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PYRUVATE CARBOXYLASE : ASPECTS OF THE REACTION MECHANISM

A thesis submitted by

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CONTENTS

SUMMARY

1. The interaction between pyruvate carboxylase and Mg^{2+} and acetyl CoA has been investigated. Using indirect binding studies the k_a and n_H values for acetyl CoA have been determined in the presence and absence of Mg^{2+}. The results have shown that the activator which is present in the enzyme assay solution is Mg^{2+}-acetyl CoA. This allosteric effector binds to the enzyme in a Michaelis-Menten manner. The site of interaction of Mg^{2+} with acetyl CoA is the 3' ribose phosphate on the adenosine moiety.

It has also been shown that acetyl CoA also binds to the enzyme non-cooperatively and thus the observed Hill coefficient has been attributed to be due to a function of the non-saturation of substrates and dilution inactivation of the enzyme at low concentrations of acetyl CoA.

2. The kinetics of oxalate inhibition have been characterized in order to gain further insight on the possible role of the bound manganese metal ion in the catalytic reaction. There is evidence to suggest that biotin$\sim CO_2$ binding sites exist and that the bound metal is in close proximity to both the pyruvate binding site and the biotin$\sim CO_2$ binding region within the transcarboxylation site. Since oxalate exhibited competitive

kinetics with respect to pyruvate and biotin$\sim CO_2$, it was postulated that oxalate was in fact a transition state analogue of the carboxyl transfer intermediate.

3. The biotin carboxylation reaction has been reviewed in an attempt to explain the enzyme specific ATP/ADP exchange and the facile reactivity of the biotin cofactor. Phosphointermediates have subsequently been isolated by trapping with diazomethane. These products have been identified as phosphoserine and phosphobiotin. A new reaction mechansim for pyruvate carboxylase has therefore been proposed to incorporate this additional information.

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.

JENNY A. DUC

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ABBREVIATIONS

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

AcCoA	acetyl Coenzyme A.
ANS	1-analinonaphtalene-8-sulphonate.
AOPCP	α,β-methylene adenosine diphosphate.
APS	adenosine 5'-phosphosulphate.
bio	biotin.
C.D.	circular dichroism.
CoA	coenzyme A.
DTE	dithioerythritol.
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
Enz	enzyme.
NEM	N-ethylmorphiline.
NMR	nuclear magnetic resonance.
OAA	oxaloacetate.
P_i	orthophosphate.
PMR	phosphorus magnetic resonance.
Ser	serine.
TNBS	2,4,6-trinitrobenzene sulphonic acid.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Pyruvate carboxylase (pyruvate:CO_2 ligase (ADP) EC.6.4.1.1) is a member of the group of enzymes containing covalently bound biotin. Several of these enzymes have been studied in detail and strong similarities are apparent. Therefore it is useful to be aware of the properties of other biotin carboxylases when considering the action of pyruvate carboxylase. The biotin containing enzymes have been the subject of a recent comprehensive review by Wood and Barden, (1977).

1.1 SOME GENERAL PROPERTIES OF PYRUVATE CARBOXYLASE

Since pyruvate carboxylase was first discovered in 1960 (Utter and Keech, 1960) it has been extensively studied, therefore only a few relevant sections will be discussed herein.

1.1.1 Quaternary structure

Chicken liver pyruvate carboxylase had been shown by Valentine et al., (1966) to have a tetrameric structure with the subunits arranged in the corners of a square. The structure was found to be dissociated by incubating at 2°C, in agreement with ultracentrifugal studies which indicated that the native enzyme dissociated into inactive monomers under these conditions (Scrutton and Utter, 1965). Similar structures had subsequently been found in turkey and calf liver preparations of the enzyme (Utter, et al., 1975). The enzyme isolated from

yeast however, was different, bearing a rhomboidal arrangement of subunits (Valentine, 1968).

1.1.2 The bound metal content

Since pyruvate carboxylase isolated from chicken liver mitochondria was shown to be a manganese metalloprotein (Scrutton et al., 1966) a number of investigators have shown that the enzyme isolated from a variety of other sources is also a metalloprotein, with manganese, magnesium or zinc as a tightly bound constituent (Scrutton and Young, 1972). The metal ion content has been measured to be 4 g atoms per mole of enzyme. However, in some species of pyruvate carboxylase, the metal ion content is different, for example, rat and calf liver and sheep kidney have been reported to contain 2 g atoms of manganese per mole of enzyme (McClure et al., 1971a; Scrutton and Young, 1972; Bais, 1974) and the enzyme isolated from Pseudomonas citronellolis does not even appear to be a metalloprotein (Taylor B. unpublished results).

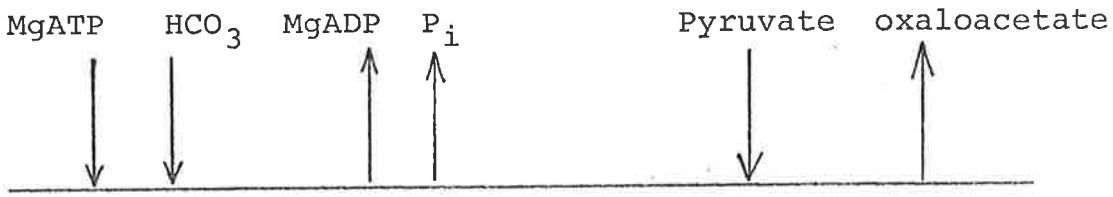
1.2 REACTIONS CATALYSED BY PYRUVATE CARBOXYLASE

1.2.1 The pyruvate carboxylation reaction

The major reaction catalysed by pyruvate carboxylase is represented in equation 1.1.

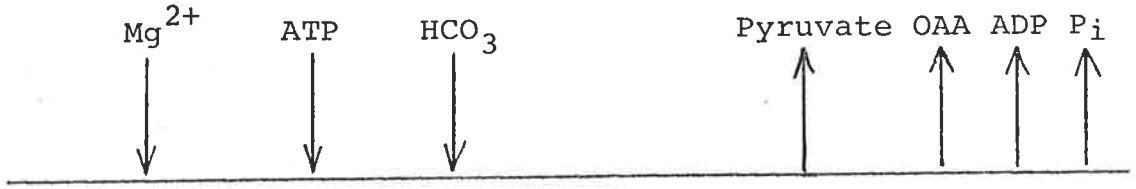
$$MgATP + HCO_3 + \text{pyruvate} \xrightarrow{Mg^{2+},\ \text{acetyl CoA}} MgADP + P_i + \text{oxaloacetate} \qquad \ldots\ldots\ldots 1.1$$

From initial rate kinetics and product inhibition studies, elucidation of some of the reaction mechanism has been obtained. With respect to rat, chicken and sheep liver pyruvate carboxylases, the overall reaction (equation 1.1) was considered to comply with a non-classical bi-bi-uni-uni ping-pong sequence (McClure et al., 1971b, Barden et al., 1972, Ashman and Keech, 1975) as represented below.



The non-classical nature of the sequence arises from the requirement that the binding sites for the two partial reactions must bind their substrates at equilibrium and must be totally independent of each other.

However, Warren and Tipton (1974a,b,c), using pyruvate carboxylase from pig liver, have suggested that equation 1.1 may be described by a sequential sequence in which no products of the reaction are released until all the substrates have bound.



The binding of MgATP and bicarbonate is an ordered process (c.f. Ashman and Keech, 1975, published a rapid equilibrium ordered process for sheep liver pyruvate

carboxylase) and occurs after rapid random equilibrium binding of Mg^{2+} and acetyl CoA.

The active species of carbon dioxide has been shown to be indeed the bicarbonate anion (Cooper et al., 1968) for the chicken liver pyruvate carboxylase reaction and also in reactions catalysed by another biotin enzyme, propionyl CoA carboxylase (Kaziro et al., 1962).

An intermediate in the pyruvate carboxylation reaction has been shown to be biotin$\sim CO_2$, since this intermediate has been able to be isolated (Lynen, 1961). In view of this finding and also the suggestion that the overall reaction proceeds by a ping-pong mechanism, two partial reactions can be written,

$$MgATP + HCO_3 + E^{bio} \overset{Mg^{2+},\ acetyl\ CoA}{\rightleftharpoons} MgADP + P_i + E^{bio\sim CO_2} \qquad .....1.2$$

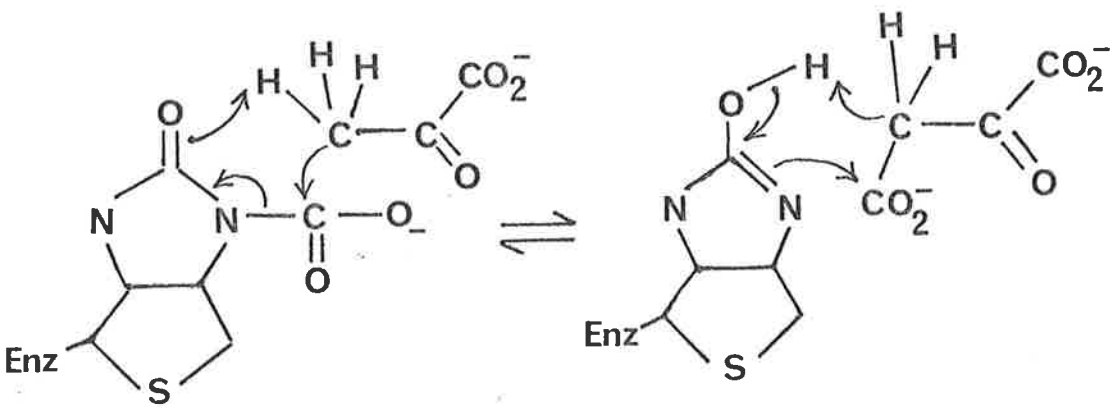
$$E^{bio\sim CO_2} + pyruvate \rightleftharpoons oxaloacetate + E^{bio} \qquad .....1.3$$

Carboxylation of the biotin occurs on the 1'-nitrogen position in the ureido functional group (Lynen, 1961, Guchhait, 1974).

To account for the participation of biotin$\sim CO_2$ in equations 1.2 and 1.3, and their mutual independence, Lynen (1961), proposed that although the biotin molecule is anchored to the enzyme through a covalent linkage at its side chain, it is free to migrate between two

catalytic sites. It was also postulated that biotin∿CO_2 had a distinct binding site at each of these partial reaction sites and it could be envisaged that the binding of MgATP and bicarbonate promoted biotin to move into the first partial reaction site where it would be readily carboxylated, and then biotin∿CO_2 could pass into the transcarboxylation site where it would in turn carboxylate pyruvate. The fact that a dissociable biotin∿CO_2 binding site could exist was substantiated by the observation that free biotin could be carboxylated by β-methyl-crotonyl CoA carboxylase (Lynen, 1961).

Biotin∿CO_2 thus interacts with pyruvate to produce oxaloacetate with retention of configuration around the substituted carbon (Rose, 1970). This would imply that the carboxylation of pyruvate is a concerted process and no significant intermediates would occur. One model regarding the mechanism of carboxylation has been proposed by Rétey and Lynen (1965) and involves decarboxylation of biotin in the enol form.



A mechanism for the stabilization of the enol form of biotin was not discussed by these workers.

1.2.1.1 A role for the bound metal

A catalytic role for the bound metal ion has been proposed for the reactions catalysed by the enzyme isolated from chicken liver (Scrutton et al., 1966), yeast (Scrutton et al., 1970) and calf and turkey liver (Scrutton et al., 1972). The observation that chicken liver pyruvate carboxylase was a manganese metalloprotein, coupled with extensive NMR studies of the enzyme prompted the suggestion that the bound metal participated in the pyruvate carboxylation process by forming a bridge between pyruvate and the enzyme (Mildvan et al., 1966; Mildvan and Scrutton, 1967). Furthermore, it was proposed that the metal ion facilitated pyruvate transcarboxylation because of its electron withdrawing properties (Mildvan et al., 1966).

NMR studies have shown that substrates and inhibitors of the transcarboxylation reaction specifically perturb the enhancement of the water proton longitudinal relaxation rate caused by the enzyme-bound manganese (Mildvan et al., 1966). This led to the suggestion that pyruvate was directly chelated to the manganese,

forming a bridge type structure.

However, recent studies using [^{13}C]pyruvate have shown that the distance between pyruvate and manganese is too large for direct chelation to occur (Fung et al., 1973). The metal activation model was therefore revised; it was proposed that there is a tightly bound water molecule between the metal and pyruvate. According to this scheme, the manganese ion promotes the acidity of the water molecule which is then responsible for the electrophilic activation of pyruvate.

As pointed out by Utter et al., (1975), the validity of this model has not been unequivocally established. For example, pyruvate may form a slowly exchanging inner sphere complex with manganese which could not be detected by the NMR techniques that have been used (Reed et al., 1972; Mildvan, 1974).

There is however, strong evidence that the bound metal atom has no direct role in the pyruvate carboxylase catalysed reaction. Substitution of different metal ions into the enzymes isolated from yeast (Co^{2+} for Zn^{2+}, Scrutton and Young, 1972) and chicken liver (Mg^{2+} for Mn^{2+}, Scrutton et al., 1972) results in only small changes in catalytic properties

of these enzymes.

In view of this evidence, it would seem that although the bound metal would appear to be near the transcarboxylation site, it would not seem to participate directly in the catalytic reaction.

1.2.1.2 Free metal ion requirement

The biotin carboxylation reaction catalysed by all pyruvate carboxylases examined so far has been shown to depend on the presence of both monovalent and divalent metal ions. Substantial differences in the extent of monovalent ion activation and specificity have been observed, (Scrutton and Young, 1972) and it has been suggested that factors such as, the associated anion play an important part in determining the specific monovalent ion role in the overall reaction.

The divalent cation appears to have two separate roles. Firstly, pyruvate carboxylase has been shown to utilize MgATP rather than ATP as a substrate (Keech and Barritt, 1967; Cazzulo and Stoppani, 1967, 1969; Feir and Suzuki, 1969; Cazzulo *et al.*, 1970; McClure *et al.*, 1971a; Bais and Keech, 1972; Warren and Tipton, 1974b). Secondly, Mg^{2+} seems

also to activate the enzyme in some way, since the optimal divalent metal ion concentration is several fold higher than the concentration of nucleotide required for maximal activity.

1.2.1.3 Activation by acetyl CoA

Initial velocity kinetic studies have shown a marked activation of reaction velocity upon addition of acetyl CoA, (Scrutton and Utter, 1965; Barritt et al., 1966) and also a decrease in the k_m values for pyruvate and bicarbonate (Ashman et al., 1972). When reaction rate was measured at varying concentrations of acetyl CoA, and the results were plotted graphically, a sigmoid curve was obtained. A Hill coefficient constant (n_H) was calculated from this data, and a value of approximately 3 was obtained for the avian enzyme and a value of approximately 2 for the mammalian enzyme. This has been interpreted to indicate that acetyl CoA binds to the enzyme with homotropic cooperativity.

The existence of discrete activator and catalytic sites within pyruvate carboxylase was therefore postulated. Ashman et al., (1973) and Scrutton and White (1973), have shown that the sheep and rat enzymes respectively can be desensitized to acetyl CoA activation by treatment with the chemical modifier, trinitrobenzene sulphonic

acid (TNBS). Since the substrates did not offer any protection against this modification, this data was the first direct indication of an allosteric site in this enzyme.

1.2.2 Acetyl CoA independent pyruvate carboxylation

Pyruvate carboxylases from a number of sources exhibit considerable variation in the degree of dependence on acetyl CoA for catalytic activity. Avian enzymes are nearly entirely dependent on the presence of acetyl CoA. The pyruvate carboxylases from mammalian origin are less dependent on acetyl CoA for catalytic activity (Ashman et al., 1972; Scrutton and White, 1972).

It has been observed that the acetyl CoA independent activity may approach approximately 25% of the activity in the presence of acetyl CoA. The enzyme from yeast shows a further decrease in dependence on an acetyl CoA activator (Cazzulo and Stoppani, 1969) while pyruvate carboxylase from Pseudomonas citronellolis has apparently no dependence on acetyl CoA whatsoever (Seubert and Remberger, 1961). Conditions when acetyl CoA independent activity is optimal require much higher concentrations of substrates and also a substantial increase in ionic strength. Treatment of the sheep liver enzyme with TNBS results in an increase in acetyl CoA independent activity (Ashman et al., 1973) however no significant effect was observed with TNBS modified rat liver enzyme.

1.2.3 The isotopic exchange reactions

As discussed previously, the ping-pong mechanism for pyruvate carboxylation predicts the release of MgADP and orthophosphate prior to the binding of pyruvate, and thus two independent partial reactions as depicted in equations 1.2 and 1.3 would exist. Therefore isotopic exchange between [^{32}P]orthophosphate and MgATP, and [^{14}C]pyruvate and oxaloacetate would be expected. A number of isotopic exchange reactions have been characterized for chicken, sheep and rat liver enzyme (Scrutton and Utter, 1965; Ashman, 1973; McClure et al., 1971c). Collectively these can be represented as follows,

$$[^{32}P] - P_i + MgADP + E^{bio\sim CO_2} \underset{\rightleftharpoons}{\xrightarrow{Mg^{2+},\ acetyl\ CoA}} [^{32}P]\text{-}MgATP + HCO_3 + E^{bio}$$

$$[^{14}C]\text{-}MgADP \underset{\rightleftharpoons}{\xrightarrow{Mg^{2+}}} [^{14}C]\text{-}MgATP$$

$$[^{14}C]oxaloacetate + E^{bio} \underset{\rightleftharpoons}{\xrightarrow{acetyl\ CoA}} E^{bio\sim CO_2} + [^{14}C]\text{-}pyruvate$$

$$[^{14}C]\text{-}HCO_3 + MgADP + P_i + E^{bio} \underset{\rightleftharpoons}{\xrightarrow{Mg^{2+},\ acetyl\ CoA}} E^{bio\sim {}^{14}CO_2} + MgADP + P_i$$

$$\downarrow\uparrow\ pyruvate$$

$$[^{14}C]\text{-}oxaloacetate + E^{bio}$$

The ATP/ADP has been shown to be specific for chicken liver pyruvate carboxylase (Scrutton and Utter, 1965). It is known to still occur in the presence of

avidin and essentially the only requirement for this exchange to take place is the presence of Mg^{2+}. The ATP/P_i exchange is totally biotin dependent and in the absence of acetyl CoA essentially no exchange takes place. Under optimal conditions, the rate of exchange of ^{32}P-orthophosphate into MgATP is approximately 2% of the overall forward reaction. Acetyl CoA is not an essential activator of the pyruvate/oxaloacetate exchange although this compound and also K^+ ions substantially stimulate this partial reaction allowing the exchange rate to proceed at approximately 50% of the overall reaction rate. The HCO_3/oxaloacetate exchange is entirely dependent on the presence of MgADP and is essentially an equilibrium of the total forward reaction and the complete reverse reaction.

1.2.4 The acetyl CoA deacylase activity

Highly purified preparations of pyruvate carboxylase from chicken liver (Scrutton and Utter, 1967), and sheep liver (Ashman et al., 1972) catalyse the slow deacylation of acetyl CoA. Maximal stimulation of this activity requires the presence of pyruvate, ATP and Mg^{2+}. Specific inhibitors of pyruvate carboxylase, for example, avidin or oxalate, do not effect the rate of hydrolysis with the exception of DTNB which causes partial inhibition.

1.3 RECENT DEVELOPMENTS IN SHEEP LIVER PYRUVATE CARBOXYLASE

1.3.1 Quaternary structure

Recently Goss et al., (1977), came to the conclusion that both sheep and chicken liver pyruvate carboxylases are tetramers with the subunits arranged in a rhombic configuration. The square planar tetrameric structure of Valentine et al., (1966) was attributed to a contaminant in the preparation of the enzyme which fortuitously behaved very similarly to pyruvate carboxylase. A number of important implications arise from this discovery. Firstly, if it is to be assumed that the subunits of the enzyme are identical (Bais, 1974), then a rhomboid tetrameric arrangement could only be generated by heterologous binding domains. This type of binding site is much less stable than homologous binding sites,and thus the maintenance of a relatively unstable structure during the course of evolution is in itself a phenomenon. However, Goss (1978), has presented evidence which suggests that the subunits are not identical. This fact would easily account for a rhomboid structure of the enzyme. Very few proteins have been shown to possess all subunits to be non-identical.

1.3.2 An explanation for the downward turning pyruvate kinetics

The double reciprocal plot of velocity against pyruvate concentration is concave downward (Scrutton and

Utter, 1965; Ashman, 1973; Nakashima et al., 1975). This was initially interpreted to indicate either negative cooperativity or that pyruvate bound to different forms of the enzyme. While measuring the rate of pyruvate/oxaloacetate exchange, Easterbrook-Smith et al., (1976b), observed that at low concentrations of pyruvate there was a nett hydrolysis of biotin∿CO_2 at the transcarboxylation site. Usually the pyruvate carboxylase reaction velocity is determined by measuring the rate of production of oxaloacetate. Therefore at low concentrations of pyruvate it was now realized that the overall reaction velocity was being underestimated due to a possible hydrolysis of biotin∿CO_2.

Therefore it was decided to measure the reaction velocity with varying pyruvate concentrations by the rate of orthophosphate release. The double reciprocal plots obtained from such an experiment were linear. Furthermore, when the ratio of the amount of orthophosphate released to the amount of oxaloacetate produced is plotted as a function of pyruvate concentration, at saturating levels of pyruvate, the ratio approached unity but as the pyruvate concentration was decreased the ratio increased.

1.3.3 A reappraisal of the reaction pathway

The reaction pathway catalysed by pyruvate carboxylase has been re-examined by Easterbrook-Smith et al., (1978) using two independent experimental approaches not previously applied to the enzyme. When the reaction

velocity was determined as a function of varying MgATP concentrations using pyruvate, 3-fluoropyruvate and 2-ketobutyrate as alternative carboxyl acceptor substrates, the slopes of the double reciprocal plots were significantly different. This result clearly indicated a sequential mechanism since a non-sequential or ping-pong pathway would have yielded plots with identical slopes.

The dependence of initial velocity as measured by orthophosphate release, rather than oxaloacetate formation, using varying concentrations of either MgATP or HCO_3 at various fixed concentrations of pyruvate was investigated. With the variable stoichiometry of oxaloacetate formation no longer a consideration, the double reciprocal plots obtained were families of straight intersecting lines indicative of a sequential mechanism.

1.3.4 A specific ATP/ADP exchange for sheep liver pyruvate carboxylase

Ashman (1973) working with sheep kidney pyruvate carboxylase, attributed the measurable ATP/ADP exchange in the presence of only enzyme and Mg^{2+} and no other reaction components to be due to adenylate kinase which would be a contaminant in the preparations of sheep kidney pyruvate carboxylase. The main reasons for this conclusion were that higher specific activity enzyme showed a lower rate of this exchange, and also that incubation of the enzyme with [^{14}C]ADP resulted in the incorporation of

radioactivity into both ATP and AMP in a 1:1 ratio.

However this appears not to be the case for sheep liver pyruvate carboxylase. Goss and Hudson (unpublished results) were able to detect an ATP/ADP exchange under the same conditions as Ashman, (1973), which could not be attributed solely to adenylate kinase contamination. These workers observed that the ATP/ADP exchange rate was approximately twice the rate of the ATP/P_i exchange. In the presence of avidin, although the ATP/P_i exchange was completely abolished, the ATP/ADP exchange was only reduced by half. These conclusions were deduced after adenylate kinase activity was taken into account.

1.3.5 Acetyl CoA spans the active site

While monitoring the rates of both the ATP/P_i and pyruvate/oxaloacetate exchange reactions, Clements *et al.*, (1978), observed that acetyl CoA was not the sole activator of these individual partial reactions. The ATP/P_i exchange was found to be stimulated equally well by CoA and other analogues which contained the complete adenine moiety of acetyl CoA. On the other hand the pyruvate/oxaloacetate exchange was stimulated by analogues which contained the acetyl pantetheine portion of acetyl CoA. These results could therefore be accounted for by postulating that acetyl CoA spans the active site with the adenine portion binding in the first partial reaction site and the acetyl pantetheine

segment binding at the transcarboxylation site.

Studies on the exchange reactions have also generated some light on the cause of inactivation of the enzyme upon dilution to below 4 U/ml. It was found that only the carboxylation site was inactivated and this effect could be decreased by introducing a non-polar environment around the enzyme, for example by the addition of polyethylene glycol. Therefore at this stage it seems likely that dilution inactivation is due to hydration of the carboxylation site of the enzyme, and that acetyl CoA protects against this process by blocking or "capping" the site thereby prohibiting the entry of water molecules into this region which would result in disruption of protein structure.

In view of these recent developments in areas which were thought to be reasonably well understood, it was decided to reinvestigate some other aspects of the pyruvate carboxylase reaction in the hope of obtaining a better understanding of the mechanism of catalysis of this enzyme.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Enzymes and proteins

Malate dehydrogenase (L-malate:NAD oxidoreductase, EC 1.1.1.37), from pig heart, lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27), type I from rabbit heart, glutamate:pyruvate transaminase (L-alanine:2-oxoglutarate aminotransferase, EC 2.6.1.1), glyceraldehyde, 3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD oxidoreductase (phosphorylating), EC 1.2.1.12), from rabbit muscle, 3-phosphoglycerate kinase (ATP:3-phospho-D-glycerate phosphotransferase, EC 2.7.23), bovine serum albumin, fraction V, and Protease from Streptomyces griseus, type VI were supplied by the Sigma Chemical Co., St. Louis, Mo., USA. Avidin was supplied by Worthington Biochemical Corporation, New Jersey, USA. Trypsin (3 x Cryst, Sterile) from beef pancreas was supplied by the Nutritional Biochemicals Corporation, Cleveland, Ohio.

2.1.2 Radioactive chemicals

Sodium [2-^{14}C]pyruvate, sodium [^{14}C]bicarbonate, [2-^{14}C]biotin, [1-^{14}C]acetyl CoA were obtained from the Radiochemical Centre, Amersham, England. [^{32}P]orthophosphate was supplied by the Australian Atomic Energy Commission, Lucas Heights, Australia.

2.1.3 General chemicals

ATP (disodium salt, Grade I), ADP (disodium salt, Grade I, AMP, NADH, Co-enzyme A (Grade I), oxaloacetic acid, pyridoxal phosphate, sodium pyruvate

(type II, dimer free), 3-phosphoglycerate, 2-mercapto-ethanol, DTE, and Trizma base, L-alanine, L-aspartic acid, o-phospho-L-serine, DL-o-phosphothreonine, guanidine-HCl (practical grade), were supplied by Sigma Chemical Co., St. Louis, Mo., USA. N-ethylmorpholine was obtained from Eastman Organic Chemicals, and polyethylene glycol (molecular weight 20000) from Union Carbide Corporation. Sodium glutamate and L-lysine hydrochloride were obtained from British Drug Houses. 1,4-bis-2(4-methyl-5-phenox-azolyl)-benzine and 2,5-diphenyloxazole were supplied by Koch-Light Laboratories Ltd., Bucks., England. Triton X-100 was supplied by ICI (Australia) Ltd., Melbourne. Sodium oxalate was obtained from Mallinekrodt Chemical Works, St. Louis, Mo., USA. $MnCl_2$ was prepared from spectrographically standard manganese (Matthey Garrett Pty. Ltd., Lond. U.K.) and redistilled HCl, and was standardised by titration against EDTA, using Eriochrome Black as an indicator (Vogel, 1967). Sodium bicarbonate and $MgCl_2$ were supplied by Anax Chemical Ltd., Sydney, Australia.

2.1.4 General materials

Sephadex G-10, G-25, DEAE A50, and NAD^+-agarose, were supplied by Pharmacia Fine Chemicals, Uppsala, Sweden. Chromatography paper was obtained from Whatman made by Balston Ltd., Eng. Silica gel for thin layer chromatography was manufactured by Eastman Kodak Company, Rochester N.Y. Dioxane, chloroform, methanol were obtained from May and Baker (Aust.) Ltd., West Footscray, Vic., while benzene was supplied by Ajax

Chemicals Ltd., Sydney, Aust.

2.2 METHODS

2.2.1 Purification of sheep liver pyruvate carboxylase.

Pyruvate carboxylase was purified from freeze-dried sheep liver mitochondria by the following procedure. The mitochondria (120 g) were suspended in 1750 ml of extraction buffer which contained 25 mM tris acetate, pH 6.7, 3.5 mM $MgCl_2$, and 1.7 mM ATP. The pH was maintained between 6.5 and 6.7 during addition of the mitochondria. The suspension was stirred for twenty minutes, and undissolved material removed by centrifuging (23000 g, for ten minutes, at 4°C).

Ammonium sulphate was added to the supernatant to give a final saturation of 33% (19.6 g ammonium sulphate per 100 ml solution). The pH was maintained between 6.9 and 7.1 during addition of the ammonium sulphate. The suspension was stirred for twenty minutes and precipitated protein collected by centrifuging (23000 g for twenty minutes at 4°C). The precipitate was suspended in 26% saturated ammonium sulphate to a final volume of 5 ml per 10 g of mitochondria. This procedure removed a substantial proportion of the contaminating glutamate dehydrogenase.

The precipitated protein was collected by centrifuging (23000 g, for ten minutes, at 4°C) and suspended in 25 mM potassium phosphate, pH 7.2, containing

1 mM EDTA, 0.1 mM DTE and 0.5% saturated with ammonium sulphate, to give a final protein concentration of 10 mg/ml. This suspension was desalted by addition of 14.5 g polyethylene glycol/100 mls. The suspension was stirred for thirty minutes and precipitated protein was then collected by centrifuging (23000 g, for twenty minutes, at 4°C). The protein was then suspended in 5 - 6 ml of the phosphate buffer described above, per 10 g of mitochondria, and undissolved protein removed by centrifuging (23000 g, for ten minutes, at 4°C). The supernatant, containing essentially all the pyruvate carboxylase was loaded on to a DEAE-Sephadex A-50 column (14 x 5 cm) previously equilibrated with 25 mM potassium phosphate, pH 7.2, containing 1 mM EDTA, 0.1 mM DTE, and 1% saturated with ammonium sulphate. The column was eluted with a linear gradient of 1 - 5% saturated ammonium sulphate, the solvent blending being 25 mM Tris-Cl, pH 8.0 containing 1 mM EDTA, 0.2 mM DTE and 5% saturated with ammonium sulphate. Pyruvate carboxylase of specific activity 15 - 20 units/mg protein was routinely obtained using this procedure.

2.2.2 Preparation of pure acetyl CoA

Acetyl-CoA was prepared from CoA by the method of Simon and Shemin (1953). Purification from salts was achieved by chromatography on Sephadex G-10 equilibrated in H_2O and 10^{-4} M EDTA at 4°C. The acetyl CoA purified by this method had an absorbance ratio 232/260 nm of 0.55 and contained no detectable free CoA-SH after testing with the DTNB assay of thiol groups as described by Butterworth *et al.*, (1967).

2.2.3 Determination of radioactivity

Samples dried on to solid supports (1" x 1" squares of Whatman's 3 MM paper) were placed in vials containing 2 ml scintillation fluid (0.3% ($^{W}/v$), 1,4-bis-2 (4-methyl-5-phenoxazolyl)-benzene, in sulphur-free toluene; Bosquet and Christain, 1960) and counted in a Packard Scintillation Spectrometer. When the samples contained coloured material, (as was the case in the radiochemical pyruvate carboxylase assay) correction was made for colour quenching using the channels ratio method (Herberg, 1965). Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid, as above, containing Triton X-100, 7:3 $^{V}/v$), and counted in a Packard Scintillation Spectrometer.

2.2.4 Protein estimation

Protein concentrations were routinely determined from absorption at 260 and 280 nm using a Varian Techtron 635-0 spectrophotometer and applying the values to the formula, for a 1 cm light path,

$$mg/ml = 1.55\ A_{280} - 0.76\ A_{260}$$ (Layne, 1957).

2.2.5 Measurement of pyruvate carboxylase activity

2.2.5.1 The spectrophotometric assay

This continuous assay procedure, based on that described by Utter and Keech (1963) involves reduction of the oxaloacetate produced by the

pyruvate carboxylase reaction, using malate dehydrogenase, with concommitant oxidation of NADH to NAD^+. Assay solutions contained, (in μmoles) in a final volume of 1.0 ml; tris Cl, pH 8.4, (100); ATP, (2.5); $MgCl_2$, (5); HCO_3^-, sodium salt, (20); pyruvate, sodium salt, (10); acetyl CoA, (0.25); NADH, (0.125); malate dehydrogenase, (5 units); and pyruvate carboxylase (0.025 - 0.1 units).

The reaction was followed at 340 nm, using either a Unicam SP800 spectrophotmeter, or a Varian-Techtron 635-0 spectrophotometer. The cell block was thermostatted at 30°C. The rate of oxaloacetate synthesis was calculated assuming an extinction coefficient at 340 nm for NADH of 6.22 mM^{-1} cm^{-1} (Dawson et al., 1969).

2.2.5.2 The radiochemical assay

In this procedure [^{14}C]bicarbonate fixed in an acid stable form is measured, while unreacted bicarbonate is driven off by acidification and subsequent drying on paper squares. Assay solutions contained, (in μmoles), in a final volume of 0.5 ml; tris Cl, pH 8.4, (50); ATP, (1.25); $MgCl_2$, (2.5); [^{14}C]HCO_3^-, sodium salt, 5 x 10^5 cpm/μmole, (5);pyruvate, sodium salt, (5); acetyl CoA, (0.125); and up to 0.06 units of pyruvate carboxylase. The reaction was initiated

by addition of enzyme and allowed to proceed for a time up to five minutes at 30°C before being quenched by addition of 50 µl of a saturated solution of 2,4-dinitrophenylhydrazine in 6 M HCl. As well as terminating the reaction, this reagent drives off unreacted [^{14}C]-bicarbonate and stabilises the oxaloacetate formed as the dinitrophenylhydrazone. Triplicate samples (0.05 ml) were applied to one inch squares of Whatman's 3 MM paper, dried at 90°C for five minutes, and the remaining radioactivity determined as described in Section 2.2.3.

The acetyl CoA-independent assay system used was that developed by Ashman (1973). The assay solutions contained, (in µmoles), in a final volume of 0.5 ml; tris Cl, pH 8.4, (50); ATP, (1.25); $MgCl_2$, (4); [^{14}C]HCO_3^-, sodium salt, 5×10^5 cpm/µmole, (40); pyruvate, sodium salt, (20); NH_4Cl, (100), and 2 units of pyruvate carboxylase. [^{14}C]oxaloacetate was determined by the procedure described above for the acetyl CoA-dependent assay.

2.2.6 ATP/Pi isotopic exchange reaction assay

Reaction mixtures contained, (in µmoles), in a final volume of 0.5 ml:tris Cl, pH 8.4,(50),ATP,(1);ADP,(1);

$MgCl_2$, (4); HCO_3^-, potassium salt, (1); acetyl CoA, (0.125); phosphate, potassium salt, (5), and pyruvate carboxylase, 1 - 2 units. In the controls, pyruvate carboxylase that had been specifically inactivated by previously treating with avidin was used. The reaction was initiated by addition of carrier-free [^{32}P]ortho-phosphate (1 - 2 x 10^6 cpm) after incubating the reaction mixtures at 30°C for ten minutes. Samples (0.2 ml) were taken at various time intervals and quenched with 0.1 ml 5 M formic acid, at 0°C.

Separation of ATP and orthophosphate was achieved by an activated charcoal separation procedure. A 0.1 ml sample of the formic acid quenched sample was diluted with 1 ml potassium phosphate (0.1 M, pH 4.4). 0.2 ml of an activated charcoal suspension in 6 N HCl (250 mg/ml) was then added and the mixture applied to a glass fibre disk. After six washes with 0.1 M HCl, and one wash with water, the disk was dried by a vacuum,put in a glass test tube, and boiled for 20 minutes with 3 mls crude HCl. The mixture was centrifuged for 2 minutes and two mls of supernatant were counted at 50% gain. Total radioactivity in each sample was obtained by counting 10 µl of the formic acid quenched samples in water at 50% gain.

2.2.7 Pyruvate/oxaloacetate isotopic exchange reaction assay

Assays contained, (in µmole), in a final volume of 0.5 ml:tris Cl, pH 8.4, (50); oxaloacetate, (1); pyruvate, (unless otherwise stated), (1), acetyl CoA, (0.125); and

pyruvate carboxylase, 0.1 units. Usually the reaction was initiated, after five minutes incubation at 30°C, by addition of sodium [2-^{14}C]pyruvate (0.005 µmole, 4 x 10^7 cpm/µmole), dissolved in 0.02 M HCl.

When the pyruvate concentration was varied, the specific activity of the pyruvate was held constant, and the reaction initiated by addition of the enzyme.

At various time intervals, samples (0.1 ml) were withdrawn, and the reaction quenched with 0.01 ml avidin (10 mg/ml in 0.02 M sodium phosphate, pH 7.4). After two minutes, pyruvate and oxaloacetate were converted to alanine and aspartate by the addition of 0.05 ml of 0.5 M sodium glutamate, 0.02 ml of 2 mM pyridoxal phosphate, and 0.02 ml sodium phosphate, 0.1 M, pH 7.4, containing one unit each of glutamate/pyruvate transaminase, and glutamate/oxaloacetate transaminase. The transamination reaction was allowed to proceed for fifteen minutes at 30°C and then quenched with 0.025 ml of 5 M formic acid. Alanine and aspartate were separated by electrophoresis on a water cooled flat-bed apparatus in 0.04 M sodium acetate (pH 5.5). Samples (0.02 ml) were applied to Whatman 3 MM papers which included lanes for marker alanine and aspartate samples. Electrophoretograms were developed for 45 minutes at 2600 V, 60 mA (40V/cm, 2 mA/cm). After drying, the aspartate and alanine markers were located by spraying with 0.5% ninhydrin in acetone. Sample lanes were cut into 1 cm pieces and radioactivity determined as described in Section 2.2.3. Since contaminant

parapyruvate moved very close to aspartate on the electrophoretograms, correction was made for it by subtracting controls from which enzyme was omitted.

2.2.8 Calculation of the rate of isotopic exchange

Consider the reaction $A^* + X \rightleftharpoons B + Y$.

The approach to isotopic equilibria is exponential and the amount of label converted to B* will be linear only over the initial few percent of conversion.

The rate of exchange at any time, however, was calculated by the formula:-

$$R = \frac{-[A].[B]}{[A] + [B]} \cdot \frac{\ln.(1 - F)}{T}$$

when $F = \frac{\text{percent of label in B at time T}}{\text{percent of label in B at equilibrium}}$

For the pyruvate/oxaloacetate exchange, the precent of label in B at equilibrium will be 50% since the initial concentrations of pyruvate:oxaloacetate were identical. This was confirmed by allowing the exchange to proceed to equilibrium as a control.

CHAPTER 3

ALLOSTERIC EFFECTS OF THE MAGNESIUM ION

3.1 INTRODUCTION

3.1.1 Classical vs. non-classical kinetics

An enzyme can be influenced by the binding of a ligand which is not structurally related to the substrates of the reaction which is catalysed. This would imply that there are two different types of binding sites within the enzyme, namely, a catalytic site and an allosteric site. The binding of a ligand at the allosteric site causes a change in either the affinity of the enzyme for substrates or the overall rate of catalysis.

