KINETIC STUDIES ON PROPIONYL-CoA CARBOXYLASE FROM PIG HEART

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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 any other person, except where due reference is made in the text.

JOHN BRIAN EDWARDS.

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SUMMARY.

Propionyl-CoA carboxylase which catalyses the reaction:

$$CH_3.CH_2.CO.SCoA + ATP + HCO_3^+ \overset{Mg^{2-}}{\rightleftharpoons} CH_3.\overset{\cdot}{C}H(CO_2H).CO.SCoA + ADP + Pi$$

was purified from pig heart and a series of experiments were carried out to investigate some of the chemical and kinetic properties of the enzyme.

The function of the essential thiol group in propionyl-CoA carboxylase was investigated using N-ethyl maleimide as the thiol modifying reagent. The inhibition of the enzyme was first order with respect to time and inhibitor concentration. From an analysis of the data it was concluded that only one molecule of N-ethyl maleimide reacted with the enzyme. The rate of inhibition remained constant between pH 7.0 and 8.0 then increased very rapidly with increasing pH. The inflection point at pH 8.2 in the pK_m/pH plots indicated that the thiol group was involved in the formation of the enzyme-propionyl-CoA complex. This was confirmed by a comparison of the kinetic constants of the native and the chemically modified enzyme which showed that the apparent K_m value for propionyl-CoA increased while the values for ATP and HCO_3^- remained constant. By measuring the rate constant of the inactivation process in the presence of varying propionyl-CoA concentrations, it was

concluded that the inhibitor could not react with the enzyme-propionyl-CoA complex. The inhibited amino acid was identified as cysteine by reaction with ^{14}C-N-ethyl maleimide and the subsequent isolation of the radio-active cysteine-inhibitor complex. From this evidence it was concluded that a cysteine residue was involved in binding propionyl-CoA to the active site.

The enzyme is also stimulated in the presence of certain univalent cations. In the presence of K^+ ions the V_{max} values for all substrates increased. In addition, the apparent K_m value for HCO_3^- was decreased although the affinity for the other substrates was unaffected. Kinetic evidence thus suggested that K^+ caused a conformational change on the enzyme, a result that was supported by the calculation of the entropy changes induced in the protein by this univalent cation.

This hypothesis was further substantiated by showing an increase in the rate of inhibition by N-ethyl maleimide in the presence of K^+ ions. This accumulated data provided strong evidence that a conformational change accompanied the reaction of alkali metals with propionyl-CoA carboxylase.

The allosteric activation of propionyl-CoA carboxylase by Mg^{2+} and $MgATP^{2-}$ was also examined. The results suggest

that Mg^{2+} has at least two roles in the reaction mechanism. Firstly, it forms a complex with ATP^{4-} to form the true $MgATP^{2-}$ substrate and secondly, it forms a complex with the enzyme to activate the enzymic reaction. The $MgATP^{2-}$ complex deviates from Michaelis & Menten kinetics in such a way as to indicate a homotropic co-operative effect involving at least two molecules of ligand. Free Mg^{2+} reduces the slope of the Hill plot for $MgATP^{2-}$ thus suggesting either a change in the order of the reaction with respect to $MgATP^{2-}$ or a reduction in the interaction between the $MgATP^{2-}$ sites. It is proposed that one site acts as an effector site and the other, a catalytic site.

Furthermore, ATP^{4-} is a competitive inhibitor with respect to $MgATP^{2-}$ and since Mg^{2+} has been shown to alter the kinetic properties, it could be suggested that this cation is binding at the effector site for $MgATP^{2-}$ and mimics the action of $MgATP^{2-}$ at this site. On the other hand, ATP^{4-} could be binding at the catalytic site.

Two substrate kinetics were also carried out in an endeavour to substantiate previous reaction mechanism proposals. Although the evidence is only preliminary, the data obtained does not conform to the simple reaction scheme proposed by other investigators.

ENZYMES.

For the sake of simplicity, the enzymes have been referred, in the text of this thesis, by their trivial names. A list of the enzymes that have been mentioned follows, together with the numbers by which they are designated in the Report of Commission on Enzymes of the International Union of Biochemistry (1961).

Propionyl-CoA carboxylase	Propionyl-CoA : carbon-dioxide ligase (ADP) 6.4.1.3.
Acetyl-CoA carboxylase	Acetyl-CoA : carbon-dioxide ligase (ADP) 6.4.1.2.
2-methyl crotonyl-CoA carboxylase	2-methyl crotonoyl-CoA : carbon-dioxide ligase (ADP) 6.4.1.4.
Pyruvate carboxylase	Pyruvate : carbon-dioxide ligase (ADP) 6.4.1.1.
Transcarboxylase	Methyl malonyl-CoA : pyruvate carboxyltransferase 2.1.3.1.

ABBREVIATIONS.

The following abbreviations have been used in this thesis:

ATP	:	adenosine 5'-triphosphate
EDTA	:	ethylene diamine tetra-acetate
Tris	:	tris (hydroxy-methyl) aminomethane
CoA and acyl-CoA	:	coenzyme A and its acyl derivatives
V_{max}	:	maximum velocity
Pi	:	orthophosphate
ADP	:	adenosine 5'-diphosphate
u.v.	:	ultra-violet

C H A P T E R O N E

GENERAL INTRODUCTION

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INTRODUCTION:

A "primary CO_2 fixation has been defined by Wood & Stjernholm (1962) as a reaction by which CO_2 is combined with some acceptor molecule to form a new carbon-carbon bond which results in a net fixation of CO_2". In the last twenty years many primary CO_2 assimilation reactions have been described. These reactions have been classified by various investigators, but in this review, the scheme of Ochoa & Kaziro (1965) will be used.

(a) Carbon-carbon bond forming carbon dioxide ligases.

(1) Propionyl-CoA carboxylase

(2) Acetyl-CoA carboxylase

(3) Methyl-crotonyl-CoA carboxylase

(4) Pyruvic carboxylase

The enzymes in group (a) catalyse an ATP dependent carbon dioxide fixation, and contain biotin as a prosthetic group.

(b) Carboxyl transferases.

(1) Methyl-malonyl-CoA : pyruvate carboxyl transferase

The transcarboxylating enzyme of group (b) is responsible for the transfer of a carboxyl group from methyl malonyl-CoA to pyruvate to form propionyl-CoA and oxaloacetate. This enzyme also contains biotin.

(c) Enoyl carboxy-lyases.

(1) Phospho-enol pyruvate carboxylases

(a) Phospho-pyruvate carboxylase (phosphorylating)

(b) Phospho-pyruvate carboxylase (transphosphorylating)

(c) Phospho-pyruvate carboxylase (transphosphorylating)

(d) Phospho-pyruvate carboxylase (transphosphorylating)

(2) Ribulose diphosphate carboxylase

(3) Phospho-ribosyl-amino-imidazole carboxylase

The carboxylases of group (c) do not require an external source of energy, because the substrate is already in an "active" form. Phospho-enol pyruvate is carboxylated with the concomitant release of a phosphate group. This group can be transferred to GDP or IDP (b), ADP (c), inorganic phosphate (d) or water (a). Ribulose diphosphate is carboxylated to yield two molecules of 3-phospho-glycerate (2). A carboxyl group is bound to 4-amino-imidazole-ribotide at position 5 of the imidazole ring to yield 4-amino-5 carboxy-imidazole-ribotide, which is an intermediate in the biosynthesis of purine nucleotides (3).

(d) Reductive carboxylases.

(1) Malate dehydrogenase (decarboxylating)

(2) Isocitrate dehydrogenase

(3) Phospho-gluconate dehydrogenase (decarboxylating)

The carboxylases of group (d) utilise reduced nicotinamide-adenine dinucleotide as the source of energy.

This present thesis is concerned with the enzymes of group (a) and (b) i.e. the biotin enzymes. Recently, several reviews, Ochoa & Kaziro (1965), Mistry & Dakshinamurti (1964) and Kaziro & Ochoa (1964), have been written about this particular group of enzymes; however, this introduction will consider only certain aspects of the literature covering this group, viz.

(a) Chemistry of the active centre including the role of biotin in the reaction sequence

(b) The reaction mechanism including the roles of various metal ions, and

(c) Some aspects of the quaternary structure of these enzymes.

THE BIOTIN GROUP OF ENZYMES:

(1) Acetyl-CoA Carboxylase

In 1950, Brady & Gurin (1950) observed that fatty acids could be synthesized in a bicarbonate buffer but not in a phosphate buffer, a requirement that was later confirmed by Gibson, Titchener & Wakil (1958), (1958a) and Wakil, Titchener & Gibson (1958). A purified fraction from chicken liver was incubated with acetyl-CoA, ATP and bicarbonate to form an intermediate that was then converted to palmitic acid in the presence of TPNH and a second liver fraction (Wakil, 1958). The intermediate was later identified as malonyl-CoA.

$$CH_3.CO.SCoA + ATP + HCO_3^- \overset{Mn^{2+}}{\rightleftharpoons} \overset{CO_2H}{\underset{\bullet}{CH_2}}.CO.SCoA + ADP + Pi \quad ...(1.1)$$

The purified enzyme from chicken liver contains 1 gram molecule of biotin per 350,000g. of protein and is inhibited by avidin (Waite & Wakil, 1962). The enzyme is not absolutely specific for acetyl-CoA since it will carboxylate both propionyl-CoA and butyryl-CoA to some extent. Furthermore, the enzyme requires a divalent cation, Mn^{2+}, although partial substitution by Ni^{2+}, Co^{2+} or Mg^{2+} has been achieved.

