PYRUVATE CARBOXYLASE: PHYSICAL AND KINETIC STUDIES

A thesis submitted by

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CONTENTS

SUMMARY

Pyruvate carboxylase was purified from sheep kidney and liver mitochondria and some of the physico-chemical and kinetic properties of the enzyme were investigated. Both enzymes were homogeneous as judged by ultracentrifugation and polyacrylamide gel electrophoresis. Using ultracentrifugation and column chromatography the molecular weight of these enzymes was shown to be 480,000.

The sheep kidney enzyme contained 4 moles of biotin per mole of enzyme but in contrast to the published results for chicken liver enzyme which contains 4 moles of Mn^{2+}, the enzyme isolated from sheep contains only 2 moles of Mn^{2+} per mole of enzyme. These findings are similar to those reported for rat liver pyruvate carboxylase.

Contrary to an earlier report using sheep kidney pyruvate carboxylase of low specific activity, the highly purified enzyme was not cold labile. In addition, either dithioerythritol or the allosteric activator of the enzyme, acetyl-CoA, protect the enzyme against the dilution inactivation which has been shown to occur at pH 8.4.

The subunit structure of the enzyme has been studied. The native enzyme contains four subunits of molecular weight 120000 as judged by polyacrylamide gel electrophoresis and ultracentrifugation in the presence of sodium dodecyl sulphate. Similarly, equilibrium centrifugation in the presence of guanidine hydrochloride gave a molecular weight of 115,000. Amino acid analysis has shown that the enzyme contained

40 ± 3 cysteine residues. The four subunits of the enzyme were shown to be identical by labelling the cysteine residues with iodo [^{14}C] acetic acid and isolating the tryptic peptides. By this method ten labelled peptides were isolated which is a quarter of the number of cysteine residues in the the native enzyme. In addition, polyacrylamide gel electrophoresis of the S-carboxymethylated protein suggests that the subunits are identical, single polypeptide chains.

The properties of sheep pyruvate carboxylase have been compared in detail with carboxylases isolated from other sources. A number of properties, e.g., the biotin content, the molecular weight and the quaternary structure of the enzyme are very similar to the enzyme isolated from other vertebrate sources. However, the amino acid composition of the enzyme isolated from different sources has been determined and compared with the composition of many other proteins using a computer analysis. With this type of analysis it was possible to show that there were two classes of animal pyruvate carboxylases with cold lability being the distinguishing physical properties.

A detailed investigation of the divalent metal ion activation of sheep kidney pyruvate was carried out. An analysis of the initial velocity kinetic data using two independent methods showed that the enzyme has an absolute requirement for Mg^{2+}. Mg^{2+} and $MgATP^{2-}$ combine with the enzyme (E) in a random manner to form the final activated complex consisting of Mg^{2+}-E-$MgATP^{2-}$. Excess ATP^{4-} is inhibitory because it shifts the equilibrium from the active

complex Mg^{2+}-E-$MgATP^{2-}$ to inactive complexes, e.g., Mg^{2+}-E-ATP^{4-} and E-$MgATP^{2-}$ which cannot form products. Furthermore, the analysis showed that the complex E-$MgATP^{2-}$ cannot break down to yield products.

In addition to analysing the activation of sheep kidney pyruvate carboxylase by Mg^{2+}, the activation by Mn^{2+} was also studied. The maximum velocity of the enzyme in the presence of either cation was the same as was their essentiality to the reaction. However, the enzyme does reach maximum velocity at lower concentrations of Mn^{2+} when compared with Mg^{2+}. Furthermore, excess Mn^{2+} is strongly inhibitory.

When the Mg^{2+} activation of the acetyl-CoA independent reactic of pyruvate carboxylase was studied it was found that the divalent metal was not essential. This result suggested that the Mg^{2+} activation is associated with the acetyl-CoA activation. The fact that Mg^{2+} altered the apparent Km value for $MgATP^{2-}$ suggested that the divalent cation induced a conformational change in the enzyme. Evidence supporting this hypothesis was obtained, (a) by determining the entropy change (ΔS^{o} = 58.6 entropic units per mole) induced in the enzyme, (b) by the change in emission intensity of 1-anilinonaphthalene-8-sulphonic acid, and (c) the tritium back exchange rate that occurred when Mg^{2+} bound to the enzyme.

In addition to the work on pyruvate carboxylase some work has been carried out on an improved convergence in the iterative computation of maximum likelihood estimates of nonlinear parameters. The improved convergence is obtained by feeding fitted values from the maximum likelihood procedure into the initial value routine. The method described

converges over a much wider range of cases than methods normally employed for the analysis of non-linear data.

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.

RENZE BAIS

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ABBREVIATIONS

ANS	1-anilinonaphthalene-8-sulphonate
CoA	Coenzyme A
DTE	Dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulphate

PUBLICATIONS

1. PAPERS PUBLISHED

The Magnesium Ion (Mg^{2+}) Activation of Sheep Kidney Pyruvate Carboxylase
(with D.B. Keech)
J. Biol. Chem., 247, 3255 (1972).

Influence of Sodium Dodecyl Sulphate on the Sedimentation Velocity of Proteins
(with P. Greenwell, J.C. Wallace and D.B. Keech)
FEBS Letters, (1974) in press.

2. PAPERS PRESENTED AT MEETINGS

Activation of Sheep Kidney Pyruvate Carboxylase by Magnesium Ions
(with D.B. Keech)
Proc. Aust. Biochem. Soc., 5, 30 (1972)

The Sedimentation of Pyruvate Carboxylase and Other Proteins in the Presence of Sodium Dodecyl Sulphate
(with J.C. Wallace)
Proc. Aust. Biochem. Soc., 6, 12 (1973).

Evidence for Identical Single Chain Monomers of Pyruvate Carboxylase
(with J.C. Wallace and D.B. Keech)
Proc. Aust. Biochem. Soc., 7, in press (1974).

CHAPTER ONE

INTRODUCTION

In recent years a number of reviews covering the physical, chemical and kinetic properties of pyruvate carboxylase and other biotin-containing carboxylating enzymes have been published (Utter et al., 1964; Scrutton and Utter, 1968; Utter and Scrutton, 1969; Moss and Lane, 1971; Scrutton and Young, 1972). Therefore, this Introduction will focus on only those aspects of these enzymes directly relevant to the work reported here, viz., the metal ion activation and the physical properties of pyruvate carboxylase.

A. THE ROLE OF DIVALENT METAL IONS IN CARBOXYLATION REACTIONS

All pyruvate carboxylases studied thus far catalyse the reaction

$$ATP + HCO_3 + \text{pyruvate} \xleftarrow[\text{acetyl-CoA}]{Mg^{2+}} \text{oxaloacetate} + ADP + Pi \quad (1.1)$$

This equation represents the overall reaction which is dependent on the presence of divalent cations. The overall reaction has been shown to proceed in two stages

$$ATP + HCO_3 + \text{E-biotin} \xleftarrow[\text{acetyl-CoA}]{Mg^{2+}} ADP + Pi + \text{E.biotin}\sim CO_2 \quad (1.2)$$

$$\text{E-biotin}\sim CO_2 + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + \text{E-biotin} \quad (1.3)$$

For chicken liver pyruvate carboxylase the E-biotin$\sim CO_2$ complex has been isolated (Scrutton et al., 1965) and the isotopic exchange reactions between ATP and [^{32}Pi] in the absence of pyruvate, and between [^{14}C]pyruvate and oxaloacetate have been studied in detail (Scrutton and Utter, 1965b). The enzyme also catalyses an exchange reaction between [^{32}P]ADP

and ATP which is dependent on the presence of Mg^{2+} and Pi. Recently, it has been shown that these exchange reactions are also catalysed by the enzymes isolated from rat liver (McClure et al., 1971a) and sheep kidney (Ashman, 1973).

Biotin-containing enzymes which catalyse reactions involving the coupling of the hydrolysis of ATP to the fixation of CO_2 exhibit a requirement for divalent cations (Hatch and Stumpf, 1961; Utter and Keech, 1963; Kaziro et al., 1961). This type of activation and/or stimulation of enzymes is indeed widespread and the possible interactions involved have been treated in many reviews (Williams, 1954; Malmstrom and Rosenberg, 1959; Steinhardt and Beychok, 1964; Vallee and Wacker, 1970; Mildvan, 1970).

Like protons, metal ions can behave as lewis acids or electrophophiles, i.e., they can share an electron pair to form a σ bond. However, in contrast to protons, metals can also function as a three-dimensional template for the binding and orientation of bases either independently or as chelate complexes. In addition, because of their filled electron orbitals, metal ions are larger and more polarized than protons and can therefore donate electrons to form π bonds as well as form σ bonds.

The characteristic physiological properties of the complexes formed by the interaction of metals with complex biological ligands including proteins, arise from three sources (Vallee and Riordan, 1959);

(1) the properties of the metal ion altered by binding to the ligand

(2) the properties of the ligand altered by binding the metal ion, and

(3) specific de novo properties of the resultant complex.

The identification of metal ion-protein interaction sites has been largely deduced from observations involving the mode of interaction of metal ions with amino acids and their derivatives. Such studies have led to the conclusion that the amino acid side chains of proteins having dissociable hydrogen ions serve as the sites for cation interactions, although peptide nitrogens may also participate. However, the binding groups of a protein are not always free to cluster around a metal ion; thus, while the protein may contain many potential cation binding sites, each metal may only combine with one or a small number of such sites.

However, in addition to interacting directly with proteins, divalent metal ions can also interact with bases such as ATP which is a substrate for the pyruvate carboxylase reaction. It has been recognised for many years that ATP and its analogues exist in solution, and especially in biological fluids as a mixture of variously ionised metal complexes and structurally conformed species (Neuberg and Mandel, 1949; Hers, 1952; Spicer, 1952). The existence of these various species is important when considering their role and biological effect, such as their rate of hydrolysis (Hoch and Huber, 1965; Liebecq and Jacquemotte-Louis, 1958), the thermodynamics of

this hydrolysis (Phillips et al., 1966), enzyme activation (Bronk and Kielley, 1957), inhibition (Kaye, 1955; McCormick and Levedahl, 1959) and enzyme kinetics (Brintzinger et al., 1960; Kerley and Leaback, 1957; Squires, 1965).

Divalent metal ion coordination with nucleotides has been demonstrated using techniques such as ^{31}P-nuclear magnetic resonance (Cohn and Hughes, 1962; Sterlicht et al., 1968), ion exchange chromatography (Walaas, 1958), electron spin resonance (Maling et al., 1963) and Raman studies (Rimai and Heyde, 1970). These studies have shown that metal ions can interact at various parts of the nucleotide molecule with the ratio of the concentrations of the complexes present depending on the experimental conditions (Phillips, 1966). The combination of metal ions with nucleotides depends on the nature of the cation and the ligand binding groups of the nucleotide. Once the complex is formed its stability depends on such factors as the relative solvation of the cation, of the ligand group and of the complex, the ionic radius of the cation, the geometry of the ligand and the configuration of the complex.

It has been proposed that the chief function of metal ions in catalysing the enzymatic reactions involving polyphosphates is to neutralise the negative charges of the polyphosphate and thus permit the approach of a nucleophile. However, the interaction of metal ions and ATP leads to the 'formation of an activated metal-ion-ATP complex', the reactions of which are not yet fully understood.

For sheep kidney (Keech and Barritt, 1967), yeast (Cazzulo and Stoppani, 1969), rat liver (McClure et al.,

1971b) and chicken liver (Barden et al., 1972) pyruvate carboxylases, the complex $MgATP^{2-}$ has been shown to be the true substrate. In addition, it was found that in the absence of excess Mg^{2+}, sheep kidney pyruvate carboxylase exhibited sigmoidal velocity versus substrate concentration plots which were explained by a homotropic cooperative effect involving at least two molecules of $MgATP^{2-}$ (Keech and Barritt, 1967). When excess Mg^{2+} was present the enzyme exhibited hyperbolic velocity curves and it was concluded that Mg^{2+} exhibited a heterotropic cooperative effect with respect to $MgATP^{2-}$. Later, Cazzulo and Stoppani (1969) using the enzyme isolated from yeast observed similar kinetic behaviour but interpreted their results to mean that the sigmoidicity may reflect a variation in the concentration of the substrate, $MgATP^{2-}$ with respect to the inhibitor ATP^{4-}. In this case, addition of excess Mg^{2+} would remove the inhibitor giving rise to normal hyperbolic kinetics. However, none of these investigators interpreted their results by considering that under all conditions the three species Mg^{2+}, ATP^{4-} and $MgATP^{2-}$, are present and thus some doubt must exist as to the validity of their conclusions.

For optimal assay conditions of carboxylating enzymes, the total divalent metal ion concentration must be in excess of the total ATP^{4-} concentration (Gregolin et al., 1966; Kaziro et al., 1961; Ling and Keech, 1966). This suggests that besides being necessary for the formation of the substrate, i.e., $MgATP^{2-}$, free Mg^{2+} may also be required to activate

ATP^{4-}-dependent carboxylases. For sheep kidney pyruvate carboxylase, Keech and Barritt (1967) and McBlair (1969) who re-examined their results, both concluded that Mg^{2+} and $MgATP^{2-}$ were required for full enzymic activity. Recently, similar conclusions have been reached for pyruvate carboxylases isolated from yeast (Cazzulo and Stoppani, 1969), rat liver (McClure et al., 1971a), chicken liver (Barden et al., 1972) and Aspergillus niger (Feir and Suzuki, 1969). However, in the case of the rat liver enzyme, McClure et al. (1971a) also concluded that because at infinite $MgATP^{2-}$ concentration the Michaelis-Menten constant for Mg^{2+} was zero, there was no absolute requirement for free Mg^{2+}.

Although the requirement for free-divalent cations appears to be characteristic of all pyruvate carboxylases, different specificities are observed. Mg^{2+}, Mn^{2+} and Co^{2+} activate the enzyme from chicken (Utter and Keech, 1963) and rat liver (McClure et al., 1971a) but only Mn^{2+} is effective as an activator of the enzyme from Bacillus stearothermophilus (Sunderam et al., 1969) and only Mn^{2+} and Mg^{2+} for the enzyme from Saccaromyces cerevisiae (Losoda et al., 1964). Other divalent metal ions, e.g., Ca^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} act as competitive inhibitors with respect to free Mg^{2+} (Scrutton et al., 1969; McClure et al., 1971a).

B. METALLOENZYMES

In addition to being activated by exogenous metal ions, all pyruvate carboxylases which have been adequately examined have been shown to be metalloenzymes, i.e., they contain metal ions which are not easily dissociated from the protein. These complexes are characterised by having very low dissociation constants (e.g., 3.2×10^{-10} M for zinc in carboxypeptidase at pH 8.0 (Coleman and Vallee, 1960). Reviews on the properties of these metalloproteins have recently been written by Vallee and Wacker (1970) and Mildvan (1970).

An interesting feature of metalloenzymes is that it has been possible to replace the 'native' metal with atoms not normally associated with the protein but capable of yielding a biologically active complex (Vallee et al., 1958; Lindskog and Malmstrom, 1962; Plocke and Vallee, 1962). The metal ions of metalloenzymes may be regarded as site specific, selective reagents for the identification of such loci which are part of the active enzymic centre. Moreover, since the chemical properties and reactivity of the metal ions may differ distinctly from those of amino acid side-chains of proteins which may participate in catalysis, metalloenzymes have provided convenient models for the study of the mechanism of enzyme action in general. The abundance of different metal ions in biological systems, the permeability of cellular and subcellular membranes and the distribution of metals among a large number of competing ligands dictate the specificity of association between various metals and a given protein. Further, the 'partition' of a given metal ion between competing protein and nonprotein ligands governs the formation

of a particular metalloprotein.

With the removal of the metal ion, the three-dimensional structure of a number of metalloenzymes undergoes marked, and sometimes apparently irreversible alterations (Kagi and Vallee, 1960), while others yield stable, metal-free, inactive apoenzyme to which activity can be fully restored by the readdition of the metal ion. This category now encompasses many proteins (Vallee and Wacker, 1970). The metal ion may stabilise the secondary, tertiary or quaternary structure of the protein. However, the same metal ion species may also serve several different capacities in the same protein. A number of mechanisms by which metal ions may affect the activity of metalloenzymes have been postulated (Vallee and Wacker, 1970);

(a) the metal ion may participate in binding substrates, cofactors or both,

(b) it may activate the enzyme-substrate complex once it is formed

(c) it may serve in both the above functions

(d) metal ions have been thought to be involved in maintaining the secondary, tertiary or quaternary structure of proteins or to interact with side chains such that the ensuing coordination complex can function catalytically

(e) certain metals undergo oxidation-reduction during the enzymic reaction

(f) the metal ion may cause conformational changes which are either functionally or structurally advantageous

(g) the coordination complex of the metal ion with the protein may be constituting an energetically poised domain.

Rapid progress is being made to allow detailed microscopic differentiation of the possibilities.

Pyruvate carboxylase purified from chickens raised on a commercial diet contains four atoms of bound manganese (Scrutton et al., 1966) whereas the enzyme from Saccharomyces cerevisiae contains four atoms of zinc (Scrutton et al., 1970). Rat liver pyruvate carboxylase also contains manganese but in a stoichiometry of two moles of metal per four moles of biotin (McClure et al., 1971a).

Studies made using the chicken liver enzyme have led to the conclusion that the tightly bound cation functions in the second partial reaction (Reaction 1.3). Using data obtained from nuclear magnetic resonance studies, Mildvan et al. (1966) have proposed a partial reaction mechanism showing the interaction of pyruvate with the tightly bound manganese. These investigators proposed a mechanism in which the binding of pyruvate causes a conformational change which brings the biotin moiety in close proximity to the bound manganese atom. Studies on the relaxation rates of the methylene protons of the substrates have led to the proposal that there is the formation of an enzyme-Mn^{2+}-pyruvate and an enzyme-Mn^{2+}-oxaloacetate bridge complex (Mildvan and Scrutton, 1967; Scrutton and Mildvan, 1970).

However, recent advances in nuclear magnetic resonance and proton relaxation rate techniques have cast some doubt on the original interpretation of the data obtained using chicken liver enzyme (Scrutton et al., 1973). It has been found that all the nuclear magnetic relaxation and electron paramagnetic resonance data suggests is that

the bound manganese is 'buried' in the enzyme in the vicinity of the site at which the carboxylation partial reaction occurs. No evidence could be cited to indicate that Mn^{2+} reacts directly with the components of the transcarboxylation partial reaction (Scrutton et al., 1973). In addition, fully active pyruvate carboxylase preparations have been isolated from Mn^{2+}-deficient avians in which the Mn^{2+} in the enzyme has been replaced by Mg^{2+} (Scrutton et al., 1972). Similarly, the enzyme isolated from Saccharomyces cerevisiae is able to substitute cobalt for the normal cation, zinc (Scrutton et al., 1970). Thus, since the metal ion is able to be exchanged in this way, and the enzyme retains full enzymic activity, it is unlikely that it has such a crucial role in the reaction mechanism as was originally thought.

C. PHYSICAL PROPERTIES OF PYRUVATE CARBOXYLASE

Examination of pyruvate carboxylase isolated from chicken, turkey, calf (Scrutton and Utter, 1965; Utter and Scrutton, 1969), rat liver (McClure et al., 1971a) and Saccharomyces cerevisiae (Young et al., 1969) by analytical ultracentrifugation reveals a major component with a sedimentation coefficient of 15 - 17S. The chicken liver preparations also show a variable amount of a minor 7S component which does not possess pyruvate carboxylase activity (Taylor et al., 1972). The 7S component is thought to consist of monomers formed by the dissociation of the 15S tetramer. The evidence for this conclusion is that when the chicken liver enzyme is exposed to temperatures below 10^{o}, there is a loss of enzymic activity accompanied by a decrease

in the amount of the 15S species and an increase in the 7S component (Valentine et al., 1966; Irias et al., 1969). On raising the temperature to 20°, the enzyme regains activity and the 15S species reappears. Recent examination of the reacting species of chicken liver and yeast pyruvate carboxylases by the 'reacting enzyme sedimentation technique' of Cohen et al. (1967) has confirmed that the major active species for these enzymes is the 15 - 17S species (Taylor et al., 1972). This method was also used to show that the active species of the enzyme from Pseudomonas citronellolis has a sedimentation coefficient of 12.9S.

Examination of yeast and chicken liver pyruvate carboxylase by both ultracentrifugation and polyacrylamide gel electrophoresis suggests that the true molecular weight of these enzymes is 450,000 - 520,000 (unpublished observations cited by Taylor et al., 1972). The previously reported molecular weight for the chicken liver enzyme of 600,000 - 660,000 (Scrutton and Utter, 1965; Scrutton et al., 1970) may have been due to the presence of an active 22S aggregate which can form under certain conditions (Taylor et al., 1971). A minor amount of this aggregate could cause the calculations to give a higher value for the molecular weight when the approach to equilibrium technique is used. The molecular weight of the turkey and calf liver enzymes are also about 500,000 as determined by gel filtration on Sephadex G-200 (Utter and Scrutton, 1969). However, the molecular weight of pyruvate carboxylase isolated from Pseudomonas citronellolis when determined by the method of Archibald (1947) was 265,000 (Taylor et al., 1972) and

this enzyme was postulated to be a dimer. The molecular weight of the enzyme isolated from Bacillus stearothermophilus was found to be 350,000 - 400,000 as determined by gel filtration on Sephadex G-200 (Cazzulo et al., 1971).

Pyruvate carboxylase has been examined in the electron microscope using negative staining. The enzyme from chicken liver (Valentine et al., 1966) and turkey and bovine liver (Scrutton et al., 1968) form a tetramer with the protomers arranged in the shape of a square; the centre to centre distance between adjacent subunits being about 75 Å. However, the yeast enzyme when examined by the same technique shows a different monomeric organisation with the four monomers of the enzyme arranged at the corners of a rhombus (Utter et al., 1967). Further evidence for a difference in quaternary structure between the enzymes isolated from yeast and other sources is the occurrence of dimers as intermediates in the dissociation of the yeast enzyme by maleic anhydride (Young et al., 1969; Scrutton and Young, 1972), whereas the chicken liver enzyme appears to dissociate directly to monomers under a variety of conditions (Scrutton and Utter, 1965a; Irias et al., 1969). In addition, Sumper and Rierpertinger (1972) have shown the existence of trimers as well as monomers and dimers on limited dissociation of the yeast enzyme.

The subunit structure of chicken liver pyruvate carboxylase has been examined by ultracentrifugation in the presence of sodium dodecyl sulphate and the 2.7S species which was observed was postulated to be subunits of molecular weight, 40,000 - 50,000 (Scrutton and Utter, 1965).

However, recent examination of chicken liver enzyme by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate and ultracentrifugation in guanidine hydrochloride has revealed only a single species of molecular weight, 110,000 - 120,000 (Barden and Taylor, 1973). Evidence for smaller subunits has only been obtained when the rat liver enzyme is subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate after extensive denaturation in guanidine hydrochloride and subsequent carboxymethylation (McClure et al., 1971a).

The structure of pyruvate carboxylase from yeast and mammalian sources is similar to other biotin-containing enzymes. Acetyl-CoA carboxylase from avian liver (Gregolin et al., 1966) and adipose tissue (Moss et al., 1972), propionyl-CoA carboxylase from pig heart (Kaziro et al., 1960) and bovine liver (Hegre and Lane, 1966) are all large enzymes (~15S) with a tetrameric structure. In addition, several of these enzymes do not appear to have subunits smaller than of molecular weight, 100,000 (see Moss and Lane, 1971). This structure is very different from that of acetyl-CoA carboxylase from E. coli (Alberts et al., 1969; Guchhait et al., 1971) and transcarboxylase isolated from propionibacterium shermanii (Jacobsen et al., 1970). These enzymes have been shown to be composed of small peptides which can be isolated and then reconstituted into active enzyme. Acetyl-CoA carboxylase has three distinct subunits; viz.

(a) a biotin-containing carboxylase carrier protein (Nervi and Alberts, 1970)

(b) a biotin carboxylase which is free of biotin and

catalyses the carboxylation of the carboxyl carrier protein or free biotin (Alberts et al., 1969; Dimroth et al., 1970)

(c) a protein, carboxyl transferase presumably, to catalyse the carboxyl transfer to acetyl-CoA (Alberts and Vagelos, 1968).

Transcarboxylase, which has a sedimentation coefficient of 18S, has been shown to contain a biotin-containing peptide of molecular weight, 11,000 (Gerwin et al., 1969), a metal containing subunit and a 12S subunit (Ahmad et al., 1972). Thus, it may be that there are two classes of biotin-containing enzymes; one which can readily be dissociated into small peptides and then the enzyme reconstituted and one class of enzymes which cannot undergo these processes.

D. AIMS OF THIS PROJECT

The formation of carbon-carbon bonds is fundamental to life and the understanding of how these bonds are formed is of great importance. Thus the enzyme pyruvate carboxylase, which forms a four carbon compound from a three carbon compound has been studied in an endeavour to understand this process more fully. In addition this enzyme is an important regulatory enzyme in the gluconeogenic pathway and control of the isolated enzyme has been studied in an effort to understand its control *in vivo*.

As pointed out in the previous discussion, it has been shown that all biotin-containing enzymes which catalyse reactions involving the coupling of the hydrolysis of ATP^{4-} to the fixation of CO_2 exhibit a requirement for divalent

cations. It has been known that Mg^{2+} complexes with ATP^{4-} to form $MgATP^{2-}$ the substrate for pyruvate carboxylase and that Mg^{2+} also activates the enzymic reaction by complexing with the enzyme. However, no indication has previously been given as to where Mg^{2+} exerts its influence or the nature of the effect induced by the divalent cation. Thus the role of metal ions in the reaction has been investigated further to determine

(a) whether the requirement for Mg^{2+} was absolute

(b) the physical nature of the Mg^{2+}-activation.

In addition, it has been suggested that Mn^{2+} may be a more effective activator of pyruvate carboxylase than Mg^{2+} and the activation of the sheep kidney enzyme by these two divalent cations has been investigated in detail. During the course of these studies it was also discovered that pyruvate carboxylase can catalyse an acetyl-CoA independent reaction and the effect of Mg^{2+} on this reaction has been studied to determine whether the divalent metal ion activation is related to the acetyl-CoA activation of the enzyme.

The second aspect of this work concerned the physical properties and structure of pyruvate carboxylase. Biotin-containing enzymes can be divided into two classes of proteins depending on whether they can be dissociated into small subunits possessing specific functions. Thus sheep kidney pyruvate carboxylase has been examined to determine which class of biotin-containing enzymes it belongs. The aims were

(a) to purify sheep kidney pyruvate carboxylase and compare its physical properties with the enzyme isolated from other sources

(b) to determine the subunit structure of the enzyme and discover whether the subunits are identical

(c) to use the amino acid composition of pyruvate carboxylase isolated from various sources and determine whether differences in properties could be explained by some simple genetic variant.

CHAPTER TWO

MATERIALS AND GENERAL METHODS

A. MATERIALS

1. Enzymes and Proteins

Albumin : Bovine serum, fraction V. Sigma Chemical Co., St. Louis, U.S.A.

Avidin : Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Catalase : Beef liver, crystalline suspension in water with 0.1% thymol. Sigma.

α-Chymotrypsin : Bovine pancreas. Three-times crystallised. Worthington.

Cytochrome c : Horse heart. Boehringer Mannheim Biochemical (London) Ltd.

β-Galactosidase : E. coli. Worthington.

Glutamic acid dehydrogenase : Bovine liver, Type I. Sigma.

Glutamic-oxaloacetic aminotransferase : Pig heart. Sigma.

Glutamic-pyruvic aminotransferase : Pig heart. Sigma.

Haemoglobin : Type IV. Sigma.

Lysozyme : Egg white, grade I. Sigma.

Malate dehydrogenase : Pig heart. Sigma.

Ovalbumin : Egg white, Grade V. Sigma.

Pronase : B grade. Calbiochem, Los Angeles, Calif., U.S.A.

Trypsin : Bovine pancreas, trypsin TPCK treated, Worthington.

Urease : Jack bean, type VII. Sigma.

2. Radioactive Compounds

[^{14}C]$NaHCO_3$: 40 mCi per mmole. The Radiochemical Centre, Amersham, Bucks, England.

Iodo-[^{14}C]acetic acid : mCi per mmole. Amersham.

Fluorodinitro [^{14}C]benzene. 5-20 mCi per mmole. Amersham.

[^{3}H]H_2O : 10 Ci per ml. Australian Atomic Energy Commission, Lucas Heights, Australia.

3. Chemicals for Specific Procedures

(a) Enzyme preparations

Tris : Trizma Base, reagent grade. Sigma.

Ammonium sulphate : Special enzyme grade. Mann Research Labs., N.Y., U.S.A.

Polyethylene glycol : 20,000 molecular weight. Union Carbide Corp., U.S.A.

Dithioerythritol : Sigma.

Sucrose : Analytical reagent grade. Colonial Sugar Refining Co., Sydney, Australia.

(b) Enzyme assay

ATP : Disodium salt. Sigma.

Sodium pyruvate : Sigma.

$NaHCO_3$: Anax Chemical Ltd., Sydney, Australia.

Coenzyme A : Free acid, grade I. Sigma.

NADH : Disodium salt, grade III. Sigma.

(c) Reduction and carboxymethylation of proteins

Ethanolamine : B.D.H. Ltd., Poole, England.

Iodoacetic Acid : Sigma. This compound was recrystallised from petroleum ether (B.P. to -80°) before use, and stored in the dark at room temperature.

2-Mercaptoethanol : Sigma.

Urea : Reagents puro. Carlo Erbo, Milan, Italy.

(d) Polyacrylamide gel electrophoresis

Acrylamide : Eastman Organic Chemicals, N.Y., U.S.A.

Ammonium Persulphate : By-products and Chemicals Pty. Ltd., Sydney, Australia.

Coomassie Brilliant Blue R-250 ; Mann.

N',N'-methylenebisacrylamide : Eastman.

Riboflavin : B.D.H. Ltd.

N',N',N',N'-tetramethylethylenediamine : Eastman.

(e) Measurement of radioactivity

1,4-bis-(5-phenyloxazolyl)-benzene : Packard Instruments Co. Ltd., La Grange, U.S.A.

2,5-diphenyloxazole : Scintillation grade. Packard.

Toluene : Analytical reagent grade. Carlo Erbo.

Triton X-100. Anax.

NCS Solubilizer : Amersham/Searle, Des Plaines, Ill., U.S.A.

4. Miscellaneous Chemicals

Biotin : Crystalline. Sigma.

Amino acids : Mann.

Dansyl chloride : Mann.

N-ethylmorphiline : Eastman. This reagent was distilled under reduced pressure at 33^{o}, before use, and stored under nitrogen at 2 - 4^{o}.

Ninhydrin : Pierce Chemicals.

Sodium dodecyl sulphate : Sigma.

5. Miscellaneous Materials

Chromatography paper : Whatman.

Dialysis tubing : Visking. B.D.H.

Polyamide thin layers : Chen Chin Trading Co. Ltd., Taipei, Taiwan.

Sephadex : All grades. Pharmacia, Uppsala, Sweden.

Buffer A : 0.025 M potassium phosphate, pH 7.2 containing 0.0001 M DTE, 0.0001 M EDTA and 10 ml of saturated $(NH_4)_2SO_4$ per litre.

Buffer B : As buffer A, except containing 60 ml of saturated $(NH_4)_2SO_4$ per litre.

B. GENERAL METHODS

Only the most general techniques that were used routinely throughout this work are described in this chapter. All other procedures are described in their appropriate chapters.

1. Assay of Pyruvate Carboxylase

The enzyme was assayed by following the incorporation of $[^{14}C]CO_2$ into oxaloacetate which was subsequently stabilised as the 2,4-dinitrophenylhydrazone as described previously (Taylor et al., 1969; Ashman et al., 1972) or by coupling with malate dehydrogenase and NADH and following the decrease in absorbance at 340 nm (Utter and Keech, 1963). An enzyme unit is defined as the amount of enzyme which carboxylates 1 μmole of pyruvate per min at 30° in the presence of 0.1 M Tris (Cl^-, pH 8.4) and saturating levels of acetyl-CoA and all substrates.

2. Protein Estimations

During the course of these studies it was found that protein estimations determined by the spectroscopic method described by Layne (1957) and using the equation

$$\text{protein concentration (mg per ml)} = 1.55\ A^{1\text{ cm}}_{280\text{ nm}} - 0.76\ A^{1\text{cm}}_{260\text{ nm}} \qquad (2.1)$$

gives low values for the protein concentration of sheep kidney pyruvate carboxylase when compared with other methods (Table 2.1). A similar result has been reported for chicken liver pyruvate carboxylase (Taylor et al., 1972). However, this method was convenient to use for monitoring protein

TABLE 2.1. COMPARISON OF PROTEIN CONCENTRATION OF SHEEP KIDNEY PYRUVATE CARBOXYLASE AS DETERMINED BY VARIOUS METHODS

Method	Protein Concentration (mg/ml)
$A_{280\ nm}$	1.46
Equation 2.1	1.609
Microbiuret method of Zamenhof (1957)	3.311 ± 0.163
Method of Lowry *et al.* (1953)	2.839 ± 0.156

concentrations from columns. The method of Lowry et al. (1953) was used when any stoichiometric measurements were necessary.

3. Analytical Ultracentrifugation

Analytical ultracentrifugation was carried out using a Beckman Model E centrifuge. Sedimentation velocity experiments were carried out at 56100 rpm. Either a four piece Titanium rotor or a two place aluminium rotor was used. The cells were 12 mm cells and 4^{o} sector Kel-F centrepieces were employed.

The diffusion coefficient of pyruvate carboxylase was determined by the method of Kawahara (1969). Measurements were made using a double sector synthetic boundary cell centrifuged at low speed (2531 rpm).

Sedimentation equilibrium determinations of protein molecular weight were carried out as described by Schachman (1957). Experiments were conducted at 2531 rpm in a 12 mm double sector cell using a four place rotor fitted with a counterweight and blanking pieces. This rotor was used because of its extra stability when rotated at low speeds.

Schlieren optics were used and photographs were taken using either Kodak Ortho Metallographic Plates or Kodalith Ortho Film, Type 3. In general, measurements were made from photographic enlargements.

4. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out using the half cross-linked 10% acrylamide gels described by Weber and Osborn (1969).

Polyacrylamide gel electrophoresis in the presence of urea was performed at pH 7.5 as described by Williams and Reisfeld (1964). Protein bands were stained with Coomassie Brilliant Blue R-250.

5. Reduction and S-Carboxymethylation

The preparation of reduced and S-carboxymethylated protein was based on the method described by Kemp (1972). The procedure yields complete carboxymethylation with minimal risk of protein degradation or chemical modification.

The protein was placed in a 10 ml 'Quick-fit' test tube and incubated with a solution containing 8 M urea, 0.1 M 2-mercaptoethanol and 0.5 M ethanolamine-Cl, pH 10.5 ('reducing solution'). A ratio of 0.5 ml of 'reducing solution' per 2 mg of protein was used. After 3 hr at 37°, 0.30 ml of a solution containing 3.0 M tris-Cl at pH 8.0 was added in order to lower the pH of the reaction mixture to pH 8.7 - 8.8. Carboxymethylation was affected by adding 0.1 ml of a 30% (w/v) solution of iodoacetic acid that was 0.3 M with respect to Tris and adjusted to pH 8.2 by the addition of 1 M Tris. The carboxymethylation reaction was allowed to proceed for 20 min at room temperature. 2-Mercaptoethanol (0.015 ml) was then added and the reaction mixture incubated at 37° for 30 min. A further 0.1 ml of the iodoacetic acid solution was added and the mixture was

again allowed to stand for 20 min at room temperature in order to effect a second cycle of reduction and carboxymethylation. 2-Mercaptoethanol (0.02 ml) was added to destroy excess iodoacetate. The solution was exhaustively dialysed and then freeze-dried.

When radioactive S-carboxymethylated proteins were prepared using iodo [^{14}C]acetic acid, the initial incubation was carried out with DTE at a half-molar ratio to the number of sulfhydral groups present instead of 0.1 M 2-mercaptoethanol. One cycle of carboxymethylation was carried out using the iodo [^{14}C]acetic acid and after a further 60 min incubation, two cycles of the normal reduction and carboxymethylation was carried out.

6. Amino Acid Analysis

Protein samples were hydrolysed in 6 N HCl *in vacuo* at 110° for the required time. Generally, protein samples were hydrolysed for 20 hr but those analysed in detail (sheep kidney, sheep liver and chicken liver pyruvate carboxylases) were S-carboxymethylated and hydrolysed for 20, 40 and 60 hr. This permitted correction for incomplete release and destruction of certain amino acids. A crystal of phenol was always present to prevent modification of tyrosine residues (Sanger and Thompson, 1963). HCl was removed by rotary evaporation. Hydrolysates from the protein samples (generally about 400 µg) were analysed by the method of Piez and Morris (1960) using a Beckman 120C Analyzer modified as described by Harding (1971).

7. N-Terminal Determination

N-terminal analysis by the Dansyl chloride procedure was carried out as described by Gross and Labouesse (1969). Dansyl amino acids were identified by two-dimensional chromatography on polyamide layers as described by Woods and Wang (1967).

8. Preparation of Acetyl-CoA

Acetyl-CoA was prepared from CoA as described by Simon and Shemin (1953) and purified as described by Keech and Barritt (1967).

9. Preparation of [^{3}H]Biotin Sheep Pyruvate Carboxylase

It has been reported that acetyl-CoA carboxylase from E. coli (Allberts et al., 1969; Guchait et al., 1971) and methylmalonyl-CoA pyruvate transcarboxylase from Propionbacterium shermanii (Jacobsen et al., 1970) both contain a small biotin-containing peptide. Also McClure et al. (1971a) found that when rat liver pyruvate carboxylase was carboxymethylated and electrophoresed on polyacrylamide gels containing SDS, multiple bands appeared. However, it was not possible to purify these protein species by Sephadex gel chromatography in the presence of SDS and show if one was a biotin-containing peptide. In order to determine whether sheep pyruvate carboxylase contained a small biotin-containing peptide, [^{3}H]biotin labelled pyruvate carboxylase was prepared by infusing biotin into sheep. It was proposed that highly labelled enzyme would be prepared such that this

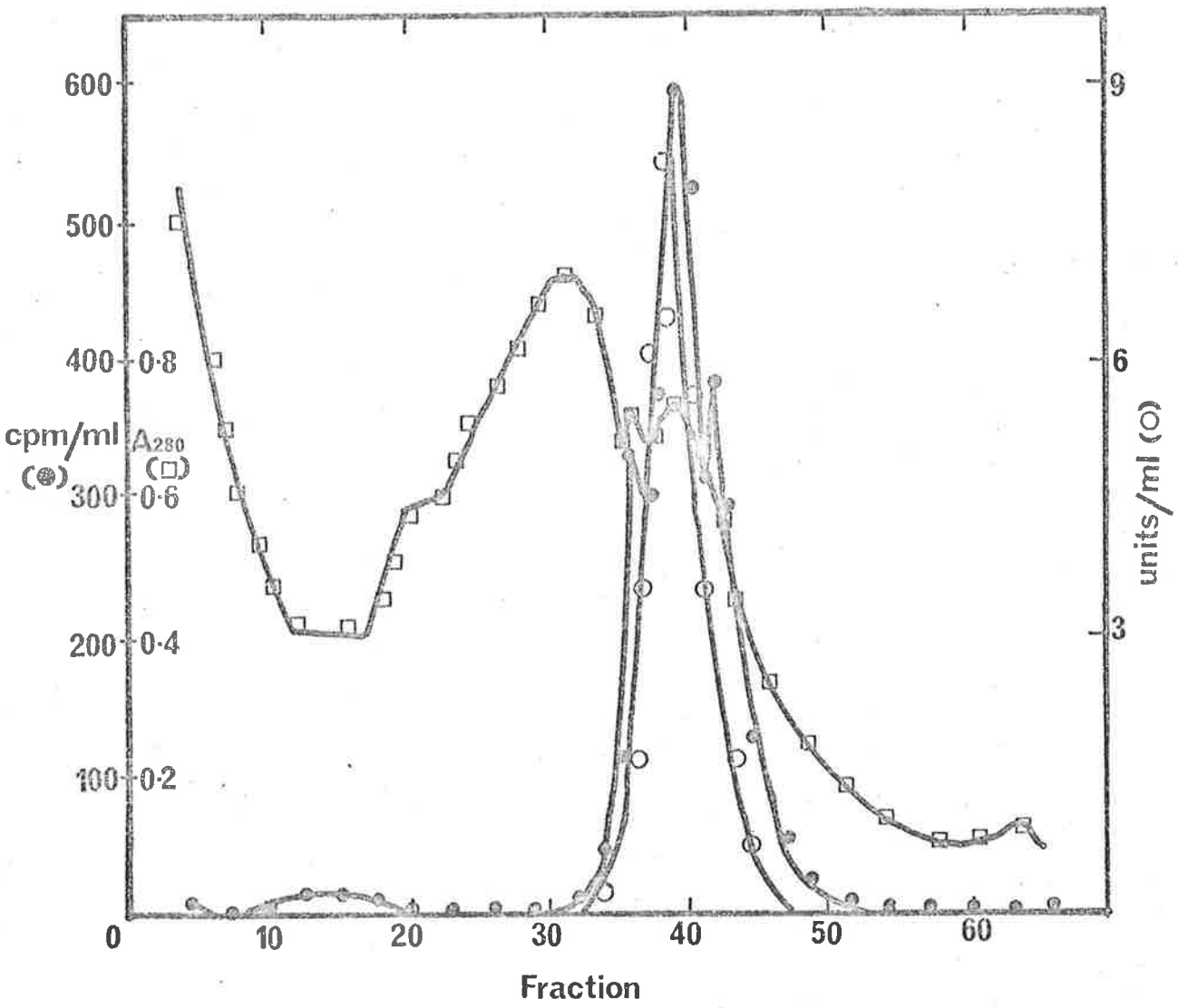


FIGURE 2.1. Preparation of ^{3}H-biotin labelled sheep liver pyruvate carboxylase. The enzyme was prepared and eluted from a DEAE-Sephadex A-50 column (22 cm x 4.6 cm) as described in the text. Protein (A_{280}), □——□ ; activity (units per ml), ○——○ ; radioactivity (cpm per ml), ●——● .

problem could be studied.