In many instances, it has been found that if the reaction velocity is plotted against activator concentration, a sigmoid velocity curve is obtained. There are a number of ways by which an enzyme may exhibit a velocity-effector profile which is sigmoid,

(1) intersubunit interaction. Examples of this type of system have been proposed by Monod, Wyman,Changeux, (1965);and Koshland, Nemethy and Filmer (1966).

(2) polymerization equilibrium. A model which obeys such a concept has been proposed by Nichol et al., (1967).

(3) alternate reaction pathways, extensively reviewed by Sweeny and Fisher (1968).

The degree of deviation of these velocity-effector plots from Michaelis-Menten kinetics can be

quantitated using the empirical Hill equation,

$$\log \frac{v}{V - v} = n_H \log [S] - \log k$$

where v, V, S and k represent the initial velocity, maximum velocity, total ligand concentration and Michaelis-Menten rate constant respectively. The n_H value or Hill coefficient, is a measure of the extent of sigmoidicity of the velocity-effector profiles since it is a function of the number of interacting substrate binding sites per enzyme molecule and the strength of the interaction between these sites.

A value for the Hill coefficient which is greater than one, has usually been interpreted to indicate cooperativity in ligand binding as predicted by either Monod *et al.*, or Koshland *et al.* The major difference between these two allosteric systems is that the Monod theory predicts that the binding of the first ligand to one of the subunits in a multimeric enzyme induces a conformational change in all the subunits. The Koshland theory, however, can account for cooperative systems and classical systems by the fact that hybrid conformational states can exist.

Although cooperative allosteric effects can successfully explain sigmoid kinetics and there are a large number of enzymes which exhibit these properties, consideration must be given with equal validity to the alternate possibilities which do also give rise to sigmoid kinetics. The polymerizing model (Nichol *et al.*, 1967) requires that an allosteric enzyme exists in monomers

and oligomers and both species can bind the substrate. If the number of binding sites is equal to the number of subunits in the polymerized form, then Michaelis-Menten kinetics would prevail. If this situation does not exist, a competition would occur between subunit binding to subunit and ligand binding to subunit which would result in a sigmoidal velocity curve. An example of this type of enzyme system is glutamate dehydrogenase with respect to binding of GTP (Freiden, 1968).

Non-allosteric and monomeric enzymes may also exhibit sigmoidal saturation curves under certain conditions. For example, enzymes which are comprised of a single site or multiple independent sites and the substrates bind via multiple reaction pathways (Sweeny and Fisher, 1968, Wells et al., 1976). A sigmoid saturation curve for a Michaelis-Menten mechanism can also be obtained if the enzyme is being gradually inactivated during the course of velocity measurement (Fischer and Keleti, 1975).

In attempting to establish whether an enzyme obeys classical or non-classical kinetics one must be certain to eliminate all possible artefacts which in themselves could give rise to non-linearity. These artefacts being, narrow ranges of substrate concentrations, saturating levels of second substrate and long assay times if the enzyme is unstable at certain concentrations of substrate. If a deviation from classical kinetics is observed after attempts have been made to remove all

artefacts,the effect can then be attributed to one of the three mechanisms discussed above, and coming to the correct conclusions concerning non-linearity, would ideally require confirmation of the result obtained using the steady state kinetic approach, by some other chemical or physical technique, for example, binding studies, circular dichroism or chemical modification.

3.1.2 Activation of pyruvate carboxylase by acetyl CoA

Pyruvate carboxylase from many sources is known to be activated by the allosteric effector, acetyl CoA. When the enzyme catalysed reaction velocity is plotted against various concentrations of acetyl CoA, a sigmoid curve is obtained. This result has been interpreted to indicate homotropic cooperativity in acetyl CoA binding. Although this explanation has been accepted by most workers in the field, it has not been unequivocally proven, and therefore the alternative explanations which could also give rise to sigmoid kinetics must be considered.

The alternatives to cooperativity as discussed in detail in the previous section are,

(1) polymerization equilibrium between monomers and oligomers.

(2) alternate pathways in the catalytic reaction.

(3) inactivation of the enzyme during the time of assay.

These general models can be applied specifically to pyruvate carboxylase and thereby the feasibility of

such events being responsible for the non-classical behaviour of acetyl CoA kinetics can be assessed.

Only chicken liver and rat liver pyruvate carboxylase have been shown to exist as monomers under certain conditions (Irias et al., 1969, Nakashima et al., 1975). The subunits of chicken liver pyruvate carboxylase, however, are inactive and cannot bind acetyl CoA (Frey and Utter, 1977). The monomers of rat liver pyruvate carboxylase on the other hand have been isolated in an active form and it has been shown that they exhibit identical sigmoid acetyl CoA saturation curves to the tetrameric species (Nakashima et al., 1975). From this evidence one can conclude that the "polymerization of proteins" model cannot be applied to pyruvate carboxylase.

The alternate pathways model could be a possible explanation for the observed kinetics but as yet, no evidence has been presented to suggest the existence of such a reaction mechanism.

Another specific property to pyruvate carboxylases, is that they undergo inactivation upon dilution to below 4 U/ml (Ashman et al., 1972). Saturating concentrations of acetyl CoA have been shown to protect against this process (Ashman et al., 1972). One can therefore conceive that during an initial velocity experiment where the concentration of acetyl CoA is being varied at concentrations below the k_d value, the enzyme would be undergoing dilution inactivation within the prescribed assay time. Initially,

the work of Scrutton and Utter (1967) seemed to contradict this argument, but recently, Frey and Utter (1977) have now published an observation of a lag phase in initial velocity experiments. Therefore instability of the enzyme during the assay procedure, is indeed a very real effect and could be one aspect in accounting for the observed non-classical behaviour of acetyl CoA kinetics.

Another fact which could also be consistent with a Hill coefficient greater than unity, is, that the non-variable substrates are not saturating and thus n_H would be a function of the degree of saturation of substrates. Warren and Tipton (1974a) observed that the sigmoidicity of the acetyl CoA velocity curve increased when the assay conditions were such that no substrate was in saturating conditions. Acetyl CoA is known to increase the affinity of the enzyme for pyruvate and bicarbonate (Ashman et al., 1972) and increasing the concentrations of either of these two substrates decreases the apparent k_a for acetyl CoA (Scrutton, 1974) and also lowers the n_H value (Easterbrook-Smith, 1977). Thus at low acetyl CoA concentrations one would expect either pyruvate or bicarbonate to be non saturating and thus contribute to increasing the n_H value for acetyl CoA.

Table 3.1 shows the variation in K_a and n_H values for acetyl CoA from different pyruvate carboxylase species obtained by initial rate kinetics and through

TABLE 3.1

The data compiled to produce this table has been selected from the following publications:-

1. Scrutton, M.C. (1971) Metabolism 20, 168.
2. Barden, R.E. and Scrutton,M.C. (1974) J. Biol. Chem. 249, 4829.
3. Scrutton, M.C., Pearce, P.H. and Fatebene, F (1977) Eur. J. Biochem. 76, 219.
4. Frey, W.H. and Utter, M.F. (1977) J. Biol. Chem. 252, 51.
5. Scrutton, M.C. and Utter, M.F. (1967) J. Biol. Chem. 242, 1723.
6. Scrutton, M.C. and White M.D. (1972) Biochem. and Biophys Res. Commun. 48, 85.
7. Warren, G.B. and Tipton, K.F. (1974) Eur. J. Biochem. 47, 549.
8. Barritt, G.J., Keech, D.B. and Ai-Mee-Ling (1966). Biochem. and Biophys Res. Commun. 24, 476.
9. Easterbrook-Smith S.B. (1977) Ph.D. thesis. University of Adelaide.

TABLE 3.1

Species	Initial Rate Studies: Conditions	k_a (μM)	n_H	Binding Studies: Conditions	k_a (μM)	n_H
Chicken		2.5[1]	2.7[1]	TNBS protection studies	9.3[3]	2.2[3]
	Low pyr, MgATP; high $MgCl_2$	3.0[2]	2.7[2]	No additions	13.9[4]	1.9[4]
		5.0[3]	2.8[3]			
		13.3[4]	2.9[4]			
	High pyr, MgATP and HCO_3	33.0[5]	2.9[5]			
Rat		20-25[6]	2.0[6]	Low pyr	160.0[3]	2.2[3]
		27.0[1]	2.1[1]	High pyr	80.0[3]	1.8[3]
	High pyr	55.0[3]	2.0[3]			
	Low pyr	130.0[3]	2.0[3]			

Table 3.1 cont.

Species	Initial Rate Studies Conditions	k_a (μM)	n_H	Binding Studies Conditions	k_a (μM)	n_H
Pig	Low ATP, varied Mg^{2+}	5.7[7]	2.0[7]			
	Low ATP, varied HCO_3	7.0[7]	1.6[7]			
	Low ATP, varied pyr	8.25[7]	2.0[7]			
		22.0[1]	1.6[1]			
Sheep	Low pyr, low HCO_3	41.0[8]	1.9[8]			
	Low HCO_3	24.0[9]	2.0[9]			
		30.0[1]	1.8[1]			

binding studies published by various workers. As one can see, these values depend greatly on the assay conditions used, whether acetyl CoA is the changing fixed variable or the variable substrate, and the identity of the second substrate.

3.1.3 Activation of pyruvate carboxylase by $MgCl_2$

Most biotin-containing carboxylating enzymes exhibit optimum activity when the concentration of Mg^{2+} is in excess of that required to complex ATP. Hence it seemed likely that there existed a unique relationship between MgATP as the substrate and Mg^{2+} as the activator (Table 3.2). All species exhibit a decrease in K_m for MgATP upon increasing concentrations of free Mg^{2+}. Zero or very low K_a values for Mg^{2+} have been interpreted to represent an extremely facile binding of Mg^{2+} to the enzyme at high concentrations of MgATP and it could be possible that MgATP could trap the required amounts of free Mg^{2+} on the enzyme surface.

The binding of this activator conforms to the Michaelis-Menten theory at saturating concentrations of acetyl CoA, however as acetyl CoA concentrations approach zero, the binding of Mg^{2+} to the enzyme is characterized by an n_H value of 2.2 (Barden and Scrutton, 1974). Another property of this divalent cation is that it significantly lowers the apparent K_a for acetyl CoA.

TABLE 3.2

Values quoted within this table have been taken from,

1. Barden, R.E. and Scrutton M.C. (1974) J. Biol. Chem. 249, 4829.
2. McClure, W.R., Lardy, H.A. and Kneifel, H.P. (1971) J. Biol. Chem. 246, 3569.
3. Warren, G.B. and Tipton, K.F. (1974) Biochem. J. 139, 311.
4. Bais, R. and Keech, D.B. (1972) J. Biol. Chem. 247, 3255.

TABLE 3.2

Species	Changing Fixed Substrate	Varied Substrate	app k_a (mM)	k_d (mM)	n_H
Chicken[1]	Mg^{2+}	MgATP	0.03	0.78	1.0
	MgATP	Mg^{2+}	0.15	7.4	1.0
Rat[2]	Mg^{2+}	MgATP	0.07		1.0
	MgATP	Mg^{2+}	0.0		1.0
Pig[3]	Mg^{2+}	MgATP	0.87		
	MgATP	Mg^{2+}	0.0		
Sheep[4]	Mg^{2+}	MgATP	0.63		1.0
	MgATP	Mg^{2+}	0.44		1.0

3.1.4 Evidence for an inter-relationship between acetyl CoA and Mg^{2+}

The relationship between activations produced by acetyl CoA and by Mg^{2+} has been examined by varying acetyl CoA and Mg^{2+} as activator-activator pair. Since double reciprocal plots with respect to free Mg^{2+} and fixed concentrations of acetyl CoA do not intersect on the ordinate,this could imply that both acetyl CoA and free Mg^{2+} are able to bind to the free enzyme in a random order equilibrium manner. It has been shown by several workers (Seufert et al., 1971, Dugal, 1973, Warren and Tipton, 1974a, Barden and Scrutton, 1974) that increasing concentrations of free Mg^{2+} lowers the apparent K_a for acetyl CoA. Pyruvate and bicarbonate which also cause a similar effect have been observed to lower the n_H value for acetyl CoA (Easterbrook-Smith et al., 1976c).

Furthermore, there has been a conflict in reports as to the effect of Mg^{2+} on the Hill coefficient for activation by acetyl CoA. All of the three possible effects, viz., a decrease (Dugal, 1973), an increase (Seufert et al., 1971) and no apparent effect (Warren amd Tipton, 1974a and Barden and Scrutton, 1974) on the Hill coefficient for acetyl CoA upon increasing concentrations of free Mg^{2+}, have been reported. Thus this matter is still left open to question and it was decided therefore to re-evaluate the hypothesis of activation of pyruvate carboxylase focusing on the role of free Mg^{2+}

acting in conjunction with acetyl CoA.

3.2. METHODS

3.2.1 Acetyl CoA deacylase assay

This assay procedure entails the determination of the [1-^{14}C]acetate produced on deacylation of [1-^{14}C]-acetyl CoA. The unreacted acetyl CoA is removed from solution by adsorption to activated charcoal. The assay solution contained, (in µmole), in a final volume of 0.4 ml:tris Cl, pH 8.4, 40; ATP, 1; $MgCl_2$, 2.5; sodium pyruvate, 4; [1-^{14}C]acetyl CoA, 5.6 x 10^5 cpm/µmole, as indicated, and 2 - 3 units of pyruvate carboxylase. Assay tubes were equilibrated at 30°C and the reaction was initiated by addition of enzyme. After a 20 min incubation the reaction was stopped by addition of 0.05 ml 6 N HCl. Carrier sodium acetate (15 µmoles) was added, followed by the addition of 0.3 mls of an activated charcoal suspension (100 mg/ml in 1 N HCl). The resulting suspension was allowed to stand for 5 min and the charcoal was then removed by centrifuging. Samples of the supernatant were taken and their radioactivity determined using the Triton scintillant described in Section 2.2.3. The extent of non-specific acetyl CoA deacylation was determined using control assays in which pyruvate carboxylase was omitted. Under these conditions the reaction was linear with time for at least 20 minutes. The agreement of duplicate assays using this procedure was usually better than 5%. The pyruvate carboxylase

deacylase activity of chicken enzyme was determined in the same way, except Tris-Cl pH 7.8 was substituted.

3.2.2 Analysis of data

Classical velocity substrate curves were fitted to a rectangular hyperbola using the computer program HYPER of Cleland (1963). Sigmoid velocity-substrate curves were fitted to the empirical Hill equation using the non-linear regression program developed by Vaunghn et al., (1976). Where appropriate, data was fitted to a straight line using a least mean squares analysis Fortran computer program.

3.2.3 ^{31}P NMR spectra

The ^{31}P NMR spectra were recorded at 36.43 MHz on a Bruker HX90E spectrometer at ambient temperature. The apparatus was operated in the pulsed Fourier transform mode as described in full detail by Farrar and Becker (1971).

Neither sensitivity nor resolution enhancement was used in any of the spectra. All measurements were made in a D_2O solution of 0.1 M NEM-Cl pD 8.0. The ^{31}P chemical shifts of nucleotides in Hz and ppm are measured from a trimethylated orthophosphate external reference included in an indwelling capillary tube.

3.2.4 8-Hydroxyquinoline titration method

This method has been outlined in detail by

Burton, 1959. Measurements of the spectral changes of 8-hydroxyquinoline were made at 360 nm on a Varian-Techtron 635-0 spectrophotometer. $MgCl_2$ (0.105 M) was added from an Agla micrometer syringe to a solution of 8-hydroxyquinoline (0.896 M) in NEM-Cl buffer (0.1 M) pH 8.4 at 30°C. Measurements were made in 3 ml cells of 1 cm light path. The amount of metal bound to 8-hydroxyquinoline was calculated from readings obtained with high concentrations of metal and 0.0896 mM of the fluorescent compound in 20 ml cells of 10 cm light path. For a given extinction, the amount of free Mg^{2+} ion is obtained by the difference from the total amount of Mg^{2+} added when no nucleotide is present. In the presence of nucleotide, the extra amount of metal needed to obtain the same extinction is the amount that is bound by the nucleotide. For compounds where there is no multiple binding sites for Mg^{2+}, the association constant can be calculated from equation 3.1,

$$\frac{a - m}{mb} = k \qquad \ldots 3.1$$

where a is the total concentration of metal, b, the total concentration of phosphate compound, and m is the concentration of free metal ion.

3.2.5 Circular dichroism spectra

All measurements were determined on a JASCO J40-CS spectrophotometer. The light source came from a 200 - 1000 nm probe. The beam of light passes through the sample in a 1 mm quartz cell and its path of travel

is bent. If the sample molecule possess chiral centres this rotation is recorded on a chart as a positive or negative deviation from zero.

The instrument settings were adjusted so that the scale was 10 m°/cm, the time constant was 4 sec and the slit width was 1 nm. The chart was set at a speed of 5 cm/min and a scale expansion of 1 min/cm.

3.3 RESULTS

3.3.1 Effect of Mg^{2+} binding on the conformation of pyruvate carboxylase

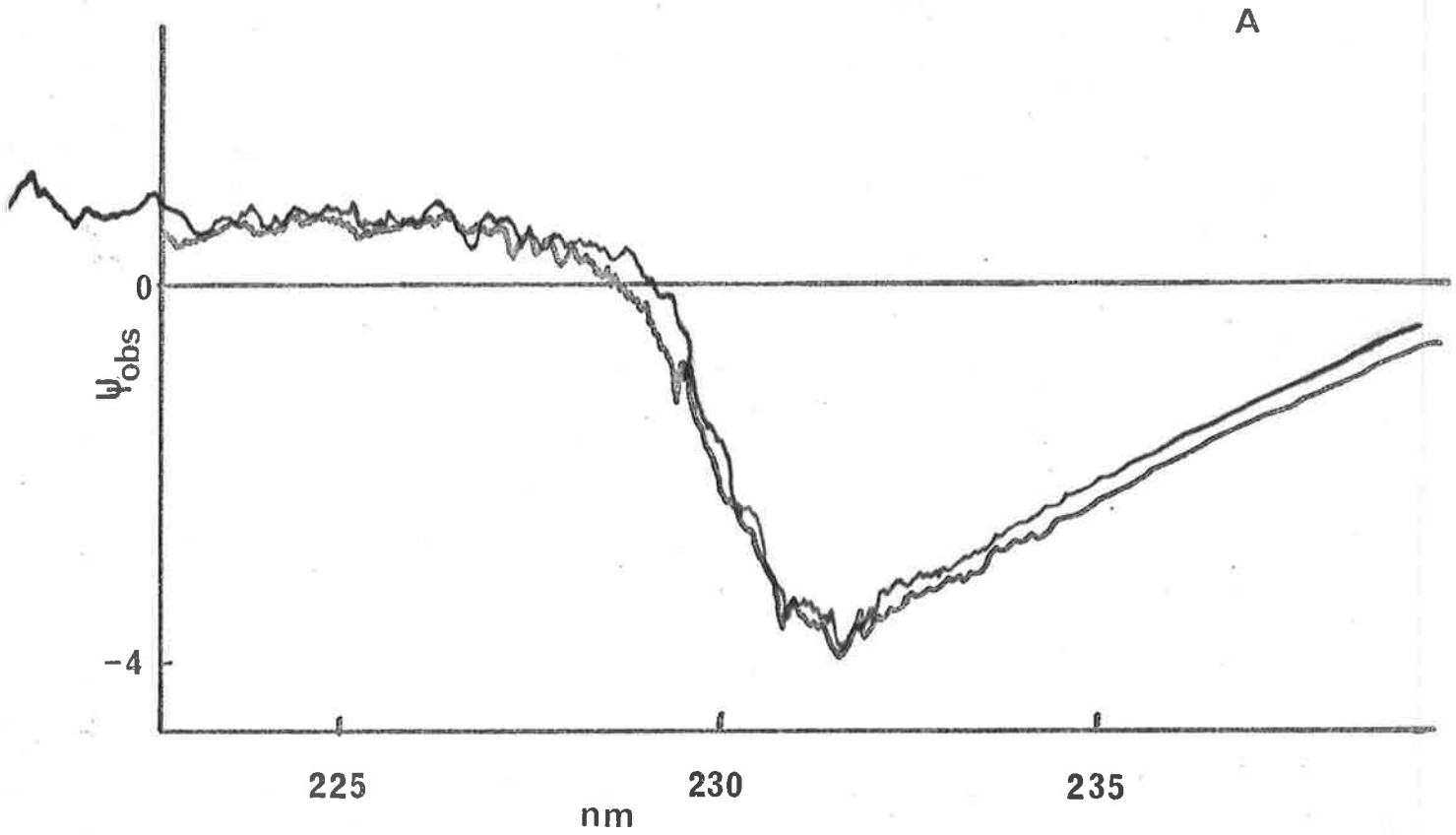
Prior to investigation of a possible relationship between Mg^{2+} and acetyl CoA activation, it was decided to determine the extent and nature of activation of the enzyme by the Mg^{2+} ion alone. Fig. 3.1 shows circular dichroism spectra of pyruvate carboxylase. A change in the conformation of the enzyme induced by the binding of a ligand, would cause a change in the pattern of light rotation and thus a different C.D. spectrum would be obtained. As one can see, the presence of Mg^{2+} does not alter the C.D. spectrum for pyruvate carboxylase implying that the binding of Mg^{2+} does not cause a conformational change in enzyme structure. This finding is contrary to that reported by Bais and Keech (1972) and a further discussion on this point is presented later. On the other hand, binding of acetyl CoA induced a large conformational change and this could be associated with the formation of a much more reactive enzyme species.

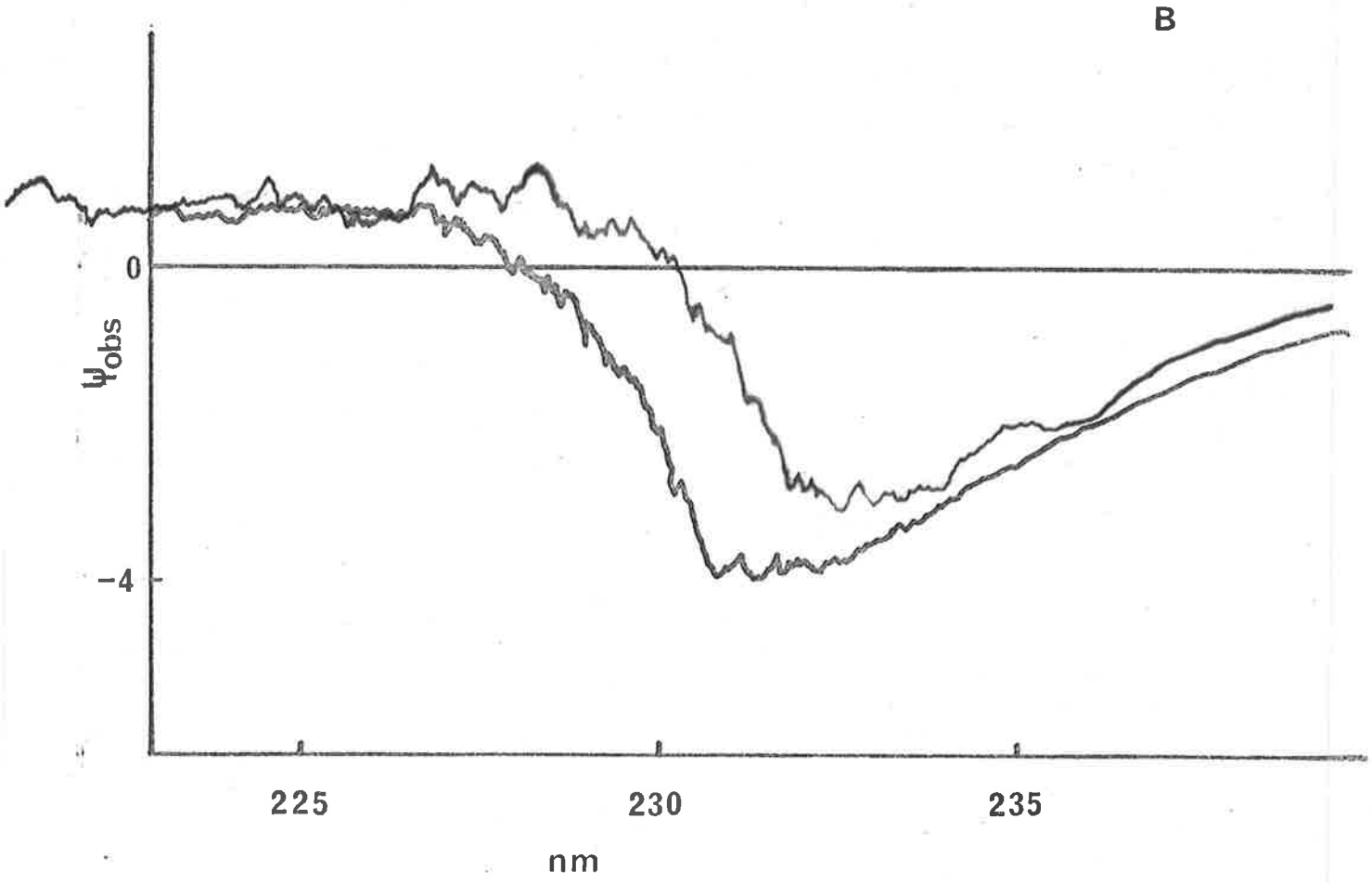
FIGURE 3.1

Spectrum of circular dichroism for pyruvate carboxylase.

Sample:	CLPC 4.5 U/ml, S.A. 30 U/mg.
Solvent:	0.1 M Tris-Cl, pH 7.8.
Cell length:	1 cm.
Temperature:	20°C.
Scale:	50×10^{-3} M^{o}/cm.
Time constant:	4 sec.
Chart speed:	5 cm/sec.
Expansion:	1 nm/cm.
Slit width:	1 nm.

In A, the overlay represents enzyme in the presence of 4 mM $MgCl_2$, while in B, the overlay shows the shift in the spectrum in the presence of 0.25 mM acetyl CoA.


A
0
ψ_{obs}
-4
225
230
235
nm


B
0
ψ_{obs}
-4
225
230
235
nm

Since the activation by Mg^{2+} cannot be attributed to conformational changes, one could postulate that its role could be in neutralizing a negatively charged amino acid group within the active site and thus facilitating the binding of MgATP and acetyl CoA. This hypothesis would imply the nucleotide portions of both these compounds bind in close proximity to each other.

3.3.2 The effect of Mg^{2+} on the acetyl CoA independent reaction

Bais and Keech (1972) stated that there is only one Mg^{2+} atom, in excess of MgATP, binding per active site. In order to establish a possible relationship between Mg^{2+} and acetyl CoA, it seemed necessary to ascertain what effects Mg^{2+} has, independently of acetyl CoA. Therefore the acetyl CoA independent reaction was measured at varying concentrations of MgATP and several fixed concentrations of free Mg^{2+} (Fig. 3.2). The result shows a significant activation of reaction velocity by Mg^{2+} and also an increase in the affinity of the enzyme for MgATP. However from the slope replot,which passes through the origin, one can infer that the enzyme does not exhibit an absolute requirement for this divalent cation in the acetyl CoA independent reaction. Since Bais and Keech (1972) came to the opposite conclusion by measuring the effect of Mg^{2+} on the acetyl CoA dependent reaction, this seems to implicate Mg^{2+} in a role which is involved with acetyl CoA.

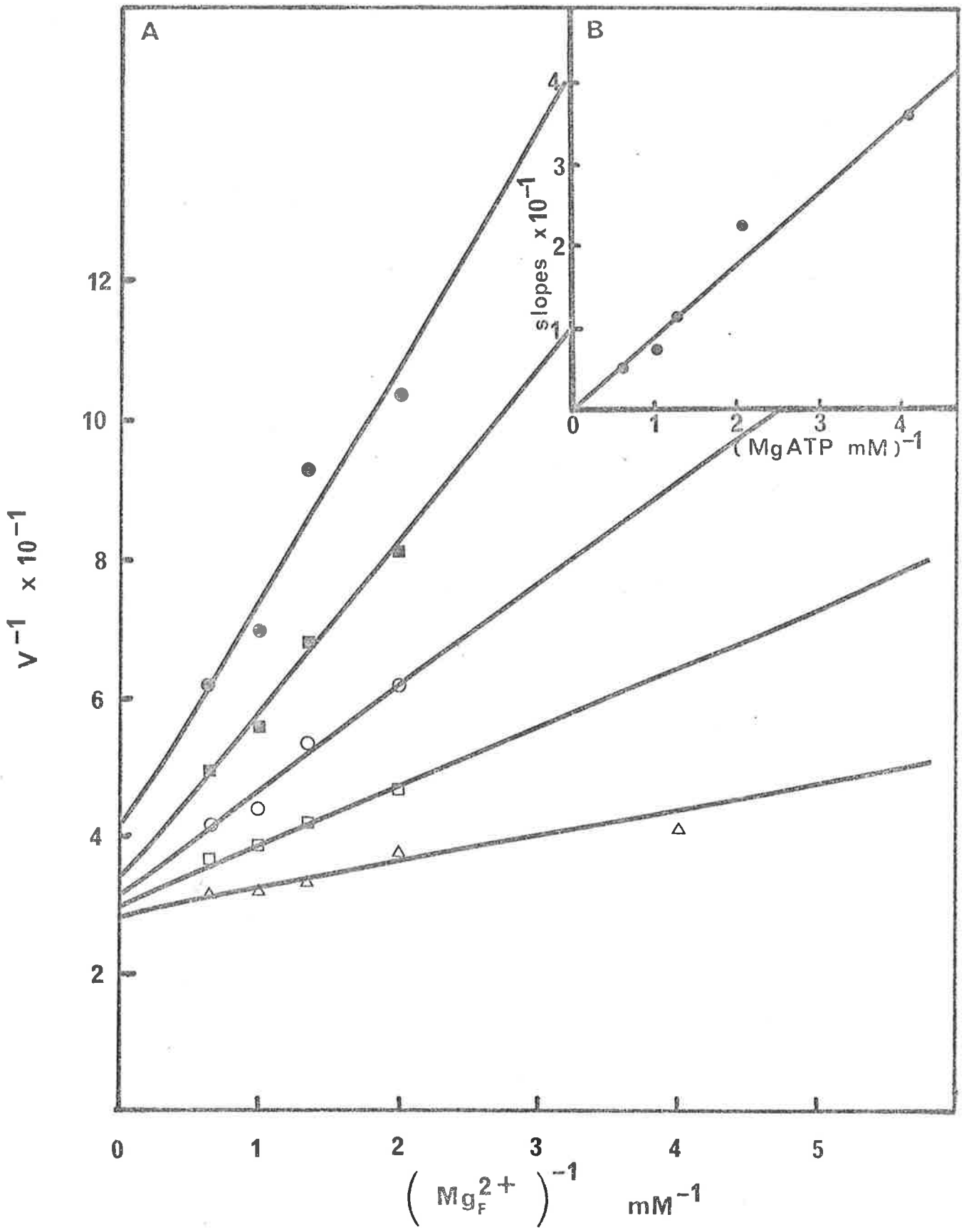
FIGURE 3.2

The non-requirement of pyruvate carboxylase for free Mg^{2+} in the acetyl CoA independent reaction.

In A the reciprocal of Mg^{2+} concentration is plotted against $^{1}/_{v}$ with the MgATP concentrations fixed at 2.5×10^{-4} M (●), 5×10^{-4} M (■), 7.5×10^{-4} M (○), 1×10^{-3} M (□) and 1.5×10^{-3} M (△).

The inset B shows the replot of the slopes from A.

Enzyme used was of S.A. 15, 3.2 U per assay, using the conditions described in Section 2.2.5.2.


A
B
slopes x10⁻¹
(MgATP mM)⁻¹
V⁻¹ x 10⁻¹
(Mg²⁺F)⁻¹ mM⁻¹

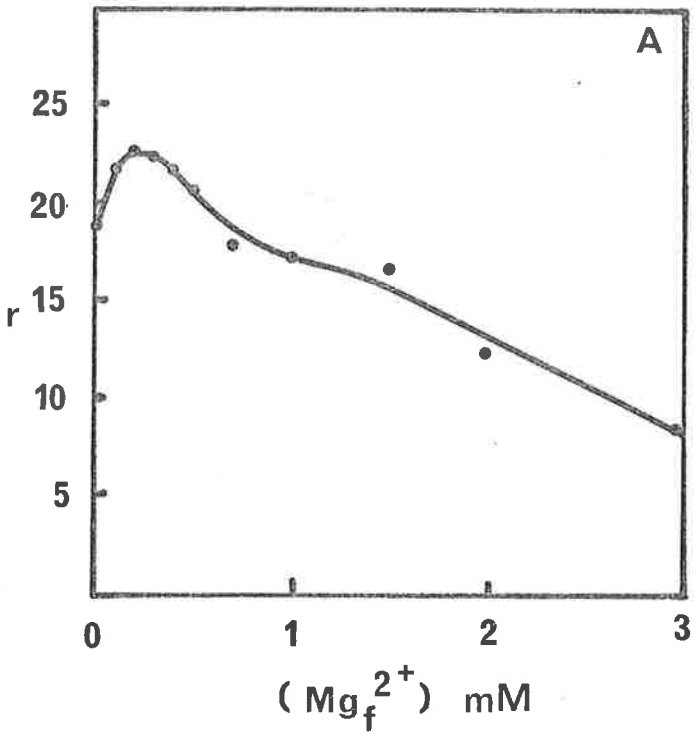
3.3.3 Divalent cation effects on the pyruvate/oxaloacetate isotopic exchange

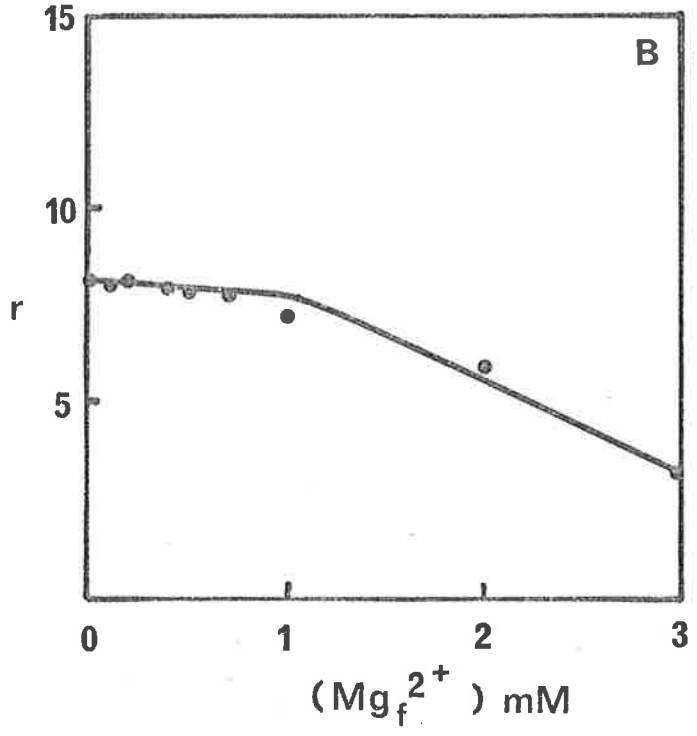
It has been shown previously (Ashman, 1973) that the presence of Mg^{2+} is essential for the occurrence of the ATP/P_i and ATP/ADP isotopic exchange activity. Initially it was thought that the locus of action of acetyl CoA was also in the first partial reaction site. However, Ashman et al., (1973) observed that acetyl CoA stimulated the pyruvate/oxaloacetate exchange some 2.5 fold. Since acetyl CoA has an effect in the second partial reaction site, it was thought this might also apply to Mg^{2+}. Figure 3.3 shows the effect of varying concentrations of free Mg^{2+} on the pyruvate/oxaloacetate exchange in the presence and absence of acetyl CoA. In the absence of acetyl CoA (Fig. 3.3B) Mg^{2+} inhibits the exchange activity only at values well above its apparent k_a of 0.44 mM (sheep kidney pyruvate carboxylase, Bais and Keech, 1972). This seems to be in agreement with the fact that it is a first partial reaction site effector. In the presence of acetyl CoA a different pattern is observed (Fig. 3.3A) At approximately 0.25 mM $MgCl_2$, a peak of stimulation is reached after which there is a gradual consistent inhibition of exchange rate as the $MgCl_2$ concentration is further increased. The concentration of acetyl CoA in the assay is also 0.25 mM and since 0.25 mM $MgCl_2$ has no stimulatory effect on the exchange rate in the absence of acetyl CoA, this could indicate a possible association between Mg^{2+}

FIGURE 3.3

The effect of Mg^{2+} on the pyruvate:oxaloacetate exchange.

Enzyme of S.A. 20, 5.6 U/ml was used in the procedure described in Section 2.2.7. The experiment was carried out in the presence (A) and absence (B) of acetyl CoA.


A
25
20
r 15
10
5
0
1
2
3
(Mg_f^{2+}) mM


B
15
10
r
5
0
1
2
3
(Mg_f^{2+}) mM

and acetyl CoA with the Mg^{2+}-acetyl CoA being a more effective activator than acetyl CoA alone.

It could be postulated that at high concentrations of Mg^{2+}, inhibition of the pyruvate/oxaloacetate exchange results from decarboxylation of oxaloacetate since Mg^{2+} has been shown to enhance the non-enzymic reaction (Warren and Tipton, 1974c). Table 3.3 shows however, that Mg^{2+} reduces the rate of enzyme catalysed decarboxylation of oxaloacetate. Since decarboxylation can only occur if biotin is situated in the second partial reaction site, the fact that this process is decreased suggests that free Mg^{2+} facilitates the removal of biotin from this particular reaction site.

3.3.4 The relationship between acetyl CoA and Mg^{2+}

Bais and Keech (1972) showed that Mg^{2+} activation was essential for enzyme activity. In their experiments they varied one activator (Mg^{2+}) and one substrate (MgATP) and found that the double reciprocal slopes, replot, did not pass through the origin. The apparent k_a for free Mg^{2+} was $(4.44 \pm 2.13) \times 10^{-4}$ M. The fact that the slope replot did not pass through zero, was stated to imply that Mg^{2+} activation was essential for pyruvate carboxylase activity.

The same type of experiment was therefore repeated but now varying two activators, Mg^{2+} and acetyl CoA. Assuming a random combination of the two activators with the enzyme, the experimental data was analysed using

TABLE 3.3

RATES OF DECARBOXYLATION OF OXALOACETATE

Experimental Conditions	Initial [Pyr] mM	Final [Pyr] mM	Δ[Pyr] mM
Pyr/OAA/Enz	0.293 ± 0.03	0.367 ± 0.031	0.073 ± 0.008
Pyr/OAA/Enz/Mg^{2+}	0.281 ± 0.054	0.473 ± 0.051	0.192 ± 0.043
Pyr/OAA	0.354 ± 0.013	0.356 ± 0.009	0.002 ± 0.008
Pyr/OAA/Mg^{2+}	0.348 ± 0.017	0.582 ± 0.028	0.177 ± 0.022

the general rate equation

$$\frac{1}{v} = \left(\frac{k_a k'_c}{V'[AcCoA]} + \frac{k_a k'_c}{V' k_c}\right) + \frac{1}{V'}\left(\frac{k'_c}{[AcCoA]} + 1\right)$$

where k_a, k'_c, and k_c are the dissociation constants for enzyme and Mg^{2+}, enzyme-Mg^{2+} and acetyl-CoA, and enzyme and acetyl-CoA respectively. V' represents the maximum velocity of the breakdown of the activated Mg^{2+}-enzyme-acetyl-CoA complex. The double reciprocal plots of velocity against free Mg^{2+} at fixed concentrations of acetyl CoA are shown in Fig. 3.4. The slopes derived from these intersecting lines were replotted against the reciprocal of acetyl CoA concentration and a straight line passing through the origin was obtained. This fact indicates that Mg^{2+} and acetyl CoA add to the enzyme in an equilibrium ordered manner. At infinite acetyl CoA concentrations, the k_a for Mg^{2+} approaches zero, and thus the presence of free Mg^{2+} becomes non-essential. This implys that Mg^{2+} is directly interacting with acetyl CoA to induce stimulation of the pyruvate carboxylase reaction.

