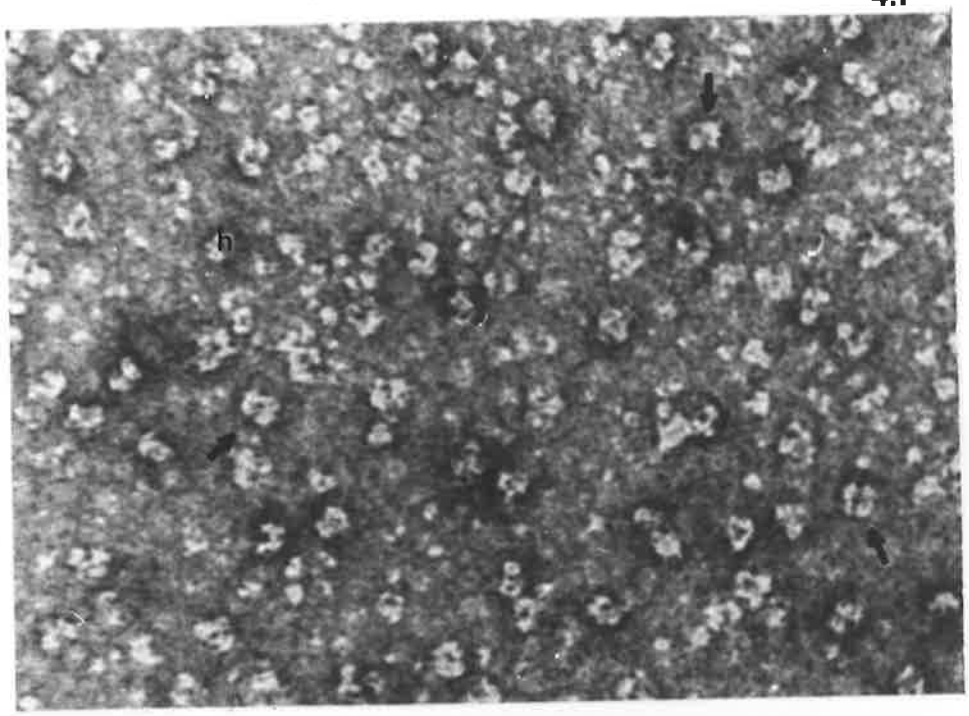
Molecules of sheep liver pyruvate carboxylase, x 300,000. Tetramers are seen (indicated by arrows) surrounded by broken particles. Plate 4.1a shows 5.6% SDS-polyacrylamide gel of the purified sheep liver pyruvate carboxylase used, with the position of the tracking dye indicated by the arrows.

PLATE 4.2

A montage of individual molecules of sheep liver pyruvate carboxylase (x 780,000). Different views of the enzyme are presented and below each is a photograph of a model of the enzyme corresponding to that image.

4.1

a



4.2

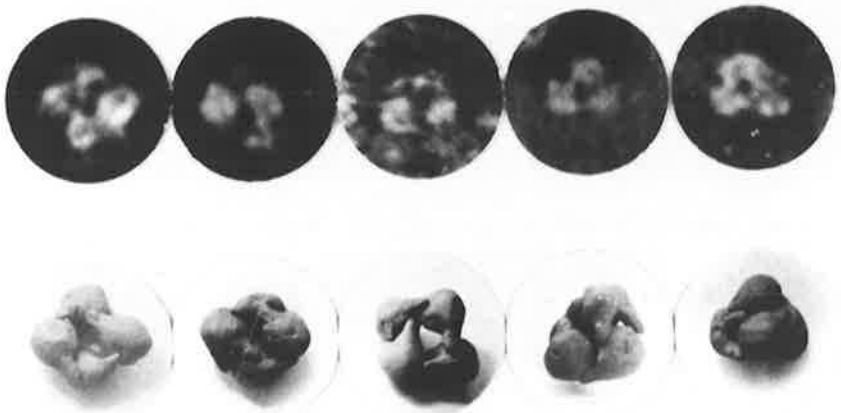


PLATE 4.3

Molecules of chicken liver pyruvate carboxylase, x 240,000. Rhombic tetramers are indicated by the arrows.

Plate 4.3a. Electrophoresis on 5.6% SDS-polyacrylamide gel of purified chicken liver pyruvate carboxylase, with the position of the tracking dye indicated by the arrow.

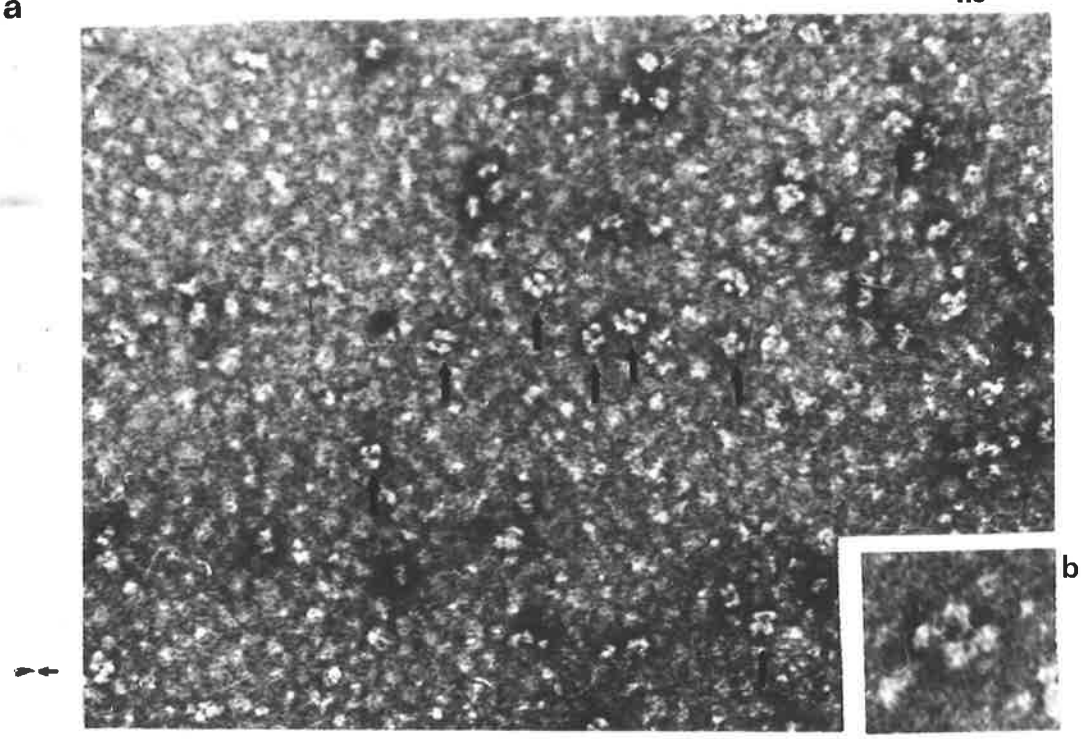
Plate 4.3b. A molecule of chicken liver pyruvate carboxylase showing the rhombic arrangement of the subunits, x 700,000.

PLATE 4.4

A montage of individual molecules of chicken liver pyruvate carboxylase (x 780,000). Different views of the enzyme are presented and below each is a photograph of a model of the enzyme corresponding to that image.

4.3

a



4.4

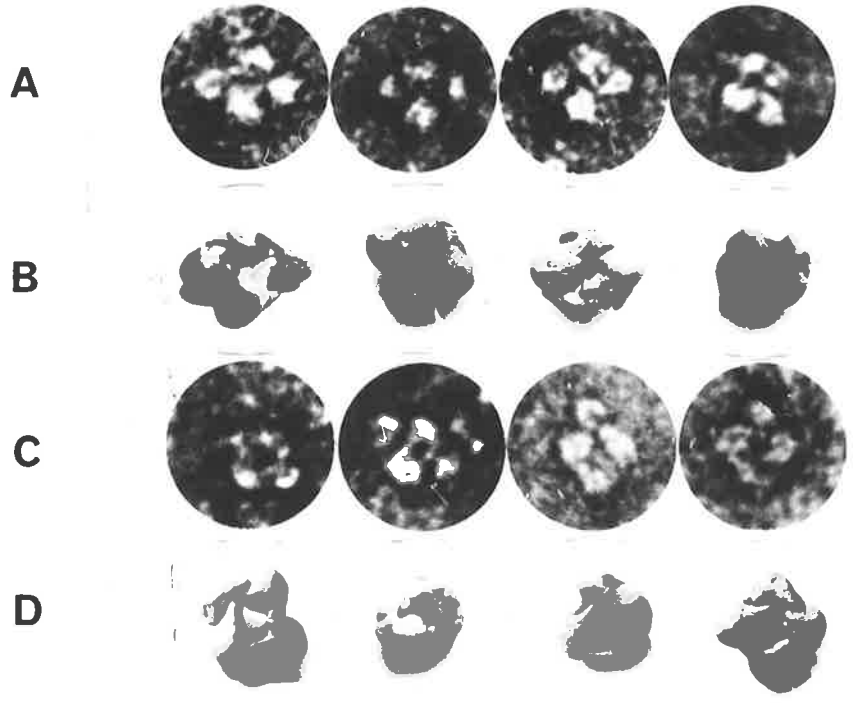


FIGURE 4.1

Ion-exchange chromatography of chicken liver pyruvate carboxylase.

Pyruvate carboxylase (≃ 10,000 units, 3.2 U/mg) was applied to a column (3.0 x 45cm) of DEAE-Sephadex-A50, and eluted with a linear gradient of ammonium sulphate from 20mM - 200mM in 25mM potassium phosphate pH 7.2, containing 0.1mM EDTA and 0.1mM DTE. Fractions of 10ml were collected at a flow rate of 1.0ml/min.

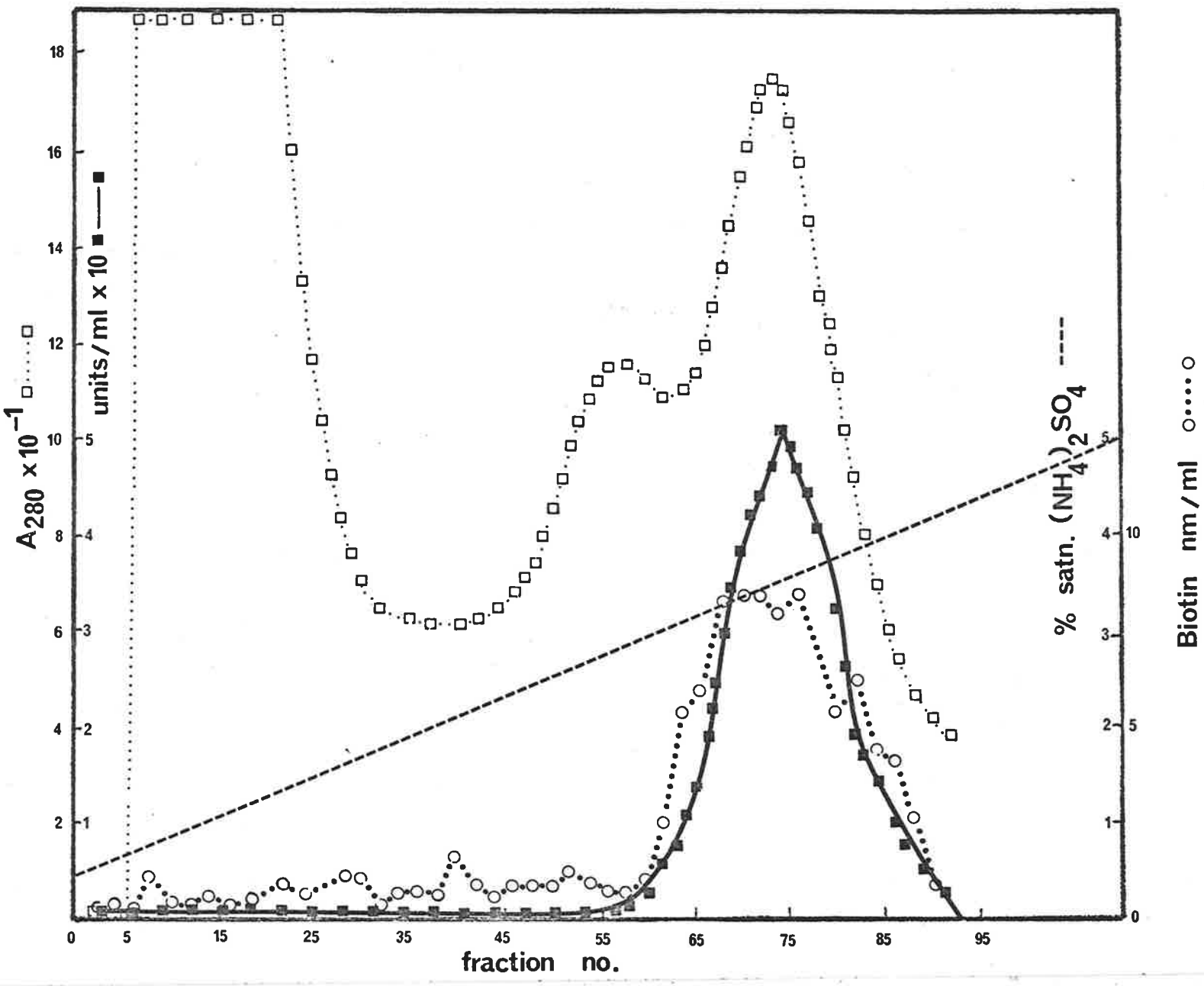

$A_{280} \times 10^{-1}$
units/ml x 10 ■——■
% satn. $(NH_4)_2SO_4$ ----
Biotin nm/ml ○····○
fraction no.
0
5
15
25
35
45
55
65
75
85
95
1
2
3
4
5
6
8
10
12
14
16
18

PLATE 4.5

A field view of the square-planar tetramers obtained from fraction 34 of the DEAE-Sephadex elution profile (fig 4.1). Magnification 240,000 x.

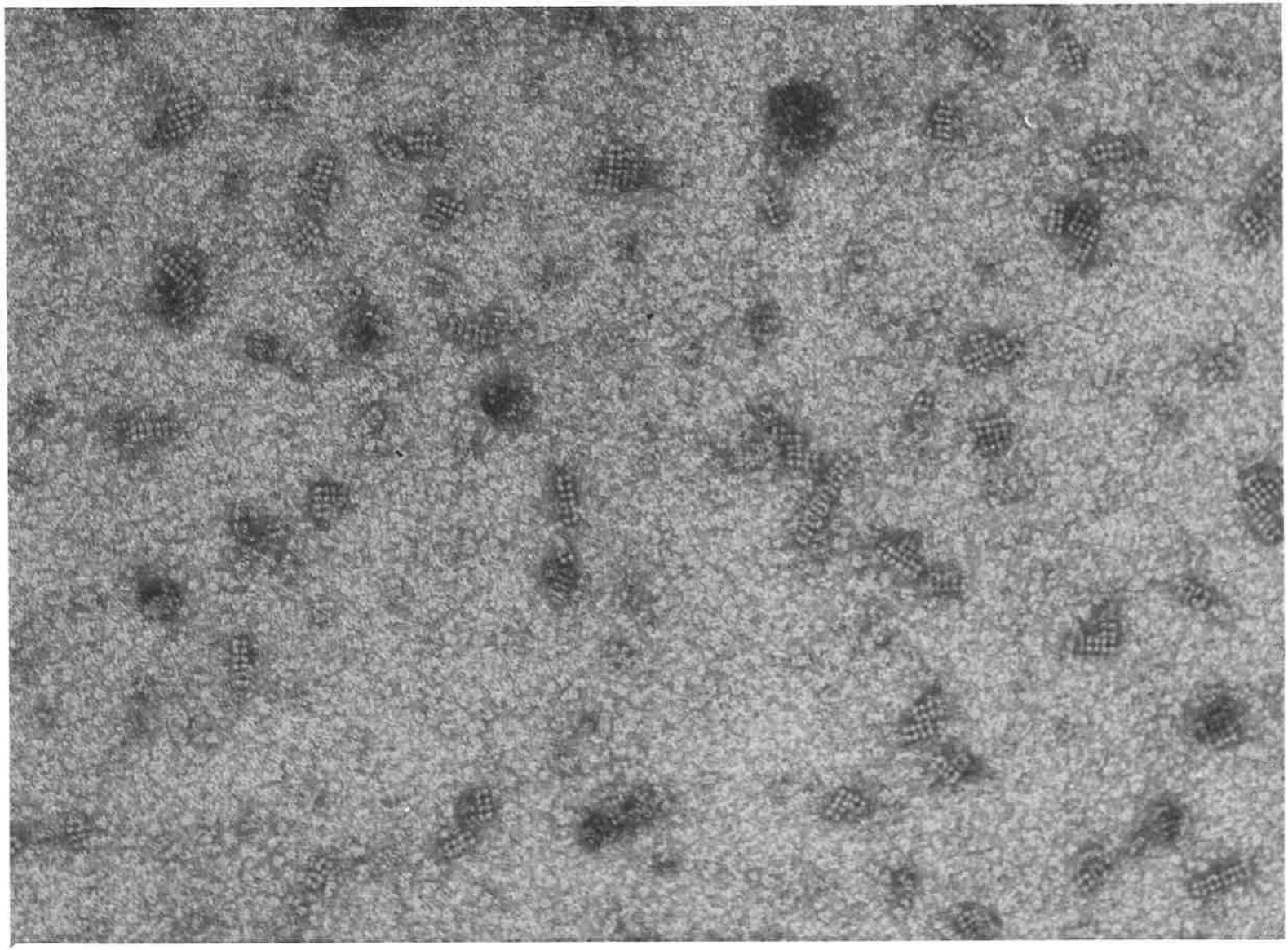


PLATE 4.6

A field view of the square-planar tetramers obtained from fraction 45 of the DEAE-Sephadex elution profile (fig 4.1). Magnification 180,000 x.

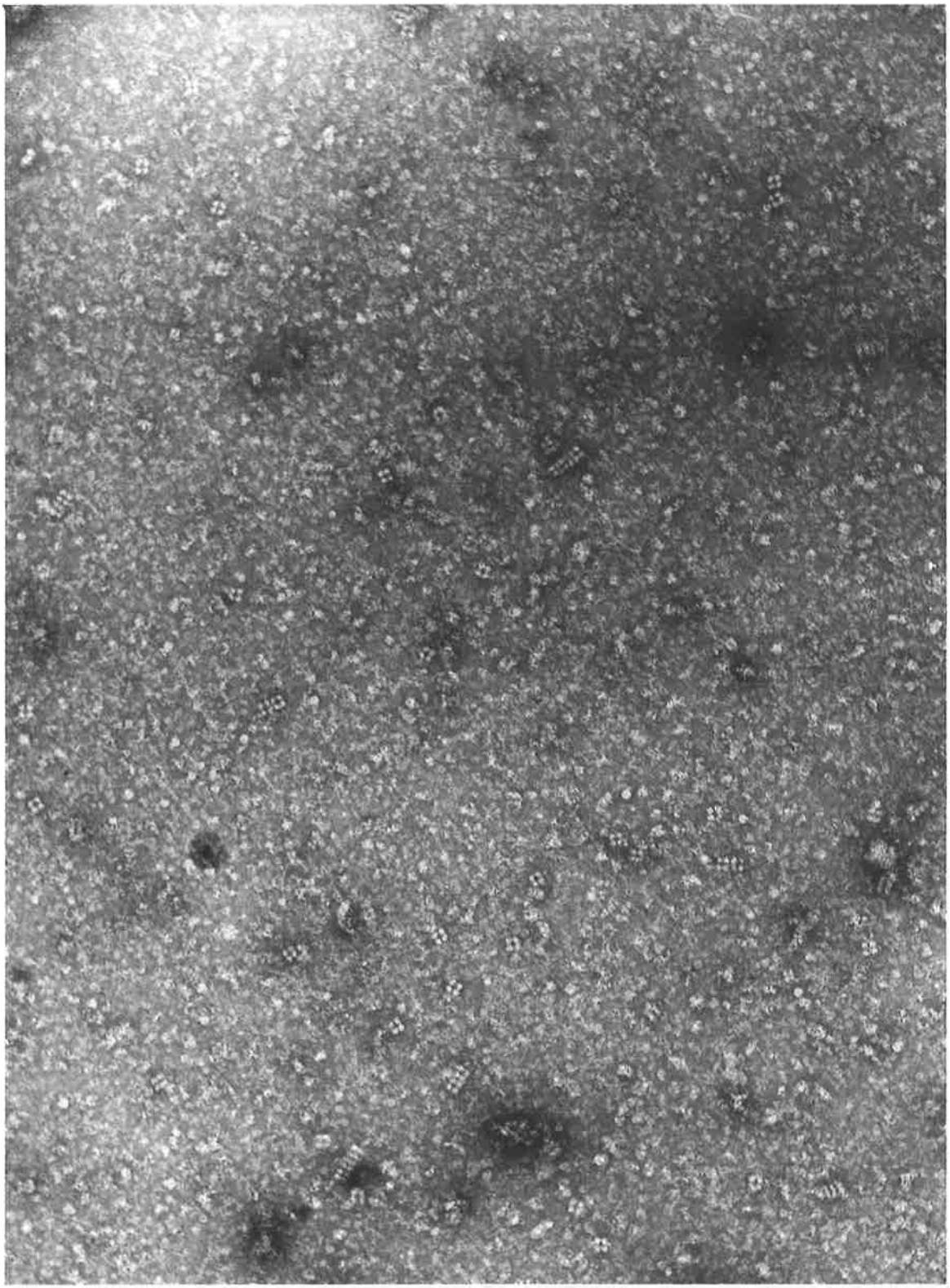


PLATE 4.7

Plate 4.7a. Square-planar tetramers from fraction 34 of the DEAE-Sephadex elution profile (fig 4.1). Magnification 300,000 x.

Plate 4.7b. The same material as shown in Plate 4.7a. The square-planar structure has been lost following incubation at $4^{o}C$ for 20 min. Magnification 300,000 x.

Plate 4.7c. The same material as shown in Plate 4.7b, after rewarming to room temperature. The tetrameric and aggregated structures have reformed. Magnification 300,000 x.

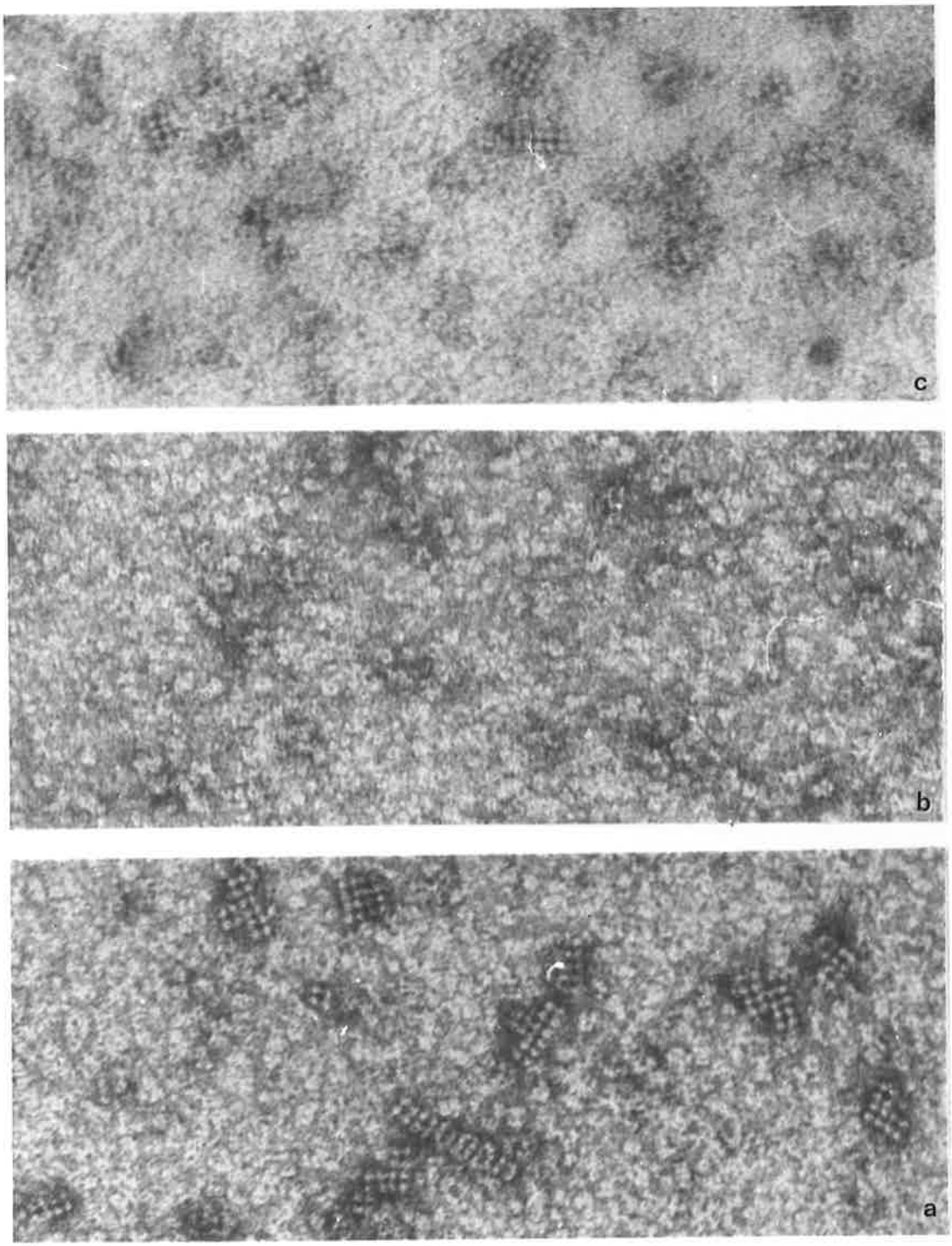

c
b
a

PLATE 4.8

Square-planar tetramers from fraction 45 of the DEAE-Sephadex elution profile (fig 4.1). The arrows indicate these structures viewed on their side, suggesting an octomeric arrangement of the subunits. Magnification 180,000 x.

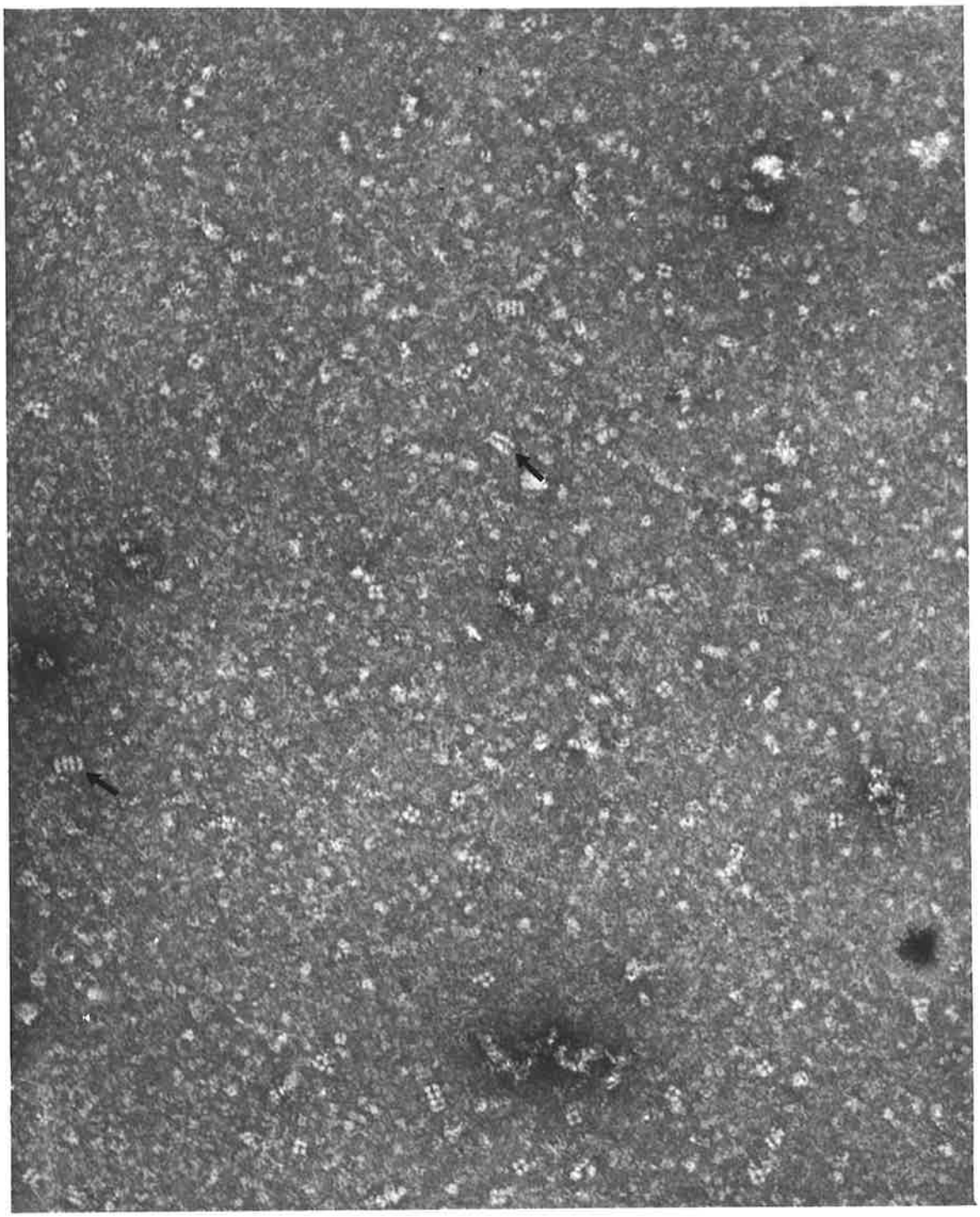


FIGURE 4.2

Chromatography of the square-planar molecules and chicken liver pyruvate carboxylase on Sepharose-4B.

The column (2.5 x 80cm) was eluted with 25mM potassium phosphate pH 7.2, containing 100mM potassium chloride, 20mM ammonium sulphate, 1mM EDTA and 1mM DTE, at a flow rate of 0.32ml/min and 4.5ml fractions were collected. The void volume (Vo) of the column was determined using Blue Dextran. The square-planar tetramers eluted in a non-aggregated state in fractions 39 - 42 as indicated by the bracket, ahead of the peak of pyruvate carboxylase activity.

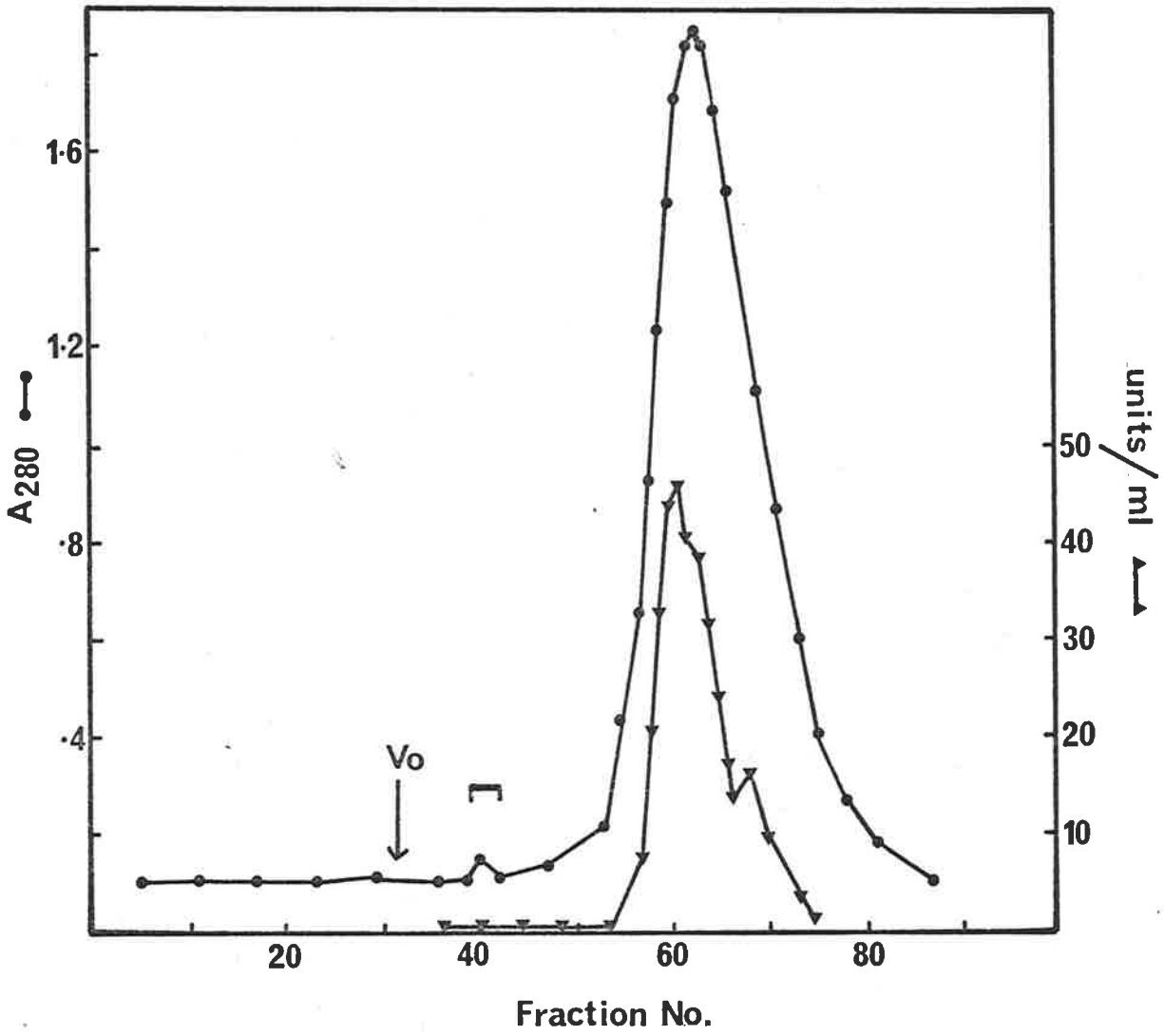

1·6
1·2
·8
·4
A280
Vo
20
40
60
80
Fraction No.
50
40
30
20
10
units/ml

PLATE 4.9

Electron micrographic images of the avidin-pyruvate carboxylase complex, x 180,000. Molecules of avidin bound to the rhombic pyruvate carboxylase molecules are indicated by the arrows and #2. Molecules on which no avidin can be seen are designated by #1.

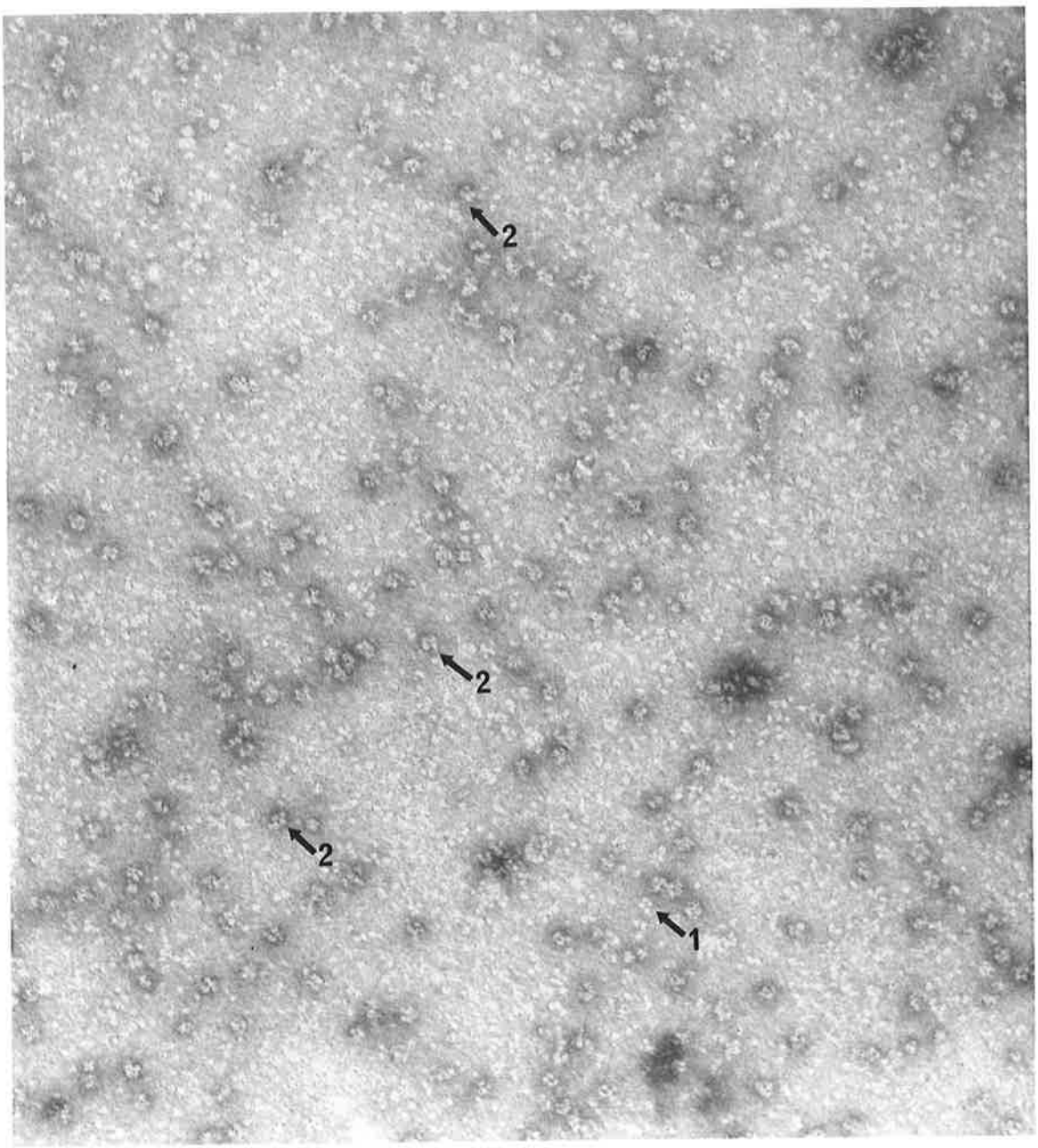
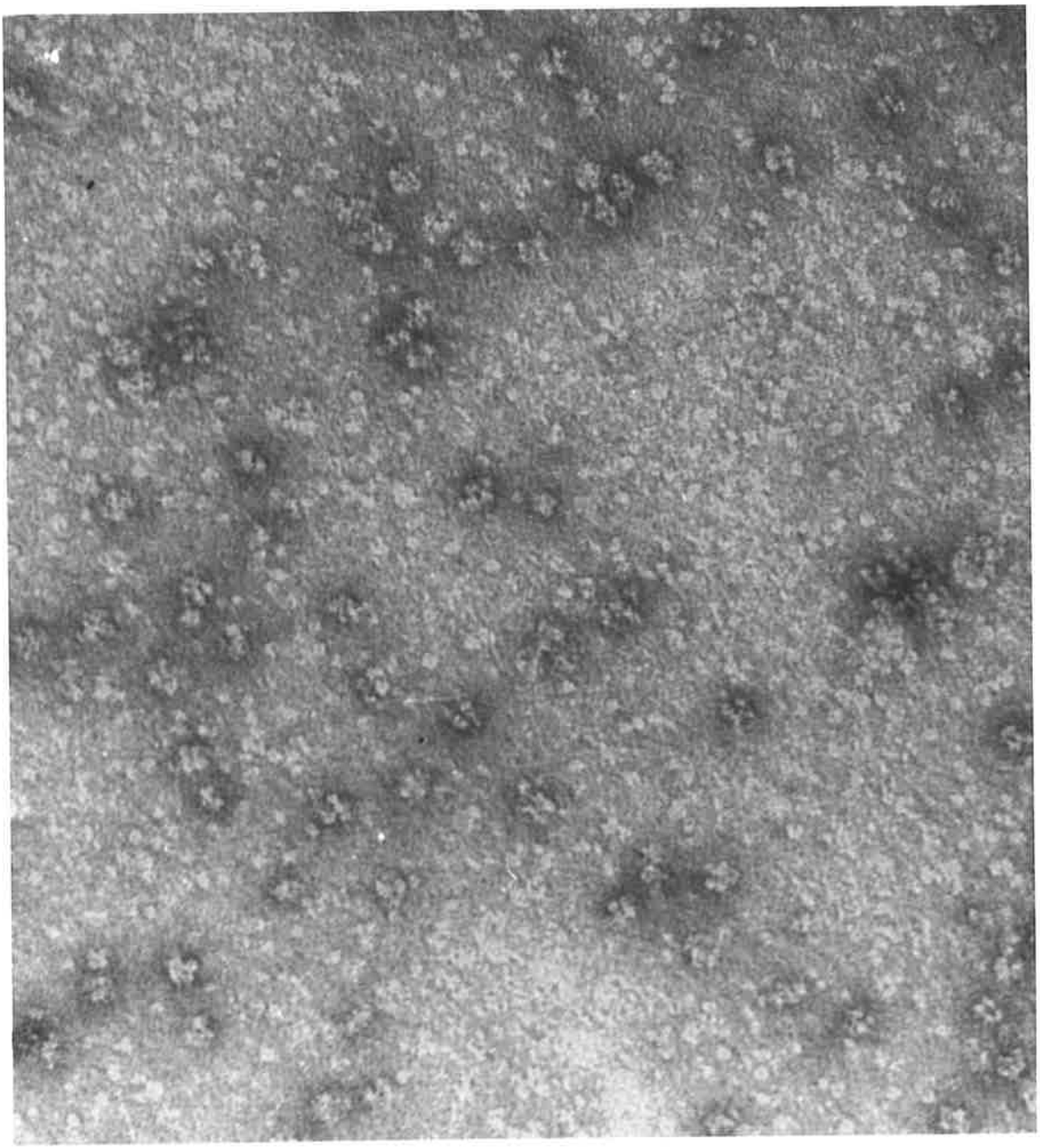

2
2
2
1

PLATE 4.10

Electron micrographic images of the avidin-pyruvate carboxylase complex x 360,000.



CHAPTER 5

CHEMICAL MODIFICATION OF PYRUVATE CARBOXYLASE

5.1. INTRODUCTION

The investigation of the chemical basis of enzyme catalysis is most readily approached through the chemical modification of amino acid residues within the enzyme. This broad field can be subdivided into two areas, dependent upon the nature of the modifier.

The first of these is the use of "group specific" reagents to achieve modification of one type of amino acid side chain (e.g. sulphydryl). A large number of chemical modifiers of this type have been described (e.g. see Table 5.1, Vallee and Riordan, 1969; Means and Feeney, 1971; Glazer et al., 1975) and yet, even though many of these reagents may react specifically with only one type of free amino acid, when applied to the modification of those residues in native proteins, other residues are often modified. The application of group specific reagents to achieve site-specific modification of a native protein is therefore largely a matter of chance and the environment around a particular amino acid residue is the primary determinant governing its reactivity in a protein.

The second, more recent, area of chemical modification studies involves the use of active site directed reagents or "affinity labels". These reagents are designed to be structurally similar to ligands known

to specifically bind to the enzyme or protein under investigation, and therefore have a high probability of combining in a non-covalent fashion with that ligand binding site. Then, by virtue of a reactive group on the affinity label, a covalent bond can be formed to an amino acid residue in the ligand binding site. Many of these reagents rely on the presence of nucleophilic amino acids in the ligand binding site to form a covalent bond with the enzyme. However with this type of affinity label it is still possible that some non-specific modification of highly reactive residues outside the ligand binding site may also occur. Furthermore, if the binding site does not contain a nucleophilic amino acid residue then no covalent attachment can occur. To overcome these problems photo-affinity labels have been employed. These reagents have the advantage that they are not reactive until subjected to irradiation and can be allowed to saturate the target site before being converted to the reactive compound (Knowles, 1972; Singer, 1970).

An alternative approach involves the design of "K_{cat} inhibitors", or "suicide substrates". These reagents are inert until acted upon by the enzyme in a manner analogous to the enzyme catalysed reaction. Upon activation they are capable of modifying amino acid residues within the ligand binding site. The use of these reagents provides methods of testing proposed reaction

mechanisms as well as specifically modifying catalytically important amino acid residues. A review of this aspect of chemical modification has recently appeared (Sigman and Mooser, 1975).

In an effort to understand the chemical mechanism of bicarbonate fixation catalysed by pyruvate carboxylase both group specific reagents and affinity labels have been used. The early work of Keech and Utter (1963) on chicken liver pyruvate carboxylase indicated that this enzyme was inactivated by certain cysteine modifying reagents. Palacian and Neet (1970) attempted to further characterize this modification process in order to understand the role that these residues played in the enzymic catalysis. The results of these studies revealed a complex situation. The type of inactivation profile observed and the effect on the physical structure of the enzyme was dependent upon the nature of the modifier used and on the composition of the buffer. The inactivation produced by p-hydroxy-mercuribenzoate, DTNB or L-cystine could be partially reversed by thiol reagents. With p-hydroxymercuribenzoate the reactivation process was shown to be accompanied by a recovery of the original sedimentation pattern of the enzyme.

A more detailed investigation (Palacian and Neet, 1972) of two of these inhibitors, NEM and L-cystine, led these authors to propose a tentative scheme for the

modification of the sulphydryl residues of chicken liver pyruvate carboxylase (fig 5.1). The model envisaged two distinct phases of modification. The first phase was accompanied by the reaction of between 4 and 32 moles of cysteine per mole of enzyme, partial or no loss of activity and no change in the sedimentation profile of the enzyme. The second phase proceeded with the reaction of an additional 16 to 39 amino acid residues per enzyme molecule, complete loss of enzymic activity and disruption of the tetrameric structure of the enzyme into higher molecular weight aggregates. The formation of monomers was observed under some conditions and so was included in the overall scheme.

These authors also investigated the function of these sulphydryl groups by both kinetic analysis of the modified enzyme and by protection studies using the substrates and the allosteric effector of the enzyme, acetyl CoA. Under some conditions of chemical modification, protection against loss of enzymic activity was observed in the presence of ATP^{-4}, oxaloacetate and acetyl CoA. However, under other conditions increased inactivation rates were observed. The dependence of the protection on acetyl CoA concentration was interpreted to indicate that the protection by acetyl CoA was a co-operative phenomenon. This co-operativity was thought to support the hypothesis of co-operative binding of acetyl CoA to pyruvate carboxylase. Recently, however, Easterbrook-Smith (1977)

has provided evidence which suggests that acetyl CoA binds to the enzyme in a classical Michaelis-Menton manner. This leads to the conclusion that interpretations of results from chemical modification studies where modification has proceeded to a large extent must be held in reservation since the disruption of the quaternary structure of the enzyme, as reported by Palacian and Neet (1972) could make valid interpretation of the data difficult.

Chemical modification studies on the enzyme isolated from both sheep kidney and sheep liver have resulted in more specific modifications. Keech and Farrant (1968) reported a single lysyl residue in the acetyl CoA binding site using FDNB and Ashman et al. (1973) confirmed this finding using the lysine-specific reagent, TNBS. A further lysyl residue in the ATP binding site was located by Easterbrook-Smith et al. (1976a) using the 2', 3' dialdehyde derivative of ATP, complexed with Mg^{2+} (Mg-oATP).

Affinity labelling was also used by Hudson et al. (1975) to identify a cysteine residue in or near the keto-acid binding site. In this instance the pyruvate analogue 3-bromopyruvate was used. Both acetyl CoA and oxaloacetate protected the enzyme against the inactivation by 3-bromopyruvate. However, when the rates of inactivation were determined in the presence of either of

these ligands and replotted in the manner described by Scrutton and Utter (1965b) it was found that neither acetyl CoA nor oxaloacetate afforded complete protection. This result may be interpreted to mean that either

(1) more than one essential amino acid residue was being modified per active site and hence at saturating concentrations of either ligand a second site was still available for modification,

or (2) the residue being modified was not in either ligand binding site but was protected to some degree by a conformational change induced by the binding of either oxaloacetate or acetyl CoA to the enzyme.

The former situation appeared to be more likely since Hudson *et al*. (1975) noted that 3-bromopyruvate modified 1.5 amino acid residues per active site in the absence of acetyl CoA but this was reduced to 1.0 residue per active site in the presence of saturating concentrations of acetyl CoA.

Recently in this laboratory Clements (1977) reported that 3-chloroacetonyl CoA functioned as an affinity label of the acetyl CoA binding site on the enzyme. Protection against inactivation of the enzyme

by this reagent was afforded by both acetyl CoA and oxaloacetate. When the degree of protection by acetyl CoA against the inactivation process was examined by the method of Scrutton and Utter (1965b) it was found that even as the concentration of acetyl CoA approached infinity a small but significant rate of inactivation still occurred. Clements (1977) concluded from this data that, in addition to modifying an amino acid residue in the acetyl CoA binding site, 3-chloroacetonyl CoA probably also modified the cysteine residue detected by Hudson et al. (1975) in the keto acid binding site. Other studies by Clements et al. (1978) had provided evidence to support the proposition that acetyl CoA was oriented with the acetyl end of the molecule close to the keto acid binding site.