Sheep were made diabetic by alloxan injection (60 g per Kg) and after 5 - 6 days were stabilised by infusion for as long as necessary (Jarrett et al., 1973). [^{3}H]-biotin in 0.9% NaCl (specific activity, 0.4 C per mole) was then infused intravenously through a jugular cannula (72 ml per day) for 3 days.

Sheep liver pyruvate carboxylase was prepared as described in Section 3.E. The results from a DEAE-Sephadex column (Fig. 2.1) show that all the radioactivity corresponded to the enzymic activity. The fractions with the highest specific activity were further purified by Sephadex G-200 chromatography. This labelled enzyme was then used for further study. However, although labelled biotin was incorporated into the enzyme by this method, the specific activity of the label was not high enough to warrant further infusions for the purpose of isolating the enzyme for in vitro studies. This was particularly noticeable for the kidney enzyme preparation because when the radioactive mitochondria was added to the normal mitochondria the label was undetectable. In addition, it was found that for in vitro experiments requiring labelled biotin enzyme, the enzyme could be successfully labelled using [^{14}C]CO_2 fixation (Section 2.11).

10. Preparation of [^{3}H]Biotin

Because pyruvate carboxylase specifically labelled with [^{3}H]biotin can be prepared by the method described above, high specific activity [^{3}H]biotin was prepared

by a modification of the method of Garnett et al. (1972); 50 mg of biotin was suspended in glacial acetic acid (2 ml) and 0.3 ml of acetic anhydride was added with 0.15 ml of ^3H_2O (10 C per ml). The acetic anhydride and water react to give acetic acid

$$H_2O + (CH_3CO)_2O \longrightarrow 2\ CH_3COOH \qquad (2.2)$$

The tube was sealed and isotope exchange achieved by heating at 120° for 4 hr. The sample was then neutralised with concentrated $NaHCO_3$ and a sample chromatographed on a G-15 column (100 cm x 1.8 cm) equilibrated with 1 M $NaHCO_3$. It was found that biotin was more soluble in 1 M $NaHCO_3$ than in water. Results of the column chromatography are shown in Fig. 2.2. Samples from fractions 28 and 36 were then chromatographed on Whatman 3 MM paper along with biotin and the original sample. After development with n-butanol : glacial acetic acid : water (2 : 1 : 1) the paper was dried and sprayed with the biotin specific dye p-dimethylaminocinnamaldehyde (p-DACA, 0.2% solution in ethanol) (McCormick and Roth, 1970). The results are shown in Fig. 2.3. Neutralisation of the acetic acid with $NaHCO_3$ results in the formation of sodium acetate which appears as a diffuse yellow spot (R_f = 0.58) when sprayed with p-DACA. Biotin appears as a pink spot (R_f = 0.84) and only the major peak from the column contained biotin. Thus this exchange method specifically labels biotin (greater than 95% of the resultant radioactivity incorporated into biotin ~5.6 x 10^6 μCi per μm).

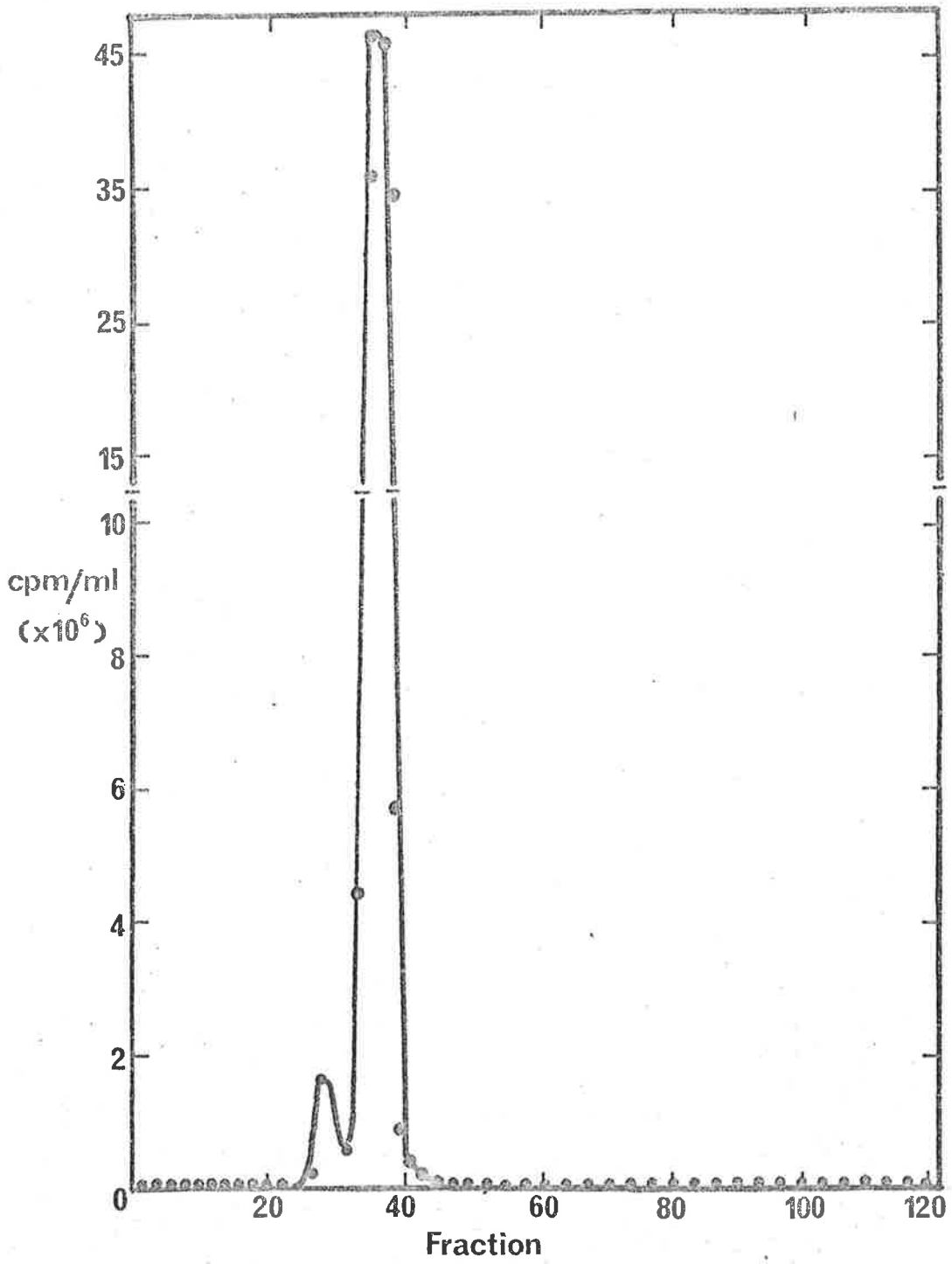
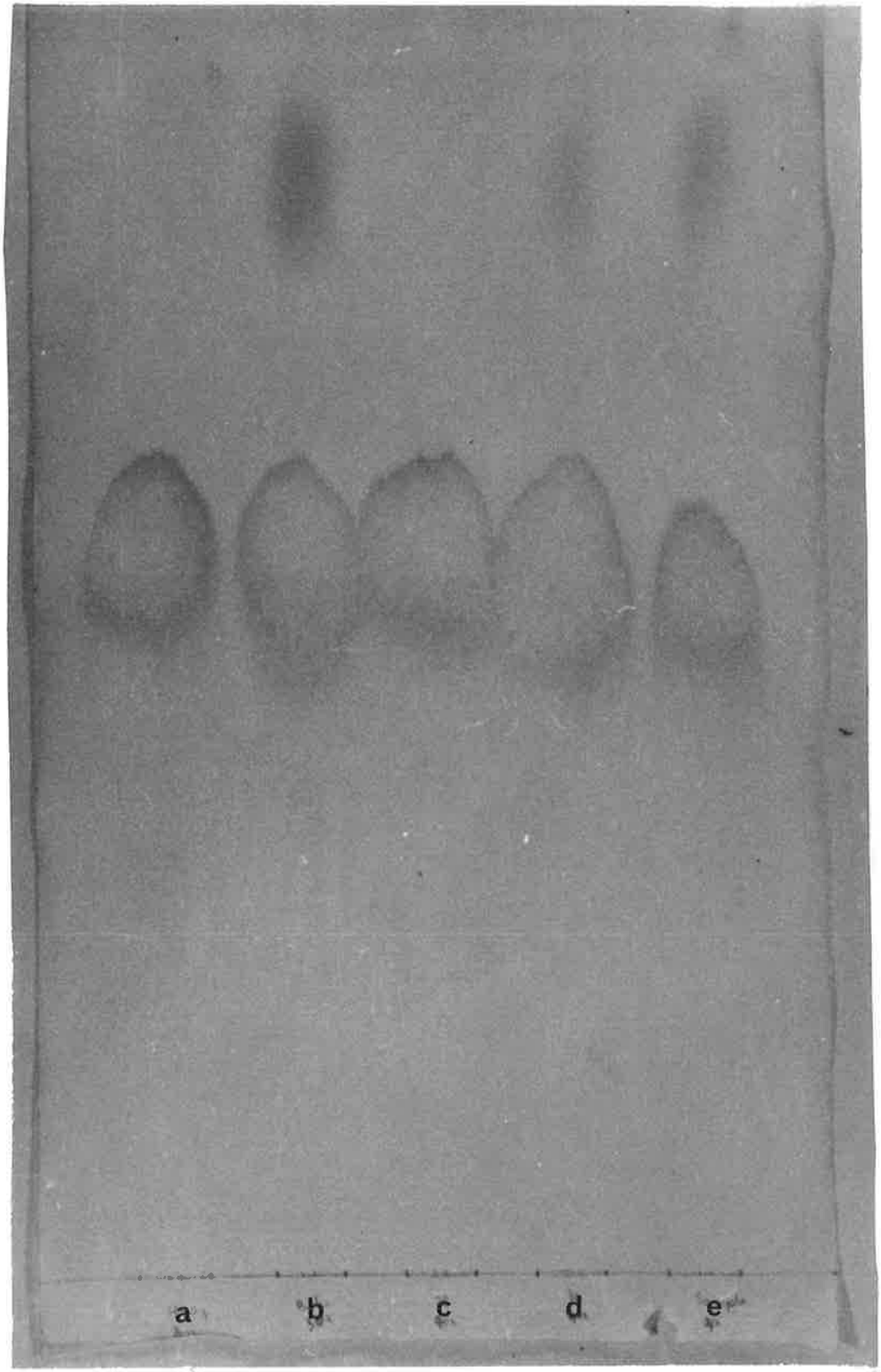


FIGURE 2.2. Chromatography of ^{3}H-biotin on a Sephadex G-10 column (100 cm x 1.8 cm). The buffer was 1 M $NaHCO_3$. The ^{3}H-biotin was prepared as described in the text and a 1 ml sample was applied to the column. Radioactivity was determined by counting samples (0.02 ml from the 2 ml fractions) in the presence of 0.2 ml of water and 2 ml of 30% (v/v) triton X-100 scintillation fluid.

FIGURE 2.3. Paper chromatography of [^{3}H]-biotin. The samples (0.02 ml) were applied to Whatman 3MM paper and the chromatogram developed using nbutanol:acetic acid: water (2:1:1) by descending chromatography for 6 hr. Biotin was located by spraying the dried chromatogram with p-DACA. The samples are a, sodium acetate, b biotin standard, c, fraction 28 from the column shown in Figure 2.2, d, fraction 36 from the column shown in Figure 2.2 and e, the sample before chromatography.


a
b
c
d
e

11. Preparation of [^{14}C]CO_2-Labelled Pyruvate Carboxylase

Pyruvate carboxylase can be labelled using [^{14}C] CO_2 (Scrutton et al., 1965) and using sheep liver enzyme it has been shown by paper chromatography that the sole radioactive product, from a pronase digest, has the same mobility as 1-N'-carboxymethoxybiocystin (D.B. Rylatt, personal communication). The procedure used for labelling was as follows.

The protein (1 - 2 mg) was incubated in 1 ml containing 0.05 M tris-Cl, pH 8.0, 0.005 M Mg^{2+}, 0.0025 M ATP and 3.4 µmoles of [^{14}C]$NaHCO_3$ (specific activity, 59 µCi per mmole). After 5 min incubation at 30°, a freshly prepared ethereal solution of diazomethane was added. The protein precipitated and was collected by centrifuging. The precipitate was washed twice with 2 ml of 0.2 M $NaHCO_3$ and once with 2 ml of water. It was then dissolved in 50% (w/v) formic acid and dialysed immediately against the appropriate buffer.

Diazomethane was prepared by adding 1 - 2 g of nitrosomethylurea to 20 ml of 50% NaOH and 20 ml of ether in a 100 ml flask. The diazomethane distilled with the ether when the mixture was warmed.

Nitrosomethylurea prepared by the method of Arndt (1943) was the generous gift of D.B. Rylatt.

12. Computer Analysis

All computer analyses were carried out using the Control Data Corporation 6400 computer of the University of Adelaide. The kinetic programs LINE, SIGMOID and HYPER were those of Cleland (1967). All other programs were written in Fortran IV.

CHAPTER THREE

PURIFICATION AND PROPERTIES OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

A. INTRODUCTION

Since its discovery in chicken liver mitochondria (Utter and Keech, 1960), pyruvate carboxylase has been shown to occur in a variety of tissues and cells (Utter and Scrutton, 1969). However, most investigators using these enzymes have focussed their attention on the kinetic properties of the enzyme with a view to relating these properties to the *in vivo* control of metabolic processes. With the exception of the chicken liver enzyme, less attention has been devoted to the physical properties of the enzyme.

Since a number of kinetic properties of the sheep kidney enzyme differ from those reported for the rat and avian liver enzymes, it was of interest to compare the physical properties of the enzymes. This chapter describes the purification and some properties of sheep pyruvate carboxylase and compares these with the properties of the enzyme isolated from other sources.

B. METHODS

1. Biotin Estimation

The biotin content was determined by the method of Green (1965). This method involved adding the biotin sample to an avidin-dye solution containing 0.06 ml of 0.1 M phosphate buffer, pH 7.0, 0.03 ml of 2,4'-hydrobenzene-azo benzoic acid (stock solution, 50 mg of dye per 100 ml of 0.1 M phosphate buffer, pH 7.0) and 0.06 ml of avidin (stock solution, 2 mg per ml of 0.1 M) which was dissolved in

phosphate buffer, pH 7.0). The addition of biotin to the avidin-dye complex causes a conformational change in the complex which results in a decrease in absorbance at 500 nm.

Enzyme samples were prepared for biotin determination by pronase digestion. The protein was placed in a stoppered test tube, heated on a boiling water bath for 10 min, cooled and pronase added (at a ratio of 10 mg of denatured enzyme to 1 mg of pronase). The digestion was allowed to proceed for 24 hr at 30^{o} after which the samples were lyophilised. The freeze-dried samples were dissolved in 0.1 M phosphate buffer, pH 7.0, centrifuged to remove insoluble material and aliquots of the supernatant taken for biotin estimation.

2. Divalent Metal Ion Estimation

Metal ion concentrations were determined using a Techtron Atomic Absorption Spectrophotometer fitted with a Techtron DI-30 Digital Indicator.

Samples were assayed directly from either the DEAE-Sephadex or G-200 column using the eluting buffer as a reference.

C. PREPARATION OF MITOCHONDRIA

All procedures for isolating mitochondria were carried out at 4^{o}. Sheep kidneys were placed in ice immediately after removal of the animal. The cortex was dissected from the kidneys and homogenised in a Waring

blendor with 3.5 volumes (w/v) of 0.25 M sucrose containing 10^{-4} M EDTA. The homogenate was centrifuged at 600 x g for 20 min to remove cell nuclei and whole cells. The supernatant fraction was centrifuged at 23,000 x g for 15 min and the precipitated material suspended in 10^{-4} M EDTA to give a final volume half that of the original 600 x g supernatant. The suspended material was centrifuged at 23,000 x g for 15 min, suspended in a minimal volume of 10^{-4} M EDTA and lyophilised. The dried mitochondria were stored at -15^{o} over silica gel.

Rat, sheep and guinea pig liver mitochondria were prepared using the same procedure. Chicken, turkey and duck liver mitochondria were prepared by the method of Scrutton et al. (1969).

D. PREPARATION OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

All operations for the purification of pyruvate carboxylase were carried out at room temperature.

1. Extraction of Soluble Protein

The mitochondria powder was extracted with 15 volumes of a solution containing 0.025 M tris acetate, 0.00175 M ATP, 0.0033 M $MgCl_2$, 0.0005 M EDTA, pH 6.7 for 15 min with stirring. During the extraction process, the pH was maintained between pH 6.7 and 6.9 by adding 1 M tris base. Insoluble material was removed by centrifuging at 23,000 x g for 15 min. After centrifuging, the pH was

adjusted to pH 7.2 with 1 M tris.

2. $(NH_4)_2SO_4$ Fractionation

To each 100 ml of the supernatant solution 14.4 g of solid $(NH_4)_2SO_4$ was slowly added while maintaining the pH between pH 7.0 and 7.2. After stirring for 20 min, the precipitate was removed by centrifuging for 15 min at 23,000 x g. To the supernatant, a further 4.9 g of $(NH_4)_2SO_4$ per 100 ml was added, and after stirring for 20 min, the precipitate was collected by centrifuging at 23,000 x g for 15 min and dissolved in buffer A at a concentration of 6 - 10 mg of protein per ml.

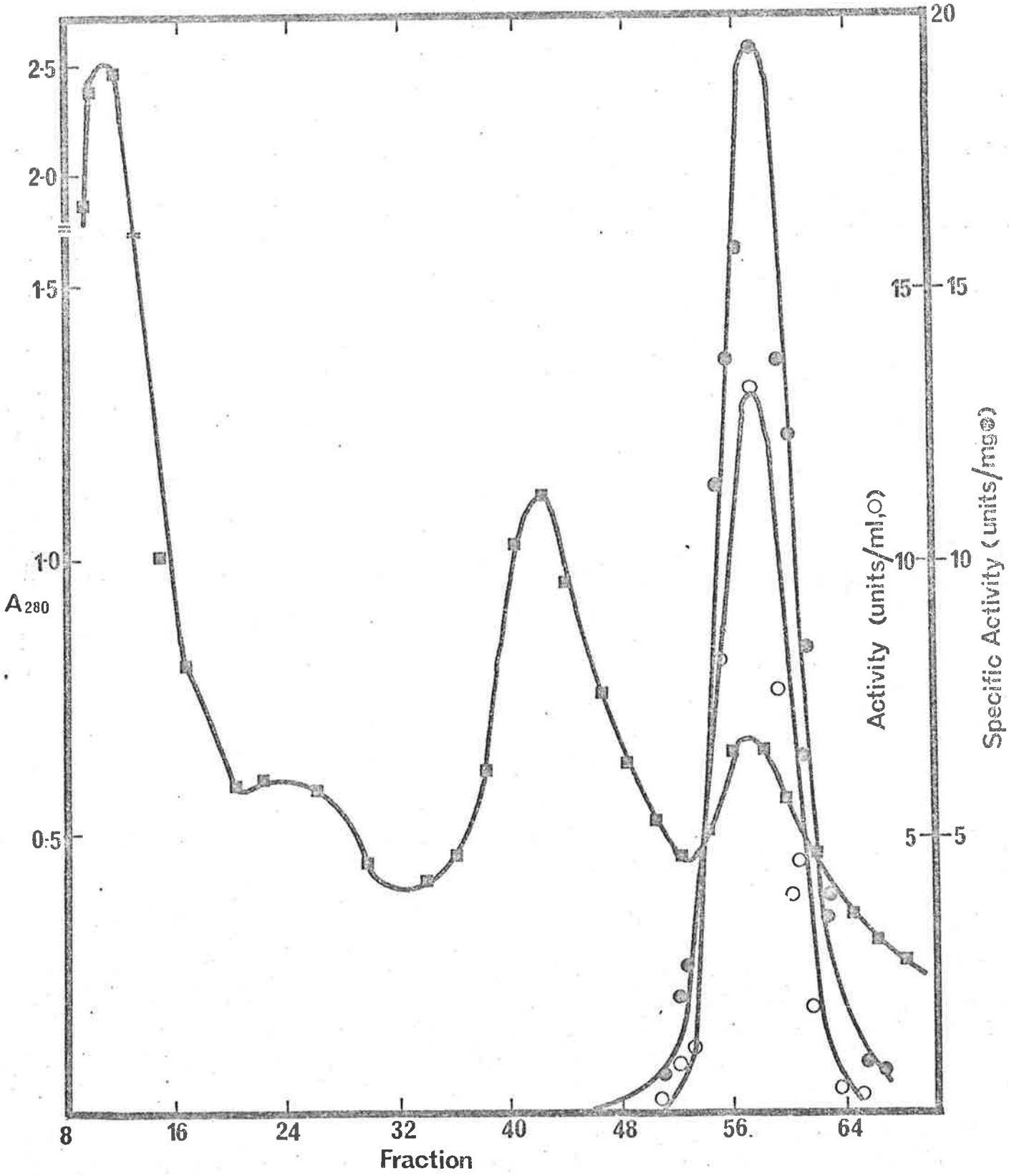
3. Polyethylene Glycol Fractionation

To each 100 ml of the protein solution was added 15.5 g of polyethylene glycol. After the polyethylene glycol had dissolved, the solution was centrifuged at 23,000 x g for 15 min. The precipitate was dissolved in buffer A (10 - 15 mg of protein per ml) with the aid of a Potter-Elvehjem homogeniser. Undissolved material was removed by centrifuging at 23,000 x g for 10 min.

4. DEAE-Sephadex Chromatography

The supernatant from the previous step was applied to a DEAE-Sephadex A-50 column (22 cm x 4.5 cm) previously equilibrated with buffer A. The protein was eluted using a linear gradient of 2 x 500 ml of buffer A to buffer B. A typical elution pattern from the column is shown in Fig. 3.1. The fractions with the highest specific activity were

FIGURE 3.1. Purification of sheep kidney pyruvate carboxylase by chromatography on DEAE-Sephadex. The enzyme (specific activity, 0.937 units per mg) was applied to the DEAE-Sephadex column (22 cm x 4.5 cm) equilibrated with buffer A. Elution of the protein was by using a linear gradient of $(NH_4)_2SO_4$, as described in the text. The volume of each fraction was 13 ml and the flow rate of the column 90 ml per hr. ■——■ , A_{280nm}; O——O , units per ml; ●——● , units per mg.


2·5
2·0
1·5
1·0
0·5
A280
20
15
10
5
Activity (units/ml,○)
Specific Activity (units/mg●)
8
16
24
32
40
48
56
64
Fraction

pooled and the protein precipitated by adding 27.7 g of $(NH_4)_2SO_4$ per 100 ml of solution. The protein was collected by centrifuging at 23,000 x g for 15 min and dissolved in buffer A (20 mg of protein per ml).

5. Sephadex G-200 chromatography

The enzyme from the DEAE-Sephadex column could be further purified by chromatography on a Sephadex G-200 column (55 cm x 2.1 cm) previously equilibrated with buffer A. Fractions containing the highest specific activity were combined and the protein was precipitated using $(NH_4)_2SO_4$ as above. After centrifuging, the precipitate was dissolved to a concentration of 20 mg of protein per ml in 0.1 M potassium phosphate, pH 7.0 containing 1.6 M sucrose and 0.6 M $(NH_4)_2SO_4$. This solution was frozen in an ethanol/dry ice bath and stored at -15^{o}.

A summary of a typical sheep kidney pyruvate carboxylase preparation is shown in Table 3.1. When stored at -15^{o} in the buffer described the enzyme remained fully active for at least 12 months.

In addition to further purifying the fractions of highest specific activity from the DEAE-Sephadex column by G-200 chromatography the other fractions with activity were also collected and stored. These could be accumulated and purified by chromatography on DEAE-Sephadex and G-200 as described.

After DEAE-Sephadex chromatography the major contaminant in sheep kidney pyruvate carboxylase preparations is glutamate dehydrogenase which elutes very close to the pyruvate carboxylase activity. Attempts have been made to remove this contaminant by using an antibody column (Carey and Wells, 1970). It was found that the antibody to bovine glutamate dehydrogenase cross reacted with the enzyme from sheep and thus this antibody was bound to a Sepharose 4B column using cyanogen bromide. Although this method was successful in removing the glutamate dehydrogenase, a G-200 column eluted under the conditions described above was equally successful in purifying pyruvate carboxylase. Thus, the G-200 column was routinely used in the preparation of the enzyme. However, towards the end of this work instead of Sephadex G-200, Sepharose 4B was routinely used for enzyme preparations because even better separation of pyruvate carboxylase and glutamate dehydrogenase could be achieved.

E. PURIFICATION OF PYRUVATE CARBOXYLASE FROM OTHER SOURCES

Pyruvate carboxylase from other mitochondrial sources was prepared by a procedure similar to that used to prepare sheep kidney pyruvate carboxylase. However, it is possible to achieve satisfactory purification of the enzyme from liver sources by using only the 33% $(NH_4)_2SO_4$ fractionation step instead of the double step described for the kidney preparation. In addition, for chicken liver pyruvate carboxylase, the published method of preparation (Scrutton and Fung,

TABLE 3.1. PURIFICATION OF SHEEP KIDNEY MITOCHONDRIAL PYRUVATE CARBOXYLASE

Details of the purification procedure, starting with 90 g of dry mitochondrial powder, are given in the text.

Step	Protein[a] mg	Enzyme units	Specific Activity	Yield %
Crude extract[b]	23218	2020	0.087	100
25% $(NH_4)_2SO_4$[b] supernatant	10847	1627	0.15	81
33% $(NH_4)_2SO_4$ precipitate	1663	1451	0.872	72
Polyethylene glycol precipitate	1216	1374	1.13	68
DEAE-Sephadex[c]	39	778	19.8	39
Sephadex G-200[c]	15	423	27.4	22

[a]Determined by the equation of Layne (1957).

[b]Assayed by the isotopic method.

[c]Highest specific activity fractions only.

1972) contains an $(NH_4)_2SO_4$ back extraction of the 33% precipitated material followed by G-25 chromatography to remove the $(NH_4)_2SO_4$ to allow the enzyme to bind to the DEAE-Sephadex. However, the method described above involves a polyethylene glycol fractionation instead of these steps. This has the advantages that instead of an $(NH_4)_2SO_4$ back extraction procedure which has been found to be unreliable, an adequate purification is achieved and secondly, it effectively lowers the $(NH_4)_2SO_4$ concentration allowing the enzyme to bind to the DEAE-Sephadex. With preparations of this type, pyruvate carboxylases have been purified from chicken liver to a specific activity of 17, from sheep liver to 16, from rat liver to 12, from guinea pig liver to 11 and pigeon liver to 9.

F. PURITY OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

The electrophoretic patterns obtained from SDS gel electrophoresis of samples taken during the purification of sheep kidney pyruvate carboxylase are shown in Fig. 3.2. As can be seen after chromatography on Sephadex G-200 (or Sepharose 4B), only one peptide band was obtained indicating a pure enzyme preparation. Similarly, chicken liver and sheep liver enzyme preparations yield only one protein band on SDS gels after purification by gel chromatography.

The sedimentation pattern for the sheep kidney enzyme Fig. 3.3A) shows that there is one sedimenting peak with $s_{20,B} \simeq$ 15S. This pattern differs from that of the chicken liver

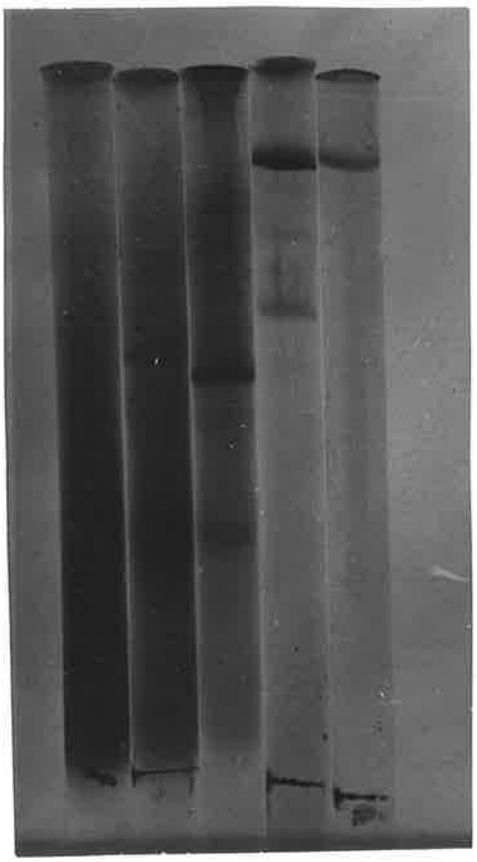


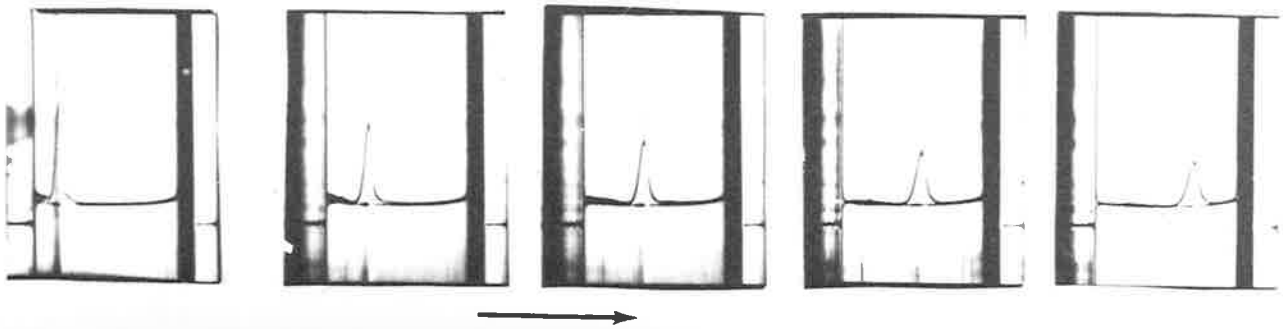
FIGURE 3.2. SDS polyacrylamide gel electrophoresis of samples taken during the purification of sheep kidney pyruvate carboxylase. The samples were A - from the initial extraction; B - after $(NH_4)_2SO_4$ precipitation; C - before application to DEAE-Sephadex; D - after elution from DEAE-Sephadex and E - after G-200 chromatography.

NOTE: SDS gels have been used throughout this work because pyruvate carboxylase aggregated when electrophoresed on either normal or urea gels and appeared as a single band at the gel surface.

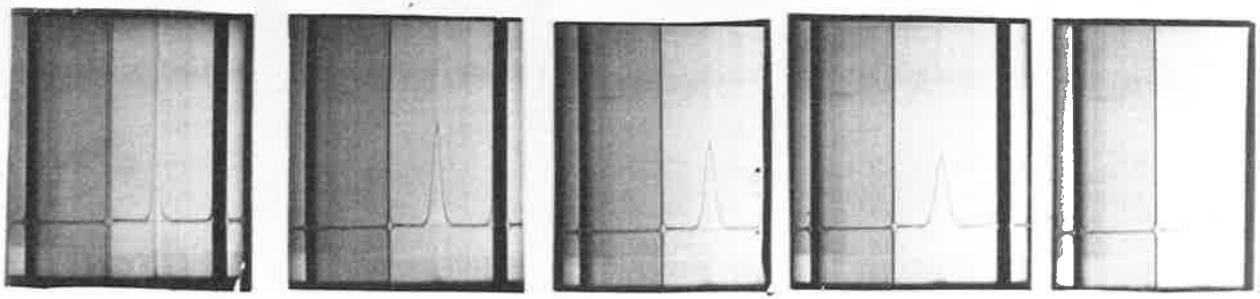
FIGURE 3.3A. Sedimentation pattern of sheep kidney pyruvate carboxylase. The enzyme (specific activity 13.2 units per mg and at 6.0 mg per ml) was sedimented in 0.01 M potassium phosphate buffer, pH 7.2 containing 0.15 M KCl, 10^{-3} M EDTA and 10^{-4} M DTE. Sedimentation was in the direction shown at 56100 rpm in a Beckman Model E ultracentrifuge. The photographs were taken at 16 min. intervals.

B. The diffusion patterns of sheep kidney pyruvate carboxylase. The enzyme (specific activity 12.9 units per mg and at 6.6 mg per ml) was centrifuged at 2531 rpm in a double sector synthetic boundary cell as described in the text. photographs were taken at 16 min intervals after the boundary was formed.

A. Sedimentation Pattern



B. Diffusion Pattern



enzyme in that chicken liver pyruvate carboxylase always has present a variable amount of 7S material (Scrutton and Utter, 1965). The pattern agrees, however, with the observations made using the rat liver enzyme for which no 7S material was observed (McClure et al., 1971a). The symmetry of the 15S peak indicates that the enzyme is homogeneous. Sheep liver pyruvate carboxylase displayed sedimentation patterns identical to those described for sheep kidney enzyme. In addition, the diffusion pattern for sheep kidney pyruvate carboxylase is shown in Fig. 3.3B.

G. PROPERTIES OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

1. Molecular Weight

Molecular weight of proteins can be determined from ultracentrifugation measurements by using the Svedberg equation (Svedberg and Petersen, 1940);

$$M = \frac{RTs}{D(1-\bar{\nu}\rho)} \qquad (3.1)$$

where R is the gas constant, T the absolute temperature, s the sedimentation coefficient, $\bar{\nu}$ the partial specific volume of the solute, ρ the density of the solvent, D the diffusion coefficient of the solute and M the anhydrous molecular weight of the protein.

The sedimentation coefficient of pyruvate carboxylase has been determined over a concentration range of 1 to 16 mg of protein per ml. For the various protein concentrations, the sedimentation coefficient, s_{obs} is related to the angular

velocity of the rotor, w (in randians sec^{-1}) and x the distance of the boundary (in cm) from the axis of rotation at time t by

$$s_{obs} = \frac{2.303}{60w^2} \quad \frac{d\log x}{dt} \qquad (3.2)$$

S_{obs} is determined from the slope of a plot of log x versus t (dlogx/dt) using the equation

$$s_{obs} = \frac{\frac{2.303}{60} \text{ slope}}{(2 \frac{rpm}{\pi 60})^2} \qquad (3.3)$$

The s_{obs} value obtained under various conditions of temperature and buffer composition is converted to standard conditions ($s_{20,w}$) by using the equation

$$s_{20,w} = s_{obs} \quad \frac{\eta_t}{\eta 20} \quad \frac{\eta}{\eta o} \quad \frac{(1 - \bar{\nu}\rho_{20,w})}{(1 - \bar{\nu}\rho t,s)} \qquad (3.4)$$

where η_t/η_o is the viscosity of water at t^o relative to that at 20^o, η/η_o is the viscosity of the solvent at t^o relative to that of water and $\rho_{20,w}$ and $\rho_{t,s}$ are the densities of water at 20^o and the solvent at t^o, respectively.

Furthermore, because of the hydrodynamic interactions between the protein molecules and the solvent displaced due to the centrifugal motion, observed sedimentation coefficients are influenced by the concentration of the protein. The absolute sedimentation coefficient is that found by extrapolating a plot of $s_{20,w}$ against protein concentration to zero concentration (Fig. 3.4). Such an extrapolation gives a value

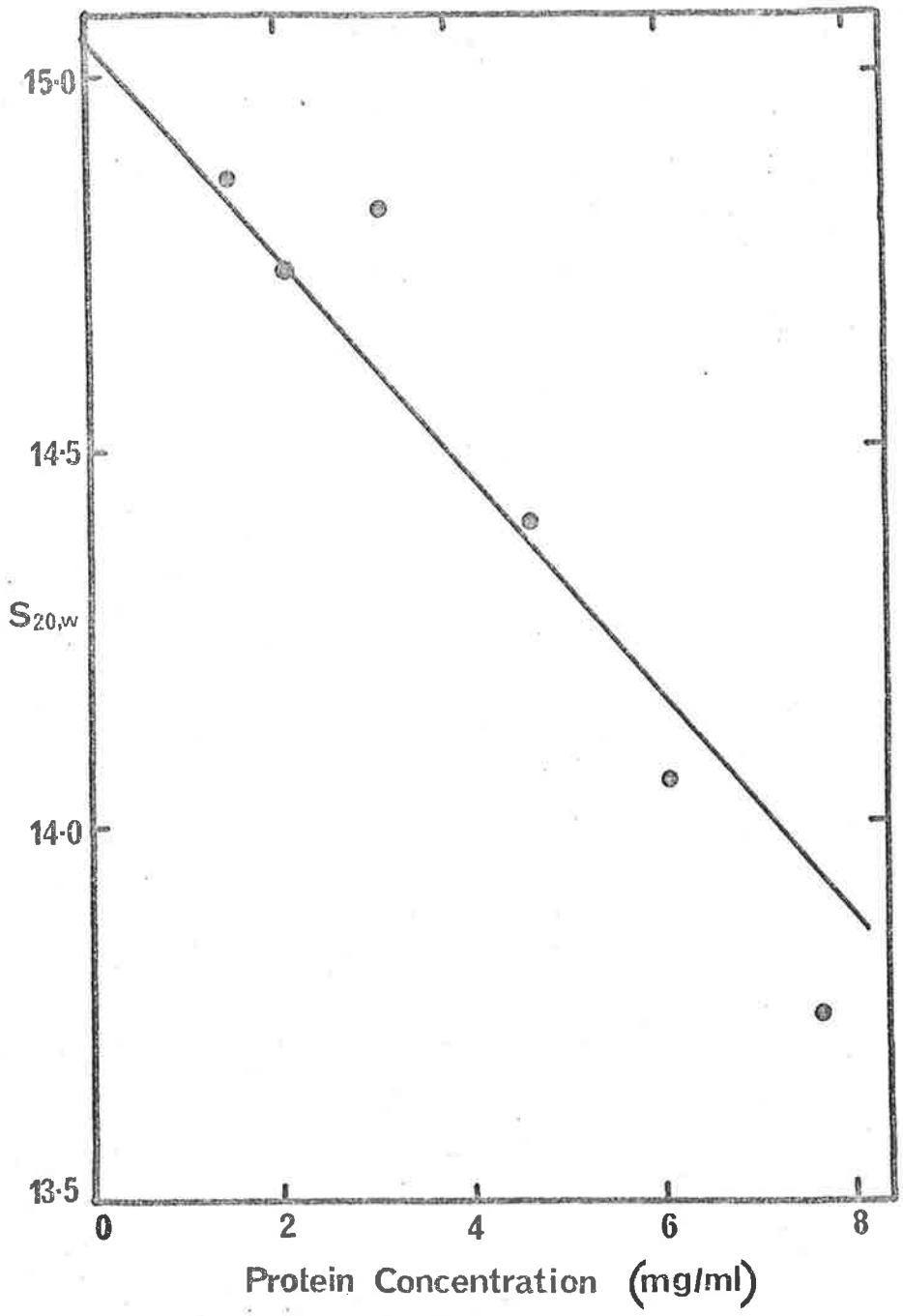


FIGURE 3.4. Determination of $s^{o}_{20,w}$ by extrapolating the $s_{20,w}$ values obtained to zero protein concentration. The intercept was determined by computer analysis of the data.

of $s^{o}_{20,w} = 15.06 \pm 0.23$ for sheep kidney pyruvate carboxylase.