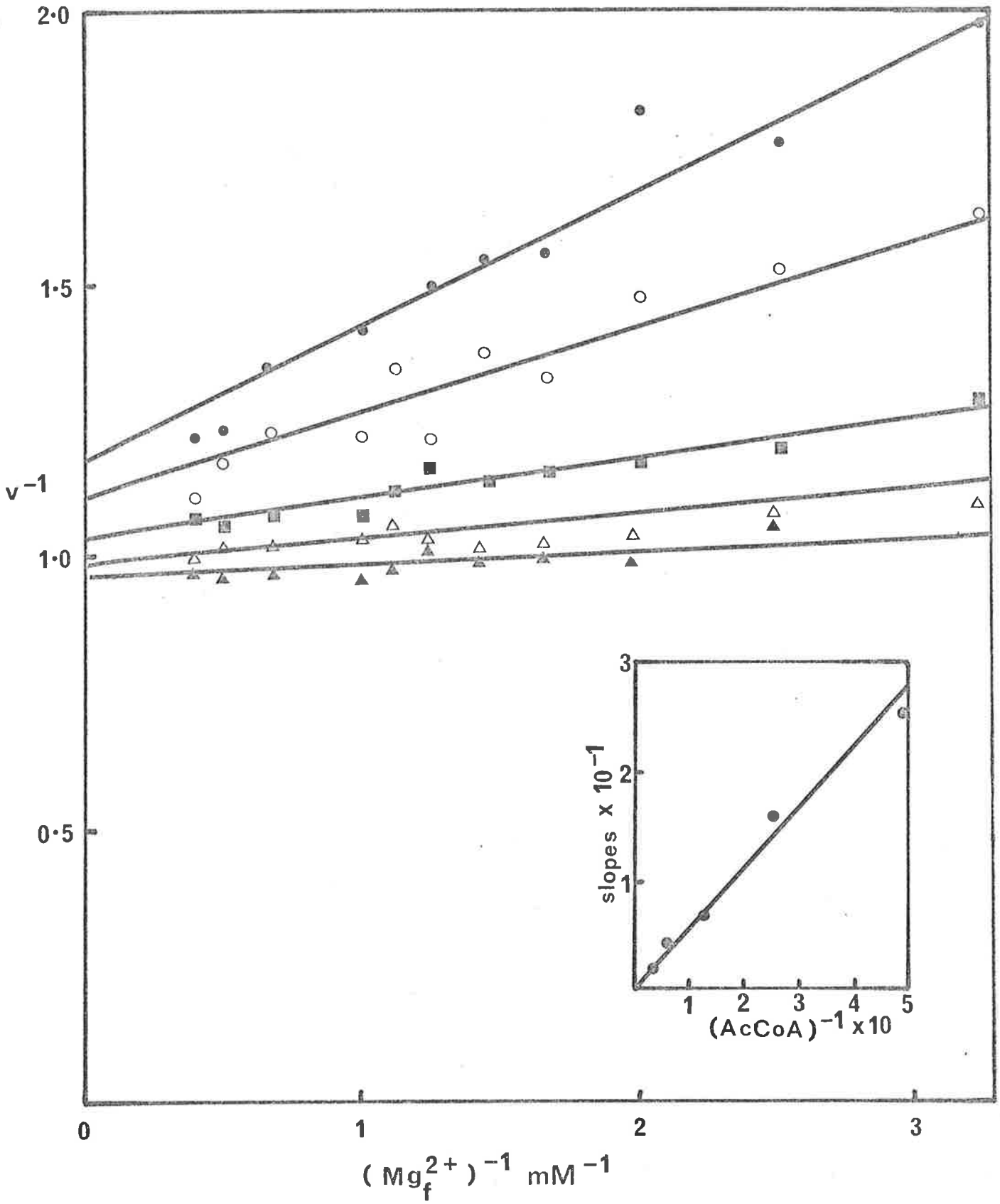
Warren and Tipton (1974a) published the results from a similar experiment but these workers did not obtain a series of straight lines in a double reciprocal plot. Upward turning curves were observed which were interpreted to indicate a cooperative interaction of free Mg^{2+} with the enzyme at sub-saturating concentrations of acetyl CoA. This discrepency should not exist since the kinetic properties of sheep liver and pig liver enzymes

FIGURE 3.4

The non-requirement of pyruvate carboxylase for Mg^{2+}.

A double reciprocal plot of velocity plotted against free Mg^{2+} with acetyl CoA fixed at 2×10^{-5} M (●), 4×10^{-5} M (○), 8×10^{-5} M (■), 1.6×10^{-4} M (△) and 3.2×10^{-4} M (▲). The inset shows a replot of the slopes against the reciprocal acetyl CoA concentrations.

The assay solution contained substrates at the following concentrations, in μmoles per ml; MgATP, (2.5); pyruvate, (20); and $NaHCO_3$ (26).


2·0
1·5
1·0
0·5
v⁻¹
0
1
2
3
(Mg²⁺f)⁻¹ mM⁻¹
slopes x 10⁻¹
1
2
3
1
2
3
4
5
(AcCoA)⁻¹ x10

have been shown to be very similar. The assay system used by Warren and Tipton contained 0.5 mM ATP, 1.5 mM HCO_3 and 5 mM pyruvate. Acetyl CoA is known to decrease the k_m for bicarbonate and pyruvate, but at the lowest concentration of acetyl CoA used (5 μM) acetyl CoA is not saturating, and thus the k_m values for the substrates would be significantly higher. Thus the non-linear double reciprocal velocity Mg^{2+} plots at various acetyl CoA concentrations observed by Warren and Tipton could be an artefact due to the lack of saturation with respect to the substrates.

To test this prediction, an exact repeat of the experiment published by Warren and Tipton was done with sheep liver pyruvate carboxylase, and the same result was obtained (Fig. 3.5). Using the same concentrations of enzyme, acetyl CoA and Mg^{2+} as these authors but now increasing the concentrations of pyruvate, bicarbonate, to 50 mM and 100 mM respectively, the result presented in Fig. 3.6 was obtained. The curvature has been reduced to linearity.

The results of Easterbrook-Smith et al., (1976c) showed that increasing the concentrations of pyruvate and bicarbonate decreased the sigmoidicity of the curves obtained when enzyme velocity is plotted against acetyl CoA concentration. In view of these experiments it was thought that increasing the concentration of free Mg^{2+} at relatively high constant concentrations of pyruvate and bicarbonate may reduce the sigmoid effect even further.

FIGURE 3.5

Warren and Tipton, Eur. J. Biochem. 47, 549 (1974).

Repeat of experiment detailed in Fig. 6 of this paper, using sheep liver pyruvate carboxylase, with concentrations of MgATP, pyruvate, HCO_3 maintained at 0.5 mM, 5 mM and 1.5 mM respectively.

The acetyl CoA concentrations used were 5×10^{-6} M (●), 7.5×10^{-6} M (■), 1.25×10^{-5} M (▲), 4.6×10^{-5} M (O).

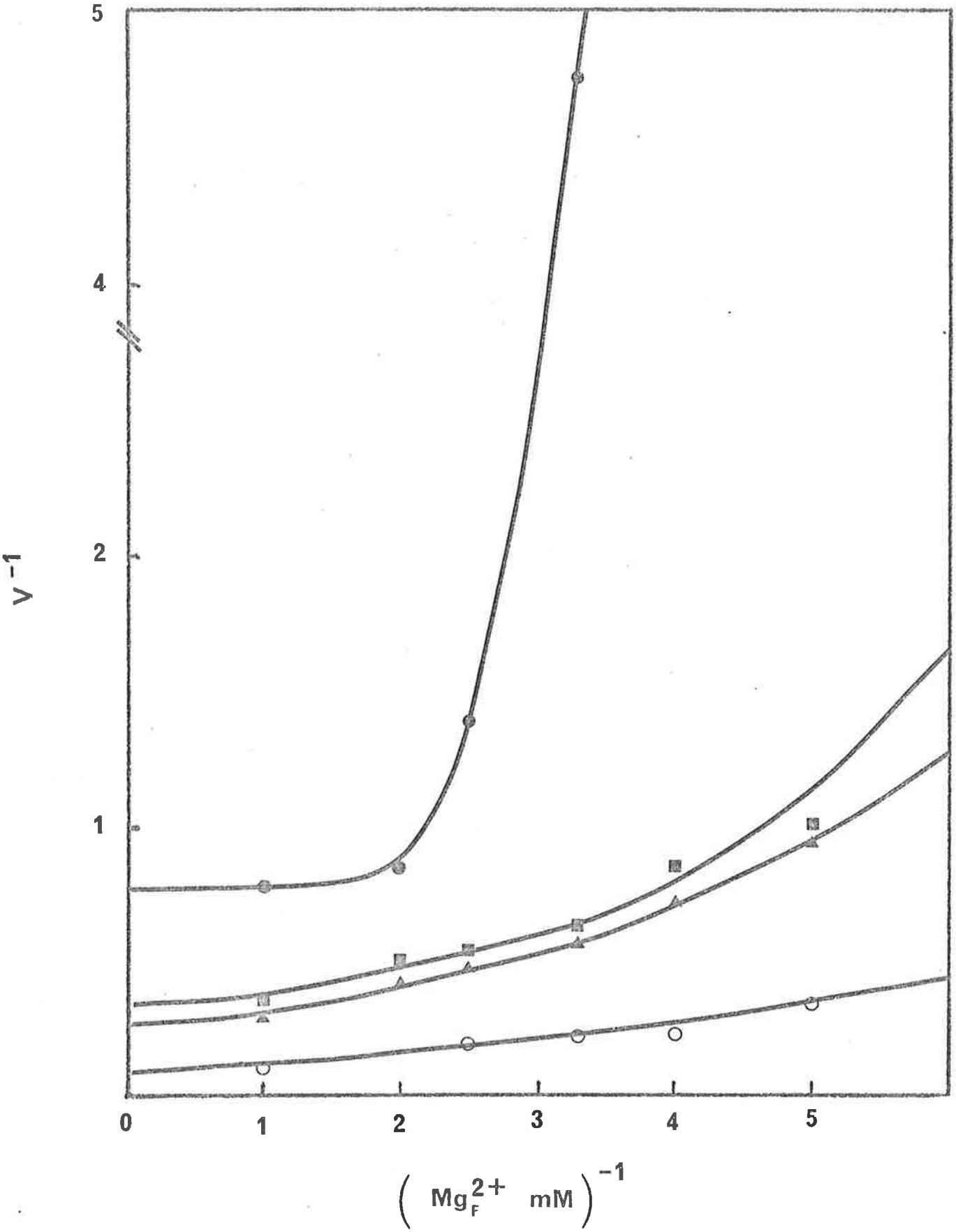
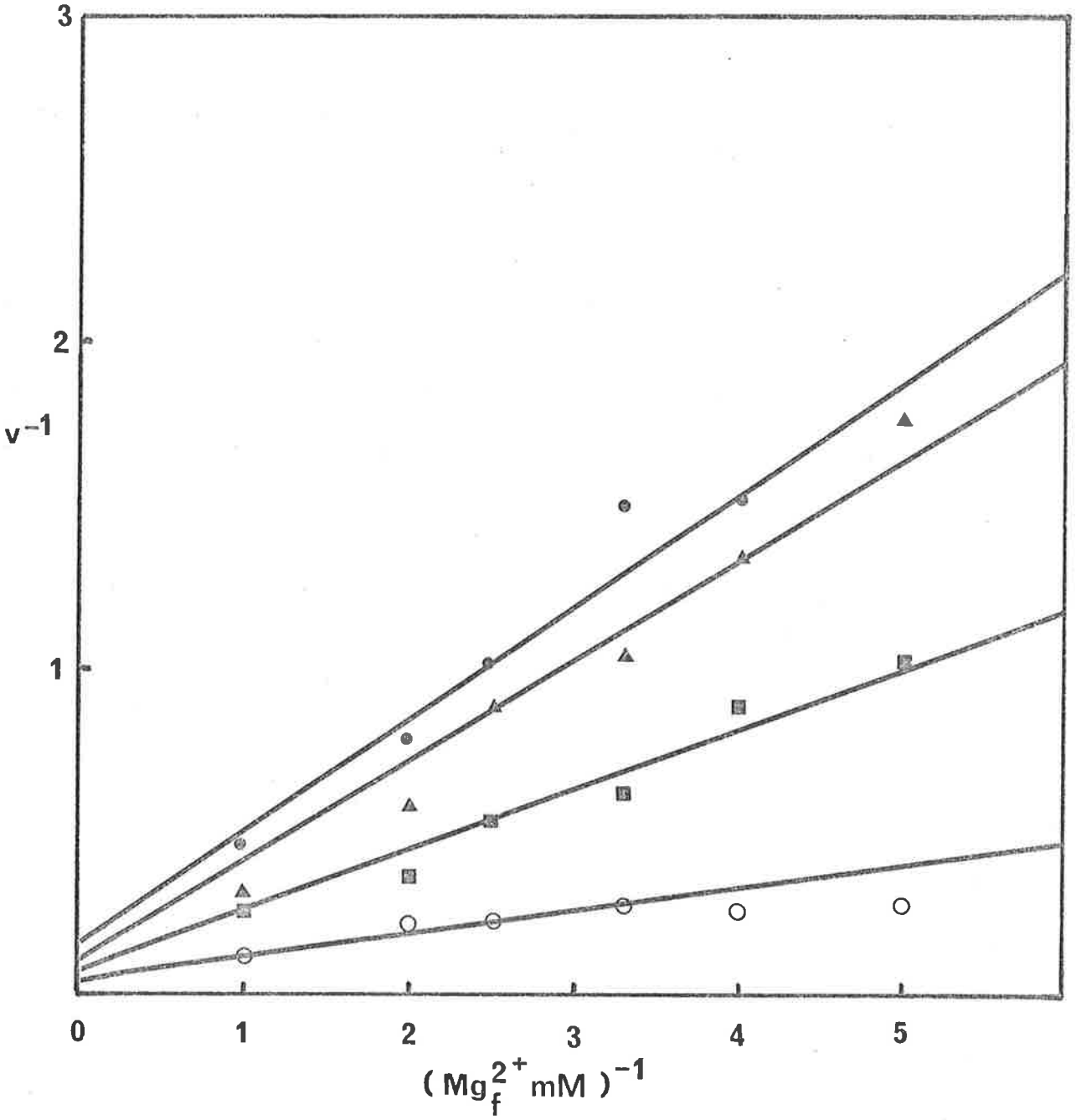

5
4
2
1
0
1
2
3
4
5
V⁻¹
(Mg²⁺F mM)⁻¹

FIGURE 3.6

Experiment described by Warren and Tipton (1974) repeated with sheep liver pyruvate carboxylase and the substrates maintained at concentrations, in μmoles per ml; MgATP, (0.5); pyruvate, (50); and $NaHCO_3$ (100).

The acetyl CoA concentrations were fixed at 5×10^{-6} M (●), 7.5×10^{-6} M (▲), 1.25×10^{-5} M (■), 4.6×10^{-5} M (○).


3
2
1
v⁻¹
0
1
2
3
4
5
(Mg²⁺f mM)⁻¹

As seen in Fig. 3.7, increasing the concentration of free Mg^{2+} caused a significant decrease in the sigmoidicity of the velocity curve. To quantitate this effect, the kinetic data were fitted to the empirical Hill equation. From Table 3.4 it can be concluded that the n_H values decrease and approach unity with increasing concentrations of free Mg^{2+}. This result indicates that since the n_H value for acetyl CoA in pyruvate carboxylase can be induced to approach unity, by increasing the concentrations of substrate and activator, this would imply that there is no cooperativity between enzyme and acetyl CoA. The fact that an n_H value slightly greater than one was still obtained can be explained by two specific properties of pyruvate carboxylase. Firstly, the enzyme would be continually undergoing dilution inactivation at low concentrations of acetyl CoA and secondly by the existence of the biotin$\sim CO_2$ leak which is maintained even at high concentrations of pyruvate (Easterbrook-Smith et al., (1976b)).

The observation that Mg^{2+} decreases the Hill coefficient for acetyl CoA is in agreement with that published by Dugal (1973), but contrary to that published by Barden and Scrutton (1974) and Warren and Tipton (1974a). A repeat of the same experiment with assay conditions identical to that used by Warren and Tipton, produced the same result (Table 3.5) as was obtained by these authors, namely, that Mg^{2+} has no effect on the n_H for acetyl CoA. The only difference between the experiments

FIGURE 3.7

Effect of free Mg^{2+} on enzyme velocity with varying acetyl CoA.

Enzyme S.A. 10, 0.06 U per assay was used in the procedure described in Section 2.2.5.2. Velocity was measured at fixed concentrations of free Mg^{2+}, 1×10^{-5} M (●), 5×10^{-5} M (▲), 1×10^{-4} M (■), 5×10^{-4} M (○) and 1×10^{-3} M (□).

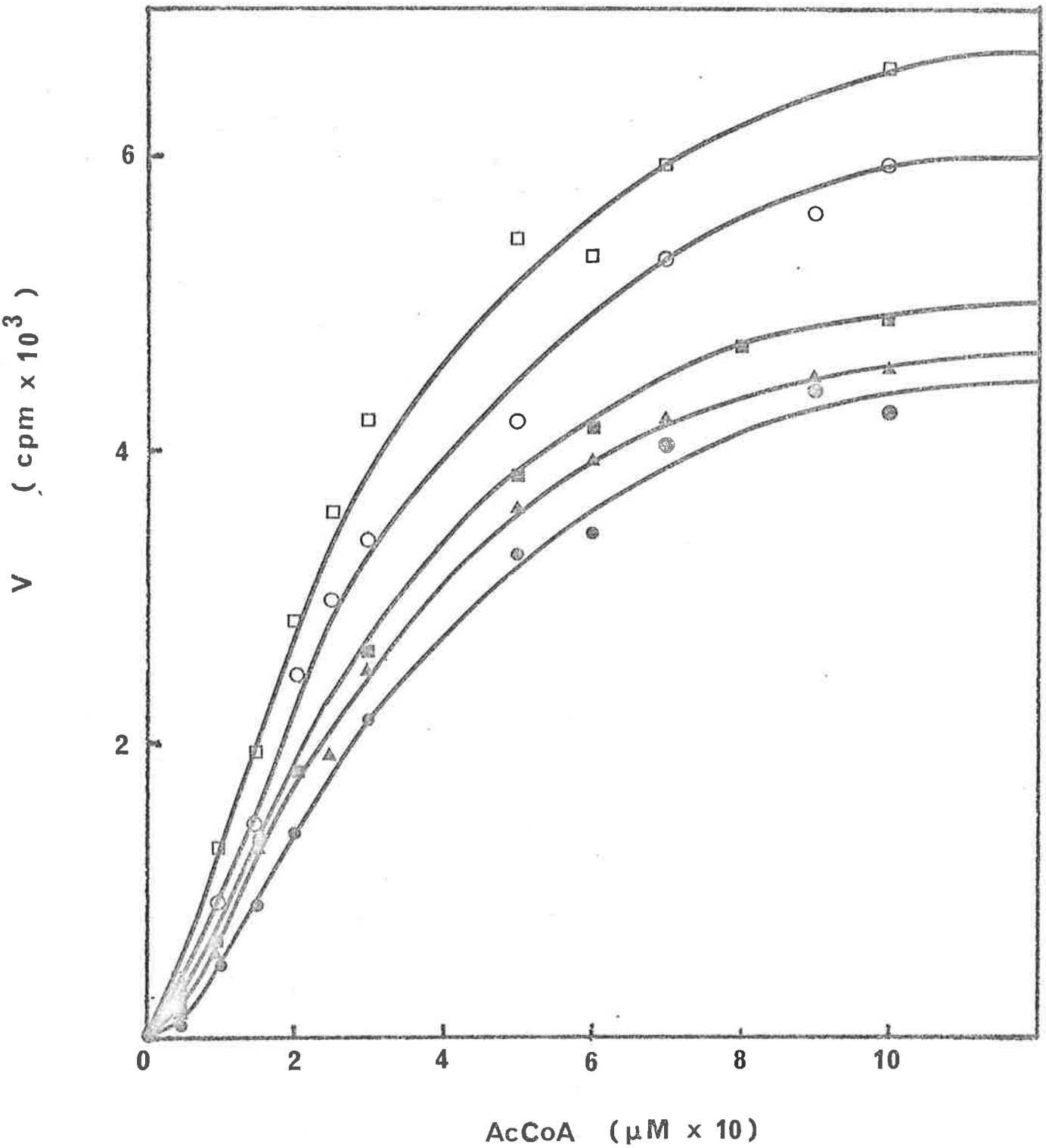

V (cpm x 10^3)
6
4
2
0
2
4
6
8
10
AcCoA (μM x 10)

TABLE 3.4

[Free Mg^{2+}] mM	n_H for acetyl CoA
0.01	2.28
0.05	2.19
0.1	2.07
0.5	1.77
1.0	1.57
1.5	1.39
2.5	1.27
3.5	1.25
4.5	1.52
5.5	1.39
6.5	1.29
7.5	1.08

The concentrations of the substrates pyruvate, bicarbonate and MgATP were maintained at 20 mM, 13 mM and 2.5 mM respectively.

TABLE 3.5

[Free Mg^{2+}] mM	n_H for acetyl CoA
0.6	2.1
0.8	2.3
1.0	2.1
2.0	2.2

The results from experiments using the assay system of Warren and Tipton (1974) containing 5 mM sodium pyruvate, 1.5 mM $NaHCO_3$ and 0.5 mM MgATP. The concentration of enzyme (S.A. 22) was 0.06 U per assay tube.

(giving rise to Tables 3.4 and 3.5) is the absolute concentrations of pyruvate, bicarbonate and MgATP. Although pyruvate, and bicarbonate may not have been saturating, the results of Easterbrook-Smith (1977) would predict that Mg^{2+} should still have had an effect since the substrates would become increasingly more saturating. Since there was no effect, one could rationalize that this could be due to the low concentration of MgATP employed. Although the concentration was approximately ten times the apparent k_m, the kinetic constant could have been underestimated since it was determined by varying MgATP at fixed concentration of Mg^{2+}, and at saturating concentrations of acetyl CoA. Mg^{2+} is known to decrease the k_m for MgATP and thus, assuming that Mg^{2+}-acetyl CoA is the true activator, at a fixed Mg^{2+} concentration and increasing concentrations of acetyl CoA, the amount of free Mg^{2+} decreases and this could result in the k_m for MgATP significantly increasing thus changing the saturation of this substrate on the enzyme. This could result in masking the decrease in Hill coefficient (Table 3.5) which is observed at higher concentration of MgATP (Table 3.4).

3.3.5 The binding of acetyl CoA to pyruvate carboxylase

Direct binding studies cannot be successfully performed up to date, since the rapid diffusion technique of Colowick and Womack, (1969) relies on radioactive substrates diffusing through the binding cell membrane and at low concentrations of acetyl CoA, [^{14}C]acetate

contamination, would result in an erroneous estimation of the amount of radioactivity unbound to the enzyme and thus the basic parameter used to construct a binding curve would be wrong. As pointed out by Easterbrook-Smith (1977), the deacylase activity of pyruvate carboxylase becomes very significant at the high concentrations of enzyme used, and thus at low concentrations of acetyl CoA, there is a large proportion of [^{14}C]acetate in the binding cell chamber and this diffuses across the membrane at a faster rate that [^{14}C]acetyl CoA. At high concentrations of [^{14}C]acetyl CoA this does not apply because the amount of [^{14}C]acetate becomes negligible compared with unbound [^{14}C]acetyl CoA and thus the estimate by Frey and Utter,(1977) that 3 - 4 moles of acetyl CoA bind per mole of enzyme is indeed correct.

The binding of acetyl CoA to pyruvate carboxylase was therefore measured using three indirect techniques:

(i) initial velocity kinetics of deacylation,

(ii) protection by acetyl CoA against enzyme inactivation by dilution,

(iii) protection by acetyl CoA against modification of the enzyme by TNBS.

3.3.5.1 Deacylase activity of pyruvate carboxylase

In view of the accumulating evidence suggesting that an n_H value greater than unity is not indicative of cooperativity of acetyl CoA binding, it was decided to test this

further using the specific acetyl CoA deacylase activity of pyruvate carboxylase. That deacylation occurs at the acetyl CoA activator site is supported by the reports of Frey and Utter (1977) that only 3 - 4 molecules of acetyl CoA bind per tetrameric enzyme molecule and also by the fact that substrates of the carboxylation reaction enhance the rate of deacylation. It was previously stated, that the sigmoid acetyl CoA curve for the carboxylation reaction could quite easily be due to the enzyme being destabilized during the assay time and also to non-saturation of substrates. The conditions of the deacylase reaction are such that enzyme concentration is greater than 4 U/ml and therefore dilution inactivation does not occur, and also, since there is only one substrate in the reaction, it is impossible to obtain a sigmoid velocity curve if the binding sites were indeed independent (Childs and Bardsley, 1975). Therefore if acetyl CoA is binding to pyruvate carboxylase in a classical Michaelis-Menten process, a hyperbolic response to the rate of deacylation would be expected.

Figures 3.8 and 3.9 present data from experiments which show the rate of deacylation by sheep and chicken liver pyruvate carboxylases respectively

FIGURE 3.8

The dependence of the rate of acetyl CoA deacylation on acetyl CoA concentration.

The rate of acetyl CoA deacylation at the acetyl CoA concentrations indicated was determined using the procedure described in Section 3.2 and 2.5 units of sheep liver pyruvate carboxylase (S.A. 19 U/mg) per assay tube.

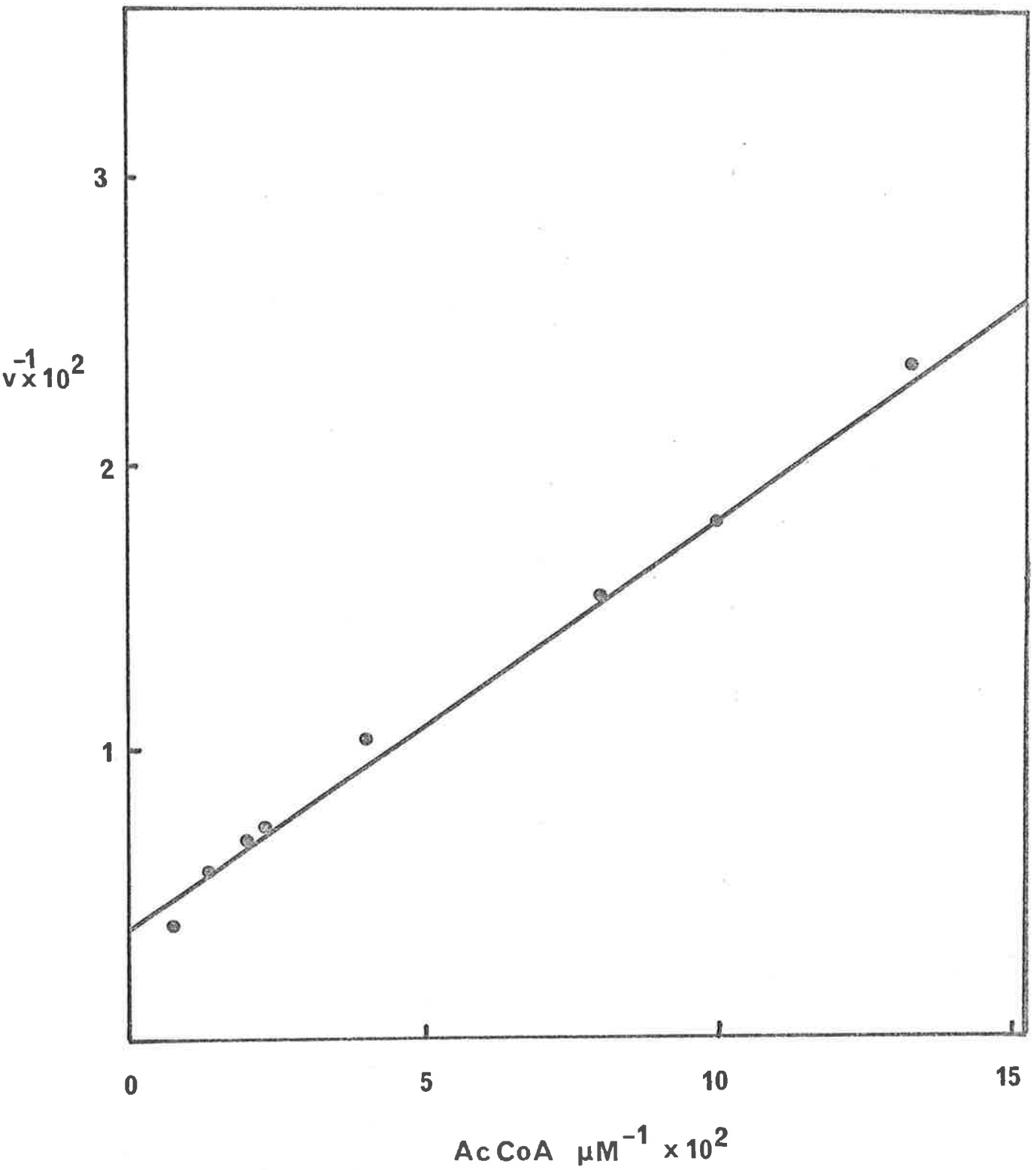
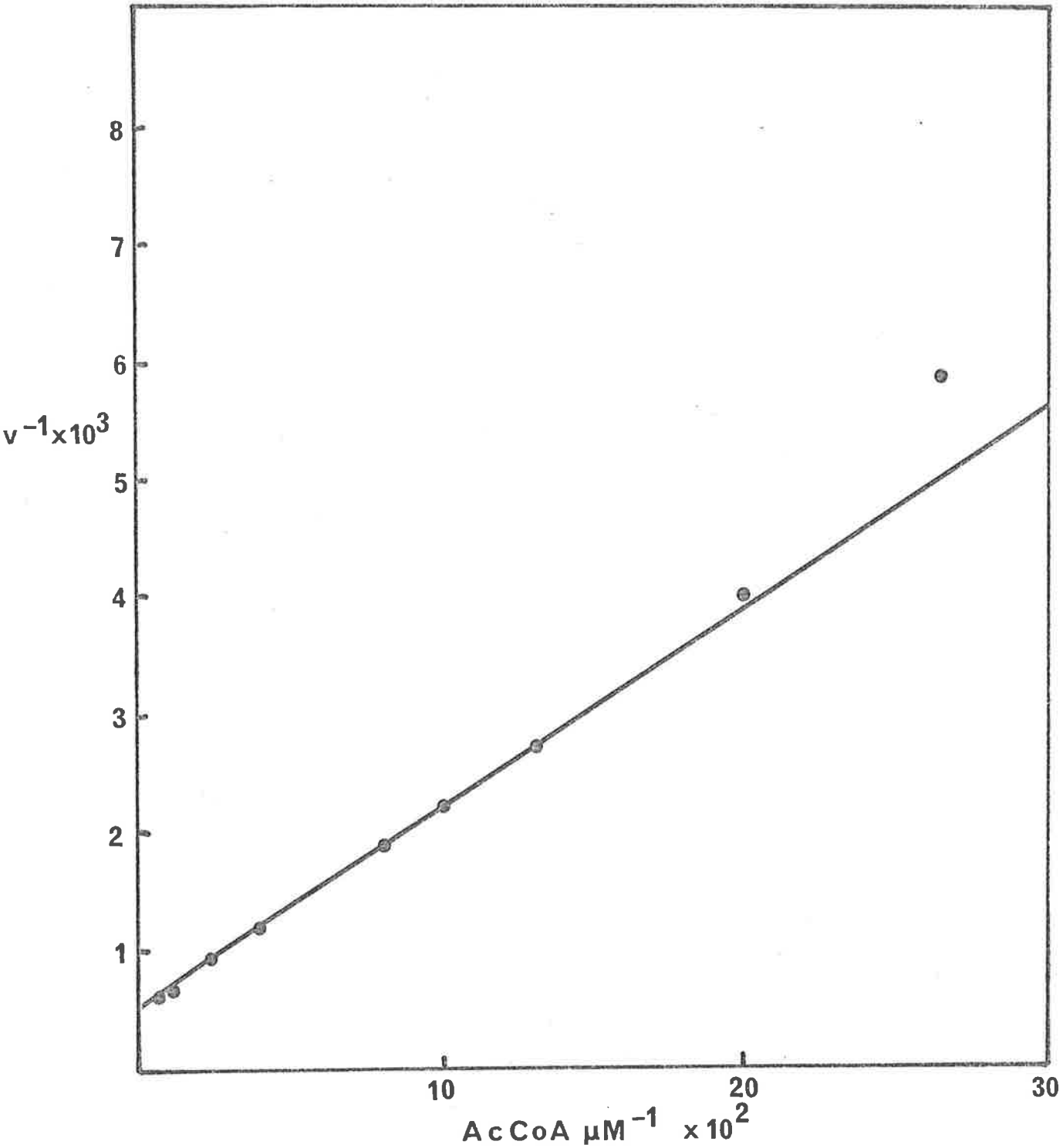

3
2
1
$v^{-1} \times 10^2$
0
5
10
15
AcCoA $\mu M^{-1} \times 10^2$

FIGURE 3.9

The dependence of the rate of acetyl CoA deacylation on acetyl CoA concentration.

A similar experiment to that described in Fig. 3.8 but using chicken liver pyruvate carboxylase (S.A. 25 U/mg, 10 units).


8
7
6
5
4
3
2
1
v⁻¹x10³
10
20
30
AcCoA µM⁻¹ x10²

as a function of acetyl CoA concentration. MgATP, Mg^{2+} and pyruvate were included in order to obtain a measure of the binding of acetyl CoA under conditions approaching those of the carboxylation assay. The k_m for acetyl CoA in this process was 40 ± 4.7 μM. The result of this experiment implies that the binding of acetyl CoA to the enzyme under these conditions is indeed a Michaelis-Menten process. The presence or absence of Mg^{2+} did not seem to alter the k_m for acetyl CoA. This result is surprising, although it could be accounted for, bearing in mind that k_a is a function of binding whereas k_m is proportional to the rate of catalysis, and thus it would seem that the reaction velocity is not significantly altered by the enhanced binding of the substrate. This postulate can be rationalized by the accumulating evidence that the extremities of the acetyl CoA molecule bind in two spatially distinct sites (Clements, unpublished results) with the acetyl end binding prior to the adenosine moiety.

3.3.5.2 Protection by acetyl CoA against dilution inactivation

It has been observed that sheep liver pyruvate carboxylase undergoes inactivation upon dilution to below 4 U/ml (Ashman et al., 1972).

Acetyl CoA protects against this process. Therefore in the presence of a certain non-saturating concentration of acetyl CoA, only the fraction of enzyme where acetyl CoA is unbound is susceptible to dilution inactivation. Using this approach, the relative $t_{\frac{1}{2}}$ values (where $t_{\frac{1}{2}}$ equals the time for 50% inactivation of enzyme) would be proportional to the amount of enzyme not bound by acetyl CoA. The rate constant for inactivation can be represented as

$$k_{inact} = k_1 E_o = \frac{\ln 2}{t_{\frac{1}{2}}}$$

where k_1 is the true first order rate constant, and E_o is the fraction of enzyme without acetyl CoA which is equivalent to

$$\frac{E_T}{1 + (\frac{[AcCoA]}{k_a})^{n_H}}$$

Therefore the rate of inactivation in the absence of acetyl CoA relative to the rate of inactivation in the presence of acetyl CoA, i.e., $\frac{k_1^o}{k_1^a}$, can be expressed as

$$\log (\frac{k^o_{inact}}{k^a_{inact}} - 1) = n_H \log [AcCoA] - n_H \log k_a$$

(Equation 3.2)

The slope of the line obtained by plotting log

$\left(\frac{k^{o}_{inact}}{k^{a}_{inact}} - 1\right)$ as a function of log [AcCoA] will be n_H and the intercept on the ordinate will be $- n_H \log k_a$ (for derivation of equation 3.2, see Legend to Fig. 3.11).

Figure 3.10, shows the protection from inactivation of the enzyme upon dilution to 0.1 U/ml by various concentrations of acetyl CoA in the presence and absence of Mg^{2+}. Mg^{2+} alone had no effect on the rate of dilution inactivation. The rate constants obtained from these plots were used to construct the linear regression according to equation 3.2. The Hill coefficient obtained in the absence of $MgCl_2$ was 2.2 $\pm$ 0.14 (Fig. 3.11). However, in the presence of $MgCl_2$, the n_H value was 1.3 $\pm$ 0.03 (Fig. 3.12). The k_a values were 9.6 µM $\pm$ 1.064 µM and 3.45 $\pm$ 1.01 µM in the absence and presence of Mg^{2+} respectively.