These findings were incorporated into a model in which Clements (1977) proposed that there were two reactive cysteine residues in close juxtaposition in the binding sites of acetyl CoA and the keto acids (fig 5.2). The model proposes that acetyl CoA is directly involved in enolizing pyruvate in the adjacent site. In doing so acetyl CoA becomes susceptible to hydrolysis of the thio-ether bond linking the acetate and CoA moieties in this molecule. This mechanism would therefore provide an explanation for the observation that pyruvate carboxylase catalyses a slow deacylation of acetyl CoA and that this deacylase activity is stimulated in the presence of

pyruvate. The role of the pair of cysteine residues in the mechanism was to act as proton donors for the enolization processes and to act in an unprotonated form as nucleophiles. Therefore, the model proposes that these cysteine residues are involved in both the binding of the ligands and the catalytic events occurring at the keto acid site on the enzyme.

The group specific chemical modification studies described in this chapter were conducted in parallel with the affinity labelling studies described above, and had as their objective a characterization of the reactivity and function of the cysteine residues in sheep liver pyruvate carboxylase. The results obtained through this approach are discussed in the light of the recent findings from the affinity labelling studies and the hypothesis presented by Clements (1977).

5.2. METHODS

5.2.1. Inactivation of sheep liver pyruvate carboxylase with chemical modifiers

The chemical modifications of sheep liver pyruvate carboxylase described in this Chapter were carried out using enzyme dissolved in Buffer A (without DTE, section 3.2.2) unless otherwise stated.

5.2.2. Preparation of NEM-cysteine (S-(N-ethyl succinimido)-cysteine)

The method of Smyth et al. (1960) was used to synthesize this compound which was used as a standard in the identification of the amino acids modified by [^{14}C] NEM. L-cysteine hydrochloride (1.35g) was dissolved in 36ml of water, followed by NEM (1.08g). The pH of the solution was adjusted to pH 6.0 by adding IN KOH and the solution was left at room temperature for 30 min. The solution was dried by rotary evaporation at 35°C. The white product was dissolved in water (5ml), precipitated with acetone and washed with ether. The S-(N-ethylsuccinimido)-cysteine thus prepared was dried in vacuo over phosphorous pentoxide. M.Pt. = 193-194°C. The compound ran as a single spot in the chromatographic system described in section 5.2.4. and was ninhydrin positive.

5.2.3. Preparation of NEM-lysine (ε-N(N-ethylsuccinimido)-lysine)

The method used for the preparation of this standard was the same as that described by Nielsen (1970). The compound was formed by reaction of the ε-amino group of lysine with NEM after the α-amino group of the amino acid had been protected by formation of the copper complex of lysine. (Greenstein and Winitz, 1961).

L-Lysine-HCl (500mg) (B.D.H.) was dissolved in 5ml of boiling water, to which was added, slowly, cupric carbonate (833mg). The mixture was refluxed for 2h and filtered while hot. The residue was washed with hot water and the filtrate and washings combined. The deep blue solution was allowed to cool and then NEM (685mg) was added. The pH was maintained at pH8 for 3h by adding 1N KOH and the solution was then left overnight at room temperature. The next day the solution was extracted three times with an equal volume of ether to remove any unreacted NEM and residual ether was removed by gentle warming on a hot plate. The copper complex of the NEM-modified lysine was decomposed by bubbling H_2S through the solution for 30 min. After removing the black CuS precipitate by centrifuging, the solution was concentrated by rotary evaporation and applied to a Sephadex G-10 column (77 x 2.8cm) equilibrated with 0.01M acetic acid. The presence of solutes in the eluate was detected by their yellow colour (A_{380nm}) and reaction with ninhydrin. The first ninhydrin-positive peak was collected and lyophillized. Yellow, ninhydrin-negative compounds were removed from the sample by washing with methanol. The residue was dissolved in 5ml of hot water and the product precipitated with methanol and collected. The precipitate was redissolved and reprecipitated with methanol, washed with methanol and dried in vacuo over P_2O_5. The product (108mg) was a white powder, charring

above 250°. The compound migrated as a single spot in the chromatographic system described in section 5.2.3. and was positive to ninhydrin.

5.2.4. Chromatographic separation of NEM-cysteine and NEM-lysine

Chromatography of NEM-cysteine and NEM-lysine in n-butanol-pyridine-acetic acid-water (30:20:6:24; v/v) on Whatman 3MM paper proved a satisfactory system for resolving these two compounds (Smyth et al., 1960).

NEM-cysteine	R_f = 0.56
NEM-lysine	R_f = 0.32

5.2.5. Enzymatic digestion of [^{14}C]NEM-labelled pyruvate carboxylase

For the identification of the amino acids modified by NEM, the [^{14}C]NEM-labelled enzyme was digested by the method of Harding (1974). The protein sample (10mg/ml) in 0.2M N-ethylmorpholine acetate (NEMA) buffer at pH 8.0 was incubated in a sealed tube at 37°C with crystalline thymol (antibacterial agent) and digested with a sequence of the following enzymes; 2% trypsin (2h), 2% chymotrypsin (16h), 10% pronase (24h), 10% protease (2h), 1% α-amino peptidase (24h).

This procedure was chosen over acid hydrolysis of the protein sample to avoid complications involved in the hydrolytic breakdown of the NEM-cysteine and NEM-lysine adducts (Smyth et al., 1960).

5.3. RESULTS

5.3.1. Chemical modifiers specific for cysteine residues

The effect of a variety of sulphydryl modifying reagents on the enzymic activity of sheep liver pyruvate carboxylase was determined and the data obtained is shown in Table 5.1. In all cases inhibition of the enzymic activity was observed, indicating that at least one sulphydryl residue was necessary for enzymic activity. NEM and DTNB were selected as reagents for further study.

5.3.2. Chemical modification of pyruvate carboxylase using n-alkyl maleimides

5.3.2.1. Inactivation of pyruvate carboxylase using NEM

Incubation of the enzyme with concentrations of NEM ranging from 0.02mM to 20mM resulted in every case in a biphasic curve when the log of the residual activity was plotted against time (fig 5.3). Examination of this biphasic profile revealed that the slower phase followed

pseudo first order kinetics (fig 5.4).

Attempts to analyse the fast phase of inactivation by subtracting the degree of inactivation corresponding to the slower phase from the experimentally determined values of the initial phase, as described by Kenney (1975) gave unreliable results, due to the velocity of the reaction. However calculations on the velocity of this fast phase at the lowest concentration of NEM used indicated that this reaction proceeded with a second order rate constant in excess of ca. $80mM^{-1}min^{-1}$.

The pseudo first order rate constants at various concentrations of NEM were calculated from the formula

$$\frac{A}{A_o} = e^{-k't} \qquad \ldots (5.1)$$

where $\frac{A}{A_o}$ represents the fraction of enzymic activity remaining after the time, t, and k' is the pseudo first order rate constant. The second order rate constant, k_1 was then determined from the relationship

$$k' = k_1[NEM] \qquad \ldots (5.2)$$

If a classical second order reaction was being observed a plot of k' against the concentration of NEM should give a straight line of slope k_1. When the data

obtained for the slow phase was plotted in this manner a value of $5.3 \pm 0.4 \times 10^{-2}$ mM^{-1} min^{-1} was obtained for the second order rate constant at concentrations of NEM below 2.0mM. Above this concentration however the value for second order rate constant decreased progressively (fig 5.5). Findings such as these are indicative of a rate limiting step in the modification process which is independent of the concentration of NEM. Two such possibilities exist:

Scheme 1. Modification of residues in the enzyme does not affect the catalytic process <u>per se</u> but instead destabilizes the enzyme so that there is a time dependent alteration of the conformation of the enzyme which results in the loss of enzymic activity. viz.

$$A + NEM \xrightarrow{kx} \underset{\text{Active}}{\text{E-NEM}} \xrightarrow{ky} \underset{\text{Inactive}}{E^{\dagger}\text{-NEM}}$$

Therefore, once the rate of formation of the E-NEM complex exceeds the rate of formation of the $E^{\dagger}$-NEM complex then the inactivation process becomes independent of the concentration of NEM.

Scheme 2. A binding site exists on the enzyme for reversible binding of NEM. In this situation, above a particular concentration of NEM (determined by the K_D of the enzyme-NEM complex) the enzyme would become

saturated and so higher concentrations of NEM would fail to increase the second order rate constant. This argument is analogous to that applied to affinity labels and is in fact one of the criteria used to determine the validity of this type of chemical modification (Singer, 1967). A scheme representing this process is shown below:

$$E + NEM \overset{K_D}{\rightleftharpoons} \underset{\text{Active}}{E...NEM} \xrightarrow{k_z} \underset{\text{Inactive}}{E\text{-}NEM}$$

If this was the case then the binding of NEM to the enzyme could be related to the structural similarity between NEM and ureido moiety of biotin (fig 5.6). This possibility was considered during the investigation of the protection afforded by the substrates and activators against inactivation of the enzyme by NEM presented in section 5.3.2.3.

The number of catalytically significant residues modified per active site during the slow phase of inactivation of the enzyme was estimated from the slope of a replot of log k' versus log concentration of the inhibitor. The rationale of this plot can be found in a report of work done in this laboratory (Keech and Farrant, 1968) in which FDNB was used to covalently modify pyruvate carboxylase. In the present study only those values in the linear region of fig 5.5 were used

since the derivation of the relationship requires that the second order rate constant be constant over the range of inhibitor concentrations used. Fig 5.7 shows the data obtained from the linear region of fig 5.5. The slope of the line was found to be 0.92 ± 0.04, suggesting that an average of approximately one molecule of NEM is involved in binding to each active site of the enzyme during the inactivation process. This may imply that only one catalytically important amino acid residue per active site is modified during this phase of inactivation of the enzyme by NEM.

From the data presented above no information can be obtained regarding the absolute second order rate constant or the number of catalytically important residues reacting during the first phase of inactivation of the enzyme by NEM. However it would appear that the residues that are modified during the first phase of inactivation of the enzyme cannot be absolutely required for catalysis since the enzyme is still partially active after this phase of inactivation has been completed. Furthermore, since the slower phase of inactivation appears to involve the modification of approximately one residue whose integrity is essential for enzymic activity then clearly the total biphasic inactivation process should be accompanied by the modification of more than one residue. To further investigate this point, the number of amino acid residues involved in the inactivation

process was determined using [^{14}C]NEM.

5.3.2.2. The stoichiometry of NEM modification

Determination of the number of residues modified by NEM on each subunit of sheep liver pyruvate carboxylase was carried out using homogeneous enzyme of specific activity 30.0 enzyme units per mg of protein. [^{14}C]NEM (3.2mM; 4.8μCi/μmole) was used in the modification procedure, and at various time intervals after the initiation of the reaction; aliquots were removed and the reaction quenched with a 30-fold molar excess of DTE. The enzymic activity remaining in these aliquots was then immediately determined. Samples of these aliquots were also removed for determination of the biotin content as described by Rylatt et al. (1977).

The remainder of the enzyme in each aliquot was precipitated with 30% trichloroacetic acid (w/$_v$) after 4mg of bovine serum albumin had been added to act as a carrier protein. The precipitates were collected by centrifuging and then washed exhaustively in 10% trichloroacetic acid until the radioactivity in the supernatant had been reduced to background levels. The denatured protein was then dissolved in formic acid (v/w), aliquots applied to 4 x 4 cm paper square, dried and the radioactivity determined using toluene scintillant as described in section 2.2.6.

Sheep liver pyruvate carboxylase has been shown to be a tetramer (Chapter 3) containing four molecules of biotin and it is therefore assumed to have four catalytic sites. Furthermore, since there is no definite procedure of sufficient sensitivity for measuring absolute protein concentrations it seemed more precise to express the stoichiometric relationship in terms of biotin content and hence by inference, active sites.

Fig 5.8A presents a plot of the residual enzymic activity versus the number of molecules of [^{14}C] NEM incorporated per active site, while fig 5.8B provides a comparison of the loss of enzymic activity with time of modification during the same experiment.

A meaningful interpretation of the data presented in fig 5.8 is difficult and clearly open to a number of alternative explanations. However, it is clear that the modification of pyruvate carboxylase with NEM results in the incorporation of greater than one molecule of[^{14}C]NEM per active site.

Further analysis of this data requires the assumption that the rates of modification of the amino acids responsible for each phase of inactivation of the enzyme be similar to the calculated rates of inactivation of the enzyme during these two phases. This is not necessarily true. Clearly the rate of modification of an

amino acid in an enzyme may proceed at a velocity greater than the resultant loss of catalytic activity. In fact, it has been concluded from the data presented in fig 5.5 that a rate limiting step is involved in the slow phase of inactivation of pyruvate carboxylase by NEM, and two possible explanations for this effect have been presented (section 5.3.2.1.) Scheme 1 is of relevance to this discussion. This scheme predicts that the modification of the amino acids in the slow phase of inactivation occurs at a faster rate than the inactivation rate of the enzyme. Therefore, if this scheme was valid any conclusions drawn from the data presented above would be of questionable significance.

To further interpret the data presented in fig 5.8A it would therefore be necessary to obtain values for the rates of modification of the amino acids involved in the two phases of inactivation of the enzyme. This could be achieved by isolating the isotopically-labelled peptides obtained from enzyme modified to different extents with [^{14}C] NEM. Quantitation of the extent of incorporation of the radioactivity into each of these peptides, when plotted as a function of time would reveal the rates of modification of the peptides, and hence the modified amino acids. However, to apply such an approach to an enzyme such as pyruvate carboxylase, on which protein chemical studies have barely begun, could result in misleading answers. In fact, the conclusions drawn from

the studies presented in Chapter 8 suggest that this would almost certainly have been the case.

Therefore, the only conclusion that can be drawn with any certainty from the studies presented above is that during the biphasic inactivation of sheep liver pyruvate carboxylase produced by NEM, more than one amino acid residue is modified.

5.3.2.3. The effect of substrates on the rate of inactivation of the enzyme by NEM

The effects of substrates at saturating concentrations on the rate of inactivation of the enzyme by NEM (0.25mM) are shown in fig 5.9. Oxaloacetate (10mM) protected against the initial fast phase of inactivation while Mg^{2+} (7mM) protected only against the slower phase. $MgATP^{2-}$ (7mM Mg^{2+}; 2.5mM ATP^{4-}) also protected against this slower phase of inactivation. However, since Mg^{2+} alone protected marginally better than $MgATP^{2-}$ it was considered more likely that the catalytically important residue modified by NEM during this slow phase was in the free divalent metal ion binding site. In separate experiments it was shown that neither bicarbonate, pyruvate nor ATP^{4-} afforded any protection against the loss of activity. These results may be interpreted to suggest that NEM modified essential residues in each of the partial reaction subsites of the enzyme.

Protection was also observed with acetyl CoA (0.25mM). This effect may be explained in terms of the existing knowledge regarding the interaction of acetyl CoA with the enzyme:

Firstly, studies on the effects of various segments of the acetyl CoA molecule on the two partial reactions catalysed by pyruvate carboxylase have enabled Clements et al. (1978) to propose that acetyl CoA is oriented with the nucleotide end of the molecule in the vicinity of the $MgATP^{2-}/HCO_3^-$ -binding site whilst the acetyl moiety influences events occurring in the pyruvate binding site. Secondly, Hudson et al. (1975) noted that acetyl CoA partially protected against modification of the keto acid binding site of the enzyme by the affinity label, 3-bromopyruvate. Finally, Duc (1978) was able to demonstrate that Mg^{2+} affected the nuclear magnetic resonance spectral position of the 3'-phosphate on the ribose ring of acetyl CoA. This suggested that the "free" Mg^{2+} was probably chelated to this phosphate group. Thus, the strong protection afforded by acetyl CoA against NEM may be viewed as a dual protection of both the keto acid and Mg^{2+} binding sites.

The data presented above could be interpreted to favour the hypothesis that NEM is acting as an affinity label of the biotin prosthetic group, as suggested in section 5.3.2.1., since the currently held mechanism of

action of the enzyme requires that biotin has a binding site in each of the two subsites. It was not possible to test this hypothesis under the conditions described for the chemical modifications given above due to the low solubility of biotin in Buffer A. However it was found that in 0.5M potassium phosphate, pH 7.2, biotin was soluble enough for these experiments to be performed. The results of inactivating sheep liver pyruvate carboxylase with 0.5mM NEM in this buffer in the presence of either 20mM or 100mM biotin are given in fig 5.10. Surprisingly, the presence of biotin increases the rate of inactivation of the enzyme, though in all cases the inhibition remained biphasic. This result would appear to negate the possibility that NEM was acting as an affinity label of the biotin prosthetic group. Furthermore, the high concentration of the buffer used would suggest that the effect observed was not due to alterations of the ionic strength of the buffer. In order to explain this effect it was necessary to postulate that the binding of free biotin to the enzyme changes the environment of the residues in the immediate vicinity of the covalently bound biotin prosthetic group, and so makes these residues more accessible to NEM. In addition, without a more detailed investigation of this effect, it is not possible to know whether the residues modified under these conditions are the same residues as those modified under the conditions described in other sections of this chapter.

5.3.2.4. The effect of NEM modification on the isotopic exchange reactions

In order to test the hypothesis that NEM modified residues in both of the partial reaction subsites of sheep liver pyruvate carboxylase the relative rates of the isotopic exchange reactions catalysed by NEM modified enzyme were determined. Table 5.2 indicates that the isotopic exchange rates of either [^{14}C]ADP or [^{32}P]orthophosphate into ATP and of [^{14}C]pyruvate into oxaloacetate were reduced to levels very similar to the reduction in the overall reaction rate.

This result is therefore consistent with the conclusion that NEM modified residues in close proximity to the two partial reaction subsites on the enzyme.

5.3.2.5. Identification of the residues modified by NEM

To determine which amino acid residues were being modified during the inactivation process homogeneous sheep liver pyruvate carboxylase (specific activity, 28.1 enzyme units per mg) was modified using [^{14}C]NEM (4.8μCi/μmole) at a concentration of 3.0mM.

The modification process was allowed to proceed until the enzyme was inactivated to 50% of the original activity. The reaction was then terminated using a 30-fold excess of DTE and the residual radioactivity not covalently bound to the protein was removed by dialysis. The protein was then digested enzymatically by the method described in section 5.2.5. and aliquots were applied to Whatman 3MM paper. Chemically prepared samples of NEM-cysteine (section 5.2.2.) and NEM-lysine (section 5.2.3.) were also applied to the paper to act as markers. The chromatography was performed in the solvent system n-butanol : pyridine : acetic acid : water (15:10:3:12:v/v/v/v) (section 5.2.4.) and after drying, the NEM-cysteine and NEM-lysine markers were located by spraying with 0.5% ninhydrin in acetone. The lanes containing the radioactive sample were cut into 1cm pieces and the radioactivity determined as described in section 2.2.6. Both [^{14}C]NEM-cysteine and [^{14}C]NEM-lysine were detected (fig 5.11).

While cysteine modification was expected, the appearance of a modified lysine residue was more unusual. The reaction of NEM with the ε-amino group of lysine would require that this side chain amino group be nucleophilic and therefore in an unprotonated form. However, in an aqueous environment, the pk_a of this group is 10.5 (White et al., 1968). Thus the environment around this lysine residue must enable deprotonation of the ε-amino

group and so facilitate the rapid reaction observed. Such a situation would exist if the lysyl residue was in a hydrophobic region, where the dielectric constant is low.

Heitz et al. (1968) have described the use of maleimides of increasing alkyl chain length as probes of the micro environment of modifiable residues in enzymes. These authors concluded that if the second order rate constant of the inactivation process increased with increasing alkyl chain length then the amino acid residue being modified was in an hydrophobic environment. The converse would imply either a polar or hydrated region around the amino acid residue or that the increased size of the reagent retarded access of it to the residue.

Therefore if the lysine residue detected in this present study was in an hydrophobic environment then a more hydrophobic chemical modifier should react with this amino acid residue at a faster rate. To test this hypothesis the inactivation of sheep liver pyruvate carboxylase in the presence of NBM was examined.

5.3.2.6. Modification of the enzyme with N-butyl maleimide

The results of modification of pyruvate carboxylase with NBM are shown in fig 5.12. The

inactivation profiles were similar to those obtained using NEM in that they were biphasic. Furthermore, accurate analysis of the first phase was again difficult due to the velocity of the reaction but, at least qualitatively, the data suggested a slower reaction than was observed with NEM. A tentative conclusion drawn from this data was that the residue(s) modified during the first phase of inactivation was in either a hypophilic region or that it was sterically less accessible to NBM than to NEM.

The second phase was more amenable to analysis (fig 5.13). The pseudo first order rate constants for this phase were obtained as described in section 5.3.2.1. for the NEM modification and, when plotted against the respective concentrations of NBM gave a non-linear profile (fig 5.14). However, below 0.025mM NBM, a linear relationship was obtained giving a second order rate constant of $2.3 \pm 0.3 \times 10^{-1}$ mM^{-1} min^{-1}. This value was 4.4 times faster than the second order rate constant obtained for the slower phase of the NEM modification, indicating that the environment around the residue modified during the slow phase of inactivation was probably hydrophobic. Furthermore when the pseudo first order rate constants (k') obtained for the three lowest concentrations of NBM were plotted in the form log k' versus log of the concentration of the inhibitor (section 5.3.2.1.) a line of slope 0.83 ± 0.05 was

obtained (fig 5.15). This suggested that an average of approximately one molecule of NBM was involved in binding to each enzyme active site during the inactivation process and was therefore in agreement with similar findings from the slower phase of inactivation of the enzyme by NEM (section 5.3.2.1.). It therefore appears reasonable to suggest that a single catalytically important residue is modified during the slow phase of inactivation of sheep liver pyruvate carboxylase by either NEM or NBM and that this residue is in an hydrophobic environment. On the other hand the environment around the residue(s) modified during the fast phase of inactivation of the enzyme by either NEM or NBM does not appear to be hydrophobic.

On the basis of these observations and the fact that the lysine residue modified by NEM would need to be in an hydrophobic region to be reactive, it can be inferred that the modification of this lysine occurs during the slow phase of inactivation of the enzyme. In addition it is possible that this lysine residue may be the one catalytically important residue that is modified during the slow phase of inactivation of enzyme by either NEM or NBM.

5.3.3. Discussion

The inactivation of sheep liver pyruvate

carboxylase in the presence of NEM occurs in two discrete phases and results in the modification of more than one amino acid residue. Kinetic analysis of the slower phase of inactivation suggested that only one catalytically important amino acid residue per active site was involved in the loss of enzymic activity associated with this phase. Furthermore, NBM reacted 4.4-fold faster during this second phase than did NEM and hence it was concluded that the essential residue modified during this slower phase was in an hydrophobic environment. An explanation for the appearance of a NEM-modified lysine therefore became apparent since an hydrophobic environment could facilitate the formation of the unprotonated form of the ε-amino group, and hence explain the hyper-reactivity of this amino group with NEM. On this basis, the modification of the lysine residue was proposed to occur during the slow phase of inactivation of the enzyme by either NEM or NBM.

If this were the case than a number of implications follow:

(1) Mg^{2+} and $MgATP^{2-}$ protect against the slow phase of inactivation of the enzyme by NEM and therefore may protect against modification of the lysine residue. The presence of two other lysine residues has been detected in

the enzyme. However the rate of inactivation of the enzyme by TNBS (Ashman et al., 1973) is not retarded by Mg^{2+} (Duc, 1978) and therefore it is unlikely to be the same lysine residue as that detected in this study. The lysine residue modified by Mg-oATP (Easterbrook-Smith et al., 1976a) is also considered not likely to be the same amino acid as that modified by NEM since, in this present study, Mg^{2+} protected marginally better than did $MgATP^{2-}$. This would not be expected if this lysine residue was in the binding site for $MgATP^{2-}$. The observed protection by $MgATP^{2-}$ in this study may be due to the excess free Mg^{2+} in the solution which is necessary to maintain ATP^{4-} fully complexed as $MgATP^{2-}$. Therefore, there may be yet another reactive lysyl residue in the enzyme.

(2) The locus of this reactive lysyl residue may be in the Mg^{2+} binding site. This follows from the work of Duc (1978) who found that Mg^{2+} had no effect on the circular dichroism spectrum of pyruvate carboxylase and therefore concluded that Mg^{2+} did not induce a significant alteration to the conformation of the enzyme upon binding. This would imply

that the binding of Mg^{2+} to the enzyme had a direct effect on the accessibility of NEM to the lysine residue rather than inducing a "protective" conformational change.

However, in contrast to the work of Duc (1978), Bais and Keech (1972) suggested that Mg^{2+} did induce a conformational change to the enzyme upon binding. This conclusion was based on the observation that the fluorescence of the 1-anilinonapthalene-8-sulphonate (ANS)-enzyme complex decreased in the presence of Mg^{2+}. An explanation for these apparently contradictory conclusions from the two different approaches used by Duc (1978) and Bais and Keech (1972) can be obtained from consideration of the properties of ANS. This compound is known to bind preferentially to hydrophobic regions on the enzyme and the intensity of fluorescence emission is related to the polarity of the environment (Aoe et al., 1970). Thus, if the Mg^{2+} binding site was hydrophobic, then ANS would be likely to bind to this site and so fluoresce. Upon binding of the Mg^{2+} ion to this hydrophobic binding site an increase in the polarity of the surrounding environment would be likely to occur and so, this would be

reflected in a decrease in the intensity of the ANS fluorescence. Alternatively the Mg^{2+} may displace the ANS from the Mg^{2+} binding site. Either possibility would be compatible with the results of both Duc (1978) and Bais and Keech (1972), and with the hypothesis that the Mg^{2+} binding site is hydrophobic in character. Thus if there was no change in the conformation of the enzyme upon binding of Mg^{2+}, the protection by this ion against the slow phase of inactivation would reflect direct protection by Mg^{2+} against modification of the essential amino acid and hence, by inference, the lysine residue.

(3) If the residue modified during the slow phase of inactivation is in an hydrophobic environment then an explanation for the progressive decrease in the values for the second order rate constants observed with increasing concentrations of either NEM or NBM may be provided. The essential point here is that both NEM and NBM are apolar reagents and therefore may bind to the hydrophobic site of the enzyme containing the reactive residue. If this was the case then as the concentration of the maleimide was increased, the hydrophobic site on the enzyme would become progressively more saturated with

the maleimide. Therefore the rate of inactivation of the enzyme would become dependent only on the rate of modification of the essential residue and independent of the maleimide concentration. Thus the inactivation process would approach zero order with respect to the maleimide concentration. Scheme 2, in section 5.3.2.1. illustrates this effect diagramatically, and appears more plausible than scheme 1 in the light of the experimental data presented.

(4) The proposed hydrophobic character of the Mg^{2+} binding site is of relevance to another property of the enzyme, namely dilution inactivation. Ovine pyruvate carboxylases undergo an irreversible loss of activity when diluted to below 4 enzyme units per ml. (Ashman et al., 1972). An extensive study of this phenomenon has recently been conducted in this laboratory by Clements et al. (1978). These workers found that dilution inactivation of the enzyme could be retarded, and under some conditions completely prevented, by decreasing the polarity of the solution into which the enzyme was diluted. Furthermore acetyl CoA or the adenosine moiety of that molecule also retarded dilution inactivation

of the enzyme. Furthermore since these investigators also showed that the adenosine moiety of acetyl CoA also influenced events occurring at the $MgATP^{2-}/HCO_3^-$ binding site on the enzyme it was proposed that dilution inactivation occurred via hydration of the first partial reaction site. It was envisaged in this model that the adenosine moiety of acetyl CoA functioned in retarding dilution inactivation by "capping" the $MgATP^{2-}/HCO_3^-$ binding site and so maintaining a pre-existing hydrophobic environment within this site. Now, since Duc (1978) has demonstrated that Mg^{2+} can chelate to the 3' phosphate on acetyl CoA, and the results presented in this section suggest that this Mg^{2+} ion may bind to a hydrophobic environment, then it is possible that the Mg^{2+} ion binding site on the enzyme is close to the first partial reaction site and that both of these ligand binding sites are in one hydrophobic region in the tertiary structure of the enzyme.

The function of the lysine proposed to be in the Mg^{2+} binding site is difficult to ascertain from the data presented. It may not perform a function in the binding of the Mg^{2+}-acetyl CoA complex and the observed inhibition of the enzymic activity during this

phase of inactivation may result only from the reduced ability of Mg^{2+} to bind to the sterically hindered site. To function as a residue involved in the binding of the Mg^{2+}-acetyl CoA complex it would seem probable that the ε-amino group of this residue would need to be ionized. The binding of Mg^{2+} in the near vicinity may provide an opportunity for this ionization to occur. As inferred from the circular dichroism studies of Duc (1978) and the ANS-enzyme fluorescence studies of Bais and Keech (1972) a change in the polarity of the Mg^{2+} binding site may occur following the binding of Mg^{2+}. This could result in an increase in the pk_a of the reactive lysyl-residue and hence an increase in the relative amount of this residue in the ionized ($-NH_3^+$) form. This ionized form of the lysine residue may then interact with one of the anionic oxygen atoms on the adenosine moiety of acetyl CoA. This situation is represented diagramatically in fig 5.16.

The results presented in the preceding sections of the chapter have provided only circumstantial evidence that the modification of the lysine residue occurs during the slow phase of inactivation of the enzyme. Direct proof of this could possibly have been obtained from quantitation of the extent of modification of the lysine with [^{14}C]NEM in the presence and absence of Mg^{2+}. Unfortunately, time did not permit this experiment to be performed. Nevertheless, this omission does not affect

the postulate that Mg^{2+} binds to an hydrophobic region in the enzyme.

From the fast phase of inactivation of the enzyme by NEM little information can be gained. The reaction occurs at too high a velocity to enable reliable estimation of pseudo first order rate constants and hence no kinetic analysis of this phase of inactivation can be obtained. However from the protective effect of oxaloacetate it is possible to suggest that a reactive residue(s) occurs in or near the keto acid binding site or that oxaloacetate induces a conformational change upon binding to the enzyme which results in protection against inactivation of the enzyme. The fact that the exchange of [^{14}C]pyruvate into oxaloacetate is reduced to a level similar to the reduction in the overall reaction is consistent with this suggestion. The modification of a cysteine residue(s) during the inactivation of the enzyme raises the possibility that this residue(s) could be either one or both of the residues modified by 3-bromopyruvate and/or 3-chloroacetonyl CoA (section 5.1) and therefore perhaps occur during the fast phase of inactivation. The investigation of the reactivity and function of cysteine residues in sheep liver pyruvate carboxylase was therefore approached through the use of the cysteine-specific chemical modifier DTNB.

5.3.4. Chemical modification of pyruvate carboxylase with DTNB

DTNB reacts with thiol groups through the energetically favourable formation of a mixed disulphide (Ellman, 1959). Since this reaction in proteins can only occur with cysteine residues, DTNB represents a truly group-specific chemical modifier. The reaction of this reagent with pyruvate carboxylase should therefore afford a more specific modification of the cysteine residues in the enzyme without concomitant modification of other amino acid residues. This approach may lead to a more complete understanding of the role of these residues in the functioning of the enzyme.

5.3.4.1. Inactivation of pyruvate carboxylase by DTNB

The inactivation profiles of pyruvate carboxylase in the presence of DTNB in the concentration range of 0.02mM to 1.0mM are shown in fig 5.17. The inactivation process followed pseudo first order kinetics with respect to time and the replot of the determined pseudo first order rate constants (k') against the respective concentration of DTNB is shown in fig 5.18. Below a concentration of 0.2mM, DTNB a linear relationship for this replot was observed, giving a second order rate constant of 2.1 ± 0.1 mM^{-1} mms^{-1}. However, above this concentration

a deviation from linearity occurred, suggesting that a rate limiting step was involved in the process leading to the inactivation of the enzyme in the presence of DTNB. Two possible schemes whereby such an effect could occur have been suggested previously (section 5.3.2.1.). A distinction between these two modes of inactivation cannot be made from the data presented above although evidence presented in latter sections of this chapter allows some speculation on this point.

The number of molecules of DTNB binding to each active site of the enzyme during the inactivation process was determined from a plot of log k' versus log concentration of DTNB (section 5.3.2.1.) (fig 5.19). The four lowest concentrations of DTNB gave a linear relationship with a slope of 0.88 ± 0.06. This result suggests that only approximately one DTNB molecule was required to inactivate each enzyme active site. One interpretation of this result is that DTNB modifies only cysteine residue in each active site, whose integrity is essential for enzymic activity. However, there are other interpretations of this result and these are given in later sections of this chapter.

5.3.4.2. The stoichiometry of DTNB modification

The number of sulphydryl residues modified by DTNB during the inactivation process was determined

using homogeneous enzyme of specific activity 25.0 units per mg. DTNB was added to give a final concentration of 0.107mM and the reaction was followed by monitoring, at various time intervals, the appearance of the TNB anion by measuring the absorbance at 412nm. The number of moles of cysteine reacting was calculated using the extinction coefficient for the TNB anion of 14050 $M^{-1}cm^{-1}$ determined in Buffer A and expressed relative to the number of moles of biotin in the solution (section 5.3.2.2.). Simultaneously, aliquots of the reaction solution were removed and their residual enzymic activity immediately determined. The data obtained is shown in fig 5.20. The initial phase of the graph was linear and extrapolated to a value of 1.3 moles of cysteine modified per mole of biotin with complete loss of activity. The departure from linearity may suggest that the initial modification of the enzyme caused exposure of other sulphydryl residues which could react with DTNB.

The fact that complete loss of enzymic activity is accompanied by the modification of 1.3 cysteine residues per active site may be compatible with the model proposed by Clements (1977) (section 5.1.). This model envisaged two essential cysteine residues in close proximity in the binding sites of acetyl CoA and the keto acids. If this was the case then the modification of any one of these two cysteine residues with DTNB

would result in reducing the accessibility of a second molecule of DTNB to the unmodified cysteine residue. This would give rise to a stoichiometry of greater than one residue modified per active site since the second cysteine residue may still react with DTNB, but at a much reduced rate. Furthermore, since the model proposes that both cysteine residues are essential for the acetyl CoA-dependent activity of the enzyme, then the modification of either of these would result in loss of enzymic activity when assayed using the acetyl CoA dependent assay. This interpretation would provide an explanation for the observation that approximately one molecule of DTNB is involved in the inactivation of each enzyme active site.

An alternative explanation is that there is only one essential cysteine residue and that the slightly larger number of cysteine residue detected in this experiment was due to minor modification of other cysteine residues.

If the former explanation was correct then protection against the inactivation would be provided by either the keto acids or acetyl CoA. Thus the effect of various reaction components on the rate of inactivation of the enzyme in the presence of DTNB was determined.

5.3.4.3. Effect of substrates on the rate of inactivation of the enzyme by DTNB

The effect of saturating concentrations of the ligands oxaloacetate (10mM), $MgATP^{2-}$ (Mg^{2+}, 7mM; ATP^{4-}, 2.5mM) and Mg^{2+} (7mM) on the loss of the catalytic activity of the enzyme in the presence of 0.1mM DTNB was determined. Fig 5.21 shows that, of these ligands, only oxaloacetate afforded significant protection. This observation was consistent with the interpretation of the data obtained from the study presented earlier using NEM (section 5.3.2.3.), that a sulphydryl residue is present in or near the keto acid binding site on the enzyme. The protection afforded by oxaloacetate was examined in greater detail using a range of oxaloacetate concentrations from 0.2mM to 15.0mM (fig 5.22). Using this data the second order rate constant for the reaction between DTNB and the enzyme-substrate complex, k_m, and the dissociation constant, K_D, for this complex can be calculated from the relationship derived by Scrutton and Utter (1965b).

$$\frac{k^a}{k^o} = \frac{k_m}{k^o} - \frac{K_D}{[\text{modifier}]}\left(\frac{k^a}{k^o} - 1\right) \quad \ldots (5.3)$$

Here k^a and k^o are the fractional order rate constants in the presence and absence of a given concentration of substrate respectively and may be derived from

equation (1) given in section 5.3.2.1. A plot of $\frac{k^a}{k^o}$ versus $-\left(\frac{k^a}{k^o} - 1\right)\Big/[\text{modifier}]$ will have a slope of K_D and an intercept on the ordinate of k_m/k^o from which k_m can be calculated.

Fig 5.23 presents the data obtained from fig 5.22 in this form. The dissociation constant, K_D, for the enzyme-oxaloacetate complex was 0.33 ± 0.05mM, in the same range as the value of 0.12mM determined by Ashman and Keech (1975) from product inhibition data using sheep kidney enzyme in 0.1M tris-Cl, pH 8.4. The second order rate constant, k_m, for the reaction of DTNB with the enzyme-oxaloacetate complex was 0.72 ± 0.04 mM^{-1} min^{-1}. This rate was only three-fold slower than the rate of inactivation of the enzyme in the absence of oxaloacetate and so reflected a fairly poor protection by oxaloacetate against the inactivation process.

Acetyl CoA was also found to protect against DTNB modification of the enzyme. Fig 5.24 shows the degree of inactivation produced by DTNB in the presence of acetyl CoA concentrations in the range 25μM to 200μM. The pseudo first order rate constants obtained from this data were inserted into equation 5.3 and the values obtained replotted (fig 5.25) in the manner described above. The dissociation constant, K_D, for

the enzyme-acetyl CoA complex was found to be 96 ± 3μM. The value obtained by Duc (1978) for this same dissociation constant using TNBS modification of the sheep liver enzyme in 0.1M tris-Cl, pH 8.4, was 27μM. The difference between the values obtained for this parameter in these two studies probably reflects the influence of the different buffer components and pH values on the ability of the enzyme to bind acetyl CoA. The second order rate constant for the reaction of DTNB with the enzyme-acetyl CoA complex was found to be 0.25 ± 0.02$mM^{-1}min^{-1}$ indicating that acetyl CoA did not completely protect against the inactivation of the enzyme by DTNB. This rate of inactivation is eight-fold slower than the rate of inactivation in the absence of acetyl CoA, indicating that acetyl CoA provides significantly better protection against the inactivation of the enzyme by DTNB than does oxaloacetate. These results are qualitatively similar to the results obtained by Hudson (1974) and Clements (1976) for the modification of the enzyme by 3-bromopyruvate and 3-chloroacetonyl CoA respectively.

Furthermore these results are consistent with the proposition that there is a reactive cysteine residue in both the keto acid and acetyl CoA binding sites (Clements, 1977). However, they would be equally consistent with the hypothesis that there is only one essential cysteine residue which may be close to both

the keto acid and acetyl CoA binding sites but is not located in either site.

In an effort to distinguish between these two possibilities, the combined effect of saturating concentrations of acetyl CoA and oxaloacetate on the rate of inactivation of the enzyme by DTNB was examined. It was reasoned that if there was only one cysteine residue being modified and that this residue was not located in either of the acetyl CoA or oxaloacetate binding sites on the enzyme then a decrease in the rate of inactivation of the enzyme in the presence of both of these ligands may not occur. Fig 5.26 illustrates that this was not the case. In fact the combined effect of both of these ligands was to completely protect against inactivation of the enzyme by DTNB.

This result does not allow an unequivocal distinction between the two possibilities given above to be made. The complete protection against the inactivation of the enzyme by DTNB in the presence of oxaloacetate and acetyl CoA could arise from either:

(1) Protection against modification of two cysteine residues located in the oxaloacetate and acetyl CoA binding sites respectively.

(2) Conformational alterations to the enzyme induced by the binding of both oxaloacetate and acetyl CoA which both partially protect an essential cysteine residue and which together fully protect against the modification of this important residue.

These two possibilities are discussed later in this chapter in the light of the evidence obtained from the affinity labelling studies described in section 5.1.

5.3.4.4. Cyanylation of the DTNB-modified enzyme

One advantage of using DTNB as a chemical modifier of sulphydryl groups in proteins is that the bulky TNB adduct can be specifically replaced by smaller nucleophiles such as the cyanide ion, to yield modified cysteine residues which exhibit very little steric hindrance (fig 5.27). This "cyanylation" reaction allows a distinction to be made between loss of catalytic activity resulting from steric hindrance afforded by the cysteine adduct and the situation where the nucleophilicity of the cysteine sulphydryl group is required for the catalytic process.

Vanaman and Stark (1970) first used the cyanylation reaction to investigate the sulphydryl

residues of the catalytic subunit of aspartate transcarbamylase. These authors found that DTNB inhibited the catalytic activity of this enzyme but that upon replacement of the TNB group with cyanide, complete recovery of activity was obtained. It was therefore concluded that the sulphydryl residue in the catalytic subunit of aspartate transcarbamylase was not involved in forming transient-state intermediates.

The technique of cyanylation was adopted in this present study as a means of assessing the relationship between the essential cysteine residue(s) and the catalytic process. The enzyme was inactivated with 0.01mM DTNB and, following 29.5% loss of activity excess DTNB was removed by gel filtration on a Sephadex G-25 column (1 x 15cm), previously equilibrated in Buffer A. The fractions collected from the column, containing the partially modified enzyme, were combined. To an aliquot of this TNB-enzyme was added sodium cyanide to a concentration of 1.0mM and aliquots were removed at various time intervals for enzymic activity determinations. The activity of the enzyme was rapidly restored and became stable after approximately ten min. The recovery of activity was expressed relative to the original activity by assuming that since the inactivation of the enzyme was kept at a low rate, the reaction was terminated instantly upon application to the Sephadex G-25 column. Thus the activity of the pooled TNB-

enzyme after gel filtration was taken at 71.5% of the original activity. As can be seen from fig 5.28 approximately 10% of the original activity was recovered following cyanylation. To establish whether the incomplete recovery of activity observed was due to partial inhibition of the enzyme by the cysteine-adduct, an aliquot of TNB-modified enzyme was incubated with 1mM DTE. Under these conditions complete reduction of the mixed disulphide would occur, regenerating the native, reduced cysteine residue. The recovered activity was essentially identical to the cyanide-treated enzyme, indicating that the incomplete recovery of the original enzymic activity was not due to partial inhibition of the enzyme by the cyano-derivative.

This result clearly illustrated that the nucleophilicity of the thiol groups on the cysteine residues were not required for maximal expression of the activity of sheep liver pyruvate carboxylase. The failure to observe complete recovery of enzymic activity in the thiol-treated sample indicated that an irreversible process had occurred during or following the modification of the enzyme with DTNB. The implications of this result are discussed in detail in the following section.

5.3.5. Discussion

The kinetic analysis of the rates of

inactivation of pyruvate carboxylase in the presence of DTNB revealed that the inactivation was accompanied by the binding of one molecule of inhibitor per enzyme active site and that the inactivation process involved a rate limiting step. The nucleophilicity of the cysteine thiol groups modified was established not to be required for the reaction catalysed by the enzyme however, since the cyano-modified enzyme exhibited the maximal potential activity. The fact that both the cyano-modified enzyme and the thiolized enzyme, in which the cysteine-TNB adduct was reduced back to the free sulphydryl group, failed to totally restore the original activity implied that some process, other than mere blocking of the cysteine thiol group was responsible for at least part of the loss of activity.

This finding had relevance to the mode of inactivation of the enzyme by DTNB. Two possible schemes whereby an inactivation process which incorporates the rate limiting step detected in the modification reaction are shown in fig 5.29. In scheme 1, DTNB is envisaged to covalently modify the enzyme, producing a TNB-enzyme complex (I) which is partially active. A subsequent conformational change, due to the addition of the TNB group to the cysteine thiol, results in a total loss of enzymic activity (II). Cyanylation or thiolysis of the TNB-cysteine adduct would result in only partial recovery of the activity of the enzyme since form II

of the enzyme has been inactivated by the conformational alteration. Implicit in this scheme is the assumption that the conformational change leading to form II of the enzyme is irreversible. Furthermore, form I of the enzyme must be partially active for this scheme to account for the experimental data. If form I of the enzyme was totally inactive then no decrease in the second order rate constant would occur at higher concentrations of DTNB. On the other hand, if this form of the enzyme was totally active then no reactivation of the enzymic activity would be observed following cyanylation of the TNB-enzyme complex. The partial inhibition of this modified enzyme form may be due to steric hindrance of some catalytic region of the enzyme.

Scheme II requires that DTNB could bind to the enzyme in a reversible manner. Following binding, the DTNB molecule could either dissociate from the enzyme surface again or react to form a covalent bond with a cysteine thiol group and so inactivate the enzyme. Thiolysis of the cysteine-TNB adduct should regenerate the native form of the enzyme and therefore total recovery of enzymic activity would be predicted. Since this result was not obtained experimentally it would appear that scheme II does not provide an adequate explanation for the events occurring during the inactivation of the enzyme. Scheme I predicts only partial restoration of enzymic activity following thiolysis of

the modified enzyme and is therefore probably a more accurate representation of the inactivation process.

The observation that the binding of one molecule of inhibitor per active site is associated with the inactivation process warrants some consideration in view of the scheme presented above. The basis on which the relationship was developed relies on the scheme shown below (Keech and Farrant, 1968).

$$E + nI \xrightarrow{k} E\,I^n$$

where E, E , n, I and k represent respectively the active form of the enzye, the inhibited form of the enzyme, the number of molecules of inhibitor involved in the reaction, the inhibitor, and the second order rate constant for the reaction. In a similar fashion the reaction proposed to occur during the inhibition of pyruvate carboxylase by DTNB can be represented as

$$E + nI \xrightarrow{k_1} E^{\dagger}I^n \xrightarrow{k_2} E\,I^n$$

Here, in addition to the terms defined above, $E^{\dagger}$, k_1 and k_2 represent the partially active form of the enzyme and the rate constants for the two processes respectively. Under conditions where the inhibitor concentration is low, the rate of formation of $E^{\dagger}I^n$ is slow relative to the rate of formation of $E\,I^n$ and so the inactivation process reduces to the same form as that used by Keech and

Farrant (1968). In this case however, the paramater, n, represents the number of inhibitor molecules which, upon covalently modifying the enzyme will subsequently give rise to inactive enzyme. Furthermore, n can be interpreted to mean that the loss of activity of pyruvate carboxylase in the presence of DTNB results from either the modification of approximately one unique cysteine residue per active site, or that the modification of more than one cysteine residue to a sum total of one molecule of inhibitor incorporated per active site is involved. While this latter possibility would seem less likely it cannot be excluded from consideration and becomes important in the following discussion in this section.

In assessing the location of the essential cysteine residue(s) in the three dimensional structure of the enzyme two factors have been considered.

(1) The protection afforded by oxaloacetate and acetyl CoA would favour the location of the cysteine residue(s) being close to the keto acid binding site. Cysteine residues have already been proposed to exist in this region of the enzyme from the affinity labelling studies using 3-bromopyruvate and chloroacetonyl CoA (section 5.1). It is therefore conceivable that DTNB could modify the same

cysteine residue(s) as the affinity labels.

(2) The conformational change proposed to occur following the modification of the cysteine residue(s) has a marked effect on the loss of catalytic activity. Such a pronounced effect would be more likely to occur if the conformational change occurred close to the active site on the enzyme, rather than being required to be transmitted from some distant region of the enzyme.

Thus it can be proposed that the cysteine residue(s) modified by DTNB are likely to be in the vicinity of the keto acid binding site and therefore, possibly the same as the cysteine residue(s) modified by 3-bromopyruvate and 3-chloroacetonyl CoA. This proposition would seem reasonable given that the enzyme is susceptible to a variety of chemical modifiers (Table 5.1) which suggests that the cysteine residue(s) are highly reactive.

The evidence presented earlier in this chapter indicated that complete inhibition of pyruvate carboxylase activity was associated with the incorporation of an average of 1.3 molecules of TNB per active site. This result was very similar to that obtained by Hudson *et al*. (1975) where 1.5 molecules of 3-bromopyruvate were incorporated per active site in the absence of

acetyl CoA. Clearly, this result indicated that more than one cysteine residue was reacting per active site. However the binding of only approximately one molecule of inhibitor was involved in the inactivation of the enzyme with DTNB. Two different explanations for this data have been presented in section 5.3.4.2. and will be reconsidered in detail here.

Model A:

This model is similar to the model proposed by Clements (1977) (section 5.1) except that no catalytic role is performed by the sulphydryl groups (as shown by the cyanylation experiments presented in section 5.3.4.4.). In it, two cysteine residues are proposed to be in close juxtaposition in the acetyl CoA and keto acid binding sites. The modification of the enzyme by 3-chloroacetonyl CoA is of particular importance to this model. Clements (1977) found that an average of 0.78 molecules of 3-chloroacetonyl CoA were bound per active site upon complete inhibition of the enzymic activity. This suggested either:

(1) 3-Chloroacetonyl CoA was a specific affinity label of the acetyl CoA binding site. In this case acetyl CoA should completely protect against the loss of enzymic activity. However, when the protective effect of acetyl

CoA was assessed using the relationship described by Scrutton and Utter (1965b) incomplete protection against the loss of enzymic activity was observed when the line fitting the data was extrapolated to infinite concentration of acetyl CoA.

(2) 3-Chloroacetonyl CoA modified the keto acid site cysteine residue exclusively or modified both cysteine residues to an extent approximately equivalent to a total of one molecule of inhibitor per active site. If chloroacetonyl CoA could modify either of the two cysteine residues then, to account for the observed stoichiometry of the reagent it appears reasonable to suggest that modification of one cysteine residue by this large reagent completely inhibits the access of a second molecule of the reagent. Therefore, in the presence of saturating concentrations of acetyl CoA complete protection against chloroacetonyl CoA should be observed since acetyl CoA would prevent access of the reagent. However, this is not observed experimentally.