Diffusion patterns for sheep kidney pyruvate carboxylase are shown in Fig. 3.3B. It can be seen that as the protein diffuses into the buffer, the height of the Schlieren peak decreases. The diffusion coefficient D, can be evaluated using the equation (Lamm, 1929);

$$(A/H)^2 = 4\pi Dt\ (1 + s^2 wt) \qquad (3.5)$$

where A is the area enclosed by the sedimenting boundary curve above its base line, H is the maximum height of this peak, t the time and w the angular velocity. However, this equation is only valid if certain boundary conditions are satisfied (Kawahara, 1969). These conditions can be satisfied within experimentation if a synthetic boundary cell rotated at low speed is employed.

Because of the dilution effect of the sector-shaped cell, the area A decreases with time. This decrease is approximately

$$(\Delta A/A) = 2sw^2t \qquad (3.6)$$

However, when the rotor speed is low, sw^2t becomes small and A is comparable with the error involved in measuring A (Kawahara, 1969). This means that A may be assumed to remain constant and its value was determined by taking the average of randomly selected diffusion patterns within one experiment. Thus the desired D can be found from a plot of $(A/H)^2$ as a function of time t when the experiment is carried out at a low angular velocity (Fig. 3.5).

The slope of such a plot is $4\pi D_{obs}$ which can be

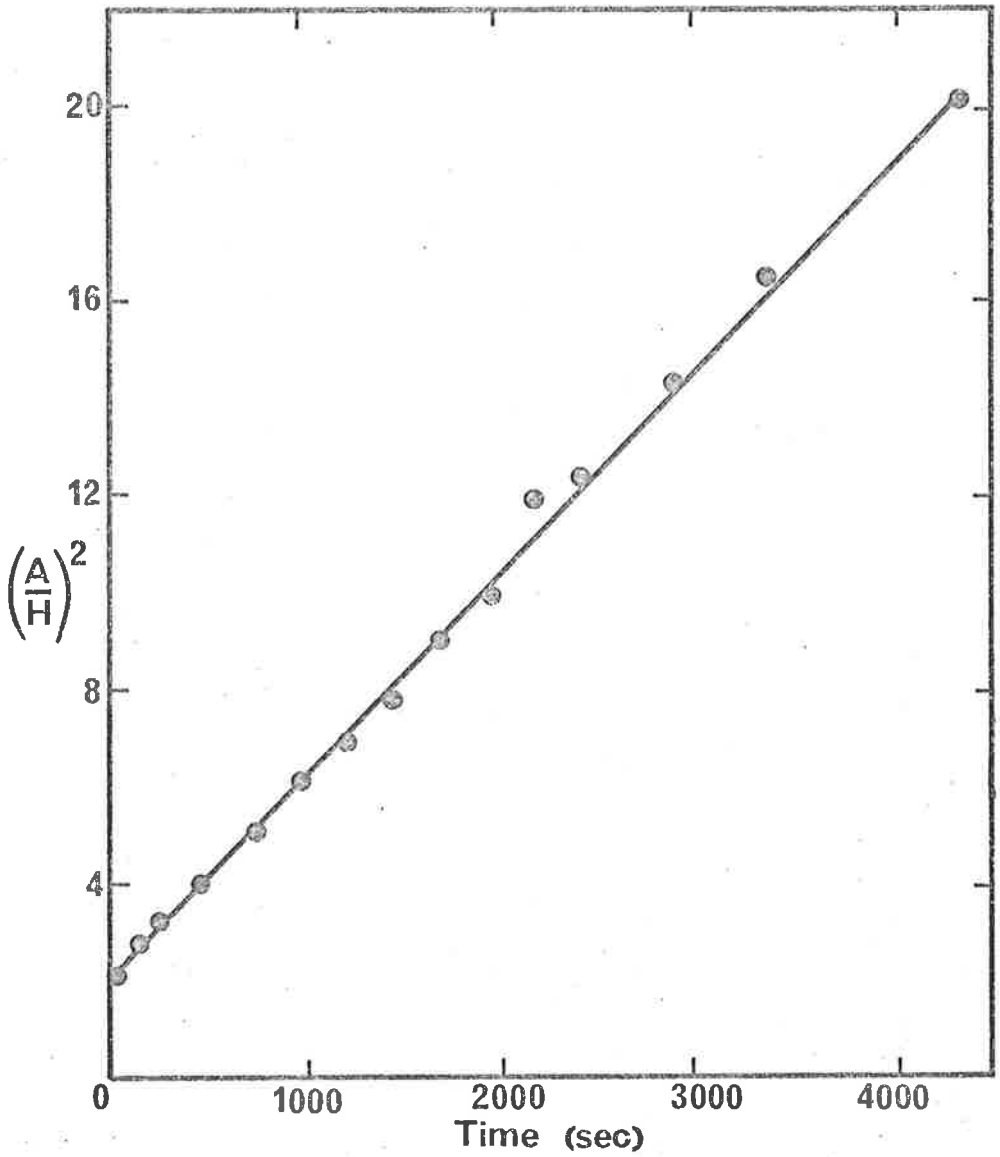


FIGURE 3.5. Determination of the diffusion coefficient of sheep kidney pyruvate carboxylase. The enzyme (2.4 mg per ml, specific activity 12.9 units per mg) was centrifuged at 2531 rpm and patterns photographed at various intervals. The area (A) and height (H) were taken from enlargements of the original photographs. The slope ($4\pi D$) was determined by a computer analysis of the data.

corrected to give $D_{20,w}$ in a manner similar to that used to correct s_{obs}. As with $s_{20,w}$, $D_{20,w}$ is dependent on protein concentration and $D^{o}_{20,w}$ was determined by extrapolating a plot of $D_{20,w}$ versus protein concentration, to zero protein concentration. Such an extrapolation gave $D^{o}_{20,w} = 3.03 \pm 0.17 \times 10^{-7}$ cm^2 sec^{-1}.

The partial specific volume ($\bar{v}$) of sheep kidney pyruvate carboxylase was determined from its amino acid composition (Table 3.2) as described by Schachman (1957). This method gave $\bar{v} = 0.733$ and when this is substituted into equation 3.1 with the $S^{o}_{20,w}$ and $D^{o}_{20,w}$ values, the molecular weight of sheep kidney pyruvate carboxylase was calculated to be 450,000 $\pm$ 30,000.

The molecular weight of sheep kidney pyruvate carboxylase has also been obtained using the approach to equilibrium method described by Schachman (1957). The protein was rotated at 2531 rpm at a concentration of 5.3 mg per ml and the equilibrium patterns analysed from photographic enlargements of the data. The meniscus and the cell bottom (oil-protein interface) were treated separately but there was no significant difference between the two calculated molecular weights indicating the homogeneity of the sample. This method gave a molecular weight of the enzyme of 480,000 $\pm$ 40,000.

In addition to using ultracentrifugation, the molecular weight of sheep kidney pyruvate carboxylase has also been determined by chromatography on Sepharose 4B. Pyruvate carboxylase eluted between *E. coli* β-galactosidase (molecular weight 520,000) and Jack bean urease (molecular

weight 483,000) indicating a molecular weight of about 500,000.

2. Amino Acid Composition

The amino acid composition of sheep kidney pyruvate carboxylase is shown in Table 3.2. For automated amino acid analysis, the cysteine content was determined from S-carboxymethylcysteine. However, to check this value, the cysteine content was also determined using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) using the method described by Means and Feeney (1971). The results are shown in Fig. 3.6. As can be seen there is an initial reaction with 8 cysteine residues without any denaturing reagent present. The presence of 8 M urea slowly exposes all the sulphydryl groups. However, in the presence of either 6 M guanidine hydrochloride or 0.1% SDS the full number of cysteine residues are accessible to the DTNB within 1 min. There appeared to be no disulphide linkages in native sheep kidney pyruvate carboxylases. The relevance of the amino acid composition of this enzyme and pyruvate carboxylase isolated from other sources is discussed in detail in Chapter 4.

3. Biotin Content

It has been shown that all ATP-requiring carboxylating enzymes contain biotin and it has been proposed that the biotin is attached to a lysine residue in such a way that it can move between the CO_2-binding site and the acceptor molecule binding site (Moss and Lane, 1971; Barden *et al.*, 1972). Chicken liver, rat liver and yeast pyruvate carboxylases contain 4 moles of biotin per mole of enzyme (Scrutton and Young, 1972). The presence of biotin in sheep pyruvate carboxylase has been shown by three methods.

TABLE 3.2. AMINO ACID COMPOSITION OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

Amino acid	Residues per mole[a]
Aspartic acid	332 ± 6
Threonine[b]	208 ± 11
Serine[b]	248 ± 9
Glutamic acid	468 ± 18
Proline	222 ± 9
Glycine	390 ± 21
Alanine	347 ± 7
Valine	290 ± 23
Cysteine[c]	44 ± 4
Methionine	80 ± 10
Isoleucine	208 ± 10
Leucine	352 ± 9
Tyrosine	106 ± 7
Phenylalanine	158 ± 8
Lysine	194 ± 8
Histidine	113 ± 8
Arginine	198 ± 3
Tryptophan[d]	62

[a]Molecular weight 480,000 [b]Corrected for destruction during hydrolysis [c]Determined as carboxymethylcysteine [d]Determined spectrophotometrically by the method of Goodwin and Morton (1946).

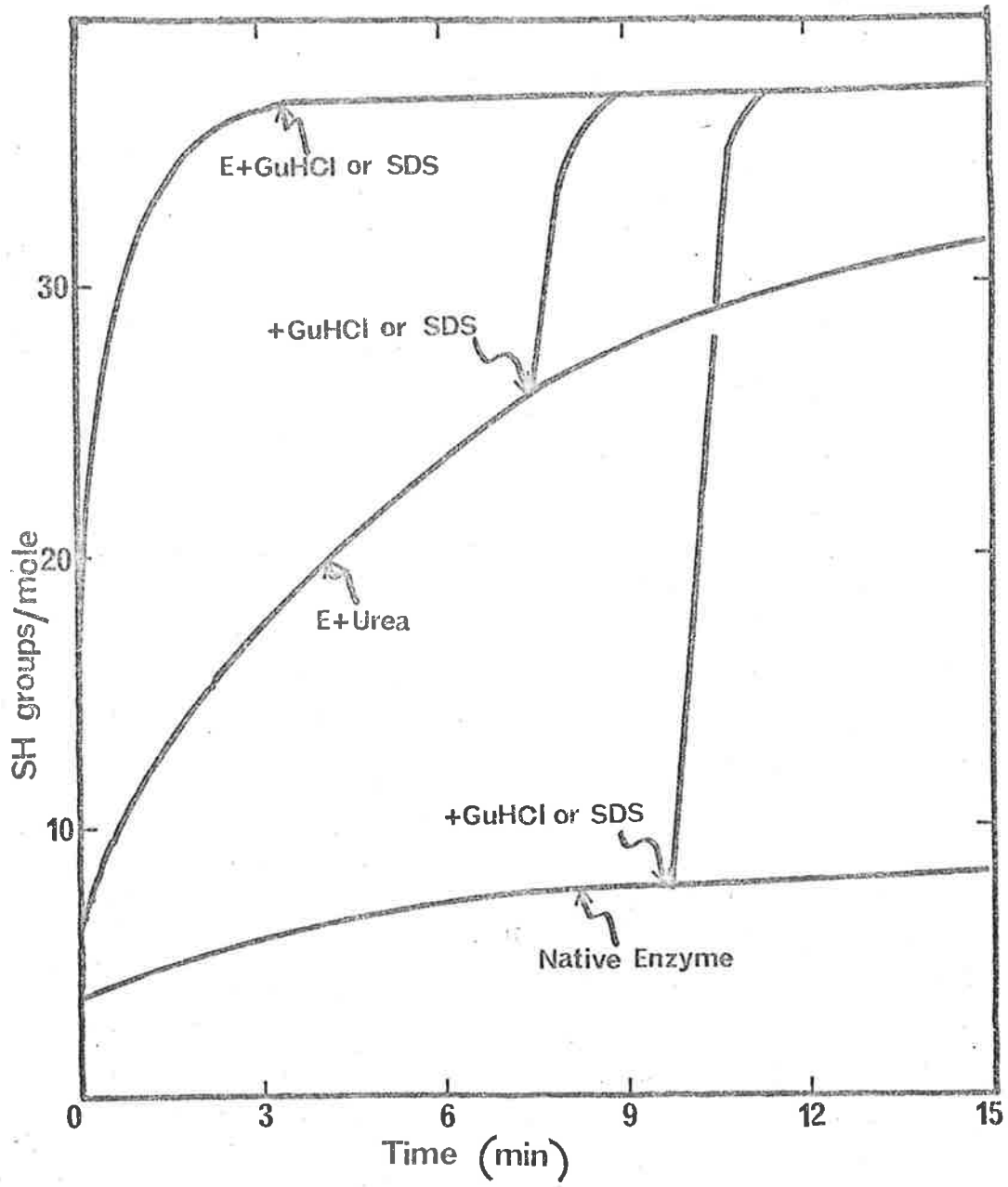


FIGURE 3.6. The kinetics of the reaction of 5,5'-dithiobis (2-nitrobenzoid acid) (DTNB) with the sulphydryl groups of sheep kidney pyruvate carboxylase. To 1 ml of the enzyme (specific activity, 13.1 units per ml and 1.2 mg per ml) in 0.1 M sodium phosphate buffer, pH 8.0 was added 0.01 ml of DTNB (39.6 mg in 10 ml of 0.1 M phosphate buffer, pH 7.0) and the optical density at 412 m μ was determined against a blank lacking protein. In addition, the reaction was carried out when the enzyme was denatured with either 6 M guanidine hydrochloride (GuHCl) or 0.1% SDS as shown.

(a) Radioactive sheep liver pyruvate carboxylase was prepared by infusion of [^{3}H]-biotin into sheep (see Section 2.9 for details). It was found that the radioactivity was associated with purified pyruvate carboxylase indicating incorporation of the [^{3}H]biotin into the enzyme.

(b) Both sheep kidney and sheep liver pyruvate carboxylases was labelled using [^{14}C]$NaHCO_3$ (Section 2.11) and using gel electrophoresis and column chromatography it was shown that the radioactivity is associated with pyruvate carboxylase (Chapter 4). In addition, the biotin moiety has been shown to be attached to a lysine residue (D.B. Rylatt, personal communication).

(c) The presence of biotin in the enzyme was demonstrated directly by using a spectroscopic assay for biotin (Green, 1965). Sheep kidney enzyme was prepared by DEAE-Sephadex chromatography and the samples prepared as described in Section B-1 of this chapter. The results in Fig. 3.7 show that there was a correspondence between the biotin content and pyruvate carboxylase activity.

The best samples of sheep kidney enzyme assayed for biotin (specific activity of the enzyme samples was greater than 12 units per mg) showed that the enzyme contained 4.21 $\pm$ 0.28 moles of biotin per mole of enzyme. Similarly, sheep liver enzyme was shown to contain 4 moles of biotin per mole of enzyme.

4. Divalent Cation Content

Many enzymes contain firmly bound metal ions which have been shown to be either involved in either maintaining structural conformation of the proteins or implicated in the reaction mechanism (for review, see Vallee and Wacker, 1970). Studies by Scrutton and Utter (1965), Scrutton et al. (1966), and Mildvan et al. (1966) reported that chicken liver pyruvate carboxylase normally contains 4 g atoms of Mn^{2+} per mole of enzyme, i.e., in a 1:1 stoichiometry with biotin. It has also been shown that the enzyme isolated from Mn^{2+} deficient chickens can substitute Mg^{2+} for Mn^{2+} and still retail full enzymic activity (Scrutton et al., 1972). For other types of pyruvate carboxylases, the calf liver enzyme has the total Mn^{2+} and Mg^{2+} in 1:1 stoichiometry with biotin, the turkey liver enzyme has Mn^{2+} in 1:1 stoichiometry and the enzyme from Saccharomyces cerevisiae has Zn^{2+} in 1:1 stoichiometry (Scrutton and Young, 1972). However, rat liver pyruvate carboxylase contains only Mn^{2+} and at a stoichiometry of 1 g atom per two moles of biotin (McClure et al., 1971a)

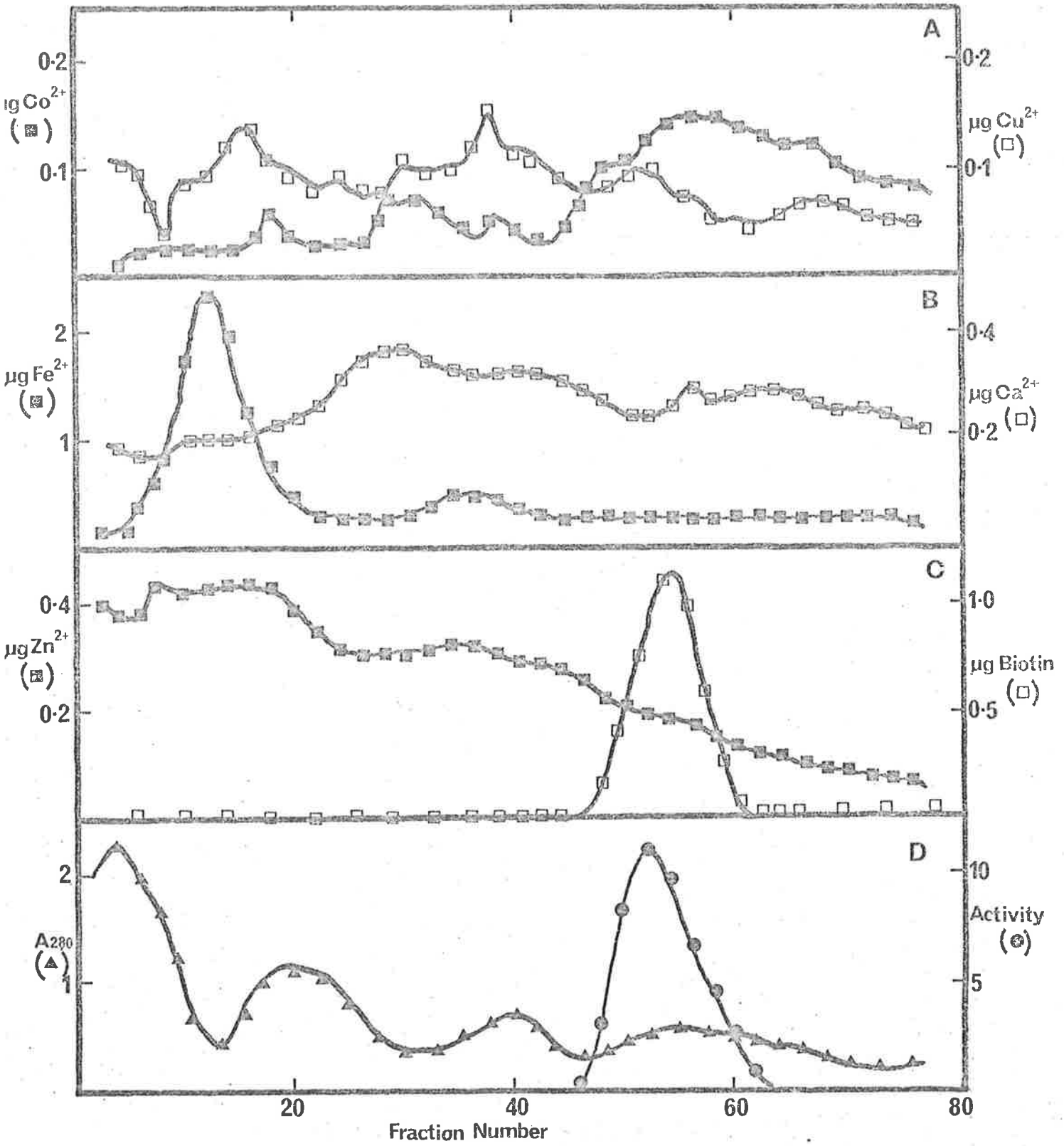
In this work sheep kidney, sheep liver and chicken liver pyruvate carboxylases have been examined for divalent metal ions by atomic absorption spectrophotometry. The previous results of Scrutton et al. (1966) were confirmed in that in the elution profile of chicken liver pyruvate carboxylase from Sephadex G-200, the Mn^{2+} cochromatographed with enzymic activity. However, the best preparations of chicken liver enzyme contained 1.6 - 1.8 moles of Mn^{2+}

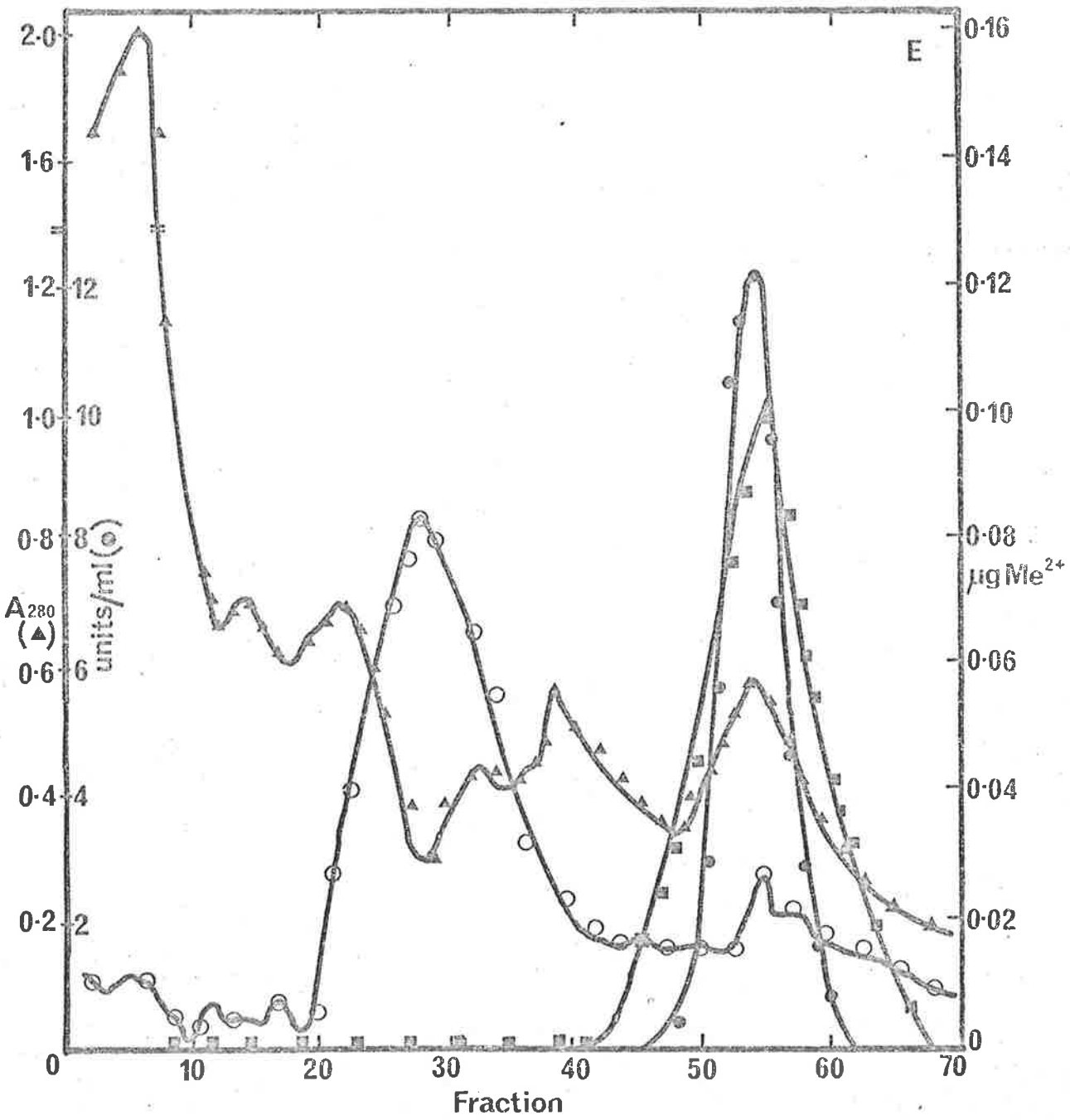
per mole of enzyme. There was no correspondence between any Mg^{2+} present and enzymic activity.

Fig. 3.7 shows the results of examining a preparation of sheep kidney pyruvate carboxylase from a DEAE-Sephadex column for various divalent metal ions. Of the cations present in the profile, only Mn^{2+} co-chromatographs with the enzymic activity. The profile also shows the existence of an iron peak and a magnesium peak but neither of them corresponded to the enzymic activity. In addition, Fig. 3.8 shows the correspondence between Mn^{2+} and enzymic activity when the enzyme is eluted from a G-200 column. The best preparations of sheep kidney pyruvate carboxylase (specific activity greater than 13 units per mg) contained 1.7 - 1.9 g atoms of Mn^{2+} per mole of enzyme. This result is similar to that found for rat liver enzyme (McClure *et al.*, 1971a) but is different from the reported value of 4 g atoms of Mn^{2+} per mole of chicken liver pyruvate carboxylase (Scrutton and Utter, 1965; Scrutton *et al.*, 1966; Mildvan *et al.*, 1966). Calf liver pyruvate carboxylase was also examined for the presence of Mn^{2+} and Mg^{2+} after the enzyme was eluted from a DEAE-Sephadex column. The results showed that there was absolute cochromatography between enzymic activity and Mn^{2+} but in addition to some correspondence between activity and Mg^{2+}, there was a large Mg^{2+} peak preceding the activity.

The results for sheep liver pyruvate are identical to those for the kidney enzyme in that the liver enzyme only has a correspondence between enzyme activity eluted from

FIGURE 3.7. The distribution of various divalent metal ions, biotin, enzymic activity and protein when sheep kidney pyruvate carboxylase was subjected to chromatography on DEAE-Sephadex. Fractions (13 ml) were collected and assayed as described in the text. In part E, 0 represents the Mg^{2+} concentration and ■ the Mn^{2+} concentration.


A
0·2
µg Co2+
(■)
0·1
0·2
µg Cu2+
(□)
0·1
B
2
µg Fe2+
(■)
1
0·4
µg Ca2+
0·2 (□)
C
0·4
µg Zn2+
(■)
0·2
1·0
µg Biotin
0·5 (□)
D
2
A280
(▲)
1
10
Activity
(●)
5
20
40
60
80
Fraction Number


E
2·0
1·6
1·2
1·0
0·8
0·6
0·4
0·2
0
12
10
8
6
4
2
0·16
0·14
0·12
0·10
0·08
0·06
0·04
0·02
0
A280 (▲)
units/ml (o)
µg Me2+
10
20
30
40
50
60
70
Fraction

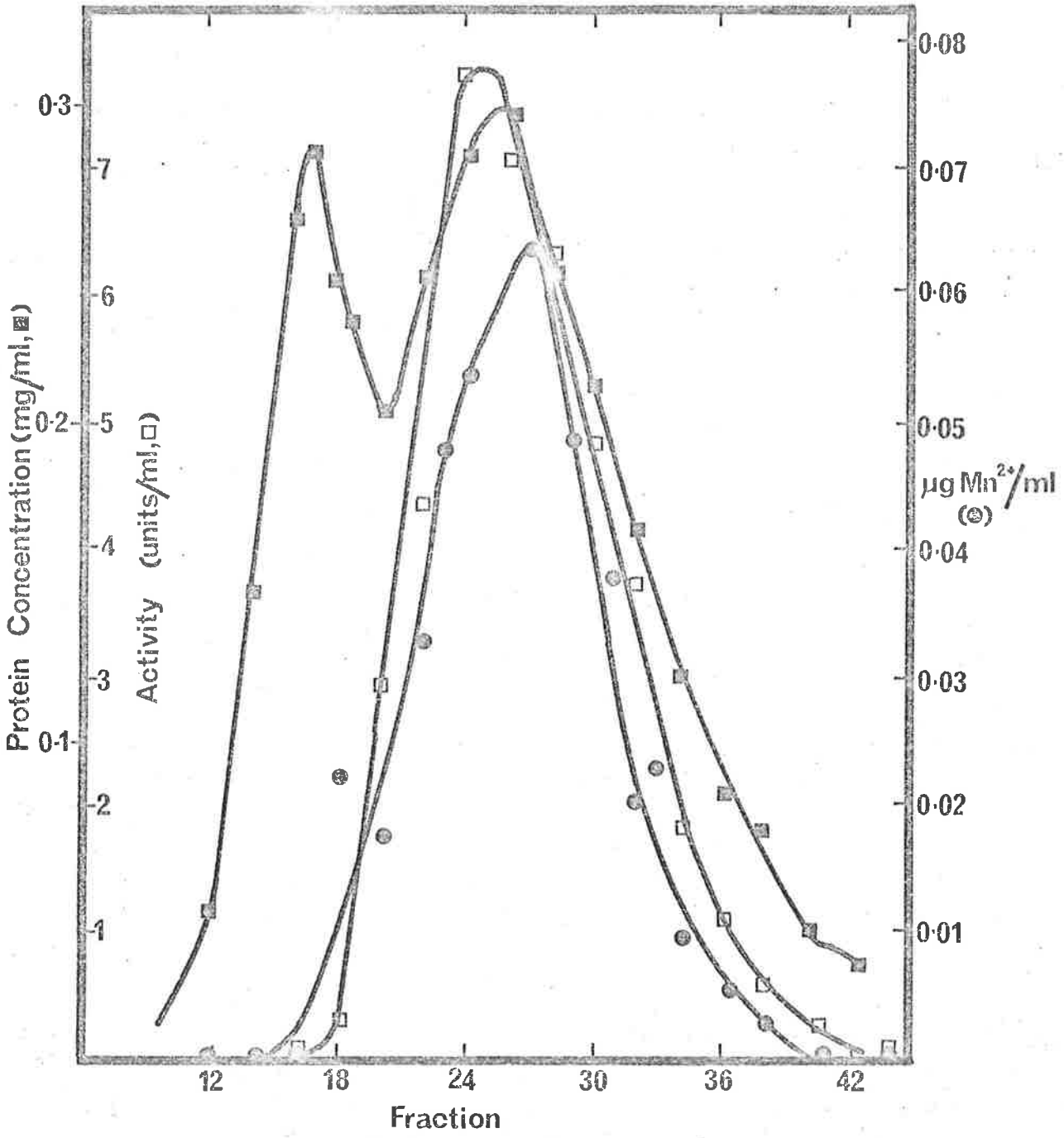


FIGURE 3.8. Distribution of manganese (●), enzymic activity (□) and protein (■) when sheep kidney pyruvate carboxylase was subjected to gel filtration on Sephadex G-200. The enzyme (specific activity, 6.7 units per mg) was applied to a column (51 cm x 2.7 cm) equilibrated with buffer A. Fractions (2.0 ml) were collected and assayed as described.

DEAE-Sephadex or G-200 and Mn^{2+} and the enzyme cont ained 1.8 - 1.95 g atoms of Mn^{2+} per mole of enzyme.

5. Enzyme Stability

(a) Temperature stability

Ling and Keech (1966) reported that sheep kidney pyruvate carboxylase was similar to the chicken liver enzyme, and loses activity at low temperatures. This phenomena has been investigated using chicken liver enzyme and coincident with the loss of enzymic activity, the enzyme dissociated into inactive 7S monomers (Irias et al., 1966).

The initial cold lability experiments reported for the sheep kidney enzyme were performed with enzyme of low specific activity (0.1 units per mg). The temperature st ability has been re-examined using highly purified enzyme (specific activity greater than 12). Fig. 3.9 shows the $t_{1/2}$ calculated from the inactivation curves of the enzyme plotted as a function of temperature. The $t_{1/2}$ for the chicken liver enzyme determined under identical conditions except that the pH of the assay mixture was 7.8, has been included for comparison. The data shows that the purified form of the sheep kidney enzyme is not cold labile. Furthermore, ultracentrifugation of the enzyme at 8^{o} showed no loss of the 15S component. It is apparent from Fig. 3.9 that the sheep kidney enzyme is also more stable when incubated at elevated temperatures ($>32^{o}$). For both sheep kidney and chicken liver pyruvate carboxylases the loss of enzymic activity that occurs at temperatures greater than 40^{o} is not reversed by cooling to 20^{o}

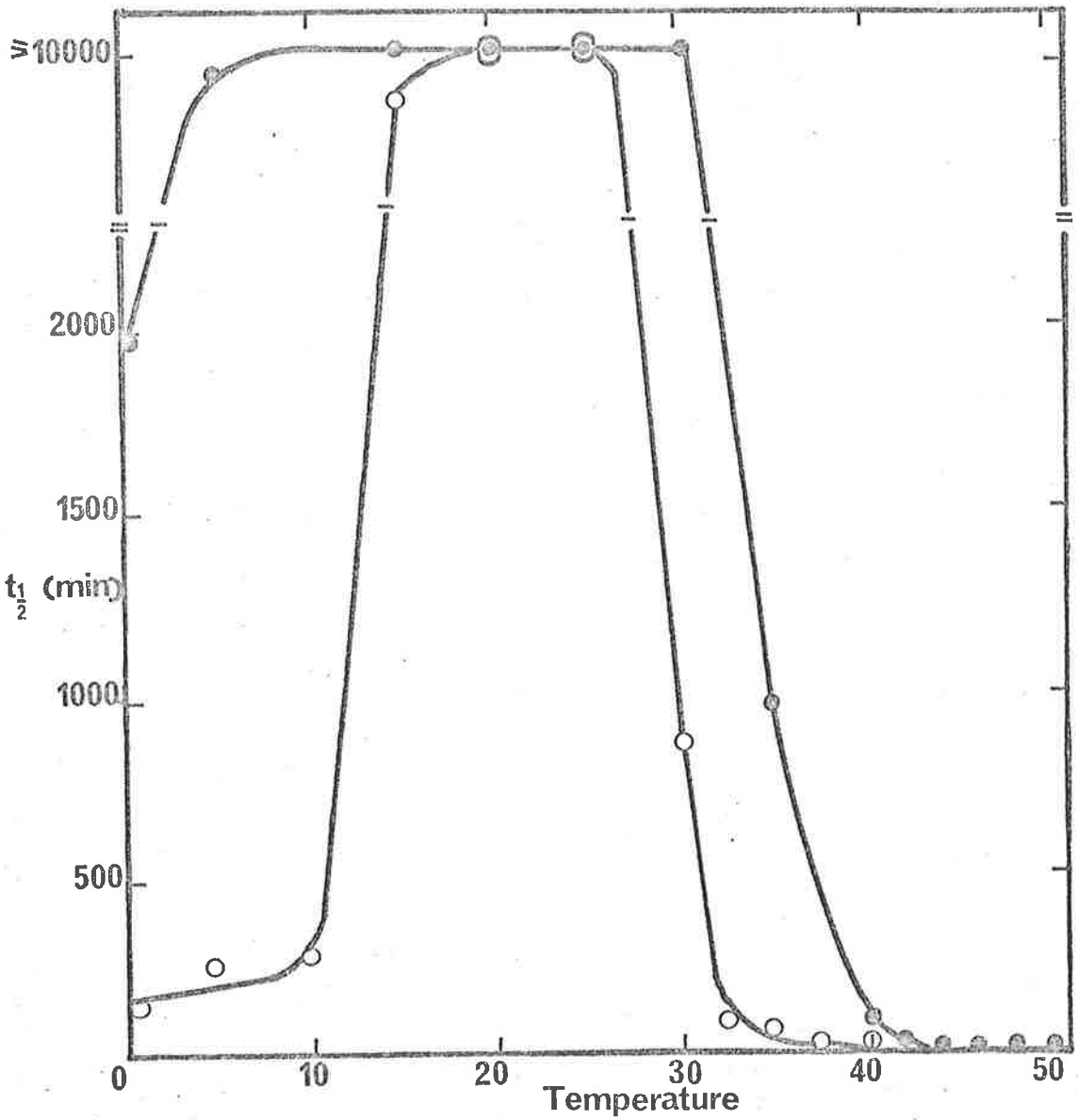


FIGURE 3.9. Temperature stability of sheep kidney (●) and chicken liver (○) pyruvate carboxylases. The enzyme (specific activity of the sheep kidney enzyme 12.4 units per mg and the chicken liver enzyme 14.0 units per mg and both at 0.5 mg per ml) was incubated at the appropriate temperature in 0.01 M potassium phosphate buffer, pH 7.2 containing 0.15 M KCl, 10^{-3} M EDTA and 10^{-4} M DTE. Time samples were removed and assayed at 30° and $t_{\frac{1}{2}}$ calculated by computer analysis of the data.

and 50 μM acetyl-CoA will not protect the enzymes against this inactivation. Irias et al. (1969) have also shown that the cold inactivation of the chicken liver enzyme is dependent on protein concentration. For sheep kidney pyruvate carboxylase there is no cold inactivation of the enzyme down to a protein concentration of 0.05 mg per ml. In addition to the chicken liver enzyme several other enzymes exhibit cold lability (Scrutton and Young, 1972) but there is no physiological explanation for this relatively unusual phenomenon.