A highly purified acetyl-CoA carboxylase from chicken liver was prepared by Gregolin, Ryder, Kleinschmidt, Warner & Lane (1966) and had a similar biotin content but the molecular weight on isolation was 7.8×10^6. This was in contrast to previous preparations from rat liver (Matsuhashi, Matsuhashi & Lynen, 1964) and adipose tissue (Martin & Vagelos, 1962) and furthermore, this preparation was not cold labile whereas the rat liver enzyme showed this characteristic.

The stimulation of fatty acid synthesis by citric acid and other carboxylic acids in cell free extracts was observed by Brady & Gurin (1952) and later confirmed by Porter, Wakil, Tietz, Jacob & Gibson (1957) and Wakil, Porter & Gibson (1957). It was thought that the generation of TPNH and CO_2 from the oxidation of isocitrate was responsible for the increased fatty acid synthesis. However, isocitrate could not

be replaced by either carbon dioxide and/or other TPNH regenerating systems. This aspect of citrate activation of fatty acid synthesis will be reviewed later in the introduction.

(2) Propionyl-CoA Carboxylase

The initial discovery that propionic acid was metabolised in animal tissues via the formation of a dicarboxylic acid led to the findings of Lardy & Peanasky (1953), that rat liver mitochondria could catalyse an ATP dependent fixation of CO_2 by propionate to form succinate. Subsequently Flavin & Ochoa (1957) showed that propionate was first converted to propionyl-CoA which was then carboxylated to yield methyl malonyl-CoA.

This enzyme has been obtained in a high state of purity from pig heart (Kaziro, Ochoa, Warner & Chen, 1961) and ox liver (Halenz, Feng, Hegre & Lane, 1962) and has been shown to contain d-biotin as the active prosthetic group. This carboxylase catalyses a reaction similar to acetyl-CoA carboxylase in that an acceptor, propionyl-CoA, is carboxylated to yield methyl malonyl-CoA, with the simultaneous hydrolysis of ATP yielding ADP and Pi.

$$CH_3.CH_2.CO.SCoA + ATP + HCO_3^- \xrightleftharpoons{Mg^{2+}} CH_3.\overset{\overset{CO_2H}{|}}{CH}.CO.SCoA + ADP + Pi \quad \ldots (1.2)$$

The methyl malonyl-CoA can be further racemized to its

optical enantiomorph (Mazumder, Sasakawa, Kaziro & Ochoa, 1961) and this in turn can be isomerized to succinyl-CoA by the enzyme methyl malonyl-CoA mutase (Stjernholm & Wood, 1961) (Mazumder, Sasakawa & Ochoa, 1963).

Propionyl-CoA carboxylase is also active in carboxylating butyryl-CoA and acetyl-CoA, although to a lesser extent (Kaziro et al., 1961) (Halenz et al., 1962).

The similarities in sedimentation coefficients, biotin content and substrate specificities of the propionyl-CoA carboxylases isolated from liver and heart indicate that the two enzymes are very similar. The biotin content of the crystalline enzyme obtained from pig heart is one gram molecule per 175,000gm. of protein or 4 molecules of biotin per mole of enzyme (Kaziro, Leone & Ochoa, 1960).

In experiments with propionyl-CoA carboxylase, Retey & Lynen (1965) and Arigoni, Lynen & Retey (1966) have shown that the transcarboxylation occurs with strict stereospecificity. In the carboxylation process that leads to the formation of the methyl-malonyl-CoA, the steric configuration is retained, indicating that the entering carboxyl group occupies the place that the hydrogen atom has vacated.

(3) 2-Methyl-crotonyl-CoA Carboxylase

In the metabolism of leucine the isopropyl portion of isovaleryl-CoA is converted to acetoacetate by a stoichiometric fixation of carbon dioxide (Coon, 1950). Plaut & Lardy (1950) observed that biotin deficient livers incorporated less radioactive bicarbonate into acetoacetate than the normal preparations. Furthermore, Lardy et al. (1953) and Fischer (1955) demonstrated that mitochondria from biotin deficient rat livers failed to oxidize isovalerate and some of its unsaturated analogues, whereas these compounds were readily converted to acetoacetate by normal mitochondria. These studies tended to involve biotin in the carboxylation reaction preceding the cleavage of 2-hydroxy-2-methyl-glutaryl-CoA to acetoacetate (Woessner, Bachhawat & Coon, 1958).

However, it was due to the work of Lynen and his associates that conclusive proof for the ATP dependent carboxylation of 2-methyl-crotonyl-CoA to 2-methyl-glutaconyl-CoA catalysed by a biotin containing carboxylase was finally obtained (Lynen, Knappe, Lorch, Jutting, Ringelmann & LaChance, 1961), (Knappe, Schiegel and Lynen, 1961).

The enzyme was purified from Mycobacterium and shown to catalyse the following reaction:

$$CH_3.\overset{\cdot}{C}(CH_3):CH.CO.SCoA + ATP + HCO_3^- \underset{\rightarrow}{\overset{Mg^{2+}}{\rightleftharpoons}} CH_3.\overset{\cdot}{C}(CH_2.COOH):CH.CO.SCoA + ADP + Pi \quad \ldots (1.3)$$

Himes, Young, Ringelmann & Lynen (1963) purified the enzyme from Achromobacter and demonstrated that the enzyme contains one gram molecule of biotin per 184,000g. of protein. The product of the reaction is the trans-isomer of 2-methyl-2-glutaconyl-CoA.

(4) Pyruvate Carboxylase

Gluconeogenesis, which is the conversion of certain amino acids or latic acid into glucose, was originally assumed to occur via pyruvic acid and from thereon by the direct reversal of glycolysis. Doubt was thrown on this concept when it was realised and especially emphasized by Krebs (1954) that three steps in the glycolytic sequence of reactions are not readily reversible. The most complicated of these steps is the sequence of reactions by which phospho-enol pyruvate is formed from pyruvate.

Two enzymes that were discovered by Utter & Kurahashi (1954) and Utter, Keech and Scrutton (1964), were thought to catalyse reactions which collectively could bypass this unfavourable pathway. One of these is the biotin-containing pyruvate carboxylase which synthesises oxaloacetate from pyruvate and bicarbonate, and requires ATP + Mg^{2+}. Hence, this enzyme is similar to the group of biotin-containing carboxylases which have been shown to

carboxylate acyl-CoA esters in the presence of Mg^{2+} and ATP. In this instance, a keto acid, viz. pyruvate, is carboxylated to yield oxaloacetate. However, a CoA ester is again necessary since the mammalian enzyme exhibits an absolute requirement for acetyl-CoA.

$$CH_3.CO.CO_2H + ATP + HCO_3^- \xrightarrow[\text{Acetyl-CoA}]{Mg^{2+}} \overset{CO_2H}{\underset{}{\overset{|}{CH_2}}}.CO.COOH \qquad \ldots (1.4)$$

Since this enzyme is atypical with respect to other biotin-containing enzymes in terms of the CO_2-acceptor, it was thought that the acetyl-CoA was functioning in two ways, i.e.

(a) To form malonyl-CoA and this in turn would trans-carboxylate the carboxyl group to pyruvate

(b) To form pyruvyl-CoA which is the true acceptor molecule.

However, investigations with isotopically labelled acetyl-CoA have ruled out these possibilities (Utter, Keech & Scrutton, 1964). The action of acetyl-CoA is now thought to involve conformational changes in protein structure; indeed the recent work of Barritt, Keech & Ling (1966) has demonstrated that at least two acetyl-CoA molecules are required to activate pyruvate carboxylase effectively.

Examination of the initial reaction rate as a function of effector concentration revealed a sigmoid relationship suggesting that more than one molecule of effector per active site is required for the activation process and that co-operative inter-

actions between the bound effector molecules have occurred. Further evidence for the non-participation of acetyl-CoA as a substrate in the carboxylation reaction was forthcoming when it was observed that the pyruvate carboxylase from Pseudomonas citronellolis did not require acetyl-CoA for activity (Seubert & Remberger, 1961).

Yeast pyruvate carboxylase differs from the mammalian enzyme in that it does not require acetyl-CoA for enzymic activity (Ruiz-Amil, de Torrontegui, Palacian, Catalina & Losada, 1965). Therefore, the enzyme could be classified along with the bacterial group of pyruvate carboxylases isolated from Aspergillus niger (Bloom & Johnson, 1962) and Pseudomonas citronellolis which do not require acetyl-CoA for activity. The purified enzyme from Pseudomonas citronellolis contains one gram molecule of biotin per 400,000g. of protein (Seubert & Remberger, 1961).

(5) Methyl Malonyl-CoA : Pyruvate Carboxyl Transferase:

This enzyme catalysed a direct carboxylation from a carboxyl donor such as methyl-malonyl-CoA to an acceptor such as pyruvate, without the intervention of carbon dioxide or the expenditure of energy to activate the system. The enzyme named methyl malonyl-CoA : pyruvate carboxyl transferase (Transcarboxylase) (Stjernholm & Wood, 1961) catalysed the following reaction:

$$CH_3.\overset{\bullet}{C}H(CO_2H).CO.SCoA + CH_3.CO.CO_2H \rightleftharpoons CH_3.\overset{\bullet}{C}H_2.CO.SCoA + \overset{\bullet}{C}H_2(CO_2H).CO.CO_2H \quad \ldots (1.5)$$

The oxaloacetate so formed was reduced to succinate, which in turn was esterified by the transfer of CoA from propionyl-CoA. Methyl malonyl-CoA was regenerated from succinyl-CoA by a vitamin B_{12} co-enzyme dependent isomerization (Swick & Wood, 1960). Thus the cycle was completed and pyruvate was reduced to propionate without the expenditure of energy.