Thus, both of these explanations appear to be incompatible with the assignment of a cysteine residue in the acetyl CoA binding site and therefore Model A reduces

to a postulate that predicts the presence of a reactive essential sulphydryl residue in the keto acid site and another essential sulphydryl residue in some other region of the protein. However, if this was the case an explanation for the appearance of only one molecule of the inhibitor being involved in the inactivation process would be required.

Two possibilities which could account for this fact are:

A (1) The rate of modification of one of the essential cysteines occurs at a rate significantly faster than the rate of modification of the second essential cysteine residue. In such a case the modification of the slower reacting cysteine residue would have little effect on the loss of activity and so not be detected in the kinetic determination of the number of molecules of inhibitor binding during the inactivation process.

A (2) The two cysteine residues are in close juxtaposition and the modification of one of these residues results in inactivation of the enzyme and, in addition, retards modification of the second adjacent cysteine residue. Thus, effectively, only one molecule of

inhibitor binds to the enzyme during the inactivation process.

Both of these forms of the original Model A predict that at least one of the cysteine residues is in the keto acid binding site. The two models are represented diagramatically in fig 5.30.

Model B:

This model predicts that the essential cysteine residues are not present in a substrate binding site but at least one is located close to the keto acid binding site on the enzyme (fig 5.30). In fact this model is very similar to final forms of Model A. The explanations for the fact that inactivation of the enzyme involves the binding of only one molecule of inhibitor are the same as for Model A viz.

B (1) A difference in the reactivities of the two essential cysteine residues. A limiting case in this situation is where the slower reacting cysteine residue is in fact a non-essential residue i.e. the modification of this amino acid residue has no effect on the catalytic activity of the enzyme.

B (2) The two essential cysteine residues are in

close juxtaposition in the enzyme.

The only difference between Model A and Model B is the exact location of one of the essential cysteine residues: Model A predicts that one essential cysteine residue is located in the keto acid site while Model B predicts that this amino acid is close to the keto acid site.

The protective effects of either oxaloacetate or acetyl CoA are of little use in discriminating between these two models. Model A predicts that partial protection against inactivation of the enzyme by oxaloacetate occurs through direct protection against modification of the essential cysteine in the keto acid site, while acetyl CoA affords protection against the other cysteine residue by inducing a conformational change which makes this second essential amino acid residue less accessible. In Model B both ligands induce conformational changes which result in the reduced accessibility of the essential cysteine residues.

The combined effect of acetyl CoA and either of the keto acids warrants some discussion however. As noted previously in this chapter, modification of the enzyme by 3-bromopyruvate in the presence of saturating concentrations of acetyl CoA reduces the total number of residues modified per active site from

1.5 to 1.0. This suggests that acetyl CoA completely protects against the modification of one cysteine (Hudson et al., 1975). Therefore it can be postulated that if the remaining residue not protected by acetyl CoA is in the keto acid binding site then, in the presence of saturating concentrations both acetyl CoA and either pyruvate or oxaloacetate, complete protection against loss of enzymic activity should occur. The results of Hudson et al. (1975) indicate that this prediction was not experimentally verified. The inactivation of pyruvate carboxylase in the presence of 3-bromopyruvate and saturating concentrations of acetyl CoA and either of the keto acids could be accounted for by either of two schemes:

(1) Upon binding of acetyl CoA and oxaloacetate a conformational change exposes yet another reactive amino acid residue which is essential for maximal enzymic activity and 3-bromopyruvate modified this reactive residue, causing the observed inactivation of the enzyme.

(2) The cysteine residue not protected by acetyl CoA is not in the keto acid binding site on the enzyme and is only partially protected by the conformational change induced by both of these ligands (i.e. Model B, presented above).

The observation reported in this chapter that saturating concentrations of oxaloacetate and acetyl CoA together protect completely against the inactivation of the enzyme by DTNB does not provide an unequivocal discrimination between these two possibilities. In either case it is necessary to propose that the smaller reagent, 3-bromopyruvate, is accessible to a sterically hindered reactive residue while the bulkier DTNB molecule is not.

It is therefore difficult to discriminate between Model A and Model B on the information obtained from the protection afforded by acetyl CoA and the keto acids against the inactivation of the enzyme by covalent modifiers. However, the situation envisaged in Model B is perhaps marginally more likely.

One piece of circumstantial information which has relevance to this discussion relies on the prediction that the mode of inactivation of the enzyme by DTNB occurs via the events depicted in scheme 1, described earlier. In this scheme it was concluded that form I of the modified enzyme should be partially active to account for the experimental observations. Now, if a reactive cysteine residue was in the keto acid binding site (Model A) and was modified by DTNB then the steric hindrance afforded by the resultant TNB-cysteine moiety would be likely to completely inhibit the binding of

the keto acids and so completely abolish the enzymic activity. On the other hand if a reactive cysteine residue was near to the keto acid site but not in that site (Model B), then the TNB-cysteine adduct may reduce the accessibility of the keto acids to the keto acid binding but may not completely inhibit the binding of these ligands. Therefore the enzyme would remain partially active. This would account for the prediction that form I of the modified enzyme in scheme 1 should be partially active.

Finally then, it is tentatively proposed that neither of the two cysteine residues are in either the acetyl CoA or keto acid binding sites but that at least one and perhaps both are located close to the keto acid binding site.

FIGURE 5.1

The scheme presented by Palacián and Neet (1972) showing the processes that accompany modification of chicken liver pyruvate carboxylase by sulphydryl-group reagents.

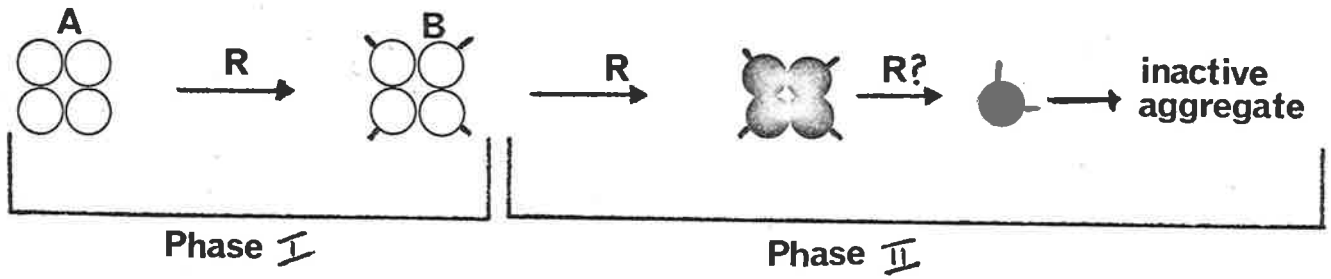
○ represents an active form of the enzyme

● represents an inactive form of the enzyme

R represents a sulphydryl-group reagent

The lines coming out of the representations of the enzyme signify the binding of the reagent.

Modification



Reactivation by Thiols

FIGURE 5.2

The model proposed by Clements (1977) to account the events occurring at the keto acid binding site on the enzyme.

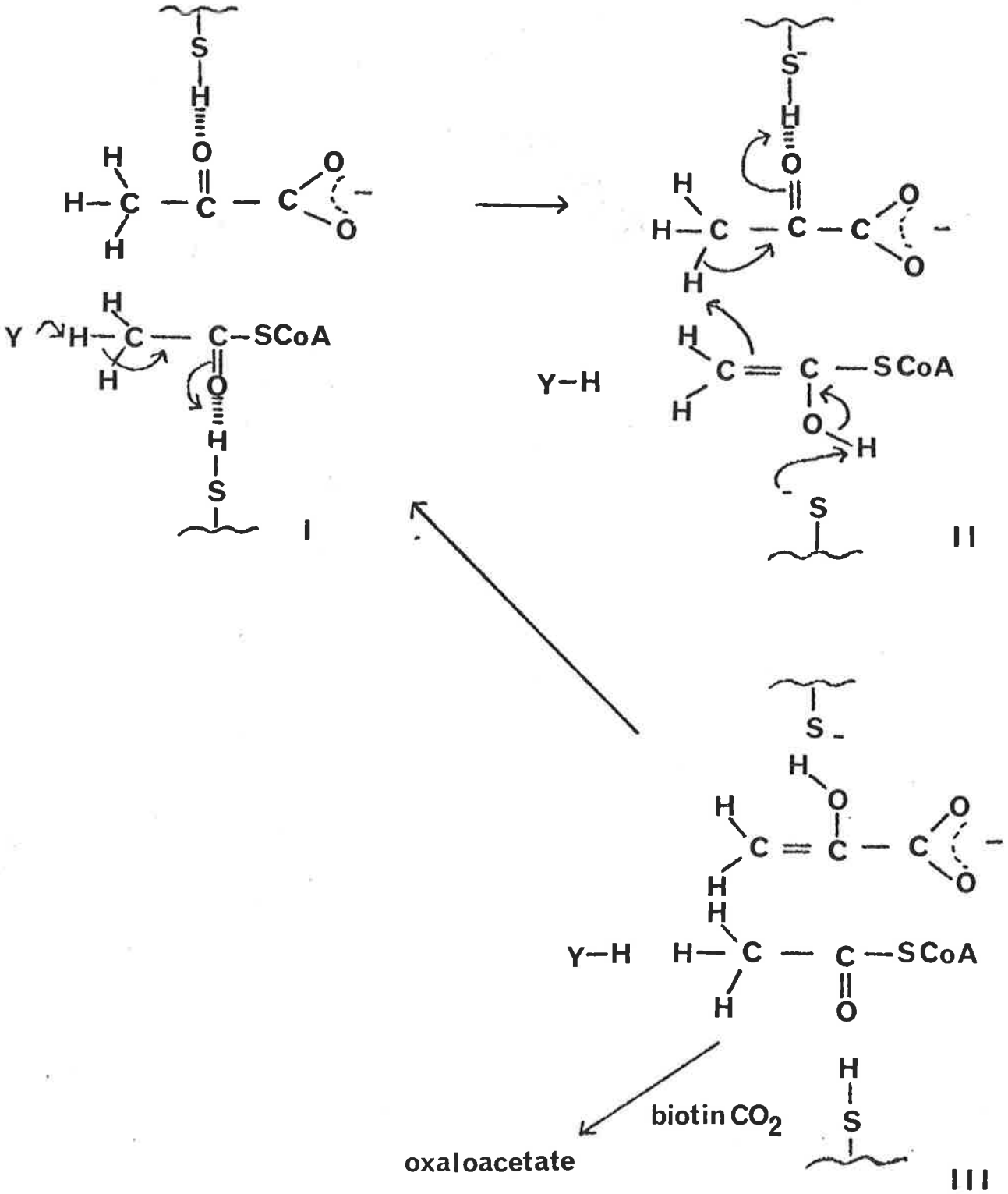

I
II
III
Y–H
biotin CO_2
oxaloacetate
+acetyl CoA
+free active site

TABLE 5.1

The effect of sulphydryl reagents on pyruvate carboxylase activity.

Pyruvate carboxylase (S.A. 14; 11 units/ml) was incubated in 0.1M Tris-Cl pH 8.4, in the presence of the chemical modifiers shown. After 10 min, aliquots were removed and the residual activity determined.

Additions	Conc^{n} (mM)	% Remaining Activity
None	-	100
N-ethylmaleimide	0.1	50.9
Iodoacetamide	10	58.0
DTNB	0.1	8.0
p-mercuribenzoate	0.1	2.0
Iodoacetate	10	74.4

FIGURE 5.3

The time dependent inactivation of pyruvate carboxylase with various concentrations of N-ethylmaleimide.

Pyruvate carboxylase (S.A. 12.6; 18 units/ml) was incubated in Buffer A (without DTE) with the concentrations of N-ethylmaleimide shown in the figure (in mM). Samples were assayed for enzyme activity remaining after the time interval designated, and expressed as the log % activity to the initial activity. In the control NEM was replaced with water.

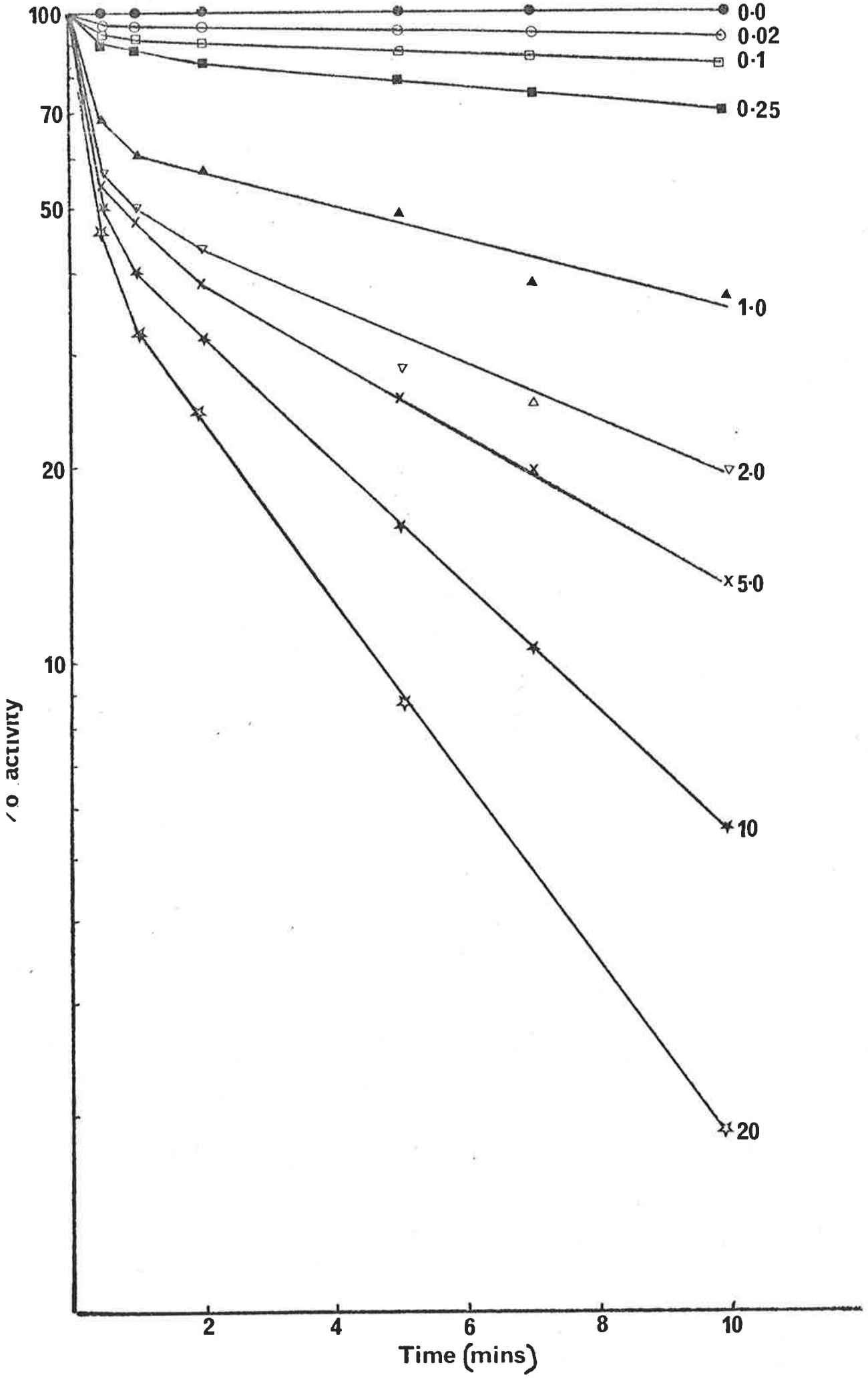

100
70
50
20
10
% activity
0·0
0·02
0·1
0·25
1·0
2·0
5·0
10
20
2
4
6
8
10
Time (mins)

FIGURE 5.4

The time dependent slow phase of inactivation of pyruvate carboxylase with various concentrations of N-ethyl maleimide.

The rate of inactivation of the enzyme at each concentration of N-ethylmaleimide was determined from the data shown in fig 5.3.

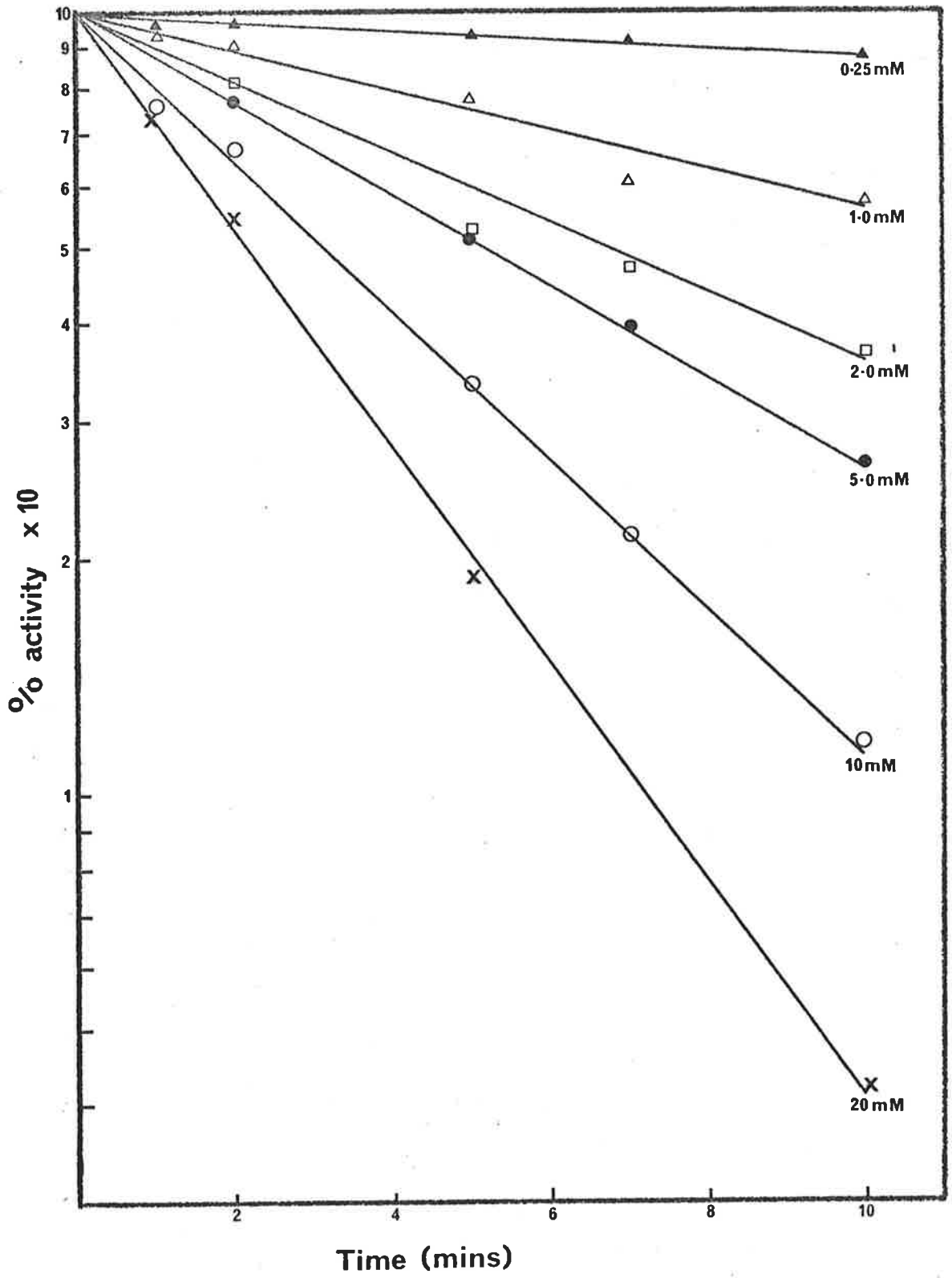

% activity x 10
Time (mins)
0·25 mM
1·0 mM
2·0 mM
5·0 mM
10 mM
20 mM

FIGURE 5.5

The pseudo first order rate constants for the slow phase of inactivation of the enzyme plotted as a function of the N-ethylmaleimide concentration. The values for k' were determined from the data presented in figure 5.4.

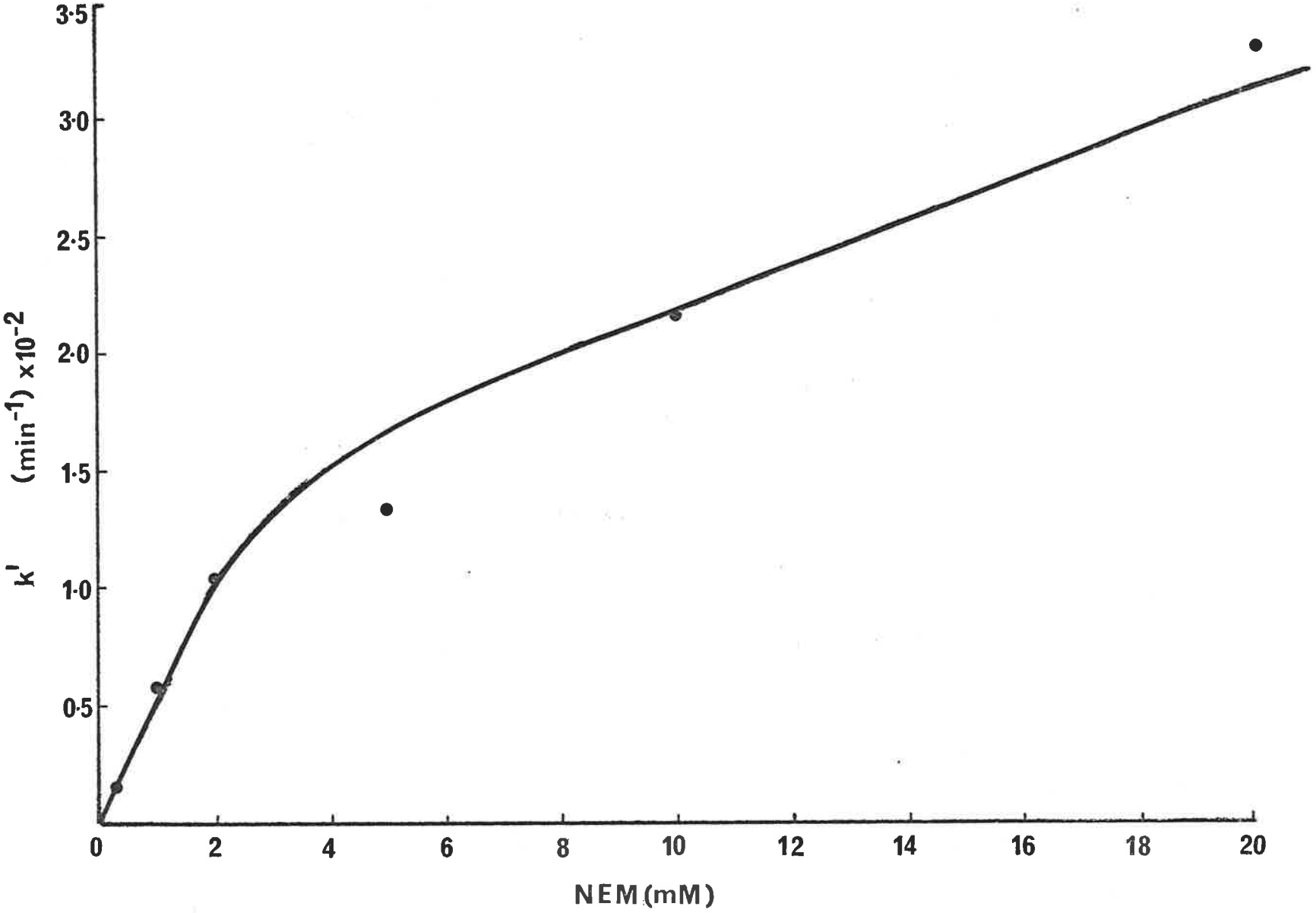
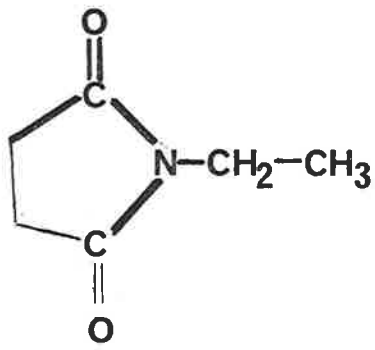
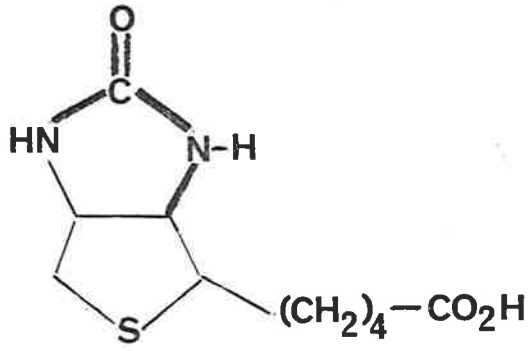

3·5
3·0
2·5
2·0
1·5
1·0
0·5
k' (min⁻¹) ×10⁻²
0
2
4
6
8
10
12
14
16
18
20
NEM (mM)

FIGURE 5.6

The structural homology of N-ethylmaleimide and biotin. The bold lines illustrate the regions of possible similarity of the structures.



N-ethyl maleimide



Biotin

FIGURE 5.7

A logarithmic plot of the data given in figure 5.5.

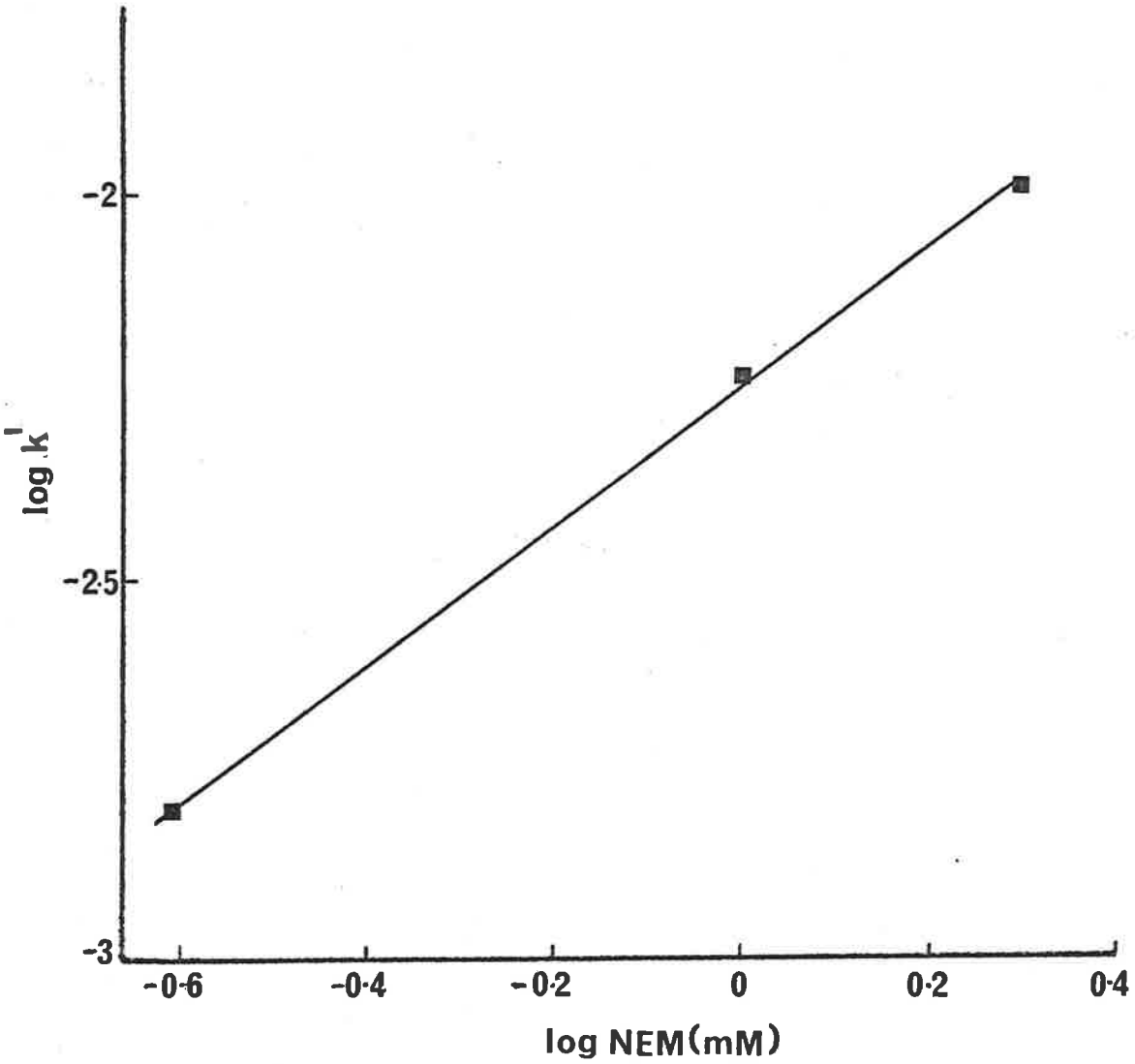

-2
-2·5
-3
log k'
-0·6
-0·4
-0·2
0
0·2
0·4
log NEM(mM)

FIGURE 5.8A, B

The stoichiometry of N-ethylmaleimide modification of pyruvate carboxylase.

Pyruvate carboxylase (S.A. 30; 42 units/ml) was incubated with [^{14}C]N-ethylmaleimide (4.8μCi/μmole; 3.2mM). The samples were removed at the time intervals indicated (figure 5.8A) and the residual activity and extent of incorporation of [^{14}C]NEM into the enzyme determined as described in section 5.3.2.2.

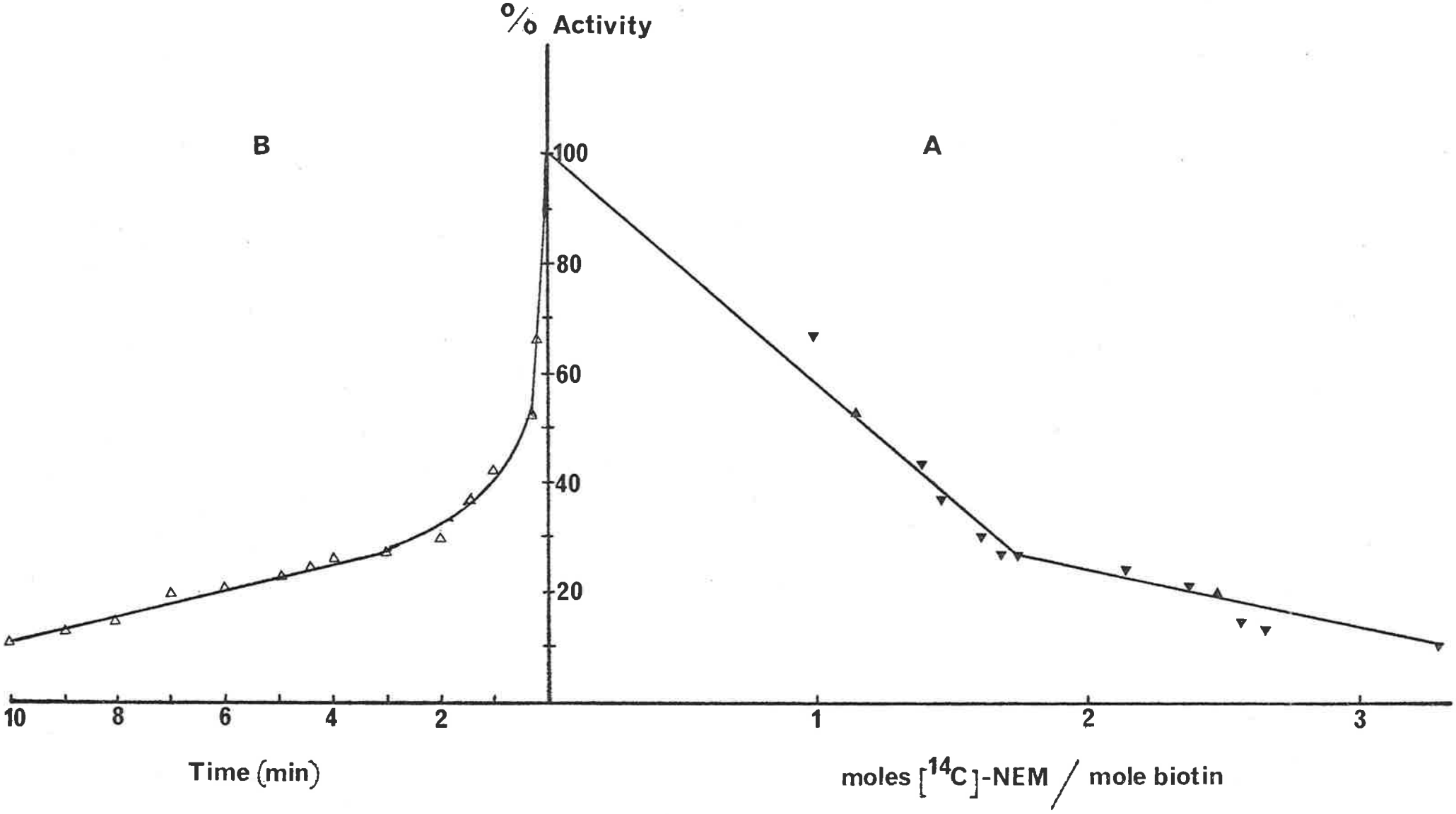

% Activity
B
A
100
80
60
40
20
10
8
6
4
2
1
2
3
Time (min)
moles [14C]-NEM / mole biotin

FIGURE 5.9

The effect of various ligands on the rate of inactivation of pyruvate carboxylase with NEM.

Pyruvate carboxylase (S.A. 12.1; 16 units/ml) was incubated with NEM (0.25mM) in the presence of the following compounds:

no additions
$MgATP^{2-}$ (7mM Mg^{2+}; 2.5mM ATP^{4-})
Mg^{2+} (7mM)
oxaloacetate (10mM)
acetyl CoA
control; water was used to replace NEM

Aliquots were removed at the times indicated and the residual enzymic activity determined.

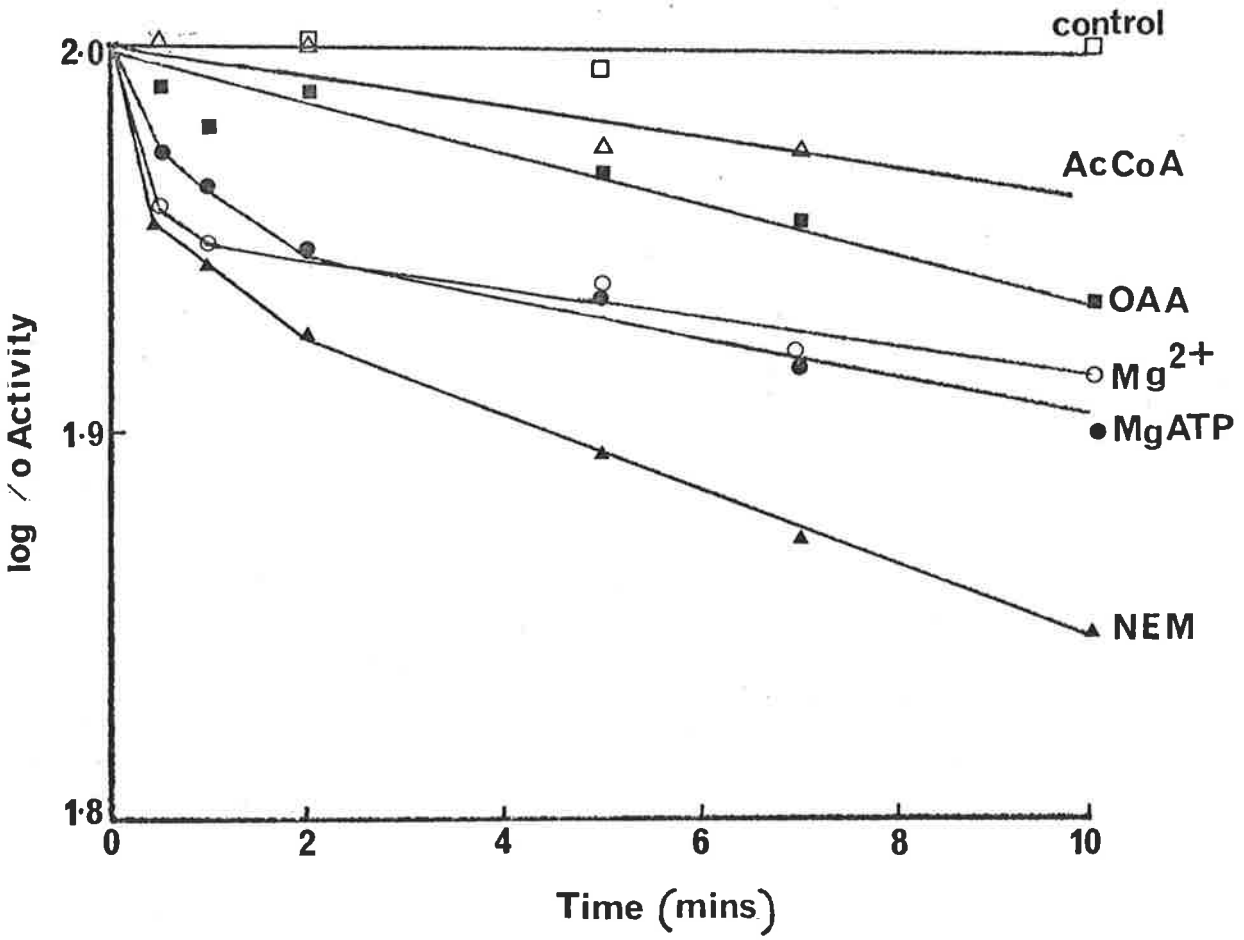

control
AcCoA
OAA
Mg2+
MgATP
NEM
2·0
1·9
1·8
log /o Activity
0
2
4
6
8
10
Time (mins)

FIGURE 5.10

The effect of biotin on the rates of inactivation of pyruvate carboxylase with NEM.

Pyruvate carboxylase (S.A. 20; 17 units/ml) was incubated with NEM (0.5mM) in 0.5M potassium phosphate buffer, pH 7.2, in the presence of the concentrations of biotin shown in the figure. At the times indicated aliquots of the solution were removed and the residual enzymic activity determined. In the control, NEM was replaced by water.

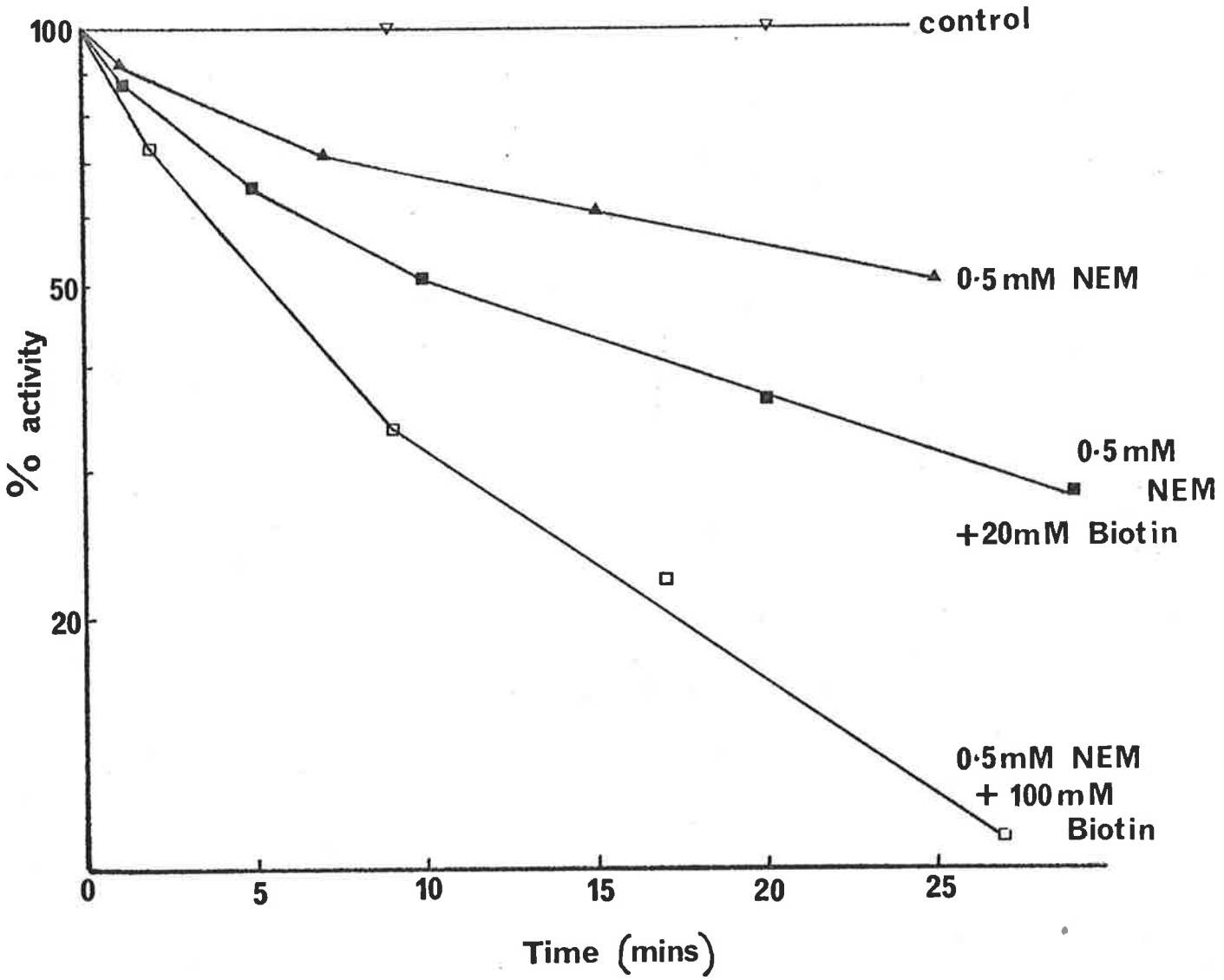

100
50
20
% activity
control
0·5 mM NEM
0·5 mM NEM +20mM Biotin
0·5mM NEM + 100 mM Biotin
0
5
10
15
20
25
Time (mins)

TABLE 5.2

The effect of modification of pyruvate carboxylase by NEM on the isotopic exchange reactions catalysed by the enzyme.

Pyruvate carboxylase (S.A. 9.8, 50 units/ml) was inactivated with 0.25mM NEM to the level of the overall activity shown and the reaction was then terminated by the addition of 50mM DTE. Aliquots of the modified enzyme were then used to initiate the isotopic exchange reactions. The rates of exchange were expressed relative to the unmodified enzyme, assigned 100%.

	Unmodified enzyme	Modified enzyme
Total Activity	100	39.4
[^{14}C]ADP/ATP exchange reaction	100	40.6
[^{32}P]/ATP exchange reaction	100	38.2
Total Activity	100	30.3
[^{14}C]pyr/OAA exchange reaction	100	44.5

FIGURE 5.11

Identification of the amino acid residues modified by $|^{14}C|$NEM.

Pyruvate carboxylase (S.A. 28.1; 80 units/ml) was incubated with $|^{14}C|$NEM (4.8μCi/μmole; 3mM) and, following 50% loss of enzymic activity, the reaction was terminated by addition of 90mM DTE. The enzyme solution was then dialysed to remove radioactivity not covalently bound to the protein, and the protein then enzymatically digested as described in section 5.2.5. Ascending chromatography was performed on aliquots of the digest as described in section 5.2.4. Authentic NEM-cysteine and NEM-lysine were run as standards on the same chromatograms and located with ninhydrin.

The bars indicate the position of these standard compounds. The arrow indicates the direction of chromatography. S.F. is the solvent front.

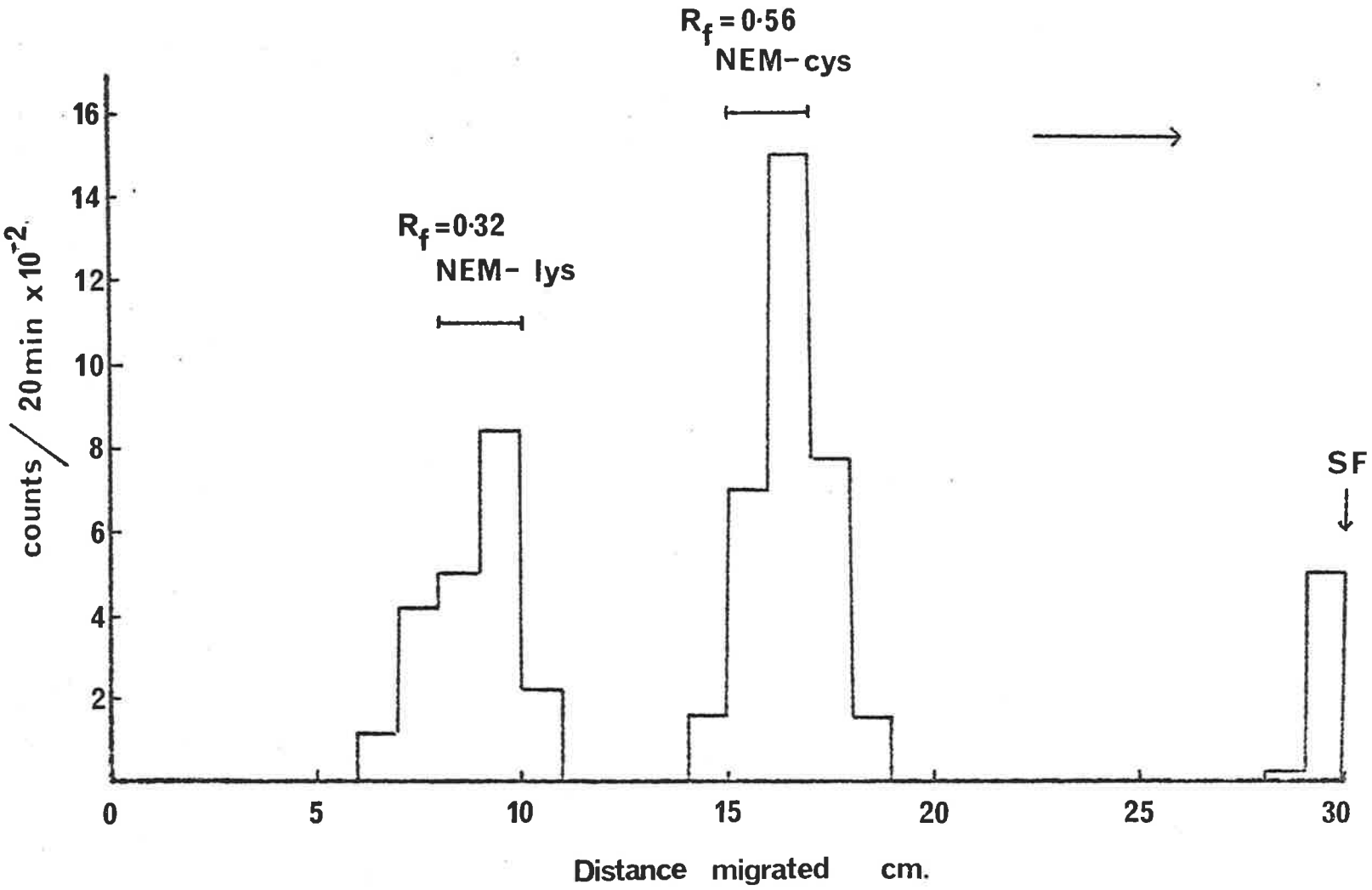

R_f = 0·56
NEM–cys
R_f = 0·32
NEM– lys
SF
counts / 20 min x 10^{-2}
16
14
12
10
8
6
4
2
0
5
10
15
20
25
30
Distance migrated cm.

FIGURE 5.12

The time dependent inactivation of pyruvate carboxylase with various concentrations of N-butylmaleimide.

Pyruvate carboxylase (S.A. 10.6; 11 units/ml) was incubated in Buffer A with the concentrations of N-butylmaleimide shown in the figure (in mM). Samples were assayed for the enzymic activity remaining after the time interval designated, and expressed as the log % activity to the initial activity. In the control water was used to replace the NBM.