(b) Dilution inactivation

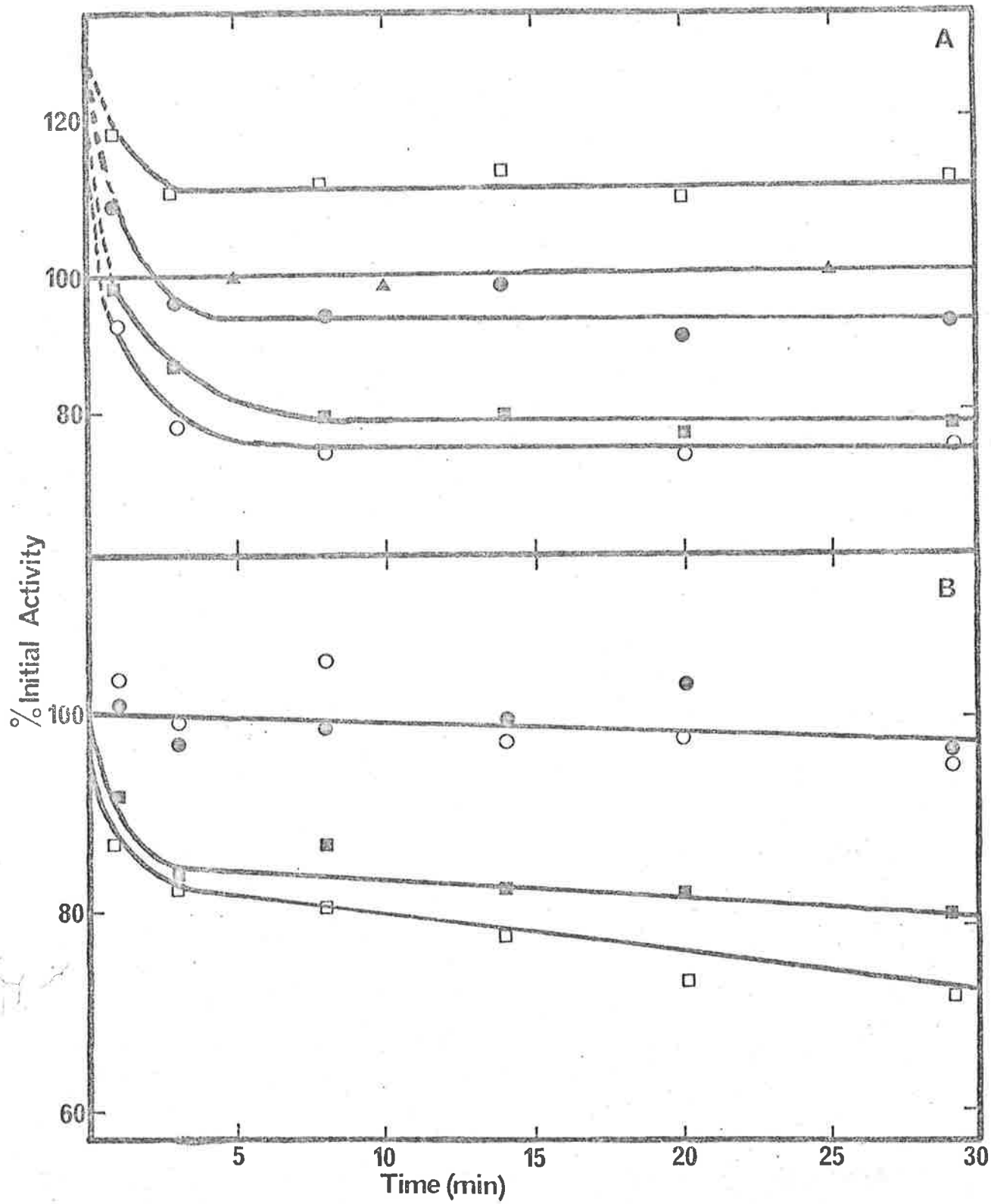
(i) Effect of dithioerythritol

Ashman et al. (1973) have demonstrated that at pH 8.4 sheep kidney pyruvate carboxylase undergoes a dilution inactivation below 4 enzyme units per ml. They postulated that this inactivation is because below 4 units per ml a substantial proportion of the enzyme exists in a less active or an inactive dissociated form.

Protection against the dilution inactivation of the enzyme by DTE has been investigated by varying the DTE concentration at low protein concentrations (Fig. 3.10A). The results show that increasing the concentration of DTE caused a decrease in the inactivation of the enzyme with the final level of activity after the dilution inactivation being proportional to the concentration of the DTE. In addition, the initial activity of the enzyme in the presence of DTE was about 20% greater than in the absence of this compound.

FIGURE 3.10A. Protection against the dilution inactivation of sheep kidney pyruvate carboxylase at pH 8.4 by dithioerythritol (DTE). Samples of the enzyme (specific activity, 8.2 units per mg) were diluted to 0.4 units per ml in 0.05 M Tris-Cl (pH 8.4) containing 0.05 M KCl and DTE at the concentrations 10^{-3} M (□), 10^{-4} M (●), 10^{-5} M (■) and no DTE (○). The control (▲) was incubated at 4 units per ml.

B. Protection against dilution inactivation of the enzyme by acetyl-CoA. The enzyme was diluted to 0.4 units per ml as described in part A except the buffer contained 10^{-5} M (○), 10^{-6} M (●), 10^{-7} M (■) and no acetyl-CoA (□). DTE was not present during the incubation.


A
B
120
100
80
100
80
60
% Initial Activity
5
10
15
20
25
30
Time (min)

(ii) Effect of Acetyl-CoA

The effect of varying the acetyl-CoA concentration on the dilution inactivation is shown in Fig. 3.10B. The results were similar to that observed with DTE in that increasing the concentration of acetyl-CoA prevented the inactivation such that at 10^{-5} M acetyl-CoA there was complete protection.

6. Energy of Activation

The carboxylation reaction rate for sheep kidney and chicken liver pyruvate carboxylases was measured over a temperature range from 16.5° to 40°. From these experiments (Fig. 3.11), the energy of activation was calculated to be 10.66 ± 0.60 kcal per mole for the sheep kidney enzyme and 8.96 ± 1.06 kcal per mole for the chicken liver enzymic reaction. The value for chicken liver pyruvate carboxylase agrees with the published value (8.1 kcal per mole) for this enzyme (Keech and Utter, 1963) and that for rat liver pyruvate carboxylase (8.5 kcal per mole) (McClure et al., 1971a).

H. DISCUSSION

It has been found that sheep kidney pyruvate carboxylase could be prepared to homogeneity by a method which with little modification could also be used to purify the enzyme from other sources. The sheep kidney enzyme has been shown to be homogeneous by ultracentrifugation and polyacrylamide gel electrophoresis. Additional evidence supporting this thesis is presented in Chapter 4.

The physical properties of the enzyme show a high degree of similarity to those of the enzyme purified from either chicken

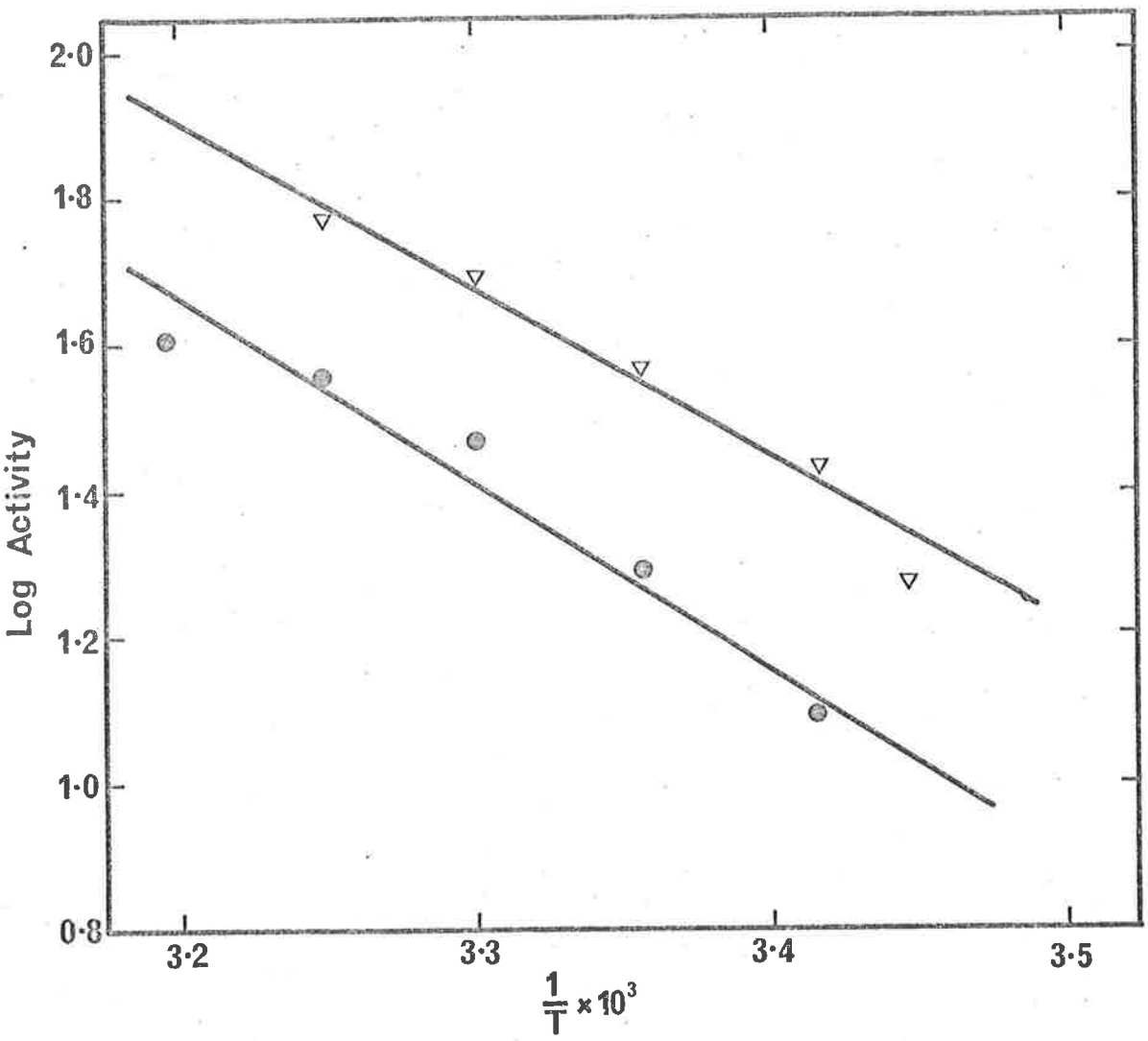


FIGURE 3.11. The effect of temperature on sheep kidney (▽) and chicken liver (●) pyruvate carboxylase assays. The assays were conducted over a temperature range from 20° to 40°. The slope of the lines were determined by a computer analysis of the data.

liver or rat liver. However, some differences do exist. By atomic absorption spectrophotometry sheep kidney and liver and chicken liver pyruvate carboxylases were shown to contain only two molecules of Mn^{2+} per four molecules of biotin. This result is similar to that for rat liver enzyme (McClure et al., 1971a) but different to the 4 g atoms of Mn^{2+} per four molecules of biotin reported for chicken liver enzyme (Scrutton et al., 1966). The stoichiometry of the metal ion is also different from that for the calf liver enzyme for which it has been reported that the total Mg^{2+} and Mn^{2+} are in a 1:1 stoichiometry with the biotin (Scrutton et al., 1972). The reason why these results for the chicken liver enzyme differ from the published work is unknown except that the original Mn^{2+} and biotin determinations were made where protein determinations were made using different methods and also using a molecular weight of 660,000 instead of the now established 500,000 (Scrutton et al., 1966). However, doubt still exists as to the role of the metal ion in pyruvate carboxylase (Scrutton et al., 1973) and thus the significance of the different metal ions which are found in the enzyme isolated from different sources is unknown.

Contrary to an earlier report (Ling and Keech, 1966) purified sheep kidney enzyme has not been found to be cold labile. The original experiments on the cold lability of the sheep kidney enzyme were carried out with enzyme purified only by an $(NH_4)_2SO_4$ precipitation. These results, showing the crude enzyme to be cold labile but the more highly purified enzyme not to be, agree with those for rat liver pyruvate carboxylase in that using only crude $(NH_4)_2SO_4$ fractionated enzyme, Wimhurst and Manchester

(1970) found the enzyme to be cold labile but using a more highly purified preparation McClure et al. (1971a) have reported that the rat liver enzyme is in fact not cold labile. Hence for the mammalian species cold lability may depend on the degree of purity of the enzyme.

However, despite these differences, most of the properties of the enzyme from sheep, rat and chicken appear to be similar. These three enzymes have a molecular weight of approximately 500,000, are made up of four subunits (see Chapter 4) and contain four moles of biotin. This is in the contrast to the bacterial enzymes which have a smaller molecular weight (Cazzulo et al., 1970; Taylor et al., 1972) and may in fact be composed of only two subunits (Taylor et al., 1972). Also supporting the similarity of the avian and mammalian enzymes is the fact that the energy of activation for the three enzymes is very similar. This indicates that the transition states for the three enzymes are similar. Further evidence has also been obtained for this postulate in that the reaction mechanism for all three enzymes are similar (McClure et al., 1971b,c; Barden et al., 1972; Ashman, 1973).

CHAPTER FOUR

THE SUBUNIT STRUCTURE OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

A. INTRODUCTION

In recent years there has been some uncertainty about the molecular weight of the polypeptide chains in pyruvate carboxylase from animal sources, although it is well established that the native enzymes have a sedimentation coefficient of about 15S, and are tetramers with four protomers of molecular weight 120 - 150,000 (Scrutton and Young, 1972). For example, McClure et al. (1971a) were able to detect only a single band corresponding to a molecular weight of 130,000 in SDS-polyacrylamide gel electrophoresis of the rat liver enzyme, but after extensive denaturation in guanidine hydrochloride and carboxymethylation, the same technique showed six or seven bands which were interpreted in terms of three or four smaller polypeptide chains. Scrutton and Utter (1965) measured the sedimentation coefficient of the chicken liver enzyme in the presence of SDS, and observed a species with an unexpectedly low sedimentation coefficient of 2.7S. Later, Valentine et al. (1966) suggested that this value corresponds to a polypeptide of molecular weight 45,000 which has since found a place in a compilation of protein molecular weights (Klotz et al., 1970).

The work described in this chapter establishes the size, number and identity of the polypeptide chains in sheep kidney pyruvate carboxylase. This objective was approached using the highly purified enzyme by carrying out SDS-polyacrylamide gel electrophoresis in the manner described by Weber and Osborn (1969). This technique has generally been accepted as a convenient method for determining the molecular

weight of protein monomers by empirical comparison with known standards, although the theoretical basis and limitations of the method have been elucidated recently (Neville, 1971). Difficulties which have been encountered with cross-linked proteins can be overcome in the case of disulphide linkages by reduction and carboxymethylation (Griffith, 1972; McDonagh et al., 1972). Molecular weights were also estimated by the related empirical technique of gel filtration in the presence of SDS (Fish et al., 1970).

The work in this chapter also confirms the finding of Valentine et al. (1966) that pyruvate carboxylase has a low sedimentation coefficient in SDS, but shows the same phenomenon with all proteins studied. The model of Reynolds and Tanford (1970a) for the structure of protein-SDS complexes allows a reinterpretation of the low sedimentation coefficient in terms of the hydrodynamic properties of the pyruvate carboxylase-SDS complex. The results show it is not necessary to postulate the existence of polypeptide chains smaller than molecular weight 120,000, and the results show no evidence for the existence of such chains.

In addition, by labelling the sulphydryl groups of pyruvate carboxylase with iodo[^{14}C] acetic acid it has been possible to show that the subunits of the enzyme are identical.

The amino acid analysis of pyruvate carboxylases from various animal sources has been determined and by a computer analysis has been used to show that there were two classes of enzymes with cold lability being the distinguishing

physical property.

B. METHODS AND MATERIALS

S-carboxymethylation of pyruvate carboxylase was carried out as described in Chapter 2. Gel filtration was carried out using a Sephadex G-150 column (80 cm x 2.2 cm) equilibrated with 0.1 M sodium phosphate buffer, pH 7.0, containing 0.5% SDS and 0.1% 2-mercaptoethanol.

Sedimentation velocity experiments in SDS were conducted at 56,100 rpm, with 2.5 mg of protein per ml of solution prepared by incubation in the above SDS-buffer for at least 12 h at 30°.

Sedimentation equilibrium measurements of the molecular weight of pyruvate carboxylase in SDS-buffer or guanidine hydrochloride were done by the approach to equilibrium technique of Archibald (1947). For this purpose, it was necessary to estimate the partial specific volume of the protein-SDS complex, $\bar{\nu}_c$. This was determined according to the equation of Hersch and Schachman (1958):

$$\bar{\nu}_c = \frac{(\bar{\nu}_p + x\,\bar{\nu}_{SDS})}{1 + x} \qquad (4.1)$$

The partial specific volume of SDS, $\bar{\nu}_{SDS}$ is 0.885 ml per g (Hersch and Schachman, 1958), and $\bar{\nu}_p$ for pyruvate carboxylase has been calculated from the amino acid content (see Chapter 3); while x is the binding ratio of SDS, known to be 1.4 g per g of protein (Reynolds and Tanford, 1970b).

For the formation of tryptic peptides pyruvate carboxylase labelled with iodo[^{14}C]acetic acid was digested at 10 mg per ml in 0.2 M N-ethylmorphiline acetate buffer, pH 8.3 for 15 hr at 30° using trypsin treated with N-tosyl-phenylalanine-chloromethyl ketone at 1% (w/w) enzyme to substrate ratio. The radioactive peptides were fractionated on a Sephadex G-25 column (115 cm x 2.4 cm) previously equilibrated with pyridine/N-ethylmorphiline/acetic acid buffer, pH 9.0 (Schroeder et al., 1962). High voltage paper electrophoresis was carried out in pyridine/acetic acid buffer, pH 6.5 as described by Offord (1965) using an apparatus similar to that described by Michl (1951).

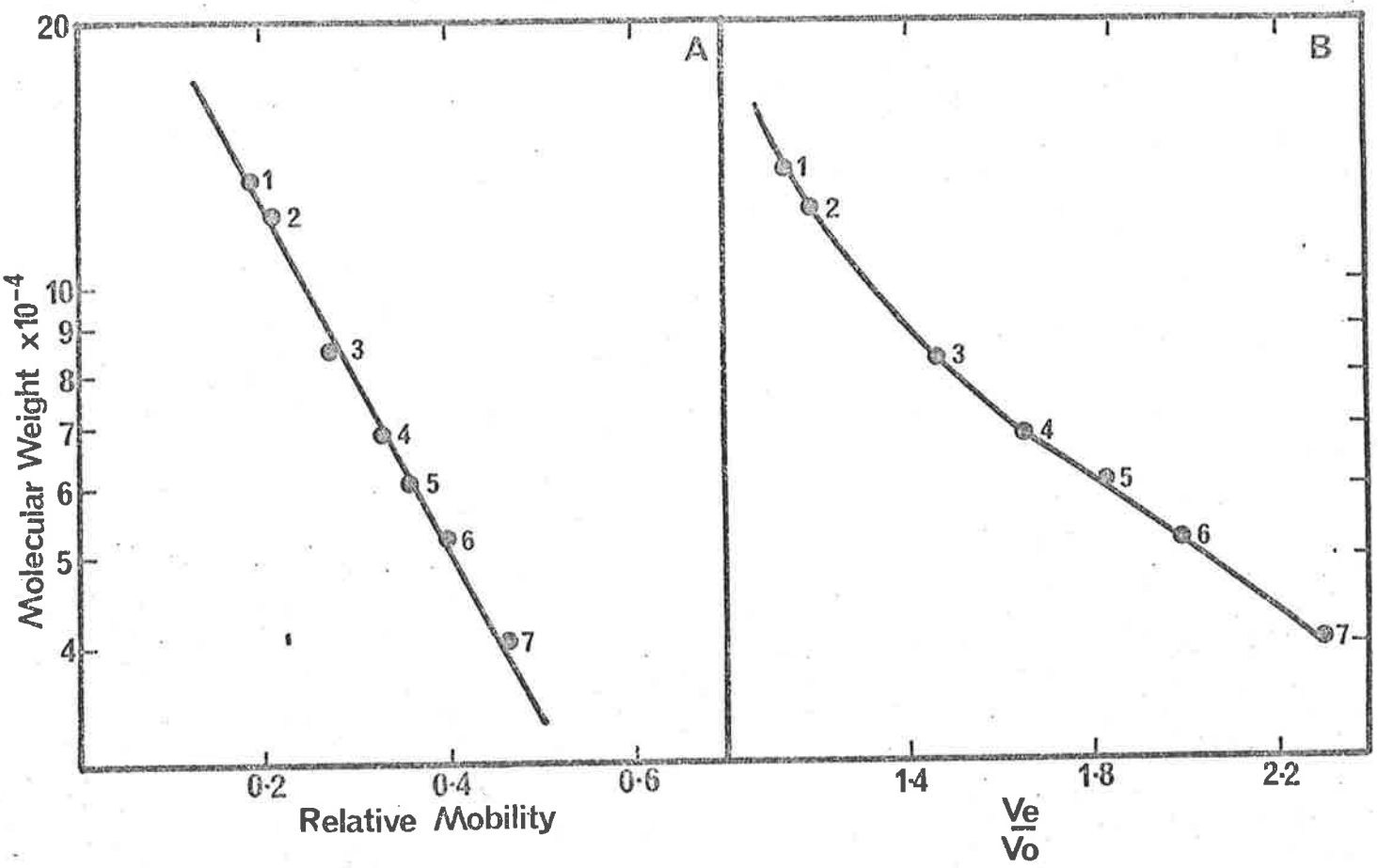
C. RESULTS

1. Subunit Molecular Weight

The SDS-polyacrylamide gel electrophoretic patterns obtained for highly purified sheep kidney and chicken liver pyruvate carboxylase (12 - 15 units of enzymic activity per mg of protein) are shown in Figure 4.1. Only one band was found for both proteins, and this pattern did not change when before electrophoresis the proteins had been reduced and S-carboxymethylated as described in Section 2. Figure 4.2A presents the results obtained when a series of proteins were electrophoresed on the same 0.1% SDS-polyacrylamide gels, and shows that over the range studied the relative mobility of the proteins through the gel was proportional to the logarithm of their molecular weights. Similarly, Figure 4.2B shows that the elution volume of the

FIGURE 4.1. Electrophoretic patterns on SDS polyacrylamide gels for A - chicken liver pyruvate carboxylase (specific activity = 14.05) and B - sheep kidney pyruvate carboxylase (specific activity = 13.3).

FIGURE 4.2. Semi-log plots of molecular weight against A - relative mobility of proteins on SDS polyacrylamide gels; B - relative elution volume of various proteins on Sephadex G-150 in SDS. The proteins are 1-β-galactosidase; 2 - pyruvate carboxylase (chicken liver and sheep kidney); 3 - urease; 4 - bovine serum albumin; 5 - catalase; 6 - glutamate dehydrogenase and 7 - ovalbumin.


A
B
20
10
9
8
7
6
5
4
Molecular Weight x10⁻⁴
1
2
3
4
5
6
7
0·2
0·4
0·6
Relative Mobility
1·4
1·8
2·2
Ve/Vo

same proteins from a Sephadex G-150 column in the presence of 0.5% SDS was a smooth function of molecular weight. Both these empirical interpolations indicated that the molecular weight of the monomers of both enzymes is $(120\pm10)\times10^3$. These results illustrate that the measured parameters, mobility and elution volume, are functions of polypeptide molecular weight (or chain length) for the group of proteins studied. Under the conditions of the present work, most proteins bind SDS with a high affinity and in approximately constant binding ratio which is 1.4 $\pm$ 0.2 g of SDS per g of protein. The critical value for saturation is that the equilibrium SDS-monomer concentration should be at least 8×10^{-4} M (0.023%) (Reynolds and Tanford, 1970b), which is well below the working concentration of 0.1% or 0.5%. Therefore, at these saturating levels of SDS, the complexes bear a constant charge and electrical force per unit mass, resulting in electrophoretic separation according to hydrodynamic properties (i.e., size) and independently of intrinsic charge.

Acetyl-CoA carboxylase from E. coli (Alberts et al., 1969) and transcarboxylase (Jacobsen et al., 1970) can both be dissociated under certain conditions into small polypeptides of specific functions. In an attempt to determine whether sheep kidney pyruvate carboxylase has small dissociable peptides, the enzyme was labelled with iodo[^{14}C]acetic acid and electrophoresed on SDS gels. After electrophoresis the gels were cut into 2 mm sections and the radioactivity determined. All the radioactivity was associated with the large molecular weight species (Fig. 4.3A)

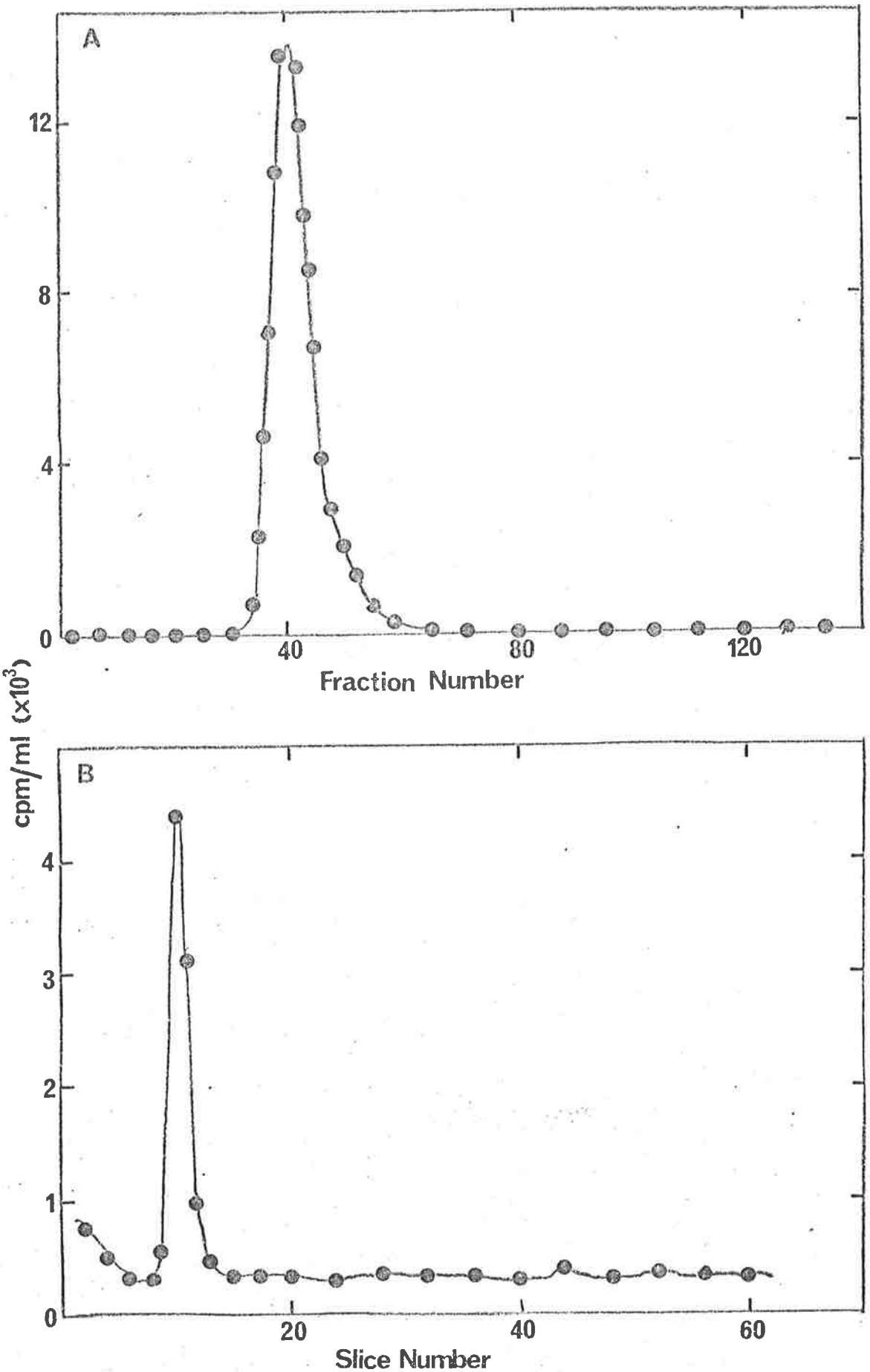
showing that the subunits did not dissociate under these conditions. In addition, when the enzyme was labelled with $[^{14}C]CO_2$ (as in Section 2.B.ii) and chromatographed on a Sephadex G-150 column in the presence of SDS (Fig. 4.3B) the radioactivity was also associated with the large molecular weight species.

McClure et al. (1971a) have found for rat liver pyruvate carboxylase that when the purified enzyme was denatured in 6.7 M guanidine hydrochloride and then carboxymethylated, they obtained six or possibly seven bands from SDS-polyacrylamide gel electrophoresis. However, when this experiment was carried out using the sheep kidney enzyme only one band of molecular weight 120,000 was observed (Fig. 4.4).

The molecular weight of the subunits of sheep kidney pyruvate carboxylase have also been determined by sedimentation equilibrium ultracentrifugation in the presence of either 0.5% SDS or 6 M guanidine hydrochloride. Analysis by this method showed that in the presence of SDS the molecular weight of the subunits was 110,000 $\pm$ 5,000 whereas in the presence of guanidine hydrochloride the molecular weight was 115,000 $\pm$ 8,000.

Since incubation with SDS or guanidine hydrochloride invariably dissociates known oligomeric proteins into monomers (Weber and Osborn, 1969; Nelson, 1971; Fish et al., 1970), the evidence strongly suggests that the above molecular weight of 120,000 truly represents the constituent polypeptide chains of the tetrameric enzymes sheep kidney and chicken liver pyruvate carboxylases, particularly as reduction and S-carboxymethylation

FIGURE 4.3. The radioactivity profiles of sheep kidney pyruvate carboxylase. A shows the profile for the $[^{14}C]CO_2$ labelled enzyme after elution from a Sephadex G-150 column (70 cm x 2.2 cm). From the 2.3 ml samples 0.2 ml were taken and the radioactivity determined in 2 ml of 30% triton X-100 scintillation fluid. B shows the radioactivity profile after iodo $[^{14}C]$ acetic acid labelled pyruvate carboxylase had been electrophoresed on SDS gels. After electrophoresis the gel was sliced into 2 mm sections and the radioactivity determined in 2.2 ml of scintillation fluid containing 0.3 ml of NCS solubiliser and 0.025 ml of 8 N NH_4OH.


A
12
8
4
0
40
80
120
Fraction Number
cpm/ml (x10³)
B
4
3
2
1
0
20
40
60
Slice Number

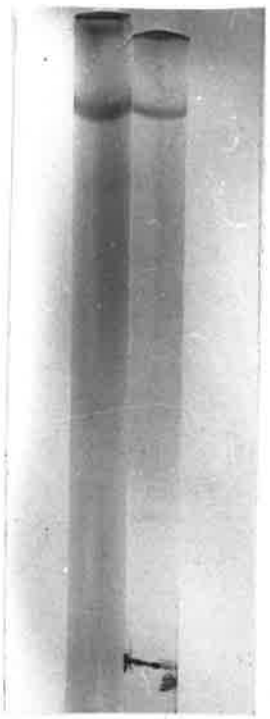


FIGURE 4.4. Electrophoretic patterns on SDS gels for A - sheep kidney pyruvate carboxylase and B - the enzyme after dialysis against 6 M guanidine hydrochloride and subsequent carboxymethylation.

did not change the result. The likelihood of this conclusion is increased by the fact that determinations using the dansylation technique have revealed alanine as the only N-terminal residue of the chains of both enzymes. The value obtained for the subunit molecular weight is also in agreement with the value of 110,000 to 120,000 recently determined for the chicken liver enzyme by Barden and Taylor (1973), who used both SDS-gel electrophoresis and ultracentrifugation in the presence of guanidine hydrochloride.

2. Sedimentation Velocity Studies of Proteins in SDS

Measurement of the sedimentation velocity of sheep kidney pyruvate carboxylase in 0.5% SDS-buffer have revealed a sedimentation coefficient of 2.5S for the complex, quite similar to the value of 2.7S found by Scrutton and Utter (1965) for the chicken liver enzyme. This is an unexpectedly low value for a globular protein of molecular weight of 120,000 but it is paralleled by values which were obtained for a series of normally globular proteins used as calibration standards in the experiments described previously. These experimental values of the sedimentation coefficients in SDS are listed in Table 4.1. They can be rationalised by reference to the general equation given by Mandelkern et al. (1952) to describe the sedimentation velocity of macromolecules which are not extremely prolate ellipsoids (equation 4.2):

TABLE 4.1. COMPARISON OF SEDIMENTATION COEFFICIENTS DETERMINED FOR PROTEINS IN SDS WITH THE EXPECTED SEDIMENTATION COEFFICIENT

Protein	Molecular weight	Theoretical 'Globular' Sedimentation Coefficients[a]	Experimental Sedimentation Coefficient
β-galactosidase monomer	134,000[b]	6.32	2.65
Pyruvate carboxylase monomer (sheep kidney)	120,000	5.88	2.51
Urease monomer	83,000[b]	4.60	2.50
Bovine serum albumin	68,000[b]	4.03	2.97
Catalase monomer	60,000[b]	3.70	2.58
Glutamate dehydrogenase monomer	53,000[b]	3.41	2.30
Ovalbumin	43,000[b]	2.96	2.69
Haemoglobin	15,500[b]	1.42	1.71
Lysozyme	14,300[b]	1.42	1.71
Cytochrome c	13,400[c]	1.36	1.80

[a] Determined from equation 4.4.

[b] See Weber and Osborn (1969).

[c] See Smith (1968).

$$\frac{s[\eta]^{1/3}}{M^{2/3}} = \frac{2.5 \times 10^{6} \ (1 - \bar{\nu}\rho)}{\eta s N} \qquad (4.2)$$

where s = sedimentation coefficient, $[\eta]$ = the intrinsic viscosity of the solute, M = the molecular weight of the solute, $\bar{\nu}$ = the partial specific volume of the solute, ρ = the solvent density, η_s = the solvent viscosity and N= Avogadro's number. The constant 2.5×10^{6} increases very slightly with axial ratio in the case of prolate ellipsoids (Sheraga and Mandelkern (1953). Halsall (1967) has pointed out that this equation is obeyed by a great many globular proteins, and that to a good approximation it may be simplified to equation 4.3.

$$\log s = \log k_1 + \frac{2}{3} \log M \qquad (4.3)$$

where k_1 is a constant. By analysis of experimental sedimentation constant data (i.e., $s^{o}_{20,w}$), an empirical form of equation 4.3 was deduced, equation 4.4.

$$\log s^{o} = \bar{3}.383 \pm 0.044 + \frac{2}{3} \log M \qquad (4.4)$$

For comparison, this equation has been used to compute the theoretical sedimentation constants in aqueous solution for the polypeptide chains listed in Table 4.1, assuming them to have globular conformations (Theoretical 'Globular' Sedimentation Coefficients). The large discrepancies between these theoretical values and the experimental sedimentation coefficients for

SDS-protein complexes are less significant than the fact that the latter values are a much less sensitive function of polypeptide molecular weight. The magnitude of the coefficients is of course different because the addition of SDS changes the values of ηs, and particularly $\bar{\nu}$ which reflects the large change in particle volume caused by solvent binding. The different molecular weight dependency is mainly due to the fact that whilst the intrinsic viscosity $[\eta]$ of globular proteins is independent of molecular weight, the intrinsic viscosity of the protein-SDS complexes has been found by Reynolds and Tanford (1970) to obey the empirical equation 4.5:

$$\log [\eta] = \log k_2 + 1.2 \log M \qquad (4.5)$$

where k_2 is a constant and M is the molecular weight of the polypeptide chain as before. Thus in the case for protein-SDS complexes I have combined the above equations and equation 4.2 is reduced to equation 4.6 which becomes equation 4.7 by substitution:

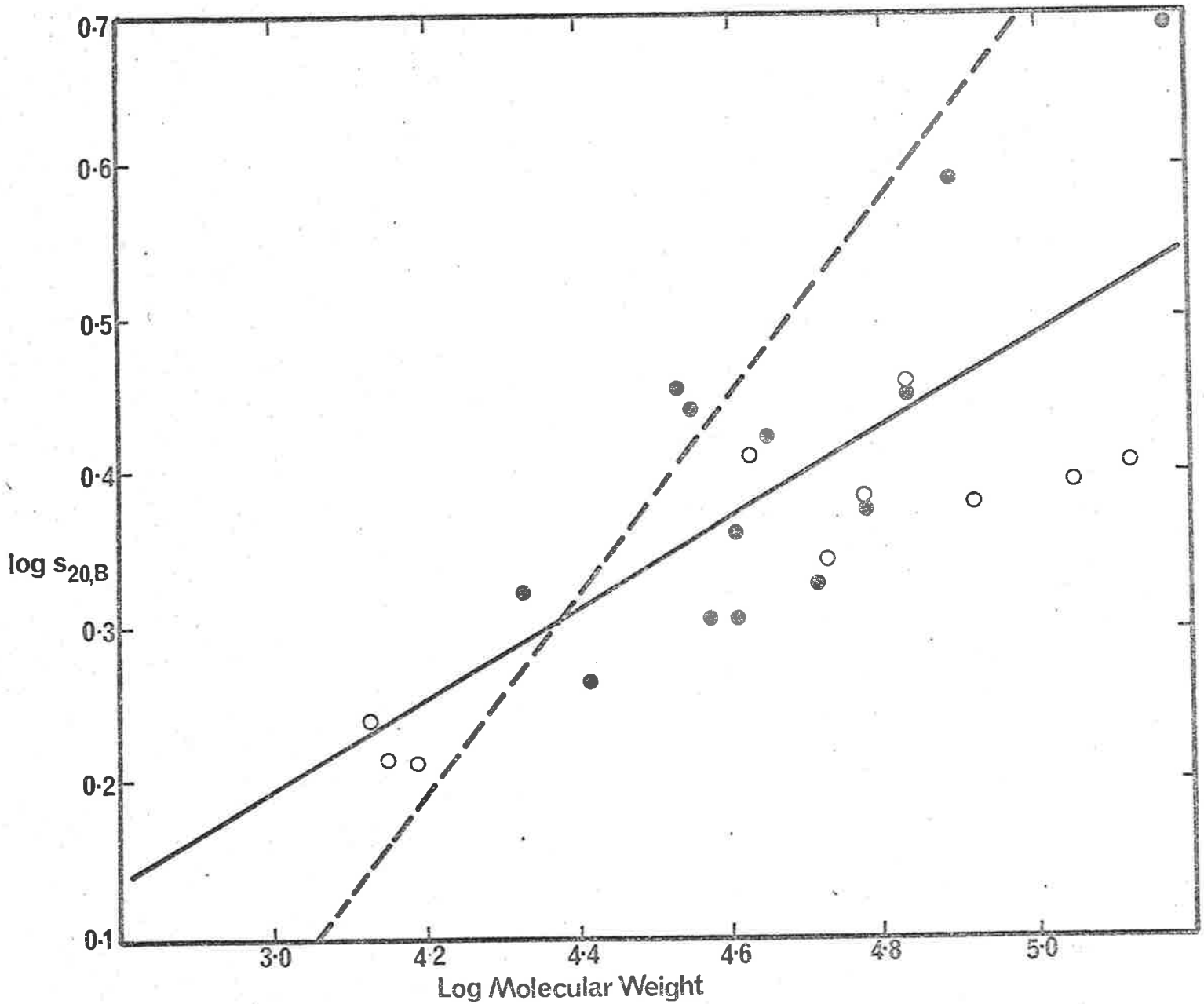
$$\log s = \log k_3 + 2/3 \log M - 1/3 \log [\eta] \qquad (4.6)$$

$$\log s = \log k_4 + 0.27 \log M \qquad (4.7)$$

where k_3 and k_4 are constants.

Equation 4.7 predicts that the plot of logs versus log M for protein-SDS complexes should be a straight line of slope 0.27. Figure 4.5 shows such a plot for the data of Table

FIGURE 4.5. The relationship between molecular weight and sedimentation, s for the protein-SDS complexes in Table 4.1 (O) and those of Nelson (1971) (●). The data have been corrected for temperature as given in the text. The data when fitted to a straight line (full line) by the method of least squares gives a value for the slope of 0.30 ± 0.06 which does not differ significantly from the theoretical value of 0.27 ($0.6 < P < 0.7$ on 22 degrees of freedom). The dashed line represents the equation of Halsall (1967) for the sedimentation of globular proteins in aqueous solution.