The role of biotin in the transcarboxylase reaction (Swick & Wood, 1960) was confirmed by Stadtman, Overath, Eggerer & Lynen (1960). Radioactive transcarboxylase was prepared by growing propionic acid bacteria in a medium containing tritiated biotin (Wood, Allen, Stjernholm & Jacobson, 1963). The biotin content, 1 gram molecule of biotin per 163,000g. of protein, was similar to the biotin content of crystalline pig heart propionyl-CoA carboxylase (Kaziro et al., 1961). With oxaloacetic acid as the carboxyl donor, the transcarboxylase displayed a broad specificity towards acetyl-CoA, propionyl-CoA, butyryl-CoA and aceto-acetyl-CoA. On the other hand, with pyruvate as the carboxyl acceptor, only malonyl-CoA and methyl malonyl-CoA could act as donors (Stjernholm & Wood, 1961) (Wood et al., 1963).

The group of enzymes summarized in the preceding section all possess biotin as the prosthetic group. The similarities in

the biotin content and molecular weights would indicate that these enzymes have comparable structures. Moreover, the common involvement of CoA esters and in most cases ATP and HCO_3^- could indicate that the active sites of these groups are very similar in nature. The pertinent literature concerning the structures and active sites of these enzymes will be presented in the following pages.

ACTIVE CENTRES OF THE BIOTIN ENZYMES:

The active centre of an enzyme may be defined as consisting of those functional arrangements of peptide linkages, hydrophobic and hydrophilic regions which belong to the enzyme proteins and which are directly involved in the construction of the transition state (or states) for the chemical transformation catalysed by the enzyme. An active site may be small and simple (perhaps a single group) or it may extend over a large region of the enzyme and itself contain many interacting groups and multiple binding sites. Although contemporary work in various laboratories is concerned with the investigation of the chemistry of the active sites of various enzymes, so far only preliminary information has been obtained about the active centres of biotin-containing enzymes. This work has been centred on the role of biotin in the reaction mechanism although other chemical information is now becoming available.

This survey will be mainly concerned with:

(a) The role of biotin and the formation of the enzyme - CO_2 complex

(b) The nature of the amino acids at the active centres.

(1) Role of biotin

In 1959, Lynen *et al.* (1959) demonstrated that free d-biotin could be substituted for 2-methyl crotonyl-CoA as the CO_2 acceptor molecule in the reaction catalysed by 2-methyl

crotonyl-CoA carboxylase.

Although the biotin-CO_2 complex is extremely unstable, it can be stabilised by esterification of the carboxyl group with diazo-methane. The product was shown to be identical with chemically synthesized 1'-N-carbomethoxy-biotin methyl ester which was prepared from biotin and chloro-methyl formate. The chemical synthesis resulted in the formation of two products,

(a) 1'-N-carbomethoxy biotin methyl ester, and

(b) 3'-N-carbomethoxy biotin methyl ester (Lynen et al., 1961).

The major product was the 1'-N-carbomethoxy biotin derivative. This was assumed to be due to the fact that the valeric acid side chain sterically hindered addition to the 3'-N position. On this basis, it was concluded that the enzymically synthesised biotin-CO_2 complex was similarly orientated (Knappe, Ringelmann & Lynen, 1961).

(2) Carboxy-Biotin Enzyme Complex

In their studies with propionyl-CoA carboxylase, Kaziro & Ochoa (1961), and Ochoa & Kaziro (1961), provided evidence for the formation of a CO_2-enzyme complex. Upon incubation of the enzyme with the necessary substrates, viz. ATP, Mg^{2+} and ^{14}C-CO_2 (but no acceptor molecule), the ^{14}C-CO_2-enzyme complex was isolated by subsequent chromatography on a Dowex-1 column. The ^{14}C-CO_2 from the isolated ^{14}C-CO_2-enzyme complex

could be transferred to propionyl-CoA to form methyl-malonyl-CoA. Although the enzyme-CO_2 complex can be formed from Mg^{2+}, ATP and HCO_3^-, it can also be synthesised from methyl malonyl-CoA (Kaziro & Ochoa, 1961). In reality, the formation of the CO_2-enzyme complex from methyl malonyl-CoA is more specific for propionyl-CoA carboxylase, since any biotin enzyme can form a CO_2-enzyme complex in the presence of ATP^{4-}, Mg^{2+} and HCO_3^-.

The structure of the carboxy-biotin enzyme complex isolated from 2-methyl crotonyl-CoA carboxylase (Knappe, Biederbick & Wrummer, 1962) has been shown to be identical with that of enzymically synthesized carboxy-biotin. After treating the carboxy-biotin-enzyme complex with diazomethane and degrading with trypsin and biotinidase, a compound, 1'-N-carbomethoxy biocytin was isolated.

Numa, Ringelmann & Lynen (1964) were able to isolate a ^{14}C-CO_2 biotin-acetyl-CoA carboxylase with preparations from rat liver after incubation of the enzyme with 3-^{14}C-malonyl-CoA and subsequent gel filtration through Sephadex. They found this complex to be acid labile and to yield on methylation and treatment with Pronase, the ^{14}C-1'-N-carbomethoxy biocytin. This compound upon digestion by biotinidase was found to yield the 1'-N-^{14}C-carbomethoxy biotin. These observations led them to conclude that acetyl-CoA carboxylase from rat liver behaves in a manner similar to the other enzymes described so far.

Waite & Wakil (1962, 1963) published observations which differed from the results of Lynen concerning the identity of the ^{14}C-CO_2-acetyl-CoA carboxylase complex. Their complex isolated by gel filtration was incubated with barium hydroxide which removes the ureido carbon of biotin. Most of the radio activity was recovered as barium carbonate; a degradation product, 3,4-diamino-2-tetra-hydro-thiophene valeric acid, was also isolated. From this evidence Waite & Wakil postulated that the ureido carboxyl group was functional in the carboxylation reactions, a scheme quite different from the proposal put forward by Lynen.

Allen, Stjernholm & Wood (1963) investigated the role of the ureido carbon of biotin in the transcarboxylase reaction by growing Propionibacterium in a medium containing ^{14}C-biotin (labelled in the ureido position). After isolation of the labelled enzyme from the cells, and by using it to catalyse the transcarboxylase reaction, they were unable to demonstrate either;

(a) The transfer of ^{14}C to the oxaloacetate, or

(b) Loss of the ^{14}C label from the enzyme.

Thus they also concluded that in the case of the transcarboxylase, the biotin does not function through the transfer of the ureido carbon.

Since their original observations, Waite & Wakil (1966)

have re-examined the basis of their hypothesis and now agree that the carboxy-biotin complex involves the carboxylation of the imino groups of biotin and does not include the reversible carboxylation of the diamino derivative of biotin.

Lynen (1967) has described the reactivity of the carboxy-biotin as being due to the weakly acidic nature of the biotin, which when carboxylated behaves much like an acid anhydride. The bond between the carbon dioxide and the nitrogen atom becomes polarized, which strengthens the electrophilic nature of the carboxyl group and, therefore, the ability to enter transcarboxylation reactions (Knappe & Lynen, 1963). The reactivity of the biotin-bound carbonic acid may be expressed in thermodynamic terms. Wood, Lochmuller, Riepertinger & Lynen (1963) determined the free energy of cleavage of the carboxy-biotin enzyme according to the equation:

$$H^+ + \text{Enzyme-Biotin-}CO_2 \rightleftharpoons \text{Enzyme-Biotin} + CO_2 \quad \ldots (1.6)$$

The observed value $\Delta F = -4.7$kcal. per mole at pH 7.0, is sufficient to allow the compound to act as a carboxylating agent with suitable acceptor molecules. The exergonic nature of the cleavage of the carboxy-biotin enzyme also explains the ATP requirement for its formation from bicarbonate and biotin-enzyme.

(3) Substrate sites

The table of properties of the biotin group of enzymes listed by Ochoa & Kaziro (1965), indicated that several members of the group are susceptible to inactivation by thiol group reagents.

A variety of thiol inhibitors has been used to inactivate propionyl-CoA carboxylase from pig heart (Kaziro et al., 1961) and ox liver (Halenz et al., 1962), and the protection of the enzyme against these inhibitors by propionyl-CoA would seem to link this substrate with the thiol group. Hegre (1964) working with the liver enzyme has also demonstrated that propionyl-pantetheine can also partially protect against thiol group reagents. Furthermore, CoA and 3'AMP were found to be competitive inhibitors of propionyl-CoA. This indicated that a multipoint attachment of this substrate is needed for binding and that the propionyl-pantetheine moiety of the substrate could be bound to a cysteine residue.

With regard to the other biotin-containing enzyme, inhibition studies with 2-methyl crotonyl-CoA carboxylase (Himes et al., 1963), and acetyl-CoA carboxylase (Waite et al., 1962), have also indicated the presence of an essential thiol group. Pyruvate carboxylase from chicken liver (Keech & Utter, 1963) has been reported to be susceptible to thiol group reagents, yet the experimental conditions under which these

reagents reacted may have given an ambiguous result. The auxiliary enzyme used in the assay system, viz. malate dehydrogenase, a thiol containing enzyme, may have been inhibited in the process. Bacterial (Seubert et al., 1961) and sheep kidney pyruvate carboxylases (Ling & Keech, 1966) are not inhibited appreciably by thiol group reagents. Wood et al. (1961) have shown that the transcarboxylase can be inhibited slowly by thiol reagents.