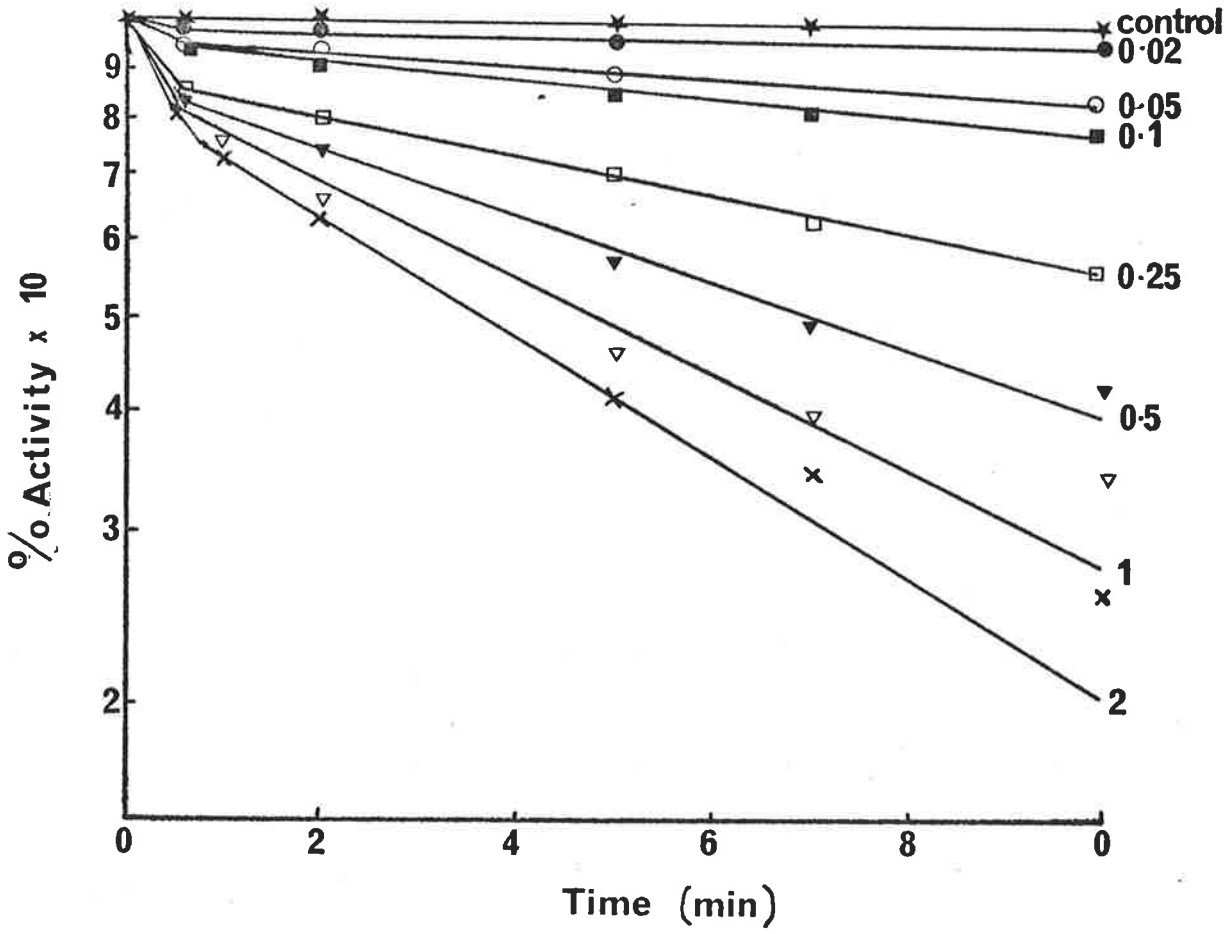

control
0·02
0·05
0·1
0·25
0·5
1
2
% Activity x 10
9
8
7
6
5
4
3
2
0
2
4
6
8
0
Time (min)

FIGURE 5.13

The time dependent slow phase of inactivation of pyruvate carboxylase with various concentrations of N-butylmaleimide.

The rate of inactivation of the enzyme at each concentration of N-butylmaleimide shown (in mM) was determined from the data shown in figure 5.12.

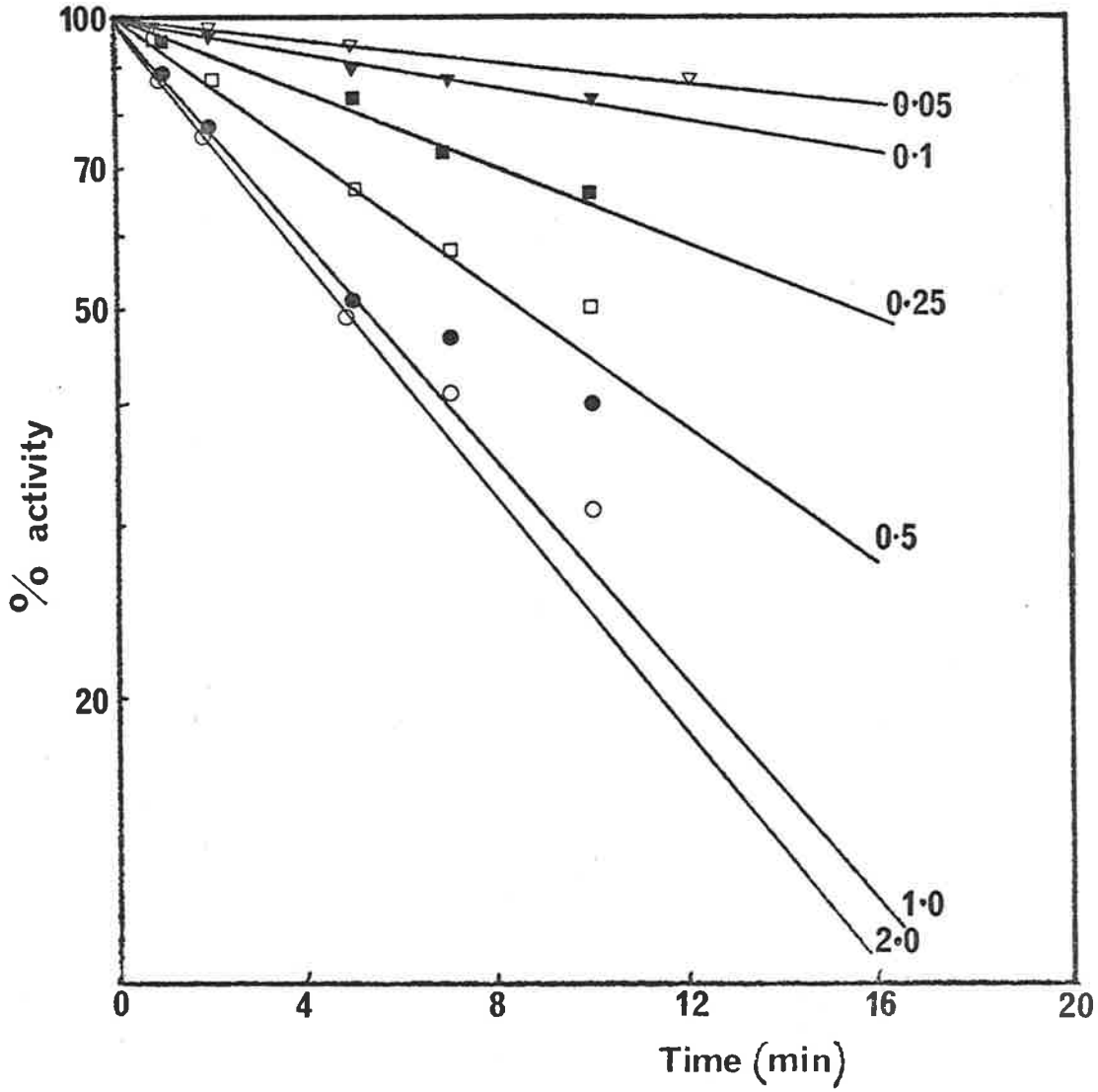

100
70
50
20
% activity
0
4
8
12
16
20
Time (min)
0·05
0·1
0·25
0·5
1·0
2·0

FIGURE 5.14

The pseudo first order rate constants for the slow phase of inactivation of the enzymc plotted as a function of the N-butylmaleimide concentration. The values for k' were determined from the data presented in fig 5.13.

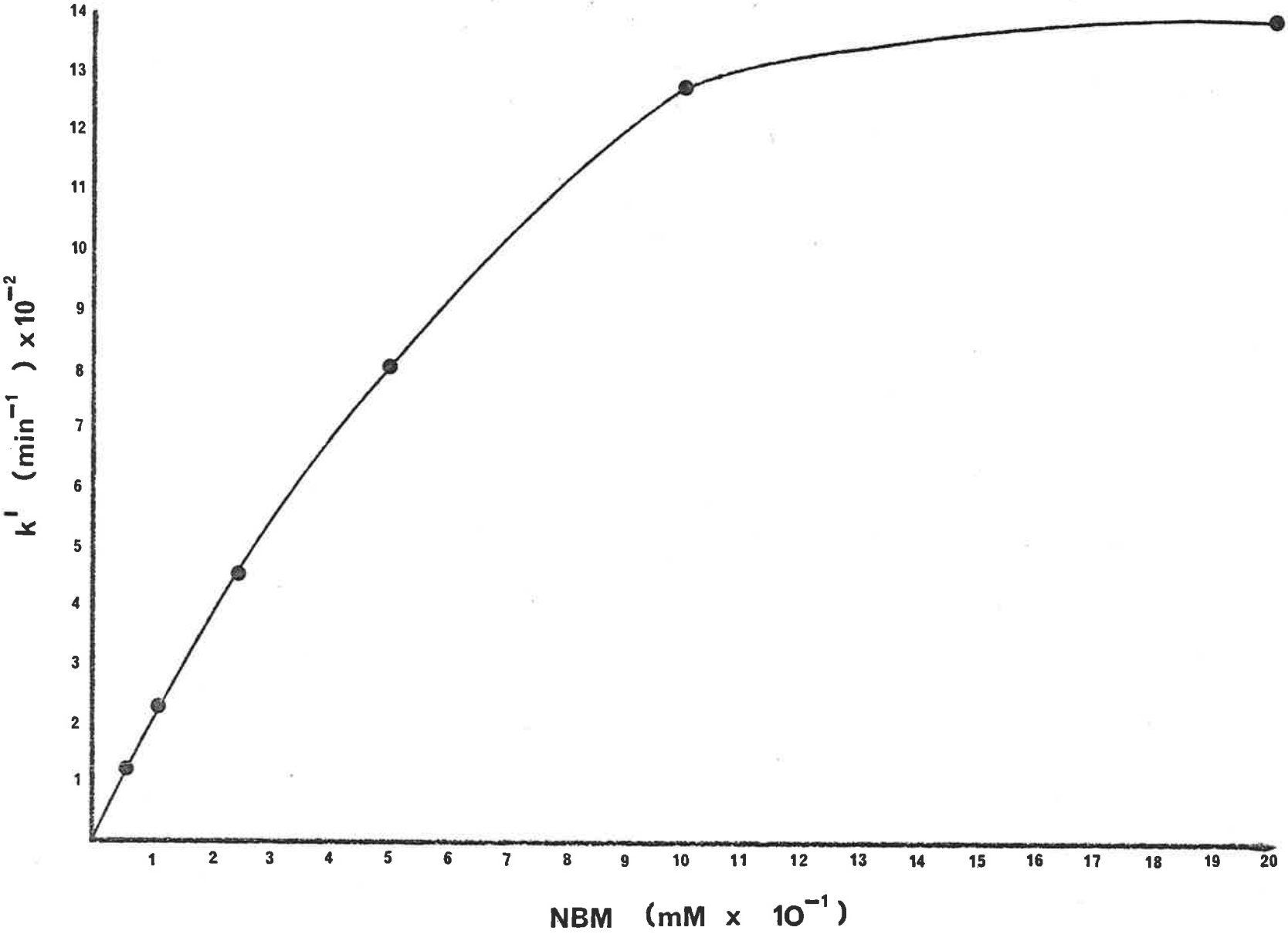

14
13
12
11
10
9
8
7
6
5
4
3
2
1
k' (min⁻¹) x 10⁻²
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
NBM (mM x 10⁻¹)

FIGURE 5.15

A logarithmic plot of the data given in figure 5.14.

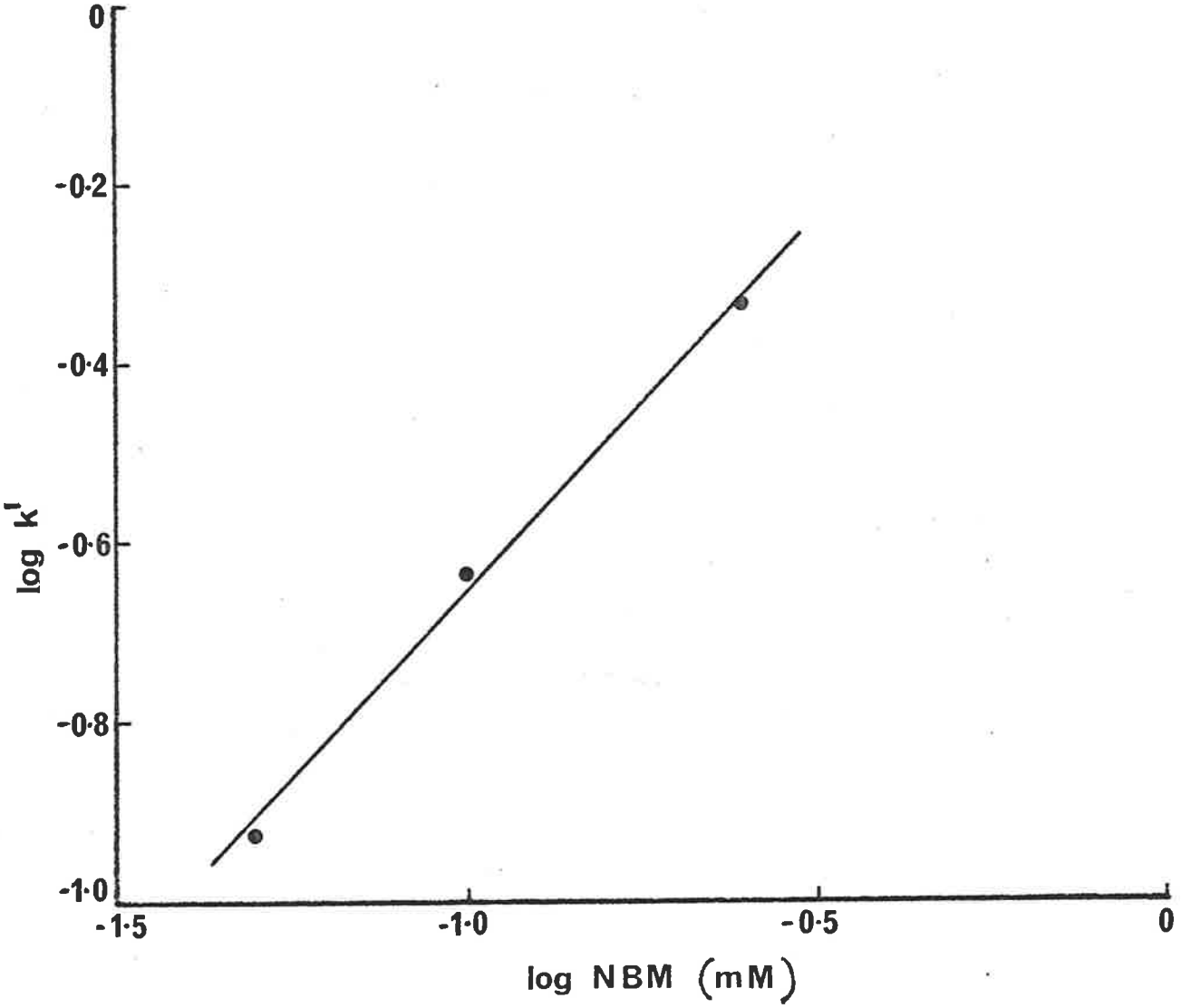

0
-0·2
-0·4
-0·6
-0·8
-1·0
log k'
-1·5
-1·0
-0·5
0
log NBM (mM)

FIGURE 5.16

A scheme showing the possible involvement of a lysine residue in the binding of the Mg-acetyl CoA complex to the enzyme.

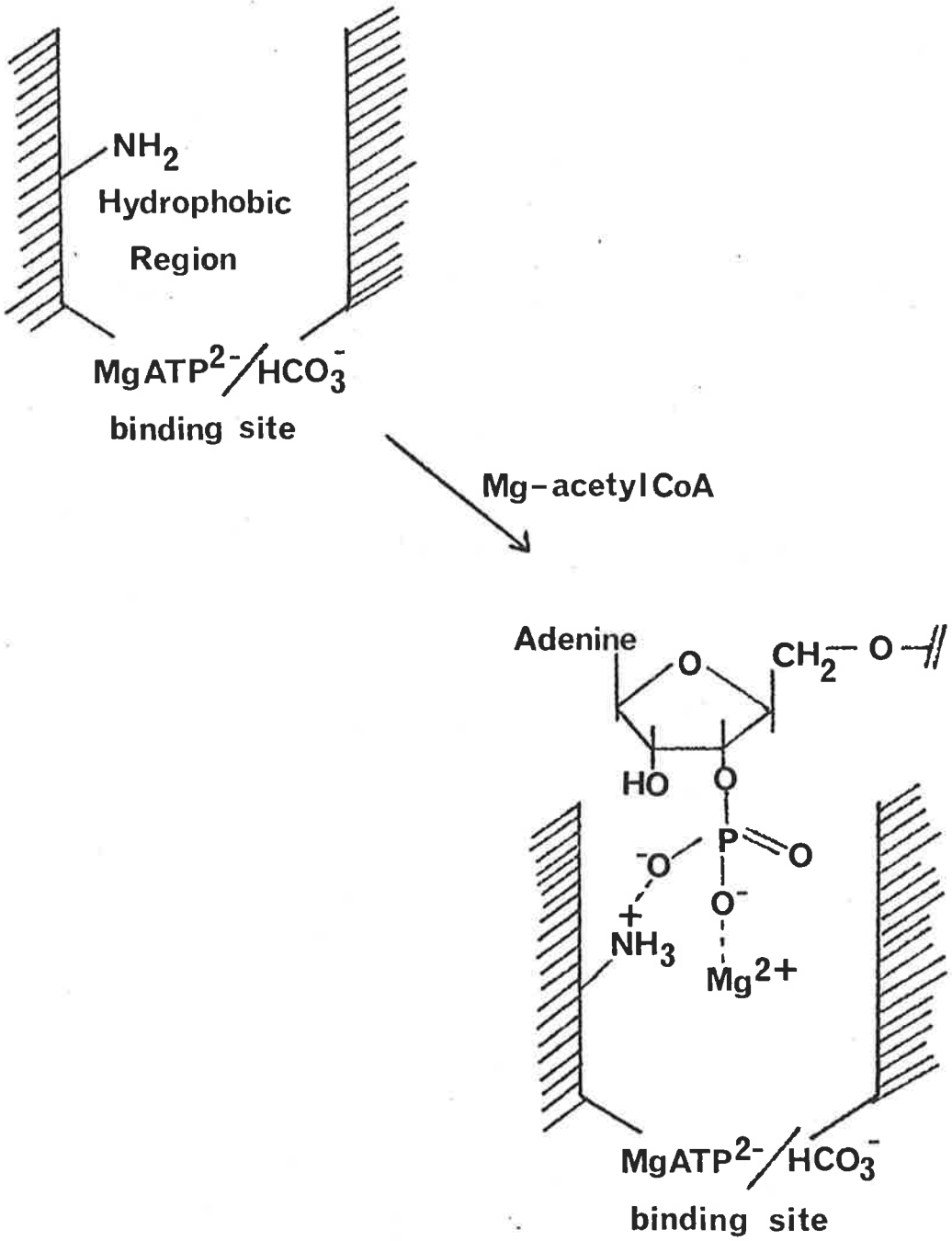

NH2
Hydrophobic
Region
MgATP2-/HCO3-
binding site
Mg-acetylCoA
Adenine
O
CH2
O
HO
O
P
O
-O
O-
+
NH3
Mg2+
MgATP2-/HCO3-
binding site

FIGURE 5.17

The time dependent inactivation of pyruvate carboxylase with various concentrations of DTNB.

Pyruvate carboxylase (S.A. 12.1; 11 units/ml) was incubated with the concentrations of DTNB shown in the figure (mM) and at the time intervals indicated samples were removed for the determination of the residual enzyme activity. In the control water was used to replace DTNB.

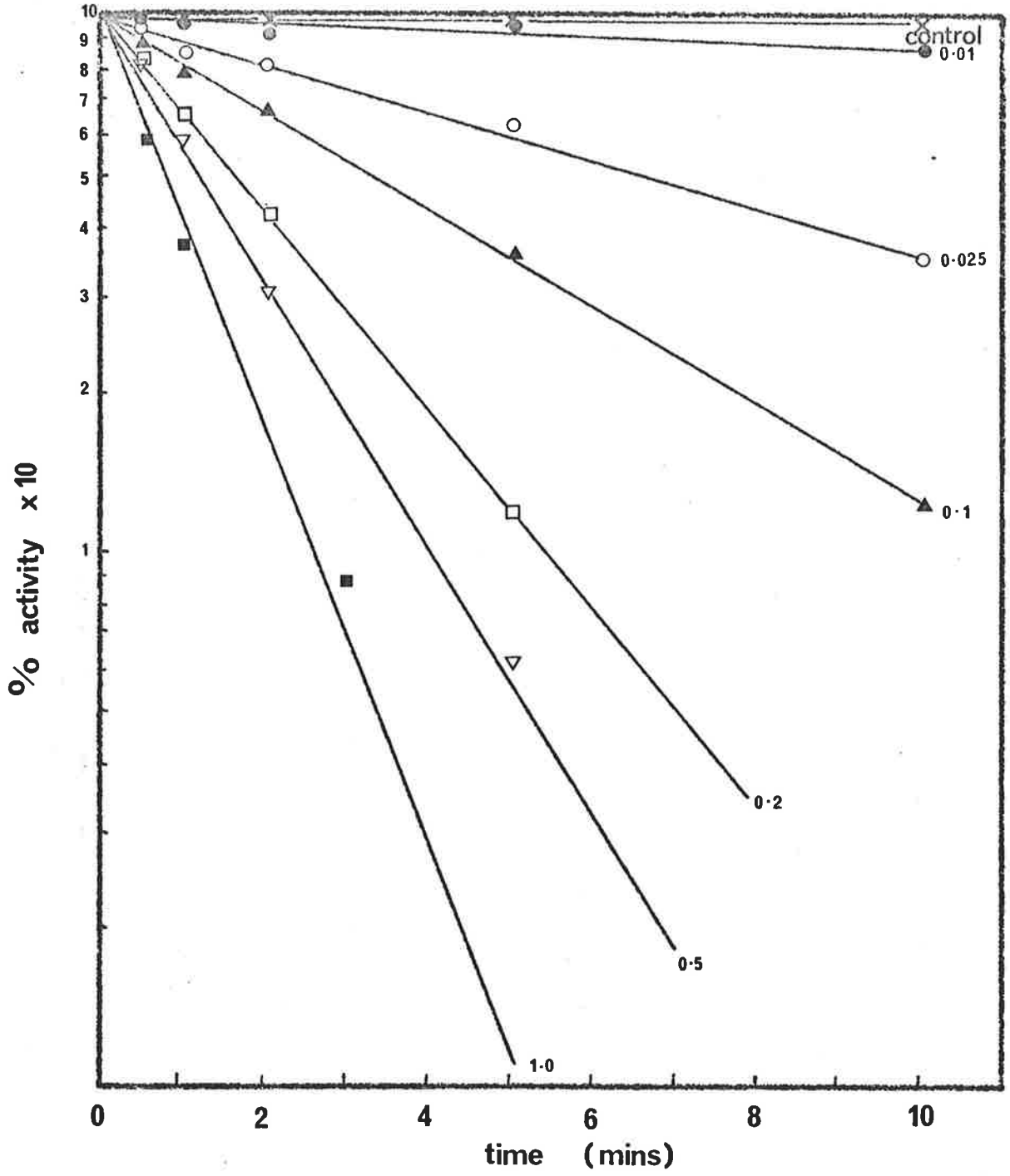

% activity x 10
time (mins)
control
0·01
0·025
0·1
0·2
0·5
1·0
0
2
4
6
8
10
1
2
3
4
5
6
7
8
9
10

FIGURE 5.18

The pseudo first order rate constants for the inactivation of pyruvate carboxylase plotted as a function of DTNB concentration. The values for k' were determined from the data presented in figure 5.17.

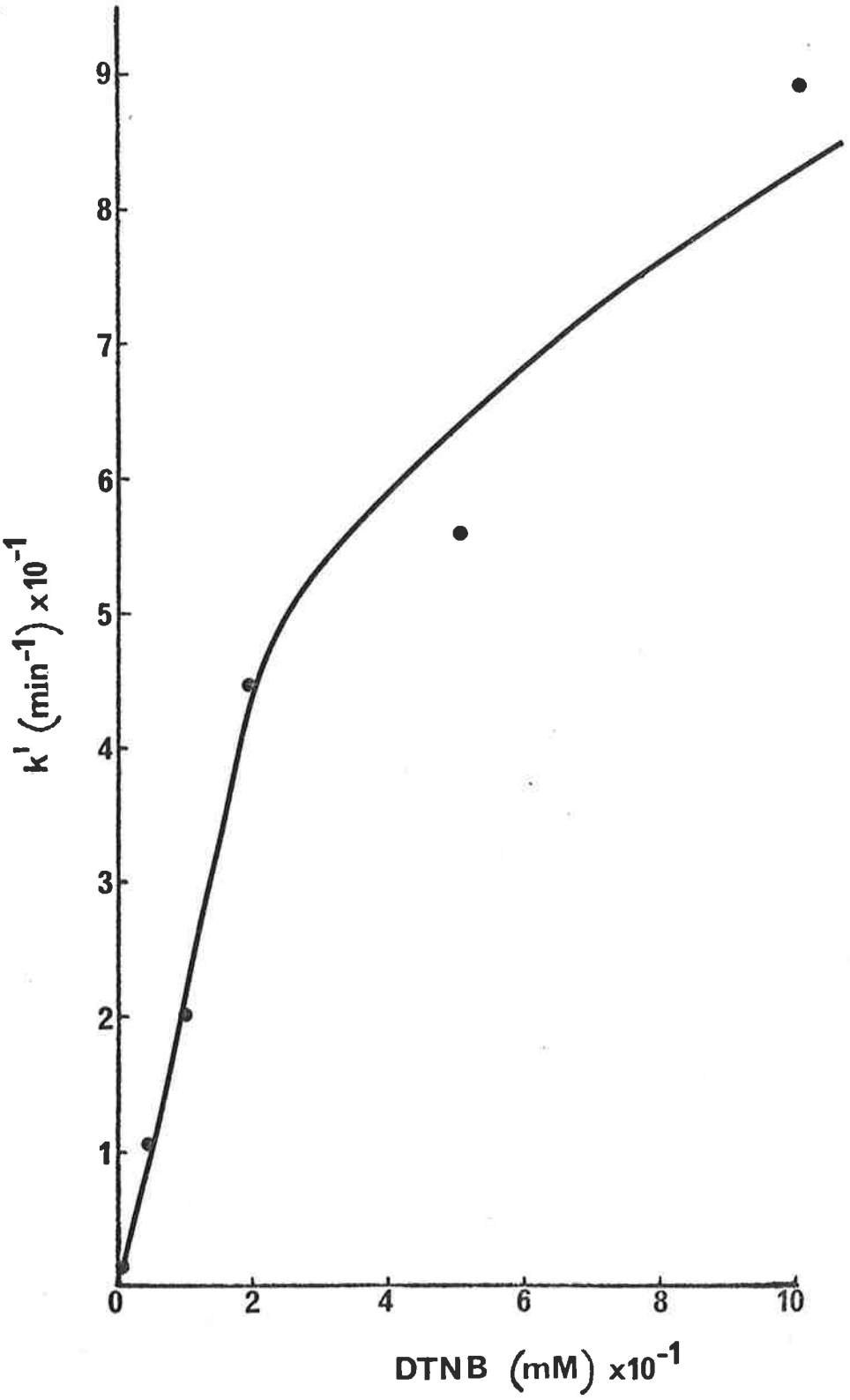

9
8
7
6
5
4
3
2
1
0
2
4
6
8
10
k' (min-1) x10-1
DTNB (mM) x10-1

FIGURE 5.19

A logarithmic plot of the data given in figure 5.18.

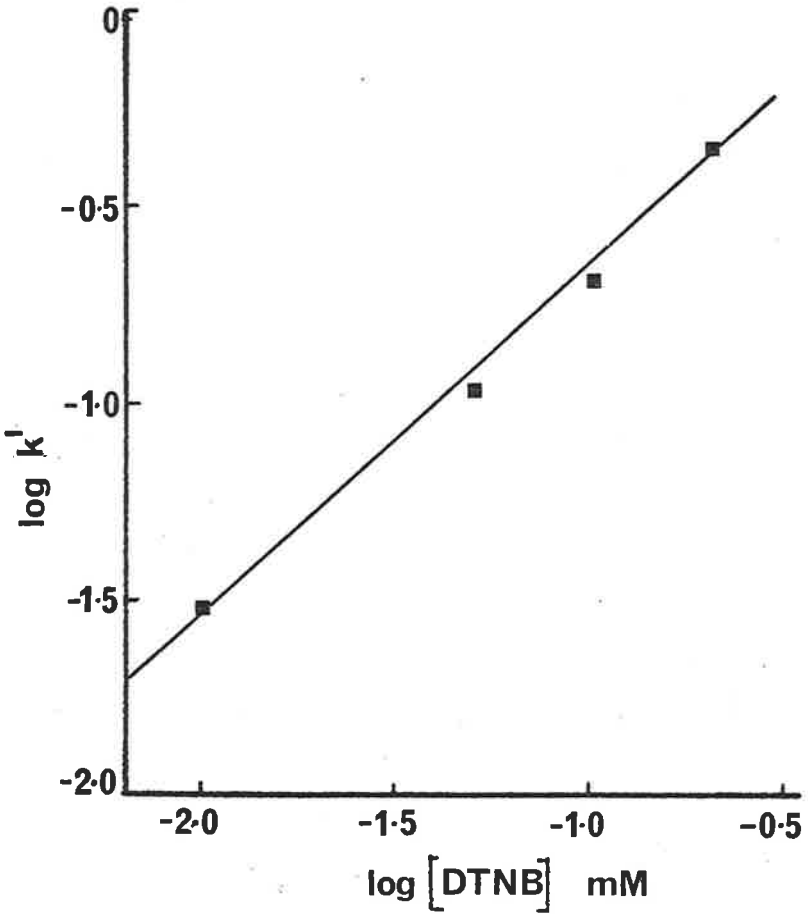

0
-0·5
-1·0
-1·5
-2·0
log k'
-2·0
-1·5
-1·0
-0·5
log [DTNB] mM

FIGURE 5.20

The stoichiometry of DTNB modification of pyruvate carboxylase.

Pyruvate carboxylase (S.A. 25.0, 18 units/ml) was incubated in 0.107mM DTNB. The reaction was followed spectrophotometrically at 412nm. At various time intervals samples were removed and the residual enzyme activity determined. The number of moles of TNB incorporated into the enzyme was determined from the absorbance at 412nm using the ε_{412} = 14050 $M^{-1}cm^{-1}$ of the TNB anion in Buffer A and expressed relative to the amount of biotin in the enzyme solution.

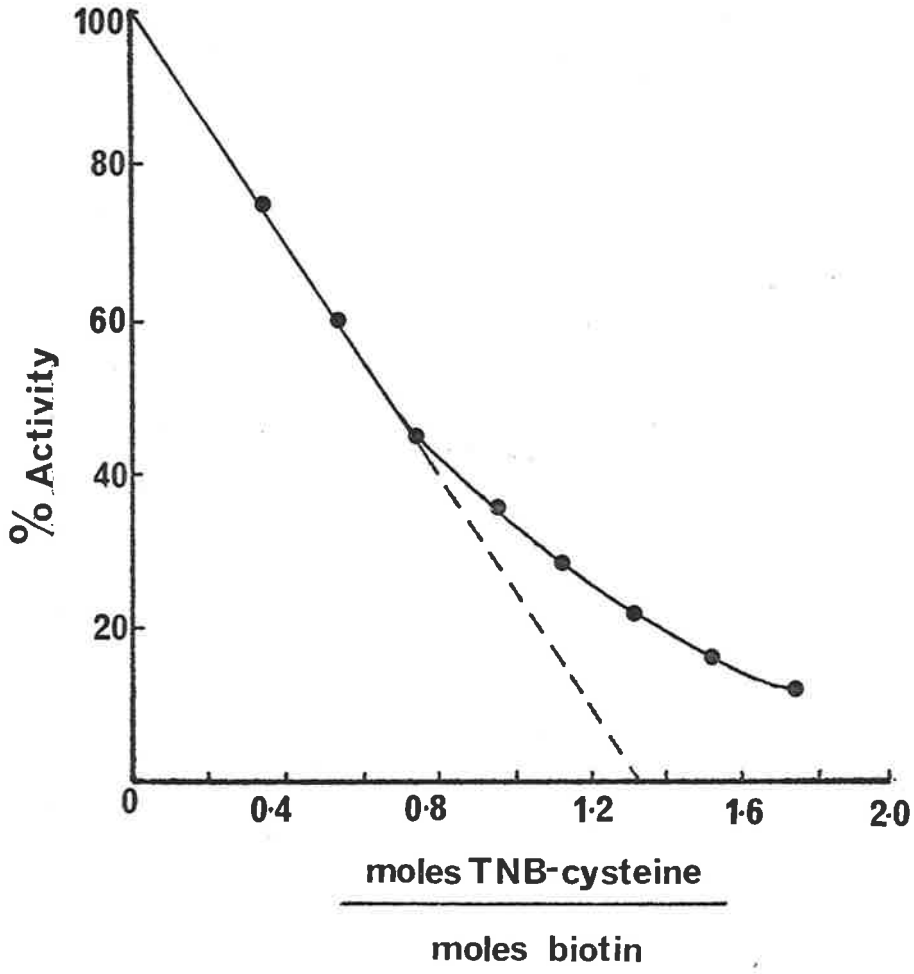

100
80
60
40
20
0
% Activity
0·4
0·8
1·2
1·6
2·0
moles TNB-cysteine
moles biotin

FIGURE 5.21

The effect of substrate of the pyruvate carboxylase reaction on the rate of inactivation of the enzyme in the presence of DTNB.

Pyruvate carboxylase (S.A. 6.1; 11 units/ml) was incubated with DTNB (0.05mM) in the presence of the following compounds:

oxaloacetate (10mM)
$MgATP^{2-}$ (7mM Mg^{2+}; 2.5mM ATP^{4-})
Mg^{2+} (7mM)
no additions

In the control, DTNB was replaced with water. Samples were removed at the times indicated for the determination of the residual enzyme activity.

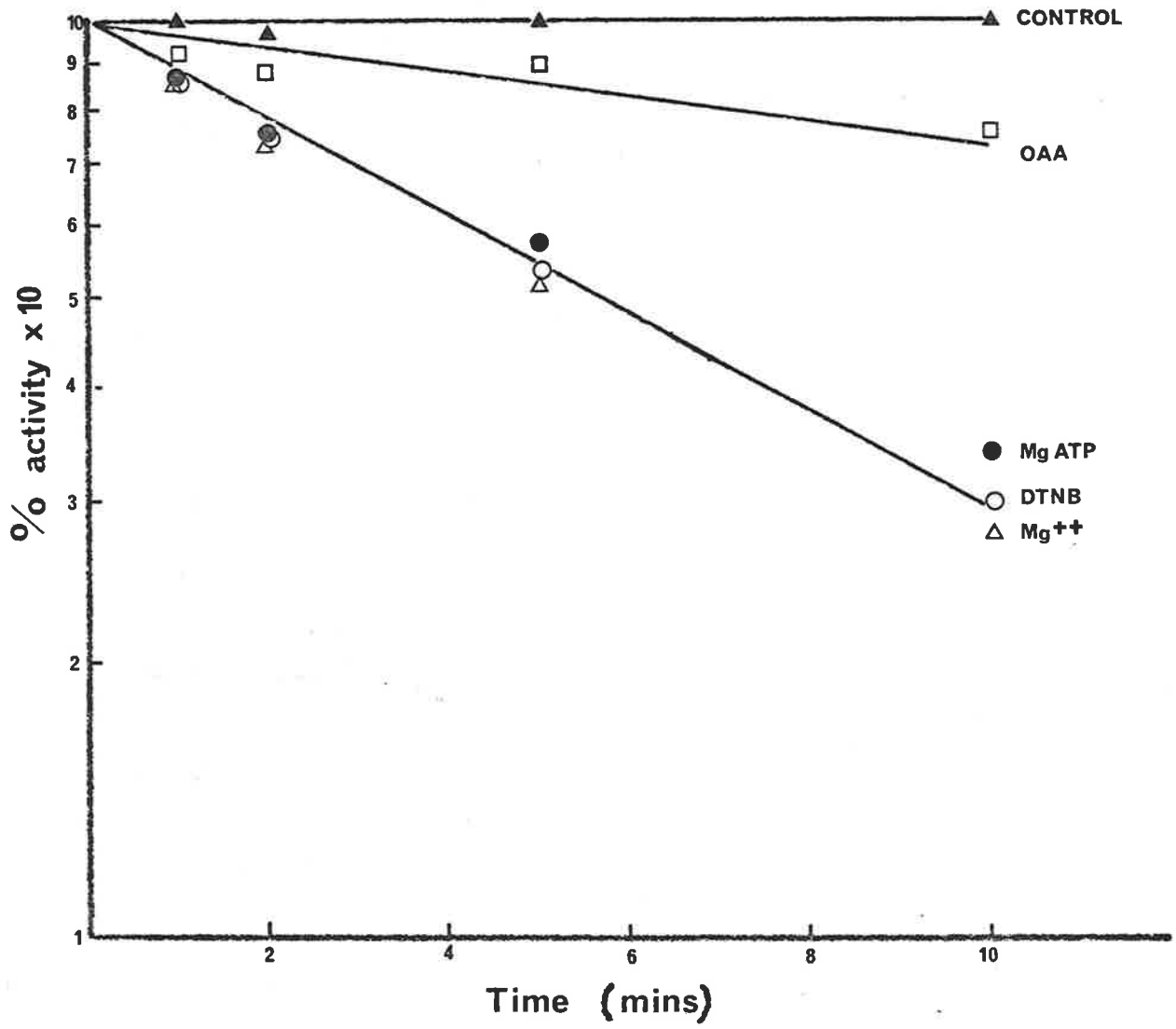

CONTROL
OAA
Mg ATP
DTNB
Mg++
% activity x 10
Time (mins)
10
9
8
7
6
5
4
3
2
1
2
4
6
8
10

FIGURE 5.22

The effect of various concentrations of oxaloacetate on the rate of inactivation of pyruvate carboxylase with DTNB.

Pyruvate carboxylase (S.A. 12.7; 13 units/ml) was incubated with DTNB (0.1mM) in the presence of the concentrations of oxaloacetate (in mM) shown in the figure. In the control DTNB was replaced by water.

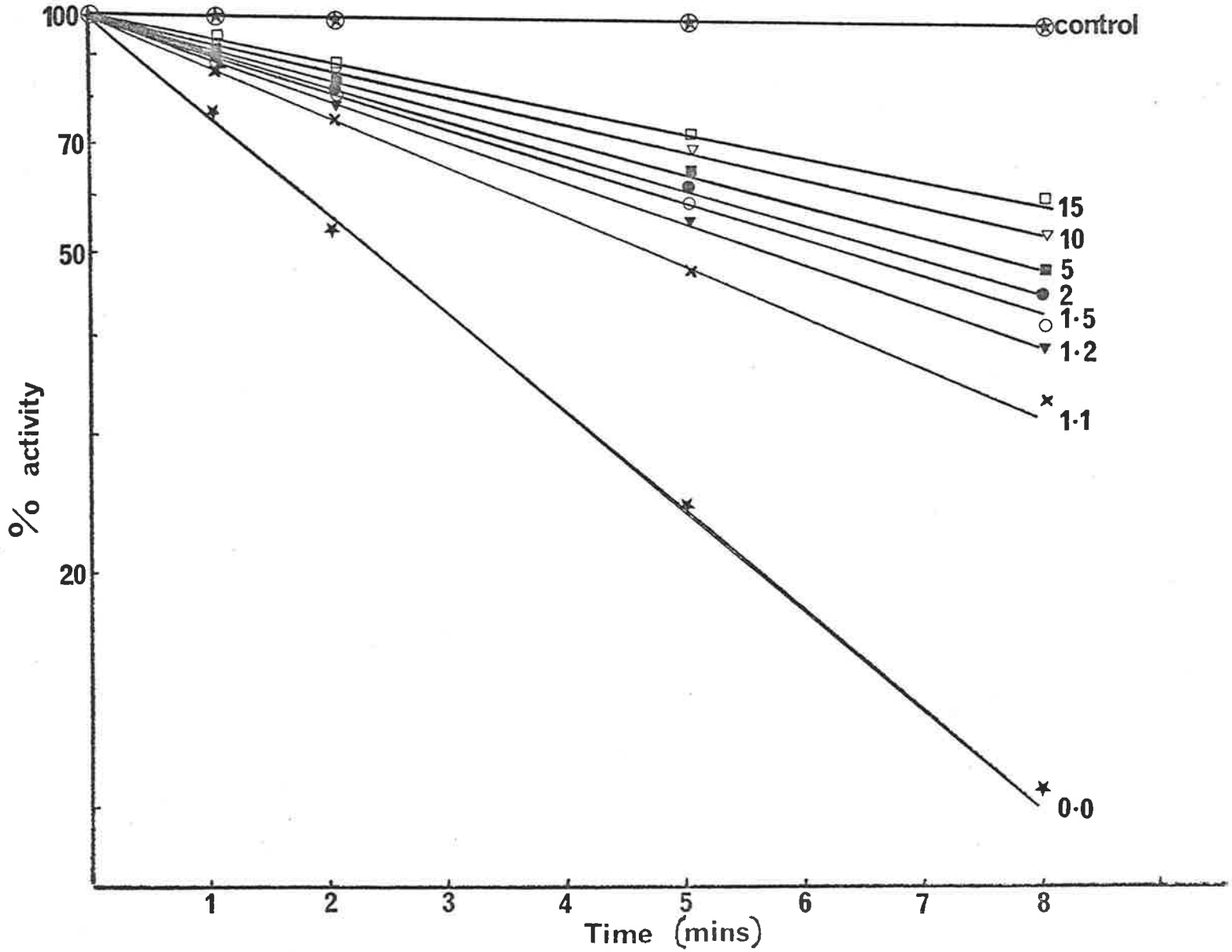

100
70
50
20
% activity
control
15
10
5
2
1·5
1·2
1·1
0·0
1
2
3
4
5
6
7
8
Time (mins)

FIGURE 5.23

The pseudo first order rate constants for the inactivation of the enzyme obtained from figure 5.22 were replotted according to equation 5.3.

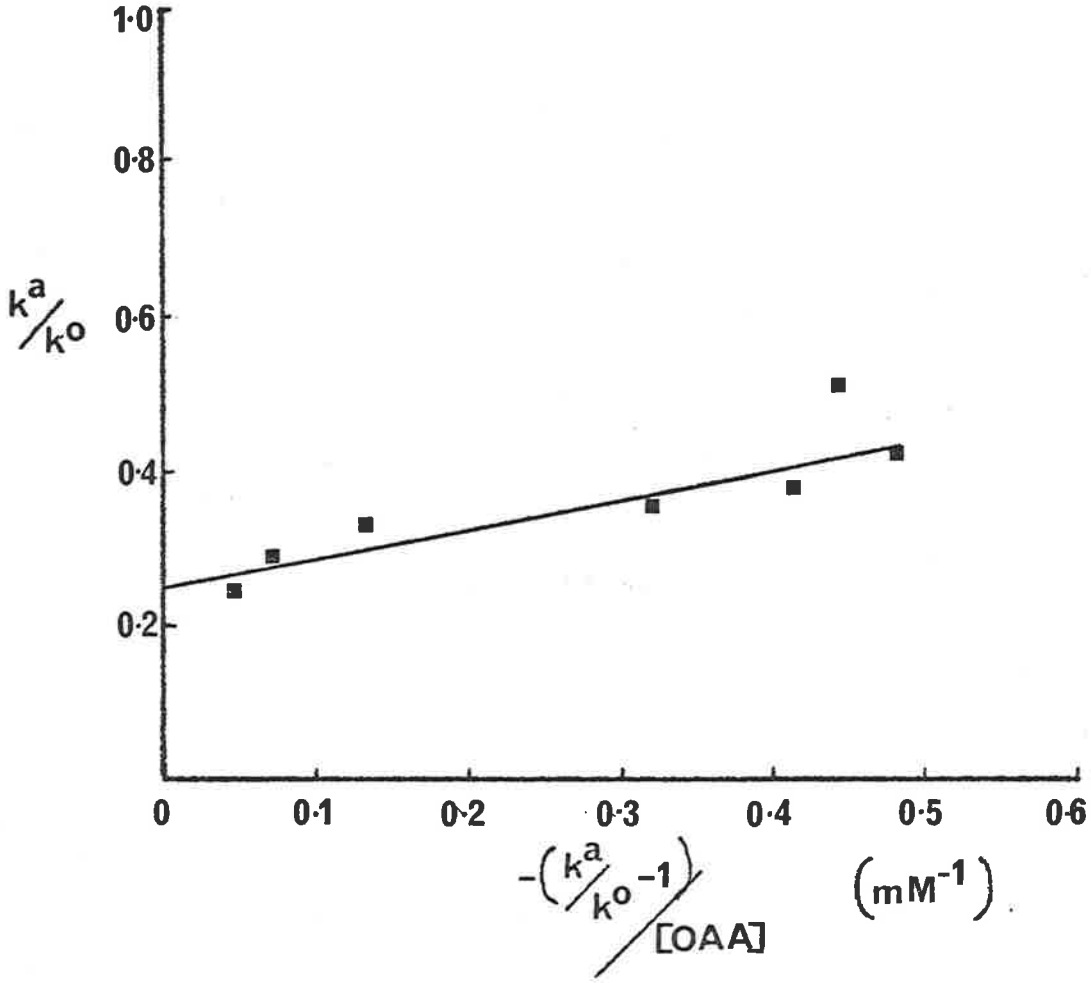

1·0
0·8
0·6
0·4
0·2
k^a/k^o
0
0·1
0·2
0·3
0·4
0·5
0·6
$-\left(k^a/k^o - 1\right)/[OAA]$
(mM^{-1})

FIGURE 5.24

The protection by acetyl CoA against inactivation of the enzyme by DTNB.

Pyruvate carboxylase (S.A. 12.7; 13 units/ml) was incubated with 0.05mM DTNB in Buffer A in the presence of various concentrations of acetyl CoA, as shown in the figure. At the time intervals indicated aliquots were removed and the residual enzyme activity determined.

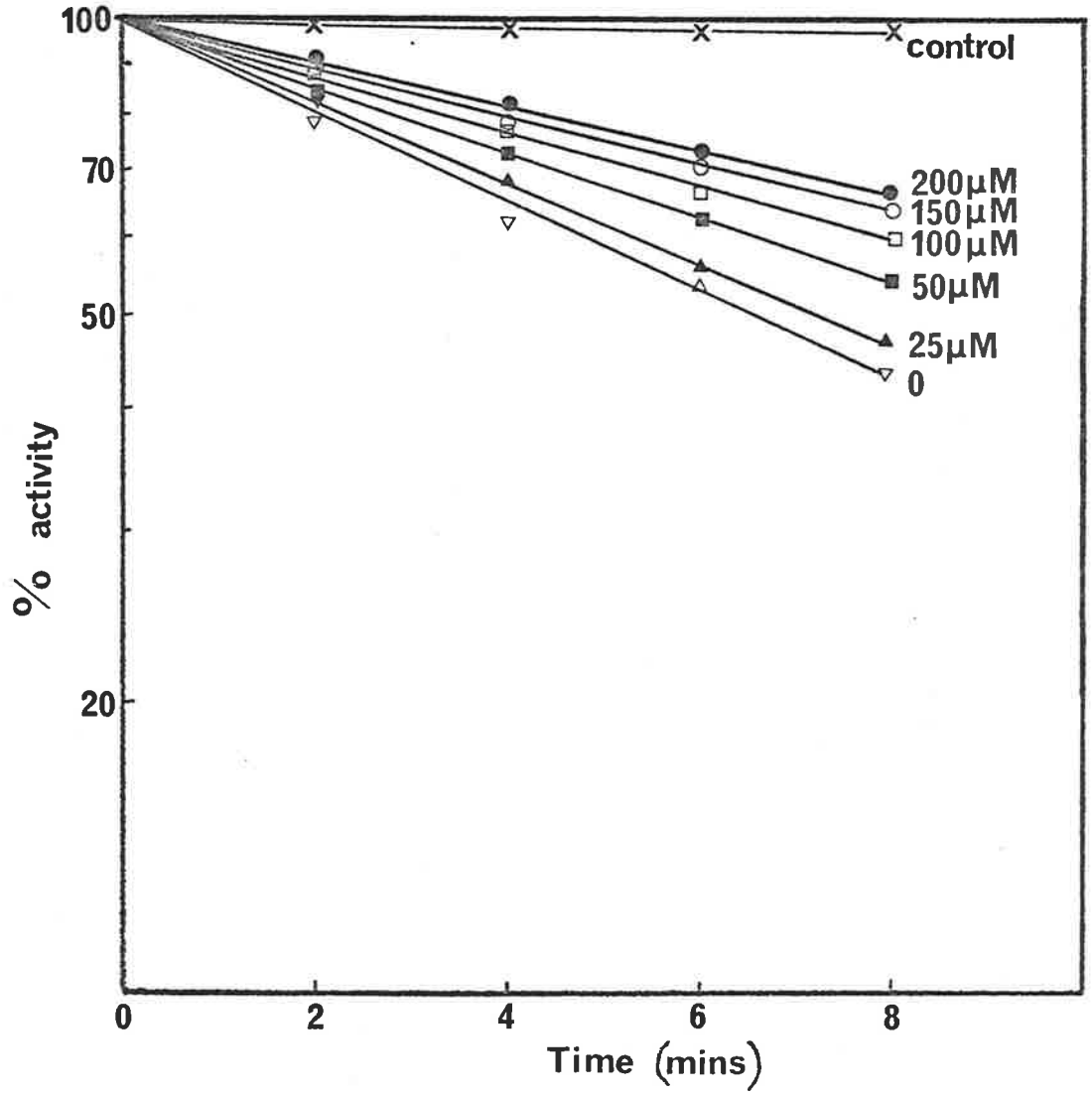

100
70
50
20
% activity
0
2
4
6
8
Time (mins)
control
200µM
150µM
100µM
50µM
25µM
0

FIGURE 5.25

The pseudo first order rate constants for the inactivation of the enzyme obtained from figure 5.24 were replotted according to equation 5.3.