3.3.5.3 Protection against TNBS modification

Sheep liver pyruvate carboxylase is desensitized against acetyl-CoA by incubation with TNBS since this chemical interacts with a lysyl group whose integrity is essential for full enzymic activity. The decline of catalytic activity in the standard assay of pyruvate carboxylase obeyed pseudo-first order kinetics at pH 8.4. Semi-logarithmic

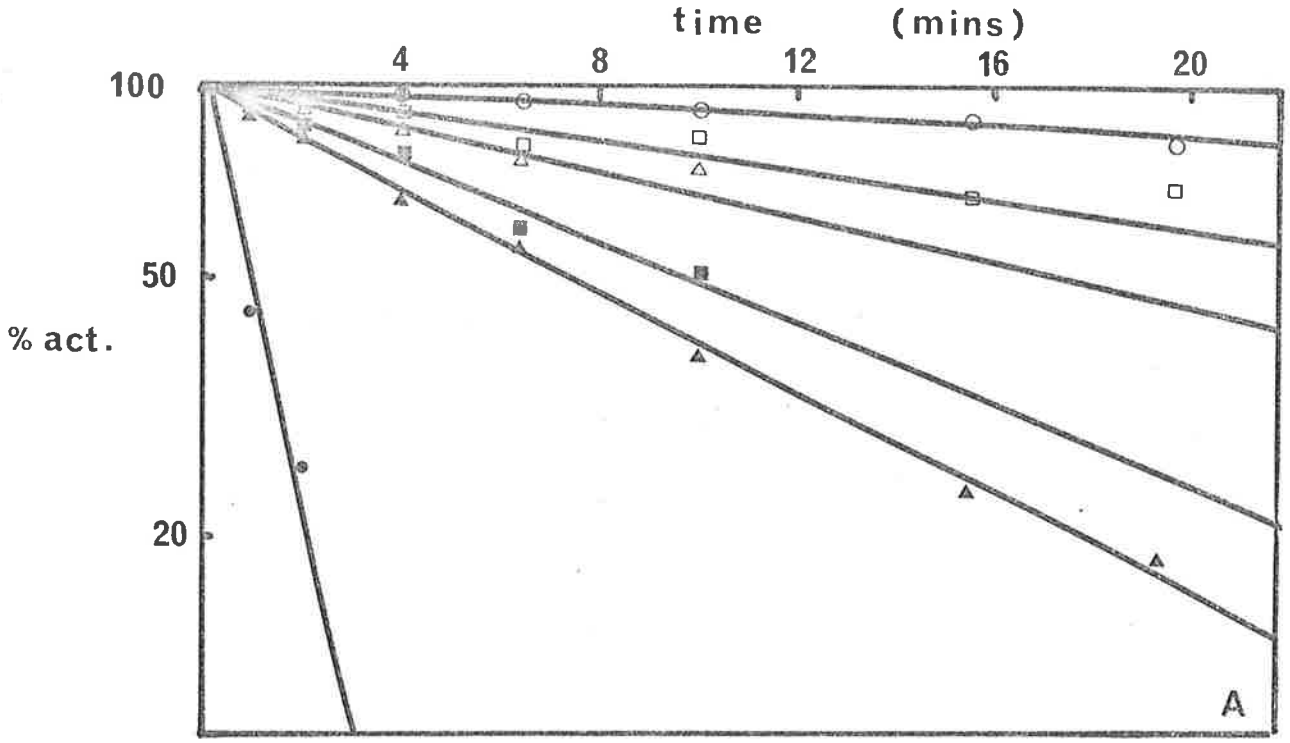
FIGURE 3.10

Protection against dilution inactivation at 0.1 U/ml in 10 mM KP_i pH 7.5.

Sheep liver pyruvate carboxylase (S.A. 5 U/mg, 5.8 U/ml) was diluted into incubation tubes containing potassium phosphate buffer and various concentrations of the protecting species. At certain time intervals aliquots were removed and assayed for remaining activity.

In Fig. A, the concentrations of acetyl CoA used were 0 (●), 2.5×10^{-5} M (▲), 5×10^{-5} M (■), 7.5×10^{-5} M (△), 1×10^{-4} M, (□) and 1.25×10^{-4} M (○).

In Fig. B, the concentrations of Mg^{2+}-acetyl CoA used were 0 (●), 2×10^{-6} M (▲), 4×10^{-6} M (■), 8×10^{-6} M (△) and 1×10^{-5} M (○).


time (mins)
4
8
12
16
20
100
50
20
% act.
A

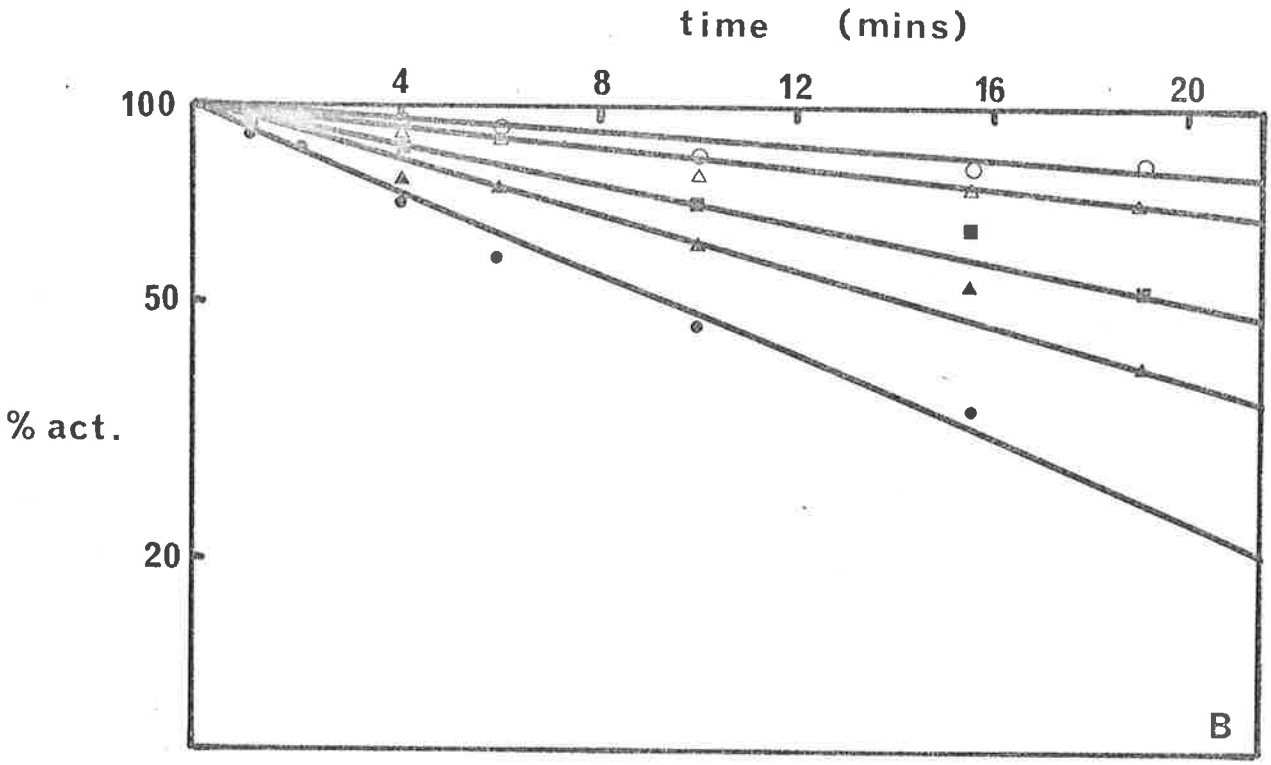

time (mins)
4
8
12
16
20
100
50
20
% act.
B

FIGURE 3.11

Derivation of Equation 3.2

In theory, only the fraction of the enzyme without bound acetyl CoA is susceptible to dilution inactivation.

If the saturation of pyruvate carboxylase with acetyl CoA can be described by the Hill coefficient equation, then the dependence of enzyme saturation ($\bar{Y}$) on acetyl CoA can be written as,

$$\bar{Y} = \frac{1}{1 + (\frac{k_a}{A})^{n_H}}$$

where k_a, A and n_H represent the dissociation constant, the acetyl CoA concentration and the Hill coefficient.

Since $\bar{Y} = \frac{EA}{E_T}$

where EA and E_T represent the concentration of enzyme bound with acetyl CoA and the total enzyme concentration respectively,

then $E_F = \frac{E_T}{1 + (\frac{A}{k_a})^{n_H}}$

where E_F is the concentration of free enzyme.

For an inactivation process,

$$k_{inact} = k_1 E_F$$

where k_{inact} is the pseudo first order rate

Figure 3.11 cont.

constant which may be derived from the expression,

$$k_{inact} = \frac{\ln 2}{t_{\frac{1}{2}}}$$

where $t_{\frac{1}{2}}$ is the time for inactivating 50% of the enzyme; k_1 is a true first order rate constant

$$\frac{k^{o}_{inact}}{k^{a}_{inact}} - 1 = \frac{A^{n_H}}{k_a^{n_H}}$$

where k^{a}_{inact} and k^{o}_{inact} are the rate constants for the inactivation process in the presence and absence of acetyl CoA.

Therefore,

$$\log\left(\frac{k^{o}_{inact}}{k^{a}_{inact}} - 1\right) = n_H \log A - n_H \log k_a$$

Figure 3.11 cont.

Replot of Fig. 3.10 A using the equation

$$\log \left(\frac{k^{o}_{inact}}{k_{inact}}_{a} - 1\right) = n_H \log AcCoA - n_H \log k_a$$

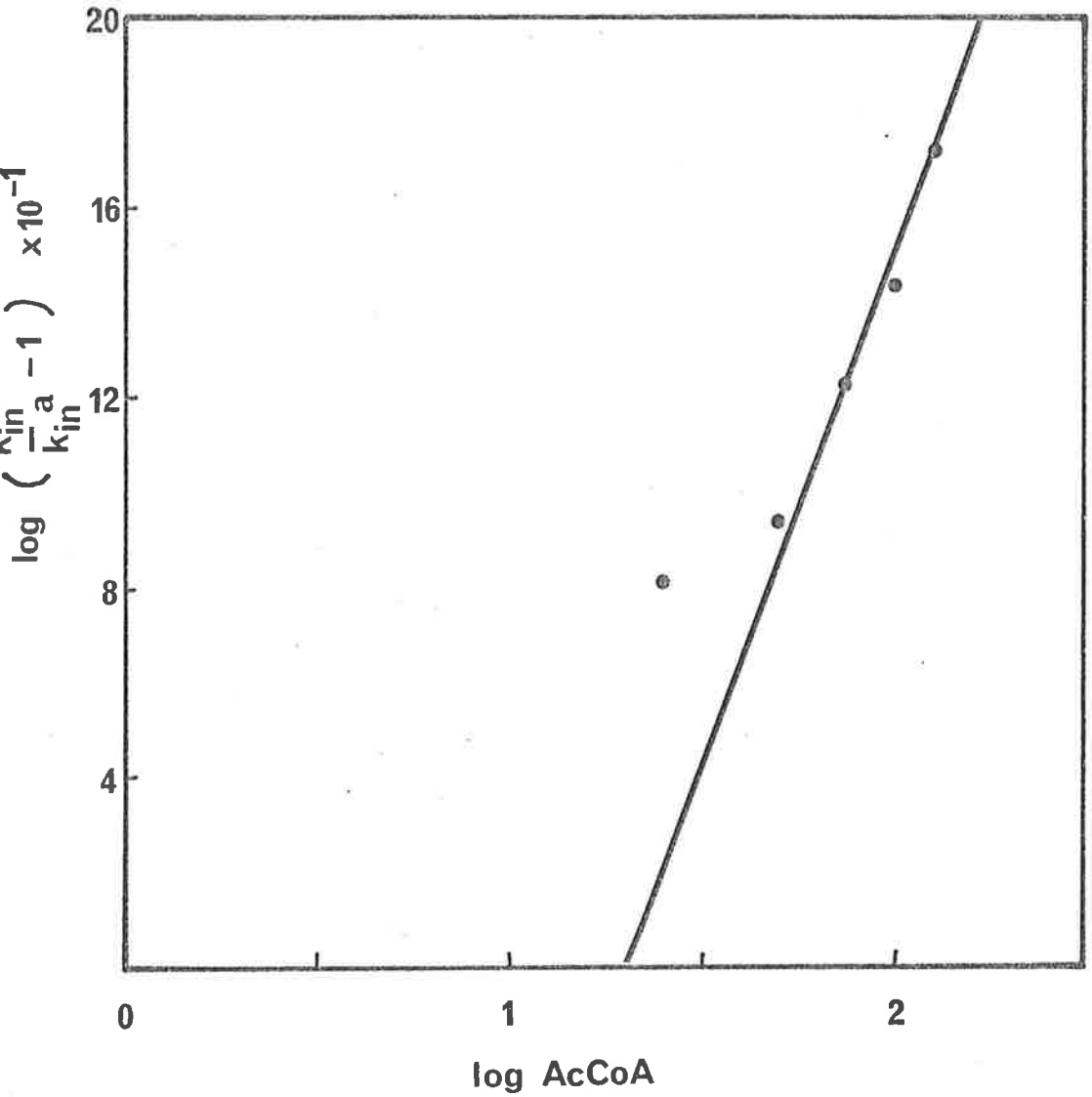
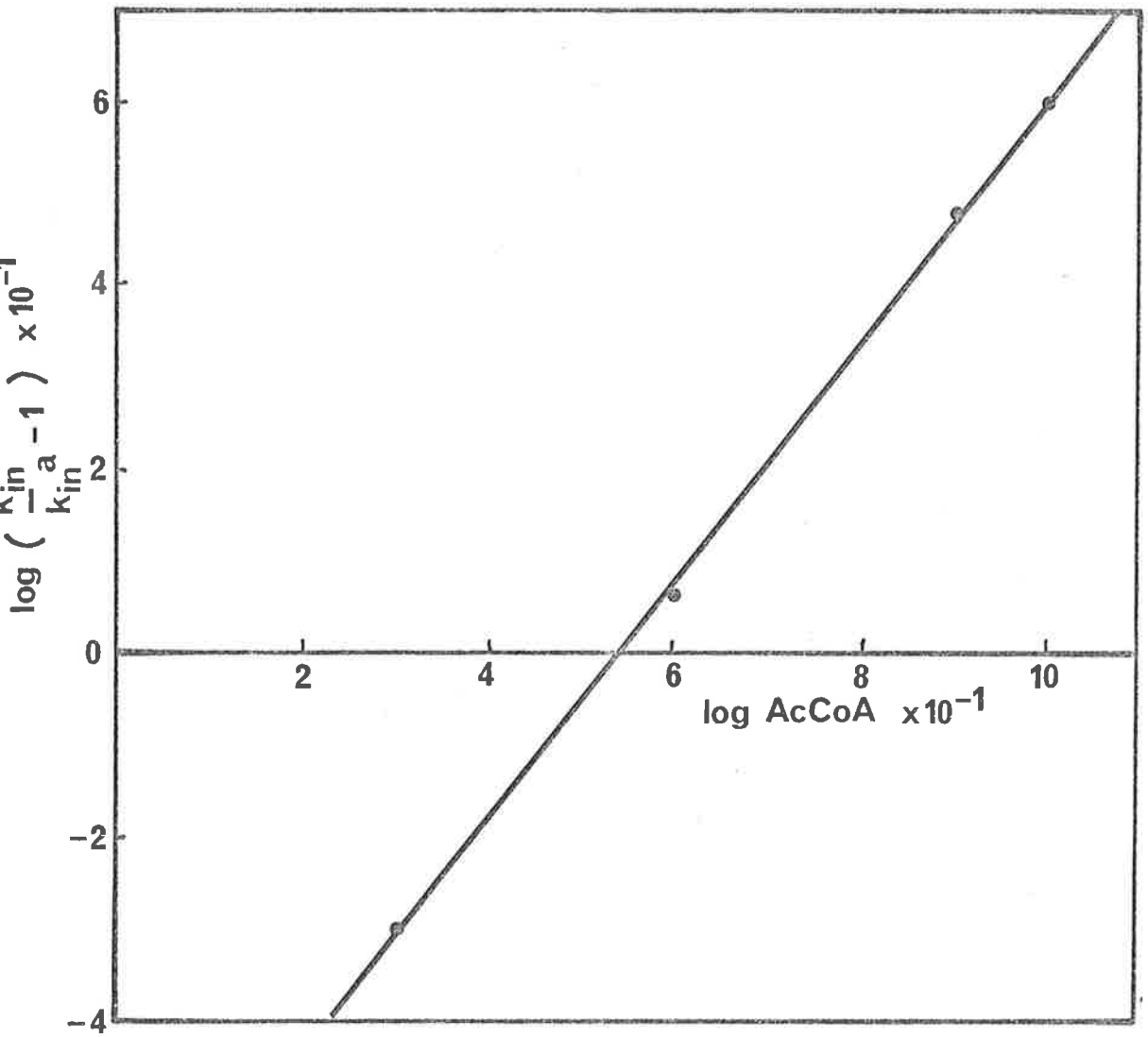

20
16
12
8
4
0
1
2
log AcCoA
log ($\frac{k_{in}^{o}}{k_{in}^{a}}$ − 1) x10^{-1}

FIGURE 3.12

Replot of Fig. 3.10 B using the equation

$$\log \left(\frac{k_{inact}^{o}}{k_{inact}^{a}} - 1\right) = n_H \log AcCoA - n_H \log k_a \ .$$


6
4
2
0
−2
−4
2
4
6
8
10
log AcCoA x10⁻¹
log (k°in / k^a_in − 1) x10⁻¹

plots of residual activity as a function of time were linear down to 10% of initial activity.

The effect of various concentrations of acetyl CoA against TNBS inactivation is shown in Fig. 3.13. It is evident that as acetyl CoA concentration increases, the $t_{\frac{1}{2}}$ values for inactivation also increase quite markedly. Determining the $t_{\frac{1}{2}}$ values at all the acetyl CoA concentrations and applying the formula $k_{inact} = \frac{\ln 2}{t_{\frac{1}{2}}}$ and using equation 3.2, the Hill coefficient was calculated to be 1.6 ± 0.11 and a k_a value of 27.5 μM $\pm$ 1.66 was obtained (Fig. 3.14). These results are in agreement with those obtained by Scrutton and White (1973).

Mg^{2+} alone does not exert any protection against TNBS modification (Fig. 3.15) but acetyl CoA in the presence of Mg^{2+} offered a greater degree of protection than just acetyl CoA alone (Fig. 3.16). Again using equation 3.2, an n_H value of 0.75 ± 0.075 and a k_a value of 10.9 $\pm$ 1.7 μM were obtained (Fig. 3.17). These chemical modification studies are in agreement with the findings from dilution inactivation protection studies since they suggest that Mg^{2+} together with acetyl CoA provide k_a values which are

FIGURE 3.13

Protection against chemical modification with TNBS by acetyl CoA.

Enzyme (S.A. 26) was used at a concentration of 0.55 mg/ml. TNBS was freshly made preceding each experiment to a concentration of 0.6 mM. The enzyme was preincubated with the protecting species for 15 mins prior to addition of the TNBS.

Modification was allowed to proceed for certain time intervals after which aliquots were removed and assayed for activity.

The concentrations of acetyl CoA used were 0 (●), 5×10^{-5} M (▲), 7.5×10^{-5} M (■), 1×10^{-4} M (○), 1.25×10^{-4} M (□), 1.5×10^{-4} M (△), 1.75×10^{-4} M (▼), 2×10^{-4} M (◒).

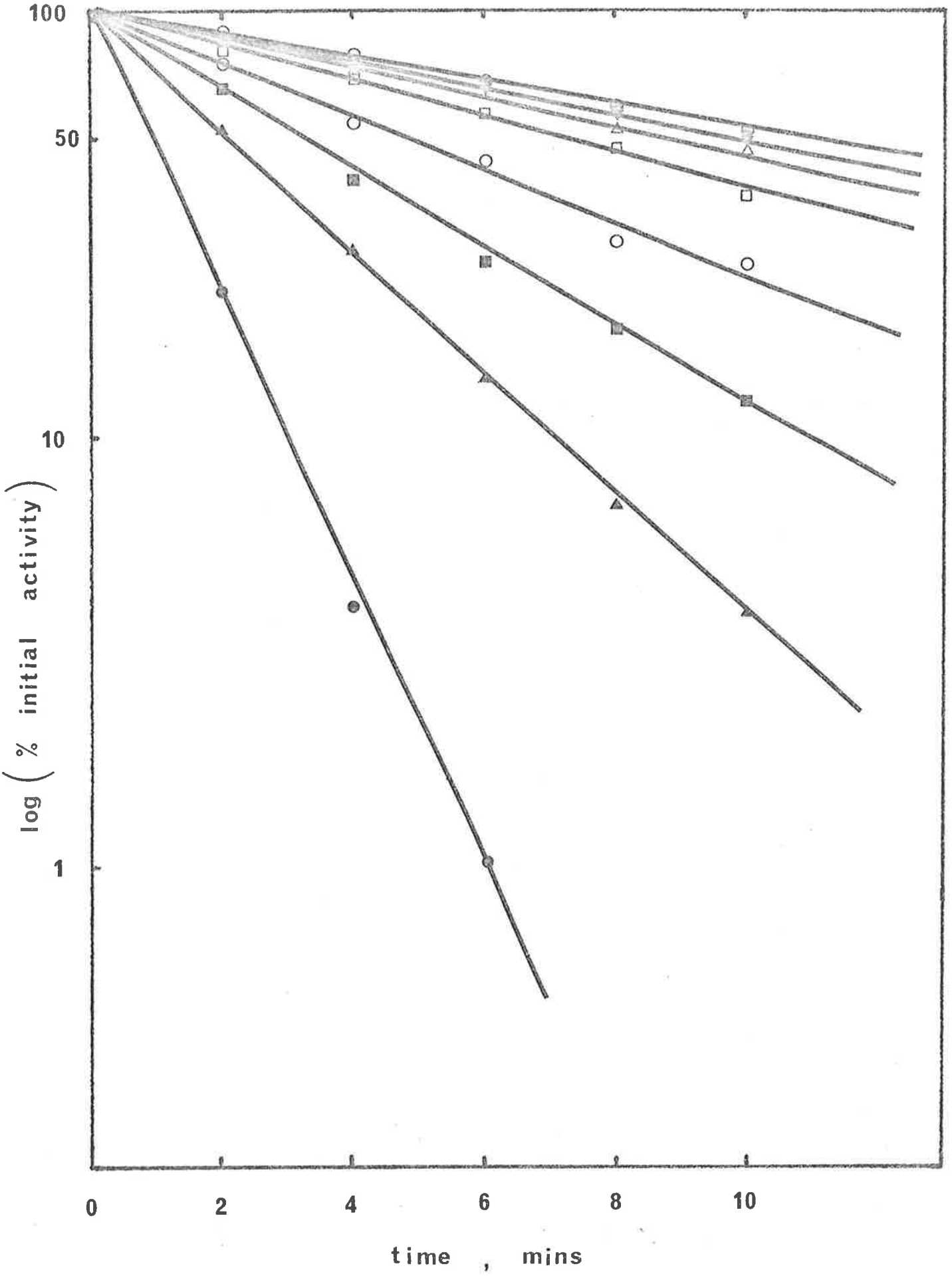

100
50
10
1
log (% initial activity)
0
2
4
6
8
10
time , mins

FIGURE 3.14

Replot of Fig. 3.13 using the equation log

$$\left(\frac{k_{inact}^{o}}{k_{inact}^{a}} - 1\right) = n_H \log AcCoA - n_H \log k_a.$$

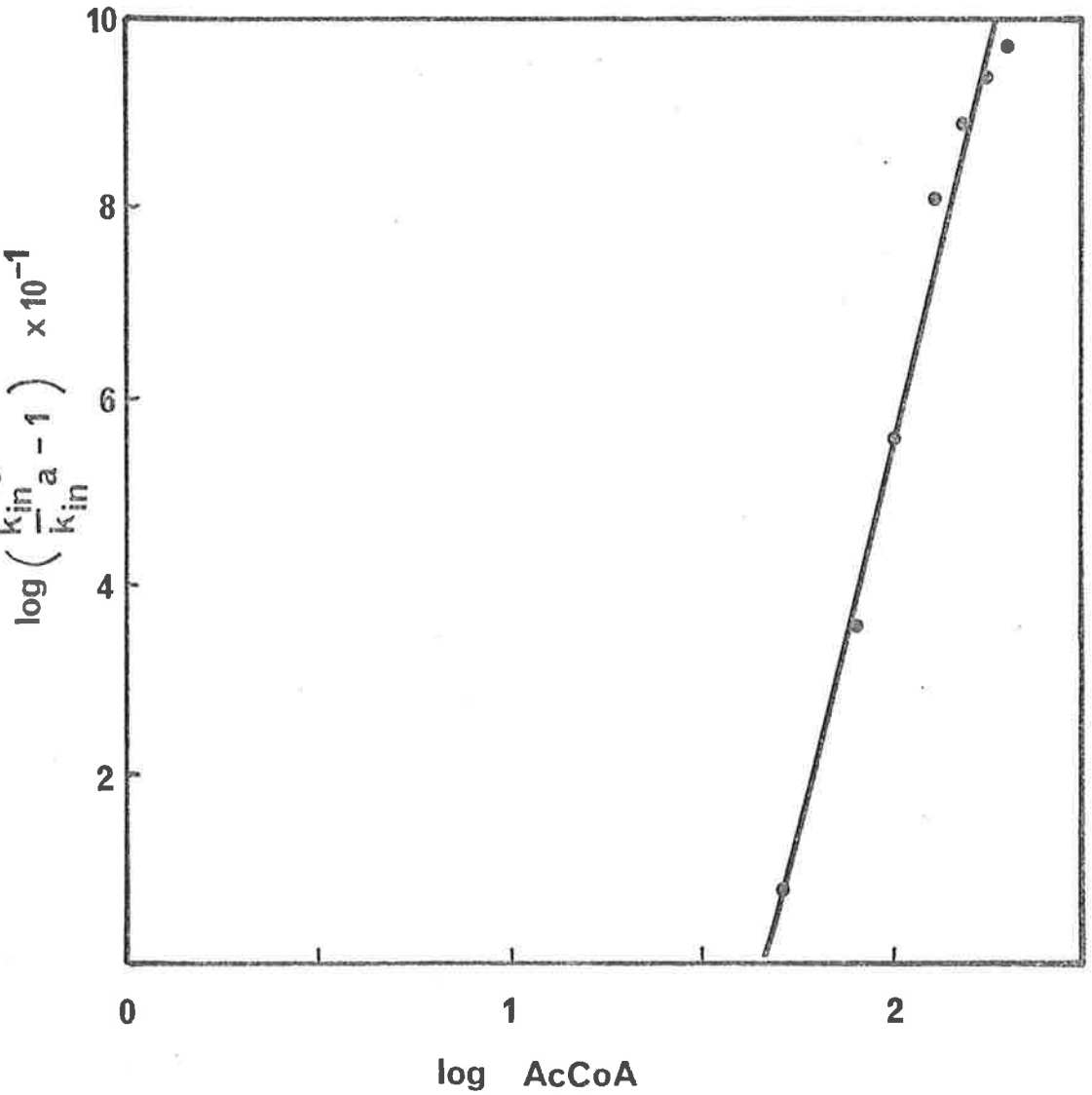

10
8
6
4
2
0
1
2
log AcCoA
$\log\left(\frac{k_{in}^{o}}{k_{in}^{a}} - 1\right) \times 10^{-1}$

FIGURE 3.15

Protection against TNBS inactivation by Mg^{2+}.

The data shows no protection by the divalent cation at all the concentrations used which were, 0 (●), 1×10^{-4} M (■), 5×10^{-4} M (▲), 1×10^{-3} M (□), and 4×10^{-3} M (○).

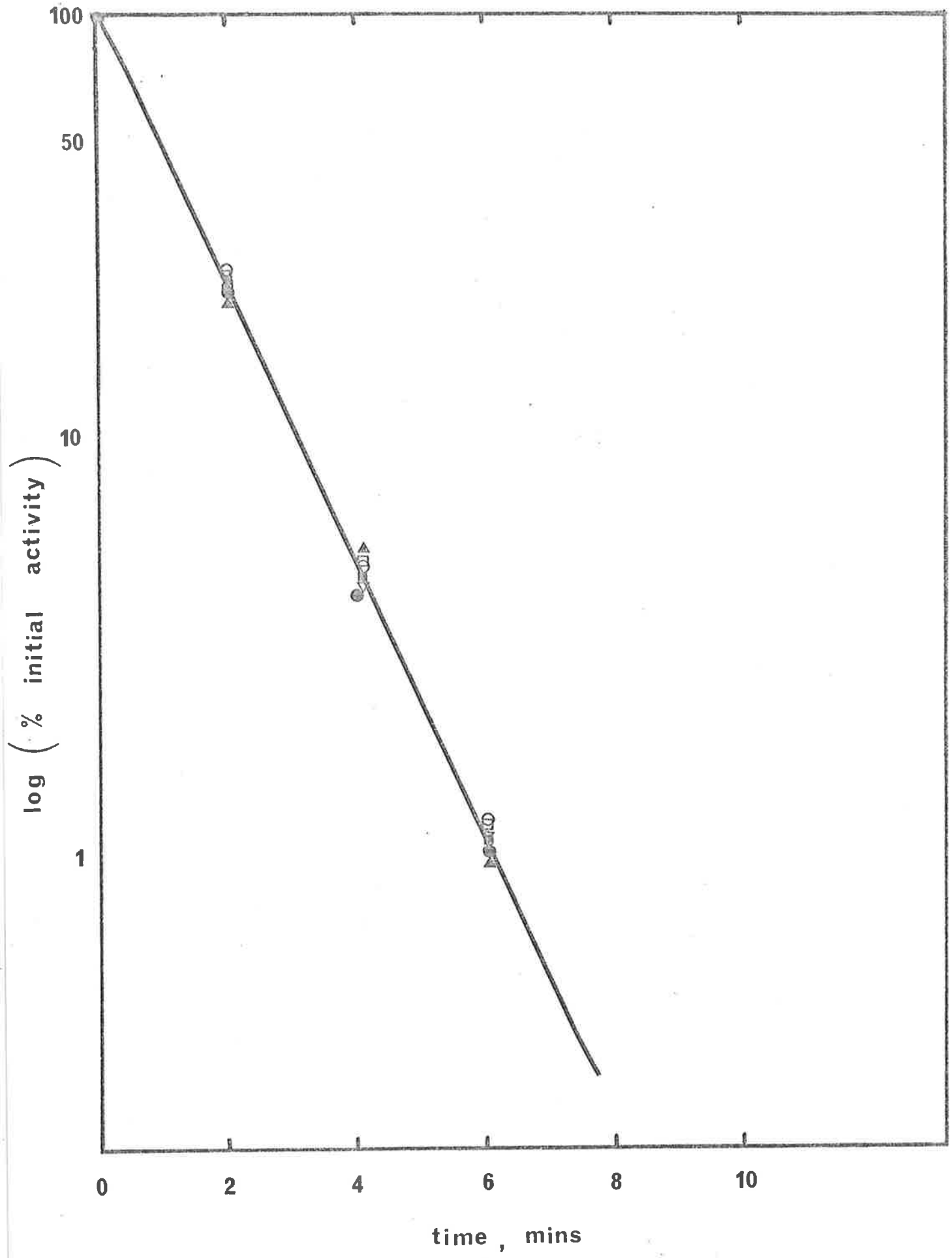

100
50
10
1
log (% initial activity)
0
2
4
6
8
10
time , mins

FIGURE 3.16

Protection against TNBS modification by acetyl CoA in the presence of Mg^{2+}.

Enzyme (S.A. 26 U/mg, 0.64 mg/ml) was pre-incubated in buffer containing 4 mM $MgCl_2$ and various concentrations of acetyl CoA prior to addition of TNBS.

The acetyl CoA concentrations used were, 0 (●), 5×10^{-5} M (■), 7.5×10^{-5} M (▼), 1×10^{-4} M (□), 1.25×10^{-4} M (○), and 1.5×10^{-4} M (▲).

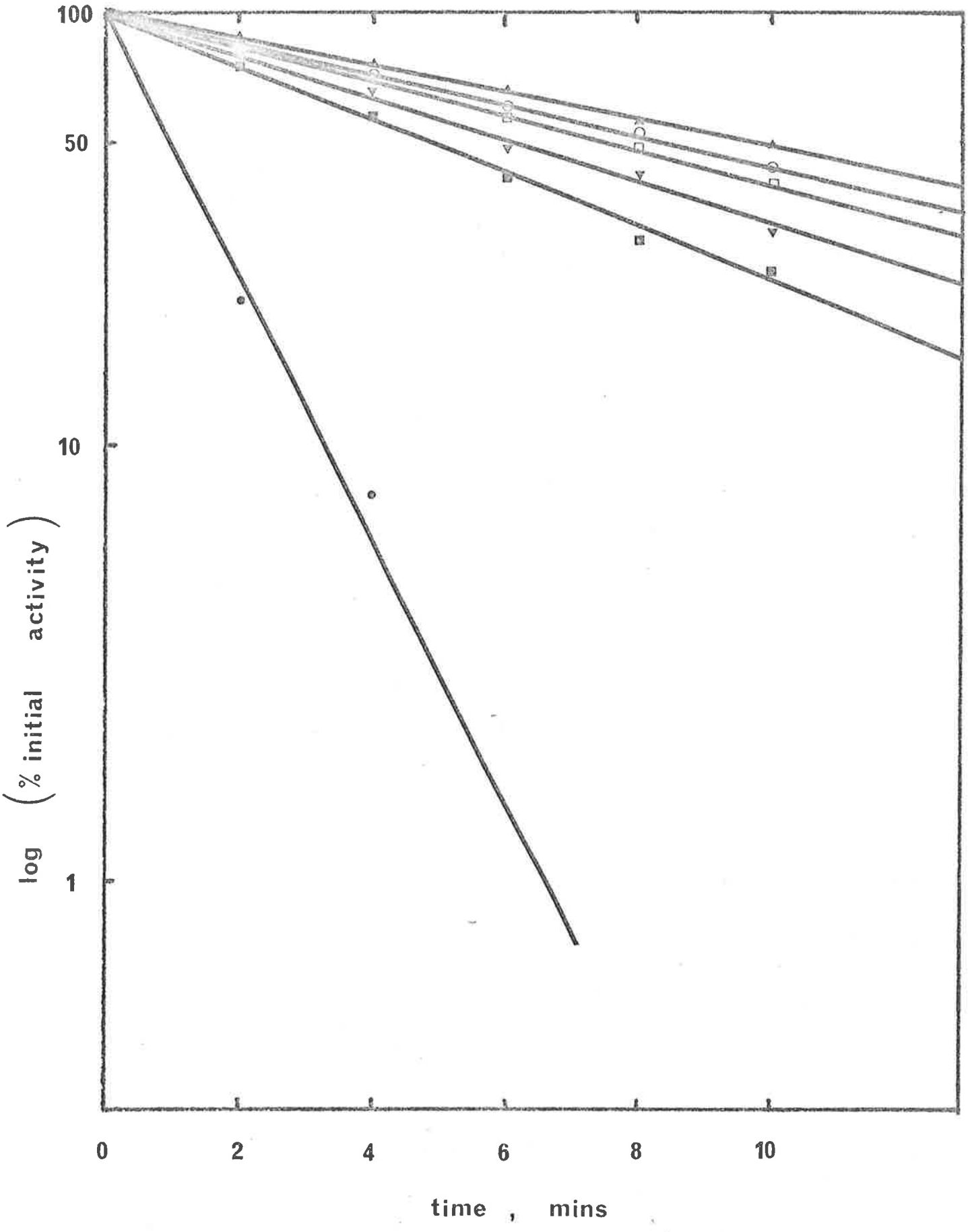
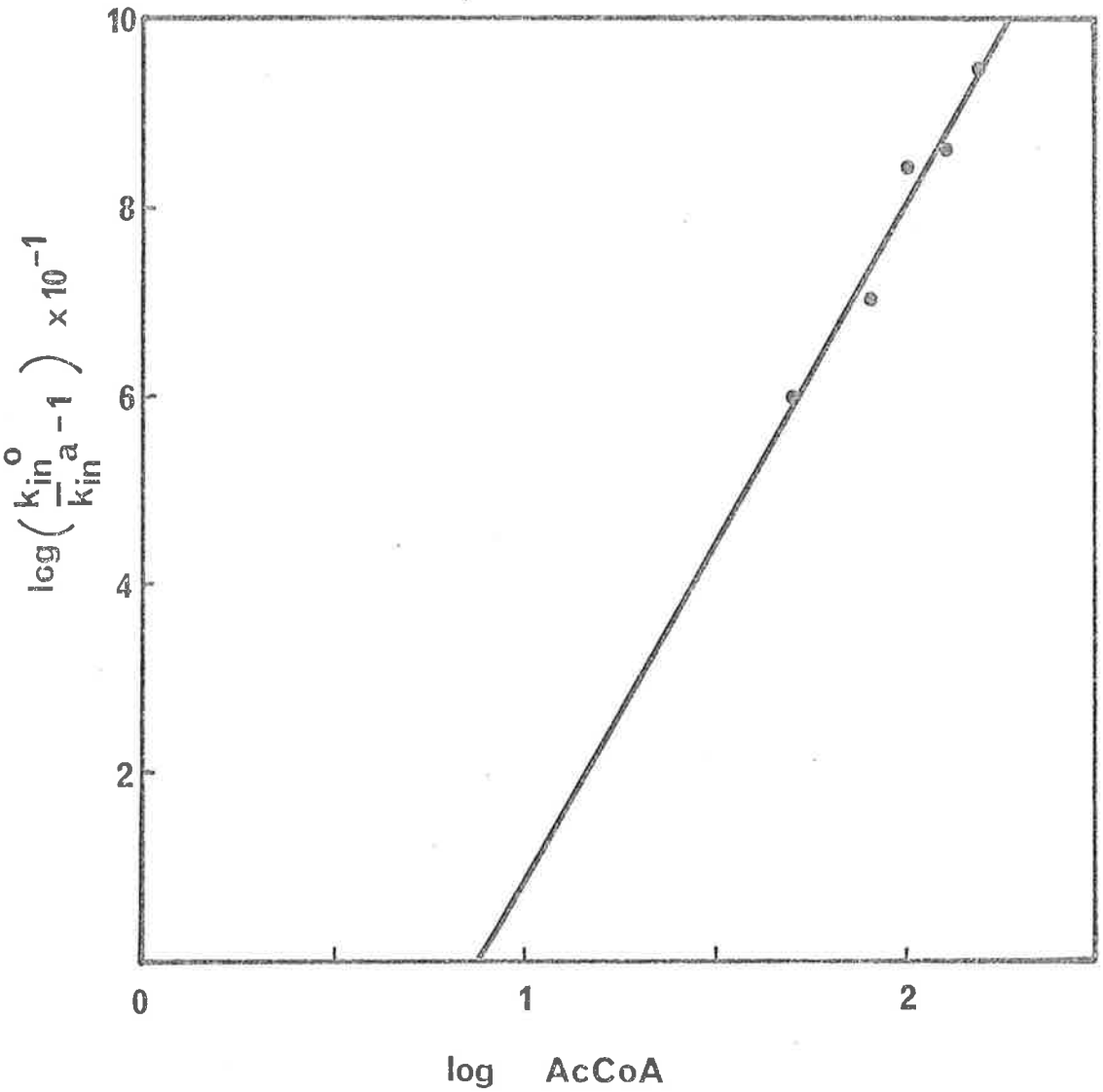

100
50
10
1
log (% initial activity)
0
2
4
6
8
10
time , mins

FIGURE 3.17

Replot of Fig. 3.16 using the equation log

$$\left(\frac{k_{inact}{}^{o}}{k_{inact}{}^{a}} - 1\right) = n_H \log AcCoA - n_H \log k_a.$$


10
8
6
4
2
0
1
2
log AcCoA
$\log\left(\frac{k_{in}^{o}}{k_{in}^{a}}-1\right) \times 10^{-1}$

comparable with the k_a value for the activator obtained from initial velocity kinetics. The n_H value for Mg^{2+} and acetyl CoA is approximately 1.0, whereas the n_H value for acetyl CoA alone, in the carboxylation reaction, is approximately 2.0. One could therefore postulate that Mg^{2+}-acetyl CoA is the true activator for the enzyme catalysed carboxylation reaction.

3.3.6 The association constant for Mg^{2+}-acetyl CoA

To test the feasibility of a Mg^{2+}-acetyl CoA complex existing in the assay solution, the association constant for Mg^{2+} and acetyl CoA was determined using the 8-hydroxyquinoline titration method of Burton (1959). A k_a value of 4600 $\pm$ 130 M^{-1} was obtained (Fig. 3.18). Using this value, a calculation can be made of the amount of Mg^{2+}-acetyl CoA present in the sheep liver pyruvate carboxylase assay solution which is used for initial rate kinetics. In the standard assay mix as described in Section 2.2.5, 94% of the acetyl CoA would exist as Mg^{2+}-acetyl CoA.