Although some biotin enzymes are inhibited by thiol group reagents, especially those requiring acyl-CoA esters as CO_2-acceptor molecules, those using keto acids do not appear to be susceptible to these reagents. Thus, it could be visualised that the binding of the acyl-CoA ester requires the presence of a free thiol group at the active site. The binding of the keto acid acceptor molecules may be achieved through the presence of a divalent cation, which was recently shown to be present in chicken liver pyruvate carboxylase (Scrutton, Utter & Mildvan, 1966). The metal was shown to be Mn^{2+} by chemical, neutron activation, emission spectral and atomic absorption spectral analyses. Mildvan, Scrutton & Utter (1966) have provided evidence that the cation may be involved in the binding of the keto-acid pyruvate to the enzyme because of the change in proton relaxation rate when the enzyme is perturbed with pyruvate. No other reaction component had this effect. Furthermore, manganese showed

an enhanced NMR signal when bound to the enzyme as compared to that of the free metal and denatured enzyme. Studies on the effect of added substrates and inhibitors show that the carboxylation of pyruvate by the enzyme-CO_2 complex causes a reduction in the proton relaxation rate. Rate of inhibition and analysis of kinetic constants obtained by enhancement values have agreed well with initial rate studies and inhibition studies obtained by the normal assay procedure. This data strongly supports the idea that bound manganese plays a functional role in the transcarboxylation portion of the pyruvate carboxylase reaction i.e. the transfer of a carboxyl group from the enzyme-CO_2 complex to pyruvate.

These results, together with Caplow's studies (1965), suggested that bound metal ions may be present in other biotin enzymes. For example, the calf liver pyruvate carboxylase also contains Mn, whereas the yeast enzyme does not appear to contain Mn but Co and Zn (Utter, 1967). Furthermore, the transcarboxylase was subjected to the proton relaxation rate method described above. While a small effect was observed, it appeared that although Mn was not involved, the presence of other metal ions could not be excluded. Indirect evidence from inhibitor studies affords some support for this postulate, since yeast pyruvate carboxylase (Losada et al., 1964), rat liver pyruvate carboxylase (Seubert & Huth, 1965) and methyl malonyl-CoA transcarboxylase from Propionibacterium shermanii (Scrutton & Utter, 1965) are inhibited

by oxalate.

(4) Activator Sites

Keech & Utter (1963) showed that acetyl-CoA was required in catalytic quantities for pyruvate carboxylase activity. Recently, Barritt et al., (1966) demonstrated that acetyl-CoA was an effector of the enzyme and consequently the manner by which this acyl-CoA ester is bound to the effector site is of major importance in terms of allosteric activation.

In avian liver pyruvate carboxylase, acetyl-CoA can be replaced by propionyl-, crotonyl-and formyl-CoA (Keech & Utter, 1963; Utter, Keech & Scrutton, 1964) but not by butyryl-, methyl malonyl-and 2-hydroxy 2-methyl-glutaryl-, aceto-acetyl-CoA or by acetyl-glutathione, acetyl pantetheine and acetyl glutamate (Keech & Utter, 1963). Similar results were obtained for sheep kidney pyruvate carboxylase (Ling & Keech, 1966) and so the requirement for an acyl-CoA is highly specific. No reaction occurred in the presence of both reduced CoA and sodium acetate, indicating that the stereochemical shape of the acetyl-CoA molecule is critical.

The chemical nature of the essential amino-acids involved in the activator sites of pyruvate carboxylase is the subject of investigation in Keech's laboratory.

Acetyl pantetheine is a competitive inhibitor of acetyl-

CoA whilst both alanine and thio-ethandamine can cause marked inhibition (Barritt, 1966). It would seem that for pyruvate carboxylase, as is the case with propionyl-CoA carboxylase, the pantetheine portion of the acyl-CoA molecule is involved in binding to the enzyme. Investigations into the activator site on the kidney enzyme by Nielsen & Keech (1967) have revealed a tryptophan residue, which is necessary for binding acetyl-CoA to the molecule. By the use of specific tryptophan inhibitors, N-bromosuccinimide and N-1,4-pyridyl-pyridinium chloride, enzyme activity is decreased. Examination of the kinetics of the reversibly inhibited enzyme revealed that the K_m for acetyl-CoA is altered, thus reflecting that the binding of acetyl-CoA may be affected.

Other investigations have revealed that sheep kidney pyruvate carboxylase possesses a lysine residue whose integrity is essential for enzymic activity (Keech & Farrant, 1967). Modification of the enzyme with amino group reagents such as 1-fluoro 2,4-dinitro-benzene, trinitro-benzene sulphonic acid etc. resulted in loss of activity. The ability of the allosteric effector, acetyl-CoA, to protect the enzyme against this inhibition suggested that the E-amino group of lysine may be involved in the enzyme-acetyl-CoA interaction.

This brief review of the active centres of the biotin enzyme has enabled one perhaps to generalise on their respective

reactive groups.

(a) A thiol group is required by those biotin-containing enzymes using an acyl-ester as the CO_2-acceptor. This statement has only been suspected by inhibition studies, yet the partial protection afforded by propionyl-CoA in the propionyl-CoA carboxylase reaction and the lack of inhibition shown by those enzymes using a keto acid as an acceptor molecule, suggests that this inhibited group may be important in the reaction mechanism.

(b) A metal ion, manganese, has been found in avian liver pyruvate carboxylase and, although all pyruvate carboxylases do not contain this particular metal, preliminary investigations suggest that other metal ions may be part of the enzyme molecules. This metal could function at or near the pyruvate binding site, but the evidence accumulated so far requires some further confirmation.

(c) An interesting problem, as regards active site investigations, has arisen concerning the activator sites of pyruvate carboxylase. The identification of a lysine residue at the effector site and the tentative demonstration of a tryptophanyl group could mean that the knowledge of operational function of this site may soon be forthcoming. On the other hand, it should be emphasised that no knowledge is yet available on whether the two proposed sites are identical as regards amino acid makeup or have individual characteristics brought about by a dissimilar amino acid sequence.

REACTION MECHANISM:

The primary CO_2-fixations catalysed by the biotin-containing enzymes are complex reactions involving three substrates and three products.

Methods of obtaining relevant information concerning reaction pathways include the analysis of initial velocity studies and product inhibition patterns obtained from steady state kinetic data. In addition, information can be obtained by means of isotopic exchange studies. For example Kaziro et al. (1962) showed that propionyl-CoA carboxylase catalysed an ATP-ADP exchange reaction which required the presence of Pi. In similar experiments, ADP was found to be essential for the ATP-Pi exchange reaction. A special requirement for the ATP-Pi exchange was the presence of bicarbonate ions. In further experiments, an exchange between methyl-malonyl-CoA and propionyl-CoA was also demonstrated. It should be noted that the specificity of the avidin inhibition tends to rule out any non-specific exchanges conducted by contaminating enzymes.

On these bases, the partial reaction sequence for propionyl-CoA carboxylase was assumed to be:

$$ATP + HCO_3^- + E \rightleftharpoons E\text{-}CO_2 + ADP + Pi \quad \ldots (1.7)$$

$$E\text{-}CO_2 + \text{propionyl-CoA} \rightleftharpoons \text{methyl malonyl-CoA} + E \quad \ldots (1.8)$$

The presence of magnesium ions was shown to be necessary for the first partial reaction (1.7) although not required for the second step (1.8). Furthermore, the formation of the E-CO_2 complex is inhibited by the presence of either p-hydroxy-mercuri-benzoate or avidin.

Lynen et al. (1961) using 2-methyl crotonyl-CoA carboxylase and Seubert et al. (1961) using pyruvate carboxylase isolated from Pseudomonas citronellolis presented evidence supporting the first partial reaction. Since neither of these exchange reactions i.e. the ADP-ATP exchange and the ATP-Pi exchange, could function without the presence of added intermediates, the possibility of an intermediate such as enzyme-Pi or enzyme-ADP seemed unlikely. However, Scrutton & Utter (1965) using avian pyruvate carboxylase could not demonstrate an absolute requirement for Pi in the ADP-ATP exchange reaction. The formation of a phosphorylated-biotin intermediate has been raised and although the majority of evidence would not favour its existence, this possibility cannot be discarded (Lynen, 1967). Moreover, the ATP-Pi exchange reaction of propionyl-CoA carboxylase only proceeds at 0.2% the rate of the overall forward reaction and since the CoA-ester exchange rate is quite rapid, it is apparent that those exchanges concerned in the first partial reaction are not working at an expected rate (Kaziro et al., 1962).

The occurrence of the second step in the reaction sequence

i.e. the transcarboxylation, was demonstrated by Lynen et al. (1959). The enzyme, 2-methyl-crotonyl-CoA carboxylase, was incubated with ^{14}C-2-methyl-glutaconyl-CoA and non-labelled 2-methyl-crotonyl-CoA. The latter compound, i.e. 2-methyl-crotonyl-CoA, was then isolated and shown to be labelled. There was no requirement for either Mg^{2+} or Pi in this exchange reaction. Similar exchanges between propionyl-CoA and methyl malonyl-CoA were catalysed by propionyl-CoA carboxylase (Halenz et al., 1962) (Halenz & Lane, 1961) (Friedman & Stern, 1961) (Kaziro & Ochoa, 1961) (Kaziro, Hass, Boyer & Ochoa, 1962). The isotope exchange between ^{14}C-pyruvate and oxaloacetate has been demonstrated in both bacterial and avian pyruvate carboxylases (Seubert & Remberger, 1961) (Scrutton & Utter, 1965). Once again these exchanges are inhibited by avidin, p-hydroxy-mercuri-benzoate, but not by EDTA, indicating that the divalent cation is not required for the transcarboxylation step.

Methyl malonyl-CoA : pyruvate carboxyl-transferase catalyses two partial reactions, one between the CoA-esters methyl malonyl-CoA and propionyl-CoA and the other, between the respective keto acids; pyruvate and oxaloacetate (Wood & Stjernholm, 1966). As expected, both these exchanges proceed in the absence of additional substrates.