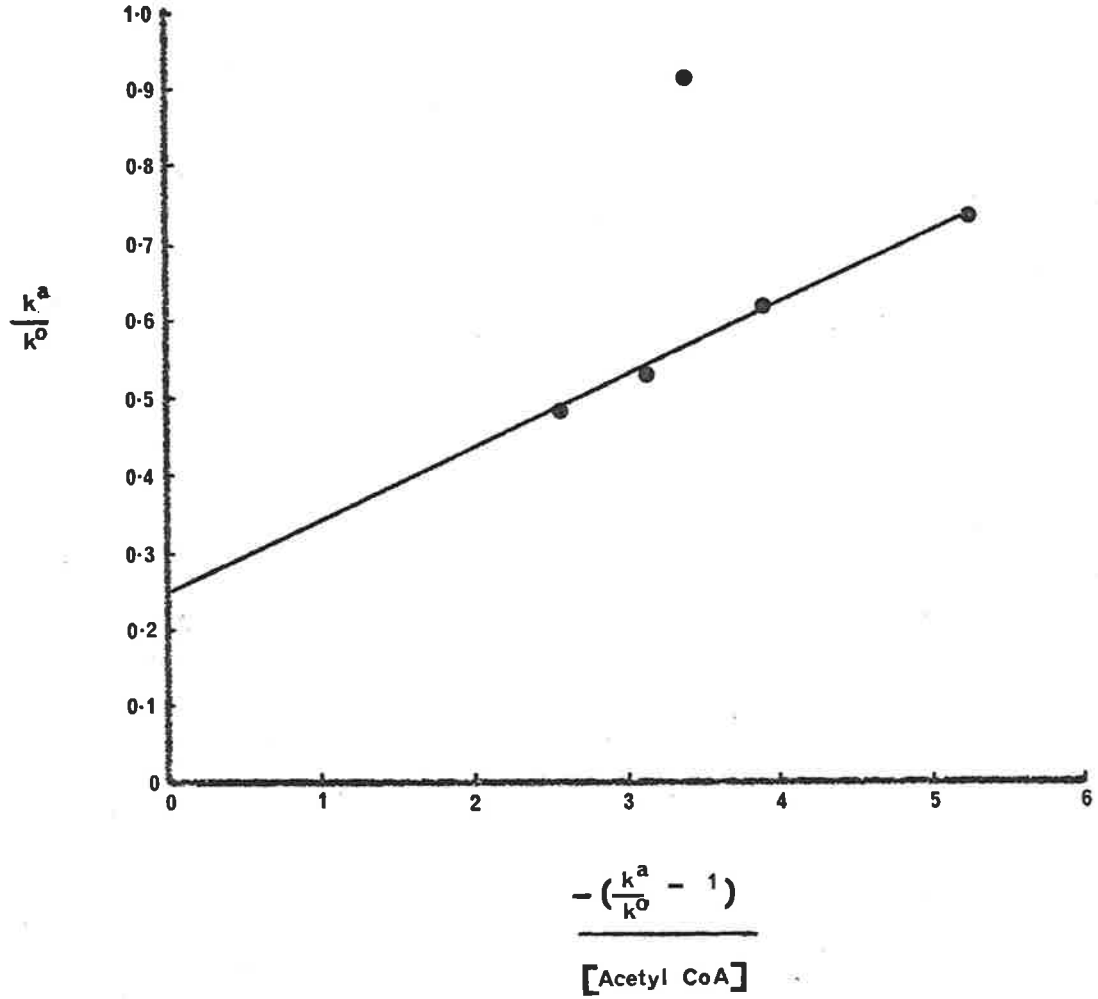

1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
0
1
2
3
4
5
6
$\frac{k^a}{k^o}$
$-(\frac{k^a}{k^o} - 1)$
[Acetyl CoA]

FIGURE 5.26

The effect of oxaloacetate and acetyl CoA on the rate of inactivation of pyruvate carboxylase by DTNB.

Pyruvate carboxylase (S.A. 12; 11 units/ml) was incubated with DTNB (0.1mM) in the presence of the following compounds:

no additions
acetyl CoA (0.25mM)
acetyl CoA (0.25m) and oxaloacetate (10mM)

Samples were removed at the times indicated and the residual enzyme activity determined.

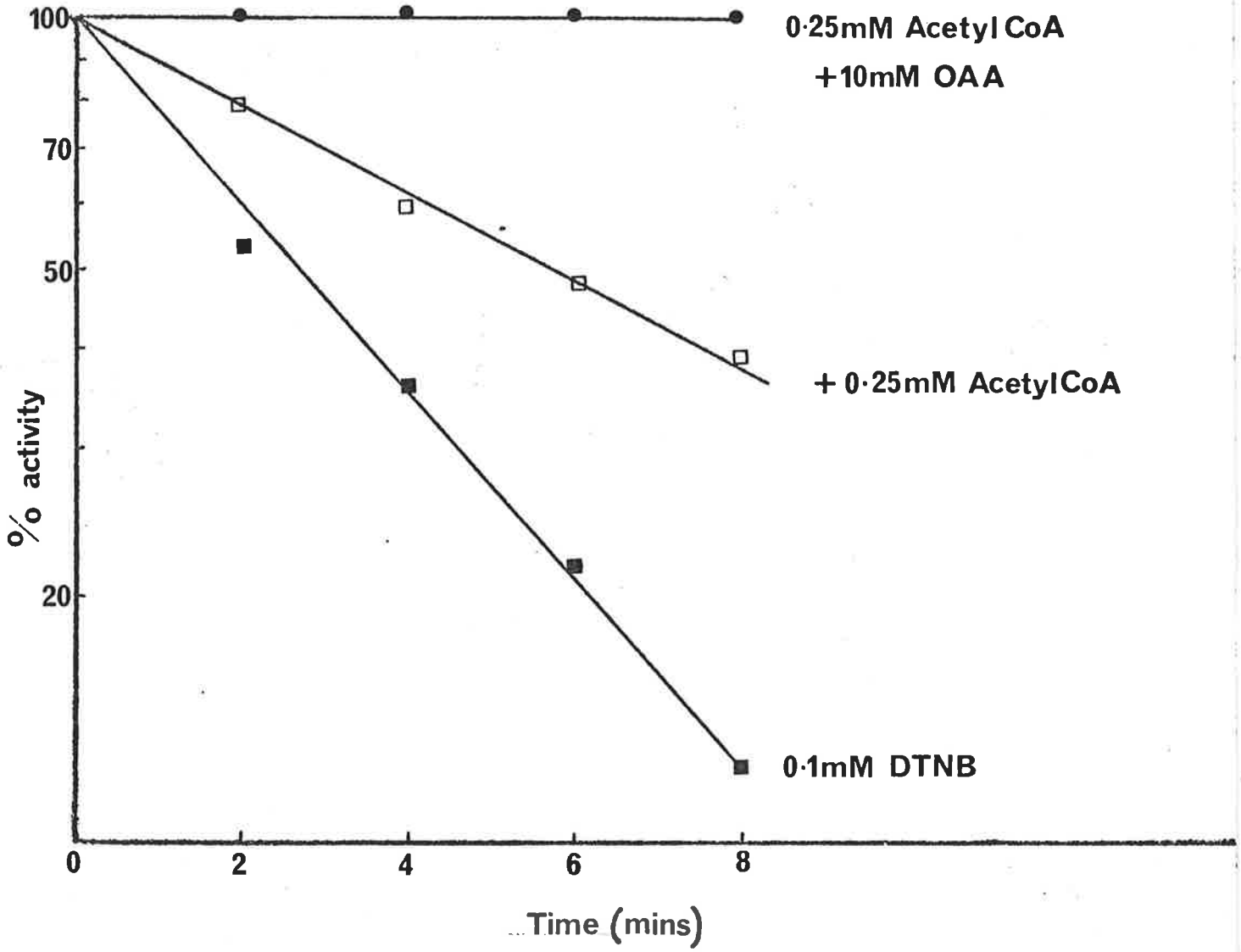

100
70
50
20
% activity
0
2
4
6
8
Time (mins)
0·25mM Acetyl CoA
+10mM OAA
+ 0·25mM AcetylCoA
0·1mM DTNB

FIGURE 5.27

The mechanism for the cleavage of the mixed disulphide bond between the protein thiol and 5-thio-2-nitobenzoic acid by cyanide.

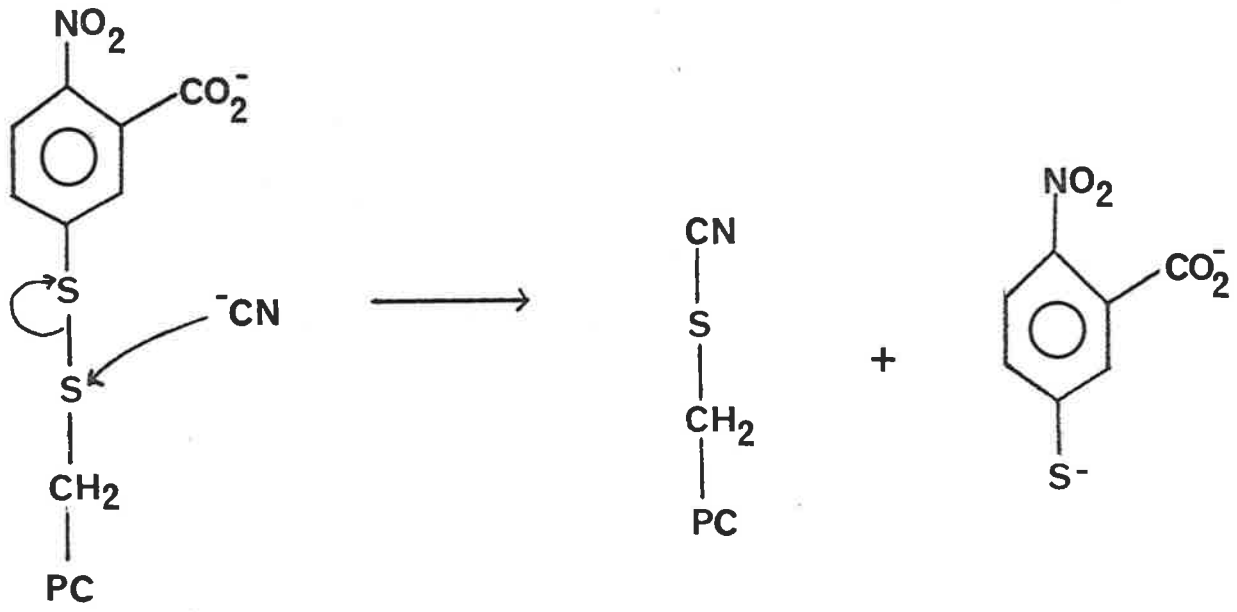

NO2
CO2-
S
-CN
S
CH2
PC
CN
S
CH2
PC
+
NO2
CO2-
S-

FIGURE 5.28

The effect of cyanide on the enzymic activity of the TNB-modified enzyme.

Pyruvate carboxylase (S.A. 25.2; 181 units/ml) was incubated with DTNB (0.01mM) and samples were removed at various times for the determination of the residual enzymic activity. At the time indicated by A the reaction was terminated by application of the solution to a Sephadex G-25 column (1 x 15cm). Following chromatography, the partially active enzyme was pooled and to aliquots were added either cyanide (○, 1mM) or DTE (●, 1mM). The recovery of enzymic activity was followed by removing samples and determining the enzymic activity as described in the text.

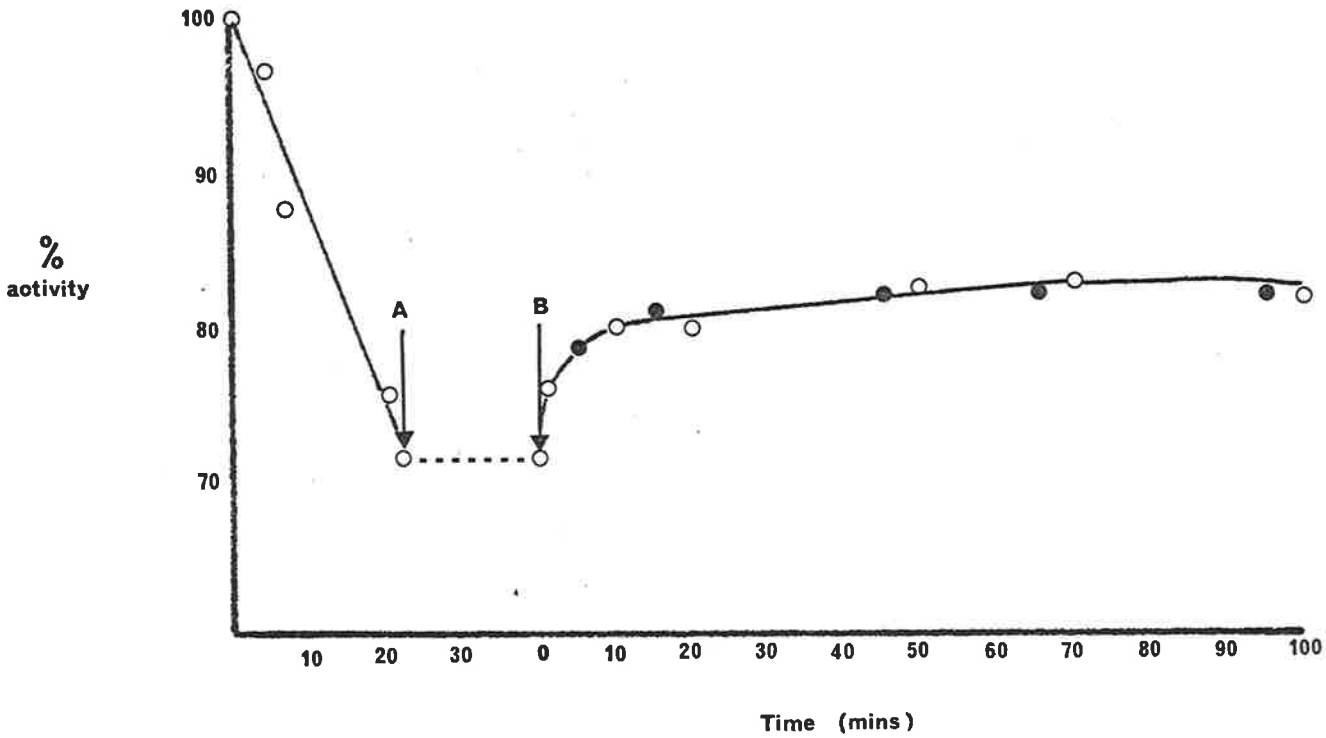
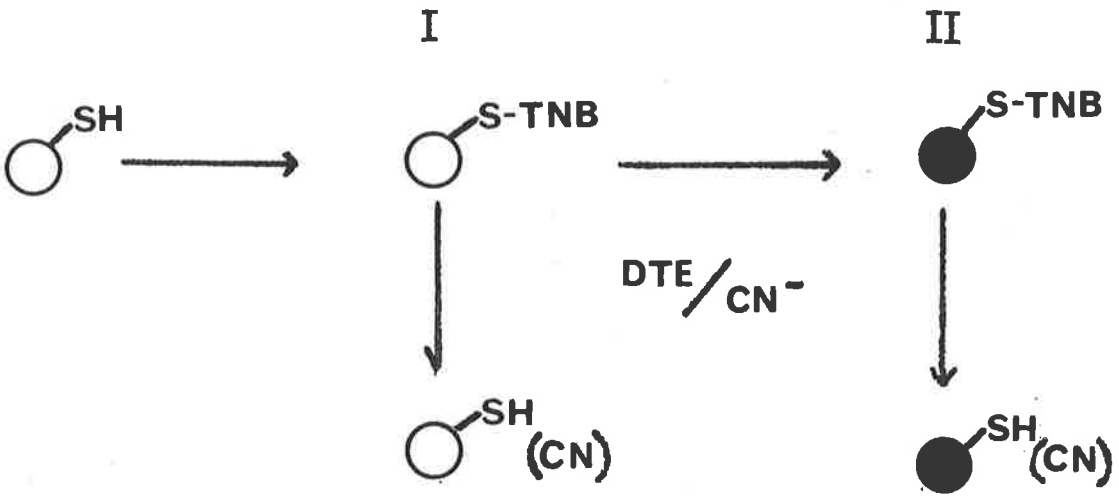

%
activity
100
90
80
70
A
B
10
20
30
0
10
20
30
40
50
60
70
80
90
100
Time (mins)

FIGURE 5.29

Two possible modes of inactivation of pyruvate carboxylase by DTNB and reactivation by thiolysis or cyanylation.

In the two schemes, ○ represents an active form of the enzyme and ● represents a toally inactive form of the enzyme.

Scheme 1



Scheme 2

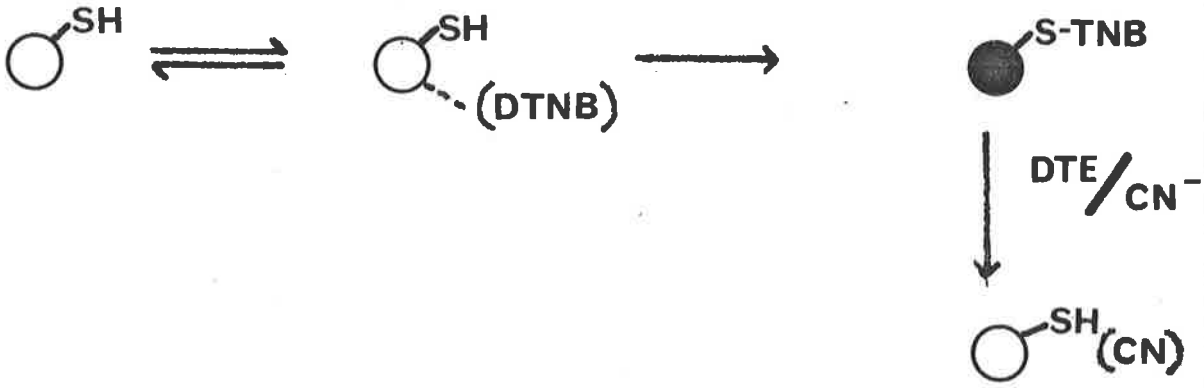


FIGURE 5.30

Models which account for the data regarding the location of the reactive cysteine residues in pyruvate carboxylase and the effect of the ligands on the extent of modification of the amino acids.

Discussion of these models is presented in the text.

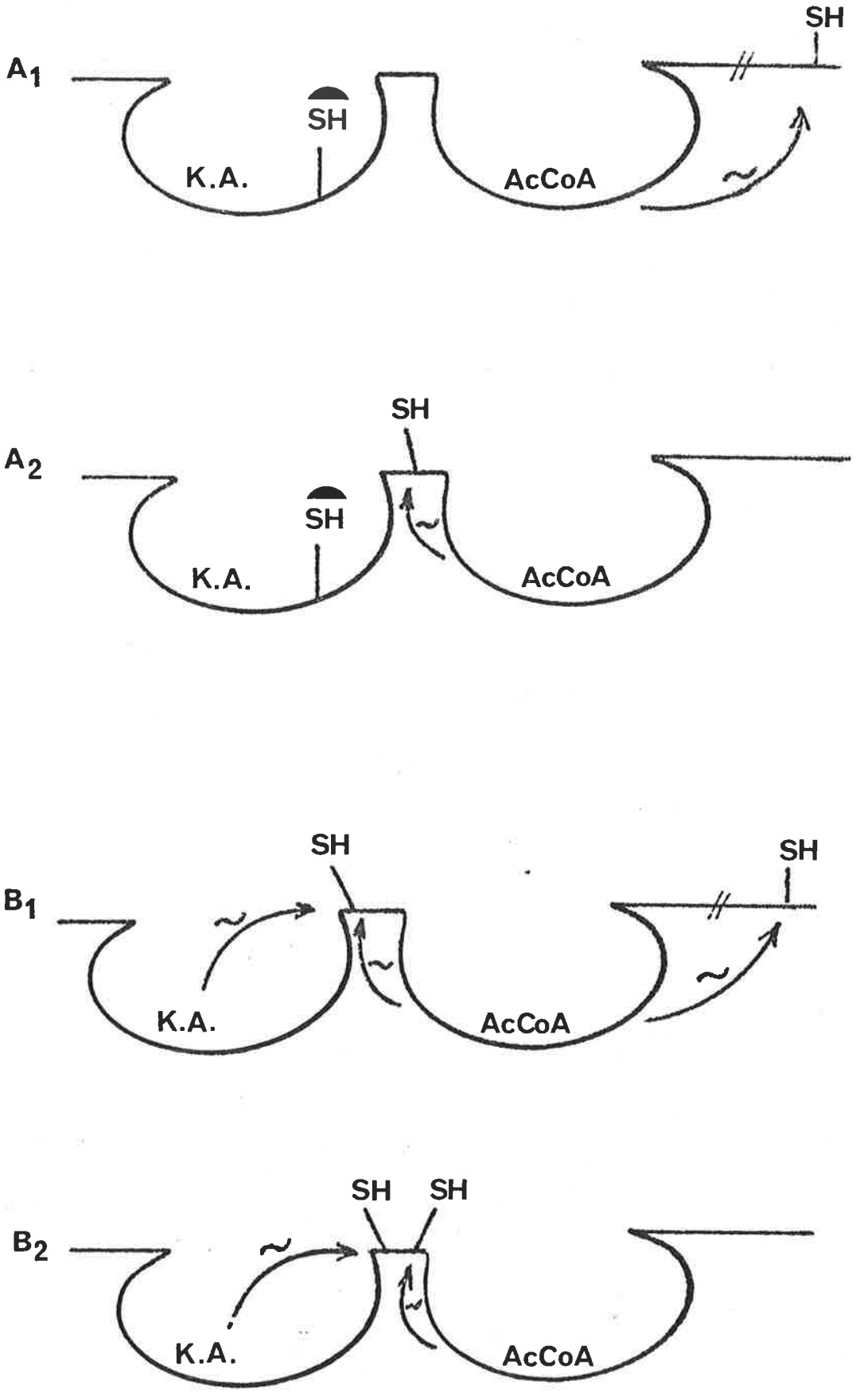
Symbols used:

K.A. = keto acid binding site

AcCoA = acetyl CoA binding site

⌒ = direct protection of the cysteine residue by a keto acid

∿ = conformational change induced in the enzyme upon ligand binding


A1
SH
SH
K.A.
AcCoA
A2
SH
SH
K.A.
AcCoA
B1
SH
SH
K.A.
AcCoA
B2
SH
SH
K.A.
AcCoA

CHAPTER 6

AFFINITY LABELLING OF TRANSCARBOXYLASE

6.1. INTRODUCTION

Transcarboxylase (E.C.2.1.3.1.) is a biotin-dependent enzyme which catalyses the reaction

methylmalonyl CoA + pyruvate $\rightleftharpoons$ propionyl CoA + oxaloacetate ... (1)

The overall reaction can be divided into two partial reactions

methylmalonyl CoA + E-biotin $\rightleftharpoons$ propionyl CoA + E-biotin $\sim CO_2$ ... (2)

E-biotin $\sim CO_2$ + pyruvate $\rightleftharpoons$ E-biotin $\sim CO_2$ + oxaloacetate ... (3)

and the intermediate biotin $\sim CO_2$ complex has been isolated (Wood *et al.*, 1963).

The enzyme can be dissociated under mild conditions into three subunits; the "head" subunit, the "ear" subunit and the biotin carboxyl carrier subunit. The head subunit has a sedimentation coefficient of 12S and contains the CoA-ester binding site while the ear subunit has a sedimentation coefficient of 5S and contains the binding site for the keto acids (Chuang *et al.*, 1975). The biotin carboxyl carrier protein subunit (1.3S) is involved in transferring the carboxyl group between the active sites on the head and ear subunits. These subunits can be further dissociated in their constituent polypeptide chains or can aggregate to produce catalytically active 18S or 26S enzyme complexes. The relationship between the

constituent polypeptide chains and the active forms of the enzyme is diagramatically represented in an article by Green et al. (1972). The properties of the enzyme and its relationship to the other biotin containing enzymes are comprehensively covered in two recent reviews (Wood and Zwolinski, 1976; Wood and Barden, 1977).

Transcarboxylase is unique amongst the biotin carboxylases, being the only well studied member of this class of enzymes which does not utilize bicarbonate as the carboxylating substrate. Instead, the enzyme generates the biotin $\sim CO_2$ complex by decarboxylation of methylmalonyl CoA, the reversal of the second partial reaction of propionyl CoA carboxylase (Kaziro et al., 1962). The biotin $\sim CO_2$ complex can then carboxylate pyruvate to form oxaloacetate. This reaction is identical to the second partial reaction catalysed by pyruvate carboxylase. Thus transcarboxylase may be viewed as a hybrid of the subunits catalysing the second partial reactions of pyruvate carboxylase and propionyl CoA carboxylase. Such a hybrid could be formed if a primordial genome contained individual genes which coded for subunits catalysing these different partial reactions. This situation is represented schematically in fig. 6.1. One of the most attractive features of this model is that, in an organism containing a limited amount of coding potential, a large number of enzymes, catalysing

different reactions, could be formed from a common pool of interchangeable gene products. Clearly, the scheme represented in fig. 6.1 could be expanded to incorporate other biotin utilizing enzymes.

An implication of the model presented above is that the subunits catalysing the same reaction in different enzymes should be derived from the same primordial gene. This would imply that essential regions within these subunits should have very similar amino acid sequences, since mutations within these regions could lead to non-functional subunits and result in the organisms possessing such mutations being selectively disadvantaged during evolution.

Rylatt et al. (1977) have recently shown that the amino acid sequences around the biotin prosthetic group of chicken liver, sheep liver and turkey liver pyruvate carboxylase are highly conserved and, furthermore, are very similar to the sequences around the biotin prosthetic group of transcarboxylase and E. coli acetyl CoA carboxylase (Wood and Zwolinski, 1976). These authors therefore concluded that the polypeptide chain containing the biotin cofactor probably evolved from a common ancestral gene. This evidence strongly supports the general scheme shown in fig 6.1. To test this model further however it would be necessary to examine the degree of homology between the amino acid sequences

in the common substrate binding sites of different members of the biotin enzymes. It was in this regard that the similarity between the second partial reactions of transcarboxylase and pyruvate carboxylase was of great interest.

Hudson et al. (1975) had reported that 3-bromopyruvate modified a cysteine residue and inactivated sheep liver pyruvate carboxylase. It was suggested by these authors that this reactive amino acid residue may have been in the keto acid binding site. The studies presented in Chapter 5 of this thesis indicate that this cysteine residue is not directly involved in the reaction catalysed by pyruvate carboxylase but support the view that it may be in or near the keto acid binding site on the enzyme. If the keto acid binding sites of pyruvate carboxylase and transcarboxylase evolved from the same primordial gene then it was possible that the cysteine may have been conserved for structural reasons. It was quite apparent however that any evaluation of the model presented earlier in this section would rely heavily on the existence of a sulphydryl residue in the keto acid binding site of transcarboxylase. The experiments described in this chapter were designed to test for this possibility using the affinity label 3-bromopyruvate.

6.2. METHODS

6.2.1. Measurement of transcarboxylase activity

The enzyme was assayed spectrophotometrically in the direction of oxaloacetate formation as described by Wood et al. (1969).

6.2.2. Synthesis of 3-bromo-2[^{14}C]pyruvate

3-Bromo-2[^{14}C]pyruvate was prepared by direct bromination of pyruvic acid using the method described in section 7.2.2. The specific activity of the compound was 5.5 x 10^6cpm/μmole.

6.2.3. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed in the absence of urea on 7.5% acrylamide gels as described by Berger and Wood (1975). Unmodified transcarboxylase was dialysed for 16 h against 0.05M Tris-Cl pH 9.0 and, following electrophoresis, the 5S ear and 6S head subunits were located using the staining system described by Fairbanks et al., (1971). The polyacrylamide gels containing the radioactively labelled subunits were sliced into 1.5mm sections and the radioactivity in the slices determined as described by Schwinghamer and Symons (1975).

6.2.4. Fluorescence of the ANS-transcarboxylase complex

Examination of the fluorescence spectrum of the ANS-transcarboxylase complex was performed using a Perkin-Elmer Fluorescence Spectrophotometer Model 203. The excitation wavelength used was 380nm. Each sample contained 0.48mg of transcarboxylase and a final concentration of 0.042mM ANS in a final concentration of 83mM potassium phosphate pH6.3. The volume was made up to 0.6ml using either water, pyruvate or oxaloacetate. The oxaloacetate was neutralized with IN sodium hydroxide immediately prior to use.

6.3. RESULTS

6.3.1. Bromopyruvate modification

Modification of transcarboxylase with 3-bromopyruvate resulted in loss of enzyme activity only when high concentrations of the modifier were used. Fig 6.2 shows the effect of 50mM 3-bromopyruvate on the enzymic activity at various pH values. Lower concentrations of this modifier at pH8.0 produced correspondingly less inhibition (Table 6.1). The inactivation profiles were routinely observed to be biphasic. Since the Michaelis constant for pyruvate in the overall reaction has been shown to be 0.77 mM (Northrop, 1969), the high concen-

trations of 3-bromopyruvate required to produce inactivation implied either that bromopyruvate had an extremely poor affinity for the keto acid site or that it did not act specifically at this site. The fact that Hudson et al. (1975) had observed pseudo first order inactivation of pyruvate carboxylase at much lower concentrations of 3-bromopyruvate suggested that the keto acid binding sites of these two enzymes may be different.

6.3.2. Effect of the keto-acid substrates on inactivation rate

One of the criteria of a specific affinity label is that the extent of modification of the protein should be reduced in the presence of the corresponding ligand. The effect of saturating concentrations of either pyruvate or oxaloacetate on the inhibition of enzymic activity in the presence of 3-bromopyruvate was therefore examined and the results are shown in Table 6.2. Both keto acids caused an increased rate of inhibition. This result clearly suggested that 3-bromopyruvate was not acting as an affinity label of the keto acid binding site of transcarboxylase.

The reason for the increase in the rate of inactivation of transcarboxylase in the presence of the keto acids is difficult to assess. One possible cause

of this effect however may be that the keto acids induce changes in three dimensional structure of the enzyme upon binding to the ear subunits. Such changes may result in increasing the accessibility of essential amino acid residues to 3-bromopyruvate and so increase the rate of inactivation of the enzyme in the presence of this modifier.

6.3.3. Effect of the keto acids on the conformation of transcarboxylase

To test whether pyruvate or oxaloacetate induced any change in the conformation of transcarboxylase upon binding the fluorescence spectra of the ANS-transcarboxylase complex was examined in the presence and absence of these keto acids. Fig 6.3 indicates that both compounds significantly affected the intensity of the fluorescence emission of the ANS-enzyme complex. However, while 10mM pyruvate increased the fluorescence emission approximately 10%, oxaloacetate (10mM) decreased the fluorescence emission by 11%. This difference between the effects of the keto acids on the enzyme may have been due to the fact that oxaloacetate, apart from binding to enzyme, would also carboxylate the biotin prosthetic group on the carboxyl carrier protein, and this may cause additional changes not induced by pyruvate.

Changes in the intensity of fluorescence emission

of an ANS-protein complex reflects changes in hydrophobic regions of the protein (section 5.3.4.), but this information does not allow any prediction of where those changes are occurring or by what mechanism they are induced. In the case of transcarboxylase, the changes in the intensity of the fluorescence emission of the ANS-enzyme complex upon the binding of the keto acids may reflect conformational alterations to the three dimensional structure of the enzyme. Examination of the circular dichroism spectra of transcarboxylase in the presence and absence of these ligands would provide an alternative method of evaluating this hypothesis. If this hypothesis was correct then the explanation for the increased rate of inactivation of the enzyme in the presence of the keto acids and 3-bromopyruvate would seem at least feasible.

6.3.4. The site of modification of transcarboxylase

Since transcarboxylase can be resolved into the 5S ear subunit and the 6S head subunit by polyacrylamide gel electrophoresis at pH8.9 (Berger and Wood, 1975) it was possible, by using radioactively labelled 3-bromopyruvate, to examine which of these subunits was being modified. Furthermore the effect of pyruvate on the extent of modification of the two subunits could be evaluated directly. If 3-bromopryvuatc was modifying the keto acid site on the 5S ear subunit then, in the

presence of saturating concentrations of pyruvate, a reduction in the radioactivity incorporated into this subunit should be observed.

The modifications were carried out at pH6.3 since at higher pH values some dissociation of the ear subunits from the 26S enzyme complex occurs and this may have given rise to spurious labelling patterns. The reactions were terminated by addition of a 10-fold molar excess of DTE over the bromopyruvate concentration used, and the protein was then dialysed against 0.05M Tris-Cl pH9.0 for 16 h to dissociate the enzyme into the subunits. Aliquots of this solution were then electrophoresed and the radioactivity associated with each subunit determined as described in section 6.2.3.

Table 6.3 shows the results obtained from modification of the enzyme in the presence and absence of pyruvate as described above. In the absence of pyruvate the enzyme lost only 5% of the initial enzymic activity during a sixty min modification period. During an identical modification, carried out in the presence of 16mM pyruvate the enzyme lost 28% of the initial enzyme activity. This effect was qualitatively the same as observed in previous studies (section 6.3.2.). Determination of the radioactivity in the two protein samples indicated that 0.773 nmoles of bromopyruvate was bound per nmole of biotin in absence of pyruvate

whereas 0.738 nmoles of bromopyruvate per nmole of biotin was incorporated in the presence of pyruvate. This result indicated that even though the rate of inactivation was increased in the presence of pyruvate, less residues were modified. Pyruvate also had a significant effect on the labelling pattern of the subunits. Table 6.3 shows that in the presence of pyruvate the extent of labelling of the 5S ear subunit was reduced but that the subunit was labelled more heavily.

The decrease in labelling of the 5S ear subunit in the presence of pyruvate may suggest that some protection against modification of amino acid residue(s) in the keto acid binding site was occurring. However it was quite clear that other residues on the 5S ear subunit were also being modified. The increased labelling of the 6S head subunit suggested that the binding of pyruvate to the 5S ear subunit may have induced changes which altered the environment of modifiable residues in the 6S head subunit. Such changes in the modification pattern of the 6S head subunit may be responsible for the increased rate of inactivation of transcarboxylase by bromopyruvate in the presence of the keto acid substrates. This interpretation of the results would imply that transcarboxylase has an essential amino acid residue which is on the 6S head subunit. The reaction of

this amino acid residue with bromopyruvate could cause inactivation of the enzyme and so effectively mask any effect of bromopyruvate modification at the keto acid binding site on the overall enzymic activity.

6.4. DISCUSSION

The use of 3-bromo-pyruvic acid as a specific affinity label of the keto acid binding site of trans-carboxylase has been shown in this chapter not to be possible. Both the 5S ear and the 6S head subunits have been shown to be modified by this reagent and since a specific affinity label of the keto acid binding site should modify only the 5S ear subunit it was clear that 3-bromopyruvate was acting, to a large extent, as a non-specific alkylating agent. The observation that pyruvate, at saturating concentrations, reduced the level of modification of the 5S ear subunit may be interpreted as evidence for a reactive residue in the keto acid binding site. However it is considered equally probable that conformational changes induced upon binding of pyruvate to the 5S ear subunit may have a similar effect. This view is strengthened by the fact that high concentrations of the reagent are necessary to produce any significant reduction in the enzymic activity, even though the K_m value for pyruvate in the transcarboxylase reaction is only 0.77mM (Northrop, 1969). This is in contrast to the effect of 3-bromopyruvate on the activity

of pyruvate carboxylase where concentrations of the order of 1mM produce a significant loss of enzymic activity, even though the K_m value for pyruvate in this enzymic reaction is, in the absence of acetyl CoA, 6mM (Ashman et al., 1972).

The much greater loss of transcarboxylase activity in the presence of both pyruvate and bromopyruvate correlates with a heavier modification of the 6S head subunit. This result may suggest that an essential residue on the 6S head subunit is more accessible to the aqueous environment following binding of pyruvate to the 26S complex, and implies that when pyruvate binds to the 5S ear subunit, some alteration of the conformation of the 6S head subunit is also induced.

These results clearly do not allow any evaluation of the model presented in section 6.1 of this chapter. The absence of a modifiable residue in the keto acid site of transcarboxylase can be interpreted in two ways. Firstly, since the investigations of the cysteine residue in the keto acid site of pyruvate carboxylase indicate that this residue is not required for catalysis (Chapter 5), then it is possible that even though the enzymes have evolved from the same primordial genome, a mutational event may have eliminated this cysteine residue from the keto acid binding site of transcarboxylase. Alternatively, the two primitive keto

acid binding sites on these two enzymes were derived from different ancestral genes and evolved to utilize the same biotin carboxyl carrier protein.

The question of the origin of these keto acid binding sites therefore remains an open one. A more specific affinity label is clearly required to unequivocally answer this question. One such possibility is the photoaffinity label 3-azido-pyruvate, which may be able to be synthesized in a similar fashion to 8-azido-AMP (Haley and Hoffman, 1974), by replacement of the bromine atom of 3-bromopyruvate with an azide functional group.

FIGURE 6.1

A possible scheme for the evolution of three biotin-utilizing enzymes from a common ancestral gene.

In this diagram

A is the gene coding for the protein containing the ATP/HCO_3 binding site

B is the gene coding for the protein containing the covalently bound biotin

C is the gene coding for the protein containing the keto acid binding site

D is the gene coding for the protein containing the CoA-ester binding site.

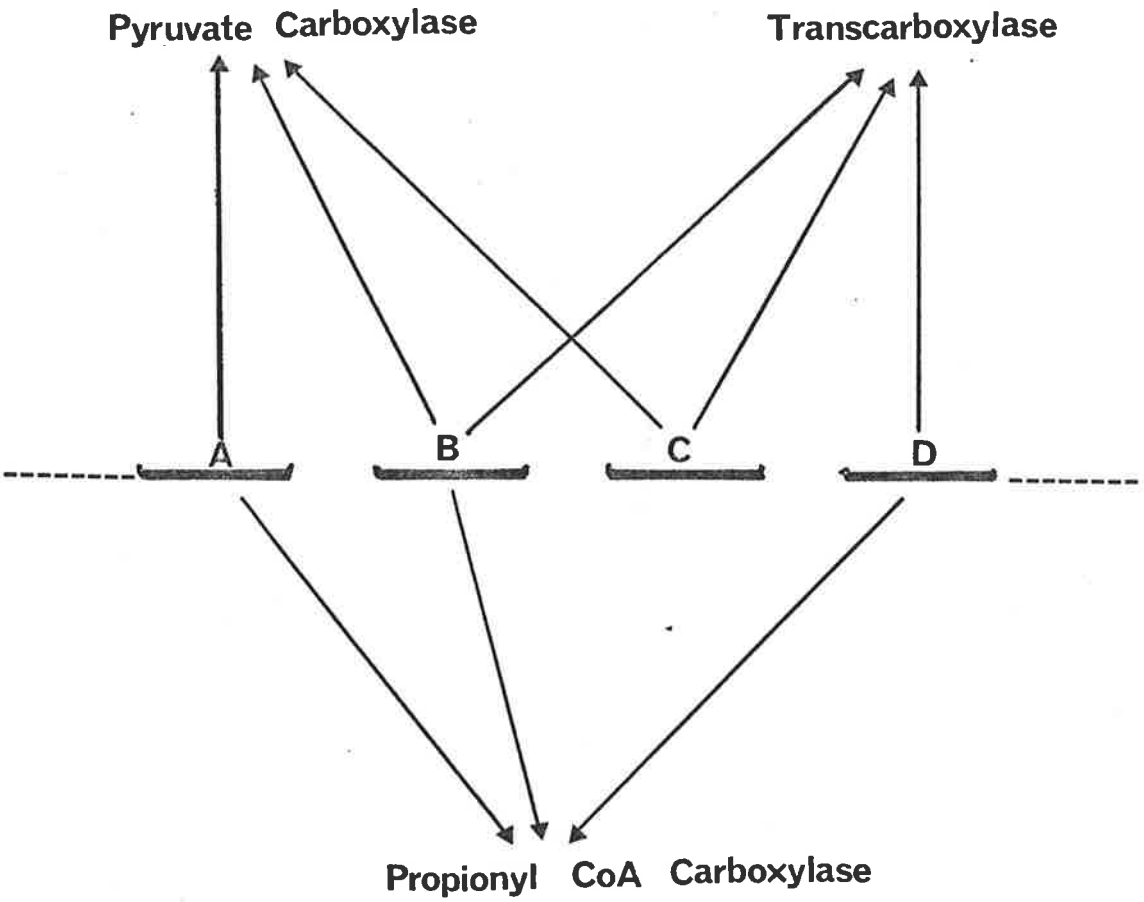

Pyruvate Carboxylase
Transcarboxylase
A
B
C
D
Propionyl CoA Carboxylase

FIGURE 6.2

The effect of pH on the rate of inactivation of transcarboxylase by 3-bromopyruvate.

Transcarboxylase (S.A. 23.5; 0.44 units/ml) was incubated with 50mM 3-bromopyruvate in N-ethyl morpholine-Cl buffer (0.375M) at pH 7.0, 7.5 and 8.0 as indicated in the figure. At the times designated, aliquots were removed for enzyme activity determination. In the control water was used to replace 3-bromopyruvate.

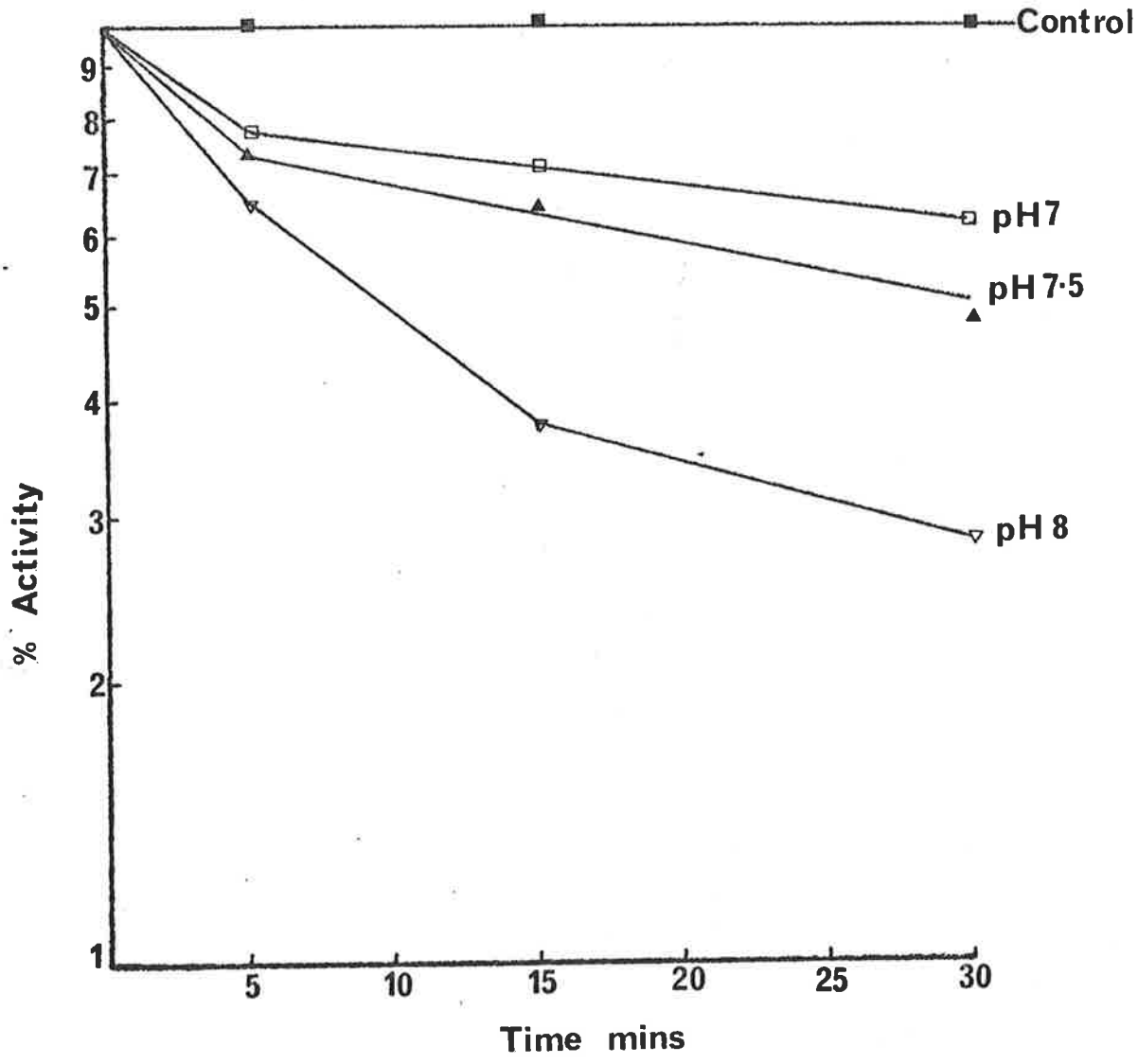

Control
pH7
pH 7·5
pH 8
% Activity
9
8
7
6
5
4
3
2
1
5
10
15
20
25
30
Time mins

TABLE 6.1

The effect of various concentrations of 3-bromopyruvate on the enzymic activity of transcarboxylase.

Transcarboxylase (S.A. 24.9, 25.5 units/ml) was incubated in 0.375M N-ethylmorpholine-phosphate buffer, pH 8.0 with the concentration of 3-bromopyruvate shown for 30 min and the residual enzymic activity determined. In the control 3-bromopyruvate was replaced with water.

TABLE 6.1

3-Bromopyruvate (mM)	% Residual Activity
0	100
2	74
10	66
25	53

TABLE 6.2

The effect of pyruvate and oxaloacetate on the enzymic activity of transcarboxylase in the presence of 3-bromopyruvate.

Transcarboxylase (S.A. 24.9; 25.2 units/ml) was incubated in 0.375M N-ethylmorpholine-phosphate buffer, pH 8.0 with the concentrations of 3-bromopyruvate, pyruvate or oxaloacetate shown in the figure. After 30 min samples were removed and the residual enzymic activity determined.

TABLE 6.2

3-bromopyruvate (mM)	Keto Acid (mM)	% Residual Activity
0	0	100
43	0	72
43	pyruvate (21.8)	63
10.8	0	74
10.8	oxaloacetate (21.6)	65

FIGURE 6.3

The effect of pyruvate and oxaloacetate on the intensity of the transcarboxylase-ANS complex fluorescence. The experiment performed as described in section 6.2.4. using either

▲—▲ transcarboxylase-ANS complex

□—□ transcarboxylase-ANS complex + 10mM oxaloacetate

●—● transcarboxylase-ANS complex + 10mM pyruvate

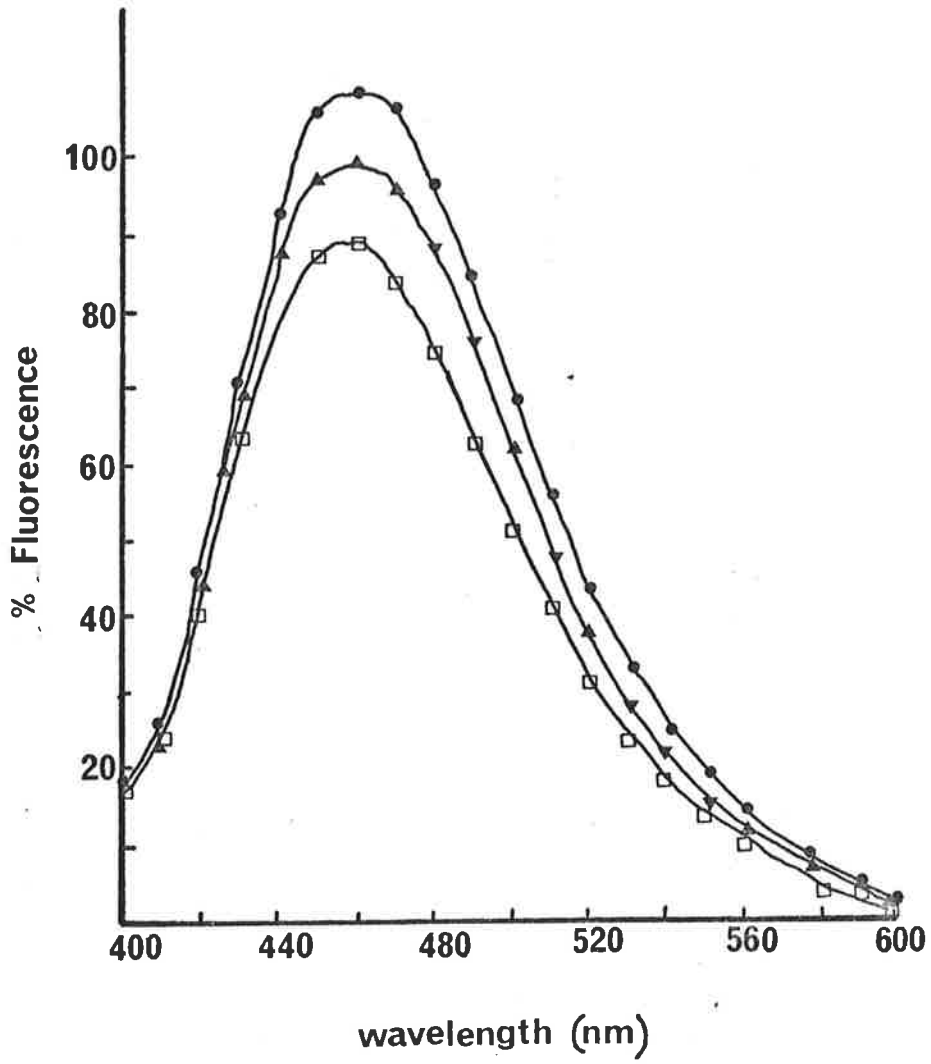

100
80
60
40
20
% Fluorescence
400
440
480
520
560
600
wavelength (nm)

TABLE 6.3

The site of modification of transcarboxylase.