0·7
0·6
0·5
0·4
0·3
0·2
0·1
log $s_{20,B}$
3·0
4·2
4·4
4·6
4·8
5·0
Log Molecular Weight

4.1, and also the similar data obtained by Nelson (1971). The sedimentation coefficients of Table 4.1, determined at 21.5°, and those of Nelson at 25° have both been corrected to 20°, the temperature used in the experimental determination of equation 4.5, by using the approximation given by Schachman (1959):

$$s_{20,w} \simeq s_{obs} \left(\frac{\eta t,w}{\eta 20,w} \right) \qquad (4.8)$$

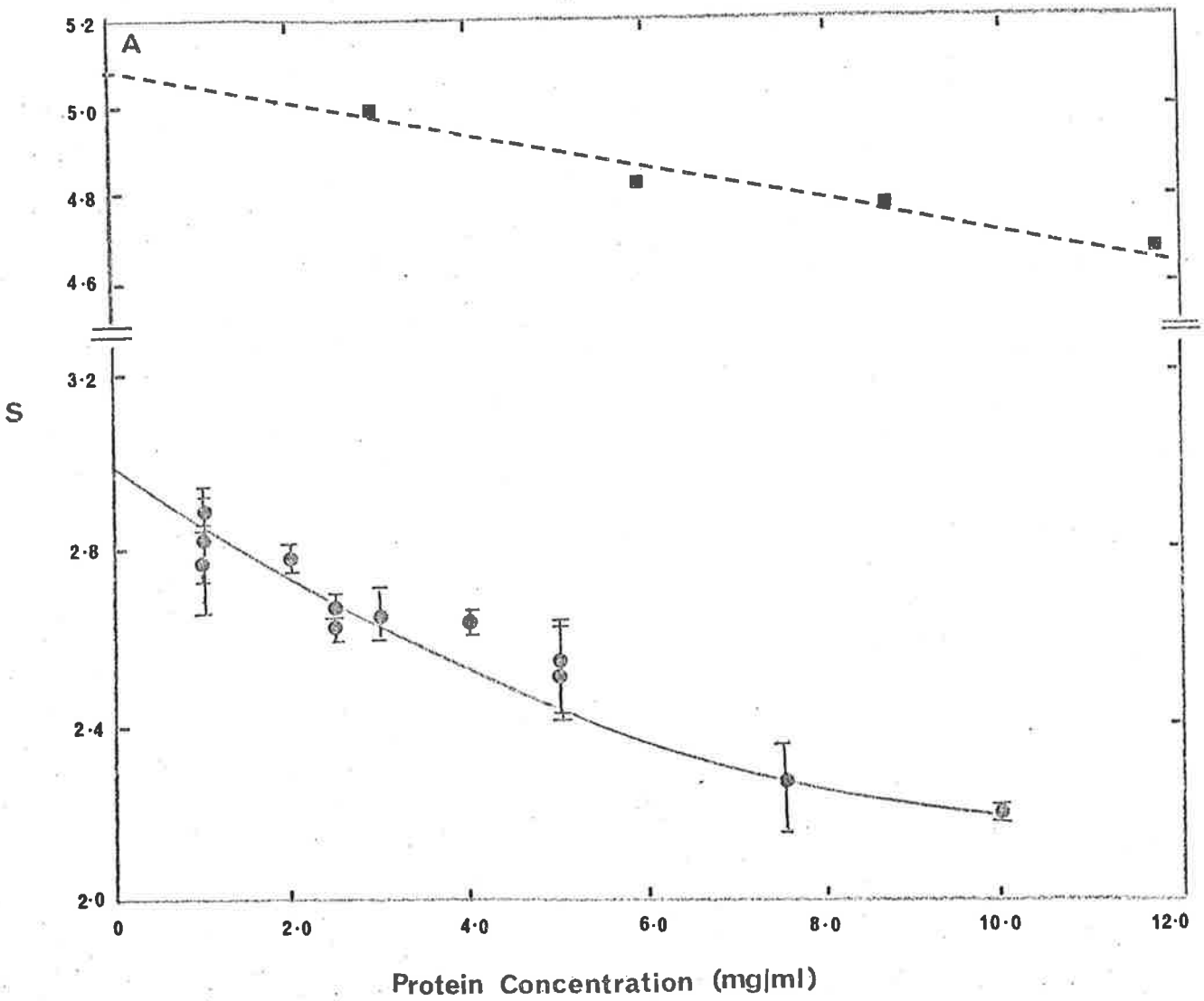
where $\eta_{t,w}$ and $\eta_{20,w}$ are the viscosity of water at t° and 20° respectively. When the points shown in Figure 4.5 were fitted to a best straight line, shown in the diagram, the slope was found to be 0.30 $\pm$ 0.06 which is not significantly different from the value of 0.27 predicted by equation 4.7. It is substantially different from the slope of equation 4.4 for globular proteins which is shown in Figure 4.5 for comparison.

The wide scatter of the points in Figure 4.5 emphasises that a precise analysis of the sedimentation velocity of protein-SDS complexes has not been attempted. Reynolds and Tanford (1970a) have presented theoretical arguments and experimental evidence which suggests that protein-SDS complexes are long, thin, rod-shaped particles. Interpretation of the sedimentation of such particles is discussed at length by Creeth and Knight (1965). The situation is complicated by the uncertain effects of the binding of large amounts of the solvent component, SDS, to give a highly electrically charged complex. However, the agreement between predicted and observed slopes in Figure 4.5

shows that the molecular weight dependence of the sedimentation coefficient of protein-SDS complexes is at least consistent with their hydrodynamic properties, and hence with the rod-shaped model of Reynolds and Tanford. There is no need to postulate dissociation into smaller polypeptide chains, particularly in the absence of independent evidence for such dissociation. With regard to pyruvate carboxylase from both sheep kidney and chicken liver it is concluded that the native enzyme is a tetramer with each subunit consisting of only one polypeptide chain.

Probably a major source of the scatter of points in Figure 4.5 is the fact that the sedimentation coefficients are not the constants obtained by extrapolation to zero protein concentration, because as Creeth and Knight (1965), have pointed out, asymmetric (or expanded) macromolecules exhibit a pronounced decrease in sedimentation coefficient with increasing macromolecule concentration. This has been confirmed for the case of a protein-SDS complex by means of the results presented in Figure 4.6A for a readily available and well-characterised globular protein, bovine serum albumin. The results are compared with the data of Baldwin (1957) for the sedimentation of the native protein, which exhibits less than half the concentration dependence.

Creeth and Knight (1965) further state that the ratio of K_s (the coefficient of concentration dependence of the reciprocal sedimentation coefficient, see Figure 4.6B and Table 4.2) to the intrinsic viscosity $[\eta]$ for a particle, is indicative of


A
5·2
5·0
4·8
4·6
3·2
2·8
2·4
2·0
S
0
2·0
4·0
6·0
8·0
10·0
12·0
Protein Concentration (mg/ml)

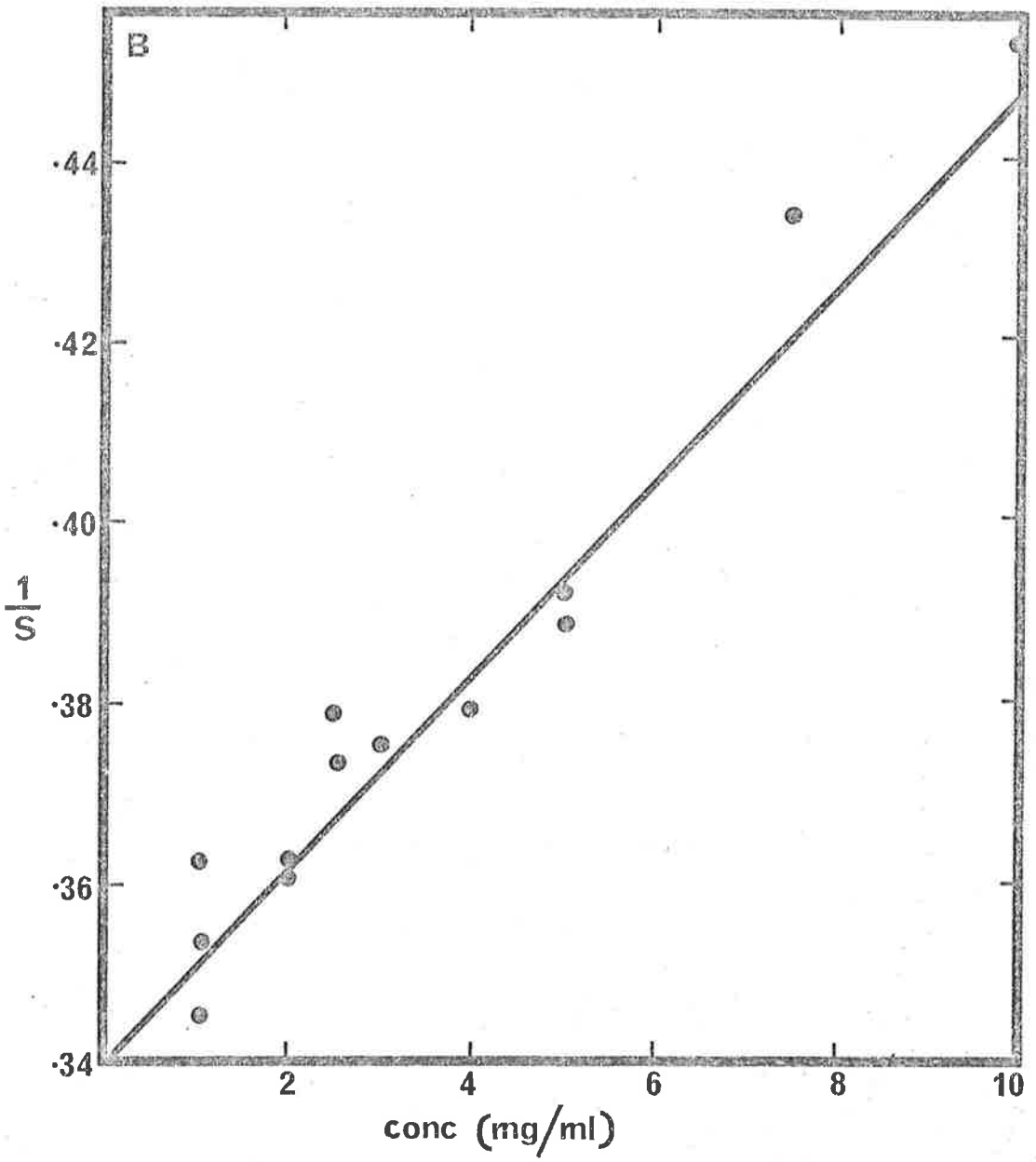


FIGURE 4.6. The dependence on protein concentration of the sedimentation coefficient of bovine serum albumin-SDS complexes at 21.5° in 0.1 M sodium phosphate buffer, pH 7.1 containing 0.5% SDS and 0.1% 2-mercaptoethanol. Part B shows the reciprocal of the sedimentation coefficient plotted as a function of concentration in order to determine the constants from the equation

$$\frac{1}{s} = \frac{1}{s^\circ} (1 + KsC)$$

as given by Creeth and Knight (1965). Also shown in Part A is the data of Baldwin (1957) for the sedimentation of native bovine serum albumin in 0.02 M sodium acetate, 0.10 M KCl, pH 4.55 at 25°.

TABLE 4.2. COMPARISON OF SEDIMENTATION DATA FOR BOVINE SERUM ALBUMIN IN THE PRESENCE AND ABSENCE OF SDS

	s^o	Ks^a ($mg.g^{-1}$)	$[\eta]$	$\frac{Ks}{[\eta]}$	Axial ratio[c]
Native protein	4.60	5.4	3.9	1.38	3.2
Protein-SDS complex	2.93	34	54.2[b]	0.63	45

[a]Determined from the data of Figure 4.5, using the equation given in Creeth and Knight (1965).

$$\frac{1}{s} = \frac{1}{s^o} \quad (1 + KsC).$$

[b]From Reynolds and Tanford (1970a).

[c]From the empirical equation given in Creeth and Knight (1965).

$$\text{Log}_{10} \text{ (axial ratio)} = 1.56 \left(1.7 - \frac{Ks}{[\eta]}\right).$$

asymmetry, if the value is substantially below that of about 1.6 found for compact spherical macromolecules. The combinations of data presented in Table 4.2 show that the ratio is 1.38 for native bovine serum albumin at 25°, but 0.63 for the protein-SDS complex at 20°, and therefore, also suggests that the detergent induces substantial asymmetry in the molecule.

If the empirical equation for axial ratio given by Creeth and Knight (see Table 4.2) may be applied to protein-SDS complexes, then a very tentative value for the axial ratio of the bovine serum albumin-SDS complex is 45, while that of the native protein is 3.2.

3. Evidence for Identical Subunits

In section 4.1 it has been shown that sheep kidney pyruvate carboxylase is a tetramer with subunits of molecular weight, 120,000. However, no indication was given as to whether the four subunits are identical.

The radioactivity profile from Sephadex G-25 chromatography of the trypsin digest of sheep kidney pyruvate carboxylase which had been treated with iodo[^{14}C]acetic acid is shown in Figure 4.7. Fractions containing radioactivity from this column were then freeze-dried and redissolved in a minimal amount of 0.1 M NH_4Cl. These samples were then subjected to high voltage paper electrophoresis for 1 h and the results analysed by autoradiography. This technique has the advantage that although the trypsin digest would cleave the enzyme at the carboxyl terminal side of arginine and lysine to form a large number of peptides only the relatively few radioactive peptides

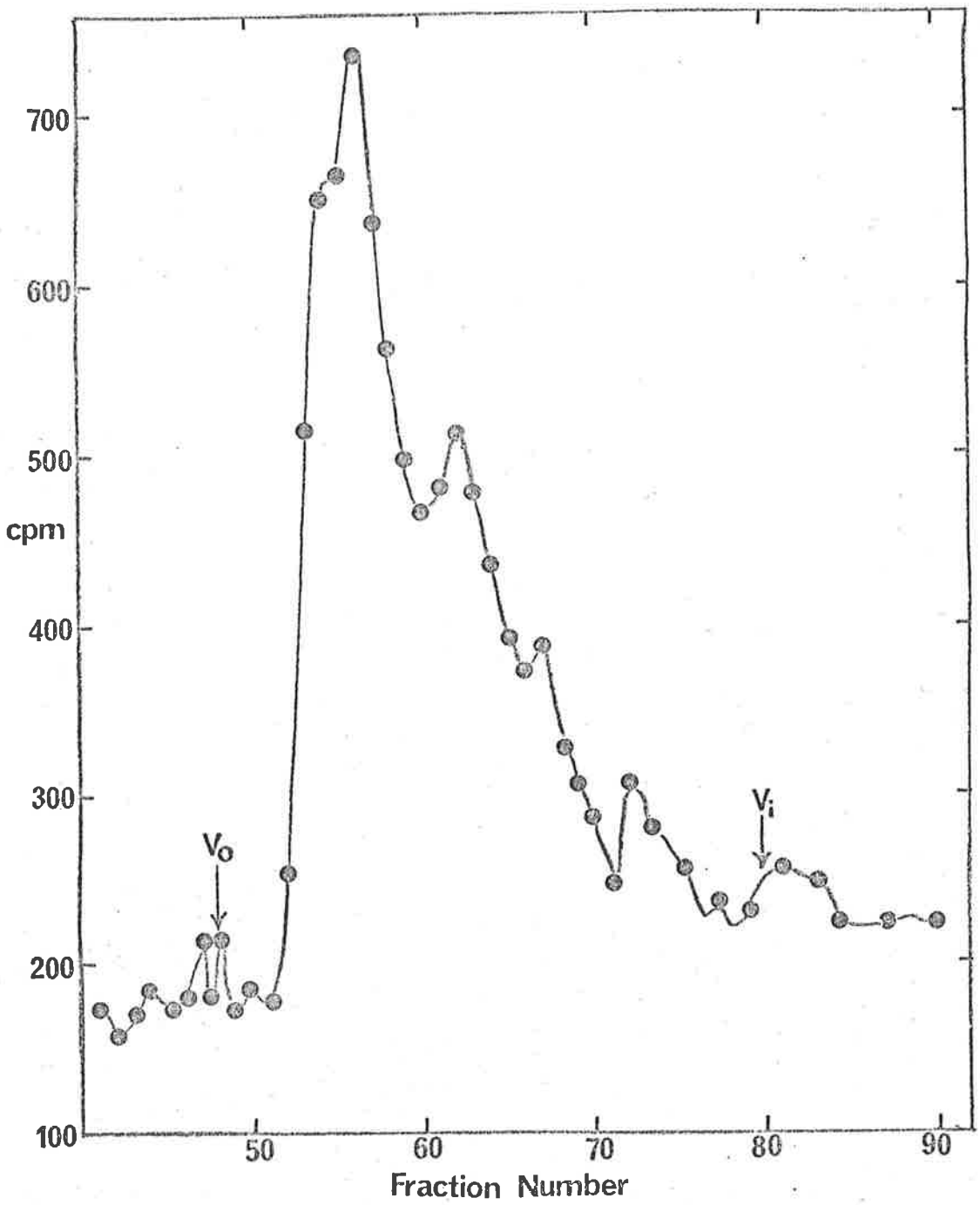
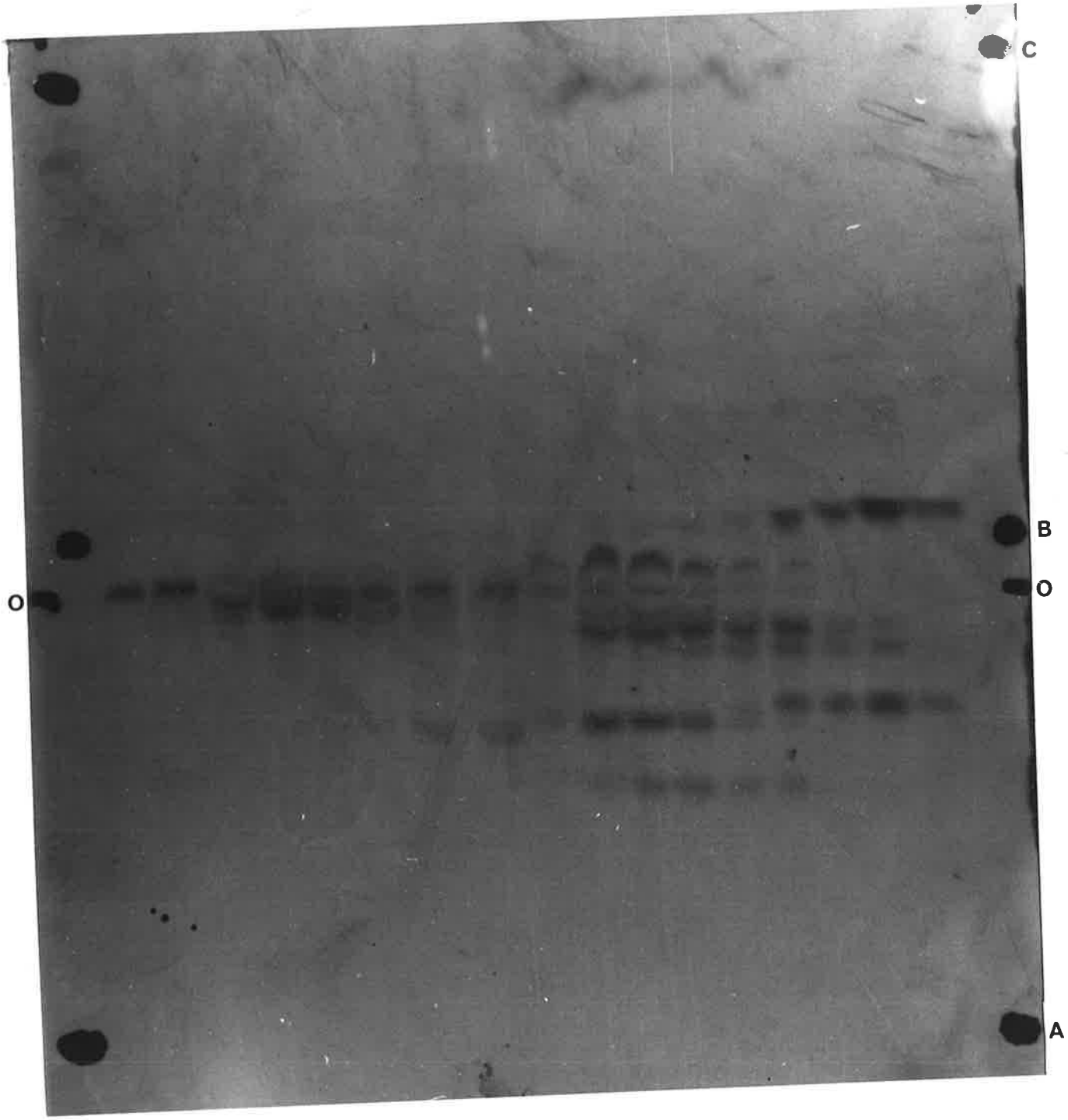


FIGURE 4.7. The radioactivity profile for trypsin digested iodo[^{14}C]acetic acid labelled sheep kidney pyruvate carboxylase eluted from a Sephadex G-25 column (115 cm x 2.4 cm) which had been previously equilibrated with pyridine/N-ethylmorphiline/acetic acid buffer, pH 9.0. 0.2 ml were taken from the 3.5 ml samples and counted in 2 ml of 30% triton X-100 scintillation fluid.

FIGURE 4.8. Autoradiograph of the tryptic digest of sheep kidney pyruvate carboxylase. Samples containing radioactivity from the Sephadex G-25 column shown in Figure 4.7 were subjected to high voltage paper electrophoresis as described in the text and then autoradiographed for 4 days. The radioactive markers were a aspartic acid, b isoleucine and c lysine.


C
B
O
O
A

would be detected. The autoradiogram (Fig. 4.8) shows that there are ten radioactive peptides separated by this method. Furthermore, the amount of radioactivity in each of the labelled peptides was approximately equivalent. From these findings, coupled with the fact that both amino acid analysis and DTNB titration indicated that sheep kidney pyruvate carboxylase cont ains forty cysteine residues, it would appear that the four subunits are identical. If they were not identical the tryptic digest would result in many more radioactive peptides. In addition, because it was possible to isolate ten different labelled peptides, it is unlikely that there would be homology around the cysteine residues such that the subunits were in fact non-identical.

4. Amino Acid Analyses of Pyruvate Carboxylases

In general terms, many of the properties of pyruvate carboxylases isolated from animal sources are similar (e.g., molecular weight, quaternary structure). However, there are differences between these enzymes and apart from cold lability the main differences are exhibited by the kinetic properties of the enzyme, e.g., n value for acetyl-CoA, acetyl-CoA independent activity. Thus it was of interest to see if their amino acid composition was also similar and if there was any single genetic variant to explain differences in the enzymic properties.

The amino acid analysis of pyruvate carboxylase isolated from a variety of sources is presented in Table 4.3. Included in this Table is the published analysis for the chicken liver enzyme and the analysis carried out in this laboratory. The reason for such a large difference is not understood. All these analyses were subjected to a computer analysis to compare them

TABLE 4.3. AMINO ACID COMPOSITION OF PYRUVATE CARBOXYLASE ISOLATED FROM VARIOUS SOURCES

Amino acid	Residues per mole[a]						
	SKPC[b]	SLPC[c]	RLPC[d]	GPLPC[e]	CLPC[f]	PLPC[g]	PCL[h]
Aspartic acid	332±6	353±24	336	341	302±14	396	447
Threonine	208±11	234±9	201	213	165±13	171	207
Serine	248±9	232±7	223	245	210±11	253	208
Glutamic acid	468±18	446±23	458	542	461±23	509	420
Proline	222±9	215±25	219	197	257±12	269	112
Glycine	390±21	349±21	346	390	421±26	428	503
Alanine	347±7	345±15	347	319	439±12	437	630
Valine	290±23	281±14	276	245	317±9	276	451
Cysteine[i]	44±4	46±5	nd	nd	44±3	nd	55
Methionine	80±10	83±10	102	64	70±8	61	73
Isoleucine	208±10	209±13	206	191	168±11	172	200
Leucine	352±9	329±18	344	296	392±13	340	499
Tyrosine	106±7	118±4	111	98	91±8	97	203
Phenylalanine	158±8	162±6	166	165	156±6	141	290
Lysine	194±8	201±16	196	202	145±5	155	165
Histidine	113±8	114±14	118	98	105±7	70	124
Arginine	198±3	217±15	209	192	258±10	278	322

[a] Molecular weight 480,000
[b] sheep kidney
[c] sheep liver
[d] rat liver
[e] guinea pig liver
[f] chicken liver
[g] pigeon liver
[h] published analysis for chicken liver (Scrutton and Utter, 1965)
[i] Determined as carboxymethyl-cysteine.

with the amino acid analysis of a large number of other proteins. The analysis was carried out using the program MULTCLAS developed to compare complex groups of data (Lance and Williams, 1967a,b). This program classifies the proteins by the nearness or 'relatedness' of their amino acid analyses in such a way that the proteins are listed according to the degree of relationship.

The results were such that all the pyruvate carboxylases except the published analysis for the chicken liver enzyme fell into one class of proteins which included various other carboxylases including ribulose 1,5-diphosphatate, acetyl-CoA and phosphoenolpyruvate carboxylases. However, within this larger class, the various pyruvate carboxylases could be further divided into two groups, i.e., the mammals and the avians.

D. DISCUSSION

By using a variety of techniques it has been possible to show that the subunits of sheep kidney pyruvate carboxylase are of molecular weight, 110 - 120,000. In addition, these subunits have been shown to be identical by using a size-charge peptide mapping procedure.

The molecular weight of the subunits are the same as for the chicken liver enzyme as has been shown in this laboratory and by Barden and Taylor (1973) but disagrees with the results for the rat liver enzyme. For rat liver pyruvate carboxylase, McClure *et al.* (1971a) were able to identify six or seven bands on polyacrylamide gel electrophoresis in the presence of SDS after

the enzyme had been exhaustively dialysed against 6.7 M guanidine hydrochloride and then carboxymethylated. However, repetition of this experiment with the sheep kidney enzyme did not result in any dissociation of the 120,000 species.

The subunit structure of sheep kidney pyruvate carboxylase appears to be similar to a variety of biotin-containing enzymes. Pyruvate carboxylase, acetyl-CoA carboxylase and propionyl-CoA carboxylase isolated from animal sources are all tetrameric proteins of molecular weight, 500 - 600,000. In addition, several of these do not appear to have subunits of less than 100,000 (Moss and Lane, 1971). However, this structure is very different from bacterial pyruvate carboxylases for which the species from Pseudomonas citronellolis has a molecular weight of 265,000 (Taylor et al., 1972) and that from Bacillus stearothermophilus a molecular weight of 350,000 (Cazzulo et al., 1971). Both these enzymes are probably dimers. The subunit structure is also different from acetyl-CoA carboxylase isolated from E. coli (Alberts et al., 1969; Guchhait et al., 1971) and transcarboxylase isolated from Propionibacterium shermanii (Jacobsen et al., 1970) both of which can be dissociated into small subunits of specific functions.

The amino analyses of pyruvate carboxylases isolated from various animal sources showed that there are two classes of enzymes; one class being cold labile whereas the other is not. If one considers the sheep kidney or liver and chicken liver enzymes, i.e., the ones analysed in the greatest detail, the main differences between those are the threonine, alanine, lysine and arginine content (difference greater than 15%).

For the sheep enzymes threonine and lysine are present in greater proportions whereas alanine and arginine are present in greater proportions in the chicken liver enzyme. The other amino acids are present in very similar amounts.

The codons for these variant amino acids are

threonine	ACU	alanine	GCU
(CLPC <SKPC)	C	(CLPC >SKPC)	C
	A		A
	G		G
lysine	AAA	arginine	AGA
(CLPC <SKPC)	G	(CLPC>SKPC)	G

Thus a mutation involving the replacement of alanine by threonine and arginine by lysine could be made simply by an A $\longleftrightarrow$ G substitution. Thus it is of interest to speculate whether the differences in properties have resulted from a change in the pyruvate carboxylase gene during evolution. Because chicken liver pyruvate carboxylase contains a greater proportion of alanine, this evidence is consistent with this enzyme being a more hydrophobic protein than the sheep kidney enzyme. Thus the chicken enzyme structure is more likely to involve hydrophobic interactions and these have been shown to be involved in cold inactivation of proteins (Tanford, 1962; Feldberg and Datta, 1971). In addition, the relatedness of the various carboxylases has also been postulated by Sumper and Rierpertinger (1972) who showed that the structure of acetyl-CoA and pyruvate carboxylases from yeast are very similar.

CHAPTER FIVE

THE DIVALENT CATION ACTIVATION OF SHEEP KIDNEY PYRUVATE CARBOXYLASE

A. INTRODUCTION

From data presented in a previous paper (Keech and Barritt, 1967) it was suggested that in the sheep kidney pyruvate carboxylase reaction mechanism, Mg^{2+} has at least two roles,

(a) it complexes with ATP^{4-} to form $MgATP^{2-}$, the true substrate for the enzyme, and

(b) it probably activates the enzymic reaction by complexing with the enzyme.

Blair (1969) fitting the same data to the rate equations for a number of model systems, pointed out that there were alternate explanations.

However, in neither communication was any indication given as to where the divalent metal ion exerts its influence on the reaction mechanism or the nature of the effect induced by the divalent cation when it complexed with the enzyme. In this chapter the role of divalent cations in the sheep kidney pyruvate carboxylase reaction mechanism was investigated to determine,

(a) whether or not the requirement for divalent cations is absolute

(b) the physical nature of the divalent cation activation and

(c) whether Mn^{2+} and Mg^{2+} have an identical activation mechanism on the enzyme.

B. METHODS

The acetyl-CoA dependent reaction of pyruvate carboxylase was assayed isotopically as described in Section 2.B1 and the acetyl-CoA independent reaction as described by Ashman et al. (1972).

The fluorescent probe 1-anilinonaphthalene-8-sulphonate (Eastman Organic Chemical Co.) was purified by the method of Aoe et al. (1970). Exposure to fluorescent light was minimised to prevent the formation of fluorescent products (McClure and Edelman, 1966). Spectrofluorometric measurements were made on a Perkin-Elmer Fluorescence Spectrophotometer 203 using an excitation wave length at 370 nm for ANS (Stryer, 1965).

Tritium exchange in pyruvate carboxylase was studied by the interrupted flow method described by Schechter et al. (1969). The protein was labelled by the addition of 5 µl of tritiated water (10 Ci per ml) per ml of protein solution (2 - 5 mg of protein per ml of buffer A). The 'in-exchange' of tritium atoms was allowed to proceed for at least two days before back exchange reaction was studied.

To study the interrupted back exchange, the enzyme was applied to a Sephadex G-25 column (100 cm x 2 cm) previously equilibrated with either buffer A or buffer A containing 0.5 mM Mg^{2+}. Buffer (40 ml) was collected and the column flow stopped for 1 h after which time a further 33 ml of buffer were allowed to flow through the column before the flow was

stopped for a further 6 hr. After a further 37 ml of buffer had been eluted the column fractions were collected and assayed for protein and counts.

C. KINETIC CONSTANTS AND RELATIONSHIPS AMONG THE VARIOUS KINETIC CONSTANTS

$$[Mg^{2+}] + [ATP^{4-}] \underset{\longrightarrow}{\overset{K_o}{\longleftarrow}} [MgATP^{2-}] \qquad (5.1)$$

$$[E] + [Mg^{2+}] \underset{\longrightarrow}{\overset{K_a}{\longleftarrow}} [Mg^{2+} - E] \qquad (5.2)$$

$$[E] + [Mg^{2+}] \underset{\longrightarrow}{\overset{K_m}{\longleftarrow}} [E - Mg^{2+}] \qquad (5.3)$$

$$[Mg^{2+}\text{-}E] + [Mg^{2+}] \underset{\longrightarrow}{\overset{K_{m'}}{\longleftarrow}} [Mg^{2+}\text{-}E\text{-}Mg^{2+}] \qquad (5.4)$$

$$[E] + [MgATP^{2-}] \underset{\longrightarrow}{\overset{K_c}{\longleftarrow}} [E - MgATP^{2-}] \qquad (5.5)$$

$$[Mg^{2+} - E] + [MgATP^{2-}] \underset{\longrightarrow}{\overset{K_{c'}}{\longleftarrow}} [Mg^{2+}\text{-}E\text{-}MgATP^{2-}] \qquad (5.6)$$

$$[E] + [ATP^{4-}] \underset{\longrightarrow}{\overset{K_s}{\longleftarrow}} [E - ATP^{4-}] \qquad (5.7)$$

$$[Mg^{2+}\text{-}E] + [ATP^{4-}] \underset{\longrightarrow}{\overset{K_{s'}}{\longleftarrow}} [Mg^{2+}\text{-}E\text{-}ATP^{4-}] \qquad (5.8)$$

M_t, S_t and C arethe total Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ concentrations respectively, V is the maximum velocity for the breakdown of the E-$MgATP^{2-}$ complex and V' is the maximum velocity for the breakdown of the activated Mg^{2+} - E - $MgATP^{2-}$ complex.

In addition, Mg^{2+}-E and E-Mg^{2+} are not the same complexes; i.e., the Mg^{2+} binding site is taken to be the left hand side of E and the $MgATP^{2-}$ site is on the right hand site. A value of 18,000 M^{-1} has been used for K_o for $MgATP^{2-}$ formation and 72,000 M^{-1} for $MnATP^{2-}$ formation (O'Sullivan and Perrin, 1964). The concentration of all species under conditions of varying Mg^{2+} and ATP^{4-} were determined using the appropriate dissociation constants and a computer program written to solve equation 5.1.

D. RESULTS

1. Initial Velocity Studies

Earlier studies in this laboratory indicated that the reaction rates of sheep kidney pyruvate carboxylase was stimulated by Mg^{2+} (Keech and Barritt, 1967). The question as to whether the binding of Mg^{2+} to the enzyme was essential for enzymic activity or simply stimulated a basic level of activity has now been pursued further.

Where there is random combination between activator (Mg^{2+}) and substrate ($MgATP^{2-}$) with the enzyme, the experimental data can be analysed using the general rate equation, Equation 5.9 (Dixon and Webb, 1964). Equation 5.9 can be rearranged in the form of a striaght line as in equation 5.10,

$$v = \frac{V'}{1 + \frac{K_a K_c'}{(Mg^{2+})(MgATP^{2-})}\left(1 + \frac{(Mg^{2+})}{K_a} + \frac{(MgATP^{2-})}{K_c}\right)} \qquad (5.9)$$

$$\frac{1}{v} = \left(\frac{K_a K_c'}{V'(MgATP^{2-})} + \frac{K_a K_c'}{V' K_c}\right)\frac{1}{(Mg^{2+})} + \frac{1}{V'}\left(\frac{K_c'}{(MgATP^{2-})} + 1\right) \qquad (5.10)$$

Double reciprocal plots of velocity plotted as a function of Mg^{2+} concentration at fixed non-saturating concentrations of $MgATP^{2-}$ for sheep kidney pyruvate carboxylase are shown in 5.1A. Because the slope and intercept terms are complex terms, very little information can be obtained from this primary plot. However, the slope and intercept terms of equation 5.10 can be rearranged in the form of linear regressions:

$$\text{i.e., Slope} = \frac{K_a K_c'}{V'}\frac{1}{(MgATP^{2-})} + \frac{K_a K'_c}{V' K_c} \qquad (5.11)$$

and if the slope is plotted as a function of the reciprocal of the $MgATP^{2-}$ concentration, the line obtained will have a slope of $\frac{KaKc'}{V'}$ and the intercept on the ordinate will be $\frac{K_a K_c'}{V' K_c}$.

When the slope is zero, then

$$\frac{K_a K_c'}{V'}\frac{1}{(MgATP^{2-})} = -\frac{K_a K_c}{V' K_c} \qquad (5.11a)$$

$$\text{and} \quad \frac{1}{K_c} = -\frac{1}{(MgATP^{2-})} \qquad (5.11b)$$

i.e, the intercept on the abscissa when the slope is zero. Similarly, a replot of the intercept term can be made by plotting intercept as a function of the reciprocal of the $MgATP^{2-}$ concentration, i.e.,

$$\text{Intercept} = \frac{K_c'}{V'} \quad \frac{1}{(MgATP^{2-})} + \frac{1}{V'} \qquad (5.12)$$

Once again, when the intercept is zero,

$$\frac{K_c'}{V'} \quad \frac{1}{(MgATP^{2-})} = - \frac{1}{V'} \qquad (5.12a)$$

and $$\frac{1}{K_c'} = - \frac{1}{(MgATP^{2-})} \qquad (5.12b)$$

The constants derived from the slope and intercept replots (Fig. 5.1B) of the data shown in Fig. 5.1A are shown in Table 5.1. The fact that K_a is not 0 at infinite $MgATP^{2-}$ concentration ($K_a = (4.44 \pm 2.13) \times 10^{-4}$ M) shows that Mg^{2+} activation is essential for sheep kidney pyruvate carboxylase activity. However, McClure et al. (1971a) using rat liver pyruvate carboxylase, arrived at the conclusion that free Mg^{2+} was not essential. However, to obtain this conclusion, McClure et al. (1971a) chose to ignore one of four points in their replot of the slope term as a function of the reciprocal of the $MgATP^{2-}$ concentration which otherwise would have given them a positive value for K_a.