Additional information supporting the proposed reaction

mechanism was obtained by Kaziro *et al*. (1962) when they investigated ^{18}O-exchange reactions. Pig heart propionyl-CoA carboxylase was assayed in the presence of either ^{18}O-H_2O or ^{18}O-$NaHCO_3$. It was observed that the oxygen incorporated during the cleavage of ATP is derived from the HCO_3^- ion, one bicarbonate oxygen atom appearing in the liberated Pi and two atoms in the free carboxyl group of methyl malonyl-CoA. Thus the bicarbonate oxygen is incorporated into the liberated Pi during ATP cleavage. These results suggest that the formation of the CO_2-enzyme complex may proceed by a "concerted" mechanism. Kaziro *et al*. (1962) also suggested from this experiment that bicarbonate rather than free carbon dioxide is the active species in the carboxylase reaction.

Additional factors which have to be taken into consideration in considering the reaction mechanism of propionyl-CoA carboxylase are the roles of the various metal ions. The reports of Neujahr & Mistry (1962) and Neujahr & Mistry (1963) that the activity of rat liver mitochondrial propionyl-CoA carboxylase could be stimulated by the *in vitro* addition of a supernatant fraction from liver which had little or no enzymic activity *per se*, eventually resulted in the identification of K^+ as the stimulating factor. Although not as marked, the activation of crystalline pig heart propionyl-CoA carboxylase by potassium and other univalent cations was also observed (Neujahr & Mistry, 1963b and 1963).

More recently (Giorgio & Plaut, 1967) demonstrated a similar stimulation of purified bovine liver propionyl-CoA carboxylase which suggested that the activity of propionyl-CoA carboxylases from several sources can be affected by the presence of univalent cations.

In addition to the stimulation of enzymic activity by univalent cations, a divalent cation, Mg^{2+} has also been essential for activity. All the biotin enzymes, except the methyl malonyl-CoA : pyruvate carboxyl transferase, require the presence of a divalent cation, in most cases, Mg^{2+}, although Mn^{2+} can occasionally be substituted. Recently Keech & Barritt (1967) have investigated the role of Mg^{2+} in the pyruvate carboxylase reaction and have postulated some interesting theories. For example, they suggest that Mg^{2+} has two separate functions; firstly, to complex with ATP^{4-} and thus form the true substrate, $MgATP^{2-}$, and secondly, to activate the enzyme as the free metal. Furthermore, the addition of the $MgATP^{2-}$ complex to the enzyme does not follow classical Michaelis-Menten kinetics but exhibits a homotropic co-operative effect. That is, plots of velocity as a function of $MgATP^{2-}$ concentration result in sigmoidal curves. When free Mg^{2+} is present, normal hyperbolic curves are obtained, which would indicate that no co-operativity now exists between the $MgATP^{2-}$ sites. Moreover, Mg^{2+} causes a decrease in the $\bar{n}$ value obtained from the Hill equation which has been used to indicate either the

number of sites or the strength of the interaction between these sites. These results suggest that Mg^{2+} can activate pyruvate carboxylase either by acting at a specific site or perhaps, acting at one of the $MgATP^{2-}$ sites.

Recently Greenspan & Lowenstein (1966) have shown that the divalent metal ion can have interesting effects on the activity of rat liver acetyl-CoA carboxylase. This enzyme is activated strongly by incubation with Mg^{2+}, although maximum activity is only obtained after a 20-30 min. incubation. The presence of citrate, although not required for the preincubation, is necessary for the complete reaction. The addition of ATP to the preincubation mixture causes a decrease in acetyl-CoA carboxylase activity; however, this inhibition is not due to the chelation of the stimulatory Mg^{2+}. These authors have suggested that certain forms of acetyl-CoA carboxylase exist which are converted from one to another by the addition of Mg^{2+}, ATP^{4-} or citrate.

In summarizing, the proposed mechanism described in equations (1.7 & 1.8) does not fit all the available evidence. For example, the exchanges, especially the ATP-ADP and ATP-Pi exchange reactions, do not proceed at a rate comparable to the overall forward reaction. This has been explained by the argument that ATP and ADP may be competing for the same sites and thus inhibiting the exchange reaction. Since the other exchange reaction i.e. in the propionyl-CoA carboxylase reaction, the propionyl-

CoA : methyl malonyl-CoA exchange is quite rapid, it could be said that the ATP-Pi and ATP-ADP exchanges are the rate determining steps. In view of the work of Keech & Barritt (1967) and Greenspan & Lowenstein (1966) which revealed that free metal could play a part in the enzymic activity, a role must be made for this in the reaction sequence. Furthermore, the double site postulated for $MgATP^{2-}$, could indicate that an activator site and a catalytic site could be operating which may also suggest that different intermediary forms of the enzyme-substrate and free enzyme may exist.

It will be our endeavour to place some meaning on several of these confusing issues and perhaps aid in elucidating a more precise reaction mechanism for the biotin group of enzymes.

QUATERNARY STRUCTURE:

Many enzymes have been shown to be composed of a number of sub-units, indeed, it now appears that most, if not all enzymes with molecular weights greater than 50,000 are composed of such sub-units. Some of these enzymes have been shown to exhibit a dissociation-association behaviour in response to certain low molecular weight compounds. It has been suggested that these substances control enzymic activity by this manipulation of the protein quaternary structure. This situation may exist in the biotin-containing enzymes, since the activation of the pyruvate carboxylase by acetyl-CoA (Barritt, et al., 1966), the homotropic co-operative effect by $MgATP^{2-}$ displayed in sheep kidney pyruvate carboxylase (Keech & Barritt, 1967) and the activation of acetyl-CoA carboxylase by citrate (Vagelos, Alberts & Martin, 1963) could be mediated by a dissociation-association process. The evidence favouring this idea will be presented in the following paragraphs.

The biotin group of enzymes have molecular weights approaching 700,000, and contain up to 4 molecules of biotin per molecule of enzyme. Therefore, it seems likely that these enzymes may consist of four sub-units, with molecular weights of 175,000, each containing one molecule of biotin (Kaziro, et al., 1961; Wood, Allen, Stjernholm & Jacobson, 1963; Himes et al., 1963; Scrutton & Utter, 1965 (a) & (b)). This concept gained

support from the work of Kaziro *et al*., (1961) who used crystalline propionyl-CoA carboxylase. They showed that on ultra-centrifugation, the native enzyme sedimented as a single peak with a sedimentation coefficient of 19.7S. Treatment with 7.0M-urea caused the protein to sediment with a coefficient of 2.6S. This suggested a dissociation of the enzyme molecule into a number of sub-units of equal size. However, this process led to the irreversible inactivation of the enzyme.

Other factors which favour the idea of dissociation-association behaviour are the stimulation of activity of acetyl-CoA carboxylase by dicarboxylic acids and the stimulation of activity of yeast pyruvate carboxylase by acetyl-CoA. The recent observations on the allosteric control of enzymic activity by small ligands other than the normal substrates and the mediation of these effects by conformational changes in the protein, have caused investigators to predict that the above enzymes are under allosteric control. These predictions are fast becoming realised and the pertinent information will be summarised in the following pages.

(1) Acetyl-CoA Carboxylase

The activation of acetyl-CoA carboxylase by citrate and isocitrate has been studied by Vagelos *et al*. (1963); Martin & Vagelos (1962); Waite & Wakil (1963) and Matsuhashi *et al*. (1964).

The stimulation of activity of the enzyme isolated from adipose tissue is 23-fold. Preincubation of this enzyme with citrate for 30 min. at 30^{o} was necessary for optimal activation; activation did not occur if the preincubation was carried out at 0^{o}. The activation process could be reversed by removing the citrate. Vagelos *et al.*, (1963); Vagelos, Alberts & Martin, (1962), showed that significant alterations in the sedimentation characteristics of the enzyme were observed during the preincubation with citrate. In the absence of citrate, the enzyme had a sedimentation coefficient of 18.9S, whilst after preincubation with citrate it increased to 43.5S, corresponding to a 3-fold increase in molecular weight of the enzyme. This suggested that a polymerization of sub-units was taking place.

An acetyl-CoA carboxylase from chicken liver (Gregolin *et al.*, 1966) has been found to resemble the other biotin enzymes with regard to enzymic characteristics andbiotin content. However, the enzyme isolated in phosphate buffer has a molecular weight approaching 8 million. The structure ofthe enzyme as seen in the electron microscope is of a filamentous type, which can be dissociated into small sub-units with concomitant loss of activity. The enzyme can be reassociated in the presence of isocitrate and other carboxylic acids. The molecular weight of the sub-unit is approx. 400,000 and the biotin content corresponds to one molecule of biotin per sub-unit.

In the presence of the assay mixture, the smaller form

of the enzyme predominates and the factor responsible for this change is MgATP. Malonyl-CoA can also cause this dissociation and it was speculated by the authors that the "carboxylated" enzyme has a greater tendency to dissociate than the "uncarboxylated" form. The addition of isocitrate to the assay mixture gives rise to the larger structure whether MgATP or malonyl-CoA is present or not and this observation is supported by the fact that isocitrate is a competitive inhibitor of the enzyme with respect to malonyl-CoA. Although certain substrates can promote the dissociation, certain factors such as pH or presence of certain salts can also effect these changes.

However, acetyl-CoA carboxylase from yeast is not stimulated by citrate (Matsuhashi & Lynen, 1964) and so it appears that the activation process is not general for all acetyl-CoA carboxylases.

(2) Pyruvate Carboxylase

The allosteric activation of enzymes by an activating compound in some cases is mediated by significant conformation alterations. In the previous section, evidence was presented for a dissociation-association behaviour promoted by citrate. Since pyruvate carboxylase has a large molecular weight, is allosterically activated by acetyl-CoA, it could be expected that conformational changes may be taking place. The evidence for this assumption will be presented in the following paragraphs.