Therefore it appears that the allosteric activator for pyruvate carboxylase is Mg^{2+}-acetyl CoA.

3.3.7 The site of interaction between Mg^{2+} and acetyl CoA

It was decided to investigate next the site

FIGURE 3.18

Titration of 8-hydroxyquinoline with $MgCl_2$

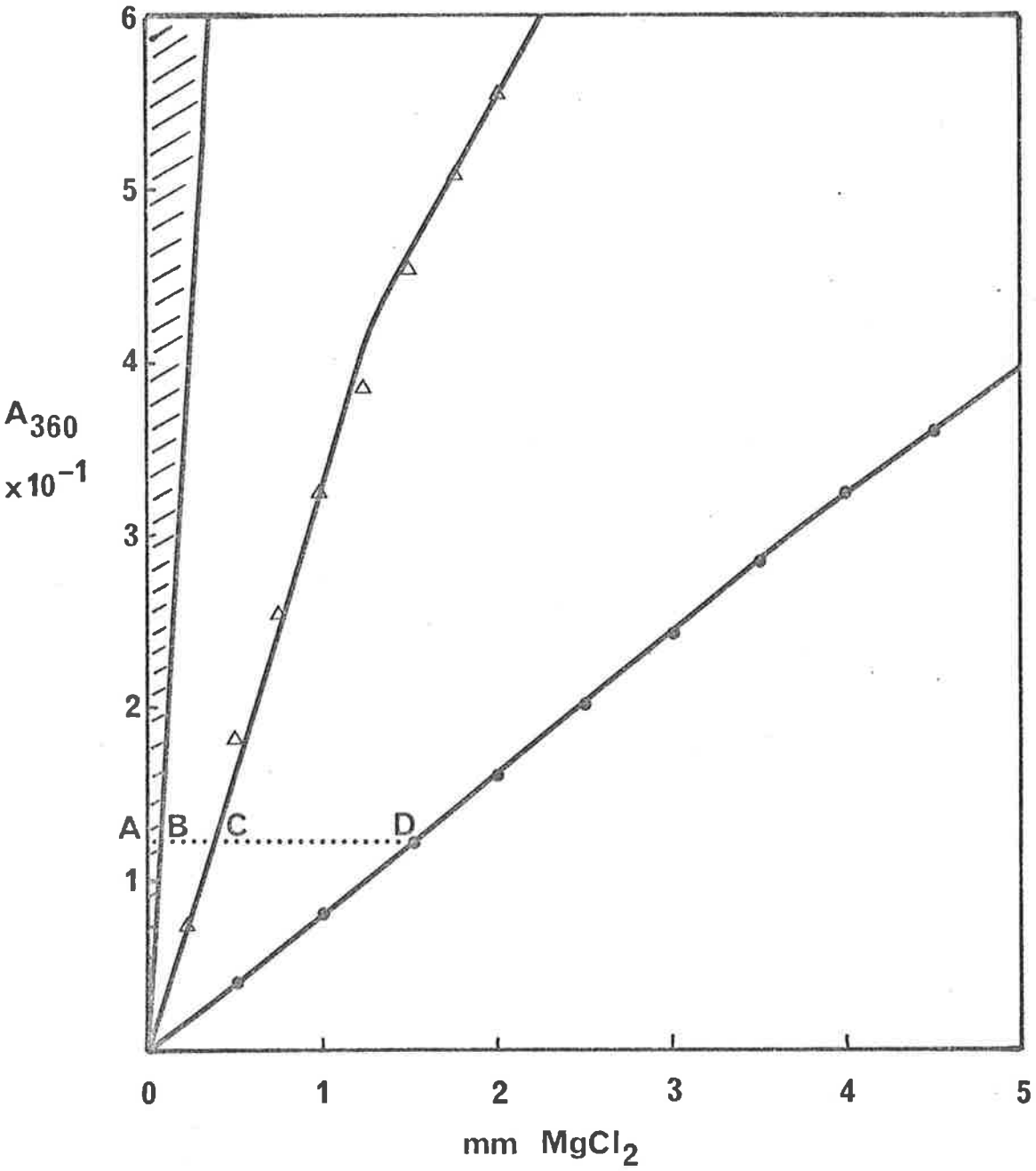
The concentration of 8-hydroxyquinoline used was 9×10^{-4} M in 0.1 M NEM-Cl pH 8.4 and the stock $MgCl_2$ concentration was 0.105 M. The solution containing divalent metal ion was introduced through a calibrated microsyringe (16.27 µl per mm). The shaded area represents the amount of metal bound to 8-hydroxyquinoline and was determined as described in Section 3.2.4. Similar results were obtained using 0.1 M Tris-Cl, pH 8.4.

Titration vessels containing

8-hydroxyquinoline only Δ—— Δ

8-hydroxyquinoline + 1.12 mM acetyl CoA ●——●

$$k \propto \frac{CD}{\overline{BC}}$$


6
5
4
3
2
A
B
C
D
1
A_{360}
$\times 10^{-1}$
0
1
2
3
4
5
mm $MgCl_2$

at which Mg^{2+} complexes with acetyl CoA. Utilizing various analogues of acetyl CoA, association constants were determined and the results shown in Table 3.6. It can be seen that the 3'-ribose-phosphate is the major determinant in Mg^{2+} binding to acetyl CoA.

Another approach used to investigate this problem was ^{31}P-phosphorous nuclear magnetic resonance. The ^{31}P NMR spectrum of acetyl CoA is shown in Fig. 3.19. Two signals are observed the integration ratio being 2:1. The assignment of these signals to the corresponding phosphate groups was verified using the acetyl CoA analogues acetyldephospho CoA and 5'-ADP (Fig. 3.20). A trimethylated orthophosphate external reference was used on all occasions. The peak at a chemical shift of -67 Hz (1.84 ppm) corresponds to the 3'-ribose-phosphate group, and the peak at 475 Hz (13.04 ppm) corresponds to the pyrophosphate group resonance. The presence of $MgCl_2$ causes a change in chemical shift of the resonance due to the 3'-ribose-phosphate group by 32 Hz (0.88 ppm). Another feature of the spectrum in the presence of $MgCl_2$ is that the pyrophosphate peak has been split indicating a change in the environment at one of the phosphate nuclei. This result verifies the site of interaction of Mg^{2+} on acetyl CoA and also implicates a slight interaction with one of the 5'-ribose-phosphate groups. A model for the binding of Mg^{2+} on acetyl CoA is presented in Fig. 3.21. The placement of a possible second site of Mg^{2+} interaction on the β-5'-ribose-phosphate was

TABLE 3.6

Compound	k_a (3 sig.Figs) (M^{-1})
Mg^{2+}-acetyl CoA	4600
Mg^{2+}-acetyldesamino CoA	4640
Mg^{2+}-acetyldephospho CoA	2487
Mg^{2+} 5'-ADP	1790

FIGURE 3.19

^{31}P-Nuclear magnetic resonance spectrum of acetyl CoA.

The spectrum shows the trimethylated ortho-phosphate marker which resonates between the two peaks which are due to the acetyl CoA resonance. The overlay shows the spectrum in the presence of a saturating concentration of $MgCl_2$.

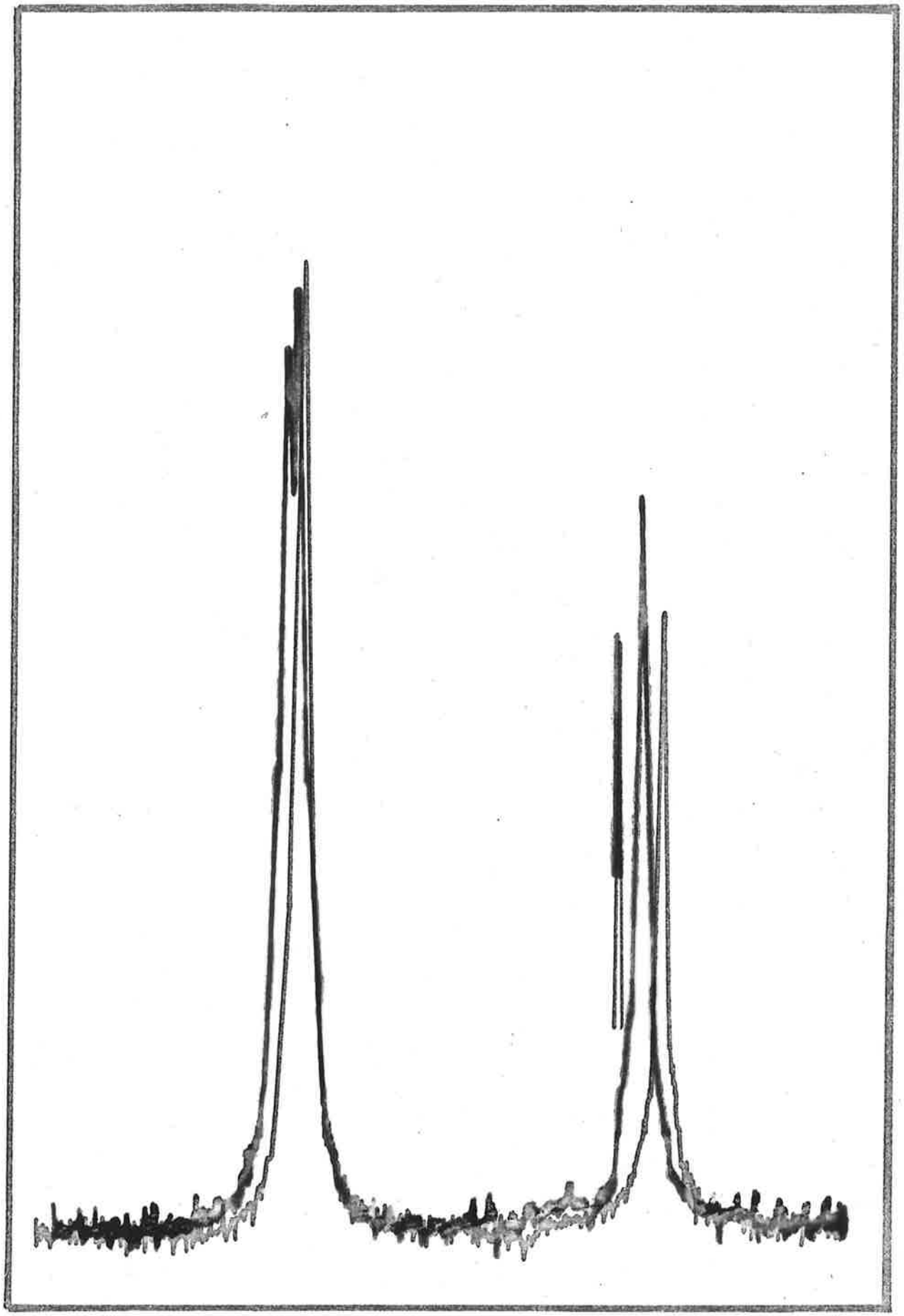


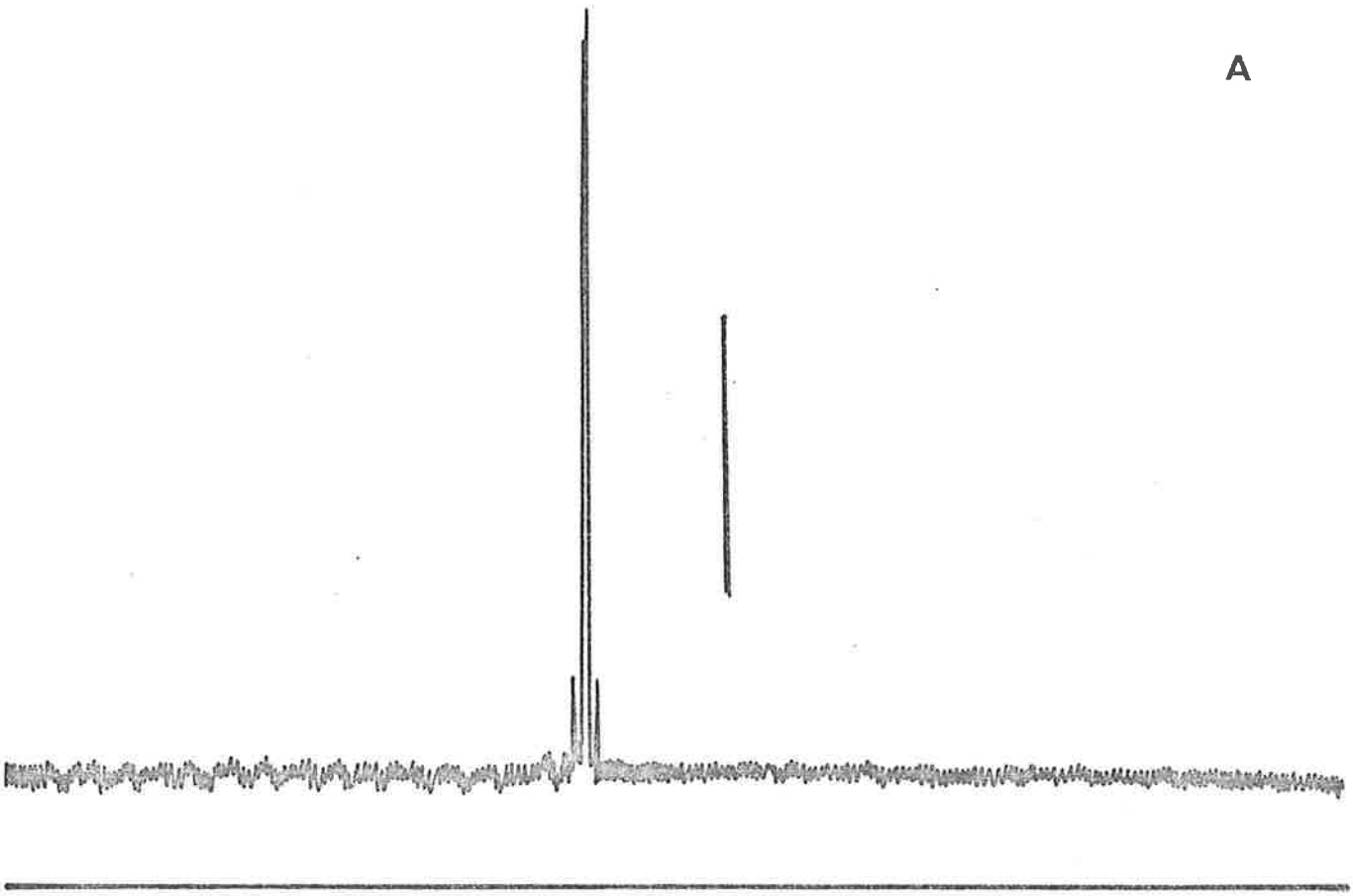
FIGURE 3.20

^{31}P-Nuclear magnetic resonance spectra of two analogues of acetyl CoA.

Fig. A represents the ^{31}P-NMR spectrum of acetyl-dephospho CoA.

Fig. B represents the spectrum of ADP.

Both spectra were performed relative to a trimethylated orthophosphate marker.


A

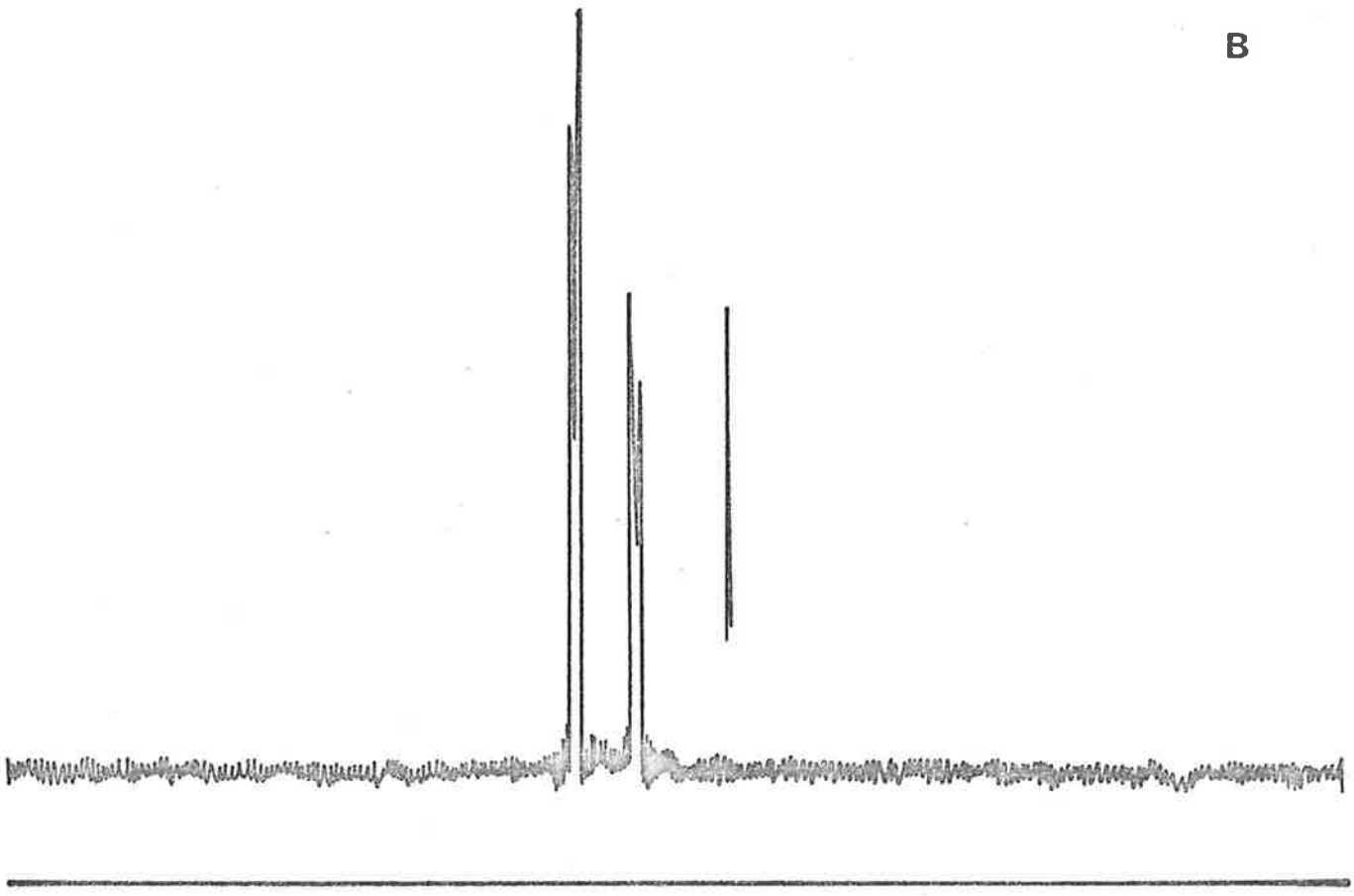
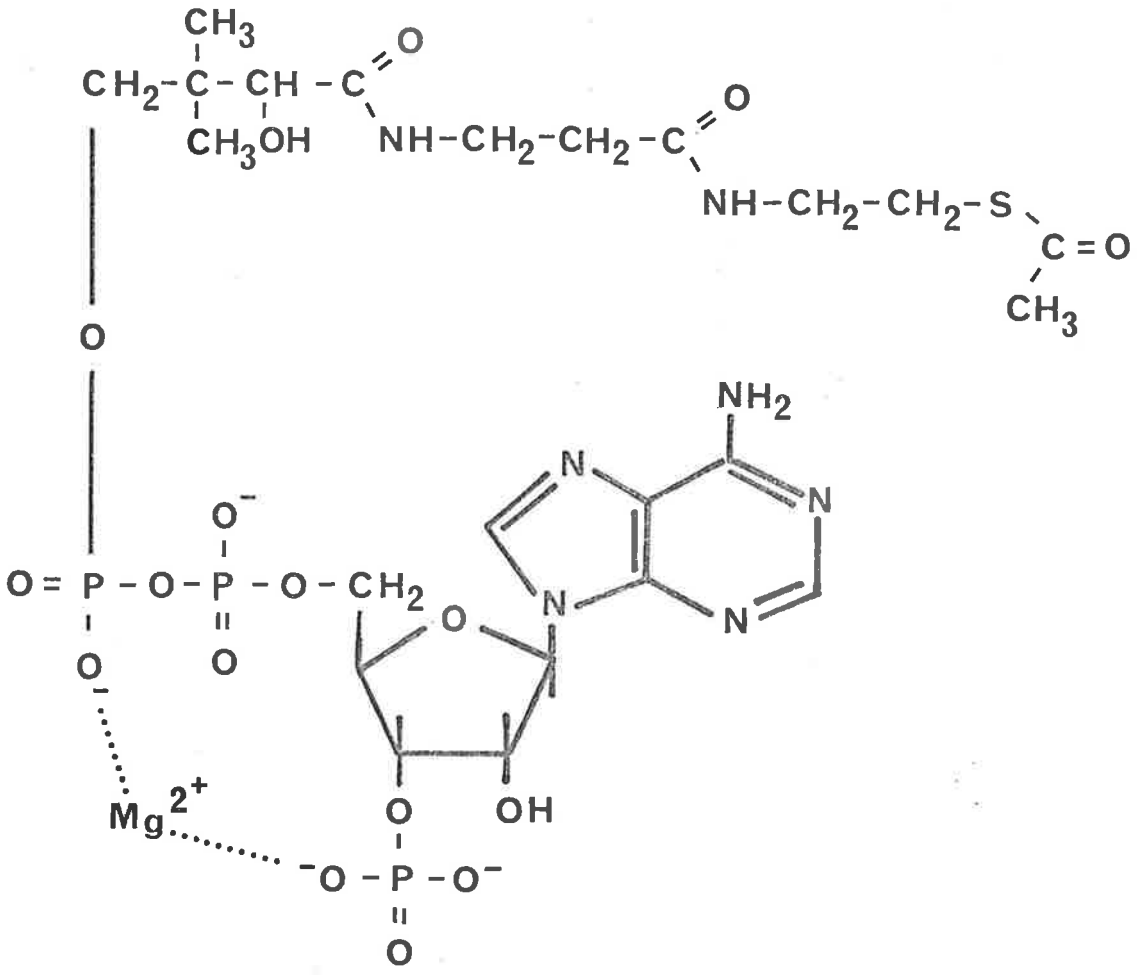

B

FIGURE 3.21

A model for the interaction of Mg^{2+} with acetyl CoA.



done in view of the report by Tran-Dinh Son et al., (1975). These workers rationalize a fixation of Mg^{2+} solely onto the β-5'-ribose-phosphate of ATP.

3.4 DISCUSSION

Evidence on the nature of activation of pyruvate carboxylase has been presented (Table 3.7). The allosteric activator seems to be Mg^{2+}-acetyl CoA which binds to the enzyme in a Michaelis-Menten manner. Acetyl CoA also binds non-cooperatively, and the value of the Hill coefficient greater than unity obtained from initial rate kinetics is probably an artefact due to its dependence on the saturation of substrates and the stability of the enzyme.

Some of the results are not in absolute agreement with the overall finding. For example the Hill coefficient for acetyl CoA as determined by protection against TNBS is 1.6. Also the n_H value determined by protection against dilution inactivation is approximately 2.2. These values theoretically should have been unity since the deacylase kinetics showed that acetyl CoA bound in a totally classical manner. TNBS modification has been studied in some detail by several workers (Scrutton and White, 1973, Ashman et al., 1973, and Scrutton et al., 1977). The lysyl residue modified by TNBS is an essential residue for full enzymic activity. However, the failure of TNBS modification to inhibit deacylase activity (Ashman et al., 1973) implies that acetyl CoA can bind to the chemically modified enzyme. Therefore the lysyl residue cannot be involved in direct

TABLE 3.7

	Acetyl CoA		Mg^{2+}-acetyl CoA	
	k_a (μM)	n_H	k_a (μM)	n_H
Binding studies (1)	9.6	2.2	3.5	1.3
(2)	27.5	1.6	10.9	0.8
Initial rate kinetics			8.0	1.9

(1) Represents protection against dilution inactivation.

(2) Represents protection against TNBS modification.

binding of acetyl CoA. Scrutton et al., (1977) also came to the same conclusion since they found that analogues of MgATP protected against TNBS modification to an extent similar to acetyl CoA.

Protection against TNBS modification by acetyl CoA is therefore not a direct binding phenomenon but a function of the binding process. An n_H value of 1.6 can be rationalized if it is assumed that in the absence of Mg^{2+}, acetyl CoA binds not as strongly (higher k_a value observed) and not as specifically, thus acetyl CoA could also bind to TNBS modified enzyme and thus the n_H value would be a function of the amount of acetyl CoA bound to free enzyme and the amount of acetyl CoA bound to TNBS modified enzyme. In the presence of Mg^{2+}, the activator binds more specifically and more strongly (lower k_a observed) and therefore cannot bind to TNBS modified enzyme and thus an n_H value of approximately 1.0 is obtained since the activator does bind non-cooperatively.

A similar argument could be applied to the dilution inactivation studies where a Hill coefficient value slightly greater than 1.0 was also obtained. One could envisage the Hill coefficient value in this case being a function of the amount of totally active enzyme bound with acetyl CoA and also partially inactive enzyme with loosely bound acetyl CoA. However, very little is known about the mechanism of dilution inactivation thus there could be conformational factors effecting the Hill coefficient which are not apparently obvious at this stage.

Although collectively the results suggest that Mg^{2+}-acetyl CoA is the enzyme activator, no prediction can be made concerning an additional role of free Mg^{2+}. Bais and Keech (1972) reported only one molecule of Mg^{2+} binding per active site which would seem to indicate that Mg^{2+} alone does not interact with the enzyme. On the other hand, they also observe a conformational change induced by the binding of this divalent cation to the enzyme. The results in Section 3.3.1 do not agree with this finding. The findings of these authors could be explained bearing in mind the fact that they used indirect methods of measurement. If, upon binding of Mg^{2+}, there is a change in the charge of a certain amino acid, this could change the hydrophobicity of a portion of the protein and this would be manifested in a change in ANS fluorescence, although a conformational change has not occurred. Similarly, Bais and Keech (1972) reported an entropy value which would seem to indicate a conformational change in the presence of Mg^{2+}. Since this value was fundamentally determined through initial rate kinetics, the effect could be associated with a Mg^{2+}-acetyl CoA interaction rather than a free Mg^{2+} effect <u>per se</u>.

Mg^{2+}-acetyl CoA has also been shown to cause a conformational change in the enzyme (Fig. 3.22). Since this compound has a Hill coefficient value of approximately 1.0 this effect can therefore be attributed to an intra-subunit process whereby the binding of this molecule causes the enzyme to adopt an activated conformation. Similar

FIGURE 3.22

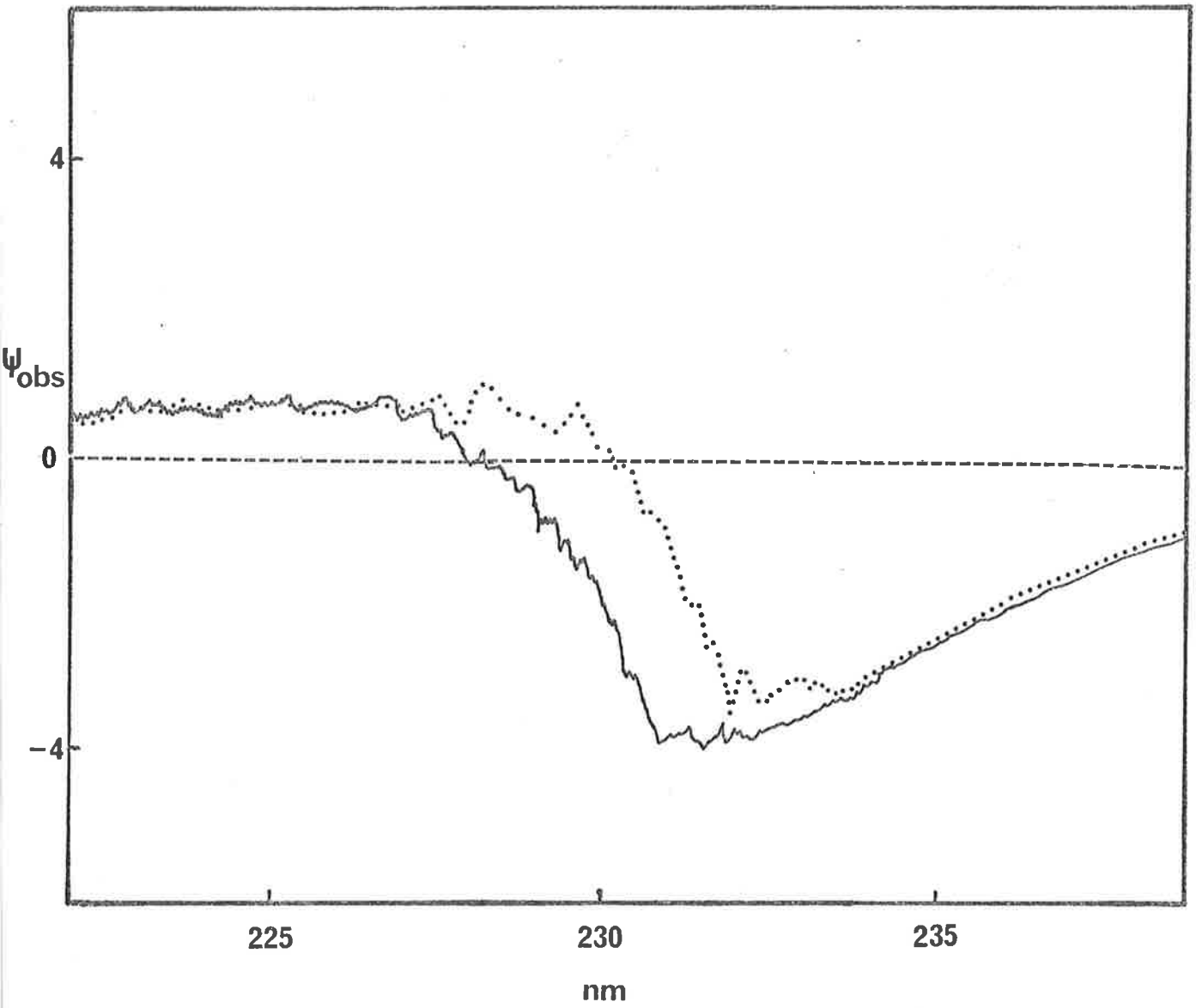
Spectrum of Circular dichroism for pyruvate carboxylase.

Sample:	CLPC 4.5 U/ml, S.A. 30 U/mg.
Solvent:	0.1 M Tris-Cl pH 7.8
Cell length:	1 cm
Temperature:	20°C
Scale:	50×10^{-3} m°/cm
Time constant:	4 sec.
Chart speed:	5 cm/sec.
Expansion:	1 nm/cm
Slit width:	1 nm.

——— Enzyme only

..... Enzyme + 0.25 M Mg^{2+}-acetyl CoA

------- 0.25 M Mg^{2+}-acetyl CoA only.


4
ψ_{obs}
0
−4
225
230
235
nm

changes in conformation of the enzyme have been observed when both pyruvate and MgATP bind (Fig. 3.23). These substrates may also cause an activated conformation of the enzyme to arise although the nature and extent of this activation is likely to differ a great deal from that produced by Mg^{2+}-acetyl CoA.

FIGURE 3.23

Spectra of circular dichroism for pyruvate carboxylase.

Sample:	CLPC 4 U/ml, S.A. 30 U/mg
Solvent:	0.1 M Tris-Cl pH 7.8
Cell length:	1 min.
Temperature:	20°C.
Scale:	100 x 10^{-1} m°/cm.
Time constant:	4 sec.
Chart speed:	5 cm/min.
Expansion:	0.5 nm/cm.
Slit width:	8 nm.

A: ——— enzyme only

..... enzyme + 30 mM pyruvate

- - - - - - - - 30 mM pyruvate only.

B: ——— enzyme only

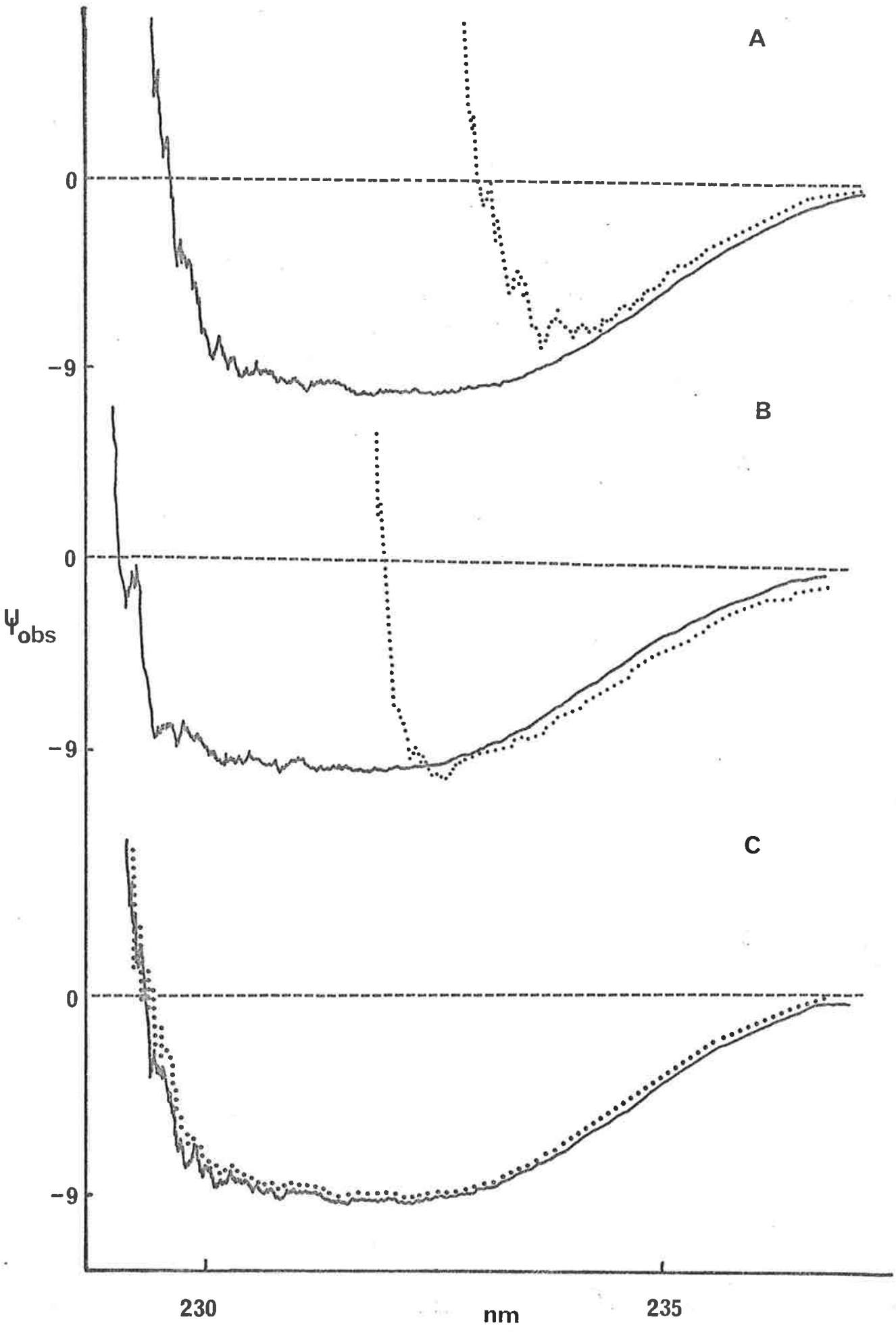
..... enzyme + 5 mM ATP

- - - - - - - - - 5 mM ATP only.

C: ——— enzyme only

..... enzyme + 20 mM HCO_3

- - - - - - - - - 20 mM HCO_3 only.


A
0
-9
B
0
-9
C
0
-9
ψ_{obs}
230
nm
235

CHAPTER 4

KINETICS OF OXALATE INHIBITION

4.1 INTRODUCTION

A general kinetic approach in attempting to elucidate the reaction pathway of a particular enzyme is by the use of substrate analogues which bind to the enzyme with an affinity approaching that of true substrate. These analogues do not participate in the reaction *per se* but merely compete for the binding sites, and therefore can be used as a probe to select possible interrelationships between substrates through analysis of analogue inhibition studies with respect to all the substrates.

One such compound which has been employed quite extensively on chicken and rat liver pyruvate carboxylases and on transcarboxylase is oxalate, an analogue of pyruvate. The outcome of inhibition studies with this compound is summarized in Table 4.1.

It has also been shown that oxalate very strongly chelates transition metal ions (Treindl *et al.*, 1975). E.S.R. studies have confirmed that oxalate interacts directly with the bound manganese ion in chicken liver pyruvate carboxylase (Reed and Scrutton, 1974). Since oxalate also binds at the pyruvate binding site this would imply that this substrate binding site is in close proximity to the bound metal ion. Therefore the kinetics of inhibition of oxalate with respect to all the substrates could yield an indication of the relative positioning between the substrate binding sites and the manganese ion.

TABLE 4.1

Species	Metal content (g atoms/mole)	Biotin content per mole	$S_{20,w}$	Properties of oxalate inhibition
Rat liver[1,2]				
pyruvate carboxylase	2	4	14.7	No effect on ATP/ADP exchange inhibits Pyr/OAA exchange non-competitive with respect to MgATP non-competitive with respect to Pyr non-competitive with respect to HCO_3.
Chicken liver[3,4]	4	4	14.8	No effect on ATP/ADP exchange
pyruvate carboxylase				inhibits the Pyr/OAA exchange protects against dissociation into monomers.

Table 4.1 cont.

Species	Metal content (g atoms/mole	Biotin content per mole	$S_{20,w}$	Properties of oxalate inhibition
Transcarboxylase[5]	4,2	4-6	18	Inhibits propionyl CoA:methylmalonyl CoA exchange inhibits pyruvate/OAA exchange. non-competitive with respect to Pyr non-competitive with respect to MM CoA protects against dissociation into smaller subunits.

1. McClure, W.R., Lardy, H.A., Wagner, M., and Cleland, W.W. (1971) J. Biol. Chem. 246, 3579.
2. McClure, W.R., Lardy, H.A. and Cleland, W.W. (1971) J. Biol. Chem. 246, 3584.
3. Scrutton, M.C. and Utter, M.F. (1965) J. Biol. Chem. 240, 3714.
4. Mildvan, A.S., Scrutton, M.C., and Utter, M.F. (1966) J. Biol. Chem. 241, 3488.
5. Northrop, D.B. and Wood, H.G. (1969) J. Biol. Chem. 244, 5820.

Since the biotin carboxylases do not all have the same ratio of manganese to biotin, (Scrutton and Utter, 1972) it would be interesting to compare the oxalate inhibition studies of sheep liver pyruvate carboxylase with the other enzymes.