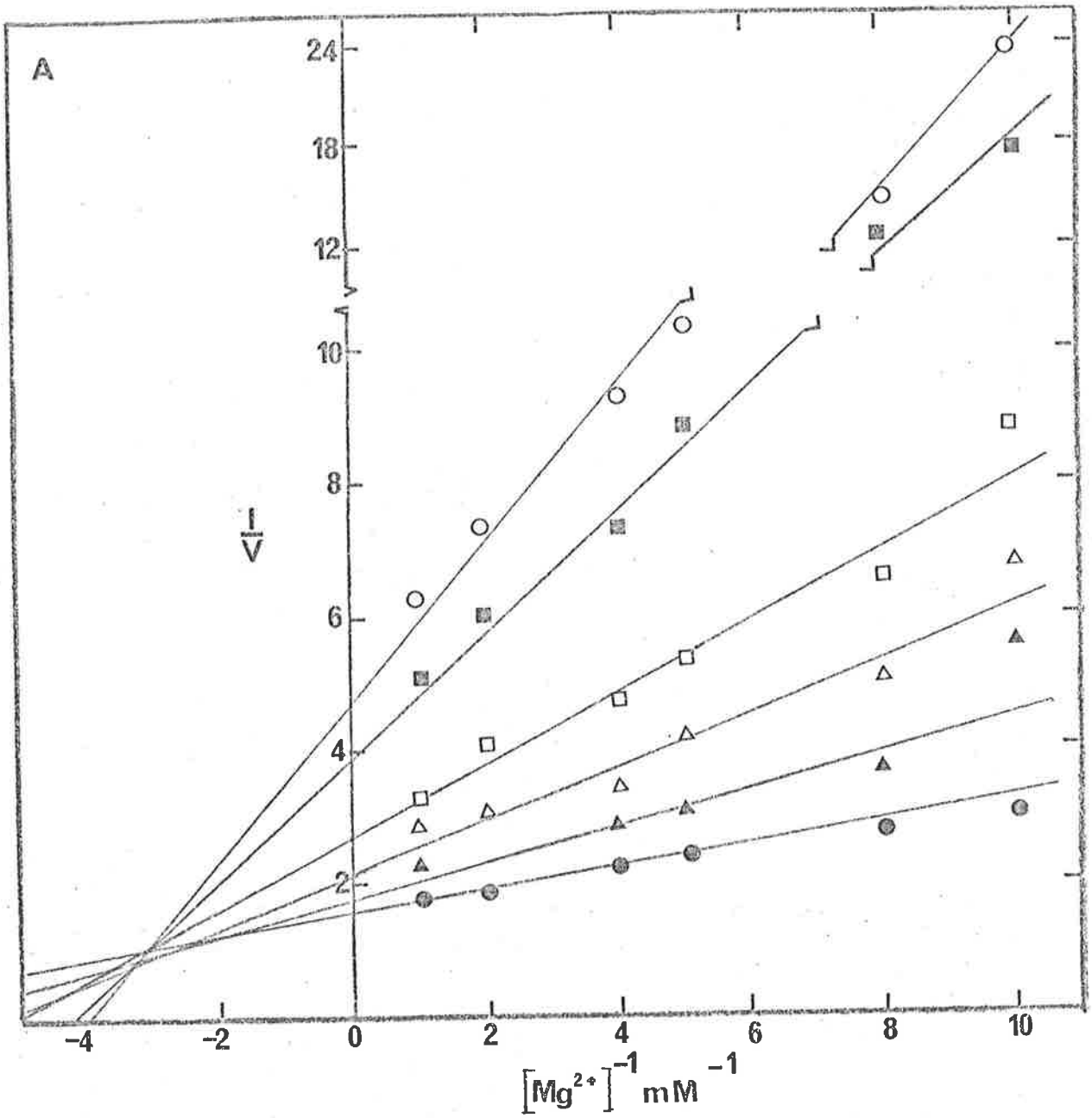
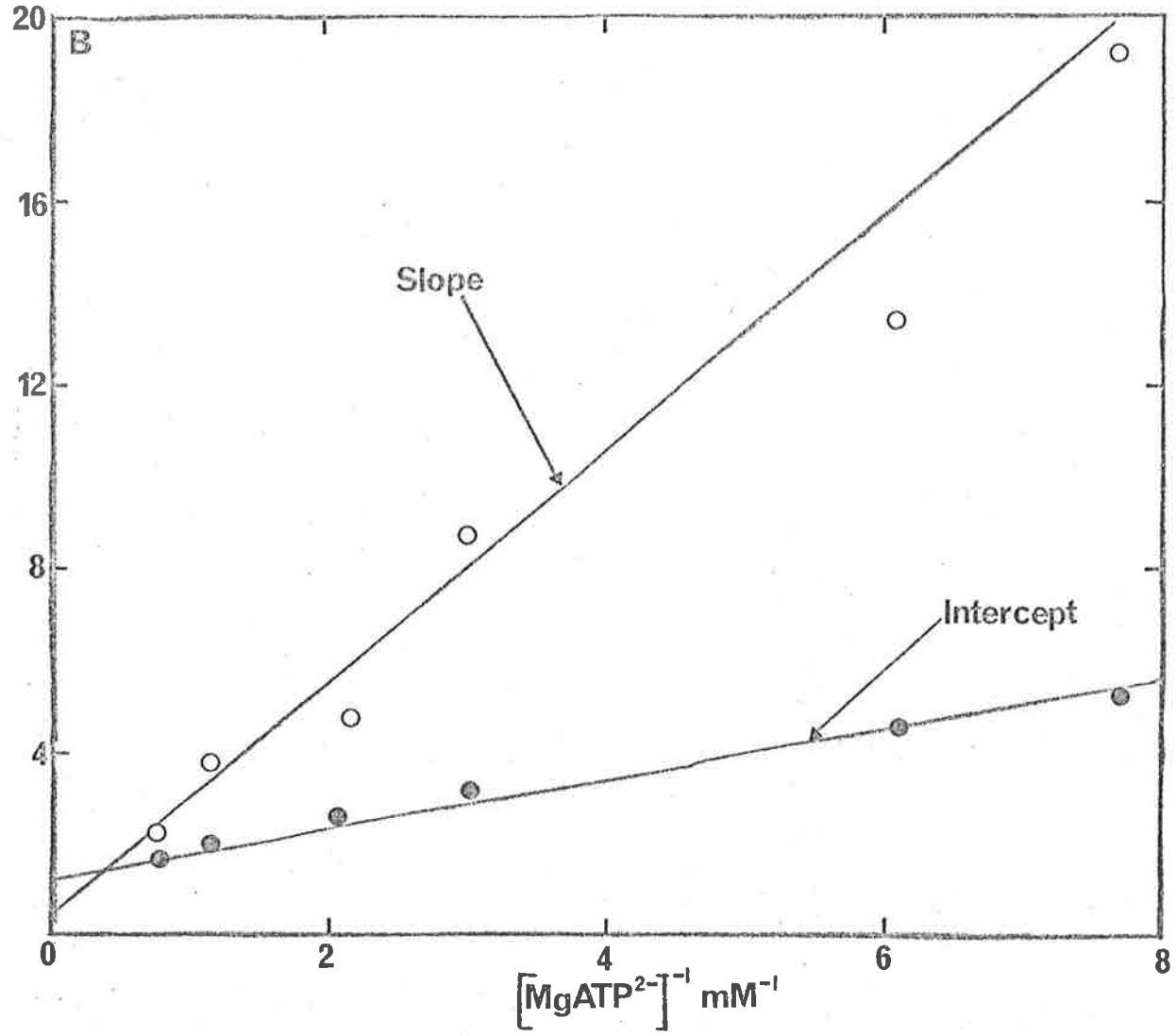


FIGURE 5.1. The requirement of pyruvate carboxylase for free Mg^{2+}. In A the reciprocal of the Mg^{2+} concentration is plotted against 1/V with the $MgATP^{2-}$ concentrations fixed at (○——○) 1.32×10^{-4} M, (■——■) 1.65×10^{-4} M, (□——□) 3.3×10^{-4} M, (△——△) 4.95×10^{-4} M, (▲——▲), 6.6×10^{-4} M, (●——●), 13.2×10^{-4} M. B.shows the replot of slopes and intercepts from A. Enzymic assays were carried out in Tris-HCl, pH 8.0, at 30° as described in the text


B
20
16
12
8
4
0
2
4
6
8
Slope
Intercept
$[MgATP^{2-}]^{-1}$ mM^{-1}

2. Models

London and Steck (1969) have described three possible models for a reaction with interacting substrate, modifier and enzyme as follows.

Model 1 - This model describes the situation where Mg^{2+}, ATP^{4-}, $MgATP^{4-}$ and E combine randomly with each other to form an active complex, $E\text{-}MgATP^{2-}$, which breaks down to form products. The equation describing this model is

$$v = \frac{VK_cC}{K_sS_t + K_mM_t + (K_c - K_s - K_m)C + 1} \qquad (5.13)$$

Model 2 - This model involves essential activation of the enzyme by Mg^{2+} which binds at a specific Mg^{2+} binding site. The activated complex is $Mg^{2+}\text{-}E\text{-}MgATP^{2-}$ and the equation describing this model is

$$v = \frac{V'K_c'K_a(M_t-C)C}{K_a(M_t-C)[K_s'(S_t-C)+K_m'(M_t-C)+K_c'C+1]+1} \qquad (5.14)$$

Model 3 - Here products can form from either $E\text{-}MgATP^{2-}$ or $Mg^{2+}\text{-}E\text{-}MgATP^{2-}$ and the equation describing this model is

$$v = \frac{VK_cC + V'K_c'K_a(M_t-C)C}{K_a(M_t-C)+K_aK_c'(M_t-C)C+K_cC+1} \qquad (5.15)$$

It is possible to distinguish between these three models by plotting velocity as a function of either total Mg^{2+}

concentration (M_t) or total ATP^{4-} concentration (S_t) at fixed concentrations of the nonvaried ligand. Results of such an experiment are shown in Figure 5.2. These data were analysed by a computer analysis based on a modified maximum likelihood analysis (see Appendix A) to fit the equations described by London and Steck (1969). According to their theory, the increase in velocity with increasing M_t concentration at low S_t concentrations, shown by the upward arrow in the S_t profile (Fig. 5.2A), indicates that for either model I or II, Mg^{2+} cannot bind to the $MgATP^{2-}$ binding site, i.e., the $E+Mg^{2+} \rightleftharpoons E\text{-}Mg^{2+}$ reaction cannot occur for Model I ($K_m = 0$) or the $Mg^{2+}\text{-}E \rightleftharpoons Mg^{2+}\text{-}E\text{-}Mg^{2+}$ reaction could not occur for model II ($K_m' = 0$). Also, since the ascending limbs of the M_t profiles (Fig. 5.2B), are sigmoidal, if model III was the correct model then $V = 0$ and thus the $E\text{-}MgATP^{2-}$ complex form products. This would have the effect of reducing model III to a special case of model II. Furthermore, when all three models were analysed by the maximum likelihood method, model II was shown to be the model of best fit. This is because this is the model with the lowest 98% confidence limits (Table 5.1). The modified equation for this model (i.e., with $K_m' = 0$) is,

$$v = \frac{V'K_aK_c'(M_t-C)C}{K_a(M_t-C)[K_s'(S_t-C)+K_c'C+1]+1} \qquad (5.16)$$

and the theoretical curves generated from this equation are shown in Figure 5.2A and B.

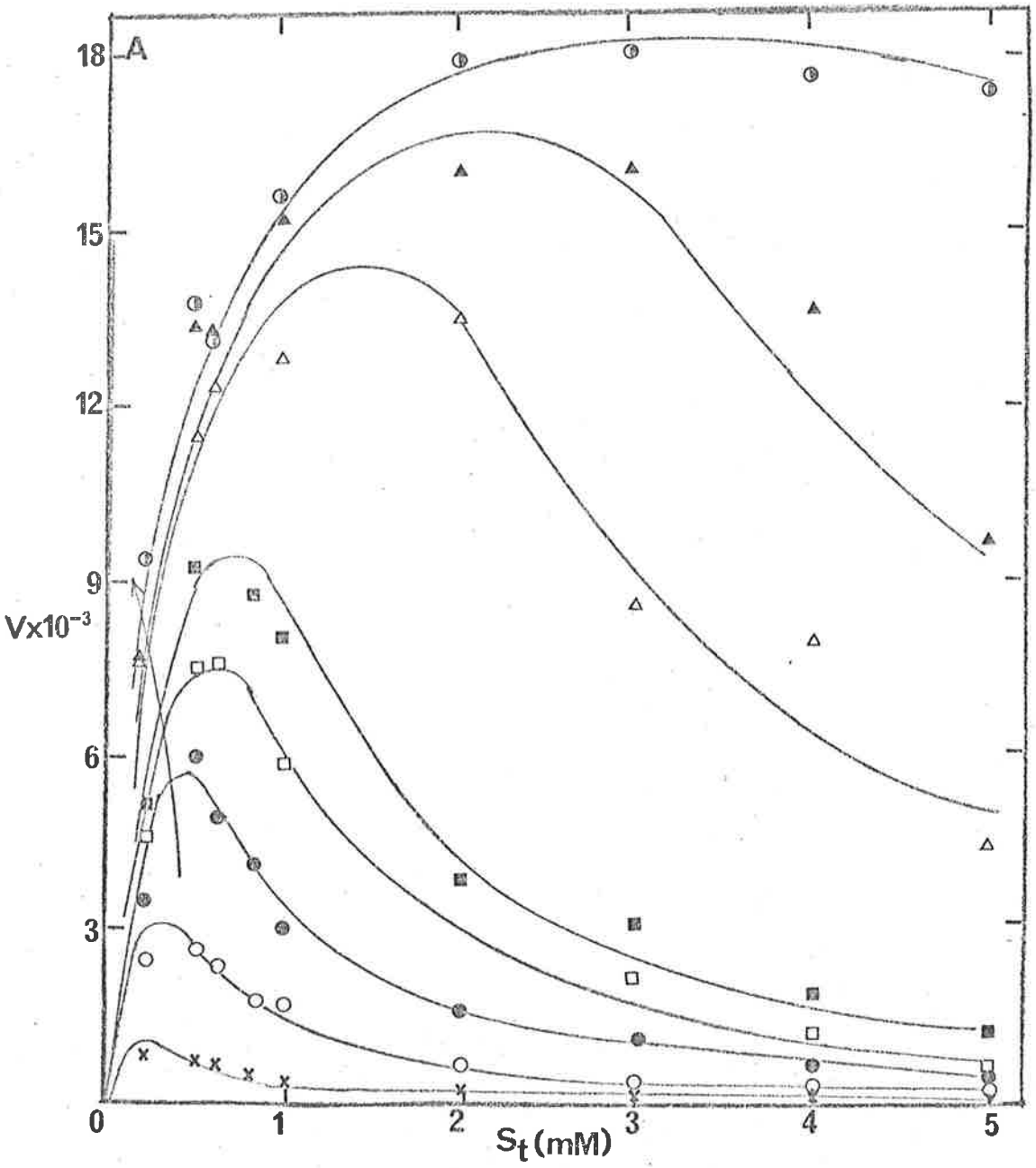


FIGURE 5.2. S_t and M_t profiles for the effect of Mg^{2+} on pyruvate carboxylase. The experimental points in A show the effect of varying S_t at fixed concentrations of M_t. ×——× , 0.2 mM; ○——○ , 0.4 mM; ●——● , 0.6 mM; □——□ , 0.8 mM; ■——■ , 1.0 mM; △——△ , 2.0 mM; ▲——▲ , 3.0 mM; ◑——◑ , 5.0 mM; B is a replot of the data in A showing the effect of varying M_t and fixed concentrations of S_t. ×——× , 0.2 mM; ○——○ , 0.5 mM; ●——● , 0.6 mM; □——□ , 1.0 mM; ■——■ , 2.0 mM; △——△ , 3.0 mM; ▲——▲ , 4.0 mM; ◑——◑ , 5.0 mM. The curves are theoretical curves generated from Equation 5.16 using the values for the constants given in Table 5.1 to describe model II.

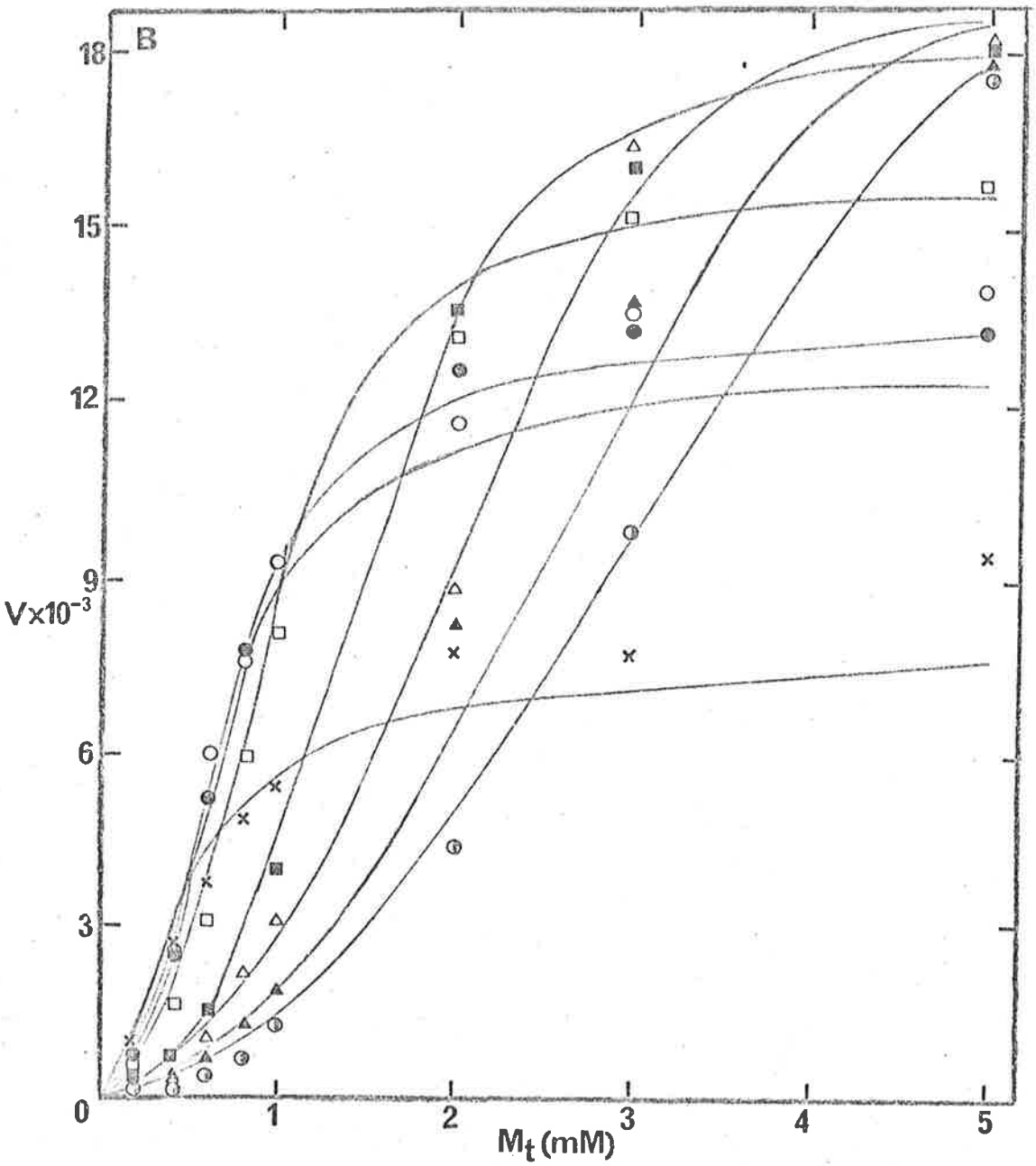

B
18
15
12
9
6
3
0
$V \times 10^{-3}$
1
2
3
4
5
M_t (mM)

TABLE 5.1. KINETIC CONSTANTS RELATED TO Mg^{2+} ACTIVATION

	Treatment			
	Equation 5.9	London and Steck		
		Model I	Model II	Model III
Ka	4.44 ± 2.13		4.30 ± 0.87	92.6 ± 88.9
Kc'	4.30		3.50 ± 0.84	1.54 ± 0.25
Kc	0.63	3.51 ± 0.88		9.02 ± 2.24
Ks'			4.89 ± 2.07	
Residual Mean Squares		0.2179	0.0340	0.0891
98% Confidence Limits				
(upper value		0.324	0.061	0.123
(lower value		0.133	0.021	0.055

All constants are x 10^{-4} M. The standard errors are only given for those constants which are directly obtained from computer program.

That model II is the correct model is supported by the fact that a plot of velocity at equimolar M_t and S_t (Figure 5.3) is sigmoidal in the region much greater than $\bar{K}_o$ ($\bar{K}_o$ = 0.056 mM). As pointed out by London and Steck (1969), for model I and III the sigmoidicity would only be apparent at concentrations in the region of $\bar{K}_o$ whereas it is at concentrations greater than this. Since model II involves the essential activation of the enzyme by Mg^{2+}, this analysis confirms the previous conclusion that Mg^{2+} activation is essential for the activity of this enzyme.

3. Comparison of Mg^{2+} and Mn^{2+} Activation

Besides containing tightly bound Mn^{2+} (see Chapter 3) pyruvate carboxylase is also stimulated by exogenous Mn^{2+}. In addition, Mn^{2+} can also form $MnATP^{2-}$ in a manner analogous to Mg^{2+} forming $MgATP^{2-}$. It has been shown that for rat liver pyruvate carboxylase, that the enzyme has a maximum velocity about 12% greater in the presence of Mn^{2+} when compared with Mg^{2+} (Wimhurst and Manchester, 1970), and in fact it has been suggested that Mn^{2+} may be the true *in vivo* metal ion activator for the yeast enzyme (Cazzulo and Stoppani, 1969). In addition, only Mn^{2+} is effective as the metal ion activator of pyruvate carboxylase isolated from *Bacillus stearothermophilus* (Cazzulo et al., 1970). It has also been shown that pyruvate carboxylase isolated from all sources is strongly inhibited by Mn^{2+} at concentrations much lower than is required for Mg^{2+} inhibition. Thus, the activation of sheep kidney enzyme by Mn^{2+} has been compared with the activation by Mg^{2+}.

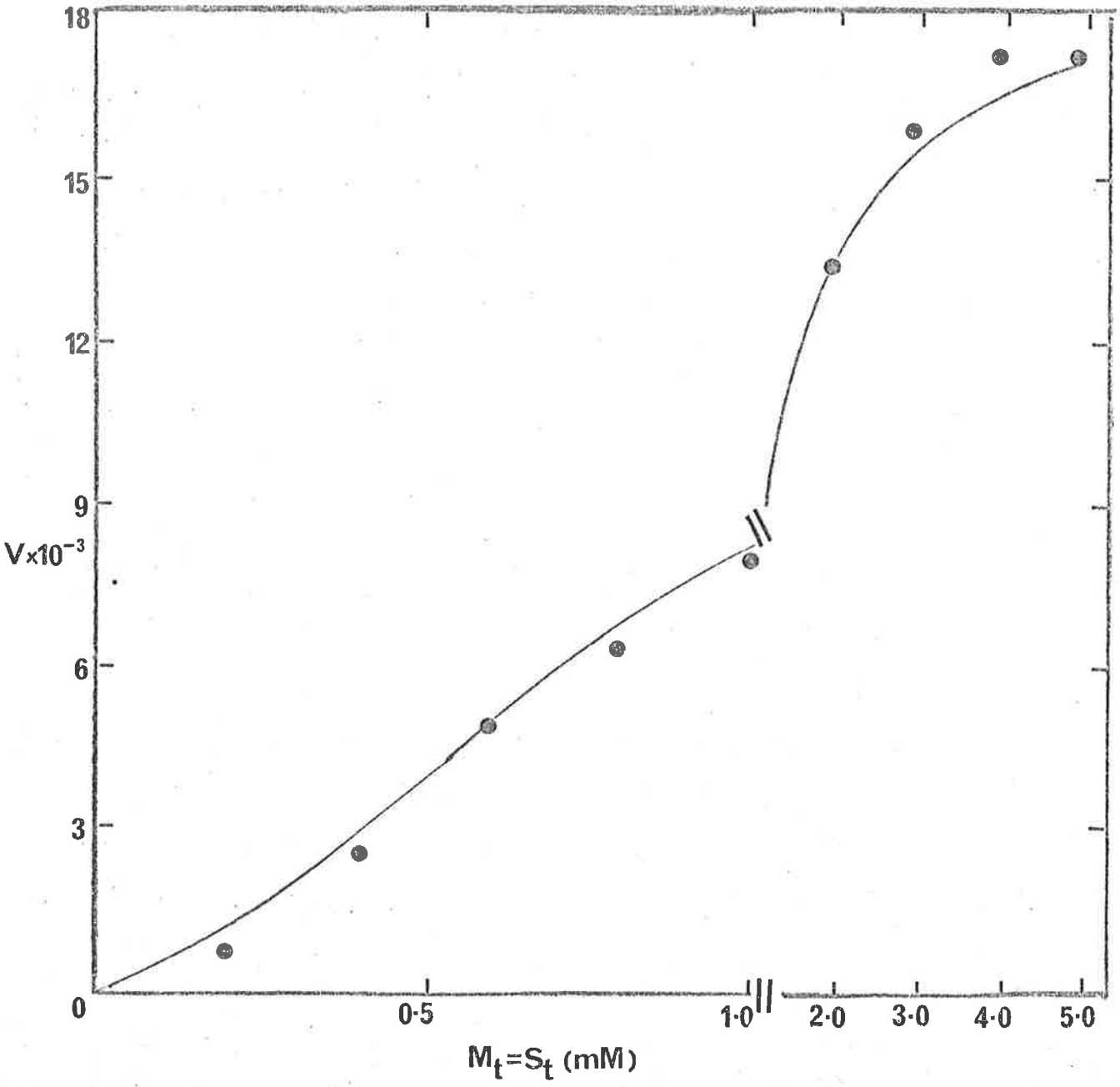


FIGURE 5.3. Velocity profiles with $M_t = S_t$ showing sigmoidicity in the range greater than $\bar{K}_o$ = 0.056 mM. The points are experimental data and the curve was generated as in Figure 5.2.

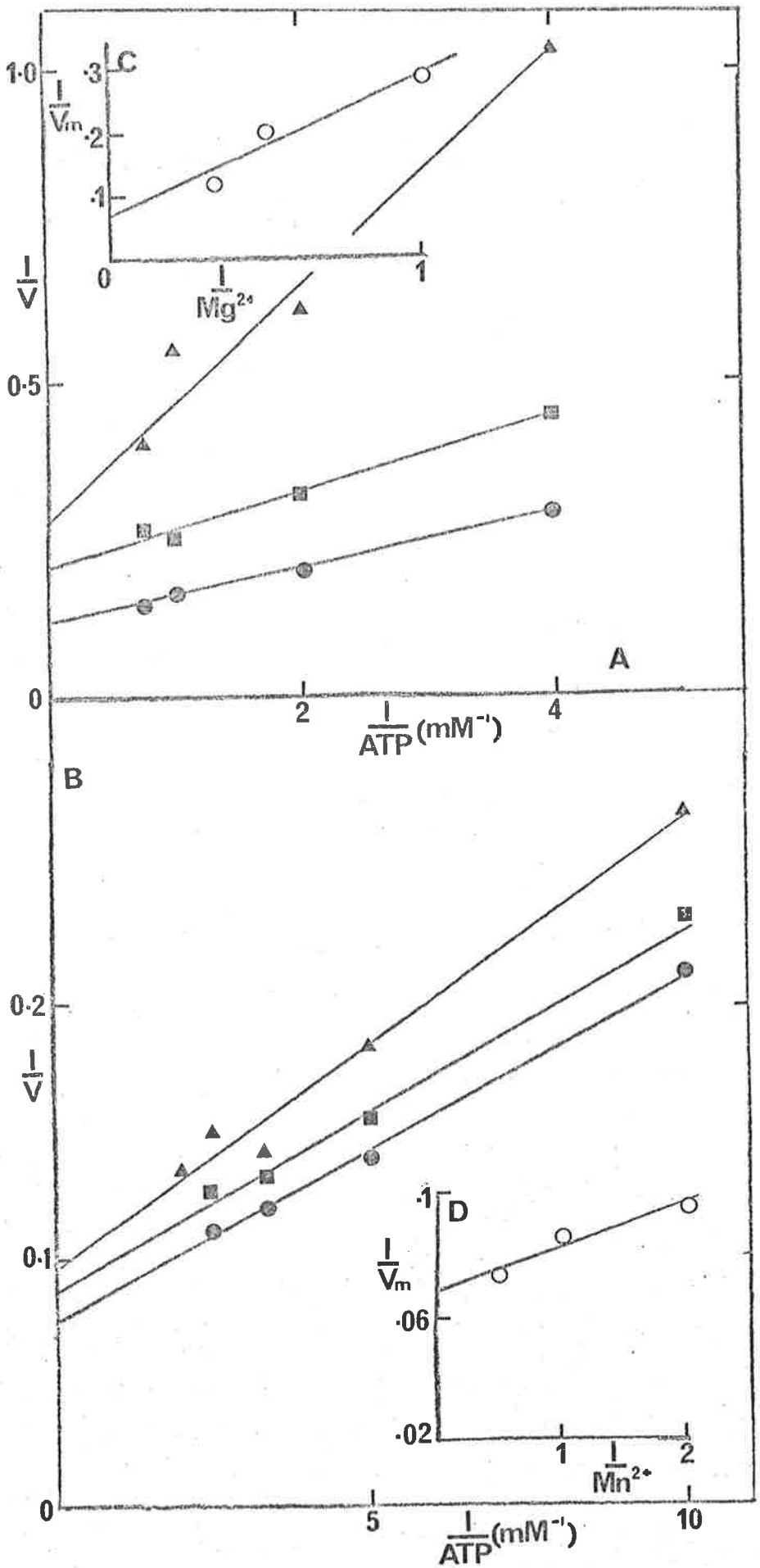
The double reciprocal plots of varying ATP^{4-} in the presence of a fixed excess of Mg^{2+} or Mn^{2+} are shown in Fig. 5.4A and 5.4B. From these data V_{obs} has been replotted as a function of excess metal ion concentration and true V_{max} has been determined for both Mg^{2+} and Mn^{2+} (Fig. 5.4C and 5.4D). The results of such plots give the maximum velocity in the presence of Mn^{2+} to be 92% of that obtained in the presence of Mg^{2+}.

The mode of action of Mn^{2+} on sheep kidney pyruvate carboxylase has been determined by varying ATP^{4-} at various concentrations of Mn^{2+} (Fig. 5.5A and 5.5B). Attempts have been made to fit this data to equation 5.16 but only the region with the Mn^{2+} concentration less than 1 mM fitted this equation. This is the region in which there is a pronounced Mn^{2+} activation with an activation constant of 8.7×10^{-6} M. The reason for only being able to fit part of the data is that once the maximum velocity has been reached, there is strong inhibition by excess Mn^{2+}. This is shown in Fig. 5.5B in which at concentrations greater than 1 mM there is inhibition of the enzymic activity. Thus there appear to be two effects of Mn^{2+} on the enzyme, i.e.,

(1) an initial activation (probably essential in a manner analogous to that exhibited by Mg^{2+}), and

(2) a strong inhibition after the maximum velocity has been attained.

FIGURE 5.4. Vm determinations for pyruvate carboxylase in the presence of Mg^{2+} and Mn^{2+}. The experimental points in A show the effect of varying ATP^{4-} at fixed excesses of Mg^{2+}; ●——● , 3.0 mM; ■——■ , 2.0 mM; ▲——▲ , 1.0 mM. B shows the effect of varying ATP^{4-} at fixed excesses of Mn^{2+}, ●——● , 2.0 mM; ■——■ , 1.0 mM; ▲——▲ , 0.5 mM. The intercepts from A and B are replotted in C and D respectively to determine true Vmax.


1·0
0·5
0
1/V
A
2
4
1/ATP (mM⁻¹)
C
·3
·2
·1
1/Vm
0
1
1/Mg²⁺
B
0·2
0·1
0
5
10
1/ATP (mM⁻¹)
D
·1
·06
·02
1
2
1/Mn²⁺

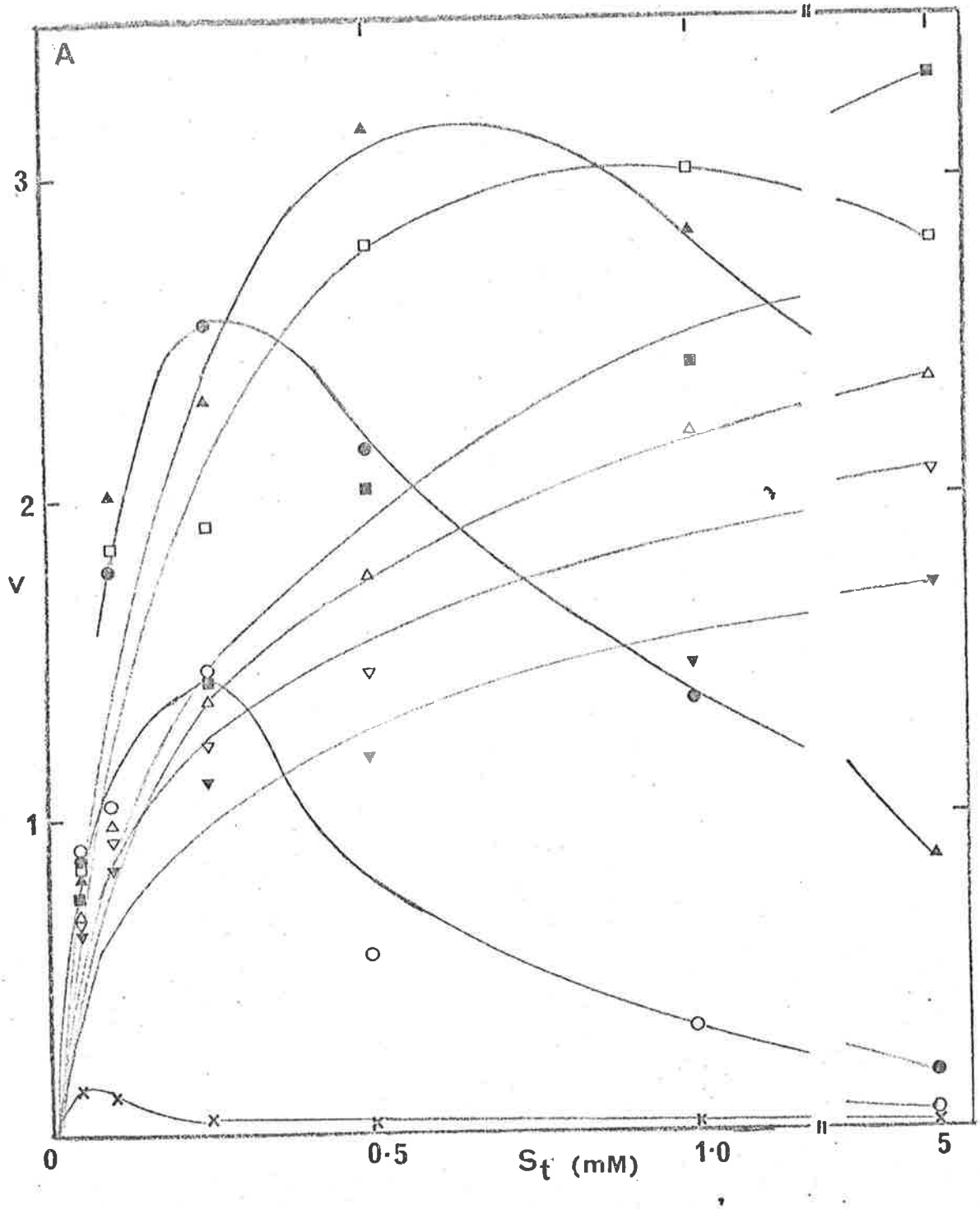
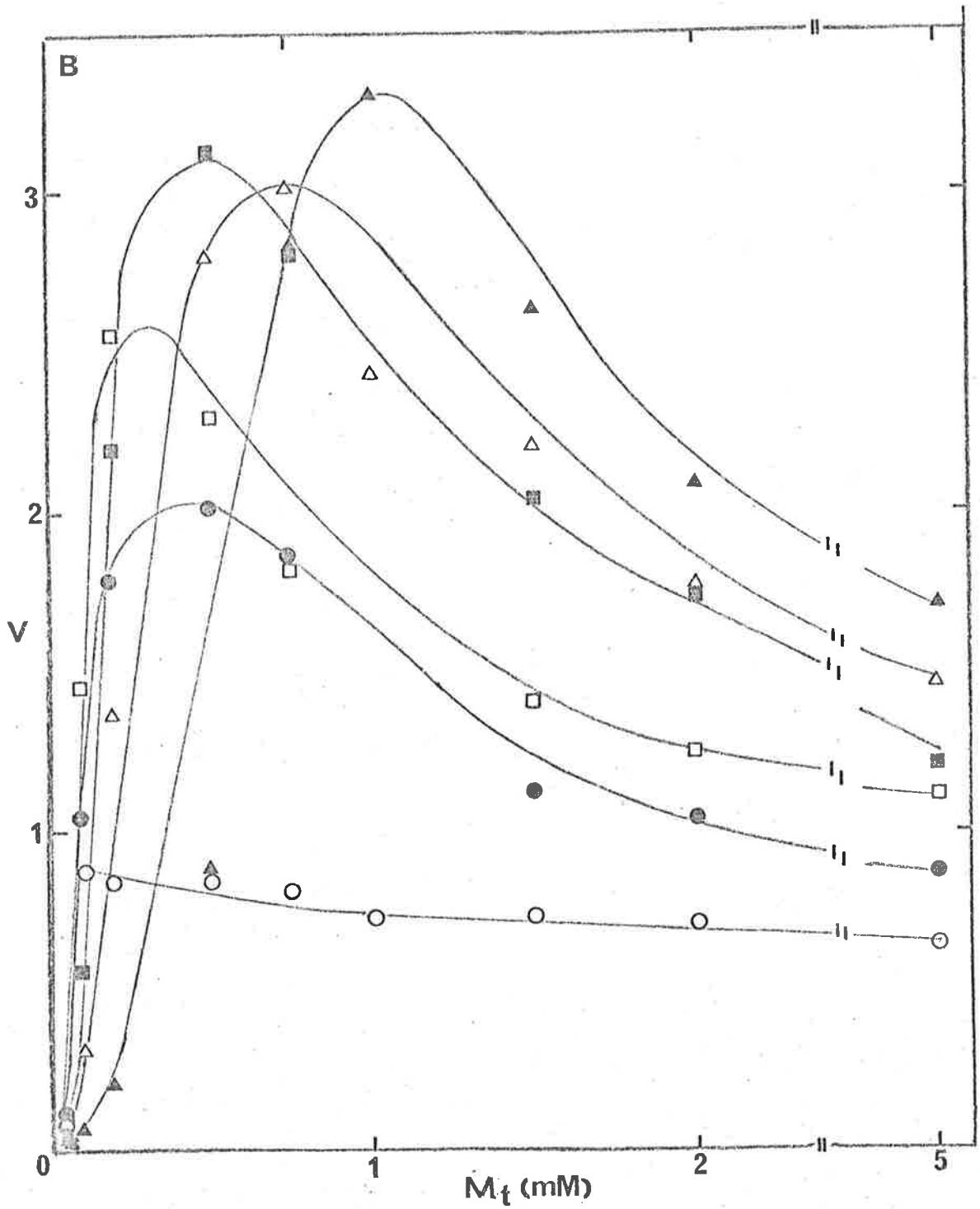


FIGURE 5.5. S_t and M_t profiles for the effect of Mn^{2+} on pyruvate carboxylase. The experimental points in A show the effect of varying S_t at fixed concentrations of M_t. ×——×, 0.05 mM; ○——○, 0.1 mM; ●——●, 0.2 mM; ▲——▲, 0.5 mM; □——□, 7.5 mM; ■——■, 1.0 mM; △——△, 1.5 mM; ▽——▽, 2.0 mM; ▼——▼, 5.0 mM. B is a replot of the data in A showing the effect of varying M_t at fixed concentrations of S_t. ○——○, 0.05 mM; ●——●, 0.1 mM; □——□, 0.25 mM; ■——■, 0.5 mM; △——△, 1.0 mM; ▲——▲, 5.0 mM. The curves are not generated from any equation.


B
V
3
2
1
0
1
2
5
M_t (mM)

4. Effect of Mg^{2+} on the Acetyl-CoA Independent Reaction

Ashman et al. (1972) have shown that sheep kidney pyruvate carboxylase can carboxylate pyruvate in the absence of the allosteric effector, acetyl-CoA. To determine whether Mg^{2+} was associated with the acetyl-CoA activation of the enzymic reaction, the effect of varying the Mg^{2+} concentration at various ATP^{4-} concentrations was studied. The S_t profile for the effect of Mg^{2+} on the acetyl-CoA independent reaction is shown in Fig. 5.6.

Ashman et al. (1972) reported that the apparent K_m value for $MgATP^{2-}$ was identical in both the presence and absence of acetyl-CoA. Thus, K_c the constant describing the binding of $MgATP^{2-}$ to the enzyme was held constant at 3.5×10^{-4} M and the data fitted to the various models described by London and Steck (1969) in a manner analogous to that described for the acetyl-CoA dependent reaction (Section 5.2). The upward arrow in the S_t profile (Fig. 5.6) indicates that $K_m = 0$. However, in contrast to the results for the acetyl-CoA dependent reaction, these data only fitted the equation for model I, i.e.,

$$v = \frac{VK_cC}{K_s(S_t-C)+K_cC+1} \qquad (5.17)$$

with V equal to 20,056, K_c as defined above and K_s equal to 5.6×10^{-5} M. These results indicate that whereas for the acetyl-CoA dependent reaction where the products can only

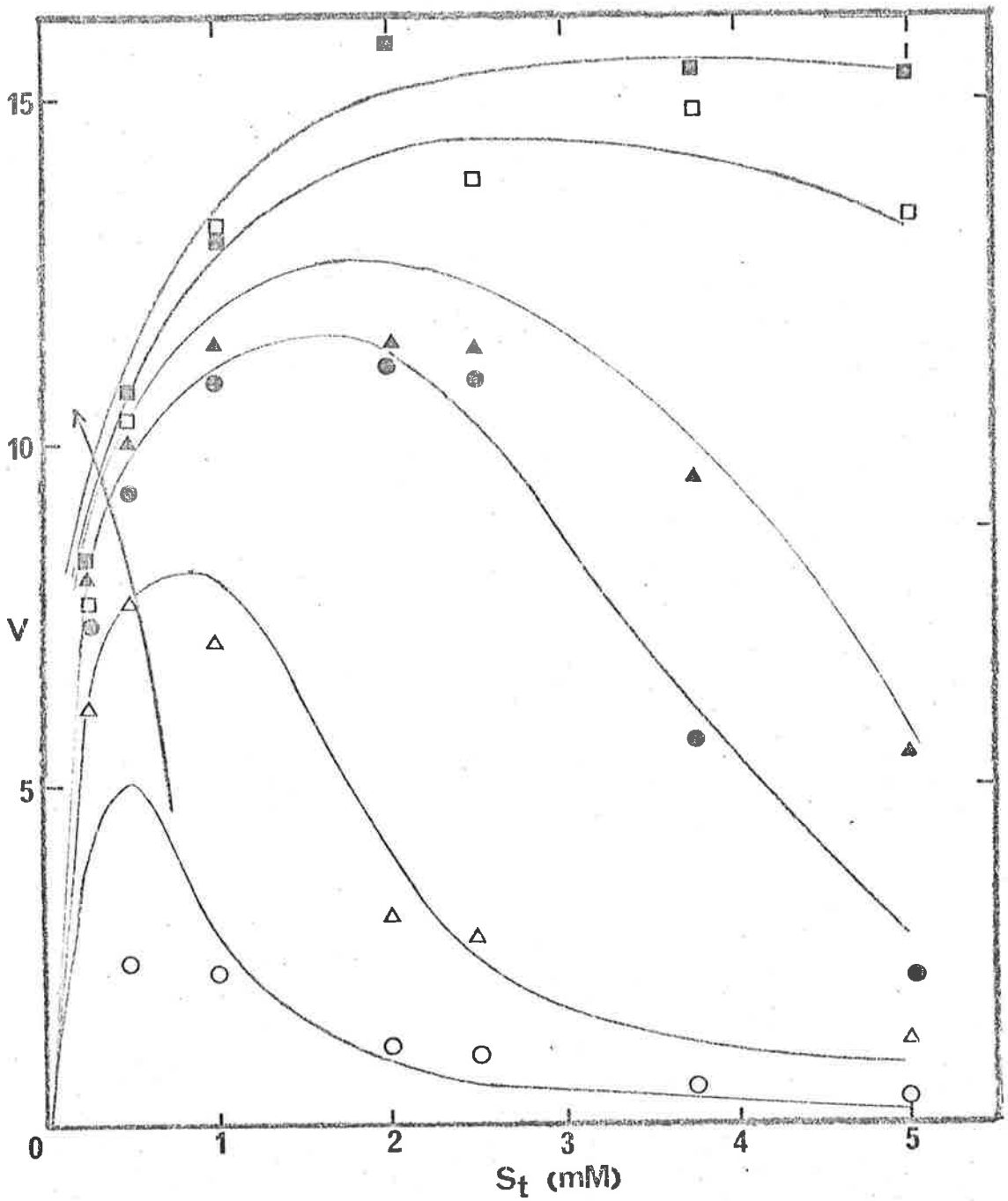


FIGURE 5.6. The effect of Mg^{2+} on the acetyl-CoA independence reaction of pyruvate carboxylase. The experimental points show the effect of varying S_t at fixed concentrations of M_t;
○——○ , 1.0 mM; △——△ , 2.0 mM; ●——● , 4.0 mM;
▲——▲ , 5.0 mM; □——□ , 7.5 mM; ■——■ , 10.0 mM.
The curves were generated from equation 5.17 as described in the text.

form from the Mg^{2+}-E-$MgATP^{2-}$ complex, for the acetyl-CoA independent reaction, products can be formed from the E-$MgATP^{2-}$ complex. This would indicate that the Mg^{2+}-activation is associated with the acetyl-CoA activation of the enzyme.