The avian pyruvate carboxylase is cold labile (Scrutton & Utter 1965a), i.e. is stable at room temperature but rapidly inactivated below 10^{o}. Protection against cold inactivation is afforded by high protein and salt concentrations or by the presence of acetyl-CoA. Reactivation can be effected by incubation of the enzyme at room temperature and this process is stimulated by MgATP. Acetyl-CoA affords almost complete protection against cold lability although the cold inactivated enzyme can still catalyse the ADP-ATP exchange reaction, indicating that not all the active sites have been affected. This cold inactivation process is accompanied by a change in sedimentation coefficient from 15S to 7.5S presumably due to labilization of bonds involved in maintaining quaternary structure. However, acetyl-CoA in the presence or absence of the other reaction components has no significant effect on the sedimentation properties of pyruvate carboxylase.

Avidin inhibition has been used successfully to identify biotin-containing enzymes (Wessman & Werkman, 1950). In the special case of pyruvate carboxylase, the inhibition by avidin is increased by the presence of acetyl-CoA but decreased by the preincubation of the enzyme with ATP, ADP or 5'AMP (Scrutton & Utter, 1965; Scrutton & Utter, 1967). These results could suggest that different conformations exist in the presence of certain substrates thus affecting the degree of inhibition by avidin.

Electron microscopy of pyruvate carboxylase isolated from chicken liver demonstrated that the enzyme molecules are arranged as tetramers with the four sub-units orientated at the corners of squares (Valentine, Wrigley, Scrutton, Irias & Utter, 1966). The hypothesis that the tetramer form is the catalytically active species is supported by sedimentation analysis. Cold lability of the enzyme results in a dissociation of the polymer into monomeric sub-units, but the enzyme can regain catalytic activity and restoration of the aggregated enzyme as typified by ultra-centrifugation, electron microscopy and enzymic assay. The molecular weight of the enzyme, as calculated by measurement of the diameter of the sub-units in the tetramer, agrees well with the value obtained by sedimentation studies.

The electron micrographs of the yeast pyruvate carboxylase vary significantly from the enzymes obtained from chicken and calf liver (Utter, 1967). This observation together with their different sensitivities to acyl-CoA's would indicate that the yeast and mammalian enzymes may have different structures. Incidentally, inhibitors of the animal enzymes such as methyl malonyl-CoA and benzoyl-CoA are as good as acetyl-CoA for activating the yeast enzyme and this becomes interesting physiologically.

AIMS OF THE PROJECT.

My first aim was to investigate the active centres of propionyl-CoA carboxylase in order to provide proof of the functional roles of certain amino acids at these areas. In particular, a reactive thiol residue which had been suggested by other workers to be essential for activity, was re-examined with a view to establishing its function in the reaction mechanism.

Another parameter that required investigation was the stimulation of enzymic activity by univalent cations. Since other enzymes of this group show unusual conformational behaviour in response to certain ligands, it could be expected that such changes may accompany this activation. Certain methods of detecting conformational changes were examined to test this hypothesis.

The roles of Mg^{2+} and $MgATP^{2-}$ were also investigated since the findings of Keech & Barritt (1967) must be supported with evidence from other members of the group to be accepted as part of the reaction mechanism of all biotin-containing enzymes. Furthermore, it was hoped to elucidate the role of the modifier site in the reaction sequence.

Finally, initial velocity data was used as a tool to

elucidate a more precise reaction mechanism. Overall this kinetic investigation was aimed at explicitly defining those areas on the protein where the substrates are bound and eventually catalysed.

C H A P T E R T W O

MATERIALS AND METHODS

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MATERIALS:

(1) Materials used for enzyme purification

(a) DEAE-cellulose (DE 50) was a Whatman product. Before use it was washed with 1N-NaOH, 1N-HCl and finally with distilled water until free of chloride ions.

(b) Calcium phosphate was prepared by the method of Keilin & Hartree (1938), washed thoroughly with water and finally stored at 4^{o} in 0.02M-phosphate buffer, pH 6.5.

(c) Protamine sulphate (from salmon sperm) was obtained from Nutritional Biochemical Corp. Cleveland, Ohio U.S.A. Aqueous solutions (1% w/v) were prepared and adjusted to pH 6.5 with 1M-tris base just prior to use. All preparations had to be tested before use since not all samples were satisfactory.

(d) Pig hearts were obtained from the Metropolitan Export & Abattoirs Board and packed in ice until required.

(e) Ethylene diamine tetra-acetic acid (EDTA) was obtained from British Drug Houses. The solutions were made up in glass distilled water, neutralized with 1N-NaOH, diluted to 0.1M and stored at 4^{o}.

(f) Reduced glutathione was obtained from Sigma Chemical Co. St. Louis Mo. U.S.A. Solutions (0.1M) were prepared and neutralized with 1M-tris base immediately before use.

(g) Ammonium sulphate. Ammonium sulphate A.R. (By products and Chemical Pty. Ltd. Australia) was purified by two recrystallisations from 10^{-4}M-EDTA in glass distilled water.

(h) Tris. Sigma tris 7-9 (Sigma Chemical Co.) was re-

crystallised twice from 85% (v/v) ethanol by the method of Sutherland & Wosilait (1956).

(i) Phosphate buffers were prepared from 1M-K_2HPO_4 and 1M-KH_2PO_4.

(j) Dialysis membrane of all sizes was supplied by the Visking Co., Division of Union Carbide Corporation, Chicago Illinois, U.S.A. and washed in EDTA at least once before using.

(k) Tris-HCl buffers. Solutions of tris prepared from purified tris base were adjusted to the appropriate pH using freshly distilled constant boiling HCl. The pH of these buffers was measured at 4^o since the temperature coefficient for tris buffers is quite significant.

(2) Materials used in assay of propionyl-CoA carboxylase

(a) Adenosine triphosphate (ATP). Purchased from the Sigma Chemical Co. Solutions were adjusted to pH 7.0 with 1M-tris base. Metal-free ATP solutions were prepared using cation resins and are described in a later section.

(b) Potassium chloride (KCl). This compound was obtained from May & Baker Ltd., England.

(c) Preparation of ^{14}C-$NaHCO_3$. ^{14}C-CO_2 was prepared from ^{14}C-$BaCO_3$ (The Radiochemical Centre, Amersham, Bucks.) and 7% (v/v) perchloric acid and distilled under vacuo at room temperature. The ^{14}C-CO_2 was absorbed by an equivalent amount of sodium hydroxide dissolved in glass distilled water from which all CO_2 had been

expelled by boiling. A trace of phenol-phthalein in the sodium hydroxide indicated that the reaction had gone to completion. The distillation flask was warmed slightly to ensure complete liberation of ^{14}C-CO_2. The solution of ^{14}C-$NaHCO_3$ was diluted to approximately 50µc. per ml. and adjusted to 0.2M-$NaHCO_3$ by addition of solid $NaHCO_3$.

The actual specific activity of the ^{14}C-$NaHCO_3$ solution was determined by dilution in tris-HCl, buffer pH 8.0. Aliquots of this solution were spotted onto Whatman 3 MM chromatography paper squares (1" x 1") previously moistened with 1% (w/v) barium chloride solution. After drying, the squares were counted by a liquid scintillation procedure in a Packard Scintillation Spectrometer.

(d) Magnesium chloride ($MgCl_2$). $MgCl_2.6H_2O$ was purchased from May & Baker Ltd., England and the concentration of the aqueous solution determined by complexometric titration using the indicator Solochrome Black 6B (Vogel, 1961).

(e) Preparation and purification of propionyl-CoA. Propionyl-CoA was prepared from Coenzyme A (CoA) (Sigma Chemical Co.) by a modification of the method of Simon & Shemin (1953).

CoA (35mg.) was dissolved in glass distilled water (0.5ml.), and a drop of bromothymol blue indicator added. The solution was kept in ice. To ensure that the CoA was in the

fully reduced form, a solution of sodium borohydride in water was added slowly until the CoA solution was approximately pH 7.0. After standing for 2 min., the solution was acidified with constant boiling HCl to destroy the excess sodium borohydride and then adjusted with 1M-tris base to pH 8.0 and allowed to stand for a further 5 min. at 0°. Propionic anhydride (0.05ml.) was then added and after 5 min. at 0°, the pH was adjusted to pH 6.0 and the solution kept frozen at -15°. The propionic anhydride used above was purified by distillation and stored in 0.5ml. quantities in sealed ampoules.

The crude propionyl-CoA solution was purified by ascending chromatography on an acid-washed Whatman 3 MM paper, using the solvent system isobutyric acid-water-conc. NH_4OH (66:33:1 by vol.) adjusted to pH 3.8 containing 10^{-5}M-EDTA (Zetterstrom & Ljunggren, 1951). The band of propionyl-CoA was located by its absorption under ultra violet light and eluted with 10^{-5}M-EDTA, pH 7.0. The presence of propionyl-CoA was verified by activation of the pyruvic carboxylase reaction. Its concentration was determined by measuring the extinction at 259mμ.

(3) Materials used in the metal-free studies.

(a) N-ethyl morpholine. N-ethyl morpholine (Eastman Organic Chemicals, U.S.A.) was purified by distillation under reduced pressure at 33°. The buffers were prepared by weighing

the appropriate amount of N-ethyl morpholine and adjusting the pH of the various solutions with constant boiling HCl.

(b) N-ethyl morpholine adenosine triphosphate (NEM-ATP).