4.2 METHODS

4.2.1 Assay for biotin

Biotin was estimated in a radioisotopic assay where a known amount of avidin was incubated with the sample and then [^{14}C]biotin was added to bind the unoccupied binding sites on the avidin. The avidin-biotin complex was precipitated with zinc sulphate enabling the [^{14}C]biotin remaining in solution to be determined. The zinc sulphate does not destroy the capacity for avidin to complex with biotin but it does precipitate the avidin-complex completely.

For the rapid determination of biotin used in these investigations the following conditions were used; 0 - 100 pmoles of biotin, 0.028 units of avidin, 100 mM potassium phosphate, pH 7.2, in a final volume of 0.50 ml. After standing for 10 min at room temperature, 50 µl [^{14}C]biotin (0.1 µCi/ml; 45 M Ci/mmole) was added and 10 min later the avidin-biotin complex was precipitated by adding 0.2 ml of 2% ($^{w}/_{v}$) zinc sulphate followed by 0.2 ml of 0.1 N sodium hydroxide. (The zinc sulphate solution is slightly acidic and the sodium hydroxide is

added to adjust the pH back to 7.0). The mixture was then centrifuged and the radioactivity in the supernatant determined by taking 0.2 ml aliquots and mixing with Triton X100 scintillant. When the biotin content of the native enzyme was required, the enzyme was digested with pronase (1 mg/ml) for 2 h at 37°C to partially digest enzyme and ensure that all the biotin was exposed to react with avidin. Prior to all sample estimations, a biotin standard curve was constructed, with unlabelled biotin in the range 0 - 100 pmoles.

4.2.2 Estimation of Mn^{2+} content

The measurement of manganese in solutions of pronase digested samples of pyruvate carboxylase was carried out by atomic absorption spectroscopy on a Varian-Techtron grating monochromator. The light source was a Neon 25 mA manganese hollow cathode discharge tube. The light passes through the absorption cell and is focused on the entrance slit (0.050 mm) of the monochromator. The fuel is a mixture of nitrogen at a pressure of 30 psi and acetylene at a pressure of 10 psi. The manganese resonance line at 2795 Å was used for analysis. The output signal of the amplifier was transferred to a digital display.

A standard curve was always obtained prior to reading sample tubes. The concentrations of $MnCl_2$ used to construct the standard curve ranged from 1×10^{-7} M to 1×10^{-5} M and were prepared in the same buffer as

the enzyme samples. Protein concentrations of sample tubes were usually diluted to approximately 0.5 mg/ml.

4.3 RESULTS

4.3.1 The manganese content of sheep liver pyruvate carboxylase

Bais (1974), reported a manganese content for sheep kidney pyruvate carboxylase of 1.8 - 1.95 g atoms per mole of enzyme. This value differed from that obtained for chicken liver and turkey liver pyruvate carboxylases although calf and rat liver enzymes have been reported to contain only 2 g atoms/mole (Scrutton and Young, 1972). Therefore it was decided to determine the Mn^{2+}:biotin ratio of the sheep liver enzyme.

Approximately 1.5 ml samples of fractions across the protein peak from a DEAE-sephadex column, which is the final step in routine enzyme purification, (Section 2.2.1),were collected from several enzyme preparations. Aliquots were taken for biotin determination and the rest used for estimation of Mn^{2+} as described in Section 4.2.1 and 4.2.2. The same techniques were employed to verify the manganese content of chicken liver pyruvate carboxylase.

A manganese to biotin ratio of 1.18 $\pm$ 0.06 was obtained for sheep liver pyruvate carboxylase, and ratio of 1.12 $\pm$ 0.25 was obtained for chicken enzyme (Table 4.2). The results did not indicate any relationship between the

TABLE 4.2*

Species	μ/ml	μ/mg	Mn^{2+}:biotin
SLPC	4.5	6.2	1.01
	4.2	6.3	0.7
	3.2	5.5	0.81
	2.6	4.9	0.68
	10.3	16.9	0.76
	12.5	18.4	0.86
	11.6	16.5	0.94
	11.3	16.6	0.62
	13.5	24.4	1.76
	15.4	25.2	0.81
	16.7	26.9	0.92
	15.4	26.3	1.12
Av. Mn^{2+}:biotin = 1.18 ± 0.06 (taken over 50 samples from 6 separate enzyme preparations)			
CLPC	15.4	9.1	0.93
	17.0	9.6	0.71
	17.7	10.8	1.11
	7.7	8.0	1.0

Table 4.2* cont.

Species	µ/ml	µ/mg	Mn^{2+}:biotin
CLPC	26.05	19.2	1.3
	32.5	23.5	1.76
	24.4	22.3	1.12
	99.6	31.3	1.3
	114.1	34.8	1.4
	90.0	37.2	0.96
	62.7	33.0	1.88

Av. Mn^{2+}:biotin = 1.12 $\pm$ 0.25 (taken over 50 samples from 4 separate enzyme preparations).

*The values shown were obtained from the peak of elution profiles from separate DEAE sephadex columns where differing specific activity enzyme was recovered.

Mn^{2+}:biotin ratio and the specific activity of the enzyme.

4.3.2 Oxalate inhibition studies of the overall reaction

Inhibition of pyruvate carboxylase by oxalate was studied as a function of the concentration of pyruvate, bicarbonate and MgATP (Figs. 4.1, 4.2, 4.3). An inhibition constant of $47.7 \pm 5.0 \times 10^{-6}$ M was calculated from the data shown in Fig. 4.2. A competitive inhibition pattern was obtained when pyruvate was the varied substrate (Fig. 4.1). Such a kinetic pattern suggests that inhibition is due to a blocking of the keto acid site by the inhibitor which prevents the binding of keto substrates. However, the effect of oxalate is more complex than this, since at low concentrations of pyruvate, the inhibition is apparently uncompetitive, and the pattern only approaches competitiveness as pyruvate exceeds k_m concentrations. Another noticeable feature of Fig. 4.1, is the fact that the extent of downward curvature of the double reciprocal plots is increased with increasing oxalate concentrations. This would suggest that there is an increase in the hydrolysis of biotin$\sim CO_2$ complex (Section 1.3.2). This result is as would be expected since the presence of a non-carboxylatable species in the transcarboxylation site would induce the biotin$\sim CO_2$ moiety to bind at this site but since CO_2 cannot be utilized, hydrolysis would take place.

FIGURE 4.1

The inhibition of the overall reaction by oxalate with varied pyruvate.

Velocity measurements were made according to the radiochemical assay described in Section 2.2.5.2. Enzyme of specific activity 10, 0.077 U per assay tube was used. The concentrations of oxalate used were, 0 (O), 2×10^{-5} M (■), 6×10^{-5} M (Δ), and 1.8×10^{-4} M (●).

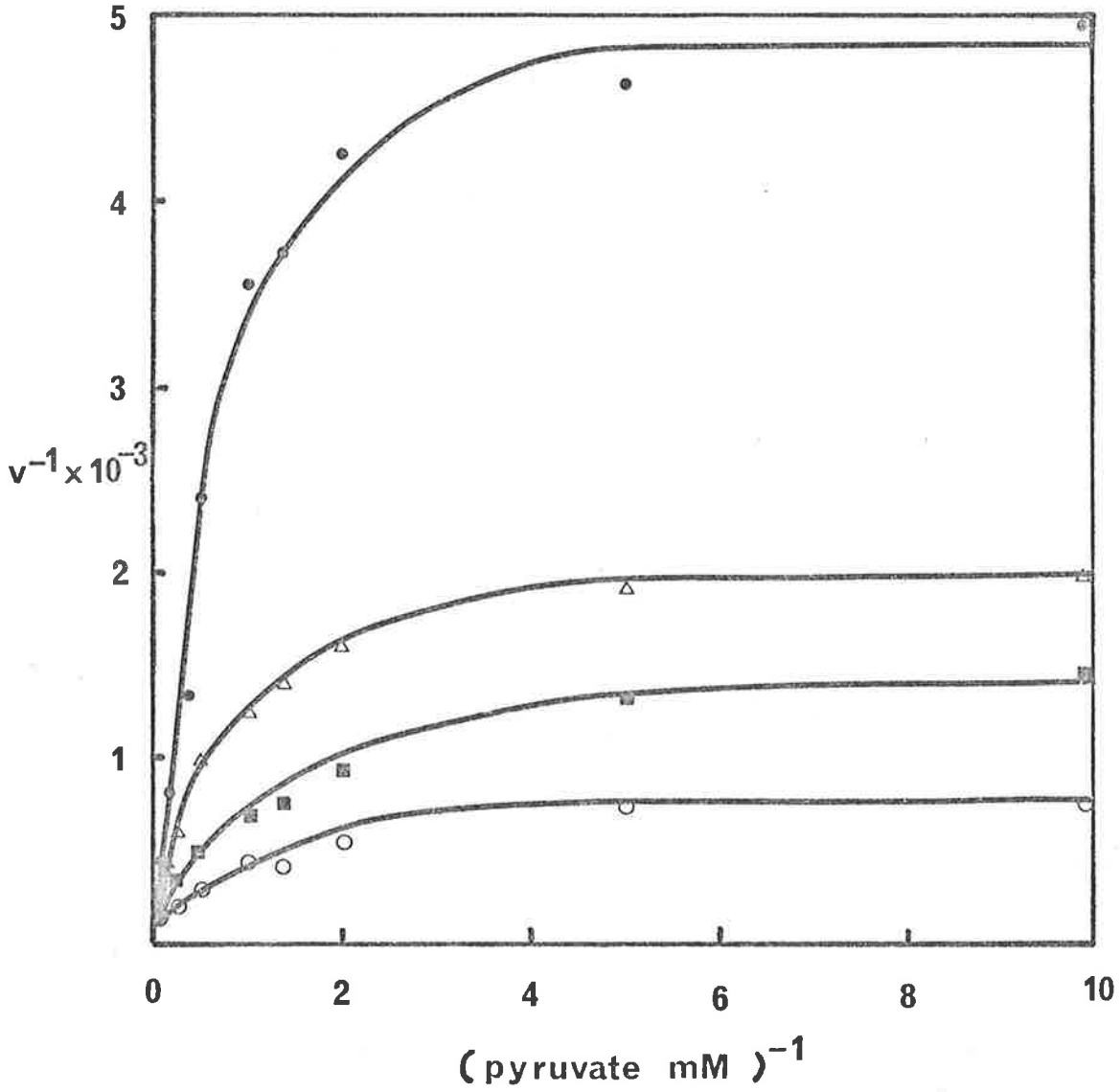

5
4
3
2
1
$v^{-1} \times 10^{-3}$
0
2
4
6
8
10
(pyruvate mM)$^{-1}$

FIGURE 4.2

Inhibition by oxalate with respect to bicarbonate.

Enzyme (S.A. 10, 0.077 U) was added to assay tubes containing solutions which were made up from CO_2-free water. In calculations of the actual concentrations of bicarbonate the small amount of endogenous carbon dioxide was taken into account.

The concentrations of oxalate employed were, 0 (□), 2×10^{-5} M (■), 6×10^{-5} M (○), and 1.8×10^{-4} M (●).

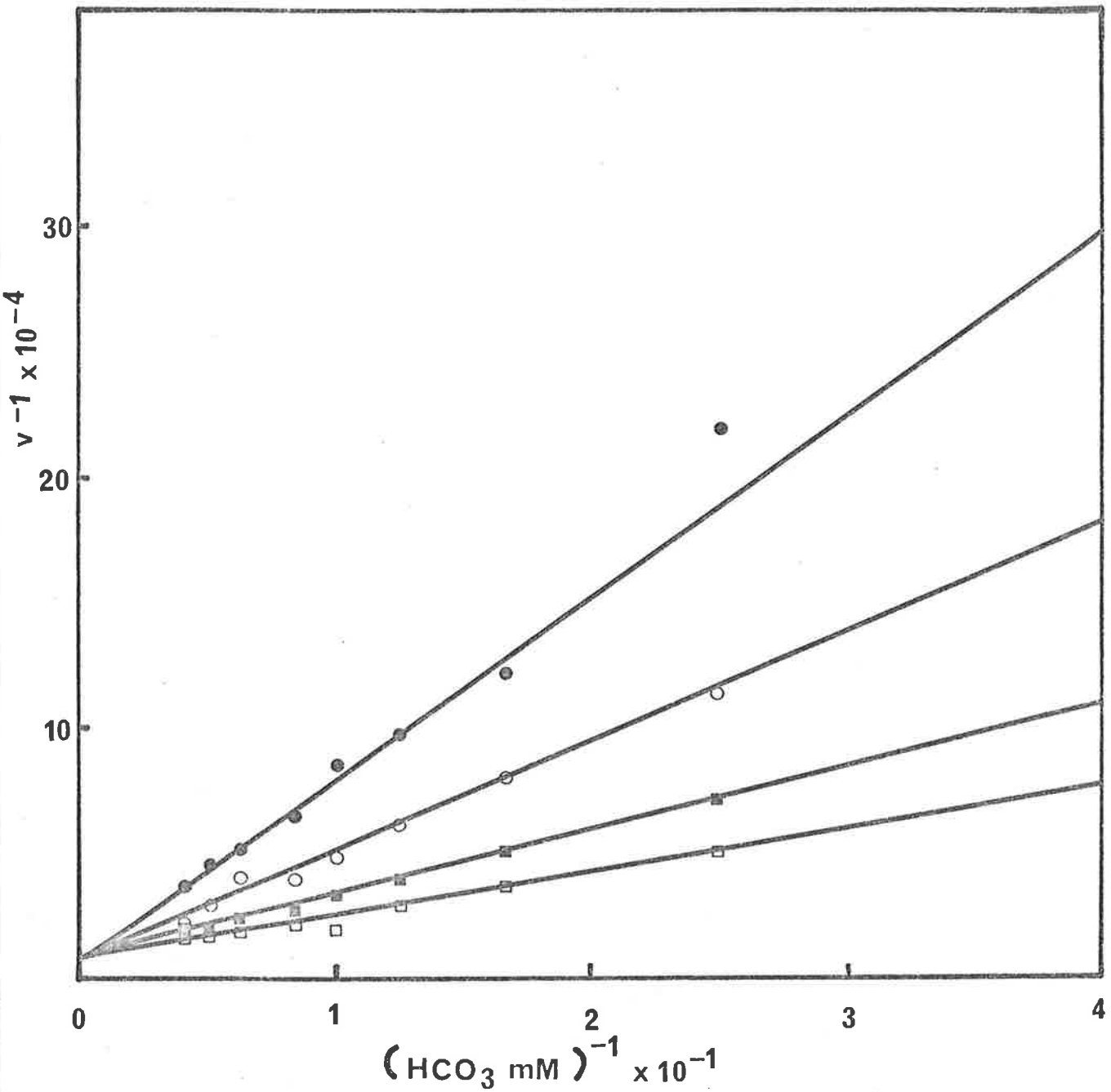
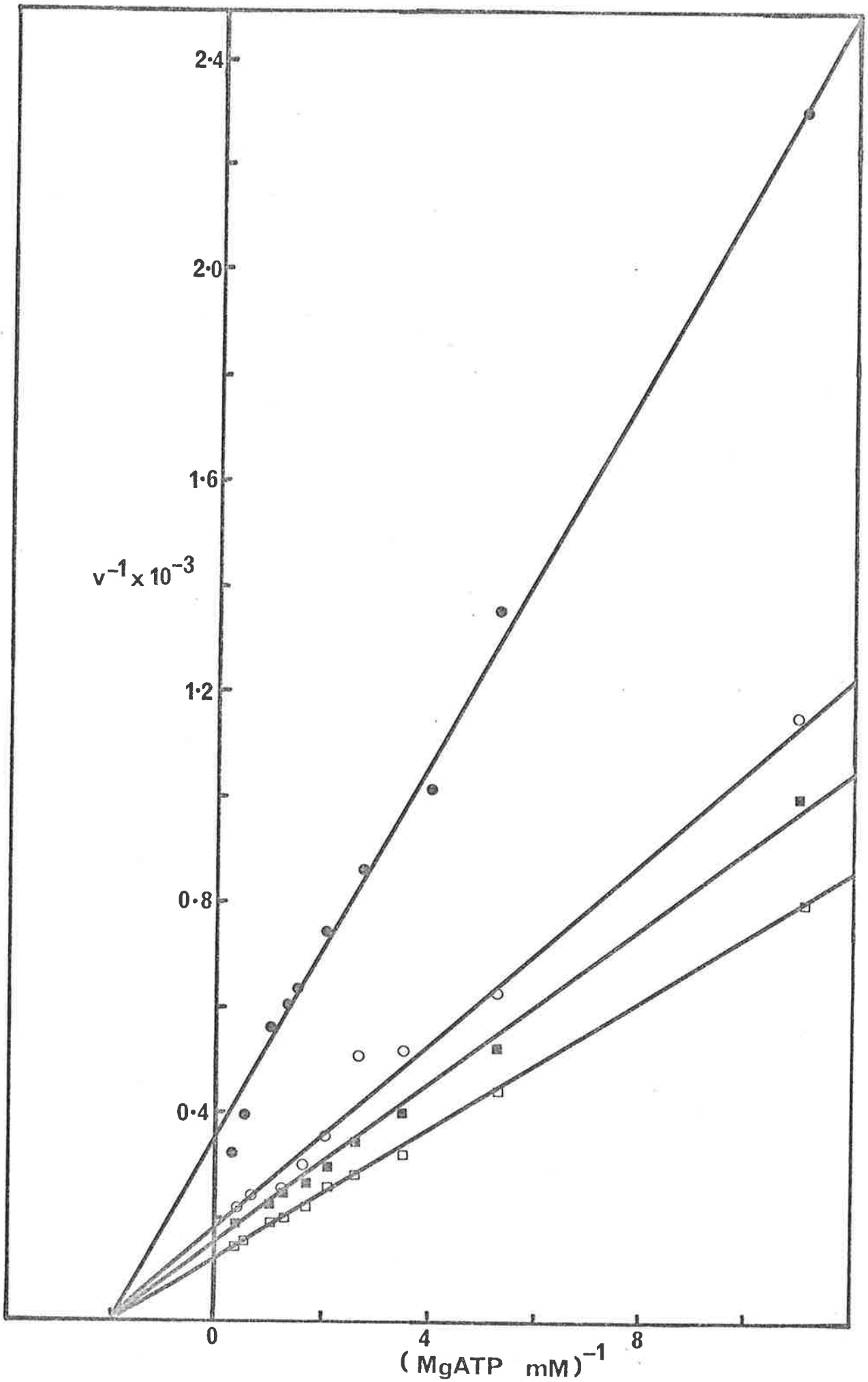

30
20
10
v −1 x 10−4
0
1
2
3
4
(HCO3 mM)−1 x 10−1

FIGURE 4.3

The effect of oxalate on the overall reaction while varying MgATP.

Assay conditions were maintained the same for this experiment as for the previous experiments described in Fig. 4.1 and 4.2. The concentrations of oxalate employed were, 0 (□), 2×10^{-5} M (■), 6×10^{-5} M (○), and 1.8×10^{-4} M (●).


2·4
2·0
1·6
1·2
0·8
0·4
$v^{-1} \times 10^{-3}$
0
4
8
(MgATP mM)$^{-1}$

The kinetic patterns produced from inhibition with oxalate when studied as functions of other substrates show that the mechanism of inhibition is indeed quite complicated. According to a sequential mechanism which prevails at relatively high concentrations of pyruvate, the pattern for an inhibitor acting simply to block the keto acid substrate site of pyruvate carboxylase would be non-competitive with respect to either MgATP or bicarbonate and competitive with respect to pyruvate. The observed pattern for inhibition as a function of varied bicarbonate shown in Fig. 4.2, is competitive and a non-competitive inhibition was observed when MgATP was varied as shown in Fig. 4.3.

The overall results obtained differ significantly from those of McClure et al., (1971b) working with rat liver pyruvate carboxylase.

Inhibition studies with oxalate

	SLPC	RLPC
Pyruvate	C	NC
HCO_3	C	NC
MgATP	NC	NC

This feature could be explained by the fact that the enzyme from rat liver differs from most of the other species with a manganese:biotin ratio of 2:1. Since oxalate strongly chelates the manganese, the metal content of these enzymes would certainly influence their interaction with this inhibitor.

4.3.3 Effect of oxalate on the isotopic exchange reactions

If oxalate binds both at the pyruvate binding site and the bicarbonate binding site, then it should inhibit both the pyruvate/oxaloacetate isotopic exchange and the ATP/P_i exchange. The results from such experiments using sheep and chicken liver pyruvate carboxylases are shown in Table 4.2. It can be seen that the second partial reaction exchange is indeed inhibited and both sheep and chicken enzymes show the same trends.

On the other hand, the ATP/P_i exchange rate was markedly stimulated. This effect appeared to increase as the concentration of oxalate was increased.

4.3.4 Dilution inactivation in the presence of oxalate

Scrutton (1974) observed that oxalate protected against loss of enzyme activity upon dissociation of chicken liver pyruvate carboxylase into monomers which accompanies incubation at 2°C. This was interpreted to indicate that this type of inhibitor stabilized the active tetrameric form of the enzyme. Therefore it was of interest to investigate the effect of this compound on the rate of inactivation which occurs upon dilution of sheep liver pyruvate carboxylase to below 4 U/ml. In this case the enzyme was diluted to 0.1 U/ml. The result from such an experiment is shown in Fig. 4.4.

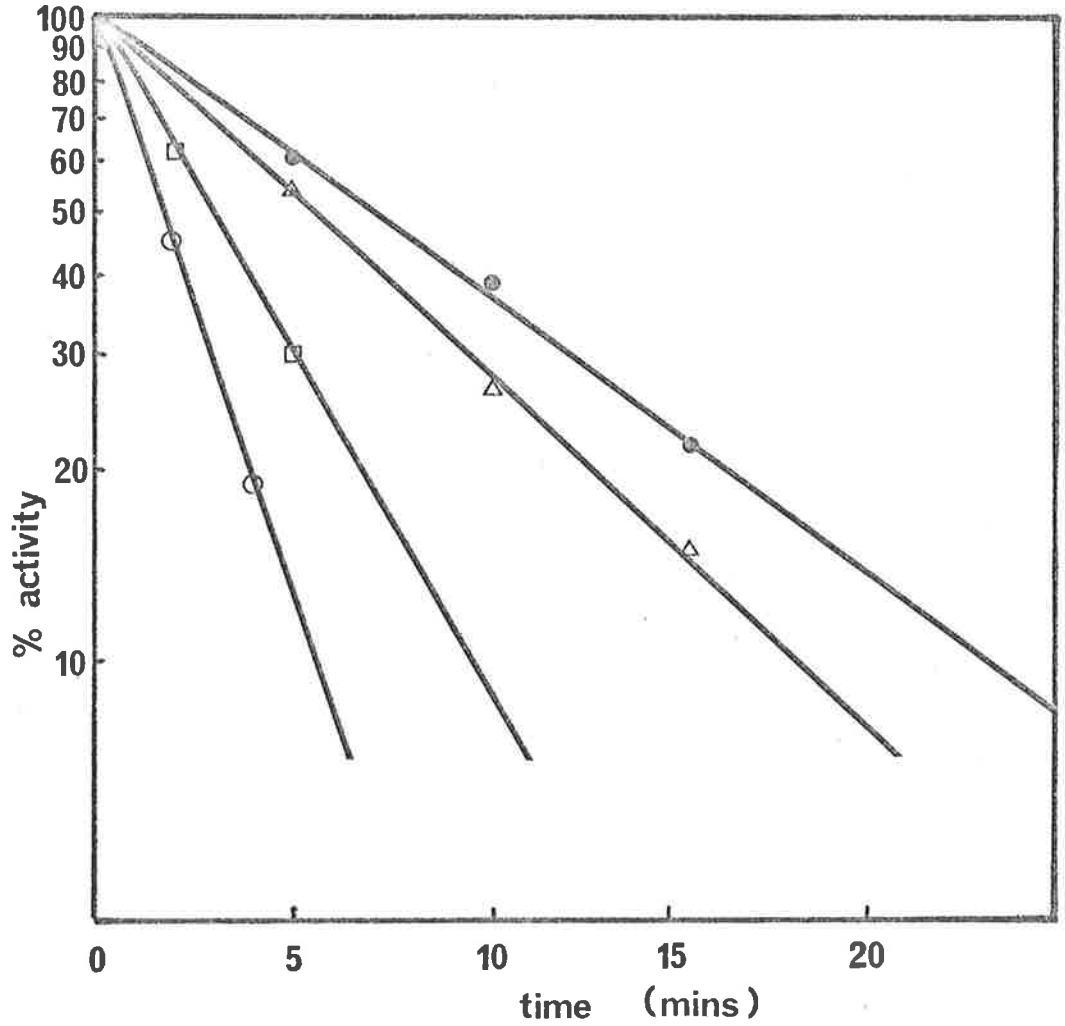
TABLE 4.2

Species		Rate of isotopic exchange	
		Pyr/OAA	ATP/P_i
		%	%
SLPC	Control	100	100
	6×10^{-5} M oxalate	76.0	116.5
	6×10^{-4} M oxalate	36.8	154.7
CLPC	Control	100	100
	6×10^{-5} M oxalate	72.0	121.6
	6×10^{-4} M oxalate	38.6	127.2

FIGURE 4.4

The effect of oxalate on the rate of dilution inactivation of sheep liver pyruvate carboxylase.

Enzyme (S.A. 15) was diluted to a concentration of 0.1 U/ml and at various time intervals aliquots were removed and assayed for activity as described in Section 2.2.5.1.(●), represents the rate of dilution inactivation in the absence of oxalate. Subsequently the concentrations of oxalate used were 2×10^{-5} M (△), 6×10^{-5} M (□), and 1.8×10^{-4} M (○).


100
90
80
70
60
50
40
30
20
10
% activity
0
5
10
15
20
time (mins)

Clearly, oxalate enhances the rate of dilution inactivation.

One could postulate from this result that the binding of oxalate would cause biotin to be displaced by a water molecule as it moves into the 2nd partial reaction site. A biotin displacement mechanism could be a control point in the enzymic reaction where hydration of the 1st partial reaction site would stop any ATP hydrolysis which could occur in the absence of biotin. Excessive hydration of the carboxylation site has been proposed to explain dilution inactivation by Clements, 1977.

4.4 DISCUSSION

Although a reappraisal of the reaction pathway for sheep liver pyruvate carboxylase has recently established that the overall reaction proceeds via a sequential mechanism (Easterbrook-Smith *et al.*, 1977), the existence of a pyruvate/oxaloacetate exchange in the absence of ATP and an ATP/P_i exchange in the absence of keto acid substrate confirms the existence of the following half reactions,

$$MgATP + HCO_3 + E^{bio} \rightleftharpoons MgADP + P_i + E^{bio\sim CO_2} \quad ..(4.1)$$

$$\text{oxaloacetate} + E^{bio} \rightleftharpoons \text{pyruvate} + E^{bio\sim CO_2} \quad ... \quad (4.2)$$

If these exchange reactions are considered independent, then pyruvate or analogues of this substrate

should not effect the ATP/P_i half reaction. It has been shown, however, that oxalate, an analogue of pyruvate, stimulates the ATP/P_i exchange. Therefore it must be assumed that equations 4.1 and 4.2 are interdependent and this could only occur through their mutual reactivity with enzyme bound biotin. Hence the presence of non-substrates could only influence a particular half reaction via its effect on the migration of the biotin ring, which only then would become kinetically sigificant.

Consequently it can be proposed that oxalate binding in the second partial reaction site would inhibit the pyruvate:oxaloacetate exchange by competing with pyruvate for the substrate binding site and also by hindering the binding of biotin$\sim CO_2$. Because of this latter effect it would in turn stimulate the ATP/P_i exchange which is entirely dependent on the presence of biotin, by making this cofactor more available to participate in the reaction.

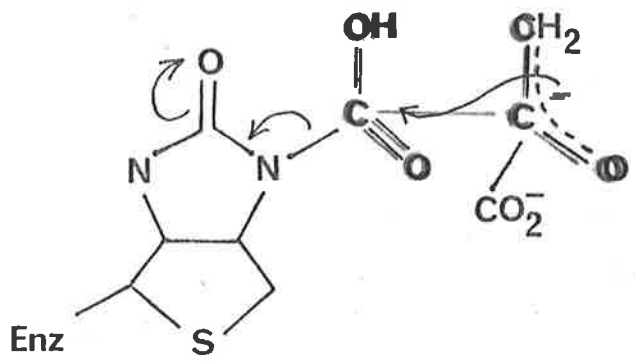
The competitive inhibition patterns observed in Figs. 4.1 and 4.2 indicate that oxalate would apparently bind at the pyruvate binding site and the bicarbonate binding site. However, the result of Fig. 4.2 more realistically suggests that oxalate is competing with the biotin$\sim CO_2$ site in the second partial reaction site. Assuming oxalate is competitive with respect to pyruvate and biotin$\sim CO_2$ this must imply that both these binding sites are in close proximity to the bound manganese since oxalate is known to also strongly chelate the bound metal.

The results of Mildvan et al., (1966) show that pyruvate and oxaloacetate significantly effect the enhancement value for the water protons around the manganese ion. It is known that enzyme bound oxaloacetate readily decarboxylates to pyruvate and biotin$\sim CO_2$ therefore these results are consistent with the above proposition of the vicinity of the pyruvate and biotin$\sim CO_2$ sites. Since in the presence of MgATP and HCO_3, where biotin$\sim CO_2$ would also be formed, no change in the enhancement value was observed, this could be interpreted to mean that biotin$\sim CO_2$ remains largely in the 1st partial reaction site which is relatively far from the manganese ion (greater than 10 Å, Reed and Scrutton, (1974)) and only in the presence of pyruvate does it migrate to the second partial reaction site.

The overall results presented are consistent with a postulate where oxalate acts as a transition state analogue of the carboxyl transfer intermediate which could be depicted as follows

Mn^{2+}

oxalate



biotin$\sim CO_2$ and pyruvate

This model incorporates 2 major assumptions, these being that biotin can migrate freely between the two partial reaction sites and that biotin$\sim CO_2$ has a dissociable binding site at each of these half sites. The mechanism of the carboxylation of free biotin by bacterial β-methylcrotonyl CoA carboxylase has been explained by Lynen et al., (1961) by suggesting these same facts, namely that biotin is bound to the enzyme not only covalently through its lysyl-carboxyl side chain linkage but also by a dissociable linkage at its ring structure.The work of Knappe (1964) has also given credence to this suggestion.

The oxalate kinetic studies and also the manganese stoichiometry for sheep liver pyruvate carboxylase are in good agreement with the observations published for pyruvate carboxylase from other sources and also for transcarboxylase and strengthens the view of the similarity between general properties of all the biotin carboxylases.

CHAPTER 5

ATP HYDROLYSIS COUPLED TO CO_2 FIXATION

5.1 INTRODUCTION

5.1.1 A number of possible mechanisms for coupling ATP hydrolysis and carbon dioxide fixation have been postulated for the biotin carboxylases. The basis for these mechanisms has generally originated from kinetic studies of the overall reaction. However this is not always reliable as has been demonstrated for succinyl CoA synthase where kinetic studies did not indicate the presence of a covalent phosphate intermediate although such a species has now been shown to exist (Moffet and Bridger, 1970). Thus any mechanism based solely on kinetic evidence must be considered with reservation until supported by other techniques.

There are three main postulates for the formation of biotin$\sim CO_2$ (Fig. 5.1) in biotin-containing carboxylases.

(1) the concerted reaction (Kaziro *et al.*, 1961)

(2) enzyme activation (Lynen, 1967)

(3) substrate activation (Polakis *et al.*, 1971, Ashman and Keech, 1975).

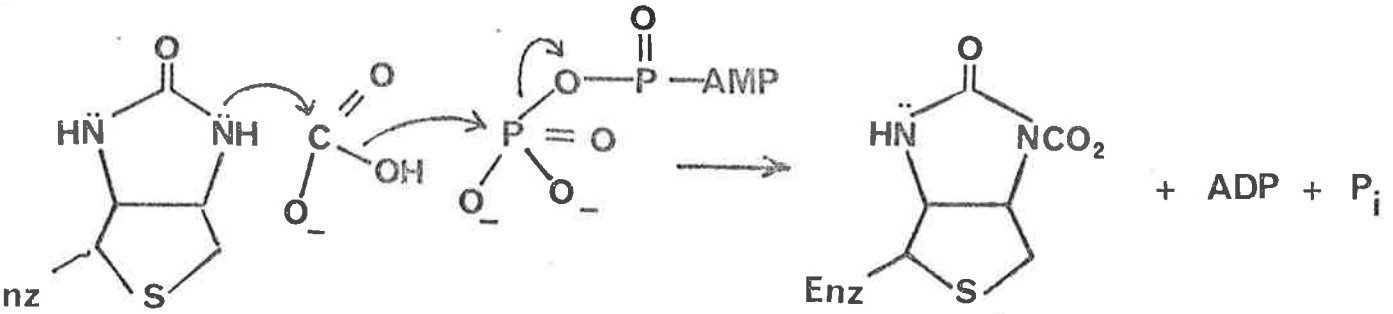
The concerted mechanism

As early as 1961, Kaziro *et al.*, observed while working on propionyl CoA carboxylase, that when $HC^{18}O_3^-$ was included in the reaction solution, the final product, oxaloacetate contained two of the labelled oxygen atoms and the third was present in the orthophosphate released as a result of the concurrent hydrolysis of ATP. This

FIGURE 5.1

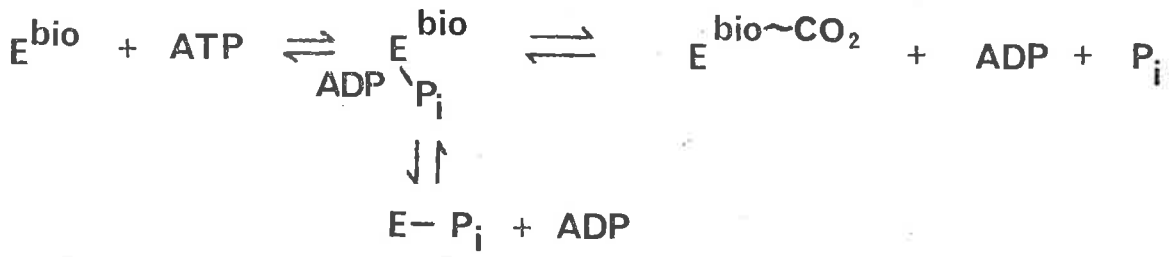
The three main mechanisms of biotin carboxylation.

1. Concerted.

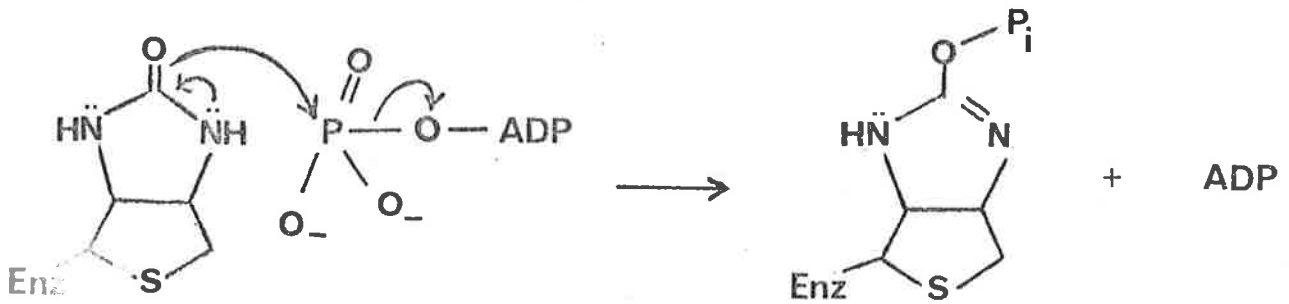


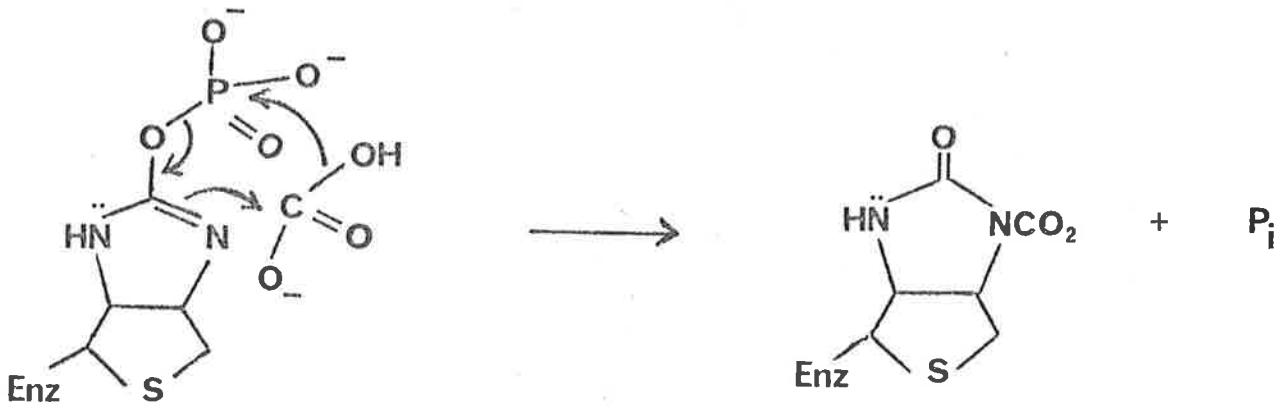
2. Enzyme activation.

a) activation of an amino acid residue

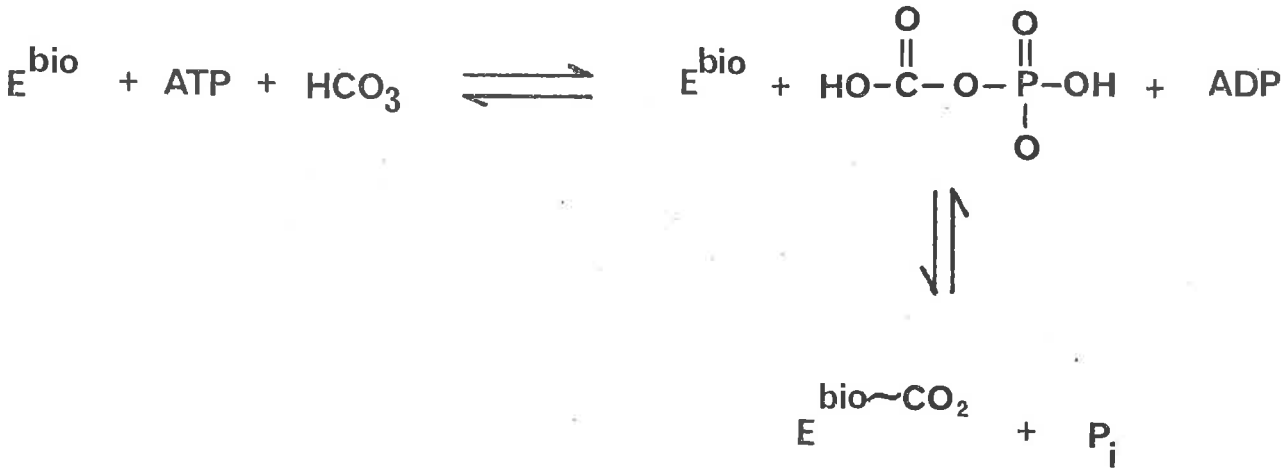


b) activation of biotin





3. Substrate activation.



result led to the postulate of a concerted mechanism for biotin$\sim CO_2$ formation. Such a mechanism would imply the following predictions; firstly, that ADP and phosphate are released after the formation of biotin$\sim CO_2$ and secondly, that biotin is an absolute requirement in both the ATP/ADP and the ATP/P_i isotopic exchange reactions. However it has been demonstrated in bovine liver propionyl CoA carboxylase (Lane et al., 1960) and chicken and sheep liver pyruvate carboxylases (Scrutton and Utter, 1965, Goss and Hudson, unpublished results) that the ATP/ADP exchange reaction is dependent only on the presence of Mg^{2+} and only partially subject to inhibition by avidin. These data show not only that biotin is not absolutely required for the ATP/ADP exchange reaction but that at least one product (MgADP) is released prior to formation of biotin$\sim CO_2$. The available evidence therefore seems to disprove the concerted model.