Transcarboxylase (S.A. 23.5; 22.5 units/ml) was incubated with 3-bromo[^{14}C]pyruvate (5500 cpm/nmole; 7.2mM) in 0.175M potassium phosphate buffer, pH 6.3, for sixty minutes and the reaction then terminated by addition of DTE to a final concentration of 72mM. The residual enzymic activity was then determined. The extent of modification was determined as described in the text and polyacrylamide gel electrophoresis performed as described in section 6.2.3.

TABLE 6.3

conditions	% residual activity	nmoles [^{14}C]bromopyruvate/nmole biotin		
		$6S_H$	$5S_E$	Total
-pyr	95	0.343	0.428	0.773
		(44.5%)	(55.4%)	
+pyr	72	0.415	0.323	
		(56.2%)	(43.8%)	

CHAPTER 7

ON THE IDENTITY OF THE POLYPEPTIDE CHAINS OF PYRUVATE CARBOXYLASE

7.1. INTRODUCTION

The electron micrographs of pyruvate carboxylase isolated from both chicken and sheep liver presented in Chapter 3 showed that the subunits of these enzymes were arranged in a rhombic configuration. Such an arrangement of subunits implied that either there were heterologous binding domains between the subunits or that the subunits were non-identical. The latter alternative was considered to be more likely, though this judgement was based only on the arguments of Monod et al. (1965). A more definitive answer was sought through investigation of the tryptic digests of pyruvate carboxylase from both the avian and ovine sources.

7.2. METHODS

7.2.1. Modification of pyruvate carboxylase with [^{14}C]IAA

Chicken liver pyruvate carboxylase was firstly dialysed against water containing 0.02% sodium azide, 10^{-4}M EDTA and 10^{-4}M PMSF and freeze-dried. The protein was then dissolved in 6M guanidine-HCl containing 0.1M tris-Cl, pH 8.4 and incubated at 37^{o}C for 1 h with an equimolar amount of DTE/SH to protein sulphydryl groups. [^{14}C]-iodoacetic acid (50μCi; 51μCi/μmole), dissolved

in 0.5ml of tris-Cl, pH 8.0 was then added to the solution and the reaction allowed to proceed for 1 h at 37°C in the dark. The unmodified cysteine residues were then converted to the S-carboxymethyl-derivative as described in Section 2.2.8.

7.2.2. Synthesis of 3-bromo[^{14}C]pyruvate

3-bromo[^{14}C]pyruvate of high specific activity was synthesised on a microscale by a method developed in this laboratory by Hudson (1975) (unpublished results). In a 5ml flask fitted with a reflux condenser and a calcium chloride drying tube, pyruvic acid (redistilled, ca. 5μmoles), sodium[^{14}C]pyruvate (50μCi; 8.2μCi/μmole) and dry bromine (ca. 15 μmoles) were reacted in 0.5ml of acetic acid (99% w/w) containing 1 drop of HCl at 65°C for 40 minutes with vigorous stirring under reflux. Chromatography of the product on silica gel thin layer plates using the solvent system chloroform/1% formic acid gave a radioactive spot of R_f 0.19, identical to authentic 3-bromopyruvate. No unreacted pyruvate (R_f = 0.5) was detected.

The specific activity of the 3-bromo[^{14}C]-pyruvate was determined using a lactate dehydrogenase assay for 3-bromopyruvate in which the irreversible inactivation of lactate dehydrogenase is minimized (Barnett et al., 1971). The assay was linear up to

30µgms of 3-bromopyruvate (Hudson, 1975) and gave a specific activity of 5.5×10^6 cpm per µmole for the synthetic 3-bromo[^{14}C]pyruvate.

Prior to modification of sheep liver pyruvate carboxylase with this reagent, the volatile solvents were removed by evaporation under a gentle stream of nitrogen and the 3-bromo[^{14}C]pyruvate was redissolved in 0.5M potassium phosphate, pH 7.2. This solution was used within fifteen minutes of preparation.

7.2.3. Modification of sheep liver pyruvate carboxylase with 3-bromopyruvate

The modification of all cysteine residues of sheep liver pyruvate carboxylase with non-radioactive 3-bromopyruvate was performed in an idential manner to the S-carboxymethylation of the enzyme (Section 2.2.8.) except that it was necessary to use 8M urea instead of 6M guanidine-HCl as the denaturant. This was because it was considered likely that the subsequent reduction of the CKM-cysteine adduct to the CHE-cysteine adduct would not proceed in guanidine-HCl due to reduction of the guanidinium group of this denaturant by the sodium borohydride. The alkylation reaction was therefore performed in 8M urea and, following the termination of the reaction with DTE, the CKM-group was reduced to the CHE-group using a 10-fold molar excess

of sodium borohydride over the final concentration of 3-bromopyruvate. Amino acid analysis of the modified protein indicated that complete modification of the cysteine residues had occurred. The CHE-cysteine derivative eluted from the column of the amino acid analyser marginally ahead of the elution position of SCM-cysteine.

7.2.4. Digestion with trypsin

The protein was suspended at a concentration of 20mg/ml in 0.1M ammonium bicarbonate and digested with 0.4mg/ml of trypsin. After 4 h, a further 0.4mg/ml of trypsin was added and digestion continued for a further 4 h. Insoluble material was then removed by centrifuging.

7.2.5. Peptide mapping procedure

Peptide maps were developed on thin layer cellulose plates using the procedure described by Vandekerckhove and Van Montagu (1974). The peptides were first separated by electrophoresis at pH 6.5 (10% pyridine: 1% acetic acid) or pH 3.5 (0.5% pyridine: 5% acetic acid) at 15 volts/cm for 1 h followed, after thorough drying under a stream of cold air, by ascending chromatography in butanol: acetic acid: pyridine: water (15:3:10:12 v/v) or butanol: acetic

acid: water (40:6:15; v/v) (Patthy and Smith, 1975). After drying, peptides were detected by spraying with 0.001% fluorescamine in acetone containing 1% pyridine, and the peptide material recovered by scraping off the spots from the plate. This material was placed in vials and the radioactivity determined as described in Section 2.2.6.

7.2.6. Preparative peptide isolation procedure

The isolation of peptides for amino acid analysis and N-terminal amino acid determination was performed on Whatman 3MM paper using as a guide the relative mobilities of the peptides in the analytical peptide maps. The same solvent systems as those described in Section 6.3.5. were used. The peptides were detected by autoradiography and, where necessary, sewn onto another piece of Whatman 3MM paper and separated in a second solvent system as described by Harris (1967). Electrophoresis was performed on apparatus similar to that described by Michl (1951). Descending chromatography was performed in a closed chromatography tank, saturated with solvent, for 16 h.

7.2.7. Identification of the N-terminal residue

Dansylation and identification of the N-terminal residues was carried out on 2-5 nmoles peptide

by the method described by Hartley (1970).

7.2.8. Calculations of the relative radioactivity in each peptide

In order to provide a basis for comparison of the amount of each peptide isolated in this study the radioactive content of each peptide has been expressed as a percentage of the total radioactivity incorporated into the protein. To do this it is necessary to assume that during any given fractionation procedure the losses observed were evenly distributed amongst all radioactive peptides (i.e. there had not been a selective or specific loss of one unique radioactive peptide). Therefore the relative ratios of the resolved peptides following any given fractionation step were assumed to be the same as prior to the fractionation step and the extent of labelling of each of individual peptides was then uniformly corrected for the loss of radioactivity during the fractionation procedure. This process was repeated for each fractionation step until unique radioactive peptides were resolved. The process is clearly only an approximation and the data obtained can be used only in a semi-quantitative or qualitative fashion.

7.3. RESULTS

7.3.1. Chicken liver pyruvate carboxylase

Palacian and Neet (1970) reported that chicken liver pyruvate carboxylase contained 55 moles of cysteine per 655,000 g of protein. Since the molecular weight of the chicken liver enzyme has recently been redetermined to be approximately 500,000 daltons (Utter et al., 1975) then, by applying a correction factor, a value of approximately 42 moles of cysteine per mole of enzyme can be obtained. Bais (1974) obtained a value of 44±4 moles of S-carboxymethyl cysteine per mole of the chicken liver enzyme by amino acid analysis. The chicken liver enzyme prepared in this laboratory has been determined to contain approximately 42 moles of cysteine per mole of enzyme by the spectrophotometric method of Ellman (1959). These three independent determinations are in excellent agreement and it can be concluded that the tetrameric form of chicken liver pyruvate carboxylase contains ca. 42 moles of cysteine and no cystine residues. Clearly then, if the chicken liver enzyme was composed of identical subunits, each subunit would contain approximately 10 cysteine residues.

One method of testing whether the subunits of chicken liver pyruvate carboxylase were identical was

therefore to modify the cysteine residues of this enzyme with [^{14}C]iodoacetic acid and then to examine the resultant tryptic digest of this material by conventional peptide isolation procedures. If the modification of the cysteine residues with [^{14}C]iodoacetic acid was performed under denaturing conditions (e.g. 6M guanidine-HCl) then it would appear reasonable to expect that each of these amino acid residues would be equally accessible to iodoacetic acid. Each cysteine residue would then contain an equivalent amount of radioactivity. The tryptic digest of the radioactively modified enzyme would therefore result in ten unique, equivalently labelled cysteine peptides if the the subunits of the enzyme were homologous.

This approach was adopted using 15 mg of chicken liver pyruvate carboxylase which was homogeneous on SDS polyacrylamide gel electrophoresis. The modification of this material with [^{14}C]iodoacetic acid (50μCi; 51μCi/μmole) was performed as described in Section 7.2.1. The protein incorporated 20.2 x 10^6 cpm. To reduce losses of this material in the subsequent purification steps non-radioactive S-carboxymethylated chicken liver pyruvate carboxylase was added to the radioactive protein to act as a carrier. The combined material contained 1359 nmoles of biotin as determined by the method of Rylatt *et al*. (1977).

The tryptic digestion of this material was performed as described in section 7.2.4. and the insoluble tryptic "core" material was removed by centrifuging. No radioactivity was detected in this insoluble material. The supernatant was applied directly to a Sephadex G-25 (fine) column (3.0 x 158cm) equilibrated in 0.1M ammonium bicarbonate and chromatographed in the same buffer at a flow rate of 1 ml per min. The elution profile is shown in fig 7.1. The majority of the SCMC-peptides chromatographed as a single broad peak. To determine the distribution of the radioactive peptides within the profile 100µl of every alternate radioactive fraction (fractions numbers 48-92) was removed, freeze dried and redissolved in 5µl of 50% pyridine. These fractions were subjected to electrophoresis at pH 6.5 for 2 hr at 50 volts/cm. The peptides were detected by autoradiography. The autoradiogram (plate 7.1) revealed approximately 12 - 14 peptides but these were clearly not labelled to the same extent. Three explanations for this result could be possible.

(1) There were contaminating proteases (e.g. chymotrypsin) in the trypsin used to digest the SCMC-enzyme.

(2) The modification of other amino acid side chains also occurred during the S-carboxy-

methylation of the protein with [^{14}C]iodo-acetic acid.

(3) The subunits of chicken liver pyruvate carboxylase are not identical in amino acid sequence.

These possibilities will be discussed in later sections of this chapter.

The remainder of the radioactive peptides were pooled into five fractions and freeze-dried. The relative amounts of radioactivity in each fraction are shown in Table 7.1. These fractions were further fractionated as described below.

7.3.1.1. Fraction A

Fraction A contained 6.6% of the original radioactivity incorporated into the protein and contained the highest molecular weight SCMC peptides. This material was not soluble in the electrophoretic or chromatographic solvents below neutrality but two radio-active peptides could be resolved by chromatography in butanol / pyridine / water (fig 7.2). The major peptide contained 5.8% of the original radioactivity while the minor peptide contained 0.8% (Table 7.?).

7.3.1.2. Fraction B

This fraction, which formed a shoulder on the major radioactive peak (fraction C), contained 9.7% of the radioactivity originally incorporated into the protein. Of this material only 61.3% was solubilized by 5mM ammonium bicarbonate however. The residual radioactivity was recovered as a precipitate by centrifugation.

The soluble peptides, (fraction B.1.), amounting to 6.0% of the original radioactivity were fractionated on a DEAE-cellulose column (1 x 13cm) using a 400ml linear gradient of ammonium bicarbonate from 5mM to 500mM (fig 7.3). Four fractions containing radioactivity were resolved. The peptides in each of these fractions were further resolved by two dimensional peptide mapping on thin layer cellulose plates (fig 7.4). The peptides were located with a fluram spray and their radioactivity determined as described in section 7.2.5. The values obtained were then expressed as a percentage of the original radioactivity as described in section 7.2.8. These percentages are shown in Table 7.3.

The insoluble fraction (B.2.) was, like fraction A, insoluble at pH values below neutrality. At pH 8.9 however this material moved as a single radioactive band with an R_m of +0.31, different to that of the

A fraction (R_m + 0.41).

7.3.1.3. Fraction C

This fraction contained 66.7% of the original radioactivity. All of this material was soluble in 5mM ammonium bicarbonate. The peptides were resolved on a DEAE-cellulose column (1.7 x 21cm) using a 1000ml linear gradient of ammonium bicarbonate from 5mM to 500mM. Twelve radioactive fractions were resolved by this process (fig 7.5). Each of the fractions was further analysed by two dimensional peptide mapping (fig 7.6) and the radioactivity corresponding to each of the SCMC-peptides was determined as described previously. These values, expressed relative to the original radioactivity are shown in Table 7.4.

Ten of the SCMC-peptides present in fraction C were able to be isolated by preparative peptide mapping on Whatman 3MM paper using conditions adapted from the analytical peptide mapping procedure. The purity of each of these peptides was established by the identification of a unique N-terminal amino acid residue. The amino acid compositions and N-terminal residues of these ten peptides is shown in Table 7.7.

7.3.1.4. Fraction D

Fraction D contained 10.7% of the original

radioactivity incorporated into the protein. Fractionation on a DEAE-cellulose column (1 x 12cm) as described for fraction B, resulted in the resolution of three radioactive fractions (fig 7.7). These fractions were further analysed by peptide mapping (fig 7.8) as described previously and the relative content of radioactivity in each of these peptides is shown in Table 7.5. The major peptide of this fraction was purified by preparative procedures identical to the procedure used for the analytical peptide mapping and the N-terminal residue and amino acid composition of this peptide is shown in Table 7.7.

7.3.1.5. Fraction E

Fraction E contained 6.2% of the original amount of radioactivity incorporated into the protein. Ion exchange chromatography on DEAE-cellulose, using a linear gradient of ammonium bicarbonate from 5mM to 300mM resolved fraction E into four radioactive fractions (fig 7.9). Analytical mapping of these fractions was again used to examine the peptide distribution in each of the resolved fractions (fig 7.10). The relative radioactive content of each of the SCMC-peptides was expressed as described previously and is shown in Table 7.6. The radioactive fractions E_3 and E_4 were single peptides and the amino acid compositions of these two peptides were identical (Table 7.7).

However, whereas peptide E_4 had a phenylalanine N-terminal amino acid residue, no N-terminal amino acid residue could be detected by dansylation of the peptide E_3. The two most likely reasons for this occurrence were that either peptide E_3 contained an N-terminal tryptophan residue, which would have been destroyed during the dansylation procedure and amino acid analysis, or that the N-terminal residue was a glutamine which had undergone cyclisation to form the pyrrolidone carboxylic acid and so block the N-terminal (see Blombäck, 1967). Either possibility allows peptides E_3 and E_4 to be regarded as separate peptides.

Another unusual feature of these two peptides was that they were eluted from the Sephadex G-25 column later than the smaller peptide, D_3. Since Sephadex is known to interact with aromatic groups to a significant extent (Eaker and Porath, 1967) it is perhaps possible that the presence of the phenylalanine residues in peptides E_3 and E_4 may have caused these peptides to be retarded on the column.

7.3.1.6. Conclusions

The results presented in the preceding sections of this chapter provide evidence in favour of the view that the subunits of chicken liver pyruvate

carboxylase are not identical in their amino acid sequences. The alternative explanations presented earlier in this chapter were that either

(1) there was contamination of the trypsin by some other protease

or (2) there was modification of other amino acid residues, in addition to the cysteine residues, by iodoacetic acid.

The former possibility would appear to be unlikely since all thirteen of the SCMC-peptides which have been isolated in a pure form contain either a lysine or an arginine residue. Therefore it would seem reasonable to suggest that these peptides have arisen primarily from the action of trypsin on the SCMC-chicken liver pyruvate carboxylase. The second possibility is considered to be unlikely since examination of the amino acid analyses of the isolated SCMC-peptides has failed to reveal the presence of any other carboxymethylated amino acid residues (see Hirs, 1967). Therefore S-carboxymethylation of amino acid residues, other than cysteine residues, would appear to have occurred to a minimal extent.

Other factors which could contribute to the appearance of the large number of radioactively labelled

peptides which are present in only trace amounts include oxidation products of the SCM-cysteine and methionine residues in the protein (Harris, 1967) or incomplete cleavage of the polypeptide chain by trypsin, due to the presence of either charged amino acid residues or proline residues adjacent to the lysine and arginine residues in the protein (Bennett, 1967). In addition some of these peptides present in only trace amounts are almost certainly tailing fractions of SCMC-peptides in adjacent fractions.

The calculations of the relative distribution of the radioactive content of the individual peptides are only approximate since these calculations require the assumption that there are equivalent losses by all peptides during the purification procedures. Furthermore, the ambiguities inherent in assigning the radioactive peptides present in trace amounts to the parent radioactive peptide further complicate the interpretation of this data. Therefore the only information that can be obtained from this data is to suggest that some of the SCMC-peptides isolated are present in amounts greater than would be expected if they were present only once in the tetrameric form of the enzyme. Therefore this would imply that there are regions of homology around some of the cysteine residues in the chicken liver enzyme.

The major result of this study is that the isolation of thirteen SCMC-peptides from a tryptic digest of SCMC-chicken liver pyruvate carboxylase appears to be incompatible with the concept of identical polypeptide chains where only approximately ten such SCMC-peptides could be formed. It is therefore suggested that the subunits of chicken liver pyruvate carboxylase may not be identical in amino acid sequence. Verification of this hypothesis would require the isolation and sequence of all radioactive peptides obtained from a trypic digest of [^{14}C]SCMC-chicken liver pyruvate carboxylase. This task is beyond the scope of the present study.

7.3.2. Sheep liver pyruvate carboxylase

Sheep liver pyruvate carboxylase can be specifically modified by 3-bromopyruvate in the presence of saturating concentrations of acetyl CoA (Hudson et al., 1975). From the evidence presented earlier in this thesis (Chapter 5) it was suggested that this same amino acid residue could also be modified by DTNB. In addition, it was shown that the cysteine residues modified by DTNB were not required to function as proton donors or nucleophiles in the reaction catalysed by sheep liver pyruvate carboxylase. It may therefore be postulated that if the subunits of the sheep liver enzyme are non-identical one such variable region may be around the reactive cysteine

residue modified by 3-bromopyruvate.

To examine this question, homogeneous sheep liver pyruvate carboxylase of high specific activity, containing 75.8 nmoles of biotin was modified with 3-bromo[^{14}C]pyruvate. The inactivation was carried out in the presence of 0.5mM acetyl CoA as described by Hudson *et al.*, (1975), using a final concentration of 0.54mM 3-bromo[^{14}C]pyruvate (specific activity 5500 cpm/nmole) and terminated by the addition of 50mM DTE (fig 7.11). The resultant CKM-cysteine derivative was reduced to the CHE-cysteine derivative using a 10-fold molar excess of sodium borohydride since the CKM-derivative is not stable over long periods of time and tends to decarboxylate. The enzyme was then extensively dialysed against water containing 0.02% sodium azide and 10^{-4}mM EDTA and freeze-dried. The remainder of the cysteine residues in the enzyme were converted to the CHE-derivatives using non-radioactive bromopyruvate as described in section 7.2.3. The protein incorporated 2.68×10^5 cpm, equivalent to 48.7 nmoles of bound 3-bromo[^{14}C]pyruvate, and lost 35% of its initial catalytic activity. These figures gave an extrapolated value of approximately 1.8 moles of 3-bromo-[^{14}C]pyruvate bound per mole of biotin. This value was higher than the value of 1.0 moles of 3-bromopyruvate bound per mole biotin in the presence of acetyl CoA, as reported by Hudson *et al.* (1975), and this discrepancy

is discussed later in this section.

To prevent losses of this material during the subsequent purification steps 200mg of sheep liver pyruvate carboxylase was modified with non-radioactive bromopyruvate, converted to the CHE-derivative, and combined with the radioactively modified protein. The tryptic digestion of this material was performed as described in section 7.2.4. and the insoluble "core" material removed by centrifuging. No radioactivity was found in this core material.

The supernatant from the tryptic digestion was applied to a Sephadex G-25 column (3.0 x 158cm), equilibrated in 0.1M ammonium bicarbonate and chromatographed in this buffer at a flow rate of 1ml per min. The elution profile showed the presence of three radioactive peaks (fig 7.12). The fractions within each of these peaks were combined and the radioactive content of each pooled fraction expressed as a percentage of the original radioactivity applied to the column (Table 7.8) (section 7.2).

These peaks were further fractionated on DEAE-cellulose columns (1.3 x 16cm) using linear gradients of ammonium bicarbonate from 5mM to 350mM. Fraction A (fig 7.13) was shown to contain two peaks of equivalent radioactive content. Fraction B (fig 7.14) and fraction C (fig 7.15) contained only one major radio-

active peak each. Chromatography in butanol : pyridine : acetic acid : water, (15:10:3:12 v/v) indicated that each of these peaks contained only one radioactive peptide (figs 7.16 - 7.19). The relative amount of radioactivity in each of these peptides was expressed as a percentage of the total radioactivity incorporated into the protein and these figures are shown in Table 7.9. Each peptide contained approximately equal percentages of the original radioactivity. The sum of the individual percentages of these four peptides was 56% of the total radioactivity originally bound to the protein. From the known specific activity of the 3-bromo[^{14}C]pyruvate it was calculated that these four peptides therefore had a sum total of 27.3 nmoles of [^{14}C]bromopyruvate covalently bound to them. Since the enzyme contained 75.8 nmoles of biotin and lost 35% of its initial activity during the modification then 27.5 nmoles of the keto acid binding site should be modified. These figures indicate that the four peptides isolated account for the loss of activity observed on the basis of one mole of bromopyruvate bound per mole of biotin, as reported by Hudson et al., (1975). Since no other major peptides have been observed in this study then it can be concluded that the additional 0.8 moles of 3-bromo[^{14}C]pyruvate incorporated per mole of biotin observed in this study were due to non-specific labelling of other amino acid residues in the protein.

The isolation of four peptides containing an approximately equal amount of radioactivity which together account for the observed loss of enzymic activity provides evidence that the sequence around the cysteine residue near the keto acid binding site on the enzyme has not been conserved during the course of evolution. Furthermore it can be concluded that the individual subunits of sheep liver pyruvate carboxylase are not identical and it would appear that this enzyme belongs to the rare class of tetrameric proteins which contain four non-identical subunits. The only other protein known to have four non-identical subunits is the E. coli Qβ replicase (see Klotz et al., 1975).

7.4. DISCUSSION

The data presented in this chapter provides evidence that pyruvate carboxylase isolated from either chicken or sheep liver contains non-identical subunits. This finding is consistent with the rhombic arrangement of the subunits of these enzyme while still permitting thermodynamically more favourable homologous binding domains (see Chapter 4). The information gained from the specific labelling of the sheep liver enzyme with 3-bromo-pyruvate suggests that there are four non-identical polypeptide chains in each tetramer. From the general approach of labelling all of the cysteine residues of the chicken liver enzyme with iodoacetic acid it has not

been possible to distinguish between

(1) two identical dimers composed of non-identical subunits

(2) four unique non-identical subunits.

The large number of peptides obtained from the tryptic digest would, however, favour the latter possibility.

Some of the peptides isolated from the chicken liver enzyme appear in quantities greater than would be predicted on the basis of their occurrence only once in the tetramer and therefore it seems possible that the four subunits contain regions of homology around some of the cysteine residues in this enzyme.

There are two possible methods whereby the degree of homology between the subunits of pyruvate carboxylase can be evaluated. The first is by approaches similar to that adopted in this present study. Such approaches require significant quantities of the enzyme and considerable expenditure of both time and labour. Furthermore, as has been illustrated in the study of the tryptic SCMC-peptides of the chicken liver enzyme in this chapter, the results obtained are equivocal until all peptides concerned have been isolated and sequenced. If these studies result in establishing that the subunits

of the enzyme have dissimilar amino acid sequences then the individual sequences of the peptides isolated during the investigation (e.g. SCMC-peptides) do not contribute to the information regarding the amino acid sequences of the individual subunits until after the subunits have been resolved as intact polypeptide chains.

The second method is to attempt to isolate the intact polypeptide chains. This approach would rely on the charge differences between the presumptive non-identical subunits since the subunits appear to be very similar in molecular weight (Chapter 3; Utter et al., 1975). Therefore ionic denaturants (e.g. SDS; guanidine-HCl) could not be used for the resolution of these subunits. Pyruvate carboxylase is however not very soluble in urea and this solvent may prove unsatisfactory. Modification of the amino groups of the enzyme with either succinic anhydride or maleic anhydride may prove useful in this regard since a number of multicomponent enzymes have been dissociated and made more soluble by this technique (Klapper and Klotz, 1972; Bulter and Hartley, 1972).

It would appear that attempts to resolve the presumptive non-identical polypeptide chains would prove worthwhile. An unambiguous answer may be obtained and in addition may provide a technique whereby the poly-

peptide chains could be resolved on a preparative basis. This would then allow comparative studies between the non-identical subunits to be performed. Alternatively, if the enzyme subunits were not resolved by several different approaches which rely on the charge of the subunit (e.g. polyacrylamide gel electrophoresis, ion exchange chromatography or isoelectric focusing), then such a result would suggest that the polypeptide chains were homologous.

FIGURE 7.1

Fractionation of the [^{14}C]SCMC-tryptic peptides of chicken liver pyruvate carboxylase on Sephadex G-25.

Flow Rate: 1 ml/min

Fraction Size: 8 ml

100µl aliquots were removed from each fraction for the determination of radioactivity and peptide material. The tubes were pooled as indicated.

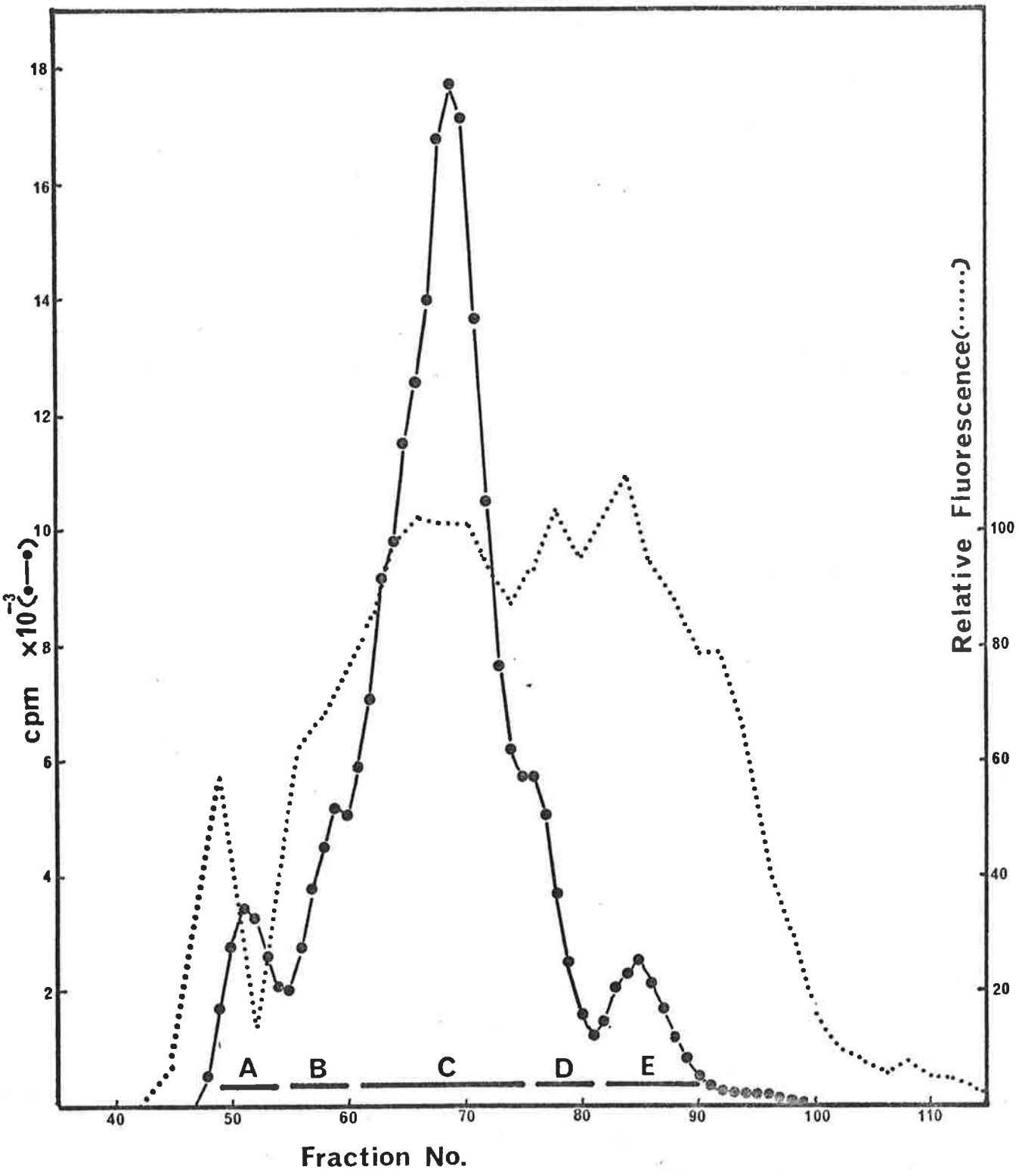

cpm x10^{-3}(•—•)
Relative Fluorescence(.....)
Fraction No.
A
B
C
D
E
18
16
14
12
10
8
6
4
2
100
80
60
40
20
40
50
60
70
80
90
100
110

PLATE 7.1

Chicken liver pyruvate carboxylase.
Electrophoresis of the [^{14}C]SCMC-peptides eluted from the Sephadex G-25 column.

100µl of every alternate fraction eluted from the Sephadex G-25 column which contained radioactivity (fractions 48 - 92 inclusive) was freeze-dried, redissolved in 50% pyridine and subjected to high voltage electrophoresis at pH 6.5 for 2 h at 50v/cm. The electrophoretogram was autoradiographed for 24h in two sections.

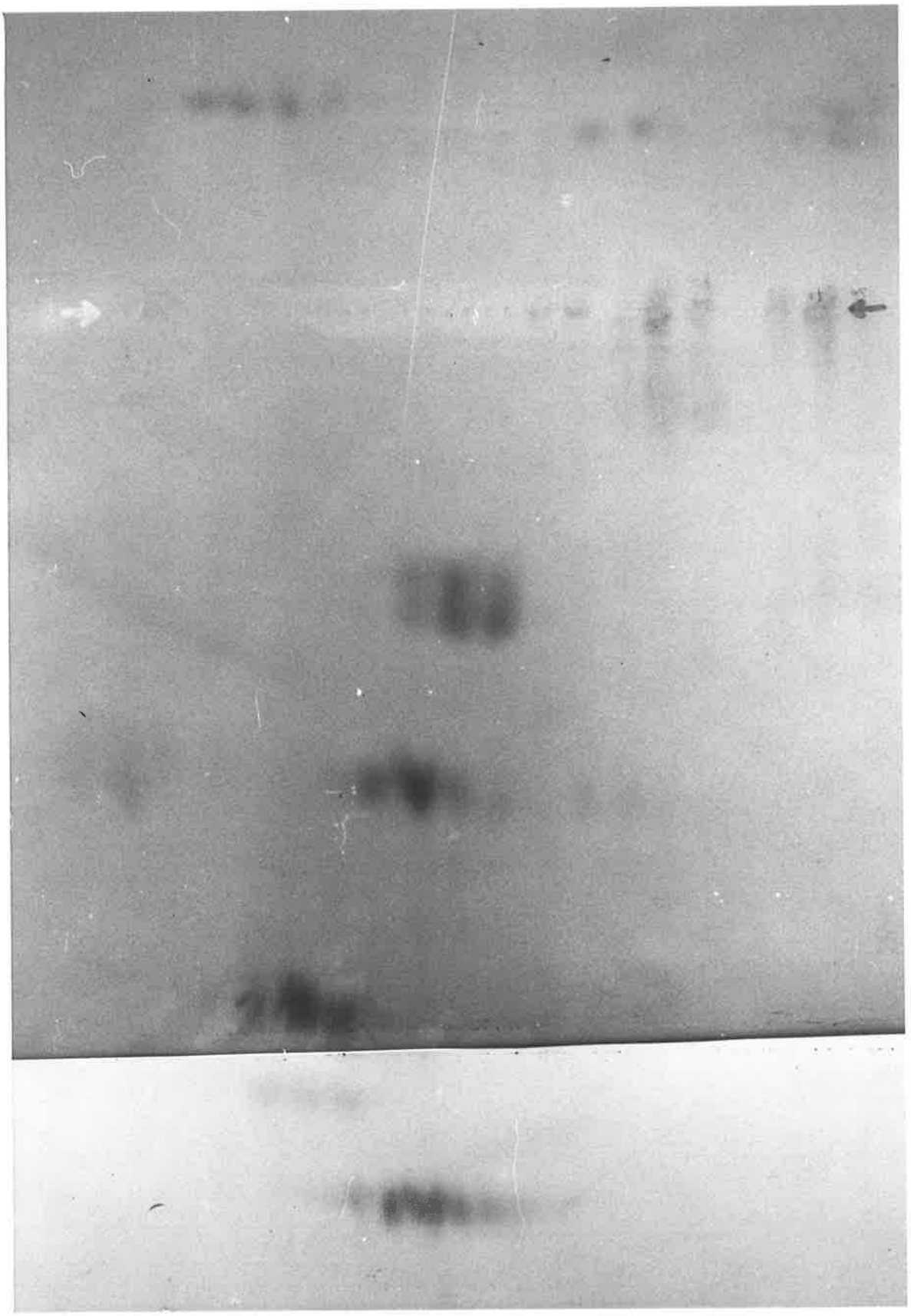


TABLE 7.1

Chicken liver pyruvate carboxylase.

The relative amounts of radioactivity in each of the fractions obtained from the Sephadex G-25 chromatography.

Fraction	% Total Radioactivity
A	6.6
B	9.7
C	66.7
D	10.7
E	6.2

FIGURE 7.2

Chromatography of Fraction A on thin layer cellulose.

A small aliquot of the peptide material in fraction A was applied to a thin layer cellulose plate and chromatographed in n-butanol : pyridine : acetic acid; 1:1:1 (v/v). The plate was then cut into 1cm strips and the radioactivity in each strip determined in toluene scintillation fluid (section 2.2.6.).

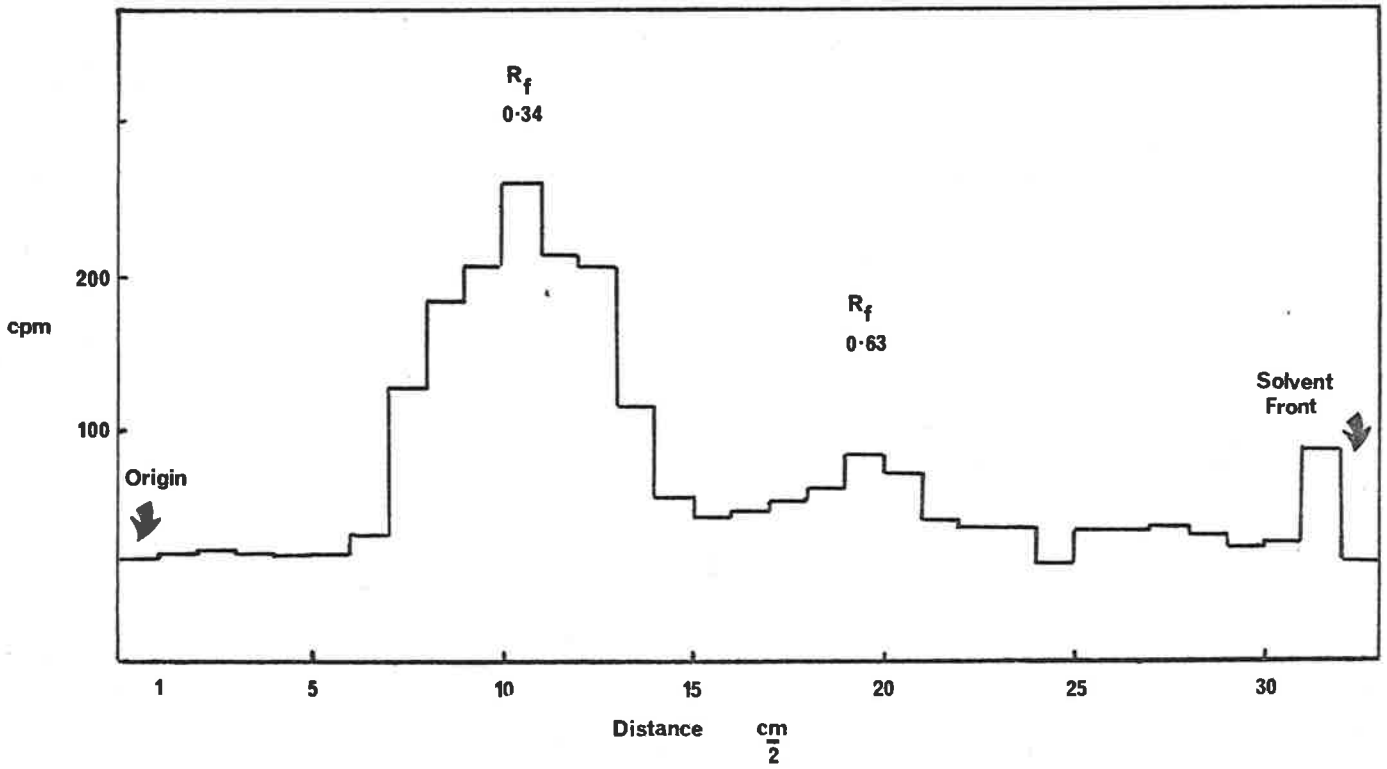

R_f
0·34
R_f
0·63
Solvent
Front
Origin
200
100
cpm
1
5
10
15
20
25
30
Distance
cm
2

TABLE 7.2

Chicken liver pyruvate carboxylase.
The distribution of radioactivity in Fraction A.

The percentage of the total radioactivity originally incorporated into the chicken liver enzyme in each radioactive peptide fraction observed during the purification of the peptides is shown.

The chromatographic solvent (1) was n-butanol : pyridine : water; 1:1:1 (v/v).

The electrophoresis (2) was performed in 1% ammonium bicarbonate, pH 8.9 and the R_m value expressed relative to the mobility of aspartic acid.

Fraction	Total % Radioactivity			
	Initial	Chromatography in (1)	$Rf^{(1)}$	$Rm^{(2)}$
A	6.6			+0.41
A_1		5.8	0.34	
A_2		0.8	0.63	

FIGURE 7.3

Chromatography of the soluble peptides of Fraction B on DEAE-cellulose.

The peptides of fraction B which were soluble in 5mM ammonium bicarbonate (fraction B_1) were fractionated on DEAE-cellulose as described in the text. Only that region of the ammonium bicarbonate gradient which contained radioactivity is shown.

Flow Rate: 0.32 ml/min

Fraction Size: 3.2 ml

50µl of each fraction was removed for the determination of radioactivity and peptide material. The tubes were pooled as indicated by the bars.

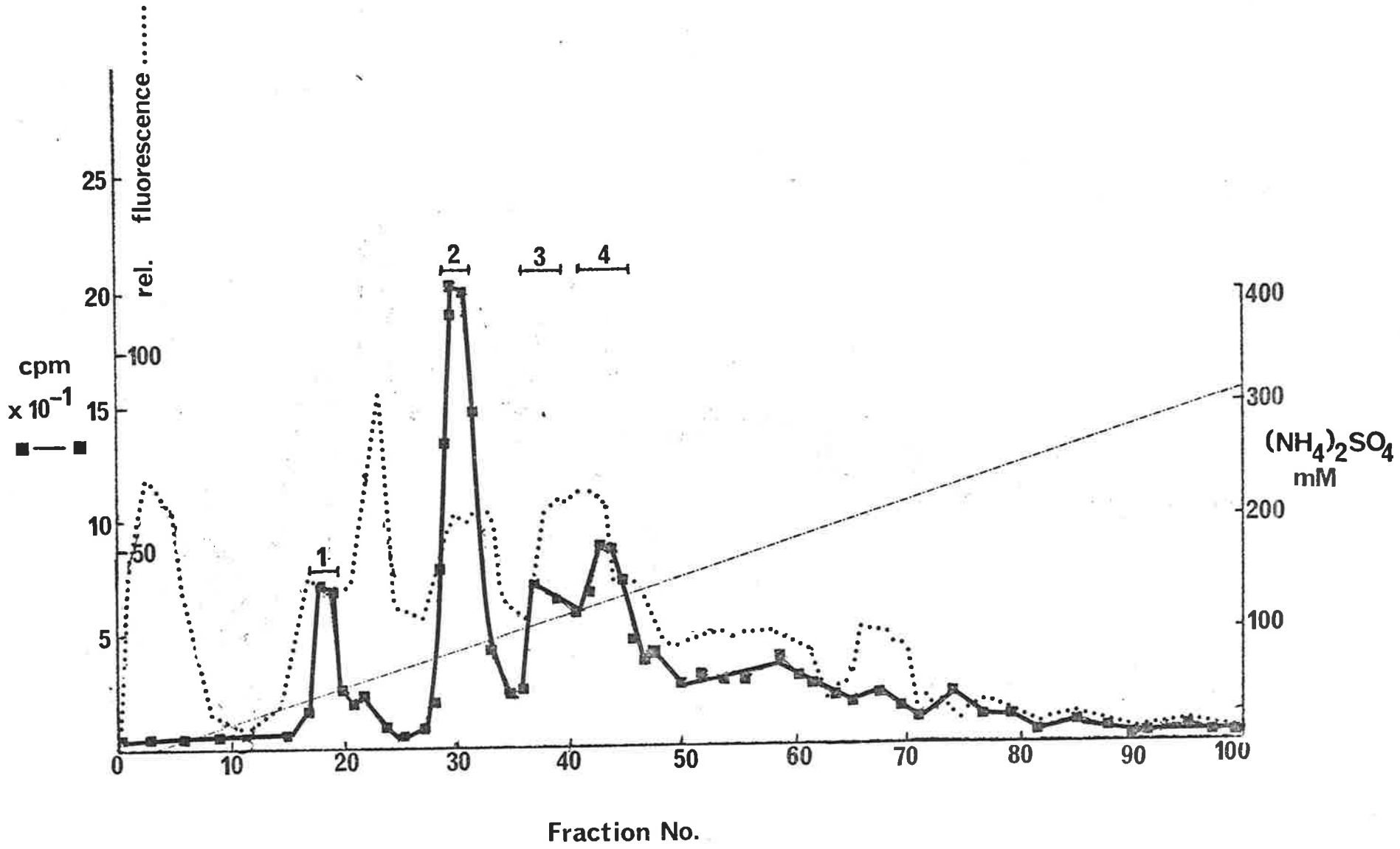
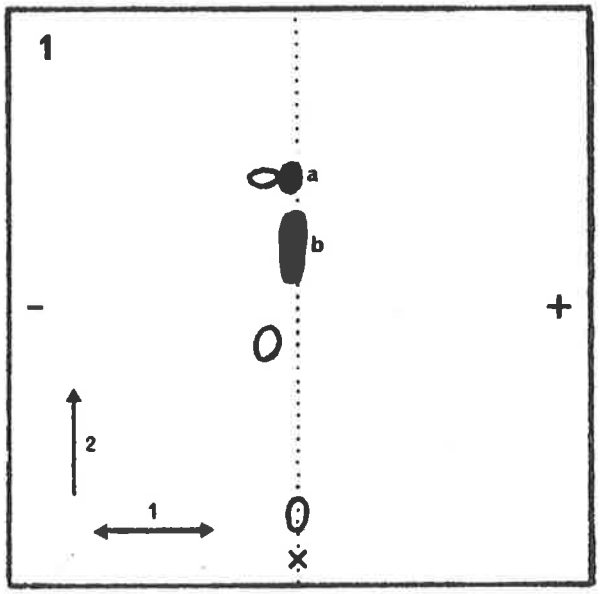
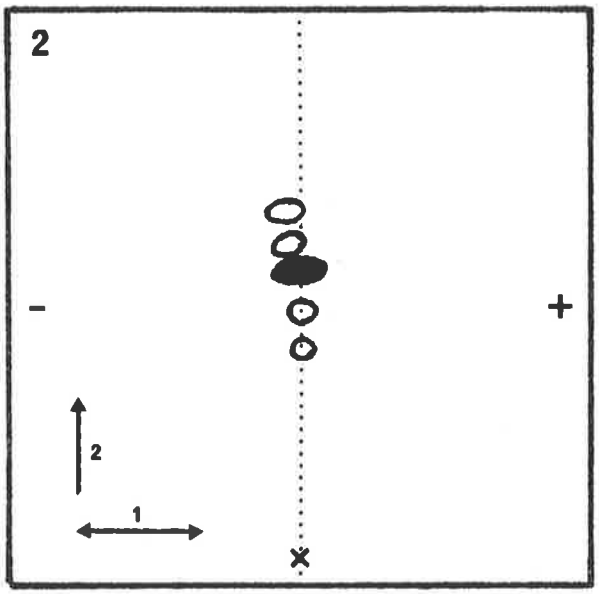

rel. fluorescence .....
cpm
x 10⁻¹
■ — ■
25
20
15
10
5
100
50
1
2
3
4
400
300
200
100
(NH4)2SO4
mM
0
10
20
30
40
50
60
70
80
90
100
Fraction No.

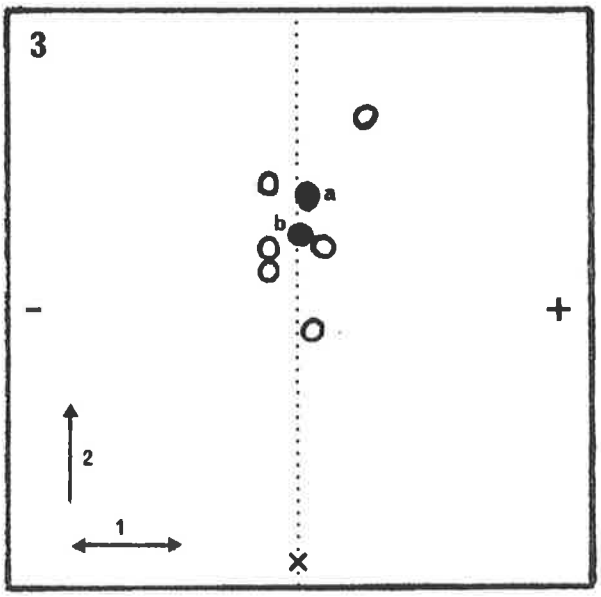
FIGURE 7.4

Two dimensional peptide maps of the soluble Fraction B peptides (Fraction B_1) obtained from DEAE-cellulose chromatography shown in figure 7.3.