ATP was purchased from Pabst Laboratories and purified as follows. ATP (66 mg.) was dissolved in water (2 ml.) and the neutralised solution added to a Dowex AG 1 x 8 (100-200 mesh) column, 24cm. x 1.2cm., which had previously been washed with 2N-HCl, 2N-NaOH and finally 3M-sodium formate to convert it to the formate form. AMP and ADP were eluted with 80ml. of 0.4M-ammonium formate at pH 4.3. The ATP was eluted with 102ml. of N-HCl and collected in 10ml. fractions at 0^{o}. The nucleotide content of each fraction was determined by measuring the extinction at 259mμ. The fractions containing ATP were combined and added to 3 vol. of 95% (v/v) ethanol containing saturated BaI_2 (0.5 ml.) at 0^{o} with stirring. After standing at 0^{o} for 30 min. the precipitate of $BaATP^{2-}$ was collected by centrifuging, washed successively with ethanol, acetone and ether then dried in a dessicator.

The dry $BaATP^{2-}$ was converted to the N-ethyl-morpholine salt by stirring an aqueous suspension with beads of the ion exchange resin Zeo Carb 225 (N-ethyl-morpholine form) for 30 min. at 0^{o}. To ensure complete removal of Ba^{2+}, the solution was finally passed down a small column of the same resin. The purity of the ATP solution was determined by ascending paper

chromatography using the solvent system isobutyric acid-water-conc. NH_4OH (66:33:1, by vol.) (Zetterstrom & Ljunggren, 1951). The concentration of the solution was determined by measuring the extinction at 259mμ and using a value of 1.54×10^4 for E, the molar extinction coefficient (Bock, Ling, Morell & Lipton, 1956).

(c) Reduced glutathione (N-ethyl morpholine salt).

Reduced glutathione was dissolved in glass distilled water and neutralised to pH 7.0 with N-ethyl morpholine base.

(d) N-ethyl morpholine bicarbonate.

N-ethyl morpholine was weighed and dissolved in an appropriate volume of glass distilled water. Carbon dioxide was bubbled through the solution under pressure until pH 8.0 was obtained. This solution was used to adjust the molarity of the ^{14}C-N-ethyl morpholine bicarbonate solution prepared below.

(e) ^{14}C-N-ethyl morpholine bicarbonate.

Instead of distilling the ^{14}C-CO_2 into sodium hydroxide as described in section 2(c) for the preparation of ^{14}C-sodium bicarbonate, the ^{14}C-CO_2 was trapped by N-ethyl morpholine. The solution was adjusted to 0.2M by addition of concentrated N-ethyl morpholine bicarbonate prepared in 3(d).

(f) Pure magnesium chloride. Spectroscopically pure magnesium metal obtained from Hilger & Watts Ltd., England was

dissolved in 1N constant boiling HCl. The solution was evaporated to dryness under reduced pressure. The solid was dissolved in water and evaporated to dryness. This process was repeated several times to ensure complete removal of excess HCl. Aqueous solutions were prepared in glass-distilled water and their concentration determined as described in section (2) (d).

(4) Materials required for scintillation counting.

(a) 1" x 1" squares of 3MM Whatman filter paper were used to absorb the radioactive material.

(b) Scintillation fluid. The Packard Instrument Co., Illinois, U.S.A. supplied PPO (2, 5 diphenyl oxazole) and dimethyl POPOP, (1,4-bis-2(4-methyl-5-phenyl-oxazoylyl)-benzene). These compounds were dissolved in sulphur-free toluene (British Drug Houses Ltd. Poole, England), according to the method of Bousquet & Christian (1960).

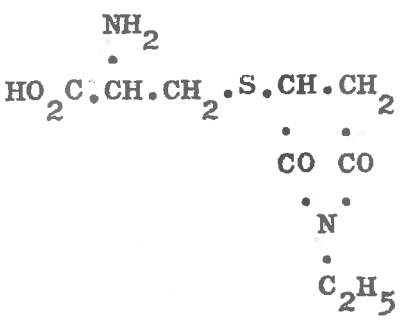
(5) Materials required for thiol group investigation.

(a) N-ethyl maleimide. This inhibitor was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. This solution was prepared just prior to use since this compound was found to be unstable especially at high pH values.

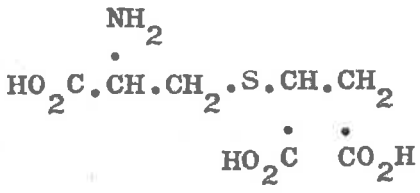
(b) ^{14}C-N-ethyl maleimide was obtained dissolved in 50% ethanol from Schwarz Bioresearch, In., N.Y., U.S.A. The solution

was evaporated to dryness under vacuo and then dissolved in the appropriate buffer solution. Radioactive N-ethyl maleimide was 97% pure as certified by the manufacturers based on paper chromatography analysis and was used without further purification.

(c) S-(1-ethyl-2,5-dioxopyrrolidino-3-yl)-L-cysteine. N-ethyl maleimide reacts with cysteine to yield a complex, S-(1-ethyl-2,5-dioxopyrrolidino-3-yl)L-cysteine, (Smyth, Blumenfeld & Konigsberg, 1964). Compound (1).



Compound 1



Compound 2

(d) S-(1,2-dicarboxy-ethyl)-L-cysteine. The above chemical (compound 1) when hydrolysed with 6N HCl for 72 hr. is converted into S-(1,2-dicarboxy-ethyl)-L-cysteine (compound 2) and ethylamine. S-(1,2-dicarboxy-ethyl)-L-cysteine was prepared from maleic anhydride and cysteine according to the method of Smyth et al. (1964).

METHODS:

(1) Protein determination

For the purpose of following the elution of protein from DEAE-cellulose columns, protein was determined by measuring the extinctions of the solutions in 1cm. quartz cuvettes at 280mμ. (Layne, 1957). This technique was used because it provided a quick and sensitive method and can be used for the measurement of proteins in solution containing large quantities of inorganic salts. Where an estimate of the protein concentration was required for the estimation of the specific activity of the enzyme in a preparation, the method of Lowry, Roseborough Farr & Randall (1951) was used. Bovine serum albumen was used as the standard protein.

(2) Propionyl-CoA carboxylase assay

For the purification of the enzyme, enzymic activity was measured by a modification of the radioactive isotopic method of Kaziro & Ochoa (1961). The reaction mixture, (total volume 0.5ml.) contained in μmoles: tris-HCl buffer, pH 8.0, 100; ATP, 1.5; $MgCl_2$, 3.0; propionyl-CoA, 0.5; ^{14}C-$NaHCO_3$ 5.0, (2.0×10^5 cpm per μmole); KCl, 4.0; reduced glutathione, 0.25. The reaction mixtures were incubated for 15 min. at 30° and the reaction stopped with 0.2ml. of 10% trichloro-acetic acid. After the solutions were treated with solid CO_2 to

displace free ^{14}C-CO_2, centrifuged to remove denatured protein, aliquots of the supernatant were counted for radio-activity.

The counting technique was a modification of the method of Wang & Jones (1959) for the measurement of ^{14}C-compounds on paper discs using scintillation counting. Aliquots of the reaction mixtures 0.05ml. were applied in triplicate to the discs using an Agla micro-syringe. The discs were dried at 100° for 5 min. and then placed into toluene phosphor contained in 3ml. scintillation vials. All samples were counted twice and the results were averaged in order to avoid complications due to the geometry of the discs. Any sample not counting 100% above the control was discarded. Reaction mixtures counted in this manner showed high reproducibility, were not affected by quenching problems and allowed recovery of scintillation fluid and vials without radio-active contamination.

(3) Modification and denaturation of the protein

Propionyl-CoA carboxylase (10mg. of sp. act. 7.0) was dialysed against 0.02M-tris-HCl, pH 7.5, containing 1×10^{-3}-EDTA and 5×10^{-5}M-glutathione for 2 hr. The protein was incubated at 22° with 1×10^{-4}M-^{14}C-N-ethyl maleimide contained in a volume of 0.5ml. of 0.05M-tris-HCl buffer, pH 8.4, for 30 min. The modification was stopped by the addition of 0.2ml. of 10% trichloro-acetic acid (w/v) containing 1×10^{-2}M reduced glutathione. The solution was immediately filtered through an

Oxoid membrane filter, the insoluble precipitate washed exhaustively with "cold" N-ethyl maleimide dissolved in 10% trichloro-acetic acid and finally with 1% acetic acid. The radio-active residue was then dissolved in 0.5ml. of 0.1M-NaOH.

(4) Hydrolysis of labelled protein precipitates

The protein to be hydrolysed was suspended in 6N-HCl in Kontes O-ring hydrolysis tubes. Nitrogen was bubbled through the solution to remove dissolved oxygen and then the tube was evacuated. The hydrolysis tubes were heated at 110° for 72 hr. The hydrolysate was evaporated to dryness several times to remove the HCl. The sample was then dissolved in small amount of the buffer used for the electrophoresis.

(5) Paper electrophoresis

High voltage paper electrophoresis was carried out using the "cooled flat plate" equipment. Whatman 3MM paper was used. The electrophoretograms were run in acetic acid-water buffer, (3% v/v) adjusted to pH 6.5 with pyridine, at 3,000 volts for 90 min. When the run was completed, the papers were dried in an oven at 80°.

(6) Detection of the separated amino acids

The amino acids were detected on developed electrophoretograms by spraying with ninhydrin-acetone (0.01% w/v) followed by heating at 80° for 5 min. (Toennies & Kolbe, 1951).

(7) Preparation of propionyl-CoA carboxylase for electron microscopy

Specimens were examined in a Siemens' Elmiskop I at 80kv. with a 50 μ objective aperture.