5. Time Course of Activation

The activation of pyruvate carboxylase by Mg^{2+} was shown to be time dependent (Fig. 5.7). The enzyme was placed into two separate reaction mixtures, one lacking pyruvate and the other without pyruvate and Mg^{2+}. The reaction was started by adding these components and the velocity followed by the removal, at 15 sec. time intervals, of standard aliquots into measured volumes of saturated 2,4-dinitrophenol hydrazine-HCl. The amount of $[^{14}C]CO_2$ fixed into acid stable oxaloacetate was then determined.

The results shown in Fig. 5.7 indicate that when the enzyme had been previously incubated with Mg^{2+}, there was no lag period, but if Mg^{2+} was added without preliminary incubation there was a lag before the enzyme became fully activated. A similar time-dependent activation has been observed for acetyl-CoA carboxylase incubated with citrate (Greenspan and Lowenstein, 1968).

6. Mg^{2+}-Induced Conformational Changes

Keech and Barritt (1967) presented evidence that one effect of Mg^{2+} on pyruvate carboxylase was to reduce the apparent K_m value for $MgATP^{2-}$ from 6.6×10^{-4} M with very low concentrations of free Mg^{2+} to 3.2×10^{-4} with 1 mM Mg^{2+}.

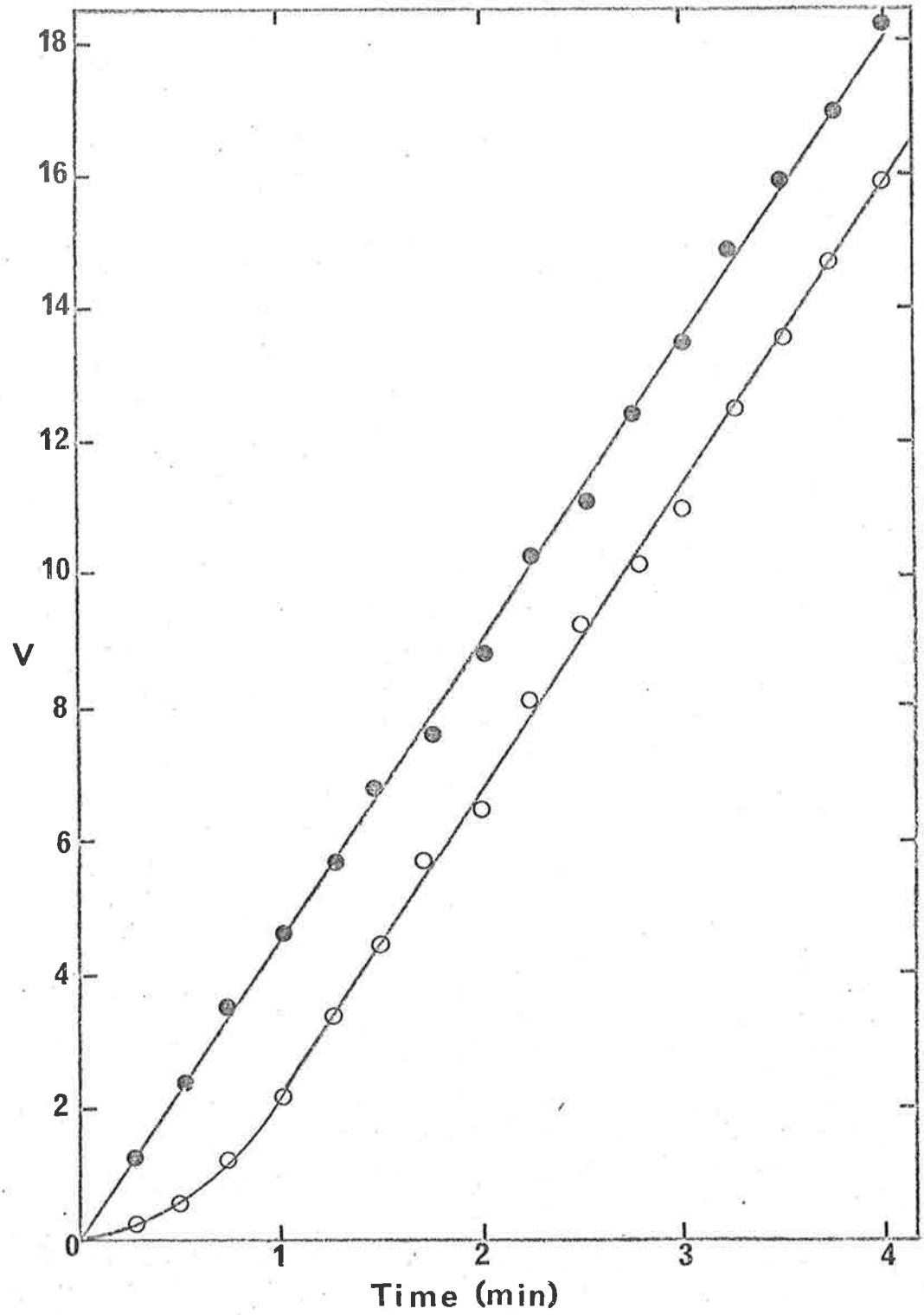


FIGURE 5.7. Time course of activation of pyruvate carboxylase by Mg^{2+}. The enzyme was previously incubated in the presence of 2 mM Mg^{2+} (●——●) and previously incubated in the absence of Mg^{2+} (O——O).

Although it has frequently been claimed that a change in the apparent K_m value for a ligand following the addition of an activator or inhibitor is due to a ligand-induced conformational change in the protein, this is not necessarily correct in a multireactant enzyme system. Therefore, to test whether the change in the apparent K_m value was due to a Mg^{2+}-induced conformational change in pyruvate carboxylase, the Mg^{2+} activation was studied using other criteria.

(a) Effect of temperature on Mg^{2+} activation

Hill plots for the activation of pyruvate carboxylase by Mg^{2+} over the temperature range 18.4° to 40° are shown in Fig. 5.8. Since the slopes of these lines over this temperature range are all approximately 1.0 it was concluded that there was only one Mg^{2+} atom binding per active site.

Table 5.2 shows that the value of K_a (see Equation 5.2) decreases with increasing temperature. From these data, thermodynamic parameters were determined. The standard entropy change ($\Delta S°$) is

$$\Delta S° = \frac{\Delta H° - \Delta F°}{T} \qquad (5.18)$$

where $\Delta H°$ is the standard enthalpy change, $\Delta F°$ the free energy change and T the absolute temperature. $\Delta H°$ was evaluated from the relationship

$$\ln K_a = \frac{\Delta H°}{RT} + \text{constant} \qquad (5.19)$$

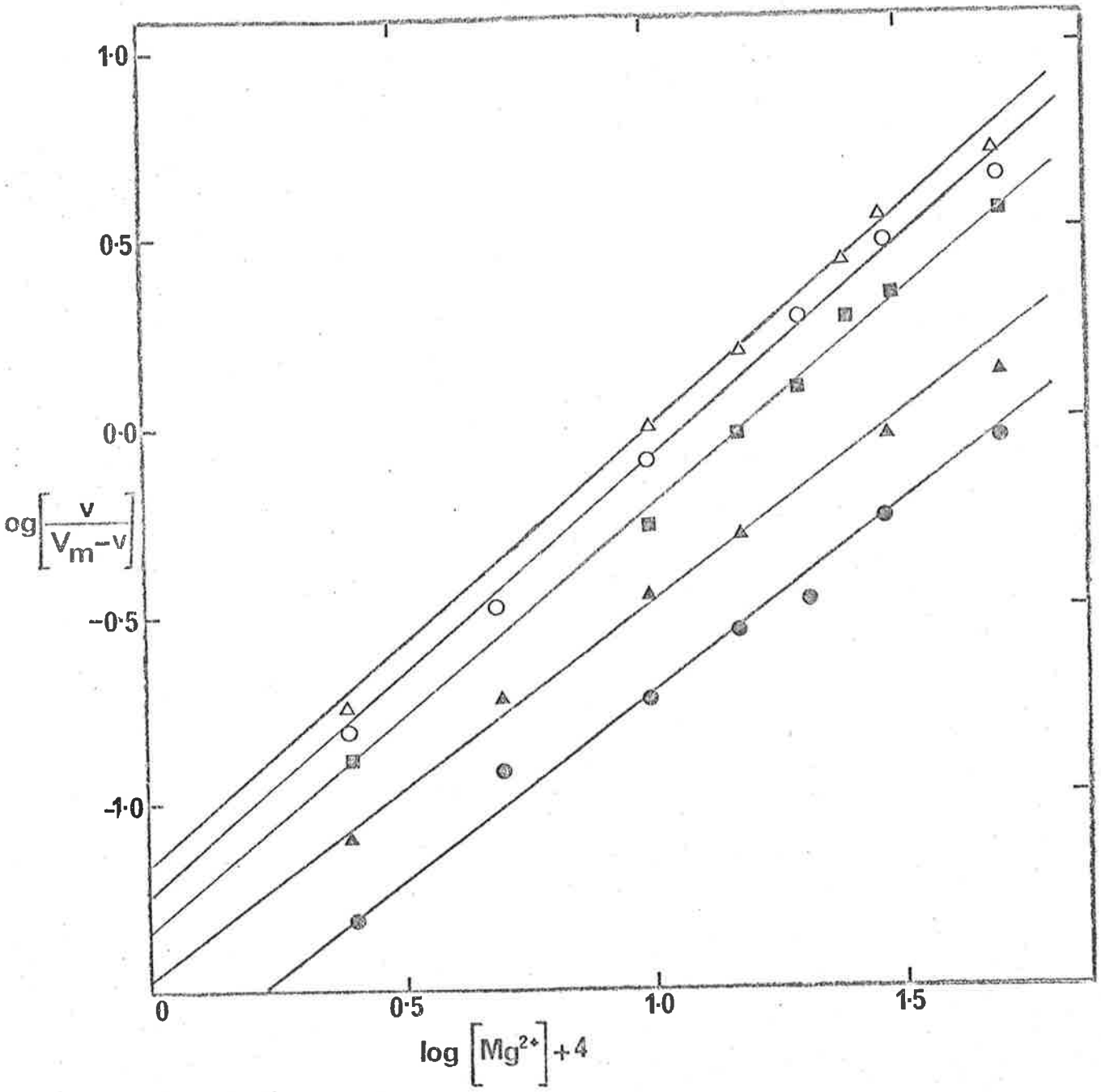


FIGURE 5.8. Hill plots for Mg^{2+} at various temperatures, showing the effect of varying the Mg^{2+} concentration with fixed $MgATP^{2-}$ concentration (6.6×10^{-4} M) at different temperatures. 18° (●——●); 24.8° (▲——▲); 30° (■——■); 35° (○——○); 40° (△——△).

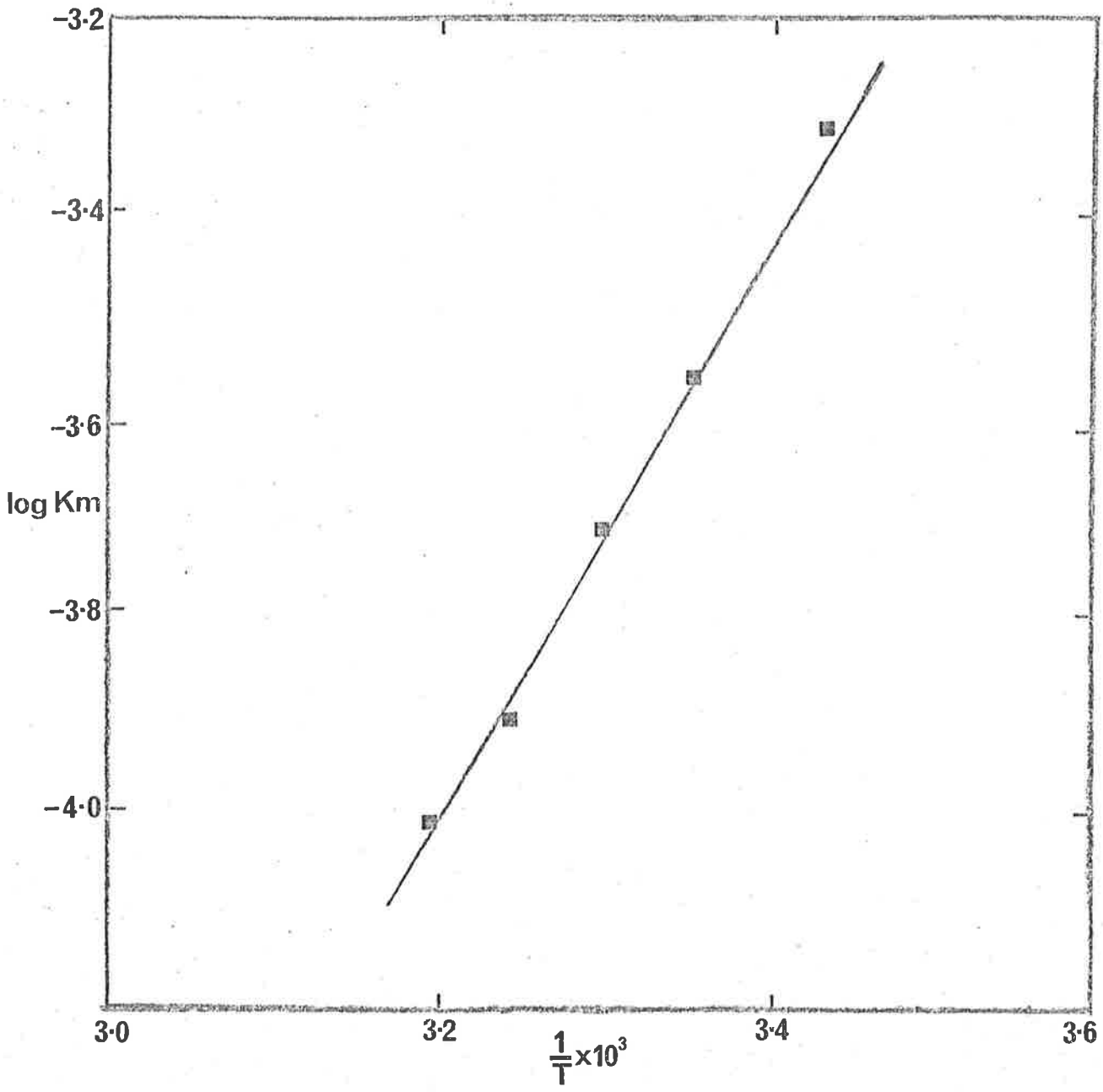


FIGURE 5.9. Determination of the ΔH° of activation for the Mg^{2+} activation of pyruvate carboxylase. Log K_a was plotted against the reciprocal of the absolute temperature. The apparent K_a values for Mg^{2+} at various temperatures were obtained from the data presented in Figure 5.8.

where R is the gas constant. A plot of log K_a as a function of the reciporcal of the absolute temperature (Fig. 5.9) gave a line with slope, $\Delta H°/2.3\ R$, from which $\Delta H°$ was calculated to be 12.6 kcal per mole. The change in free energy, $\Delta F°$ was derived from the relationship.

$$\Delta F° = -RT \ln K_a \qquad (5.20)$$

and this value together with $\Delta H°$ was used to derive $\Delta S°$ from Equation 5.18. Table 5.2 summarises the thermodynamic data showing that $\Delta S°$ was equal to 58.6 entropic units per mole, a value in the range which has been interpreted (Taketa and Pogell, 1965; Worcel, 1966; Edwards and Keech, 1968) to indicate a change in protein conformation.

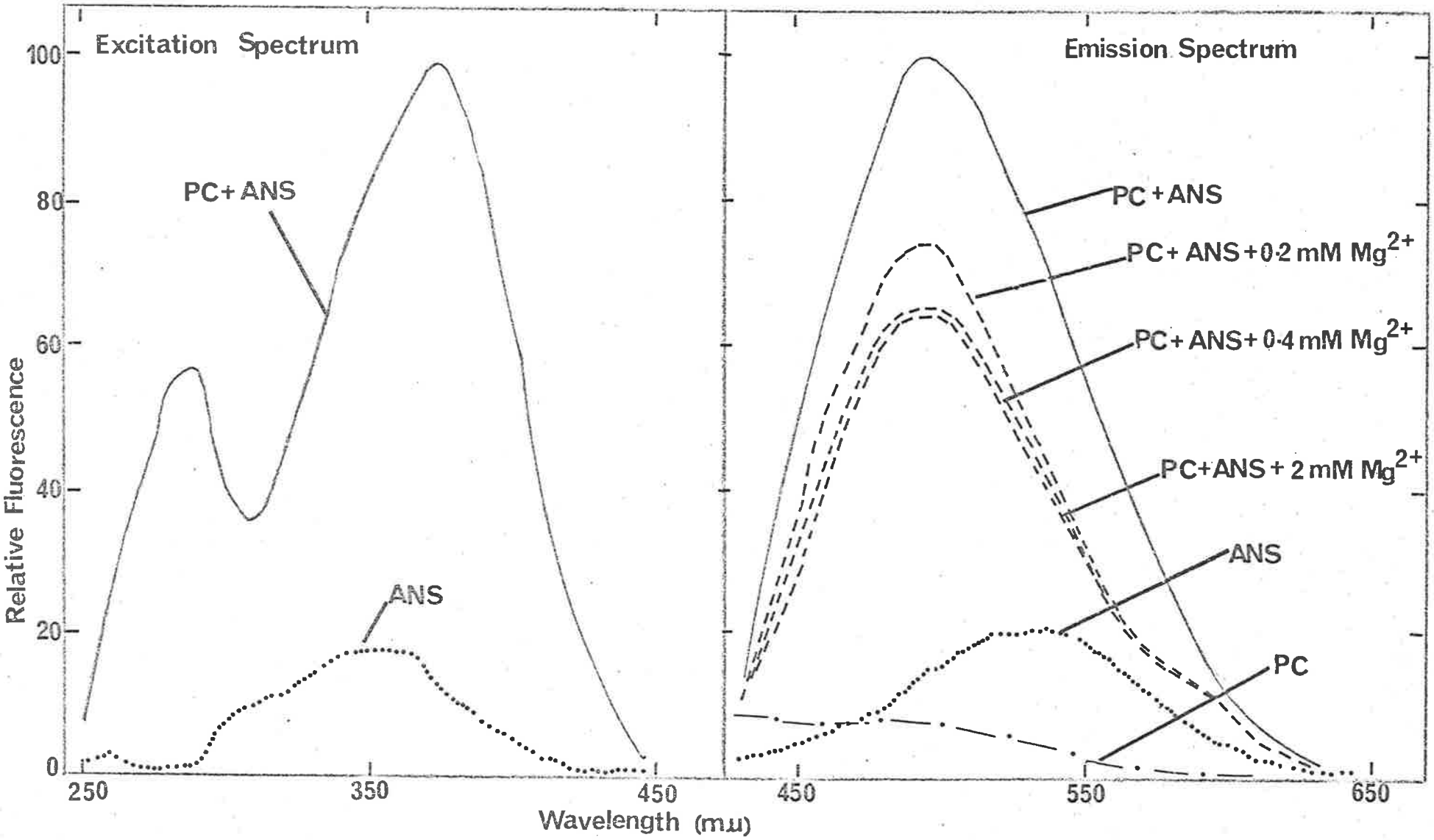
(b) Effect of Mg^{2+} on the Emission Intensity of an Enzyme-Dye Complex

The intensity of fluorescence emission of the fluorescent dye, 1-anilinonaphthalene-8-sulphonate (ANS), which is known to bind preferentially to hydrophobic regions of proteins, is related to its environment, i.e., it is sensitive to the polarity of the environment. This property makes this and other similar compounds ideal as microenvironmental probes since changes in emission intensity reflect changes in environment (Aoe et al., 1970; Edelman and McClure, 1968). The addition of pyruvate carboxylase to a dilute solution of ANS in Tris-Cl at pH 7.2 resulted in a large increase in fluorescence emission intensity (Fig. 5.10). The wavelength

TABLE 5.2. THE THERMODYNAMIC PROPERTIES OF Mg^{2+} ACTIVATION USING ΔH^{o} AS DETERMINED FROM FIG. 5.9. ΔF^{o} IS DETERMINED FROM EQUATION 5.20.

Temperature	Ka value ($\times 10^{-4}$ M)	ΔF^{o} (cal/mole)	ΔS^{o} (entropic units)
18.0	4.89	-4409	58.4
24.8	2.78	-4846	58.6
30.0	1.94	-5145	58.6
35.0	1.29	-5480	58.8
40.0	0.845	-5712	58.5

FIGURE 5.10. Fluorescence excitation and emission spectra of ANS bound to pyruvate carboxylase (PC). Emission intensity was recorded at 485 nm for the excitation spectrum. Excitation was at 370 nm for the emission spectrum. The mixture contained (final volume 0.5 ml); pyruvate carboxylase, 0.6 mg per ml; ANS, 10 μM and Tris-HCl pH 7.2, 50 mM. The spectra were also recorded at the Mg^{2+} concentrations as shown.


Excitation Spectrum
Emission Spectrum
PC+ANS
ANS
PC + ANS
PC + ANS + 0·2 mM Mg2+
PC + ANS + 0·4 mM Mg2+
PC+ANS + 2 mM Mg2+
ANS
PC
Relative Fluorescence
100
80
60
40
20
0
250
350
450
450
550
650
Wavelength (mμ)

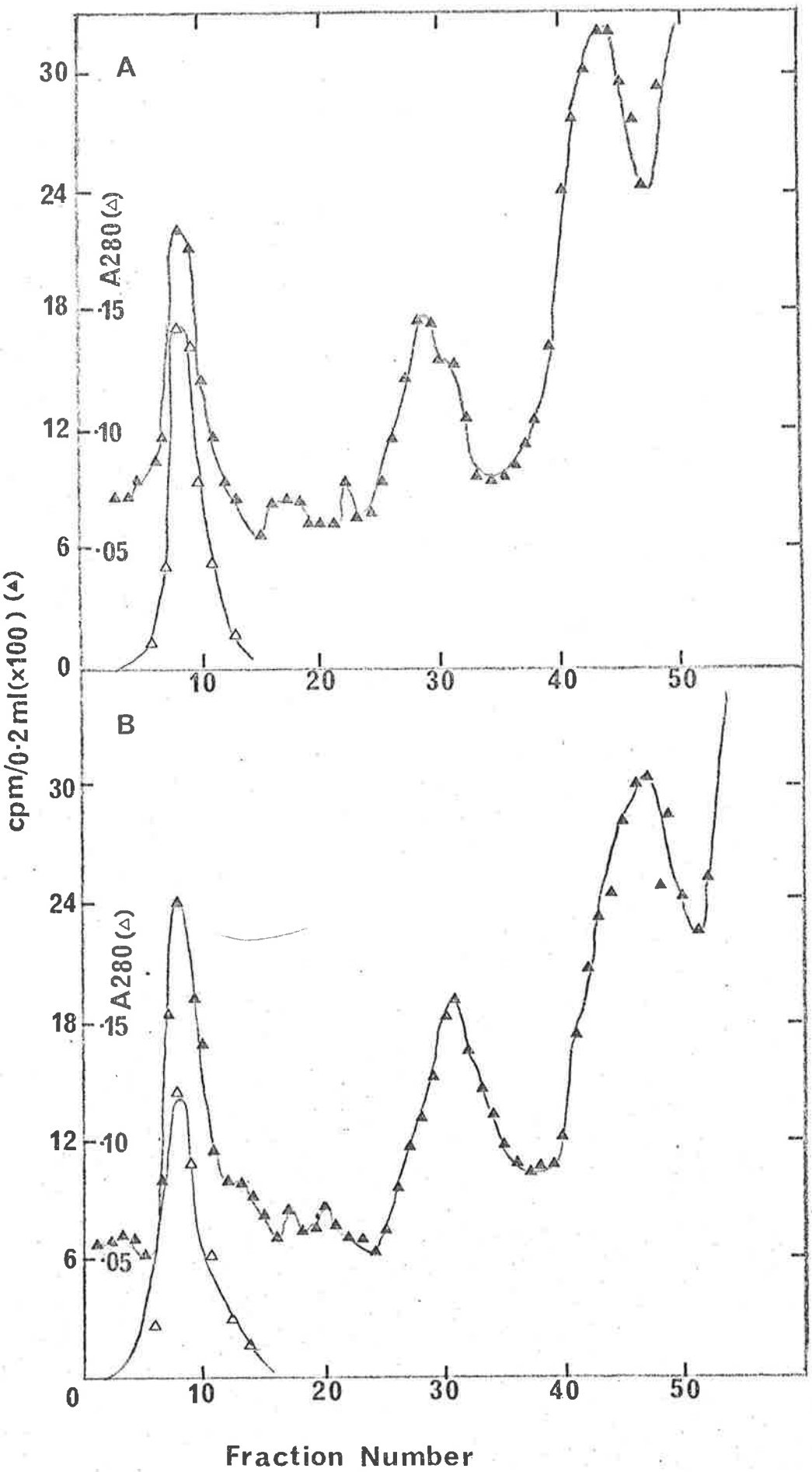
of maximum excitation was 370 nm and the emission peak appeared at 485 nm. Neither ANS enzyme, nor Mg^{2+} alone produced significant emission readings under these conditions. The fluorescence intensity of the above system was markedly lowered by Mg^{2+} with no change in the spectral position, indicating that Mg^{2+} produced a pronounced alteration in enzyme structure. It would also appear that this conformational change is a significant factor in the activation of sheep kidney pyruvate carboxylase because the change in fluorescence intensity occurs at a Mg^{2+} concentration in the region of K_a.

(c) Hydrogen exchange as a measure of solvent exclusion due to Mg^{2+}

The investigation of protein conformation by measurement of hydrogen exchange differs significantly from other approaches to the study of conformation in solution in that the extent of exchange reflects, the behaviour of protein molecules over a period of time or their history (Hvidt and Nielsen, 1966; Englander, 1967). Methods involving spectroscopic or hydrodynamic measurements give information only about the mean conformation at a specific moment. Thus because Mg^{2+} was shown to cause a conformational change, the interupted gel filtration technique of Schechter et al. (1969) has been used to obtain information about the kinetic distribution of the back exchanging hydrogen atoms.

Fig. 5.11 shows the extent of tritium back exchange in the presence and absence of 0.5 mM Mg^{+}. These data can be used to show that the ratio of radioactivity to protein

FIGURE 5.11. The tritium back-exchange of pyruvate carboxylase in the absence (A) and presence of Mg^{2+} (B). The enzyme was incubated either in the absence or presence of 0.5 mM Mg^{2+} and 5 μl of $^{3}H\ H_2O$ (10 Ci per ml) and the interrupted back exchange was studied on a Sephadex G-25 column (100 cm x 2.2) as described in the text.


A
B
30
24
18
12
6
0
·15
·10
·05
A280 (Δ)
10
20
30
40
50
cpm/0·2 ml (×100) (Δ)
Fraction Number

of the 6 hr. and 1 hr. stopped flow peaks in the presence of Mg^{2+} are 3.1 and 7.4 respectively, whereas in the absence of Mg^{2+} they are 2.8 and 6.1. This suggests that the enzyme has less exchangeable hydrogens exposed in the absence of Mg^{2+} than in the presence.

E. DISCUSSION

It has been pointed out that pyruvate carboxylases isolated from various sources require the addition of excess Mg^{2+} for maximum velocity (Keech and Barritt, 1967). Using sheep kidney pyruvate carboxylase, it was demonstrated that Mg^{2+} had at least two roles in the reaction mechanism and concluded that free Mg^{2+} stimulated the enzymic activity. The present investigation extends the previous observations and shows conclusively that Mg^{2+} activation of the enzyme is essential for enzymic activity.

The analysis of the kinetic data presented in this communication shows that the results are in agreement with a model that assumes the essential activation of pyruvate carboxylase by Mg^{2+} to form an active Mg^{2+}-E-$MgATP^{2-}$ complex. The results have been fitted to a rate equation for the model assuming essential Mg^{2+} activation as described by London and Steck (1969) and the constants describing the model show that the Mg^{2+} does not bind to the Mg^{2+}-enzyme complex and that excess ATP^{4-} can inhibit the reaction.

In addition to analysing the activation of sheep kidney pyruvate carboxylase by Mg^{2+}, the activation by Mn^{2+}

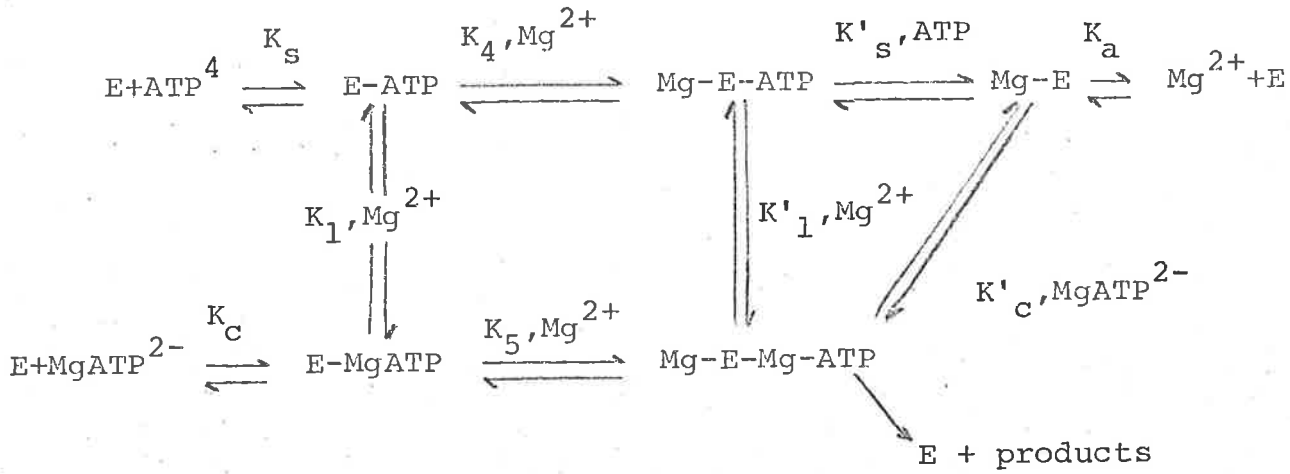
was also studied. However, in contrast to the data for the Mg^{2+}-activation only part of the data could be fitted to the essential activation model described by London and Steck (1969) and this gave an activation constant for Mn^{2+} of 8.7×10^{-6} M. Thus when compared with the activation by Mg^{2+} for which K_a is 4.30×10^{-4} M, the activation by Mn^{2+} occurs at much lower concentrations of the divalent cation. The other striking difference between the activation by the two cations is that for Mn^{2+}, once the maximum velocity has been reached there is very strong inhibition by any excess Mn^{2+}. This is evident in the M_t profile for Mn^{2+} (Fig. 5.5B) which shows that at concentrations greater than 1 mM there is inhibition of the enzymic activity.

Ashman et al. (1972) showed that sheep kidney pyruvate carboxylase can carry out an acetyl-CoA independent reaction and the Mg^{2+}-activation of this reaction has been investigated. The data fits the non-essential metal ion activation model described by London and Steck (1969). Thus because in the presence of acetyl-CoA the divalent metal ion is essential whereas for the independent reaction it is not, these results suggest that the metal ion activation is associated with the acetyl-CoA activation. A similar association between Mg^{2+} and acetyl-CoA has been found for the chicken liver enzyme for which it was shown that Mg^{2+} was cooperative at low acetyl-CoA concentrations (M.C. Scrutton, personal communication).

Keech and Barritt (1967) found that when the initial velocity of the overall reaction was plotted as a function of $MgATP^{2-}$ concentration the resultant profiles were sigmoidal.

From the model used here to establish an absolute requirement for Mg^{2+} it was not possible to make any conclusions on the number of $MgATP^{2-}$ binding sites because K_c' may be a function of more than one binding constant. The sigmoidal velocity response curves could be explained by the fact that since sheep kidney pyruvate carboxylase is a tetramer there may be homotropic cooperative interactions (Monod et al., 1965) existing between the different binding sites for $MgATP^{2-}$ on the four subunits.

However, an alternative explanation for the sigmoidicity could be as postulated by Sweeny and Fisher (1968) that there are alternate pathways to form the active complex, Mg^{2+}-E-$MgATP^{2-}$. A general mechanism to explain these results is shown in the following scheme:



This mechanism is consistent with the model used to establish the absolute requirement for Mg^{2+} because as stated by London and Steck (1969), $K_aK_c' = K_sK_c$ and $K_4K_s = K_aK_s'$. Therefore, it is not possible in this model to distinguish between the different pathways for the formation of the active complex Mg^{2+}-E-$MgATP^{2-}$ described by K_aK_s' and K_aK_s and also described by K_aK_c' and K_sK_c. The mechanism shows that all the species Mg^{2+}, ATP^{4-} and $MgATP^{2-}$ can combine with the free enzyme and that there are alternate pathways to form the only complex that will yield products, i.e., Mg^{2+}-E-$MgATP^{2-}$. The fact that values for constants for two of the pathways have been obtained suggests the validity of the above scheme.

The results from a time dependent study of the Mg^{2+} activation of sheep kidney pyruvate carboxylase suggested that Mg^{2+} induced a conformational change in the enzyme. Supporting this hypothesis is the fact that Mg^{2+} induced a $\Delta S°$ of 56.8 entropic units per mole and since the range of values for most chemical reactions is +10 to -30 entropic units per mole, this value can be interpreted to mean that Mg^{2+} caused a significant conformational change in the enzyme (Taketa and Pogell, 1965; Worcel, 1966; Edwards and Keech, 1968). Direct evidence for a conformational change has been obtained using the fluorescent probe 1-anilinonaphthalene-8-sulphonate and tritium back exchange. It was observed that when Mg^{2+} bound to pyruvate carboxylase a change in the fluorescence emission intensity of 1-anilinonaphthalene-8-sulphonate occurred thus providing additional evidence of a conformational change. This change

in fluorescence emission intensity could be due to either a change in the microenvironment of the probe or by a change in the number of dye molecules bound by the enzyme when the enzyme bound Mg^{2+}. However, both these explanations would still require Mg^{2+} to induce a conformational change in the enzyme. Also the tritium back-exchange experiment showed that when Mg^{2+} is present, pyruvate carboxylase changes conformation such that less hydrogen atoms are available for back-exchange.

The fact that sheep kidney pyruvate carboxylase has an absolute requirement for divalent metal ions in the acetyl-CoA dependent reaction may be of importance in regulating gluconeogenesis. It is known that many metabolites complex with both Mg^{2+} and Mn^{2+}, and thus the amount of free divalent metal ion may be limited in the mitochondria where sheep kidney pyruvate carboxylase is located (Ling and Keech, 1966). If this were true, then small fluctuations in the divalent metal ion concentrations could effect the activity of this enzyme as has been proposed for pyruvate carboxylase in rat liver (McClure and Lardy, 1971).

CHAPTER SIX

GENERAL DISCUSSION

A. PHYSICAL PROPERTIES

The physical studies on purified sheep kidney pyruvate carboxylase described in this work have shown the enzyme to be a tetrameric protein of molecular weight 480,000 ± 30,000. This quaternary structure and molecular weight is similar to the enzyme purified from both chicken liver (Valentine et al., 1966; Scrutton and Young, 1972) and rat liver (McClure et al., 1971a). It is also similar to other biotin containing enzymes isolated from animal sources (Moss and Lane, 1971) but differnt from pyruvate carboxylase isolated from bacterial sources (Cazzulo et al., 1970; Taylor et al., 1972) which are probably dimers.

The similarity of sheep kidney pyruvate carboxylase to other biotin containing enzymes from animal sources has been confirmed by comparing their amino acid composition using a computer analysis developed by Lance and Williams (1967a,b). This study showed that there were two classes of pyruvate carboxylases with the main distinguishing property being the cold lability of one of these groups. The difference in these two groups has been explained by mutations in the pyruvate carboxylase gene such that there is the replacement of alanine by threonine. However, it should be pointed out that this conclusion is only tentative and studies on the actual gene would be the only conclusive evidence.

The sheep kidney enzyme contains four moles of biotin per mole of enzyme but, in contrast to the enzyme isolated frm

chicken liver which contains four moles of Mn^{2+} per mole of enzyme, this enzyme contains only two moles of Mn^{2+}. This metal ion content for the sheep kidney enzyme is similar to that reported for rat liver pyruvate carboxylase (McClure et al., 1971a) which also contains two moles of Mn^{2+}. It is also apparent that Mg^{2+} does not make the metal ion content equal to the biotin content as is the case for calf liver pyruvate carboxylase (Scrutton et al., 1972).

The subunits of sheep kidney pyruvate carboxylase have been shown to be of molecular weight 110,000 - 120,000 by gel electrophoresis in the presence of SDS, ultracentrifugation in the presence of SDS or in the presence of guanidine hydrochloride.

In addition, Scrutton and Utter (1965) measured the sedimentation coefficient of the chicken liver enzyme in the presence of SDS, and observed a species with a sedimentation coefficient of 2.7S. Later, Valentine et al. (1966) interpreted this value to correspond to a molecular weight of 45,000. However, from ultracentrifugation carried out in the presence of SDS, it was found that low sedimentation coefficients are a general phenomenon of protein-SDS complexes and it is not necessary to postulate subunits of molecular weight of less than 120,000. This conclusion has recently been verified by Barden and Taylor (1973) who found that the chicken liver enzyme has subunits of molecular weight, 120,000 as determined by SDS polyacrylamide gel electrophoresis or ultracentrifugation in the presence of guanidine hydrochloride.