Enzyme activation

Several enzymes which utilize MgATP as a substrate have been shown to form enzyme phosphate complexes (Moffet and Bridger, 1970, Anthony and Spector, 1970; Plowman and Cleland,1967). In many of these enzymes, the overall reaction pathway is predominantly sequential although an alternate ping-pong pathway exists since an ATP/ADP exchange reaction is catalysed independently of the other substrates. The pyruvate carboxylase reaction mechanism has now been shown to be sequential (Easterbrook-Smith et al., 1978) although the enzyme catalyses specific exchange reactions

between ATP/ADP, ATP/P_i and pyruvate/oxaloacetate.

The existence of an activated phosphate intermediate was initially postulated by Lynen, 1961 as a general mechanism for biotin carboxylases and later Scrutton and Utter, 1965,applied this theory to pyruvate carboxylase to explain the enzyme-specific ATP/ADP exchange. The alternative would be an enzyme-ADP activated intermediate, but this is unlikely since an ADP-independent isotopic exchange reaction has not been observed. If a phospho-intermediate did exist, the overall reaction could be represented in the following manner:-

$$E + ATP \rightleftharpoons E \sim P_i + ADP \qquad (i)$$

$$E \sim P_i + HCO_3 \rightleftharpoons E \sim CO_2 + P_i \qquad (ii)$$

$$E \sim CO_2 + Pyr \rightleftharpoons E + OAA \qquad (iii)$$

Such a scheme, would lead to the prediction that a HCO_3/OAA exchange should occur in the presence of phosphate but be independent of ADP. This experiment was carried out by Scrutton and Utter and since no exchange activity was observed in the absence of ADP, these investigators concluded that no phosphorylated intermediate participated in the pyruvate carboxylation reaction.

In retrospect, one cannot rule out the possibility of a phosphorylated intermediate solely on these grounds because the function of ADP in the HCO_3/OAA exchange may not be catalytic but purely conformational. Such a situation

exists in the γ-glutamyl transfer reaction catalysed by glutamine synthetase (Meister, 1968). Here a catalytic amount of ADP is required not to participate in the chemical reaction, but so that the substrate, glutamate can bind to the enzyme. Therefore with regard to pyruvate carboxylase, ADP may only be necessary for efficient binding of one of the substrates (viz. P_i) or release of one of the products, (viz. bicarbonate).

Substrate activation

An alternative mechanism to enzymic activation is substrate activation where the enzyme reacts with an ATP-activated bicarbonate species. This hypothesis has received a great deal of support from the work done by Polakis et al., (1972) who showed that acetyl CoA carboxylase could catalyse the formation of ATP from carbamyl phosphate and ADP at 40% of the forward reaction rate. The reactive species in the carboxylation reaction was therefore postulated to be a carbonyl phosphate moiety. Similar investigations were carried out on sheep liver pyruvate carboxylase, (Ashman and Keech, 1975) and the activated substrate theory remained an attractive mechanism to explain the experimental observations.

However, any postulated mechanism for biotin carboxylation must be able to explain the following observations:

(1) an enzyme catalysed ATP/ADP exchange which has been shown to be biotin-independent.

Scrutton and Utter, (1965) have suggested that

ATP hydrolysis could result in the formation of a tight enzyme-phosphate complex and a looser enzyme-ADP complex and thus the exchange of ADP could exist but no phosphate exchange would occur. The recent data of Goss and Hudson (unpublished results) support this hypothesis. Hence one could conclude that biotin must participate in the ATP/P_i exchange, and in nett ATP hydrolysis.

The presence of a carbonyl phosphate intermediate could also support these data since it has been shown that ATP could still be generated from ADP and the analogue carbamyl phosphate even in the absence of biotin (Polakis et al., 1972).

The one definite conclusion that can be made from these findings is that biotin does not appear to participate catalytically in the hydrolysis of ATP but its presence may be a conformational requirement for the binding of other substrates or products, namely bicarbonate ADP and phosphate.

(2) the HCO_3/OAA exchange is ADP-dependent. Ashman et al., (1973), found that the analogues of ADP, which were AOPCP and APS, did not support the HCO_3/OAA exchange above background levels. This was shown to be due to the lack of their ability

to be phosphorylated. Therefore it seemed apparent that there was a need for ATP hydrolysis so that the exchange would occur. One could then deduce, that the formation of $E^{biotin\sim CO_2}$ in the forward reaction from bicarbonate has an absolute requirement for hydrolysis of ATP, possibly to supply energy for the formation of an activated intermediate complex associated with the enzyme.

(3) the possibility of more than one phosphate group capable of interacting with the enzyme, as implied by product inhibition studies (Ashman, 1973). At fixed concentrations of phosphate with varied bicarbonate, the double reciprocal plot showed a series of intersecting lines which intersected to the left of the ordinate. A secondary plot of the slope of these lines when plotted as a function of phosphate concentration resulted in a concave upward curve (Fig. 5.2) which would indicate the participation of more than one molecule of phosphate in the overall reaction. Other explanations for this data can also be given and cannot be excluded; namely that ortho-phosphate could combine with more than one form of the enzyme, or that the binding of this anion causes a change in the reaction mechanisms.

The models for biotin carboxylation which have appeared up to date do not seem to adequately

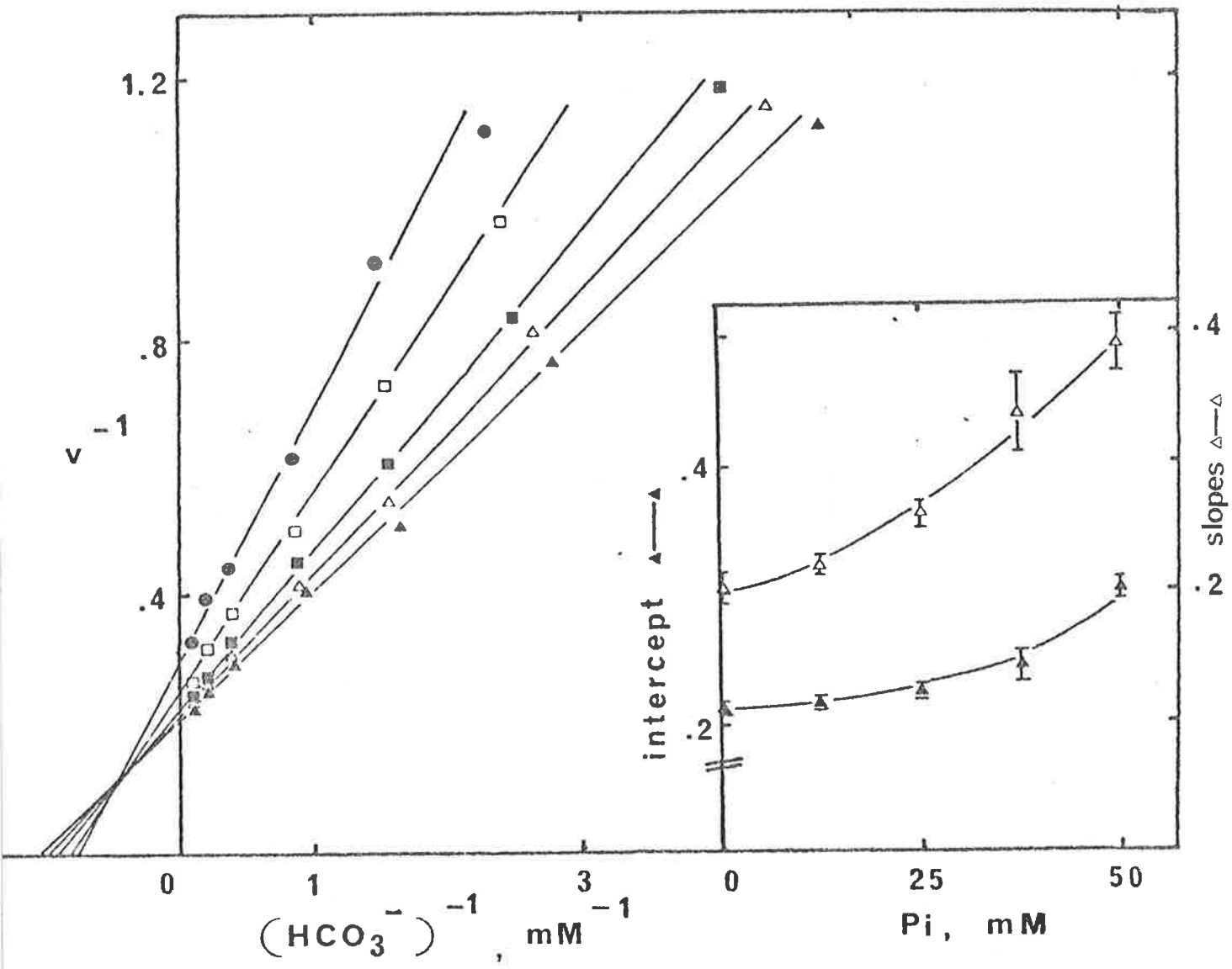
FIGURE 5.2

(Taken from Ashman, 1973)

Product inhibition by orthophosphate with HCO_3^- as varied substrate.

The enzyme (0.05 units, S.A. 12) was assayed by the spectrophotometric method for P_i as product inhibitor.

P_i (Na^+) was added at the following millimolar concentrations (with the Na^+ concentration held constant by addition of NaCl), 0 (▲), 12.5 (△), 25 (■), 37.5 (□), and 50 (●).


1.2
.8
.4
v −1
0
1
3
(HCO3−) −1, mM−1
intercept
.4
.2
0
25
50
Pi, mM
.4
.2
slopes

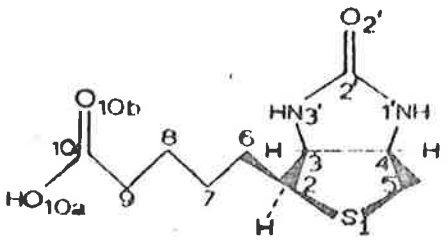
explain all the available data, thus a review of this subject would seem warranted.

5.1.2 Properties of biotin

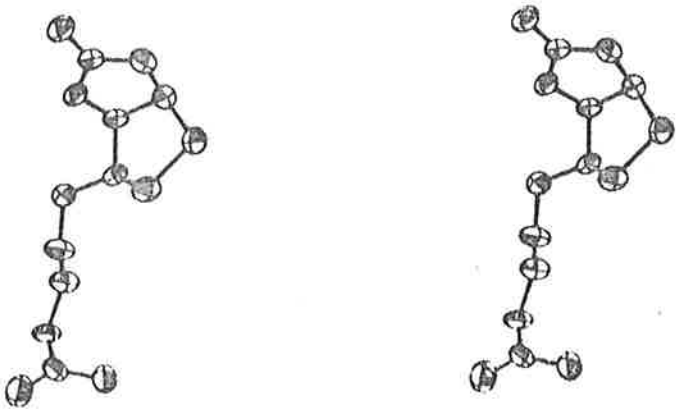
Since biotin is the species which is initially carboxylated, an understanding of the properties and reactivity of this co-factor would certainly aid in trying to elucidate the chemical mechanism of carbon dioxide fixation catalysed by biotin carboxylases. Naturally there would be significant differences in properties of biotin when it is attached to a protein and inside an amino acid environment compared with it being free in solution. However, investigation of the behaviour of this molecule or its analogues in various solvents which try to mimic the protein environment should provide valuable information which could then be taken into account when postulating an enzyme-catalysed carboxylating mechanism.

The absolute configuration of biotin is shown in Fig. 5.3. Biotin is attached to the enzyme surface by a covalent bond between the carboxylic acid group of its side chain and a lysine residue on the enzyme surface. The amino acid environment around the biotin attachment site is described in Fig. 5.4. Using the amino acid sequence, the secondary structure around this attachment site was discussed by Rylatt *et al.*, (1977), on the basis of the predictions of Lim (1974). Calculations revealed an α-helical conformation of the amino acid backbone around biotin and thus the biocytin molecule has a great deal of freedom to move.

FIGURE 5.3

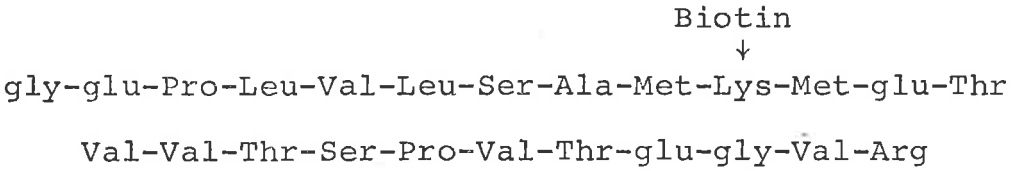


Configuration of biotin as determined from crystallographic work by Traub, W. (1956), Nature 178, 649.



The molecular conformation of d-biotin as published by De Titta, G.T., Edmonds, J.W., Stallings, W. and Donohue, J. (1976), J. Am. Chem. Soc. 98, 1920.

FIGURE 5.4



Sequence around the biotin attachment site for sheep liver pyruvate carboxylase (Rylatt et al., 1977).

It has been initially shown by Lynen et al.,(1961) for β-methylcrotonyl carboxylase and later for acetyl CoA carboxylase by Guchhait et al., (1974), that biotin is carboxylated on the 1'-N-ureido nitrogen position. This carboxylated biotin species is then thought to flip from the carboxylation site to the transcarboxylation site where it transfers carbon dioxide to an acceptor molecule. If biotin is to function as an effective nucleophilic catalyst in enzymic reactions, then it would be predicted that the cofactor would have special properties which make it highly susceptible to carboxylation by bicarbonate or ATP-activated bicarbonate. The principal determinants underlying the reactivity of biotin are as follows:-

(1) the basicity of the ureido group

(2) the nucleophilicity of the ureido 1'-nitrogen atom

(3) the electrophilic activation of the 1'-N-carboxyl group in carboxybiotin.

The relative acidity constants for biotin and two structural analogues are given in Table 5.1. These values indicate that the ureido functional group has very low basic properties and incorporation of this moiety into a ring structure further reduces its base activity. Of the compounds tested in Table 5.1, biotin is the weakest base and it is because of this feature that the carboxylated biotin intermediate in enzymic reactions is relatively stable and the transcarboxylation reaction is not extremely fast. This is not a disadvantage since biotin$\sim CO_2$ can be considered

TABLE 5.1

Acidity Constants (pK_a)

	H_2O[2]	Formic[1] Acid	3% Glacial[1] Acetic Acid
1,3 dimethylurea	-0.2	-1.33	-2.28
2-imidazolidone	-1.05	-2.57	-2.72
Biotin	-1.13	-2.79	-2.69
2-Methoxy-2-imidazoline	9.14[3]	-	-

1. Bowen, C.E., Rauscher, E., Ingrahan, L.L., (1969) Arch. Biochem. Biophys. 125, 865.
2. Caplow, M. (1969) Biochemistry 8, 2656.
3. Hegarty, A.F., Bruice, T.C., Benkovic, S.T. (1969) Chem. Commun., 1173.

as a discriminating carboxylating agent, that is, stable enough not to react non-specifically. This could be an important factor when considering the fact that biotin$\sim CO_2$ must flip from the first partial reaction site to the second partial reaction site.

If protonation of biotin could eventuate it is presumed to occur on the carbonyl oxygen atom. This was deduced by Greisser et al., (1973), by observing the change in infrared stretching frequencies when an analogue of biotin was titrated with acid. Therefore the site which has the most ability to donate electrons is the carbonyl oxygen atom.

The nucleophilicity of biotin was investigate- by Hegarty et al., (1969), using the functionally equivalent analogue 2-imidazolidone. The ureido group was found to have an extremely low nucleophilicity making model studies of enzymic carboxylation very difficult. It was observed (Caplow, 1965) that N-carboxyimidazolidone decarboxylated extremely rapidly when the species was uncharged and a uni-molecular decarboxylation mechanism was deduced from relevant entropy and enthalpy values. This led to the hypothesis that the carboxylation of 2-imidazolidone should occur by reaction with its tautomer. It was subsequently found (Hegarty et al., 1969) that a chemical model for the biotin tautomer, 2 methoxy-2-imi- dazoline possessed a nucleophilic centre, namely the carbonyl carbon atom, which had a 10^{10}-fold greater nucleophilicity

than that of 2-imidazolidone. Since biotin has approximately the same pKa values both in keto and enol forms as 2-imidazolidone one can postulate that the tautomers of biotin exhibit similar reactivity. Although the concentration of the enol component of biotin would be very low, the immense reactivity difference between keto and enol forms would appear to be consistent with a carboxylation route involving the enolized form. When biotin is enzymically bound, any tendency to shift the equilibrium between tautomers to the enol form would enhance its reactivity enormously and thus be advantageous in terms of catalysis of a carboxylation reaction.

5.1.3 Activation of biotin

Model chemical studies to elucidate the mechanism of enzymic carboxylation have provided valuable information on the participation of biotin in this process. Some reservation must be placed on the interpretation of the results of such studies since simple model compounds may not necessarily reflect the reactivity in the environment of an enzyme. Nevertheless predictions can be made since a compound participating in a chemical reaction will do so via the same mechanism whether it is in solution or bound to an enzyme. Naturally the amino acid environment of the protein plays a crucial part in determining the rate of reactivity due to the fact that within an enzyme's active site the substrate functional groups are juxtapositioned to maximize molecular interaction while in

solution a chemical reaction is dependent on the rate and position of collision of the molecules, Van der Waals forces between the molecules, and the activation energy of the chemical process.

Clearly the outcome of these chemical studies has been that biotin in its most stable configuration is unreactive even with highly activated electrophiles. Thus when biotin is bound to an enzyme, it seems reasonable to assume that some activation of its ureido functional group must occur in order to be compatible with the observed overall rates of reaction of the biotin carboxylases which have been observed. This activated biotin species would only then be able to participate effectively in any one of the three mechanisms for ATP hydrolysis coupled to carbon dioxide fixation which were discussed in Section 5.1.1.

There are several ways in which biotin could be activated through its interaction with the enzyme. Recently, α-(+)-biotin has been crystallized and X-ray diffraction techniques employed to determine various molecular characteristics (De Titta et al., 1976). Of particular relevance was the fact that the ureido carbonyl bond length is 1.25 Å which is significantly longer than the average length of carbonyl bonds which is 1.21 Å and similarly the carbon-nitrogen bonds (1.34 Å) were shorter than the average 1.37 Å. This result suggests a partial delocalization of electronic charge within the ureido

group and could support a proposed activation of biotin via a hydrogen-bonding interaction between a polar amino acid on the enzyme surface and the carbonyl oxygen atom. Greisser et al., (1973) demonstrated that hydrogen bonding does occur between biotin and the polar hydroxyl group of phenol as measured by the shift in carbonyl infrared stretching frequencies to longer wavelengths, thus indicating a decreasing double bond character. If hydrogen bonding between the ureido group and a phenolic or similar group present at a suitable position in the enzyme polypeptide chain did occur, this would greatly facilitate enolization of the biotin and thus bring about an appropriately active species to catalyse a carboxylation reaction.

Activation could also be achieved by interaction of the ureido group with a metal ion. The ability of this functional group to coordinate to a metal ion has been demonstrated by Greisser et al., (1973), as evidenced by line-broadening of the NMR spectra of biotin in the presence of differing amounts of manganese chloride. Since pyruvate carboxylase has an associated bound manganese atom, a possible function of this bound metal could be in facilitating the enolization of biotin. However, (Reed and Scrutton, 1974) have shown that the manganese is greater than 10 $\mathring{A}$ from the ATP binding site and thus the participation of this metal ion in biotin carboxylation would be difficult to rationalize.

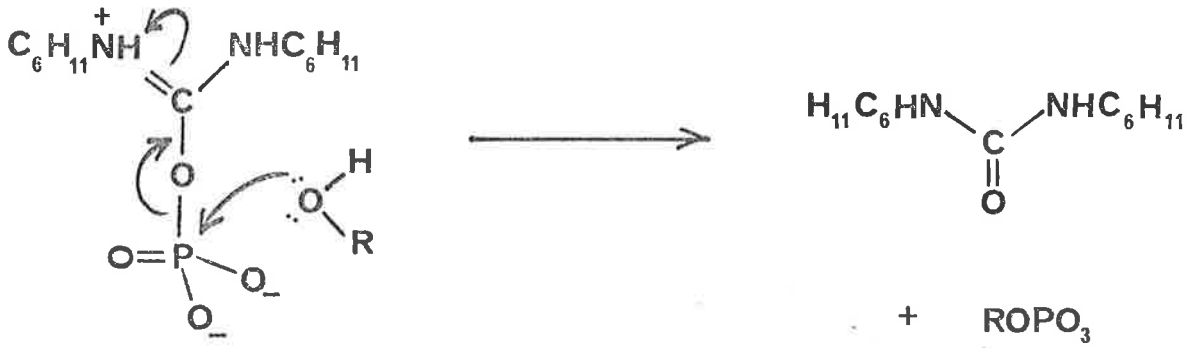
In general, activation of biotin to its high energy isourea form can be achieved by attack of an

electrophile, X, where X could be a proton, a metal ion or a phosphoryl group. In biotin-containing enzymes where the active site features a phosphate group and a urea moiety in reactive proximity, this would certainly induce a facile reaction to occur. The biotin-phosphate intermediate could be considered analogous to an o-phospho-carbodiimide species (I, Fig. 5.5, Khorana, 1961). The inter-molecular cleavage of the phospho-ester bond occurs in virtually quantitative yield. Cyclization of such a compound would result in an increase in proximity of functional groups and thus its reactivity could be more accurately related to the behaviour of a similar species in an enzyme's active site. Hydrolysis of a cyclic phosphate-urea moiety was found to be extremely rapid (Kluger and Adawadkar, 1976) and was indicative of a reaction proceeding via intramolecular nucleophilic catalysis. This would be compatible with the expected activity exhibited by the biotin carboxylases.

5.1.4 Postulate of an enzyme phosphate intermediate

From recent work done on the mechanism of biotin catalysis (Visser and Kellogg, 1977, and Kluger and Adawadkar, 1976), it can be concluded that biotin-like molecules seem to have no detectable catalytic ability in carboxylations unless they have been firstly activated to the high energy tautomer which is appreciably more nucleophilic. In applying this knowledge to the action of biotin-containing enzymes, biotin itself would not then

FIGURE 5.5



I

Dicyclohexylphosphocarbodiimide (I) reaction with alcohols. (Khorana, 1961).

"catalyse" carboxylation but merely act as a carbon dioxide transfer agent. In performing this function it would require the energy derived from ATP hydrolysis.

This hypothesis would require that the enzyme adopts an activated conformation or produces extremely active intermediates which would enable biotin to become carboxylated. As discussed in the previous section, the enzyme could activate biotin carboxylation by shifting the equilibrium between biotin tautomers through hydrogen bonding with a nearby polar amino acid or through ionic bonding with the bound manganese. Intermediate structures resulting from these types of interactions would be virtually impossible to isolate since the enol form of biotin is extremely labile. Verification of a mechanism proceeding via secondary molecular interactions would therefore be very difficult.

An alternate method of biotin activation was phosphorylation. This process would result in the formation of a phospho-ester on the ureido-carbonyl group. Enzyme bound biotin$\sim CO_2$ is known to be relatively unstable, but Lynen (1967) was able to isolate this species in the stable methyl ester form by diazomethylation. If a phospho-biotin species did exist, it would be extremely labile. It would seem reasonable to assume that the only way to trap an intermediate in a very rapid reaction is to use an equally vigorous reaction which would stabilize the transient compound. Isolation of a reactive intermedite

in the methylated phospho-ester form by diazomethylation might be suitable.

Powers and Meister (1976) have successfully used this technique in isolating an activated-CO_2 intermediate from glutamine-dependent carbamyl phosphate synthetase. This work prompted the search for a carbonyl phosphate intermediate by the same technique in pyruvate carboxylase (Goss, unpublished results) but no methylated carbonyl phosphate was detected.

However, no experimental evidence so far has been produced to discount the mechanism involving o-phosphobiotin. Therefore it was decided to attempt to isolate a phosphate intermediate of pyruvate carboxylase by diazomethylation.

5.2 METHODS

5.2.1 Preparation of sheep liver pyruvate carboxylase, specific activity 30 U/mg.

The enzyme was prepared in the manner described in Chapter 2, but an additional purification step was included (Goss, unpublished results). Sheep liver pyruvate carboxylase in NEM-sucrose storage buffer, (1.6 M sucrose; 0.1 M NEM-Cl, pH 7.0; 1% sat. ammonium sulphate) was applied to a NAD-agarose column (1 cm x 5 cm) previously equilibrated with 50 mM Tris-Cl, pH 8.4, containing 10^{-2} M DTE and 10^{-4} M EDTA. This buffer solution was pumped through the column at a rate of 0.5 ml/min and one ml fractions were collected.

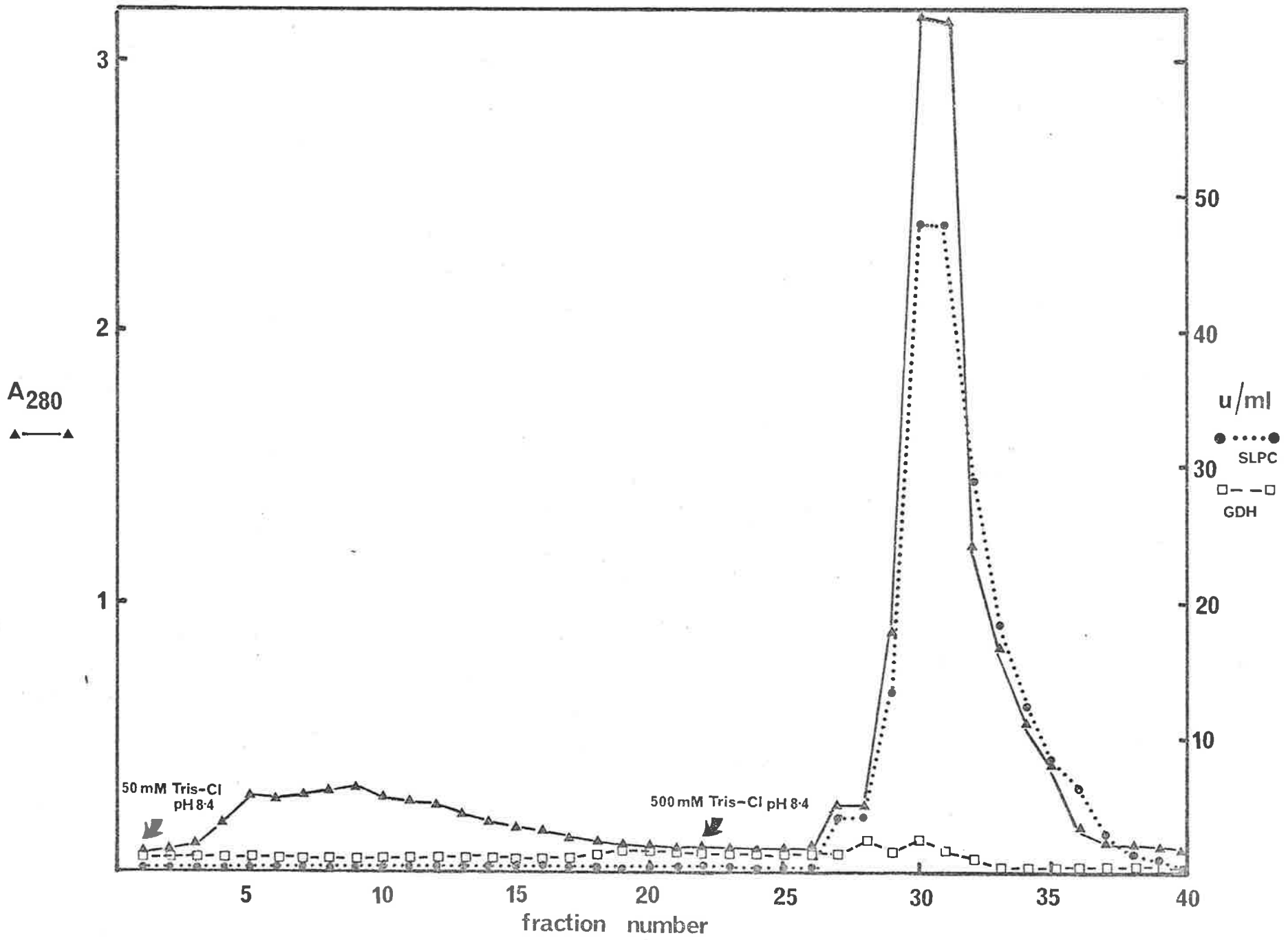
Contaminating protein was removed from sheep liver pyruvate carboxylase by this method. Enzymic activity was then eluted from the column with 0.5 M Tris-Cl, pH 8.4, 10^{-2} M DTE and 10^{-4} M EDTA (Fig. 5.6). The specific activity of the pooled enzyme containing fractions was 30 U/mg and the homogeneity was checked on 5.8% acrylamide SDS gels. The enzyme was concentrated by precipitation with ammonium sulphate (45% saturation) and redissolved in a minimal volume of 0.2 M Tris-Cl, pH 8.4.

5.2.2 Preparation of [γ-^{32}P]ATP

The procedure used for the preparation of [γ-^{32}P]ATP was a modification of the technique used by Glynn and Chappell (1964). To a sample of [^{32}P]orthophosphate, were added the following components, in μmoles/ml; Tris-Cl, pH 8.0, 40; MgCl, 20; 3-phosphoglyceraldehyde, 4; ATP, 20; potassium phosphate, 0.1; β-mercaptoethanol 0.014: To this solution was added 7 units of glyceraldehyde-3-phosphate dehydrogenase and 5 units 3-phosphoglyceric phosphokinase. Incubation at room temperature was allowed to proceed for two hours and resulted in 96% incorporation of [^{32}P]orthophosphate into ATP. The reaction was followed by thin layer cellulose chromatography in 0.8 M ammonium bicarbonate. The [γ-^{32}P]ATP was purified on Dowex-1 (1 cm x 3 cm) equilibrated in 0.2 M ammonium formate pH 4.0. The sample was diluted to a volume of 5 mls and loaded onto the column. Forty-five mls of the buffer were passed through the column at

FIGURE 5.6

NAD-agarose column purification of sheep liver pyruvate carboxylase. Specific activity initially 15 U/mg was increased to 30 U/mg.


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

a flow rate of 1 ml/min. This was followed by 25 mls of 0.4 M ammonium formate, pH 3.5. The purified [γ-^{32}P]ATP was eluted from the column with 1 M HCl. The solution was then neutralized with NaOH and desalted by passage through a Sephadex G-10 column as judged by chromatography on thin layer polyamide sheets. The [γ-^{32}P]ATP was 100% pure.

5.2.3 Preparation of diazomethane

This method was adapted from the procedure described by Vogel (1967). P-toluenesulphonylmethyl-nitrosamide (2.15 gms) was dissolved in a solution of ether (30 mls) and cooled on ice. Potassium hydroxide (0.4 gms) in redistilled ethanol (10 mls) was then added and a precipitate formed. More ethanol was added until the precipitate just dissolved. After 5 min at 4°C, the ethereal diazomethane solution was distilled off from a water bath (60 - 65°C), and collected in a vessel cooled on ice.

5.3 RESULTS

5.3.1 Trapping of an enzyme-bound phosphate with diazomethane

To a reaction solution of volume 0.1 mls containing [γ-^{32}P]ATP (10 mM, specific activity, 6000 cpm/nmole), ADP (5 mM), $MgCl_2$ (20 mM), potassium phosphate (20 mM) and acetyl CoA (0.5 mM) was added 0.1 ml pure sheep liver pyruvate carboxylase (S.A. 30 U/mg, 41 nmoles biotin in Tris-Cl

pH 8.4, 0.2 M). After incubation at 30°C for 2 mins, the solution was quenched with 5 mls of diazomethane: methanol, 9:1, which was introduced through a syringe. After standing at room temperature for 1 hr, the ether phase was removed and the enzyme precipitated by centrifugation. The precipitate was then suspended in 1 ml of 0.02 M potassium phosphate buffer pH 7.2 containing 50 µl ethanol and digested at 37°C with 2% trypsin for 2 hrs and then 1 mg per ml pronase for 24 hrs. The sample was then freeze-dried and taken up in 50 µl pyridine: H_2O, 1:1. One tenth of the volume was applied to a thin layer silica gel plate and chromatographed using benzene: ethanol, 9:1 as the developing solvent. Two radioactive bands with Rf values of 0.23 and 0.42 separated from the origin (Fig. 5.7).

In order to ascertain whether these bands were specific for pyruvate carboxylase, a number of control samples were used. For example, a system lacking enzyme, samples where unlabelled orthophosphate,and acetyl CoA, were deleted, a system where the enzyme was inactivated with avidin and the corresponding avidin control. The results from these experiments are presented in Fig. 5.8.

The system lacking enzyme or where enzyme was replaced by avidin, produced no radioactive bands separating from the origin. The presence or absence of unlabelled orthophosphate in the original reaction solution did not change the band pattern, and hence it can be concluded that non-specific phosphate binding has not occurred.

FIGURE 5.7

^{32}P-counts profile after chromatography on a silica gel plate using benzene-ethanol, 9:1 as the developing solvent.

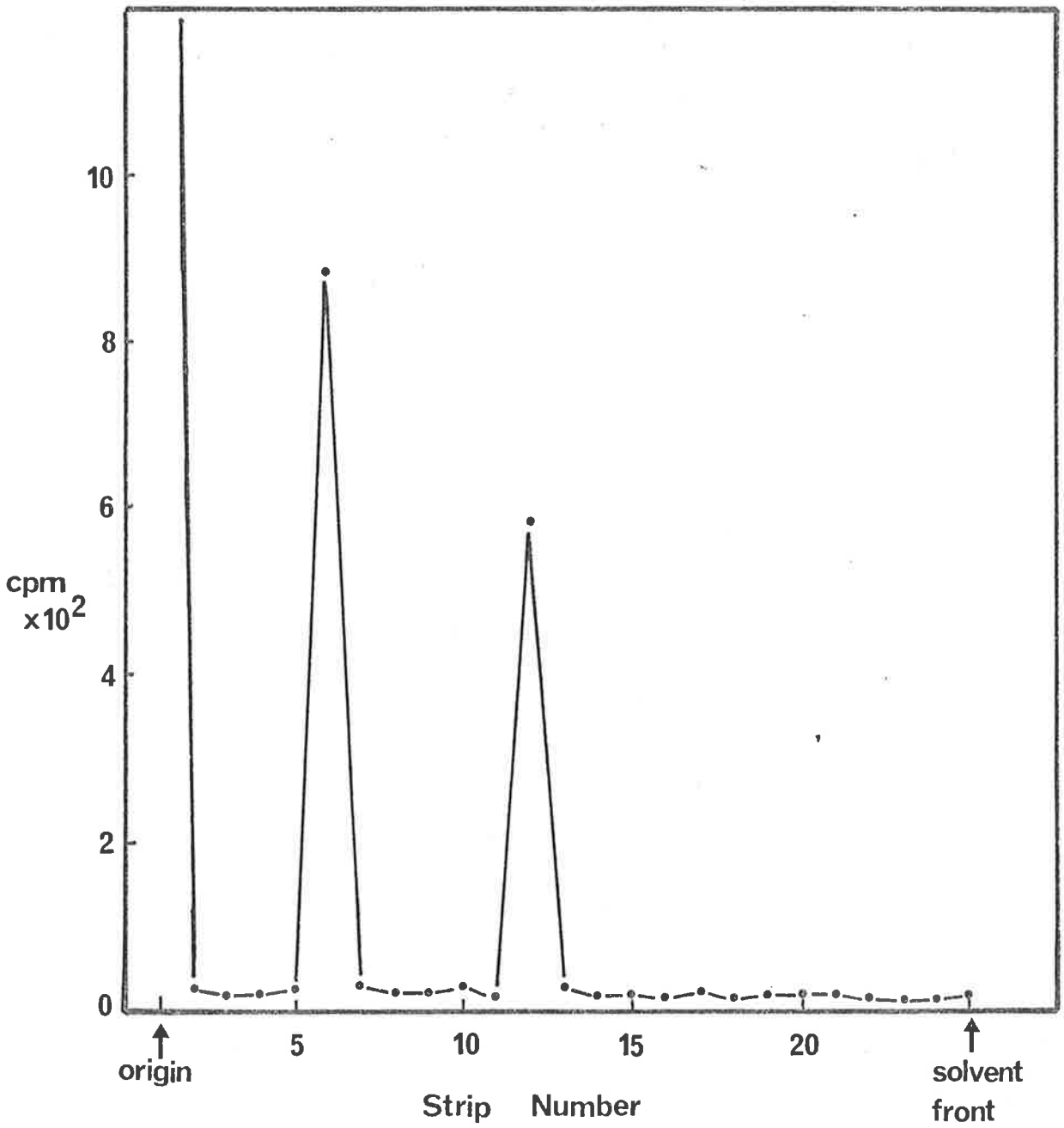
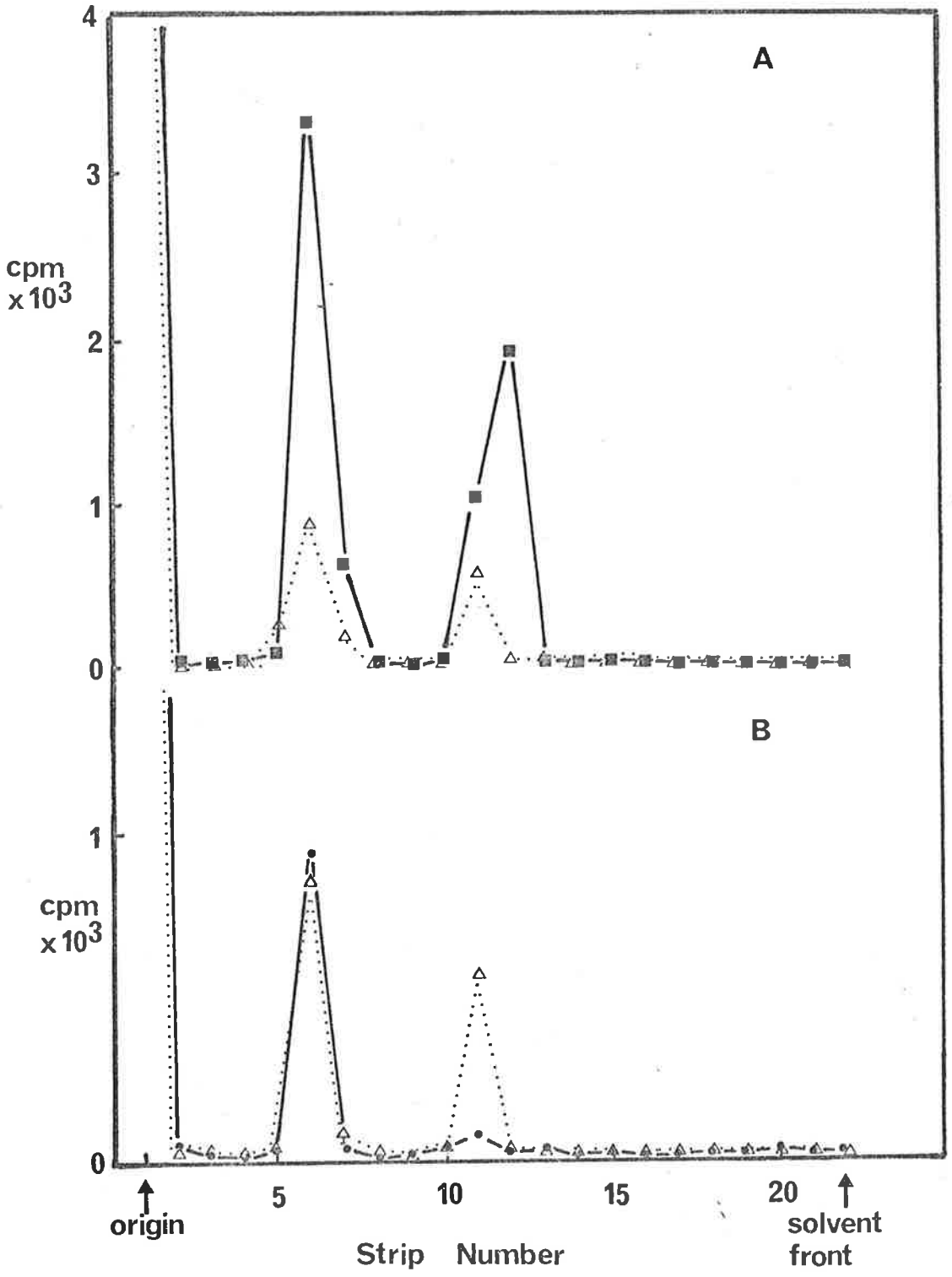

10
8
6
cpm
x10²
4
2
0
origin
5
10
15
20
solvent
front
Strip Number

FIGURE 5.8

^{32}P-counts profiles representing results from various control samples which were perfomed to verify that the radioactive bands were specific to pyruvate carboxylase.