Plates 1, 2, 3, and 4 represent aliquots (2 - 10 nmoles) from fractions $B_{1.1}$, $B_{1.2}$, $B_{1.3}$, and $B_{1.4}$ respectively developed on thin layer cellulose. X denotes the position of application of the sample. Electrophoresis in the first dimension was at pH 6.5 (◄—1—►), followed by chromatography in n-butanol : acetic acid : pyridine : water (↑2) (section 7.2.5). The radioactive peptides, located as described in section 7.2.5, are indicated by shading.


1
a
b
-
+
2
1


2
-
+
2
1


3
a
b
-
+
2
1

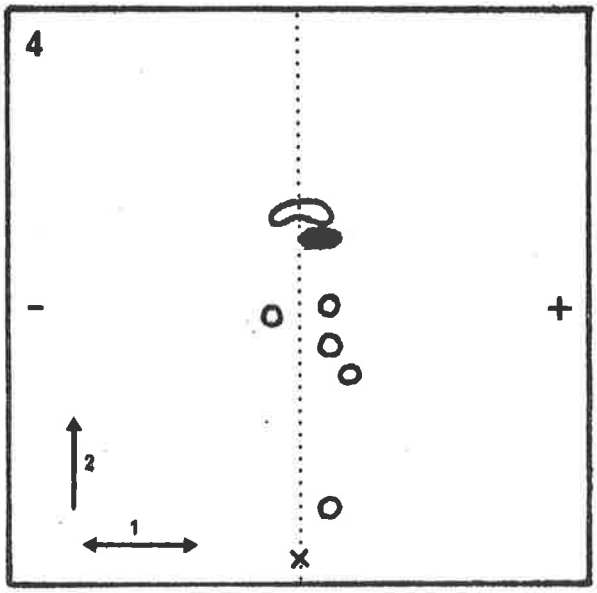

4
-
+
2
1

TABLE 7.3

Chicken liver pyruvate carboxylase.

The distribution of radioactivity in Fraction B.

The data is set out as described for fraction A. The peptide maps from which the data was obtained are shown in figure 7.4.

Fraction	% Total Radioactivity			
	Initial	5mM NH_4 HCO_3 solubilization	Ion-exchange chromatography	Two dimensional peptide mapping
B	9.74			
B_1		5.97		
$B_{1.1}$			0.511	
$B_{1.1a}$				0.277
$B_{1.1b}$				0.234
$B_{1.2}$			3.28	3.28
$B_{1.3}$			0.928	
$B_{1.3a}$				0.489
$B_{1.3b}$				0.439
$B_{1.4}$			1.24	1.24
B_2		3.77		3.77

FIGURE 7.5

Chromatography of the peptides from Fraction C on DEAE-cellulose.

The fractionation was performed as described in the text. Only that region of the ammonium bicarbonate gardient which contained radioactivity is shown.

Flow rate: 0.32ml/min

Fraction size: 4.5ml

100µl of each fraction was removed for the determination of radioactivity and peptide material. The tubes were pooled as indicated by the bars.

Fraction No.
$(NH_4)_2SO_4$ mM
rel. fluorescence
cpm x 10^{-3}
I_1
I_2
I_3
I_4
I_5
I_6
I_7
I_8
I_9
I_{10}
I_{11}
I_{12}

FIGURE 7.6

Two dimensional peptide maps of the Fraction C peptides obtained from the DEAE-cellulose chromatography shown in figure 7.5.

Plates 1 - 12 represent fractions C_1 - C_{12} respectively, developed as described previously (section 7.2.5). X denotes the position of application of the sample.

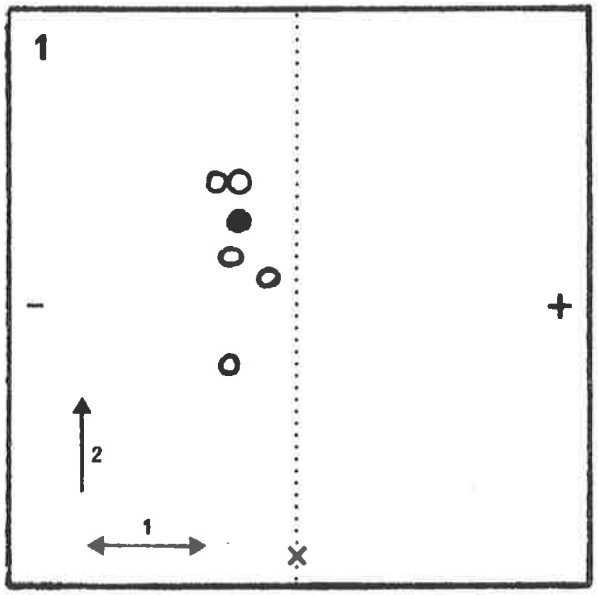
The chromatographic solvents were

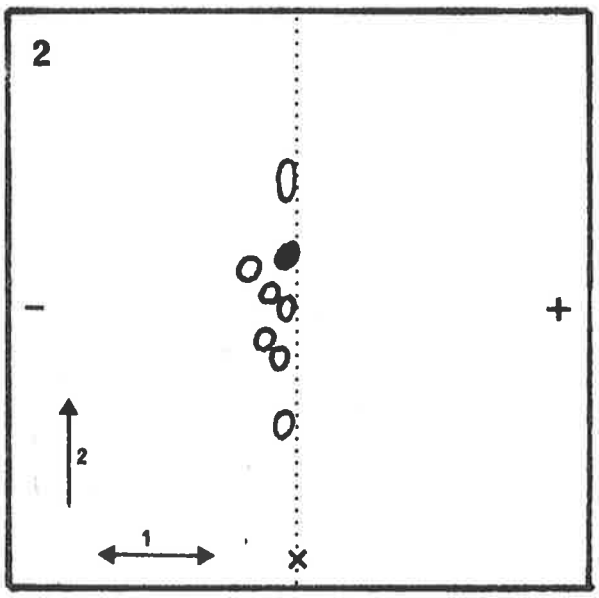
Electrophoresis: ←1→ ; pH 6.5

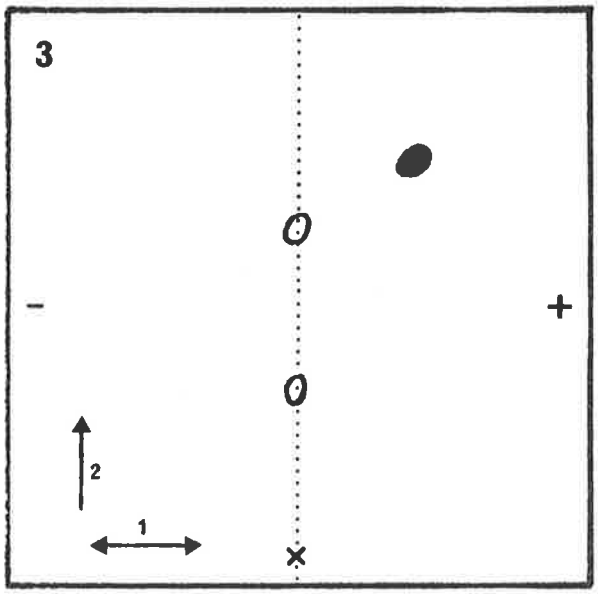
←3→ ; pH 3.5

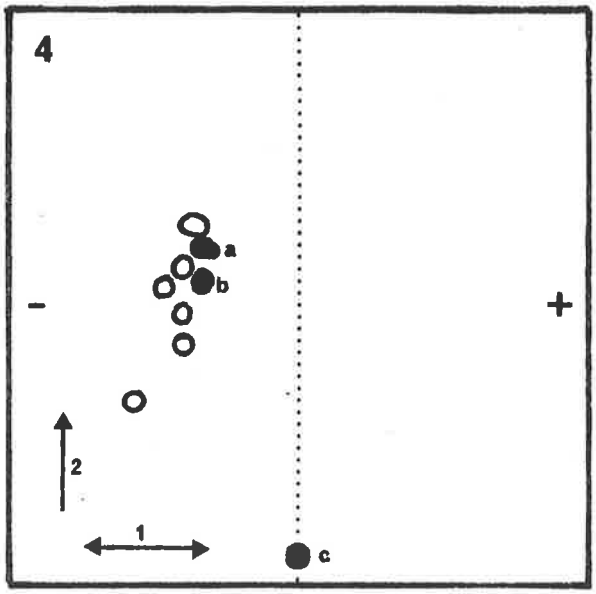
Chromatography: ↑2; n-butanol; acetic acid : pyridine : water (section 7.2.5).

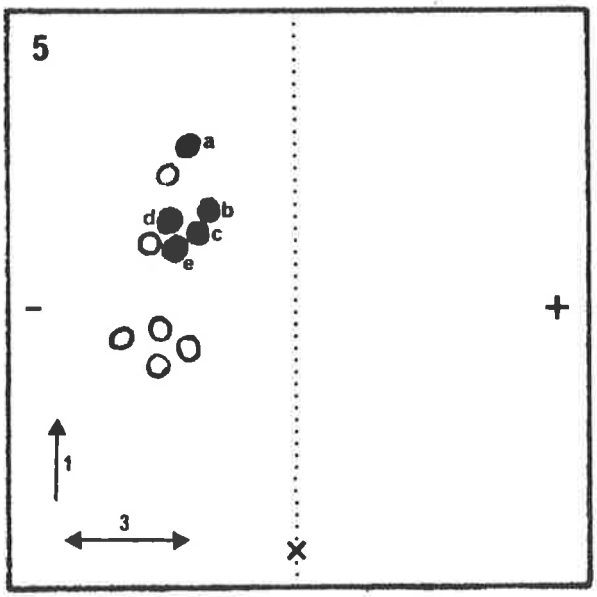
The radioactive peptides are indicated by shading.

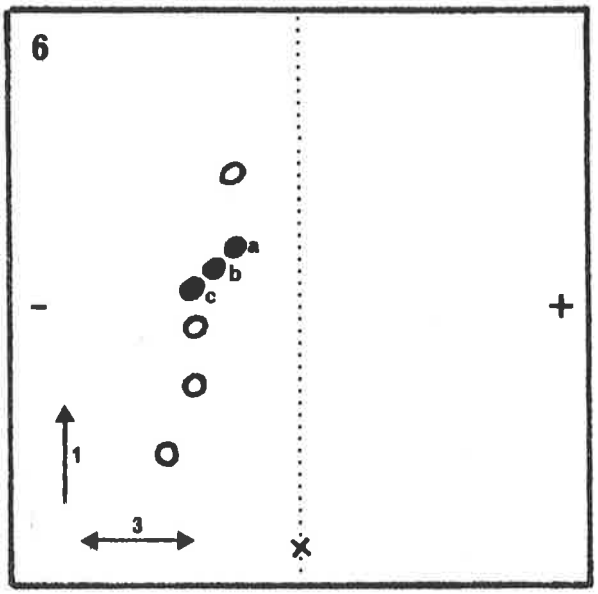

1
−
+
2
1

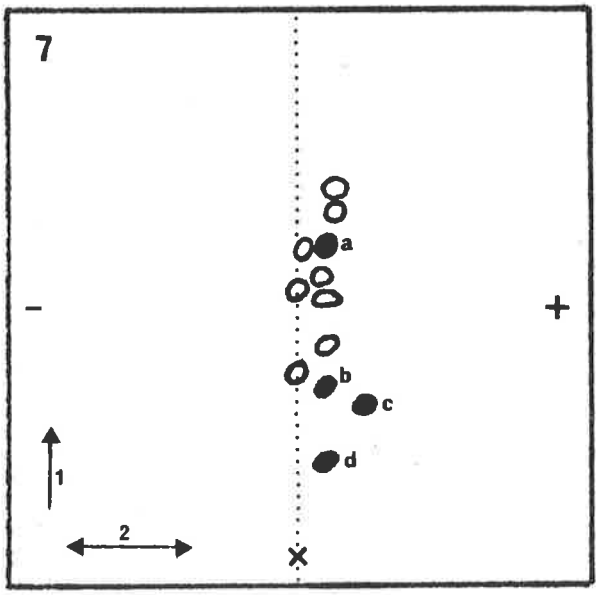

2
−
+
2
1


3
−
+
2
1


4
a
b
−
+
2
1
c


5
a
b
c
d
e
–
+
1
3


6
a
b
c
–
+
1
3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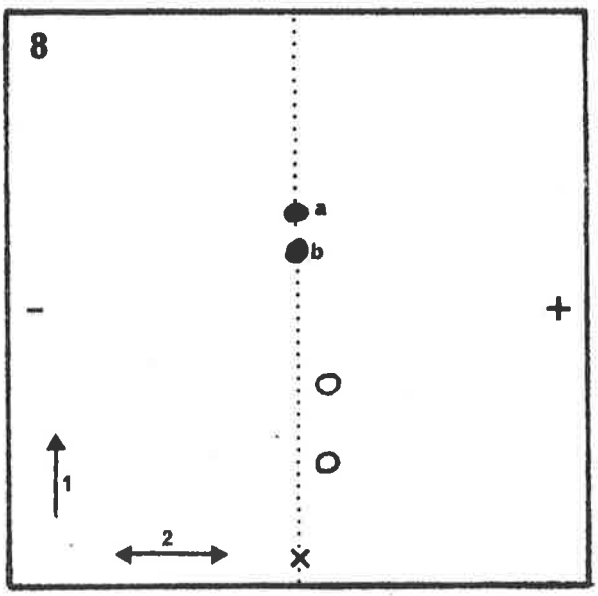
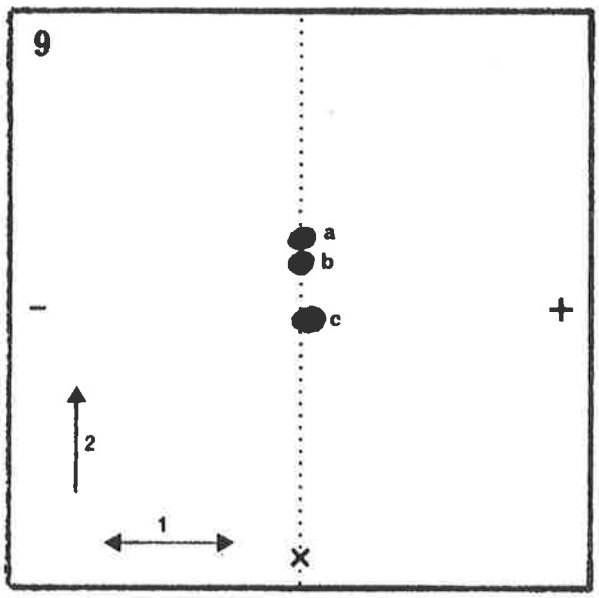
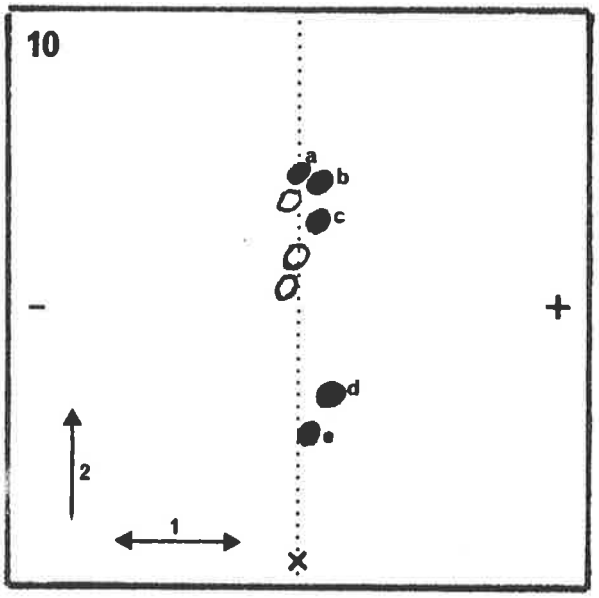
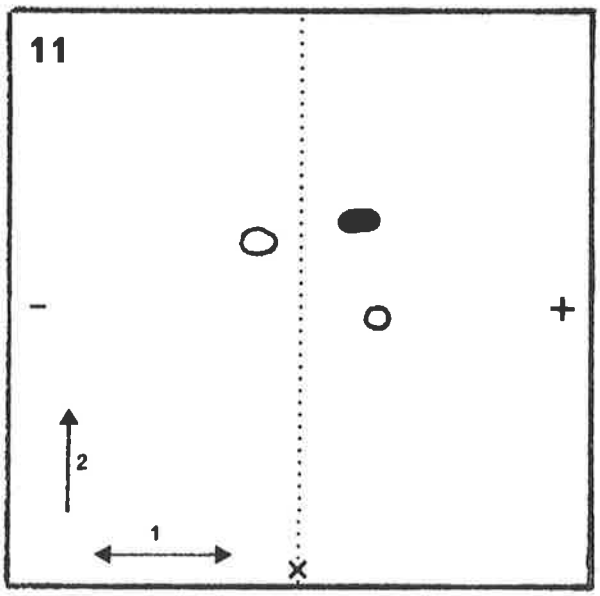
a
b
c
d
–
+
1
2


8
a
b
–
+
1
2


9
a
b
c
–
+
2
1


10
a
b
c
d
e
–
+
2
1


11
–
+
2
1

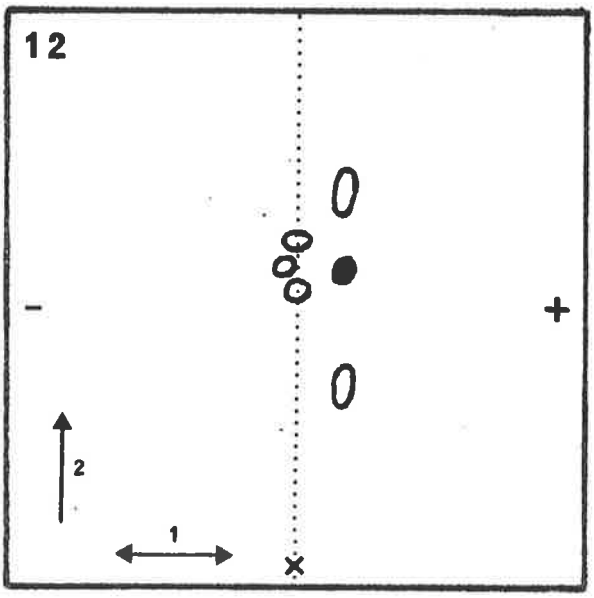

12
–
+
2
1

TABLE 7.4

Chicken liver pyruvate carboxylase.
The distribution of radioactivity in Fraction C.

The data is set out as described previously. The peptide maps from which this data was obtained are shown in figure 7.6.

Fraction	% Total Radioactivity		
	Initial	Ion-exchange chromatography	Two dimensional peptide mapping
C	66.71		
C_1		2.2	2.20
C_2		2.0	2.00
C_3		3.2	3.20
C_4		5.9	
C_{4a}			0.38
C_{4b}			0.36
C_{4c}			5.15
C_5		10.9	
C_{5a}			0.31
C_{5b}			7.54
C_{5c}			0.80
C_{5d}			0.35
C_{5e}			1.90
C_6		10.8	
C_{6a}			3.35
C_{6b}			6.90
C_{6c}			0.54
C_7		4.7	
C_{7a}			1.31
C_{7b}			0.66
C_{7c}			1.83
C_{7d}			0.89

Fraction	% Total Radioactivity		
	Initial	Ion-exchange chromatography	Two dimensional peptide mapping
C_8		8.2	
C_{8a}			1.64
C_{8b}			6.56
C_9		7.8	
C_{9a}			0.58
C_{9b}			0.55
C_{9c}			6.67
C_{10}		5.7	
C_{10a}			0.68
C_{10b}			1.01
C_{10c}			1.17
C_{10d}			2.44
C_{10e}			0.37
C_{11}		3.6	3.6
C_{12}		1.2	1.2

FIGURE 7.7

Chromatography of the peptides from Fraction D on DEAE-cellulose.

The fractionation was performed as described in the text. Only that region of the ammonium bicarbonate gradient which contained radioactivity is shown.

Flow rate: 0.32ml/min

Fraction size: 3.2ml

50µl was removed from each fraction for the determination of radioactivity and peptide material. The tubes were pooled as indicated by the bars.

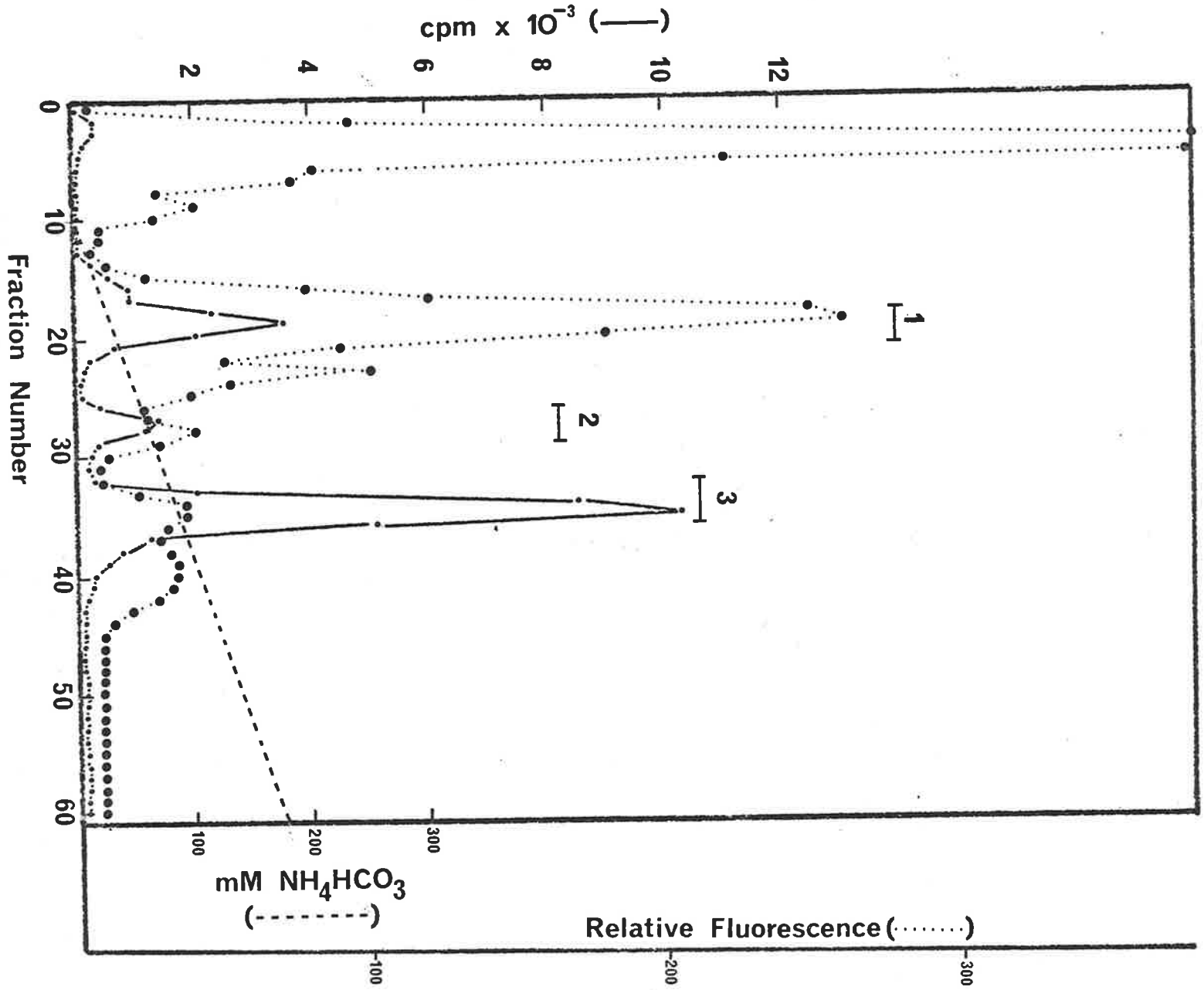

cpm x 10^{-3} (——)
2
4
6
8
10
12
0
10
20
30
40
50
60
Fraction Number
1
2
3
100
200
300
mM NH_4HCO_3
(- - - - - - -)
Relative Fluorescence (·····)
100
200
300

FIGURE 7.8

Two dimensional peptide maps of the Fraction D peptides obtained from the DEAE-cellulose chromatography shown in figure 7.7.

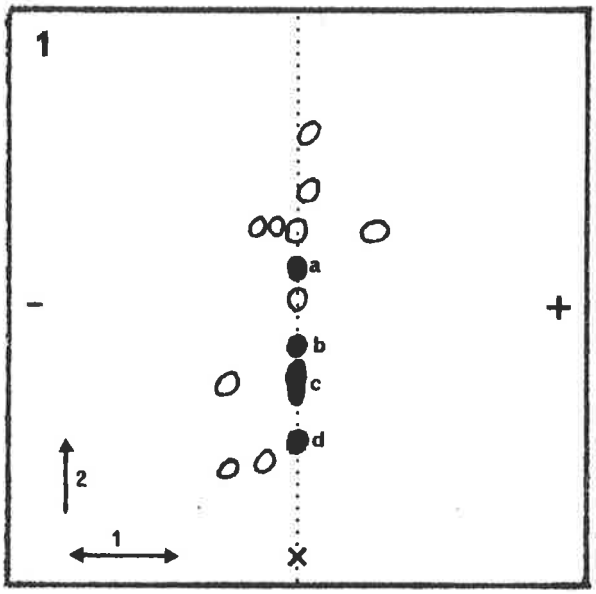
Plates 1 - 3 represent fractions D_1 - D_3 respectively, developed as described in section 7.2.5. X denotes the origin.

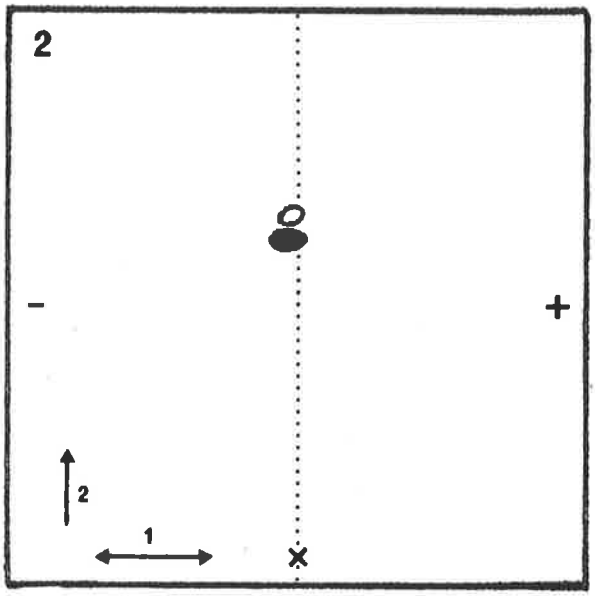
The chromatographic solvents were

Electrophoresis: ←1→ ; pH 6.5

Chromatography: ↑2; n-butanol : acetic acid; pyridine : water (section 7.2.5).

The peptides containing radioactivity are denoted by shading.


1
a
b
c
d
2
1


2
2
1

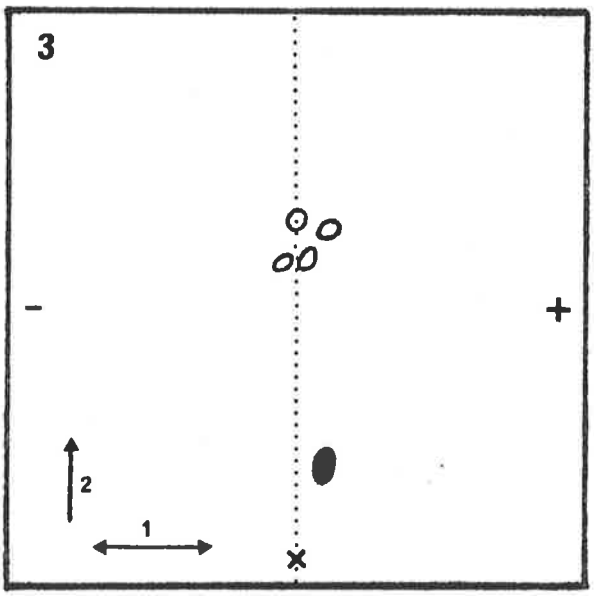

3
2
1

TABLE 7.5

Chicken liver pyruvate carboxylase.

The distribution of radioactivity in Fraction D.

The data is set out as described previously.

The peptide maps from which this data was obtained are shown in figure 7.8.

Fraction	% Total Radioactivity		
	Initial	Ion-exchange chromatography	Two dimensional peptide mapping
D	10.7		
D_1		2.3	
D_{1a}			0.1
D_{1b}			0.3
D_{1c}			1.5
D_{1d}			0.4
D_2		0.8	0.8
D_3		7.5	7.5

FIGURE 7.9

Chromatography of the peptides from Fraction E on DEAE-cellulose.

The fractionation was performed as described in the text. The figure illustrates the region of the ammonium bicarbonate gradient which contained radioactivity.

Flow rate: 0.32ml/min

Fraction size: 4.5ml

100µl of each fraction was removed for the determination of radioactivity and peptide material. The tubes were pooled as indicated by the bars.

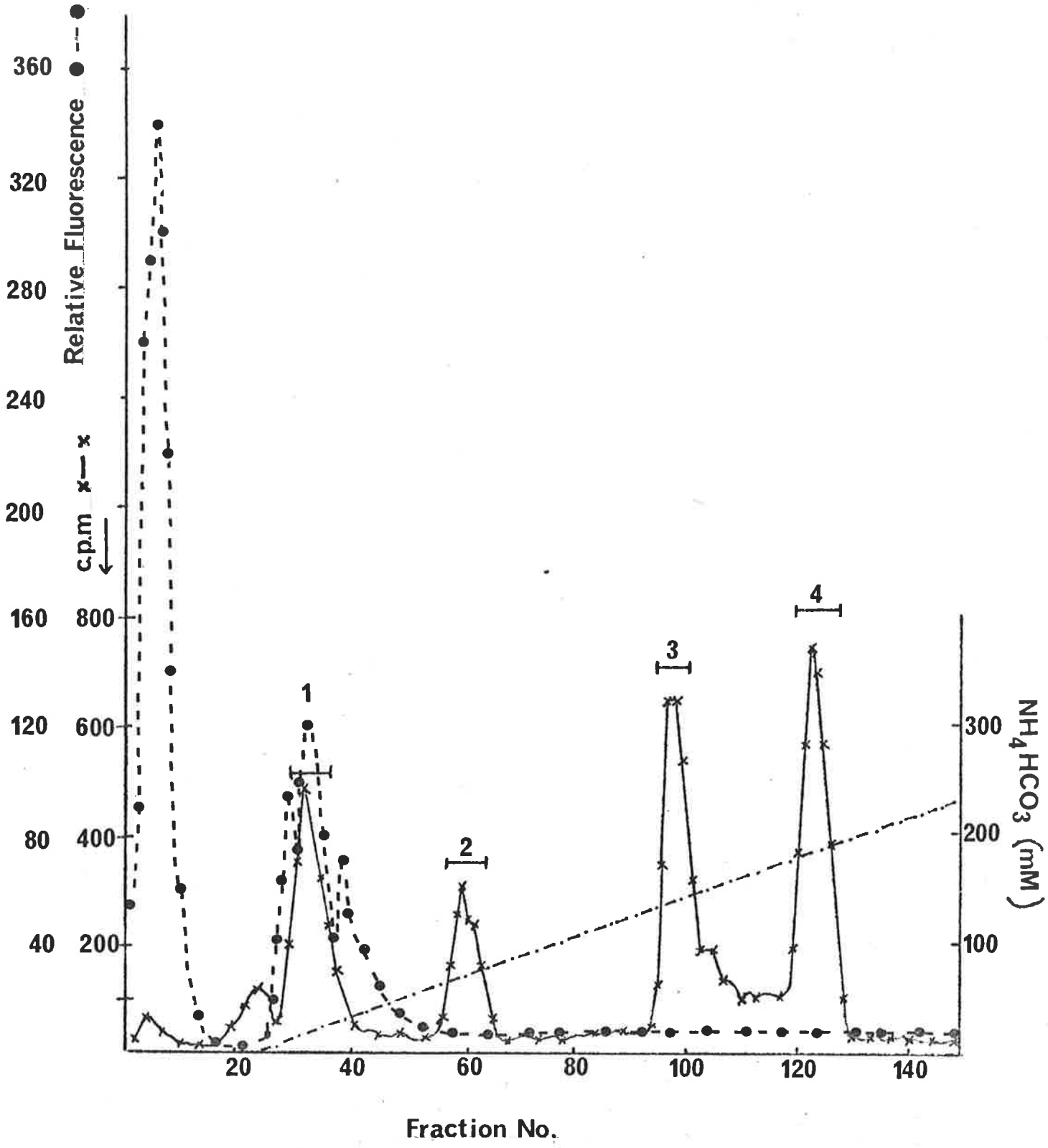

Relative Fluorescence ● --- ●
c.p.m x— x
360
320
280
240
200
160
120
80
40
800
600
400
200
1
2
3
4
300
200
100
NH_4HCO_3 (mM)
20
40
60
80
100
120
140
Fraction No.

FIGURE 7.10

Two dimensional peptide maps of the Fraction E peptides obtained from the DEAE-cellulose chromatography shown in figure 7.9.

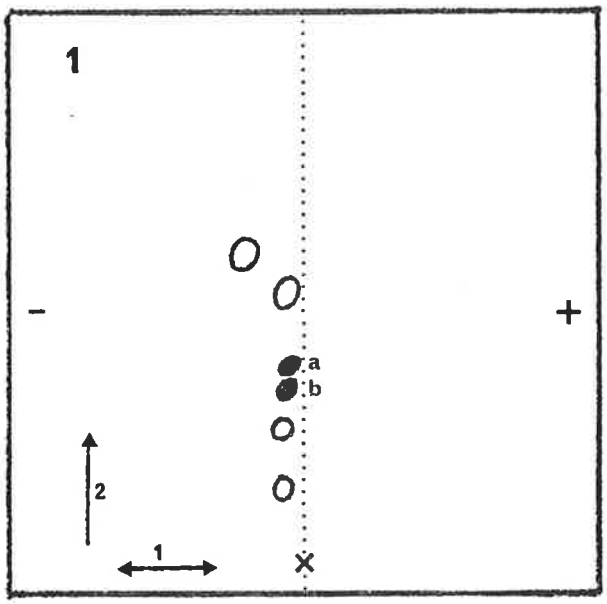
Plates 1 - 4 represent fractions E_1 - E_4 respectively, developed as described in section 7.2.5. X denotes the origin.

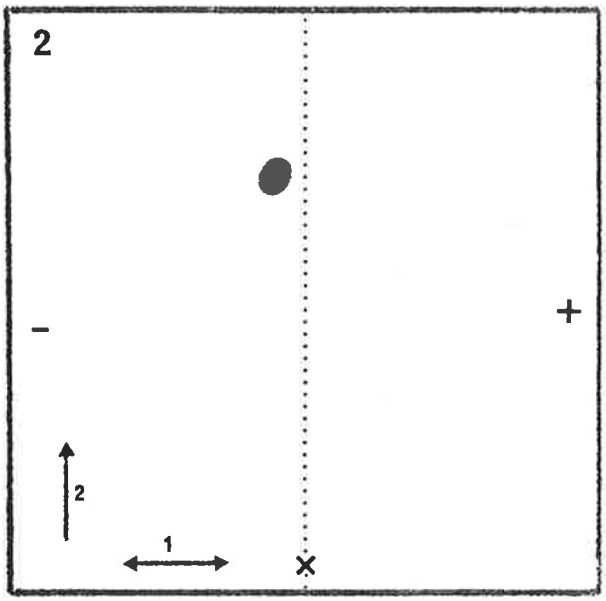
The chromatographic solvents used were

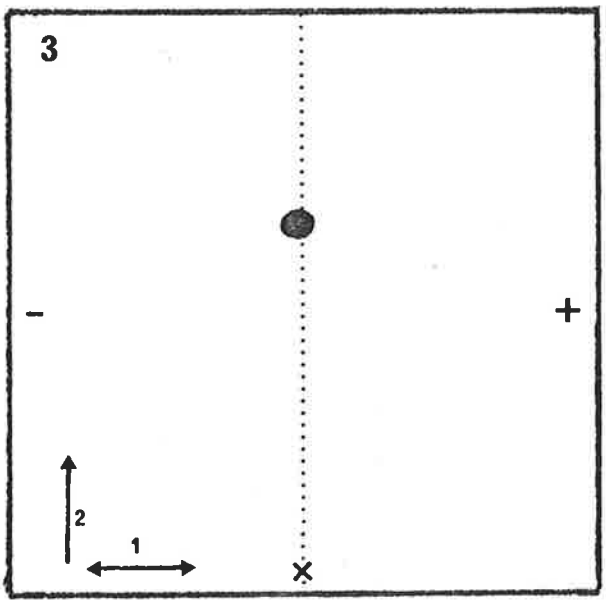
Electrophoresis : ←1→ ; pH 6.5

Chromatography : ↑2 ; n-butanol : acetic acid : pyridine : water (section 7.2.5).

The radioactive peptides are indicated by shading.


1
-
+
a
b
2
1


2
-
+
2
1


3
-
+
2
1

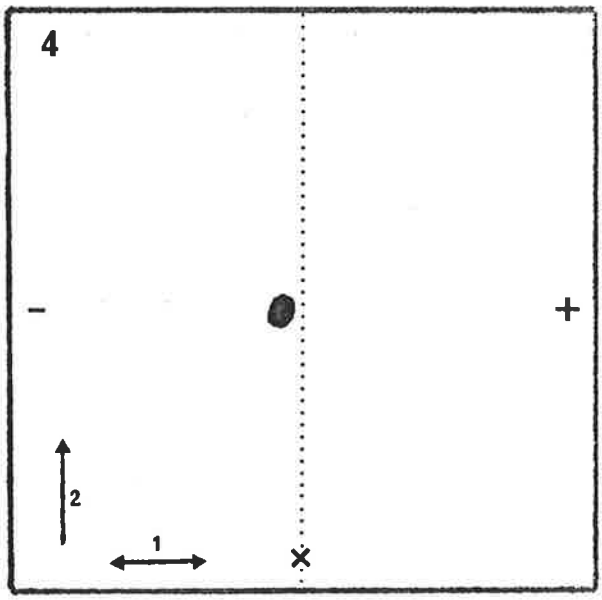

4
-
+
2
1

TABLE 7.6

Chicken liver pyruvate carboxylase.
The distribution of radioactivity in Fraction E.

The data is set out as described previously. The peptide maps from which this data was obtained are shown in figure 7.10.

Fraction	% Total Radioactivity		
	Initial	Ion-exchange chromatography	Two dimensional peptide mapping
E	6.2		
E_1		1.3	
E_{1a}			0.7
E_{1b}			0.6
E_2		0.8	0.8
E_3		1.8	1.8
E_4		2.3	2.3

Chicken liver pyruvate carboxylase

Purification of [^{14}C]SCMC-peptides on a preparative scale.

The purifications were performed on Whatman 3MM paper as described in section 7.2.6. Electrophoresis buffers were the same as described in section 7.2.5.

Chromatographic solvents were

BAW = n-butanol : acetic acid : water
(40:6:15; v/v)

BAPW = n-butanol : acetic acid : pyridine : water
(15:3:10:12; v/v)

Peptide	Electrophoresis	Chromatography
C_3	pH 6.5; 50 V/cm; 90 min	-
C_{5b}	pH 3.5; 50 V/cm; 60 min	BAW
C_{6a}	pH 3.5; 50 V/cm; 60 min	BAPW
C_{6b}	pH 3.5; 50 V/cm; 60 min	BAPW
C_{7c}	pH 6.5; 50 V/cm; 60 min	BAPW
C_{8b}	-	BAPW
C_{9c}	-	BAPW
C_{10c}	pH 6.5; 40 V/cm; 90 min	BAPW
C_{10d}	-	BAPW
C_{11}	-	BAW
D_3	-	BAPW
E_3	-	-
E_4	-	-

TABLE 7.7

The amino acid composition and N-terminal amino acid residue of the [^{14}C] SCMC-peptides isolated from chicken liver pyruvate carboxylase.

A: determined as S-carboxymethylcysteine

Amino Acid	Peptide					
	C_3	C_{5b}	C_{6b}	C_{6a}	C_{7c}	C_{8b}
Asx	1.0 (1)	1.0 (1)	0.8 (1)	1.1 (1)	1.2 (1)	1.3 (1)
Thre	-	2.9 (3)	0.8 (1)	1.9 (2)	-	1.9 (2)
Ser	-	1.9 (2)	0.8 (1)	1.2 (1)	-	-
Glx	1.1 (1)	-	1.7 (2)	0.9 (1)	0.9 (1)	-
Pro	-	-	-	1.0 (1)	-	-
Gly	1.1 (1)	-	1.3 (1)	2.2 (2)	1.0 (1)	1.3 (1)
Ala	1.0 (1)	-	1.1 (1)	0.8 (1)	1.0 (1)	3.2 (3)
½ Cys[A]	1.1 (1)	1.0 (1)	0.8 (1)	1.3 (1)	1.5 (2)	1.1 (1)
Val	1.0 (1)	1.2 (1)	0.7 (1)	0.9 (1)	1.0 (1)	1.0 (1)
Met	-	-	-	-	-	1.3 (1)
Isoleu	0.8 (1)	-	-	-	0.9 (1)	-
Leu	-	1.3 (1)	2.1 (2)	1.7 (2)	-	1.6 (2)
Tyr	-	-	-	-	-	1.1 (1)
Phe	-	1.7 (2)	-	1.3 (1)	-	1.2 (1)
Lys	-	-	-	-	-	1.0 (1)
His	-	-	-	-	-	-
Arg	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)	-
Number of Residues	8	12	12	15	9	15
N-terminal	Gly	Asp	Gly	Ala	Ile	Gly

Peptide													
C_{9c}		C_{10c}		C_{10d}		C_{11}		D_3		E_3		E_4	
2.0	(2)	1.2	(1)	1.9	(2)	2.2	(2)	1.1	(1)	-		-	
-		-		-		-		-		-		-	
-		-		-		-		0.9	(1)	-		-	
2.0	(2)	1.1	(1)	0.9	(1)	3.9	(4)	1.1	(1)	2.0	(2)	2.0	(2)
-		-		-		-		-		0.9	(1)	1.0	(1)
1.4	(1)	0.9	(1)	1.0	(1)	1.9	(2)	-		-		-	
4.2	(4)	1.0	(1)	2.3	(2)	3.0	(3)	-		-		-	
-		0.7	(1)	0.8	(1)	1.1	(1)	1.0	(1)	0.8	(1)	0.9	(1)
2.2	(2)	0.9	(1)	1.2	(1)	1.4	(1)	-		-		-	
-		-		0.7	(1)	1.7	(2)	-		-		-	
-		0.9	(1)	0.8	(1)	0.9	(1)	1.0	(1)	-		-	
-		-		-		-		-		0.9	(1)	1.0	(1)
-		-		-		-		-		-		-	
1.2	(1)	-		1.4	(1)	1.3	(1)	-		1.0	(1)	0.9	(1)
-		-		-		-		-		-		-	
-		-		-		-		-		0.8	(1)	1.0	(1)
1.0	(1)	1.0	(1)	1.0	(1)	1.0	(1)	1.0	(1)	1.0	(1)	1.0	(1)
13		8		12		18		6		8		8	
Ala		Val		Phe		Phe		Asp		?		Phe	

FIGURE 7.11

The modification of sheep liver pyruvate carboxylase with 3-bromo[^{14}C]pyruvate.

Sheep liver pyruvate carboxylase (S.A. 26.1; 75.8 units/ml) in a volume of 4.5ml of Buffer A (without DTE) was incubated with 3-bromopyruvate (5500 cpm/nmole; 0.54mM) in the presence of 0.5mM acetyl CoA. After 25 min, DTE was added to a concentration of 50mM to terminate the reaction.

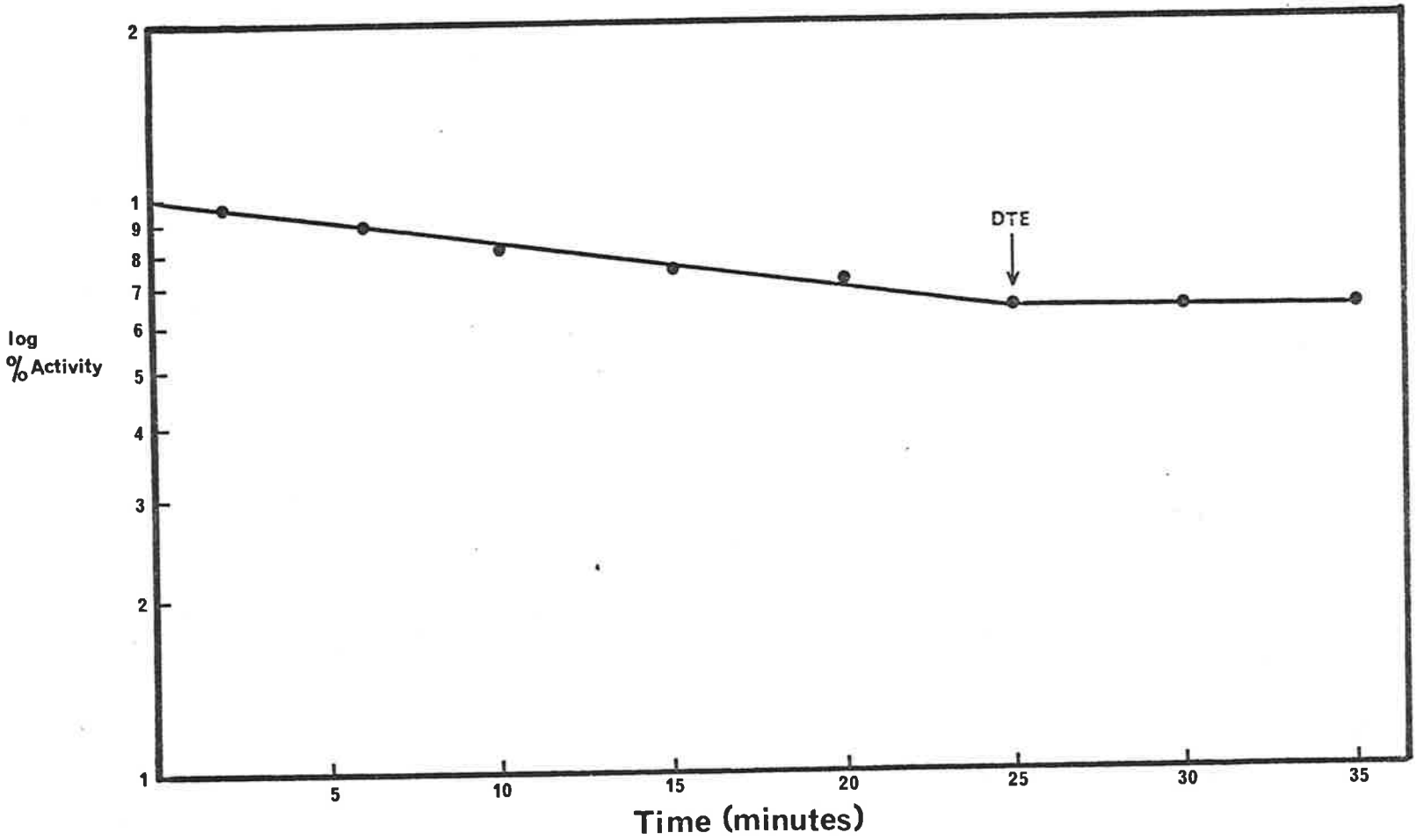

log
% Activity
2
1
9
8
7
6
5
4
3
2
1
DTE
5
10
15
20
25
30
35
Time (minutes)

FIGURE 7.12

Fractionation of sheep liver pyruvate carboxylase modified with 3-bromo[^{14}C]pyruvate on Sephadex G-25.

Flow rate: 1 ml/min

Fraction size: 9 ml

200µl was removed from each fraction for the determination of radioactivity and peptide material. The tubes were pooled as indicated.