McClure et al. (1971a) found that when rat liver pyruvate carboxylase was denatured in 6.7 M guanidine hydrochloride and then carboxymethylated, they obtained six or possibly seven bands from SDS-polyacrylamide gel electrophoresis. However, when this experiment was carried out with the sheep kidney enzyme only one band was observed.

The subunits of sheep kidney pyruvate carboxylase have been found to be identical by peptide size-charge analysis. The tryptic peptides from the enzyme labelled with iodo[^{14}C]-acetic acid have been separated on a Sephadex G-25 column and then high voltage paper electrophoresis and the number of radioactive peptides determined by autoradiography. From this analysis there were ten peptides which is a quarter of the number of cysteine residues in the native enzyme. Thus this result would indicate that the subunits are identical.

B. DIVALENT METAL ION ACTIVATION

From analysis of the divalent cation activation of sheep kidney pyruvate carboxylase it was possible to show that the enzyme has an absolute requirement for the divalent cation. This is in contrast to the work of McClure et al. (1971a) who using the rat liver enzyme interpreted their results to mean that the requirement for Mg^{2+} was not absolute.

This absolute requirement for divalent metal ions is associated with the acetyl-CoA activation of the enzyme. It has been observed that the requirement for divalent cations in the acetyl-CoA independent reaction of the enzyme was not absolute. The association of the divalent cation activation

with the acetyl-CoA activation has also been observed for the chicken liver enzyme for which it was found that Mg^{2+} was cooperative at low acetyl-CoA concentrations (M.C. Scrutton, personal communication).

The divalent cation activation of sheep kidney pyruvate carboxylase causes a conformational change in the enzyme which has been shown by using thermodynamic and physical techniques. This type of conformational change of a protein by a divalent cation is quite common (Reynolds and Schlesinger, 1969; Mildvan, 1970; Hunt and Ginsburg, 1972) and for pyruvate carboxylase it may be that a change in enzyme structure is required before acetyl-CoA can bind. This will be resolved by binding studies but as pointed out by Ashman (1973), acetyl-CoA binding studies are not feasible at present because of the acetyl-CoA deacylase activity even in the purest preparations.

APPENDIX A

AN IMPROVED METHOD OF NON-LINEAR KINETIC PARAMETERS

A. THEORETICAL

In fitting curves which depend non-linearly on parameters to be estimated the iterative maximum likelihood procedure is usually preceded by a procedure to obtain initial values of the parameters. Each iteration on the maximum likelihood procedure then produces corrections to these values. Our suggestion is that instead of adding these corrections to the initial values, as is the normal procedure, use these corrections to find fitted values for the curve, which are then fed back, in place of the original observations, into the initial value routine (Figure A-1). The new values for the parameters are more accurate than those obtained simply by adding the corrections to the initial values. The procedure iterative. In the explanation of the theory, the fitting of the Michaelis-Menten equation is described. It should be pointed out that this example rarely requires the use of the method described because the normal method should suffice. However, the practical usefulness of the method is demonstrated in the fitting of the equation describing the activation of sheep kidney pyruvate carboxylase by the metal, Mg^{2+}, where it was found that the normal maximum likelihood method did diverge whereas the modified method converged.

In fitting a Michaelis-Menten curve

$$v = \frac{V_{max}\, s}{s + K_m} \qquad (1)$$

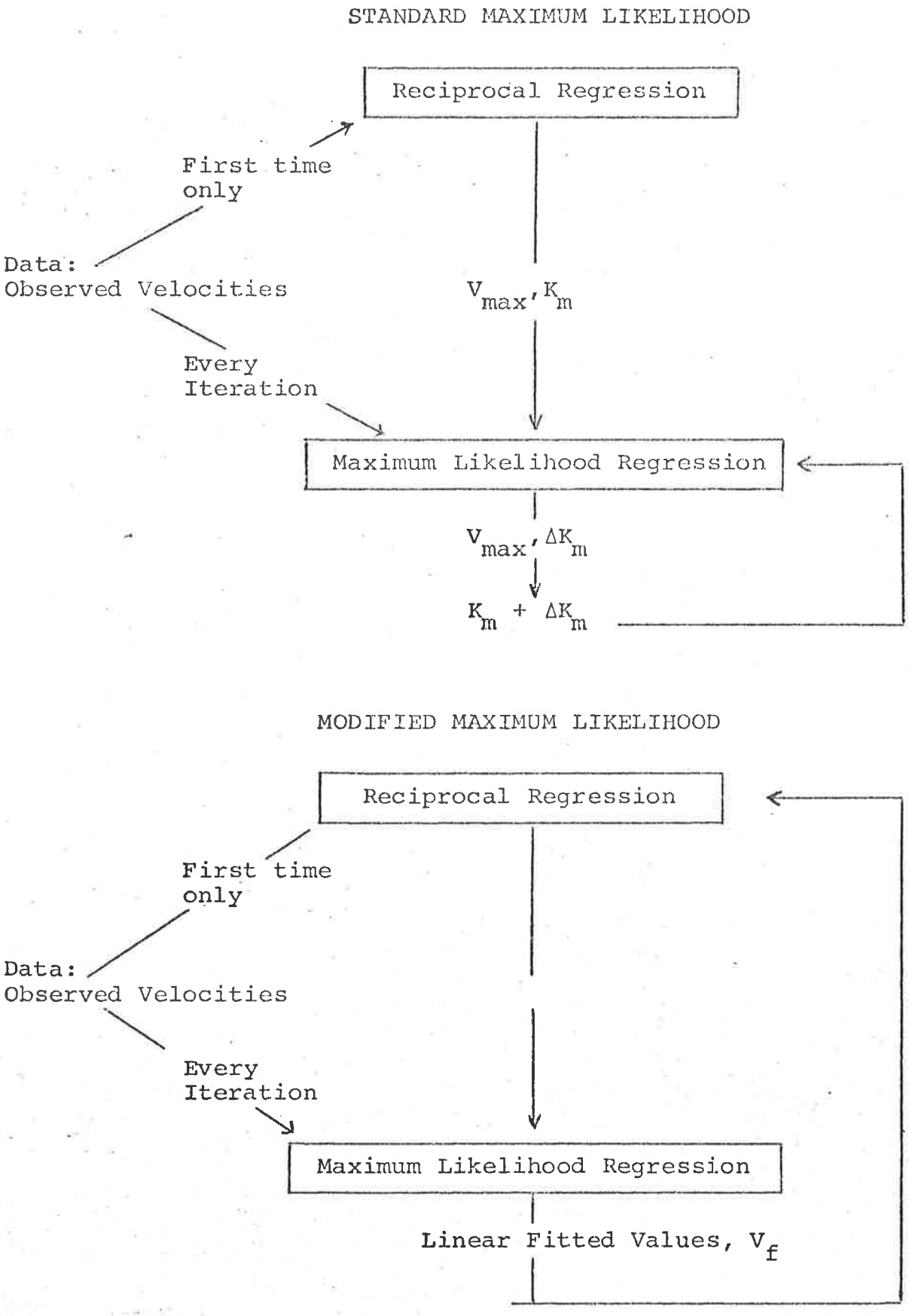


FIGURE A.1. Comparison of the normal and modified maximum likelihood methods.

to a number of observed velocities v, each corresponding to substrate concentrations s, initial values of the parameters V_{max} and K_m can be found by the usual double reciprocal regression

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{s} \qquad (2)$$

$$= \beta_o + \beta_1 z \qquad (3)$$

where $\beta_o = \frac{1}{V_{max}}$, $\beta_1 = \frac{K_m}{V_{max}}$ and $z = \frac{1}{s}$

From the estimates b_o, b_1 of β_o, β_1 obtained by running a simple regression routine of 1/v on z, the initial values

$$V_{max}^{(o)} = 1/b_o \text{ and } K_m^{(o)} = b_1/b_o \qquad (4)$$

can be obtained.

The initial value $K_m^{(o)}$ is fed into the maximum likelihood routine, which is based on the Taylor expansion of v with respect to K_m:

$$v = V_{max}\left(\frac{s}{s + K_m^{(o)}}\right) + (K_m - K_m^{(o)})V_{max} \frac{\partial}{\partial K_m}\left(\frac{s}{s + K_m}\right)_{K_m = K_m^{(o)}} \qquad (5)$$

$$= \alpha_1 z_1 + \alpha_2 z_2 \qquad (6)$$

where $\alpha_1 = V_{max}$, $\alpha_2 = (K_m - K_m^{(o)})V_{max}$, (7)

$$z_1 = \frac{s}{s + K_m^{(o)}}$$

$$z_2 = \frac{\partial}{\partial K_m} \left[\frac{s}{s + K_m}\right]_{K_m = K_m^{(o)}} \tag{8}$$

$$= - \frac{s}{(s + K_m^{(o)})^2} \tag{9}$$

From a subroutine, for the regression of the values of v on the corresponding values (z_1, z_2), estimates a_1, a_z of α_1, α_2 can be obtained from which one obtains improved estimates,

$$V_{max}^{(1)} = a_1 \tag{10}$$

and the correction ΔK_m to K_m given by

$$\Delta K_m = a_2/a_1. \tag{11}$$

The usual maximum likelihood procedure is to add ΔK_m to $K_m^{(o)}$ and use it in place of $K_m^{(o)}$ in a repetition of the second regression routine. In place of this we advocate that the estimates a_1, a_2 be used to calculate a set of fitted values of v:

$$v_f = a_1 z_1 + a_2 z_2 \tag{12}$$

and that the values of v_f so obtained be used instead of v in the initial reciprocal regression, that is, regress $1/v_f$ on z (equation 3) and from the new estimates $b_o^{(1)}$, $b_1^{(1)}$ calculate $K_m^{(1)} = b_1^{(1)}/b_o^{(1)}$.

The new value $K_m^{(1)}$ is then used together with the experimental values v in the maximum likelihood routine. The alternation of the initial routine using fitted values, v_f and maximum likelihood routine using observed values v can be continued until the sequence of values

$$K_m^{(o)}, K_m^{(1)}, K_m^{(2)}, K_m^{(3)} \quad \ldots \text{ converges.}$$

The new method combines the advantages of both reciprocal regression and maximum likehood, that is, reciprocal regression, being linear, is computationally efficient but informationally inefficient in the sense of statistical estimation theory. On the other hand, the maximum likelihood is informationally efficient, but being based on the Taylor approximation, computationally inefficient. When the results of the maximum likelihood are taken off as fitted values and put into the initial routine, they are reduced to improved estimates of the parameters in an appropriate non-linear way (the non-linear operation occurs in the taking of reciprocals $1/v_f$ of v_f).

B. FITTING A NUMBER OF PARAMETERS

The equation describing the essential activation of this enzyme by the metal Mg^{2+} which reacts with the reaction component, ATP^{4-}, is

$$v = \frac{V_{max} K_a K_c' (M_t - C)\ C}{K_a (M_t - C)[K_s' (S_t - C) + K_c' C + 1] + 1} \qquad (13)$$

where M_t, S_t and C are Mg^{2+}, ATP^{4-} and the $MgATP^{2-}$ complex respectively, and

$$K_a = \frac{(Mg^{2+})(E)}{(Mg^{2+}\text{-}E)} \qquad (14)$$

$$K_s = \frac{(Mg^{2+}\text{-}E)(ATP^{4-})}{(Mg^{2+}\text{-}E\text{-}ATP^{4-})} \qquad (15)$$

$$K_c' = \frac{(Mg^{2+}\text{-}E)(MgATP^{2-})}{(Mg^{2+}\text{-}E\text{-}MgATP^{2-})} \qquad (16)$$

and V_{max} is the maximum velocity. This is a modified version of the equation as derived by London and Steck (1969). The initial values of the parameters V_{max}, K_a, K_c' and K_s' were obtained using the reciprocal of equation (13), that is:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_s'}{V_{max}\,K_c'}\,\frac{(S_t-C)}{C} + \frac{1}{V_{max}\,K_c'}\,\frac{1}{C} + \frac{1}{V_{max}\,K_a K_c'}\,\frac{1}{(M_t-C)C} \tag{17}$$

and by doing a regression of $1/v$ on $(S_t-C)/C$, $1/C$ and $1/(M_t-C)C$ using a weighting on $1/v$ equal to v^2. This gave initial values $V_{max}^{(o)}$, $K_a^{(o)}$, $K_{s'}^{(o)}$ and $K_{c'}^{(o)}$. Corrections on these values were then obtained using the modified maximum likelihood routine, based on the Taylor expansion of v with respect to the parameters:

$$v = v^{(o)} + (V_{max}-V_{max}^{(o)}) \left[\frac{\partial v}{\partial V_{max}}\right] + (K_a-K_a^{(o)}) \left[\frac{\partial v}{K_a}\right] +$$

$$+ (K_c'-K_{c'}^{(o)}) \left[\frac{\partial v}{\partial K_c'}\right] + (K_s'-K_{s'}^{(o)}) \left[\frac{\partial v}{\partial K_s'}\right] \tag{18}$$

where $v^{(o)}$ is v evaluated at the initial values $V_{max}^{(o)}$, $K_a^{(o)}$, $K_{c'}^{(o)}$ and the derivatives

$$\frac{\partial v}{\partial V_{max}} = \frac{K_a K_c'(M_t-C)C}{K_a(M_t-C)[K_s'(S_t-C)+K_c'C+1]+1} \tag{19}$$

$$\frac{\partial v}{\partial K_a} = \frac{V_{max}\,K_c'(M_t-C)C}{\{K_a(M_t-C)[K_s'(S_t-C)+K_c'C+1]+1\}^2} \tag{20}$$

$$\frac{\partial v}{\partial K_c'} = \frac{V_{max} K_a (M_t-C) C\{K_a K_s' (M_t-C)(S_t-C)+K_a (M_t-C)+1\}}{\{K_a (M_t-C)[K_s' (S_t-C)+K_c' C+1] + 1\}^2} \quad (21)$$

$$\frac{\partial v}{\partial K_s'} = \frac{-V_{max} K_a^2 K_c' (M_t-C)^2 (S_t-C) C}{\{K_a (M_t-C)[K_s' (S_t-C)+K_c' C+1]+1\}^2} \quad (22)$$

are all evaluated at these same initial values.

Equation (18) is in the form

$$v = v^{(o)} + \alpha_1 z_1 + \alpha_2 z_2 + \alpha_3 z_3 + \alpha_4 z_4 \quad (23)$$

and from a regression of $(v-v^{(o)})$ on the corresponding values of (z_1, z_2, z_3, z_4) one can obtain $\alpha_1, \alpha_2, \alpha_3$ and α_4, the corrections to V_{max}, K_a, K_c' and K_s'. This regression was done using a weighting on v equal to $1/v^2$ and the new values of the parameters are

$$V_{max}^{(1)} = V_{max}^{(o)} + \alpha_1 \quad (24)$$

$$K_a^{(1)} = K_a^{(o)} + \alpha_2 \quad (25)$$

$$K_c'^{(1)} = K_c'^{(9)} + \alpha_3 \quad (26)$$

$$K_s'^{(1)} = K_s'^{(o)} + \alpha_4. \quad (27)$$

However, instead of using these new values in a repetition of the above maximum likelihood analysis new fitted values

v_f are obtained by

$$v_f = v^{(1)} + \alpha_1 z_1 + \alpha_2 z_2 + \alpha_3 z_3 + \alpha_4 z_4 \qquad (28)$$

and these values of v_f are then fed back into the inverse regression routine. This sequence of using fitted values for the inverse routine and the experimental data for the maximum likelihood routine is repeated until the values for all the parameters have converged (Table A-1). In this type of analysis, when the maximum likelihood procedure is done on the experimental data, the standard errors of the corrections are the standard errors of the parameters and as can be seen in Table A-1all the parameters are highly significant. The fitted curves are shown in Fig.5.2 It was found for this example that when the normal maximum likelihood routine was used, there was divergence in the analysis and true values for the parameters could not be obtained.

By fitting a series of such non-linear equations, describing different models, it was possible to show that Mg^{2+} and $MgATP^{2-}$ combine with the enzyme (E) in a random manner and form the final activated complex Mg^{2+}-E-$MgATP^{2-}$. It was also shown that excess ATP^{4-} is inhibitory because it shifts the equilibrium from the active complex Mg^{2+}-E-$MgATP^{2-}$ to inactive complexes, for example, Mg^{2+}-E-ATP^{4-}, which cannot form products. Furthermore, the analysis showed that the complex E-$MgATP^{2-}$ cannot break down to yield products.

TABLE A.1. ESTIMATION OF PARAMETERS FROM NON-LINEAR KINETIC PATTERNS

Cycle	V_{max}	K_a	K_c'	K_s'
Initial values	21665	2900	3029	3280
I	21754	2420	2735	2126
II	21562	2334	2859	2046
III	21553	2328	2868	2045
IV	21553	2327	2869	2045
V	21553	2327	2869	2045
Standard errors	2040	476	690	864
Marginal T-statistics	10.6	4.89	4.14	2.25
Probability on 59 degrees of freedom	< 0.001	< 0.001	< 0.001	< 0.05

The estimation of V_{max}, K_a, K_c' and K_s' for the activation of sheep kidney pyruvate carboxylase by Mg^{2+}. The table shows the initial values obtained for each constant and the convergence of these to their true value by using five cycles of the modified maximum likelihood routine. All the constants are statistically significant. The standard error of each constant is the standard error of the corrections given by the fifth maximum likelihood cycle on the experimental data.

C. DISCUSSION

The example that has been discussed uses reciprocal regression to estimate the initial values of the parameters. This method can be widely used to evaluate initial estimates of the parameters of rate equations because the equations describing both enzyme inhibition (Cleland, 1963) and enzyme activation (Dixon and Webb, 1964) can readily be put into linear reciprocal form. However, the method has wider application. The method has been adapted for the analysis of velocity versus substrate concentration curves which are to be fitted to the Hill equation (Hill, 1910) where the three point fit to the logistic method of estimating parameters as discussed by Bliss (1970) can be used to estimate the initial values of n, the interaction coefficient, V_{max} the maximum velocity and $K_{0.5}$ the substrate concentration required to give $v = V_{max}/2$. The modified maximum likelihood procedure can then be used to obtain the final values of these parameters and the value of n used to find whether the system was hyperbolic ($n = 1$) or sigmoidal ($n > 1$). Other examples of the uses of this procedure are the analysis of radioisotopic exchange data (Laiken and Printz, 1970) and in the analysis of macromolecule-ligand binding (Fletcher et al., 1970).

However, the method should be readily applicable to any non-linear system.

APPENDIX B.

A COMPUTER PROGRAM FOR FITTING NON-LINEAR KINETIC PARAMETERS

```
SYKABUU,T50,CM65000
RUN(S)
ALTLIB (P=STATLIB)
LGO
END OF RECORD
      FUNCTION FDIST(A,B,C) FDIST = 0 RETURN END
      FUNCTION SQRT(X)
      SQRT = 1.0
      IF(X.LT.1.E-20) RETURN
      SQRT = X** 0.5
      RETURN
      END

      PROGRAM MODEL (INPUT,OUTPUT)
      COMMON DZ(100),Z(100,4),TM(100),TS(100),C(100),V(100),X(100,4)F(1
      0)
      DIMENSION Y(100),YF(100),YF1(100),KV(100),VVF(100),SDEV(100)
      COMMON/WEIGHT/W(200)
      COMMON/REG/SSQ(10),B(30),SE(30)
      READ2,N
2     FORMAT(I2)
      READ1,(TM(I),TS(I),V(I),I=1,N)
1     FORMAT(F10.4,F10,F10.4)
      DO4I=1,N
      C(I)=(18000*TS(I)+18000*TM(I)+1)-(SQRT((18000*TS(I)+18000*TM
      (I)+1)
      **2-(4*18000*18000*TM(I)*TS(I))))
      YF(I)=V(I)
4     C(I)=C(I)/36000
      DO123II=1,8
      CALL FIRST (N,YF,TS,TM,C,100)
      CALL CORRECT (N,YF,TS,TM,C,B,II)
      PRINT 12,(B(I),I=1,4)
12    FORMAT(//,*  VB = *,E15.6,5X,*AK = *,E15.6,5X,*CK =*E15.6,5x,
      *SK = *,E15.6)
      PRINT 19,(V(I),YF(I), I=1,N)
19    FORMAT(4(2E15.6))
123   CONTINUE
      END

      SUBROUTINE FIRST(N,YF,TS,TM,C,M)
      DIMENSION BCV(4),X(100,4),YF(100),Y(100),TM(I),TS(I),C(I)
      COMMON/WEIGHT/W(200)
      COMMON/REG/SSQ(10),B(30),SE(30)
C     CALCULATE INITIAL VALUES
      DO4I=1,N
      W(I)=YF(I)**2
      X(I,1)=(TS(I)=C(I))/C(I)
      X(I,2)=1.
      X(I,3)=1/C(I)
```

```
      X(I,4)=1/((TM(I)-C(I))*C(I))
4     Y(I)=1/YF(I)
      CALL REGAL (N,4,X,100,Y,O,O,1)
      RETURN
      END

      SUBROUTINE CORECT (N,YF,TS,TM,C,BIV,M)
      DIMENSION BIV(4),DZ(100),X(100,4),YF(100),Y(100),F(100),
      VEL(100,10)
      DIMENSION TM(1),TS(1),C(1)
      COMMON/WEIGHT/W(200)
      COMMON/REG/SSQ(10),B(30),SE(30)
C     CALCULATE CORRECTIONS ON INITIAL VALUES
      VB=1/BIV(2)
      SK=BIV(1)/BIV(3)
      AK=BIV(3)/BIV(4)
      CK=BIV(2)/BIV(3)
      DO4I=1,N
      VEL(I,M)=YF(I)
      DZ(I)=(AK*SK*(TM(I)-C(I))*(TS(I)-C(I)))+(AK*CK*(TM(I)-C(I))*
      C(I))+
      (AK*(TM(I)-C(I)))+1
      F(I)=(VB*CK*AK*(TM(1)-C(I))*C(I)/DZ(I)
      X(I,1)=(AK*CK*(TM(I)-C(I))*C(I))/DZ(I)
      X(I,2)=(CK*(TM(I)-C(I))/(DZ(I)**2)
      X(I,2)=X(I,2)*VB
      X(I,3)=((AK*(TM(I)-C(I))*C(I))*((S(I)*AK*(TM(I)-C(I))*(TS(I)-
      C(I)))+
      (AK*(TM(I)-C(I)))+1))/DZ(I)**2
      X(I,3)=X(I,3)*VB
      X(I,4)=-(AK*AK*CK*(TM(I)-C(I))*(TM(I)-C(I))*(TS(I)-C(I)*C(I))/
      DZ(I)**2
      X(I,4)=X(I,4)*VB
      W(I)=1/VEL(I,1)**2
4     Y(I)=VEL(I,1)-F(I)
      CALL REGAL(N,4,X,100,Y,0,0,1)
      VB=VB+B(1)
      AK=AK+B(2)
      CK=CK+B(3)
      SK=SK+B(4)
      PRINT12,VB,AK,CK,SK
12    FORMAT(//,*  VB =  *,E15.6,5X, AK  =  *,E15.6,5X,*CK = *,
      E15.6,5X,*SK = *,E15.6
      DO11I=1,N
11    YF(I)=F(I)+B(1)*X(I,2)+B(3)*X(I,2)+B(3)*X(I,3)+B(4)*X(I,4)
      RETURN
      END
END OF RECORD
```

```
59      NO OF POINTS - FORMAT I2
0.002     0.002     888.0          DATA MG  CONC. ATP CONC. VELOCITY
                                   FORMAT F10,4,F10,4,F10.4
  "         "         "
  "         "         "
  "         "         "            TO 59TH DATA CARD
END OF RECORD
END OF FILE
```

BIBLIOGRAPHY

Ahmad, F., Lygre, D.G., Jacobsen, B.E. and Wood, H.G. (1972). J. Biol. Chem., 247, 6299.

Alberts, A.W. and Vagelos, P.R. (1968). Proc. Nat. Acad. Sci. U.S., 59, 561.

Alberts, A.W., Nervi, A.M. and Vagelos, P.R. (1969). Proc. Nat. Acad. Sci. U.S., 63, 1319.

Aoe, H., Sarngadharan, M.G. and Pogell, B.M. (1970). J. Biol. Chem., 245, 6383.

Archibald, W.J. (1947). J. Phys. Colloid. Chem., 51, 1204.

Arndt, F. (1943). Org. Syn. Coll., 2, 461.

Ashman, L.K. (1973). Ph.D. Thesis, University of Adelaide.

Ashman, L.K., Keech, D.B., Wallace, J.C. and Nielsen, J. (1972). J. Biol. Chem., 247, 5818.

Baldwin, R.L. (1957). Biochem. J., 65, 503.

Barden, R.E. and Taylor, B.L. (1973). Fed. Proc., 32, 510.

Barden, R.E., Fung, C.H., Utter, M.F. and Scrutton, M.C. (1972). J. Biol. Chem., 247, 1323.

Blair, J.M. (1969). FEBS Letters, 2, 245.

Bliss, C.I. (1970). In Statistics in Biology, Vol. 2. McGraw-Hill Inc., New York.

Bronck, J.R. and Kielley, W.W. (1957). Biochim. Biophys. Acta, 24, 440.

Brintzinger, H., Prijs, B. and Erlenmeyer, H. (1960). Experimentia, 16, 468.

Carey, W.F. and Wells, J.R.E. (1970). Biochem. Biophys. Res. Commun., 41, 574.

Cazzulo, J.J. and Stoppani, A.O.M. (1969). Biochem. J., 112, 747 and 755.

Cazzulo, J.J., Sundaram, T.K. and Kornberg, H.L. (1970). Proc. Roy. Soc. London, B, 176, 1.

Cleland, W.W. (1963). Biochim. Biophys. Acta, 67, 173.

Cleland, W.W. (1967). Adv. in Enzymology, 29, 1.

Cohen, R., Giraud, B. and Messiah, A. (1967). Biopolymers, 5, 203.

Cohn, M. and Hughes, T.R. (1962). J. Biol. Chem., 237, 176.

Coleman, J.E. and Vallee, B.L. (1960). J. Biol. Chem., 235, 390.

Creeth, J.M. and Knight, C.G. (1965). Biochim. Biophys. Acta, 102, 549.

Dimroth, P., Guchhait, R.B., Stoll, E. and Lane, M.D. (1970). Proc. Nat. Acad. Sci. U.S., 67, 1353.

Dixon, M. and Webb, E.C. (1964). In Enzymes, p.420. London, Longmans, Green & Co. Ltd.

Edelman, G.M. and McClure, W.D. (1968). Acc. Chem. Res., 1, 65.

Edwards, J.B. and Keech, D.B. (1968). Biochim. Biophys. Acta, 159, 167.

Englander, S.W. (1967). In Poly-α-amino acids, G.D. Fasman (Ed.) Dekker, N.Y., p.339.

Feir, H.A. and Suzuki, I. (1969). Can. J. Biochem., 41, 697.

Feldberg, R.S. and Datta, P. (1971). Eur. J. Biochem., 21, 447.

Fish, W.W., Reynolds, J.A. and Tanford, C. (1970). J. Biol. Chem., 245, 5166.

Fletcher, J.E., Spector, A.A. and Ashbrook, J.D. (1970). Biochemistry, 9, 4580.

Garnett, J.L., Halpern, B. and Kenyon, R.S. (1972). J. Chem. Soc. Chem. Commun., 1972, 135.

Gerwin, B.I., Jacobsen, B.E. and Wood, H.G. (1969). Proc. Nat. Acad. Sci. U.S., 64, 1315.

Goodwin, T.W. and Morton, R.A. (1946). Biochem. J., 40, 628.

Green, N.M. (1965). Biochem. J., 94, 23C.

Greenspan, M.D. and Lowenstein, J.M. (1968). J. Biol. Chem., 243, 6273.

Gregolin, C., Ryder, E., Kleinschmidt, A.K., Warner, R.C. and Lane, M.D. (1966). Proc. Nat. Acad. Sci. U.S., 56, 148.

Griffith, I.P. (1972). Biochem. J. 126, 553.

Gross, C. and Labouesse, B. (1969). Eur. J. Biochem., 7,463.

Guchhait, R.B., Moss, J., Sokalaki, W. and Lane, M.D. (1971). Proc. Nat. Acad. Sci. U.S., 68, 653.

Halsall, H.B. (1967). Nature, 215, 880.

Harding, H.W.J. (1971). Ph.D. Thesis, University of Adelaide.

Hatch, M.D. and Stumpf, P.K. (1961). J. Biol. Chem., 236, 77.

Hegre, C.S. and Lane, M.D. (1966). Biochim. Biophys. Acta, 128, 172.

Hers, H.G. (1952). Biochim. Biophys. Acta, 8, 424.

Hersch, R.T. and Schachman, H.K. (1958). Virology, 6, 234.

Hill, A.V. (1910). Biochem. J., 7, 471.

Hoch, A. and Huber, G. (1956). Biochem. Z., 328, 44.

Hunt, J.B. and Ginsburg, A. (1972). Biochemistry, 11, 3723.

Hvidt, A. and Nielsen, S.O. (1966). Adv. Protein Chem., 21, 287.

Irias, J.J., Olmsted, M.R. and Utter, M.F. (1969). Biochemistry, 8, 5136.

Jacobsen, B., Gerwin, B.I., Ahmad, F., Walgell, P. and Wood, H.G. (1970). J. Biol. Chem., 245, 6471.

Jarrett, I.G., Filsell, O.H. and Ballard, F.J. (1973). Hormone and Metab. Res. (in press).

Kagi, J.M.R. and Vallee, B.L. (1960). J. Biol. Chem., 235, 3188.

Kawahara, K. (1969). Biochemistry, 8, 2551.

Kaye, M.A.G. (1955). Biochim. Biophys. Acta, 18, 456.

Kaziro, Y., Leone, E. and Ochoa, S. (1960). Proc. Nat. Acad. Sci. U.S., 46, 1319.

Kaziro, Y., Ochoa, S., Warner, R.C. and Chen, J.Y. (1961). J. Biol. Chem., 236, 1917.

Keech, D.B. and Barritt, G.J. (1967). J. Biol. Chem., 242, 1983.

Kemp, D.J. (1972). Ph.D. Thesis, University of Adelaide.

Kerley, M. and Leaback, D.H. (1957). Biochem. J., 67, 245.

Klotz, I.M., Langerman, N.R. and Darnall, D.W. (1970). Ann. Rev. Biochem., 39, 33.

Lamm, O. (1929). Z. Physik. Chem., A143, 177.

Lance, G.N. and Williams, W.T. (1967a). Aust. Comp. J., 1, 15.

Lance, G.N. and Williams, W.T. (1967b). The Computer J., 9, 373.

Laiken, S.L. and Printz, M.P. (1970). Biochemistry, 9, 547.

Layne, E. (1957). Methods in Enzymology, 3, 447.

Liebecq, C. and Jacquemotte-Louis, M. (1958). Bull. Soc. Chem. Biol., 40, 67.

Lindskog, S. and Malmstrom, B.G. (1960). Biochem. Biophys. Res. Commun., 2, 213.

Ling, A.M. and Keech, D.B. (1966). Enzymologia, 30, 367.

London, W.P. and Steck, T.L. (1969). Biochemistry, 8, 1761.

Losoda, M., Canovas, J.L. and Ruiz-Amil, M. (1964). Biochem. Z., 340, 60.

Lowry, O.H., Nira, J., Rosenbrough, A., Farr, L. and Randall, R.J. (1951). J. Biol. Chem., 193, 265.

Maling, J.E., Tasbovich, L.T. and Blois, M.S. (1963). Biochem. J., 3, 79.

Malmstrom, B.G. and Rosenberg, A. (1959). Adv. Enzym., 21, 131.

Mandelkern, L., Krigbaum, W.R., Sheraga, H.A. and Flory, P.J. (1952). J. Chem. Phys., 20, 1392.

McClure, W.D. and Edelman, G.M. (1966). Biochemistry, 5, 1908.

McClure, W.R. and Lardy, H.A. (1971). J. Biol. Chem., 246, 3591.

McClure, W.R., Lardy, H.A. and Kneifel, H.P. (1971a). J. Biol. Chem., 246, 3569.

McClure, W.R., Lardy, H.A., Wagner, M. and Cleland, W.W. (1971). J. Biol. Chem., 246, 3579.

McCormick, D.B. and Roth, J.A. (1970). Anal. Biochem., 34, 226.

McCormick, W.G. and Levedahl, B.H. (1959). Biochim. Biophys. Acta, 34, 303.

Means, G.E. and Feeney, R.E. (1971). In Chemical Modification of Proteins. Holden-Day Inc., p.220.

Michl, H. (1951). Monatsch. Chem., 82, 484.

Mildvan, A.S. (1970). The Enzymes, 2, 445.

Mildvan, A.S. and Scrutton, M.C. (1967). Biochemistry, 6, 2978.

Mildvan, A.S., Scrutton, M.C. and Utter, M.F. (1966). J. Biol. Chem., 241, 3488.

Monod, J., Wyman, J. and Changeux, J.P. (1965). J. Mol. Biol., 12, 88.

Moss, J. and Lane, M.D. (1971). Adv. in Enzymology, 35, 321.

Moss, J., Yamagishi, M., Kleinschmidt, A.K. and Lane, M.D.

(1972). Biochemistry, 11, 3779.

Nelson, C.A. (1971). J. Biol. Chem., 246, 2895.

Nervi, A.M. and Alberts, A.W. (1970). Fed. Proc. 29, 333.

Neuberg, C. and Mandel, I. (1949). Arch. Biochem. Biophys., 23, 499.

Neville, D.M. (1971). J. Biol. Chem., 246, 6238.

Offord, R. (1965). Nature, 211, 591.

O'Sullivan, W.J. and Perrin, D.D. (1964). Biochemistry, 3, 18.

Phillips, K.C., George, P. and Rutman, R. (1966). J. Amer. Chem. Soc., 88, 2631.

Phillips, R. (1966). Chem. Rev. 66, 501.

Piez, K.A. and Morris, L. (1960). Anal. Biochem., 1, 187.

Plocke, D.J. and Vallee, B.L. (1962). Biochemistry, 1, 1039.

Reynolds, J.A. and Schlesinger, M.J. (1969). Biochemistry, 8, 588.

Reynolds, J.A. and Tanford, C. (1970a). J. Biol. Chem., 245, 5161.

Reynolds, J.A. and Tanford, C. (1970b). Proc. Nat. Acad. Sci. U.S., 66, 1002.

Rimai, L. and Heyde, M.E. (1970). Biochem. Biophys. Res. Commun., 4, 313.

Sanger, F. and Thompson, E.O.P. (1963). Biochim. Biophys. Acta, 71, 468.

Schachman, H.K. (1957). Methods of Enzymology, 4, 32.

Schachman, H.K. (1959). Ultracentrifugation in Biochemistry, Academic Press, New York and London.

Schechter, A.N., Moravek, L. and Anfinsen, C.B. (1969). J. Biol. Chem., 244, 4981.

Schroeder, W.A., Jones, R.T., Cormick, J. and McCalla, K. (1962). Anal. Chem., 34, 1570.

Scrutton, M.C. and Fung, C.H. (1972). Arch. Biochem. Biophys., 150, 636.

Scrutton, M.C. and Mildvan, A.S. (1970). Arch. Biochem. Biophys., 140, 131.

Scrutton, M.C. and Utter, M.F. (1965). J. Biol. Chem., 240, 1.

Scrutton, M.C. and Utter, M.F. (1965b). J. Biol. Chem., 240, 3714.

Scrutton, M.C. and Utter, M.F. (1968). Anal. Rev. Biochem., 37, 249.

Scrutton, M.C. and Young, M.R. (1972). The Enzymes, 6, 1.

Scrutton, M.C., Griminger, P. and Wallace, J.C. (1972). J. Biol. Chem., 247, 3305.

Scrutton, M.C., Keech, D.B. and Utter, M.F. (1965). J. Biol. Chem., 240, 574.

Scrutton, M.C., Olmsted, M.R. and Utter, M.F. (1969). Methods in Enzymology, 13, 235.

Scrutton, M.C., Reed, G.H. and Mildvan, A.S. (1973). Adv. Exptal. Med. and Res., (in press).

Scrutton, M.C., Utter, M.F. and Mildvan, A.S. (1966). J. Biol. Chem., 241, 3480.

Scrutton, M.C., Young, M.R. and Utter, M.F. (1970). J. Biol. Chem., 245, 6220.

Sheraga, H.A. and Mandelkern, L. (1953). J. Amer. Chem. Soc., 75, 179.

Simon, E.J. and Shemin, D. (1953). J. Amer. Chem. Soc., 75, 2520.

Smith, H.M. (1968). In Handbook of Biochemistry, Chemical Rubber Company, Cleveland, U.S.A. pC63.

Spicer, S.S. (1952). J. Biol. Chem., 199, 301.

Squires, R.F. (1965). Biochem. Biophys. Res. Commun., 19, 27.

Steinhardt, H., Jones, D.E. and Kustin, K. (1968). J. Amer. Chem. Soc., 90, 7110.

Stryer, L. (1965). J. Mol. Biol., 13, 482.

Svedberg, T. and Petersen, K.O. (1940). The Ultracentrifuge. Oxford University Press, New York.

Sumper, M. and Rierpertinger, C. (1972). Eur. J. Biochem., 29, 237.

Sundaram, T.K., Cazzulo, J.J. and Kornberg, H.L. (1969). Biochim. Biophys. Acta, 192, 355.

Sweeny, J.R. and Fisher, J.R. (1968). Biochemistry, 7, 561.

Taketa, K. and Pogell, B.M. (1965). J. Biol. Chem., 240, 651.

Tanford, C. (1962). J. Amer. Chem. Soc., 84, 4240.

Taylor, B.L., Barden, R.E. and Utter, M.F. (1971). Fed. Proc., 30, 1057.

Taylor, B.L., Barden, R.E. and Utter, M.F. (1972). J. Biol. Chem., 247, 7318.

Taylor, P.H., Nielsen, J. and Keech, D.B. (1969). Biochem. Biophys. Res. Commun., 37, 723.

Utter, M.F. and Keech, D.B. (1960). J. Biol. Chem., 235, PC17.

Utter, M.F. and Keech, D.B. (1963). J. Biol. Chem., 238, 2603.

Utter, M.F. and Scrutton, M.C. (1969). Current Topics in Cell. Reg., 1, 253.

Utter, M.F., Keech, D.B. and Scrutton, M.C. (1964). Adv. in Enz. Reg., 2, 49.

Utter, M.F., Scrutton, M.C., Young, M.R., Tolbert, B., Wallace, J.C., Irias, J.J. and Valentine, R.C. (1967). Proc. VII Int. Congr. Biochem. (Tokyo), 2, 247.

Vallee, B.L. and Riordan, J.F. (1969). Ann. Rev. Biochem., 38, 539.

Vallee, B.L. and Wacker, W.E.C. (1970). The Proteins, Vol. 5.

Vallee, B.L., Rupley, J.A., Coombs, T.L. and Neurath, H. (1958). J. Amer. Chem. Soc., 78, 5879.

Valentine, R.C., Wrigley, N.G., Scrutton, M.C., Irias, J.J. and Utter, M.F. (1966). Biochemistry, 5, 3111.

Walaas, E. (1958). Acta Chem. Scand., 12, 528.

Weber, K. and Osborn, M. (1969). J. Biol. Chem., 244, 2406.

Williams, D.E. and Reisfeld, R.A. (1964). Ann. N.Y. Acad. Sci., 121, 373.

Williams, R.J.P. (1954). Special Lectures Biochem., University College, London, p.100.

Wimhurst, J.M. and Manchester, K.L. (1970). Biochem. J., 120,79.

Woods, K.R. and Wang, K.T. (1967). Biochim. Biophys. Acta, 133, 369.

Worcel, A. (1966). Biochim. Biophys. Acta, 113, 178.

Young, M.R., Tolbert, B. and Utter, M.F. (1969). Methods in Enzymology, 13, 250.

Zamenhof, S. (1957). Methods in Enzymology, 3, 702.

Zikan, J. and Bennett, J.C. (1973). Biochim. Biophys. Acta, 317, 447.