Fig. A represents the intensity of bands in the presence (Δ ---- Δ) and absence (■——■) of acetyl CoA.

Fig. B shows the result using enzyme inactivated with avidin (●——●). The band pattern in the absence of avidin (Δ·····Δ) is given for reference.


A
B
cpm
x 10^3
4
3
2
1
0
1
0
origin
5
10
15
20
solvent
front
Strip Number

In the presence of avidin, the band of R_f 0.42 is not observed. This would suggest that ^{32}P-phosphate associated with biotin moves in this position. Deletion of acetyl CoA results in a six-fold increase in the intensity of both radioactive bands. Since both the specific effectors of the active site of pyruvate carboxylase, namely avidin and acetyl CoA also effect the amounts of phosphointermediates isolated, this also supports the finding that the reaction catalysed by sheep liver pyruvate carboxylase proceeds via phosphorylated intermediates.

In all radioactivity profiles shown, there is a very large band remaining at the origin. Since this was present in the system where enzyme was deleted, it can be attributed to methylated [γ-^{32}P]ATP. Attempts were made to remove this residual radioactivity at the origin. The two methods employed were:-

(1) charcoal adsorption of [γ-^{32}P]ATP; 0.2 mls of an activated charcoal suspension in 6 N HCl (250 mgs/ml) was added to the digested enzyme in 1 ml of 0.02 M potassium phosphate pH 7.2. The mixture was then centrifuged and the supernatant removed and freeze-dried. 50 µl pyridine: H_2O, 1:1 was subsequently added and the chromatography system applied.

(2) Sephadex G-25 column separation; the methylated protein prior to digestion was dissolved in 0.5 mls of 6 M guanidine HCl and applied to

a Sephadex G-25 (1.5 cm x 20 cm) column equilibrated in 6 M guanidine HCl. Flow rate was controlled at 1 ml per min, and 1 ml samples were collected. Aliquots from each fraction were taken for protein and radioactivity determinations. The profile from such a column is shown in Fig. 5.9. Fractions corresponding to the protein peak were pooled and dialysed against 0.02 M potassium phosphate buffer pH 7.2 and 0.02 M sodium azide with three changes of 100 vols. The precipitated enzyme was then digested with trypsin and pronase, freeze-dried and redissolved in 50% pyridine.

Both procedures resulted in removal of nearly all radioactivity from the origin but also suffered great losses within the moving bands.

5.3.2 Identification of the radioactive products

Likely candidates for phosphorylation were serine and threonine and therefore these phosphorylated amino acids were methylated and used as markers for identification of the radioactive bands (Markers I and II, Fig. 5.10). Since the free carboxyl group of the markers would also be esterified whereas the amino acids derived from the hydrolysis of the enzyme would still be in the acid form, the digested protein was remethylated. Aliquots were spotted on thin layer silica gel plates

FIGURE 5.9

Separation of methylated [γ-^{32}P]ATP from radioactively labelled protein by Sephadex-G25 column chromatography in 6 M guanidine HCl.

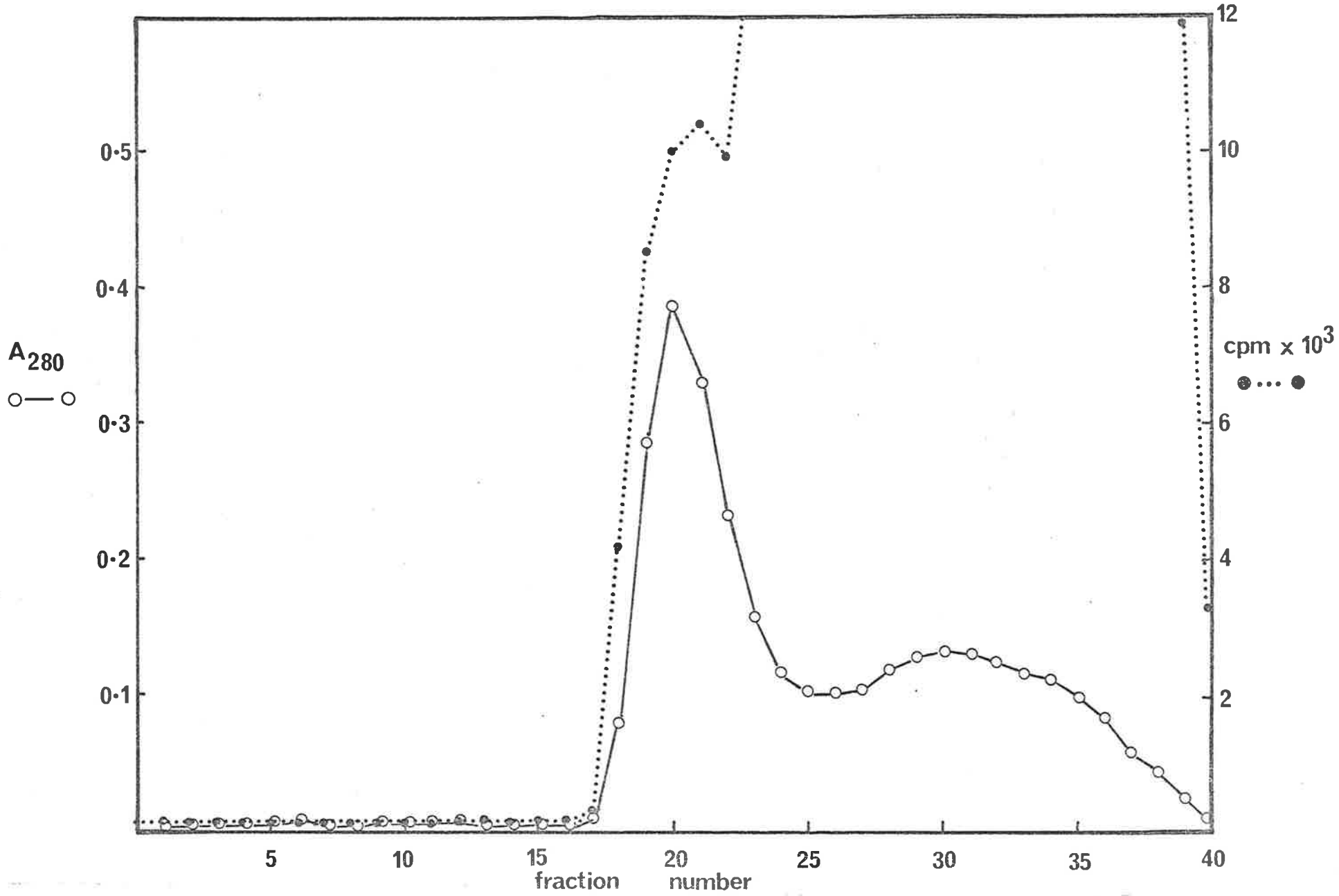
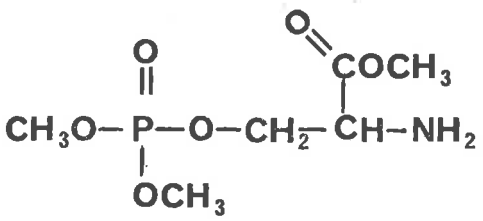
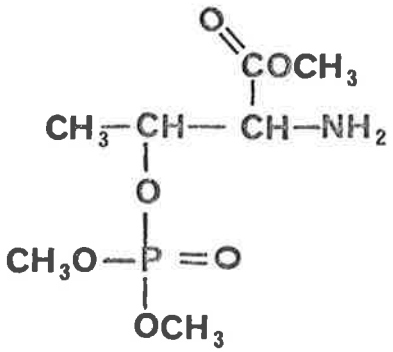

A280
○—○
0·5
0·4
0·3
0·2
0·1
12
10
8
6
4
2
cpm x 10^3
●···●
5
10
15
20
25
30
35
40
fraction number

FIGURE 5.10



Marker I

Trimethylated-phosphoserine



Marker II

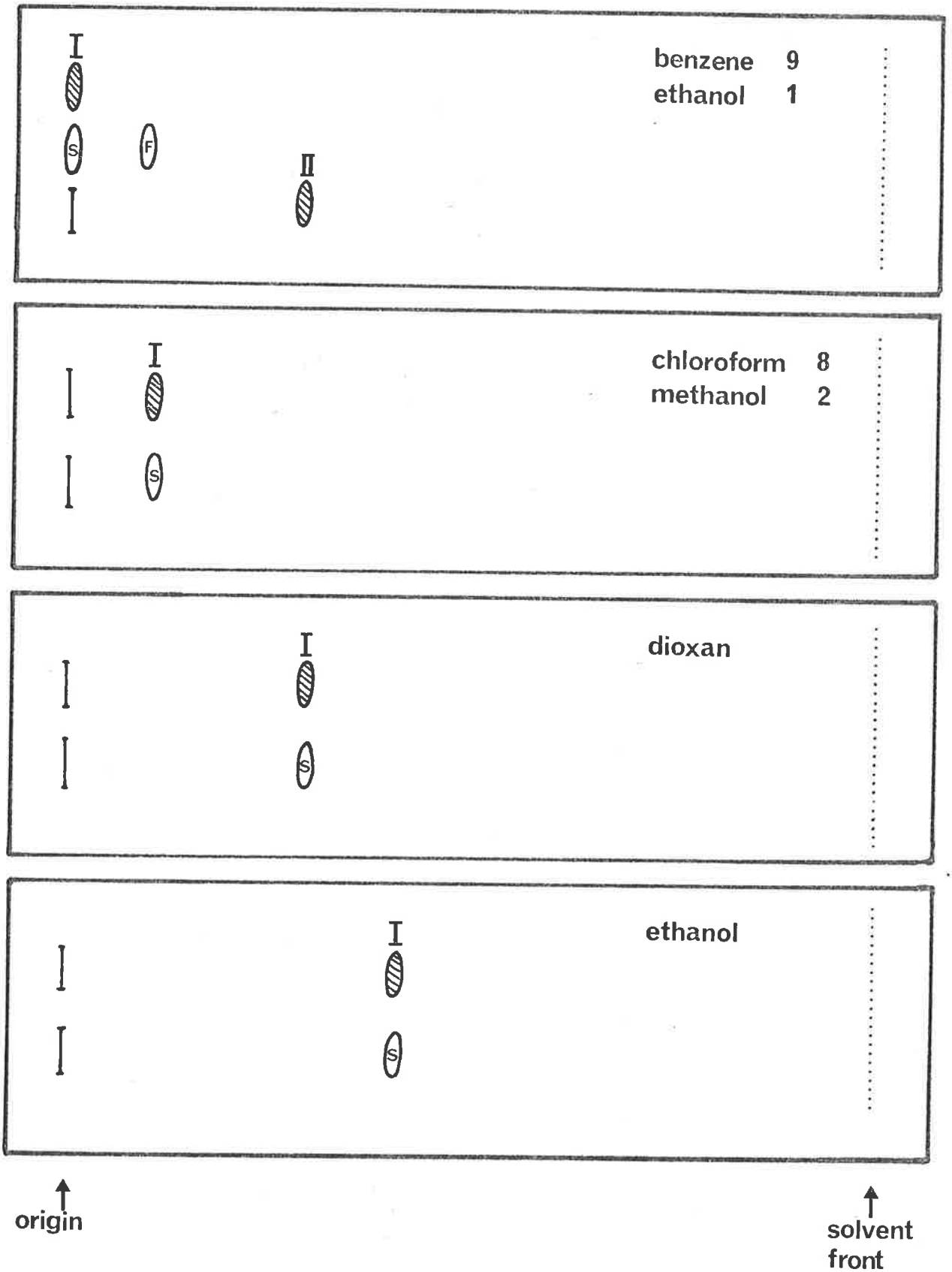
Trimethylated-phosphothreonine

and chromatographed in various solvents (Fig. 5.11). When the remethylated samples were rechromatographed in benzene:ethanol, 9:1, the separating bands had smaller R_f values indicating that the radioactive species had decreased polarity. The slow running band (Band S, R_f 0.23) after remethylation did not move from the origin while the more mobile band (Band F, R_f 0.43) has a new R_f of 0.07. In this system,marker I does not move from the origin, while marker II moves with an R_f value of 0.28. Marker II did not correspond to either of the enzyme associated bands and therefore one can conclude that phosphothreonine is not one of the radioactive products. It is known that a sample will stay with the solvent front if the solvent system is too polar, and it will not move from the origin if the solvent system is insufficiently polar. Therefore since marker I and band S did not move from the origin, solvents of increasing polarity were employed to effect clear separation (Fig. 5.11) and subsequently it was deduced that band S corresponded to phospho-serine. As the solvent polarity increased band F streaked considerably across the chromatogram and could not be located as an isolated band.

To verify the prediction that band F represented a phosphobiotin species as indicated by the studies with avidin treated enzyme, the band of R_f 0.42 was scraped off the silica gel plate and the radioactivity extracted with absolute ethanol. This solvent was subsequently removed by rotary evaporation. The residue was dissolved

FIGURE 5.11

Identification of band S using four different developing solvents and trimethylated-phosphoserine (I) and trimethylated-phosphothreonine (II) as markers.


I
S
F
II
benzene 9
ethanol 1
I
S
chloroform 8
methanol 2
I
S
dioxan
I
S
ethanol
origin
solvent
front

in 200 µl of 4 N HCl, and hydrolysed at 110°C, for 4 hrs to remove the methylated phospho-ester moiety. The HCl was then removed and the presence of biocytin was determined using the biotin assay of Rylatt et al., (1977). The result revealed that biotin occurred in stoichiometric amounts (1:1) with the phosphate present (Table 5.2).

Since the biotin assay employed relies on the counting of unbound ^{14}C-biotin, the presence of free ^{32}P-phosphate could interfere with the actual amount of ^{14}C counts which would be counted. To overcome this problem, the sample was kept at 0°C for 5 weeks so that the ^{32}P-phosphate would decay. An aliquot was recounted for ^{14}C-counts and the amount of biotin was again determined. The same result was obtained.

5.4 DISCUSSION

It has been shown that phosphorylated enzyme intermediates do exist for sheep liver pyruvate carboxylase. Previous attempts to isolate such species (Scrutton and Utter, 1965) had failed apparently because they did not compensate for the lability of such a product.

Since the previously postulated mechanisms for pyruvate carboxylase did not account for the presence of such reactive intermediates a new mechanism must be put forward to incorporate the additional information.

Recently, Kluger and Adawadkar (1976) provided support for a mechanism involving a phosphorylated

TABLE 5.2

	^{32}P cpm	nm Biotin[1]
Band F	463	0.116
Elution from silica gel with EtOH	150	0.0375
After hydrolysis in HCl and resuspended in KPi buffer	53	0.013
Biotin assay of hydrolysed sample	-	0.010

[1]As calculated from S.A. [γ-^{32}P]ATP = 4000 cpm/nm.

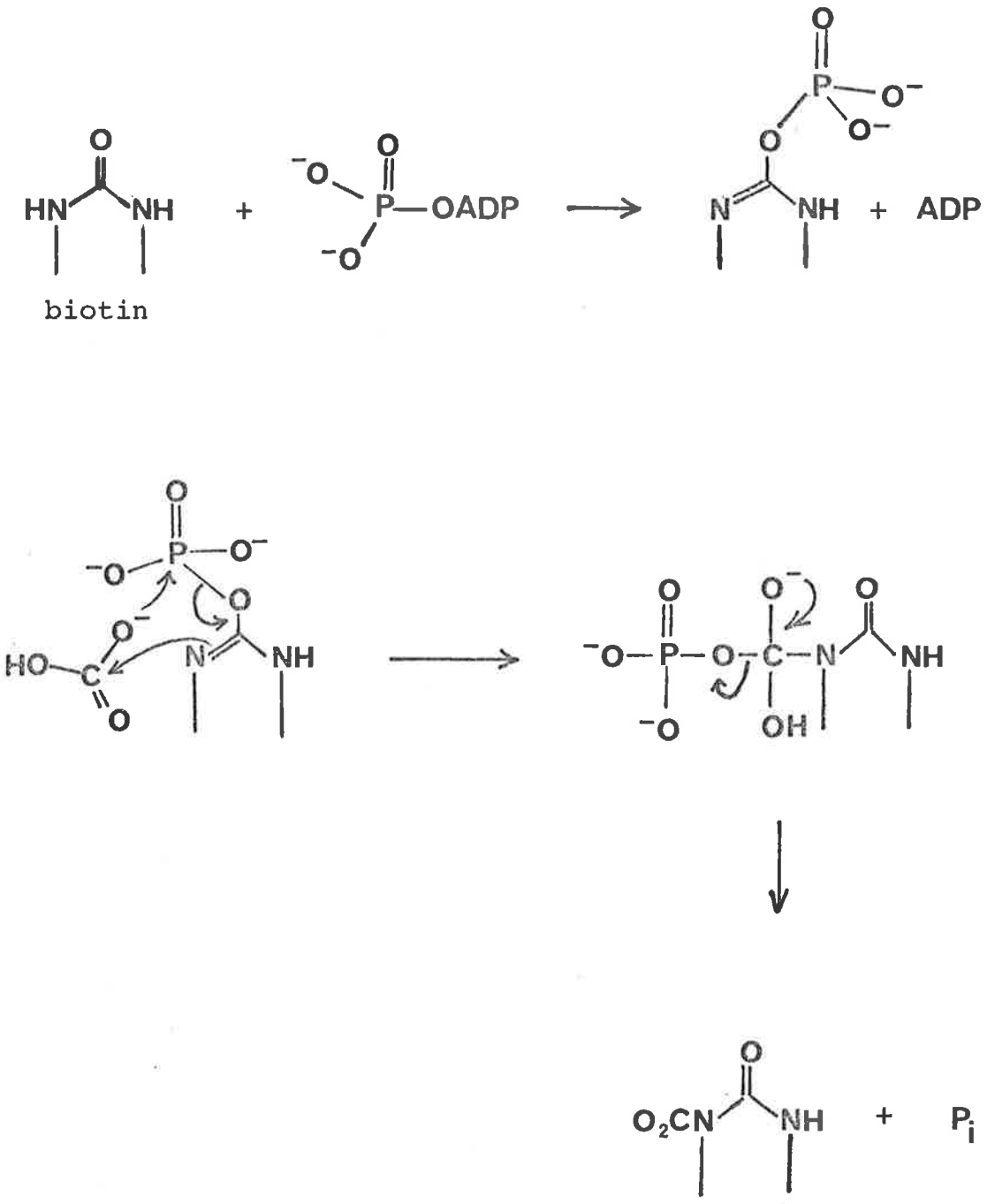
intermediate (Fig. 5.12) by using a model compound for the reactive portions of biotin and ATP. Their mechanism postulated the existence of a phosphorylated biotin intermediate which would activate this cofactor to participate in a carboxylation reaction with bicarbonate. Since phosphorylated biotin has now been isolated from sheep liver pyruvate carboxylase, this mechanism is consistent with the proposition made by these authors. Another attractive aspect of this chemical pathway is the transient existence of a carbonyl phosphate moiety attached to biotin (Fig. 5.12). This would provide a rationale for the observations of Polakis et al., (1972), and Ashman et al., (1975) with the analogue carbamyl phosphate.

Both Kluger and Adawadkar, and Lynen postulated that biotin interacted directly with ATP. This mechanism however would not explain the existence of a biotin-independent ATP/ADP exchange, and also could not account for a phosphorylated serine species which has also been isolated.

An alternate reaction mechanism has therefore been presented (Fig. 5.13) which takes into account all experimental evidence up to date. The catalytic step regulated by Mg^{2+}-acetyl CoA, could quite easily occur via the mechanism of Kluger and Adawadkar, and a possible chemical pathway for the reaction with ATP has been depicted in Fig. 5.14.

The proposed mechanism seems satisfactory

FIGURE 5.12



Proposed mechanism for biotin carboxylation by Kluger and Adavakar, (1976).

FIGURE 5.13

Proposed reaction pathway incorporating phosphointermediates and the recent findings of Easterbrook-Smith *et al.*, (1976).

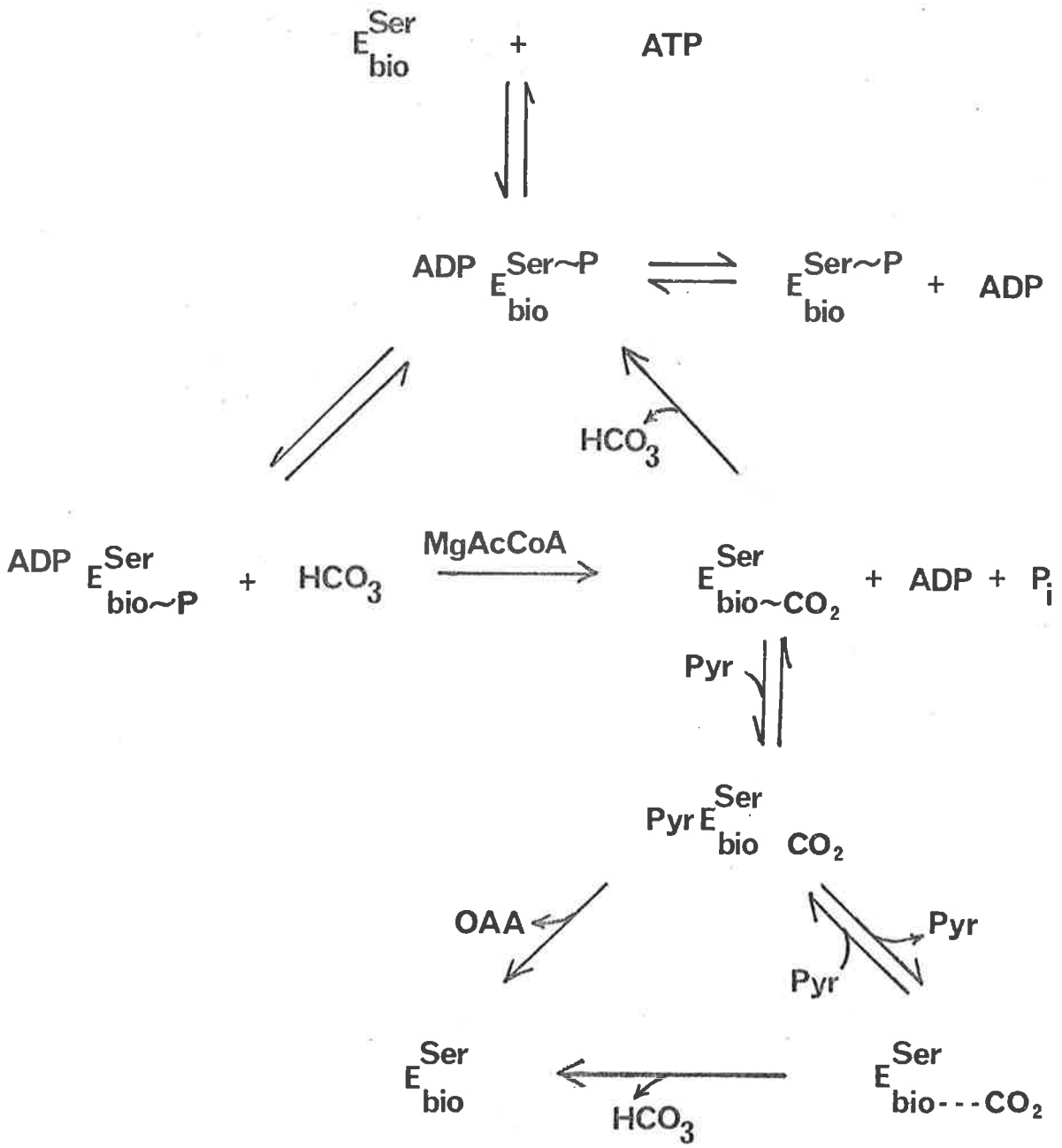
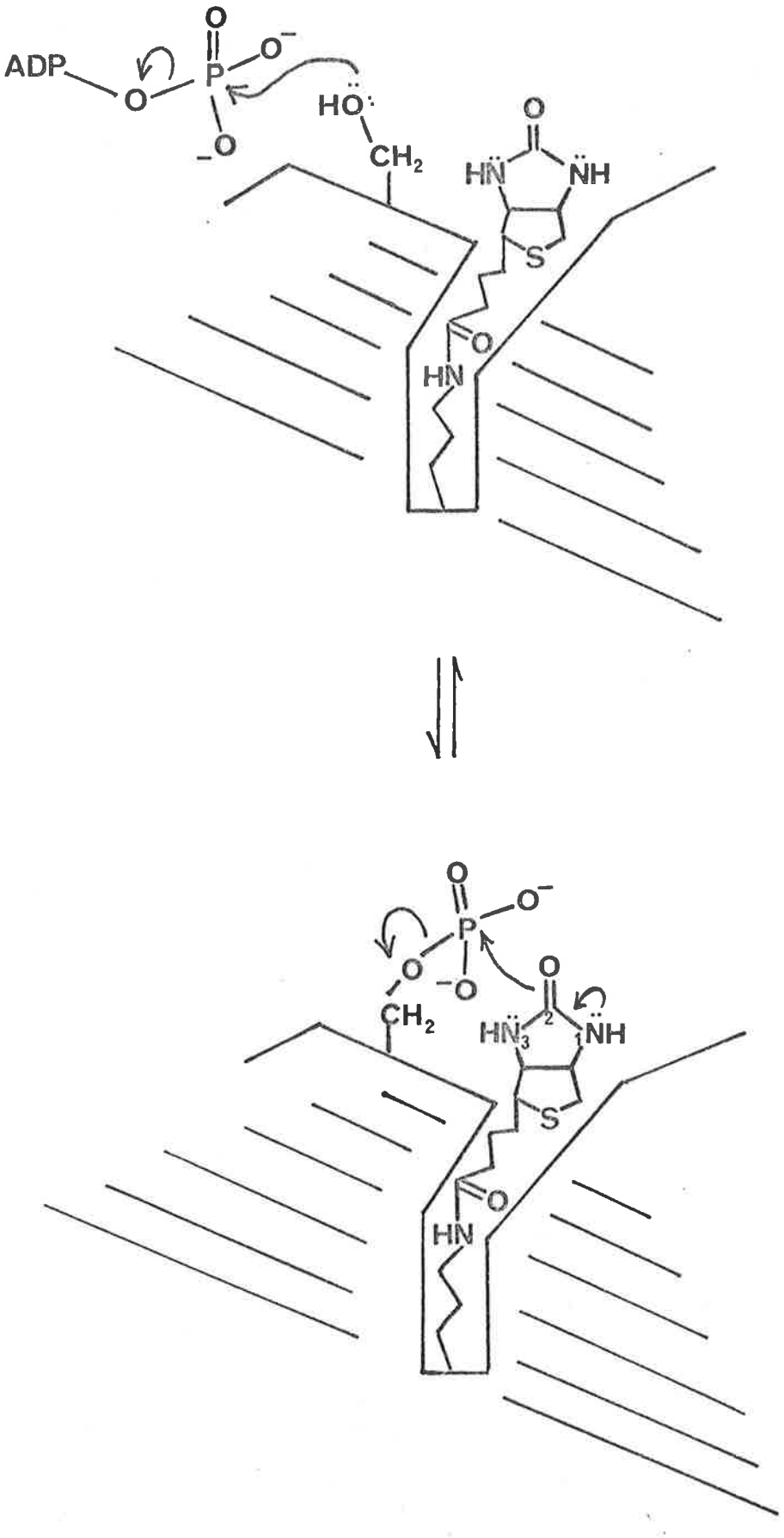

E Ser bio
+
ATP
ADP E Ser~P bio
E Ser~P bio
+ ADP
HCO3
ADP E Ser bio~P
+ HCO3
MgAcCoA
E Ser bio~CO2
+ ADP + Pi
Pyr
Pyr E Ser bio CO2
OAA
Pyr
Pyr
E Ser bio
HCO3
E Ser bio---CO2

FIGURE 5.14

Stereochemical model for the carboxylation of biotin.


ADP
O
P
O⁻
HO
CH2
HN
NH
S
O
HN
⇌
O
P
O⁻
O
CH2
HN3
2
NH
1
S
O
HN

because it can explain the necessary criteria which did not seem reconcilable with previously suggested mechanisms. A catalytic role for phosphoserine and o-phospho-biotin has been assigned since any predictions of chemical transactions must now involve these intermediate species. From Fig. 5.14, it can be seen that there would be steric hinderance to carboxylation at the 3'-nitrogen position and thus 1'N-carboxybiotin would be formed preferentially. This has indeed been shown to be the case by Lynen (1959) and more recently, Guchhait et al., (1974). An ATP/ADP exchange in the presence of avidin could also occur since the formation of the intermediate species $^{(ADP)}E_{bio}^{Ser\sim P_i}$, is biotin-independent. This exchange rate has been shown to be greater than the ATP/P_i exchange (Ashman, 1973). The deletion of acetyl CoA from an ATP/P_i exchange mixture as discussed in Section 5.3.1, is in effect monitoring the rate of formation of phosphointermediates under ATP/ADP exchange conditions. The amount of phosphoserine and phosphobiotin produced is greater under these conditions than under ATP/P_i exchange conditions, hence this observation is consistent with the higher rate of the ATP/ADP exchange. The ATP/P_i isotopic exchange is extremely low in the absence of acetyl CoA but phospho-serine and phosphobiotin are formed even to a greater extent. This would seem to implicate acetyl CoA in a role in the first partial reaction which facilitates the release of orthophosphate. The proposed reaction mechanism predicts this to be so, since there are two pathways by which ATP can be formed from ADP, whereas phosphate can only enter the scheme through one route.

In 1965, Scrutton and Utter predicted the existence of a phospho-intermediate based on the evidence for the existence of an enzyme specific ATP/ADP exchange. They dismissed the possibility of such an intermediate participating in the main reaction pathway, and suggested that the ATP/ADP exchange resulted from an abortive side reaction in which an intermediate was an enzyme-phosphate complex. The basis for their reasoning came from the finding that an HCO_3/OAA exchange was totally dependent on the presence of ADP. The proposed mechanism accounts for the absolute requirement of this nucleotide in this exchange since the back reaction from biotin$\sim CO_2$ would not occur if both ADP and P_i were not bound.

CHAPTER 6

GENERAL DISCUSSION

How does pyruvate carboxylase work? This is the fundamental question on which all study of this enzyme has been based. The main approach up to date has been kinetic, via initial velocity measurements, product inhibition studies, and protection by substrates and activators against chemical modification. This has led, in turn, to a prediction of the reaction pathway. A sound kinetic background can now induce further study into the molecular aspects of the reaction which are gained from the knowledge of the amino acid sequence of the active site.

A beneficial tool in such a project is the technique of affinity labelling. This procedure involves the synthesis of substrate analogues which would covalently bind to the enzyme and after an appropriate cleavage procedure is applied, peptides within a substrate binding site could be sequenced yielding information about the amino acids which make up certain binding regions and from this, a mechanism of enzyme catalysis could be more accurately proposed. Several affinity labels for pyruvate carboxylase have already been synthesized. Two in particular, being [^{14}C]bromopyruvate as a probe for the pyruvate binding site, (Hudson et al., 1975), and [^{14}C]-o-ATP for the ATP binding site (Easterbrook-Smith et al., 1975) are just the beginning of a large field which could be opened up by this tool. The tryptic peptide after [^{14}C]bromopyruvate modification has recently been isolated (Goss, unpublished results). Although sequences have not

yet been obtained up to date, this work has already reaped information suggesting that the polypeptide chains of sheep liver pyruvate carboxylase are not identical.

In view of the work presented in this thesis in Chapter 3, it would be valuable to synthesize affinity labels for the acetyl CoA binding site. As discussed previously the activator for the pyruvate carboxylation reaction seems to bind to the enzyme non-cooperatively. Key segments in binding would appear to be the 3'-phosphate group, the adenine moiety and the acetyl group. Already an affinity label for acetyl CoA binding in the trans-carboxylation site has been synthesized (Clements, unpublished results). This molecule, [^{14}C]chloroacetonyl CoA, is essentially the intact CoA group with the acetyl group replaced such that a stable covalent bond forms between this region of the molecule and the enzyme. It would certainly be worth synthesizing an affinity label in which the adenine moiety was modified. Scrutton et al., (1977) have observed that MgATP protects the enzyme against TNBS modification, therefore this could be interpreted to indicate that the modified lysine residue is situated in close proximity to both MgATP and Mg^{2+}-acetyl CoA binding sites. It has also been suggested by Keech and Barritt (1967) that MgATP could have more than one binding site. This would be accounted for if the MgATP site and the Mg^{2+}-acetyl CoA site had very similar amino acid sequences. What would be the advantage of having a

substrate and an activator site so closely related? One explanation could be that the first partial reaction site must be saturated with nucleotide, possibly to protect against dilution inactivation. Several potential affinity labels of ATP have been realized, these being 6-thioinosine triphosphate, 8-azido-ATP and 6-azido-ATP. Theoretically, similar modifications to the adenine portion of acetyl CoA could be performed and this would allow sequencing in this vital region of the active site to proceed.

Sequencing work is indeed very useful, but if there exists alternate chemical pathways by which a reaction can occur, the only method to resolve the question is by the isolation of reactive intermediates. Diazomethylation is a very successful technique which has been employed for this purpose, and it is through the stabilization of these reactive species by this method that the isolation of compounds such as biotin$\sim CO_2$ (Lynen, 1961) serine$\sim PO_4$ and biotin$\sim PO_4$ (Chapter 5) have been possible. Knowing the existence of these intermediates a more accurate prediction of the enzyme catalysed reaction can be made (Fig. 5.13). Table 6.1 shows the carboxylating biotin enzymes and the reactions they catalyse. It can be seen that the common link between all these enzymes is that they all catalyse identical ATP hydrolysis reactions. Thus the possibility of phosphate activation of the biotin cofactor could also occur in all of these

TABLE 6.1

Reaction catalysed by the biotin enzymes

$$E^{bio} + ATP + HCO_3 \rightleftharpoons E^{bio\sim CO_2} + ADP + P_i$$

$$E^{bio\sim CO_2} + \text{acceptor} \rightleftharpoons \text{carboxylated acceptor} + E^{bio}$$

Enzyme	Acceptor
Pyruvate carboxylase	CH_3COCO_2H
Acetyl CoA carboxylase	$CH_3COSCoA$
β-Methylcrotonyl CoA carboxylase	$CH_3-\overset{CH_3}{\overset{\vert}{C}} = CH-CO-SCoA$
Propionyl CoA carboxylase	$CH_3CH_2COSCoA$
Geranyl CoA carboxylase	$CH_3-\overset{CH_3}{\overset{\vert}{C}} = CH(CH_2)_2\overset{CH_3}{\overset{\vert}{C}} = CHCOSCoA$

enzymes. All have been shown to catalyse ATP/P_i and ATP/ADP exchange reactions (Moss and Lane, 1971). E. coli acetyl CoA carboxylase and sheep kidney pyruvate carboxylase have been shown to catalyse specifically ATP production from carbamyl-phosphate and ADP. Thus the isolation of phosphate intermediates from sheep liver pyruvate carboxylase could lead to the realization that this is a general property of all carboxylating enzymes. In agreement with this postulate is the observation that the sequence around the biotin binding site has been strictly conserved (Rylatt et al., 1977) and thus the mechanism of action of biotin could also have been conserved.

Although the preceding discussion has emphasized the advantages of protein chemistry, nevertheless, the kinetics of the catalysed reaction yield information concerning the basic events in the overall reaction pathway. Several important facts can be determined, viz.,

(1) the order of binding of substrates and release of products,

(2) reaction sequence, which could occur via a sequential or ping-pong pathway,

(3) whether or not the mechanism is concerted.

Kinetic studies do not elucidate any molecular chemistry of the enzyme catalysed reaction but they do shed light on the nature of the participants of the reaction. The overall concept of Chapter 3 was based strongly on the initial kinetic observations that the

k_a for acetyl CoA was reduced 3-fold in the presence of $MgCl_2$ and that the Hill coefficient steadily decreased towards unity as the free Mg^{2+} concentration was raised. Again, in Chapter 5, the postulated mechanism could not be as confidently presented if the kinetic studies of Goss and Hudson (unpublished results) were not taken into account although the isolation of the phosphate intermediates did provide the foundations needed for this hypothesis.

In conclusion, the work presented in this thesis was an attempt to clarify certain aspects of the pyruvate carboxylation reaction. A variety of techniques were employed with the aim of arriving at conclusions from as many avenues as possible, and thus, hopefully, increasing the probability that the conclusions are correct. Pyruvate carboxylase has been investigated for nearly two decades and considerable information has now been obtained. This basic groundwork can now pave the way to more subtle applications of kinetic studies, NMR studies, protein chemistry, etc., such that a better understanding of the action of enzymes which catalyse bicarbonate fixation in metabolic cycles, can be achieved.

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