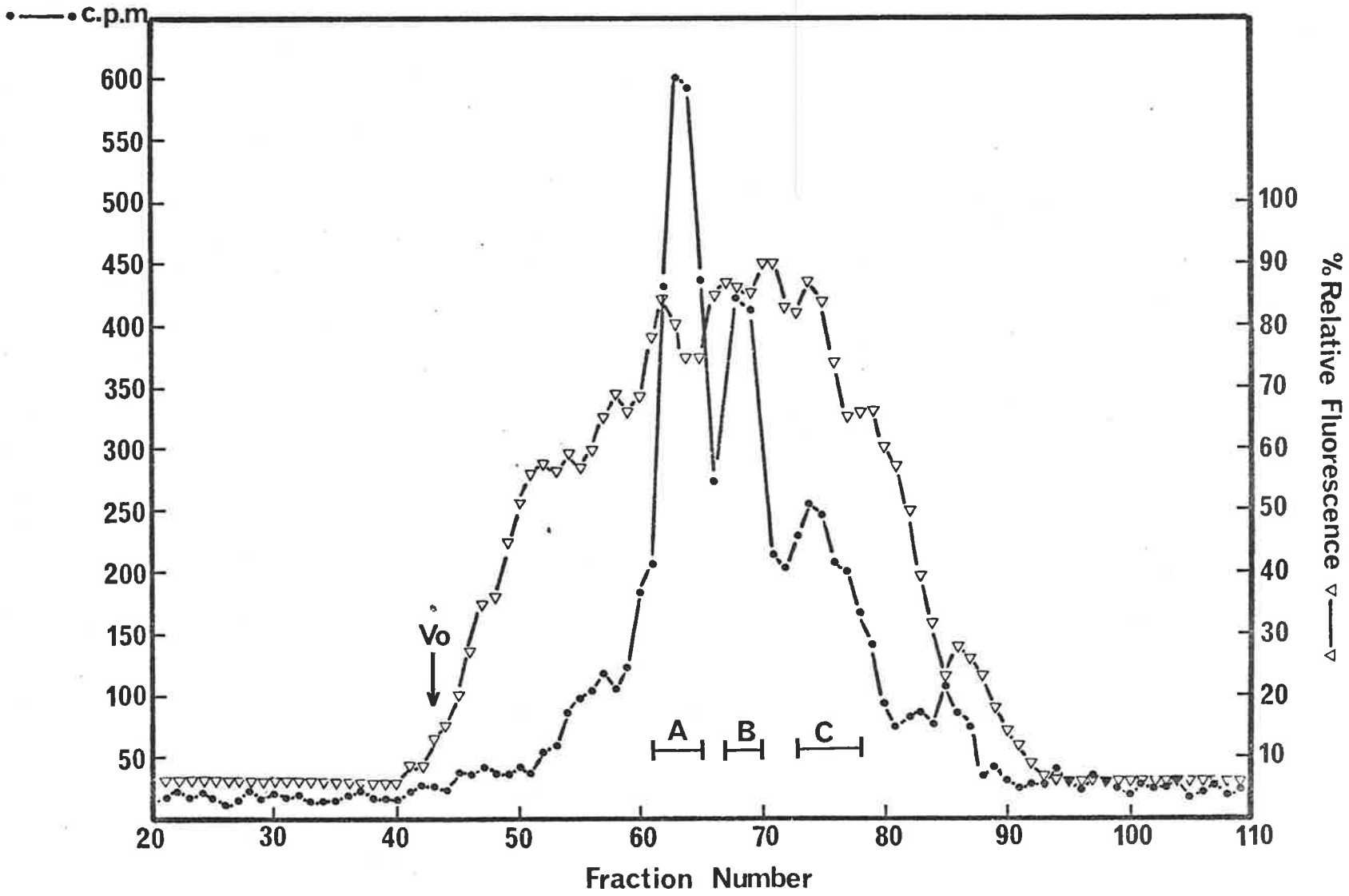

•——• c.p.m
600
550
500
450
400
350
300
250
200
150
100
50
Vo
A
B
C
20
30
40
50
60
70
80
90
100
110
Fraction Number
100
90
80
70
60
50
40
30
20
10
% Relative Fluorescence ▽——▽

TABLE 7.8

Sheep liver pyruvate carboxylase.
The distribution of radioactivity in each fraction obtained from the Sephadex G-25 chromatography.

The percentage of the total radioactivity originally incorporated into the sheep liver enzyme in each radioactive fraction is shown.

Fraction	% Total Radioactivity
A	35.9
B	23.2
C	22.1

FIGURE 7.13

Chromatography of Fraction A peptides on DEAE-cellulose.

The fractionation was performed as described in the text.

Flow rate: 0.32ml/min

Fraction size: 3.2ml

200µl aliquots were removed for the determination of radioactivity and detection of the peptide material. The bars indicate the tubes that were pooled.

CHE Peptide – Fraction A

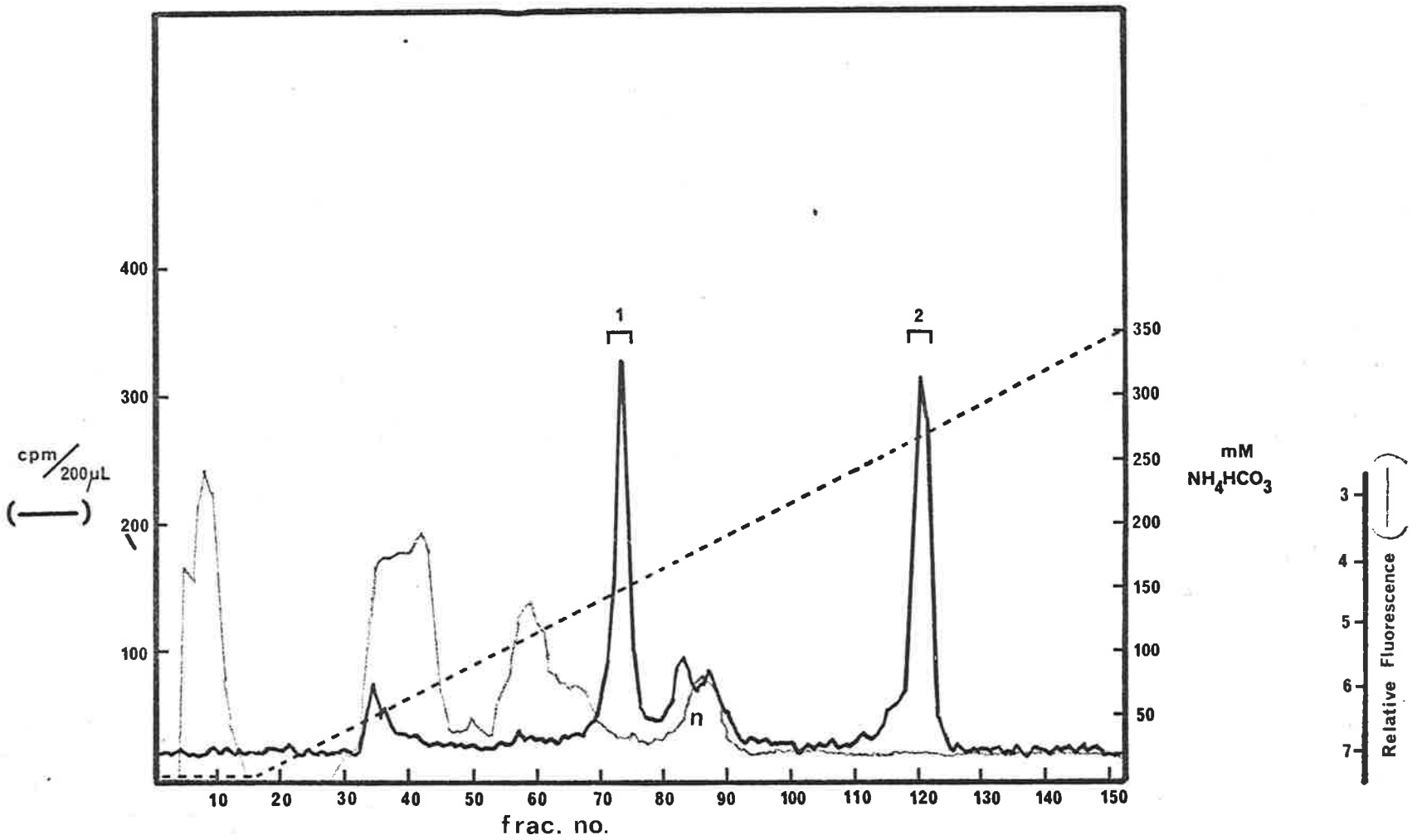


FIGURE 7.14

Chromatography of Fraction B peptides on DEAE-cellulose.

The fractionation procedure is described in the text.

Flow rate: 0.32ml/min

Fraction size: 3.2ml

200µl aliquots were removed for the determination of radioactivity and detection of the peptide material. The tubes were pooled in the region indicated by the bar.

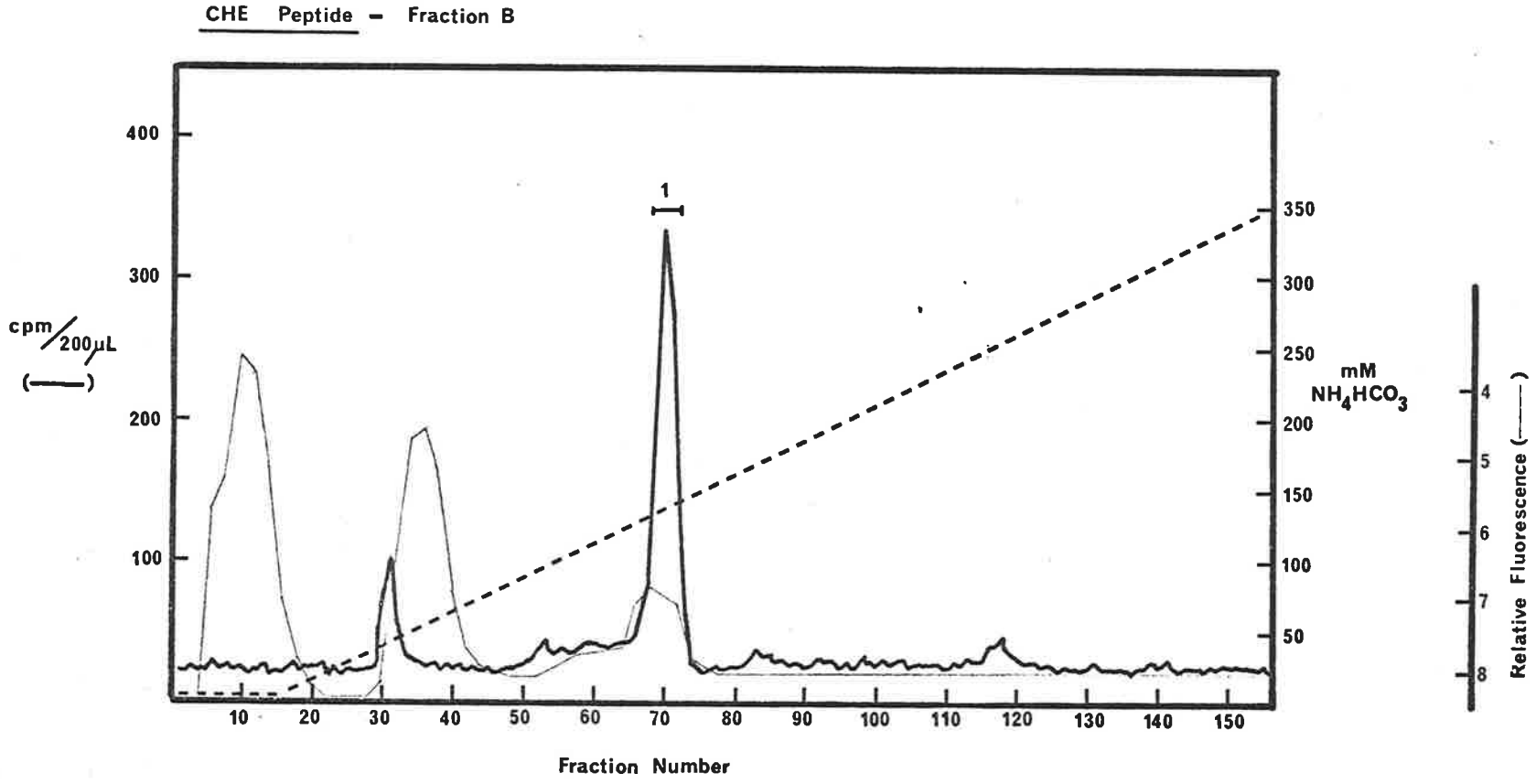

CHE Peptide – Fraction B
cpm/200µL
(——)
400
300
200
100
1
350
300
250
200
150
100
50
mM
NH_4HCO_3
4
5
6
7
8
Relative Fluorescence (——)
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
Fraction Number

FIGURE 7.15

Chromatography of Fraction C peptides on DEAE-cellulose.

The fractionation procedure is described in the text.

Flow rate: 0.32ml/min

Fraction size; 3.2ml

200µl aliquots were removed for the determination of radioactivity and detection of the peptide material. The tubes were pooled as indicated by the bar.

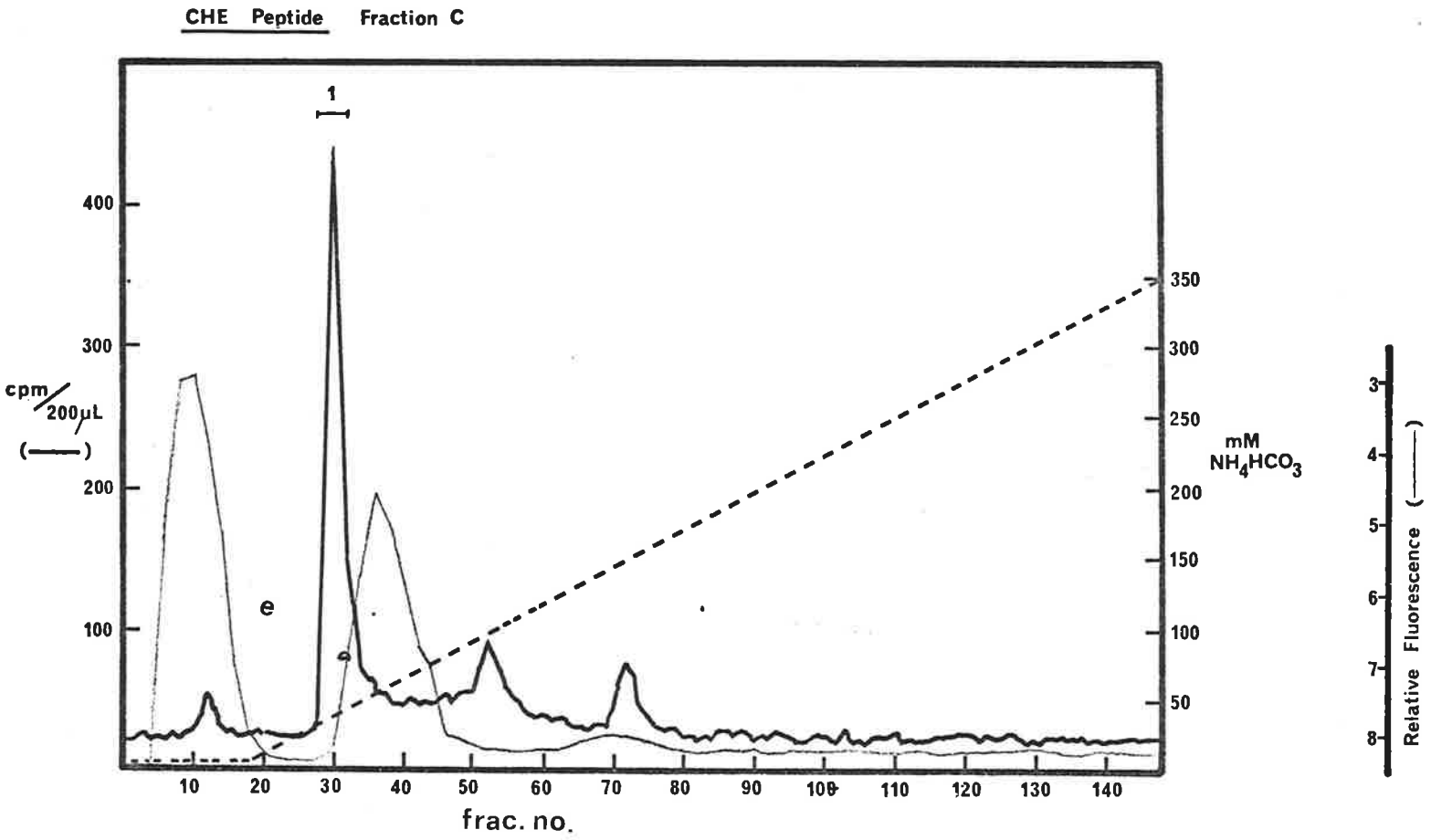

CHE Peptide Fraction C
1
400
300
200
100
cpm/200µL
(——)
e
350
300
250
200
150
100
50
mM
NH_4HCO_3
3
4
5
6
7
8
Relative Fluorescence (——)
10
20
30
40
50
60
70
80
90
100
110
120
130
140
frac. no.

FIGURE 7.16

Chromatography of CHE-peptide A_1 on thin layer cellulose.

An aliquot was spotted onto the plate and chromatographed in n-butanol : pyridine : acetic acid : water (section 7.5.2). Following chromatography the radioactivity was located by cutting the plate into half cm strips and determining the radioactivity in each strip in toluene scintillation fluid as described in section 2.2.6.

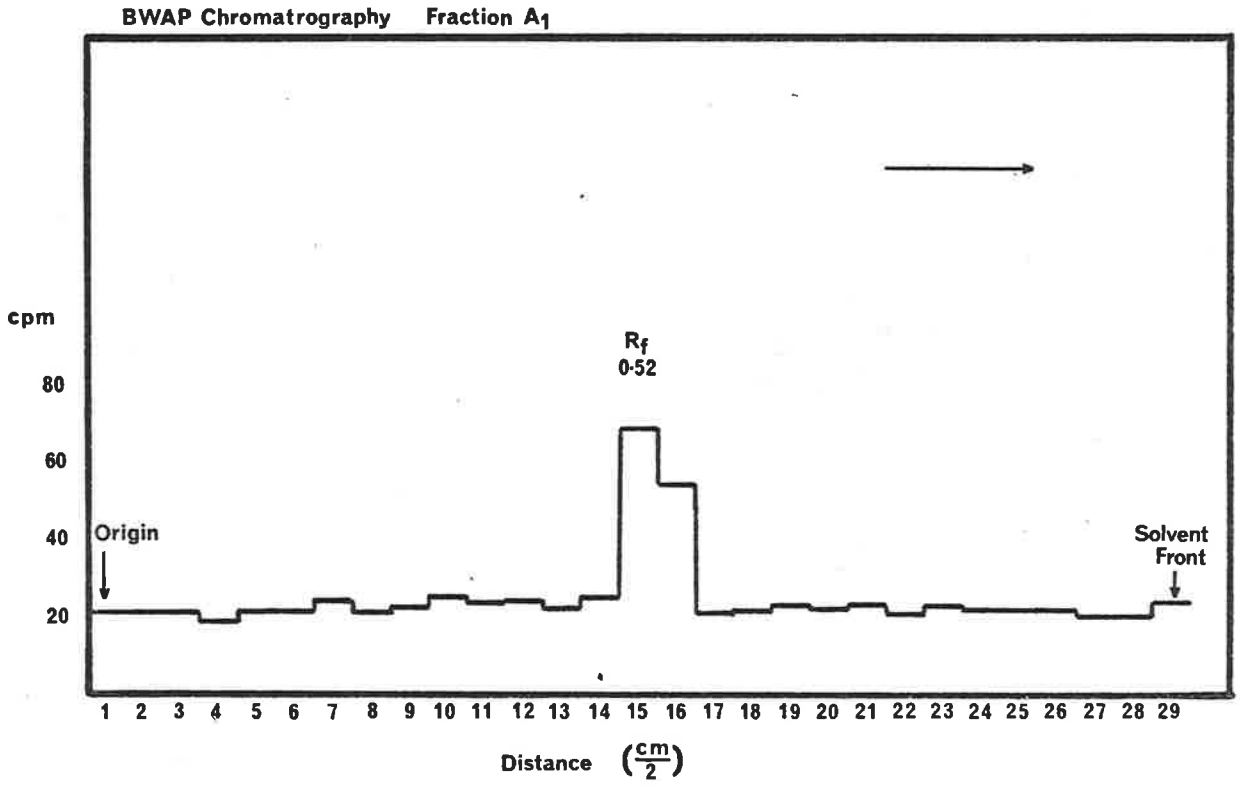

BWAP Chromatrography Fraction A1
cpm
80
60
40
20
Origin
Rf
0·52
Solvent
Front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29
Distance (cm/2)

FIGURE 7.17

Chromatography of CHE-peptide A_2 on thin layer cellulose.

The chromatography and determination of the radioactivity was performed as described for CHE-peptide A_1.

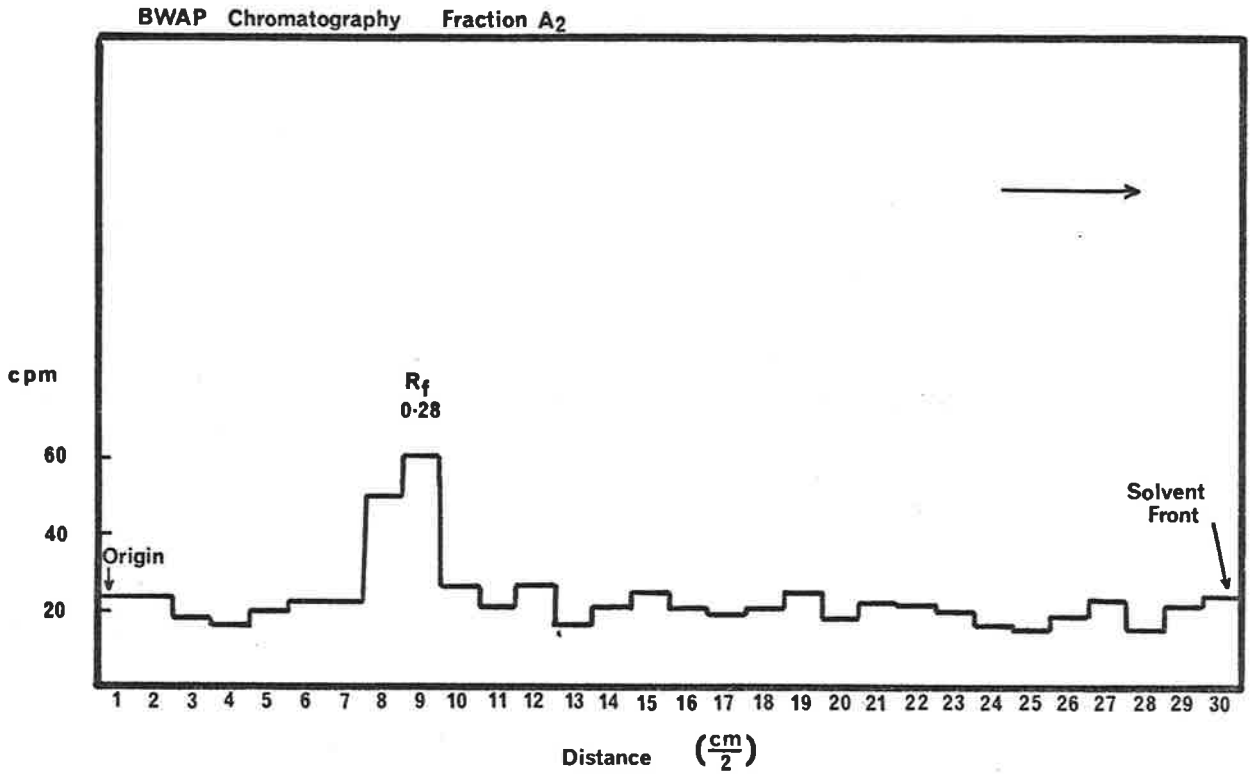

BWAP Chromatography Fraction A2
cpm
60
40
20
Origin
Rf
0·28
Solvent
Front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
Distance (cm/2)

FIGURE 7.18

Chromatography of CHE-peptide B_1 on thin layer cellulose.

The chromatography and determination of the radioactivity was performed as described for CHE-peptide A_1.

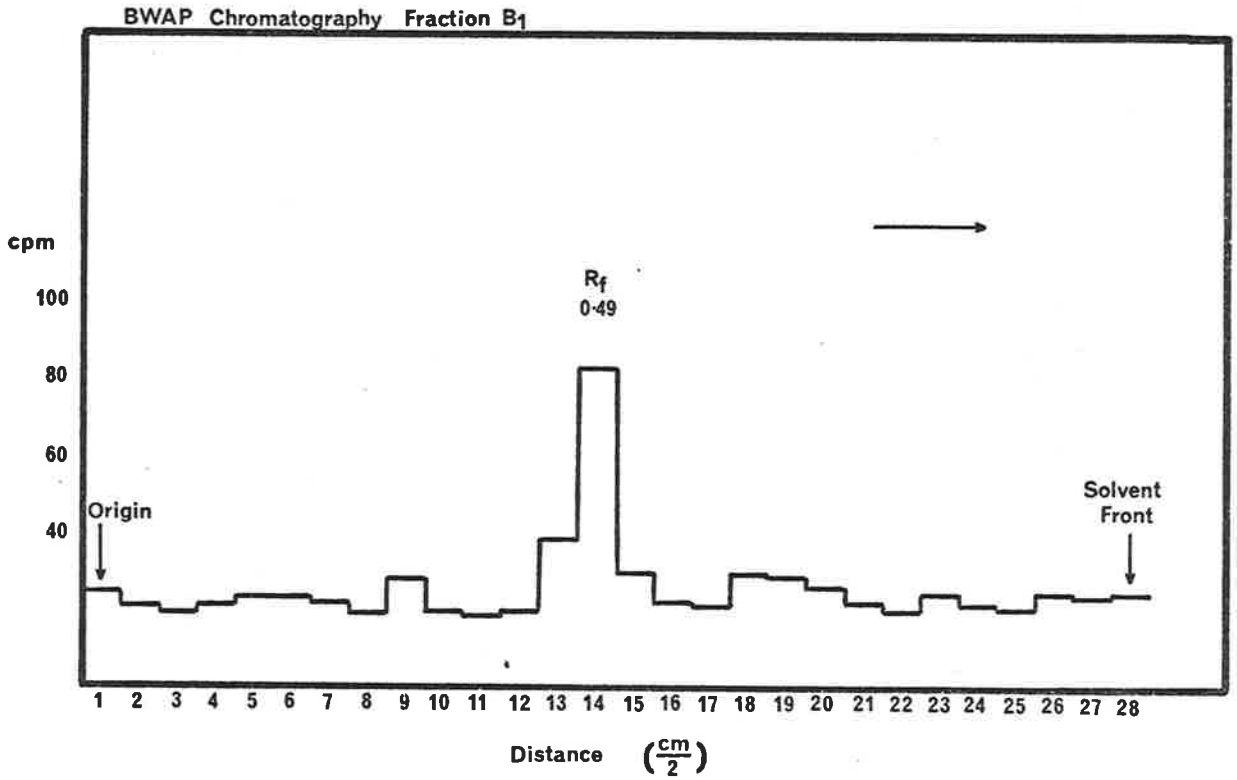

BWAP Chromatography Fraction B1
cpm
100
80
60
40
Rf
0·49
Origin
Solvent
Front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
Distance (cm/2)

FIGURE 7.19

Chromatography of CHE-peptide C_1 on thin layer cellulose.

The chromatography and determination of the radioactivity was performed as described for CHE-peptide A_1.

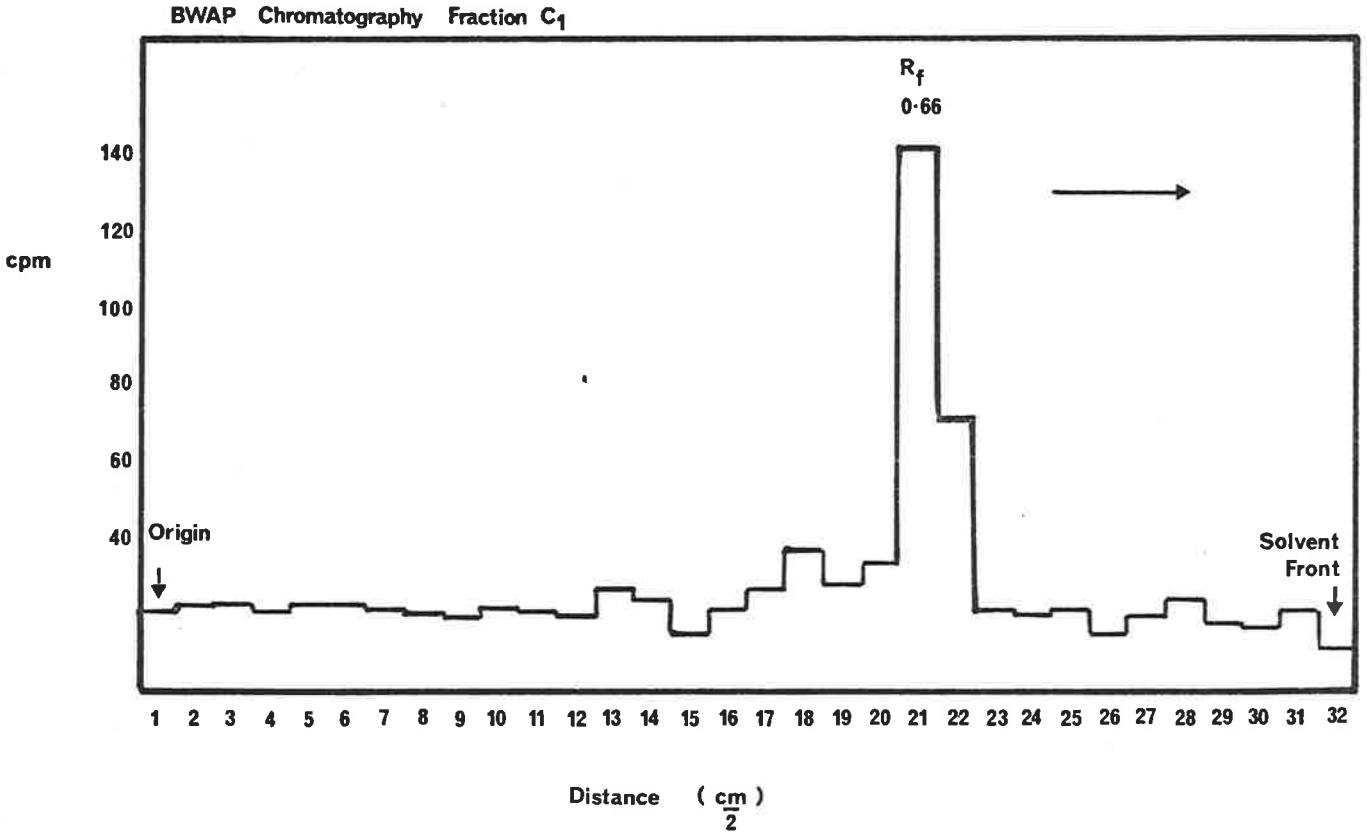

BWAP Chromatography Fraction C_1
R_f
0·66
cpm
140
120
100
80
60
40
Origin
Solvent
Front
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32
Distance (cm / 2)

TABLE 7.9

Sheep liver pyruvate carboxylase.

The distribution of the radioactivity in each of the |^{14}C|CHE-peptides.

Peptide	% Total Radioactivity
A_1	13.8
A_2	13.1
B	14.9
C	14.2

CHAPTER 8

CONCLUDING DISCUSSION

The work presented in this thesis forms part of an investigation into the structure and function of pyruvate carboxylase. The main emphasis of this investigation in the past, has centred on defining the system in precise kinetic terms, although chemical modification studies and investigations of the physico-chemical properties of the enzyme have been undertaken to some extent. The major impediment to progress in these latter two areas of investigation of the enzyme has been the lack of a routine method of obtaining homogeneous sheep liver pyruvate carboxylase in a highly active form. The method presented in Chapter 1 of this thesis, using affinity chromatography on an NAD^+-agarose column, provides the necessary final step in the purification of this enzyme. The development of this method provides the basis for an expansion into the understanding of the chemical events occurring during the enzyme-catalysed reaction and the formulation of a reaction mechanism which incorporates kinetic data, reaction intermediates, functional amino acid residues and the influence of the tertiary and quaternary structure of the enzyme.

8.1. Physical properties of sheep liver pyruvate carboxylase

It was not surprising to find that sheep liver pyruvate carboxylase had a native molecular weight of

ca. 4.8 x 10^5 daltons and could be dissociated into subunits of ca. 1.1 x 10^5 daltons. All other eukaryote pyruvate carboxylases have been shown to have very similar properties (Barden et al., 1975). The tetrameric structure of the enzyme was confirmed by examination of the images produced by the highly purified enzyme in the electron microscope. What was surprising however was that electron micrographs of either the chicken liver or sheep liver enzymes showed a rhombic arrangement of subunits very similar to the enzyme isolated from yeast (Valentine, 1968). This was in contrast to an earlier report (Valentine et al., 1966) that the enzyme isolated from chicken liver had a cyclic arrangement of subunits and appeared as a square planar tetramer in electron micrographs. The discrepancy between the conclusions of these two studies of the structure of the chicken liver enzyme was resolved when it was found that partially purified preparations of the enzyme from this source contain a contaminant which appears as a square planar tetramer in the electron microscope. The structure does not contain biotin, does not catalyse the pyruvate carboxylase reaction and can be separated from the rhomic pyruvate carboxylase structures by either ion-exchange or molecular sieve chromatography. Similar observations have recently been made by Utter and his group (Utter, 1978; personal communication). In addition this structure is probably, in fact, an octomer. The square

planar tetramer has not been further characterised in this study, although this would be of interest since it also appears to be present in preparations of pyruvate carboxylase from both turkey and bovine liver sources (Utter et al., 1975).

The similarity in the arrangement of the subunits of pyruvate carboxylase from sheep liver, chicken liver and yeast suggests that this structure has been conserved during the evolution of these enzymes. In addition the rhombic structure has one very interesting implication: a tetramer with a rhombic arrangement of subunits cannot have homologous binding domains and yet also be composed of identical subunits. The question of the degree of homology between the individual subunits of pyruvate carboxylase was therefore addressed.

A tryptic digest of the chicken liver enzyme, modified at the cysteine residues with [^{14}C] iodoacetic acid produced a large number of radioactive peptides. If the polypeptide chains of the enzyme had been homologous only 10-11 SCMC-peptides should have been isolated. However, thirteen peptides were isolated and characterized by amino acid analysis and N-terminal analysis and this suggested that the polypeptide chains of the enzyme were not identical in amino acid sequence.

However, without a complete study of all of the radioactive peptides, including their amino acid sequences, it is difficult to assess whether there are two pairs of non-idential subunits (i.e. $\alpha_2\beta_2$) of whether each subunit is to some degree unique (i.e. $\alpha\beta\gamma\delta$).

The results obtained from a study of the sheep liver enzyme were less ambiguous. When the enzyme from this source was modified using 3-bromo-[^{14}C]pyruvate, in the presence of acetyl CoA, as described by Hudson et al. (1975), and the modified enzyme digested with trypsin, four approximately equivalently labelled peptides were resolved. This result is most readily interpreted to favour the view that each subunit of this enzyme is to some extent unique, and therefore suggests that the enzyme has an $\alpha\beta\gamma\delta$-type composition of subunits.

The results of Rylatt et al. (1977) indicate that the amino acid sequence around the biotin prosthetic group in each subunit of the chicken liver, turkey liver and sheep liver enzymes is highly conserved. Thus there are clearly regions of conserved sequence in each of the subunits of pyruvate carboxylase from these sources. This is compatible with the findings presented in this thesis that the levels of radioactivity

in some of the SCMC-peptides isolated from the chicken liver enzyme were higher than would have been expected if these peptides were present only once in the tetrameric form of this enzyme. This observation would suggest that some of the amino acid sequences around the cysteine residues may have been conserved in all four subunits of this enzyme. However since at this stage it is unknown where the proposed regions of homology and non-homology exist with respect to the active centre in the 3-dimensional structure of the enzyme, the overall proposed non-homology of the polypeptide chains, and their significance to catalytic events occurring at the active centre of the enzyme cannot be assessed.

If the interpretation of the results obtained in this present study is correct then the task of obtaining the entire amino acid sequence of the tetrameric form of pyruvate carboxylase is certainly formidable, since four polypeptide chains of ca. 1100 amino acid residues will need to be sequenced. On the other hand, such heterogeneity may serve to provide a method of assessing the relative importance of a particular peptide. For example, if a chemical modifier (e.g. MgoATP) were used to modify pyruvate carboxylase and, following cleavage of the polypeptide chain, only one unique peptide was isolated then, by virtue of the conserved sequence, this would provide strong evidence

that this peptide was an important portion of the enzyme amino acid sequence.

In this regard the isolation of four equivalently labelled peptides from the tryptic digest of sheep liver pyruvate carboxylase modified with 3-bromo[^{14}C]pyruvate suggested that the peptide in which the modified cysteine occurred was not a vitally important region of the polypeptide chain where strict conservation of the polypeptide chain was essential. This postulate had bearing on the other major area of investigation of this thesis; the investigation of the reactive cysteine residues of sheep liver pyruvate carboxylase.

8.2. The reactive cysteine residues of pyruvate carboxylase

Chemical modification of sheep liver pyruvate carboxylase with DTNB results in inactivation of the enzyme which involves a rate limiting step. The modified cysteine residues were shown not to function as either nucleophiles or proton donors in the catalytic reaction since replacement of the TNB-cysteine adducts with the cyanide ion was accompanied by a restoration of enzymic activity equivalent to that observed when the modified enzyme was treated with DTE. However only partial restoration of the original enzymic activity

was observed upon treatment of the enzyme with either of these reagents. This suggested that the rate limiting step detected during inactivation of the enzyme by DTNB may have involved a conformational change in the tertiary or quaternary structure of the enzyme. A similar scheme to this was proposed by Palacian and Neet (1972) to explain the effects of a variety of sulphydryl group-specific reagents on the activity and quaternary structure of the chicken liver enzyme.

The location of the reactive cysteine residues in the 3-dimensional structure of the enzyme was difficult to assess. However, on the basis of the arguments presented in detail in Chapter 5, it was postulated that one of the reactive cysteines was close to, but possibly not in, the keto acid binding site. Furthermore it appeared possible that the cysteine residues modified by DTNB were the same as those modified by 3-bromopyruvate, 3-chloroacetonyl CoA and probably NEM and NBM.

If this assumption was correct, then it is possible to speculate on the reason for a conformational change occurring after the modification of these, apparently non-functional, cysteine residues. According to the method of Chou and Fasman (1974 a,b) for the prediction of protein conformation from

primary sequence data, cysteine residues have a high probability of being present in β-sheet regions of proteins. Negatively charged amino acids are however either indifferent β-sheet formers (Asp^-) or strong β-sheet breakers (Glu^-). Thus if the reactive cysteine in sheep liver pyruvate carboxylase was in a β-sheet region then the introduction of a negatively charged group via a chemical modifier is likely to have serious effects on the conformation of that region of the polypeptide chain. Such a situation may account for the observation that both negatively charged chemical modifiers (DTNB and 3-bromopyruvate) completely inhibit pyruvate carboxylase activity. In contrast, if the same cysteine residue is modified by a small reagent (e.g. CN^-) to produce a small neutral adduct (e.g. SCN) then little influence on the conformation of the polypeptide chain, and hence catalytic activity of the enzyme, would be expected. Modification of the enzyme with either NEM or NBM may represent an intermediate case. Both of these reagents produce inactivation of the enzyme in two phases. The rate of the first phase of inactivation is very high and produces a partially active form of the enzyme. It has been tentatively suggested in Chapter 5 that cysteine residues may be modified during this first phase of inactivation. If this is the case, then the partial activity of the enzyme modified to the end of the first phase of inactivation by either NEM or NBM may be due to

distortion of the β-sheet region by the introduction of a large neutral moiety.

8.3. Extensions of these studies

The work presented in this thesis suggests that at least one of the reactive cysteine residues in sheep liver pyruvate carboxylase may be near to the keto acid binding site on the enzyme. It may be possible to test this proposal directly by making use of the photolysable bifunctional reagent reported by Henkin (1977). This reagent is a mixed disulphide of 2-thiopyridine and 2-thiobenzyl diazoacetate (fig 8.1). Reaction of this compound with the reactive sulphydryl residues of sheep liver pyruvate carboxylase would result in the formation of a new mixed disulphide between the enzyme and 2-thio-benzyl diazoacetate (fig 8.1.I). If, after removing the unreacted compound, [^{14}C]pyruvate was added to a solution of the modified enzyme and the solution then photolysed, the carbene formed from the photolytic destruction of diazoacetate group may insert into the [^{14}C]pyruvate bound at the keto acid site on the enzyme, if the modifier is close to this ligand binding site. The [^{14}C]pyruvate not covalently linked to the enzyme could be removed by dialysis or gel filtration and the amount of radio-activity remaining would be a measure of the degree of insertion of the carbene into pyruvate. Denaturation

of the modified enzyme prior to photolysis should provide an adequate control of the amount of insertion of the carbene into unligated pyruvate.

The conformational change proposed to occur upon modification of these reactive cysteine residues may result in a significant separation of the bound diazoacetate group and the keto acid binding site prior to photolysis, in which case the experiment would fail. However, considering that the 2-thiobenzyl diazoacetate does not carry a net negative charge, the conformational change may not occur. Similar experiments could be conducted using radioactively labelled acetyl CoA.

Irrespective of the results of the experiments proposed above this compound, and its isomers, would still prove very useful as a bifunctional chemical modifier to "scan" the amino-acids in the immediate vicinity of the reactive cysteine residues. The important chemical feature of the compound is that the carbene formed upon photolysis is highly reactive and can insert into a variety of amino acid side chains, and even hydrophobic residues (e.g. alanine, see Vaughan and Westheimer, 1969). Thus this compound would be suitable for establishing whether there were two cysteine residues in close juxtaposition near the keto acid site, as discussed in Chapter 5. The photochemical Wolff rearrangement of the carbene (2) would

not be as serious a problem in this case since the ketene formed is still readily attacked by nucleophilic amino acids. Crosslinking between the reactive cysteine residues modified with this compound and some portion of the biotin peptide would have an enormous advantage with respect to rapidly isolating the peptides in the near spatial vicinity of the biotin prosthetic group.

The cysteine specific reagent 2-nitro-5-thiocyanobenzoic acid (NTCB) (Degani et al., 1970) (fig 8.2) is another chemical modifier which could prove quite valuable in the further investigation of the cysteine residues of sheep liver pyruvate carboxylase. The reaction of this reagent with the enzyme would result in direct cyanylation of the cysteine residues without the necessity to produce the TNB-adduct first. This may avoid the conformational change proposed to be caused by the negatively charged TNB moiety and so may result in quantitative modification of the cysteine residues without conformational alterations to the enzyme or any loss of enzymic activity. This would provide a direct estimation of how many cysteine residues were highly reactive in the enzyme without the complications arising from, possibly, unfolding of the polypeptide chain, which could expose other cysteine residues.

Furthermore if this reagent was successful in completely modifying the reactive cysteine residues without causing loss of enzymic activity, then the effect of other chemical modifiers could be examined in much greater detail, without the complications associated with the loss of activity due to modification of the cysteine residues (e.g. see NEM inactivation of the enzyme, Chapter 5). This may lead to the identification of other less reactive essential amino acids.

A further advantage of the use of this reagent is that under relatively mild conditions (37^{o}-50^{o}C, pH 8.0) the resultant cyano-cysteine adducts (β-thiocyanolalanine) are cleaved in high yield to form 2-iminothiazolidine derivatives (fig 8.3) (Degani and Patchornik, 1974). Following modification of the enzyme with this reagent then, the protein could be cleaved into a small number of large peptides. Unfortunately, the N-terminals of these peptides are blocked by the cyclic 2-iminothiazolindine group so that direct sequencing of these fragments would not be possible. However, these peptides could form the basis for further examinations of the homology of the poly-peptide chains and, following additional cleavage, for primary sequence studies.

FIGURE 8.1

A scheme showing the reactions of the mixed disulphide of 2-thiopyridine and 2-thiobenzyl diazoacetate (Henkin, 1977).

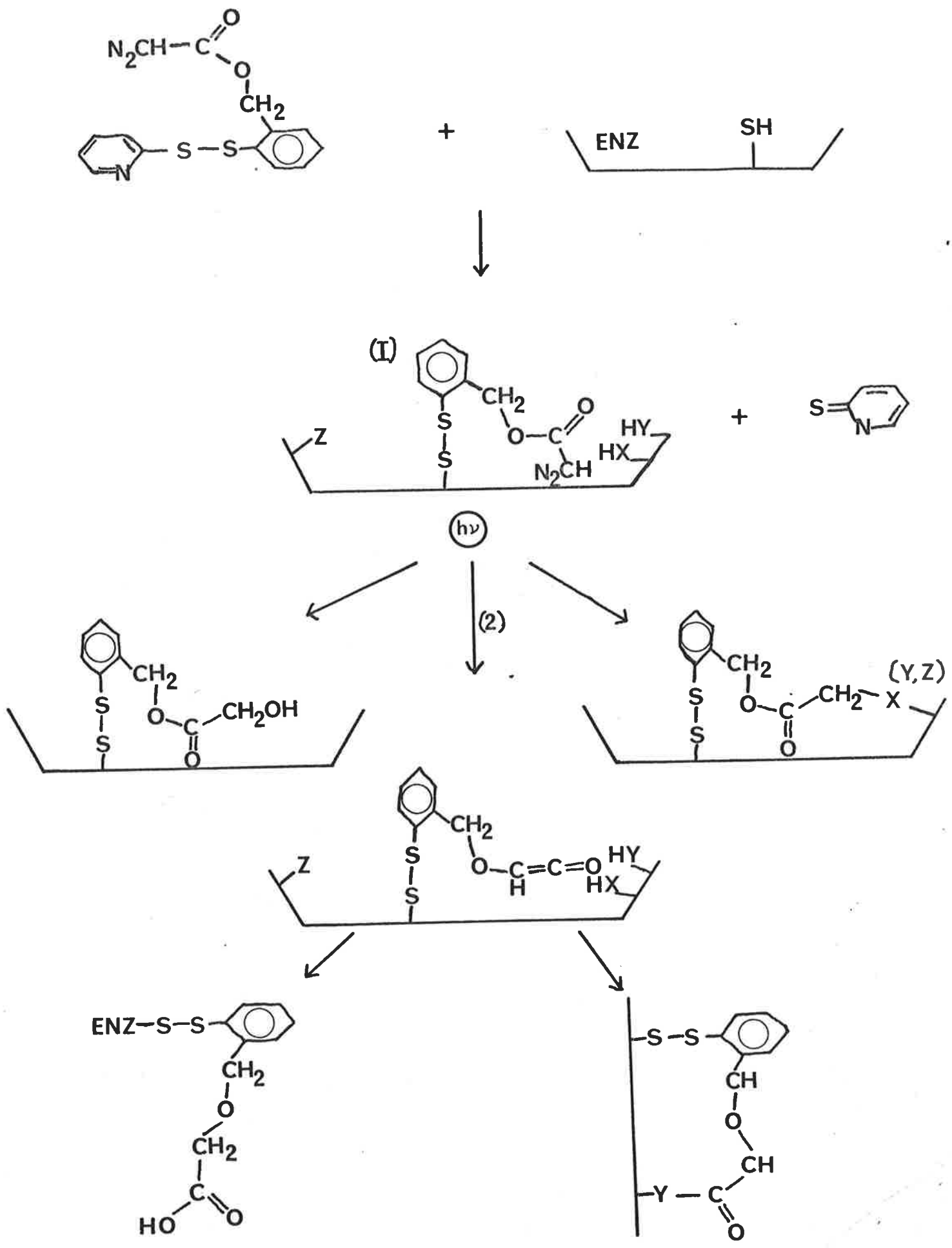
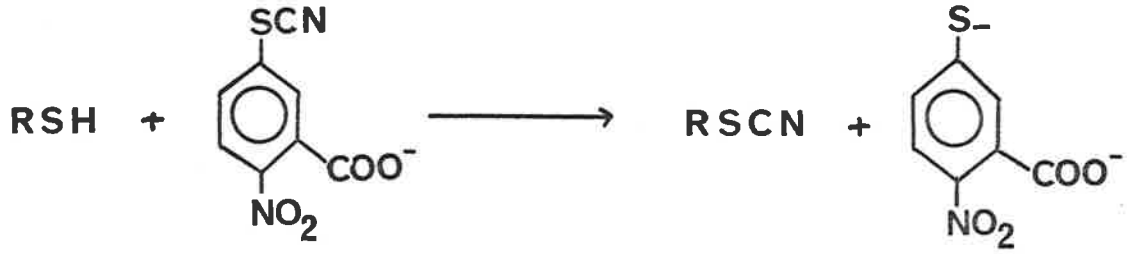

(I)
(2)
hν
ENZ
SH
HY
HX
Z
(Y, Z)
ENZ–S–S
HO

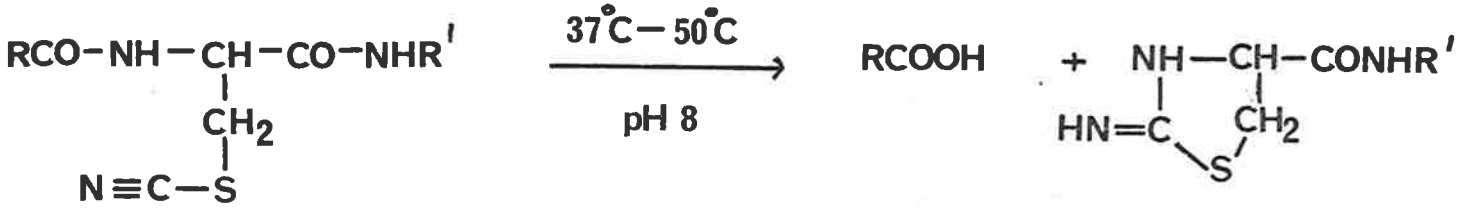
FIGURE 8.2

A scheme showing the modification of cysteine thiol groups with 2-nitro-5-thiocyanobenzoic acid (NTCB).

FIGURE 8.3

A scheme showing the cleavage of the peptide bond on the N-terminal side of the β-thiocyanoalanine derivative.





ß – thiocyanoalanine

2–iminothiazolidine

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