Enzyme preparations (Spec. activity 7.4) were fixed for 30 min. in 1:12 (V/V) 40% formalin : 0.05M veronal acetate buffer, pH 7.0. Samples were transferred onto carbon-coated grids and washed with 0.1M-KCl. These preparations were then negatively stained with 2%-uranyl acetate, pH 4.5.

C H A P T E R T H R E E

PREPARATION OF PROPIONYL-CoA CARBOXYLASE

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INTRODUCTION:

The method of isolation of propionyl-CoA carboxylase was based on the procedure described by Tietz & Ochoa (1959). The initial steps of the purification described by these authors were unsuitable for conditions existing in this laboratory, i.e., it involved centrifuging large volumes of liquid. Furthermore, the initial $(NH_4)_2SO_4$ fractionation and the subsequent dialysis proved uneconomical with respect to the amounts of both $(NH_4)_2SO_4$ and reduced glutathione required. Initially, 2-mercapto-ethanol was tried as a replacement for reduced glutathione but this was unsatisfactory. Moreover, both the yield and specific activity of the enzyme following these initial purification steps were poor compared with data obtained by Tietz & Ochoa (1959). This was probably due to our use of ^{14}C-$NaHCO_3$ instead of ^{14}C-K_2CO_3 in the reaction mixtures. At the time this did not seem important but the work of Neujahr & Mistry (1963) and Neujahr (1963) demonstrated that propionyl-CoA carboxylase was stimulated several fold by K^+. Since the purification of the enzyme on DEAE cellulose columns was reproducible, probably due to the use of KCl and KH_2PO_4/K_2HPO_4 buffers in the eluting mediums, these methods were retained, although simplified purification procedures have subsequently been published (Kaziro, Ochoa, Warner & Chen, 1961; Kaziro, Grossman & Ochoa, 1965).

The modification of the previously published procedures involved concentrating the initial cell-free extract by absorption onto protamine sulphate. This meant that all subsequent steps could be carried out using relatively much smaller volumes. These steps were primarily those of the original authors (Tietz & Ochoa, 1959). In our hands, the method described was reproducible and yielded reasonable amounts of enzyme of satisfactory specific activity. The enzyme preparations used for most of the work described had a specific activity greater than 2.0 and were free of contaminating enzymes which could have interfered with the assay method.

ISOLATION OF PROPIONYL-CoA CARBOXYLASE:

Step 1: Preparation of cell-free extract

Twelve pig hearts were trimmed of fat, washed with cold distilled water, dried and minced in a meat grinder using a 3mm. grid. The mince was washed with an equal weight of cold 0.04M-tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}M-EDTA, stirred for 30 min. and then centrifuged at 2,500 x g. for 10 min. The supernatant solution was discarded.

The mince was homogenised with 2 volumes of buffer (same as above) for 1 min. in a Waring blender. The suspension was stirred at 4^{o} for 15 min. then centrifuged at 22,500 x g. for 20 min. The supernatant solution was filtered through muslin and cotton wool to remove lipid-like material. Reduced

glutathione was added to a final concentration of 5×10^{-4}M. The protein concentration was 7.2mg. per ml. Specific activity = 0.028.

Step 2: Protamine sulphate and ammonium sulphate fractionation

Freshly prepared 1% protamine sulphate (adjusted to pH 6.8 with 1M-tris base) was added to the solution in the proportion of 1mg. protamine sulphate to 20mg. of protein and the pH adjusted to 6.5 with 1M-acetic acid. The extract was stirred at 0^{o} for 20 min., then centrifuged at 22,500 x g. for 15 min. The supernatant was discarded. The residue was washed with 250ml. of distilled water containing 5×10^{-4}M-glutathione, pH 7.0 and centrifuged at 22,500 x g. for 15 min. The supernatant was discarded.

The residue was suspended in 200ml. of 0.05M-tris-HCl buffer, pH 7.5, containing 1×10^{-3}M-EDTA, 5×10^{-4}M-glutathione and 5% $(NH_4)_2SO_4$ and homogenised using a Potter-Elvejhem homogeniser. The suspension was stirred for 15 min., then centrifuged for 30 min. at 22,500 x g.

The residue was re-homogenised with a further 250ml. of the same buffer solution and centrifuged as before. The protein concentration of the combined supernatants was adjusted to 10mg. per ml. with buffer solution. Solid $(NH_4)_2SO_4$ was added to 35% saturation, equilibrated for 30 min. and centrifuged

at 22,500 x g. for 20 min. The supernatant was adjusted to 60% saturating with solid $(NH_4)_2SO_4$ and centrifuged as before. The precipitate was dissolved in 0.02M-tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione to approximately 40mg. protein per ml. and dialysed against 2 litres of the same buffer. The buffer was changed three times during the 16 hr. dialysis. Specific activity = 0.25.

Step 3: Chromatography on diethyl-amino-ethyl cellulose

A DEAE-cellulose column (20cm. x 3 cm.) was prepared without applied pressure and equilibrated with 0.02M-tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione. The dialysed protein solution from step 2 was centrifuged to remove any insoluble material, diluted to 10mg. protein per ml. and then added to the DEAE-cellulose column. After allowing the protein solution to run into the resin, the column was washed with 500ml. of 0.05M-tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}-EDTA and 5 x 10^{-4}M-glutathione and finally with 500ml. of the same buffer containing 0.05M-KCl.

A gradient elution was carried out with 500ml. of 0.05M-tris-HCl buffer, pH 7.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione and 0.05M-KCl in the mixing chamber and 500ml. of the same buffer including 0.2M-KCl in the reservoir. Fractions of 10ml. were collected with an automatic fraction collector and assayed for protein concentration and enzymic

activity. A typical elution pattern is shown in Fig. (3.1). Only the fractions containing the highest specific activity were combined and precipitated with $(NH_4)_2SO_4$ (398g. per litre). The precipitate was collected by centrifuging at 22,500 x g. for 30 min., dissolved in 0.02M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione then dialysed against 2 x 2 litres of the same buffer. Specific activity = 2.2.

Step 4: Absorption on calcium phosphate gel

The protein concentration of the dialysed solution obtained from Step 3 was adjusted to 15mg. per ml. and $Ca_3(PO_4)_2$ gel was added slowly in the proportion 1mg. of gel to 2mg. of protein. The solution was stirred for 20 min. at 4^o and centrifuged at 22,500 x g. for 20 min. The specific activity = 3.2.

Step 5: Chromatography on diethyl-amino-ethyl cellulose

After adjusting the protein concentration to 5mg. protein per ml. the supernatant was then added to a DEAE-cellulose column (15cm. x 2cm.), equilibrated with 0.02M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione. The column was washed with 250ml. of 0.05M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione.

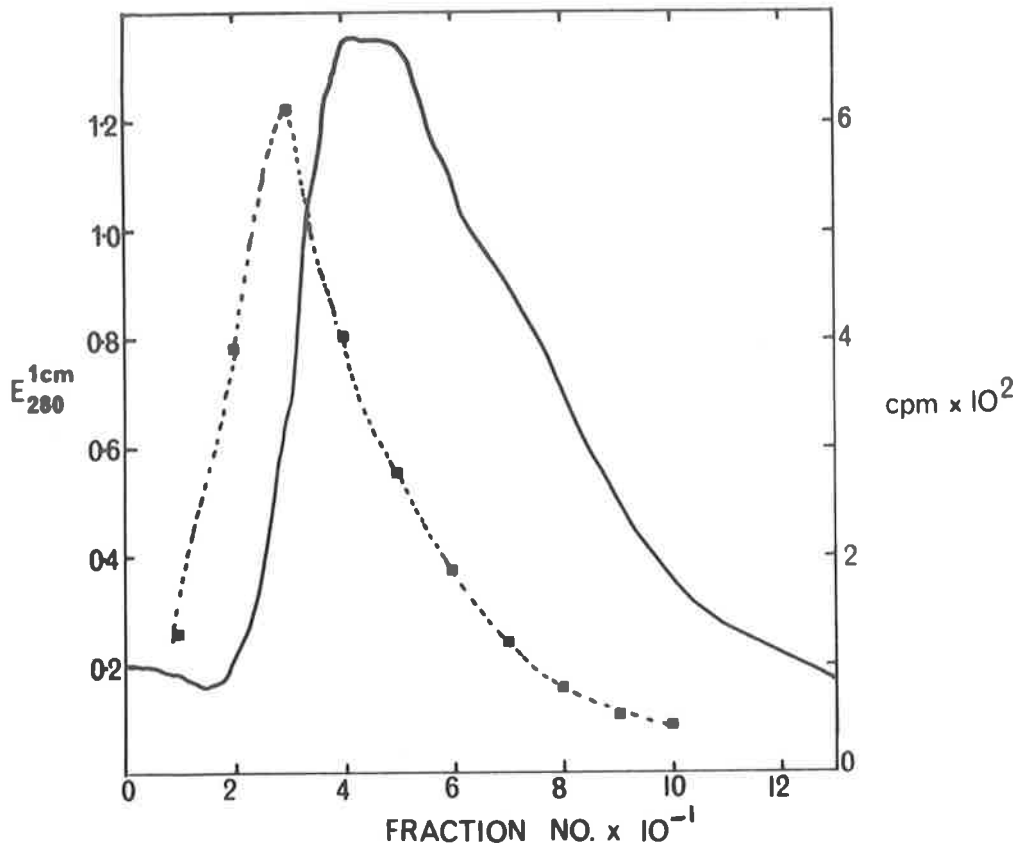

Fig. 3.1. Chromatography on DEAE-cellulose (pH 7.5).

Fig. 3.1 depicts the protein and activity profiles resulting from the chromatography of propionyl-CoA carboxylase on DEAE-cellulose, pH 7.5. Aliquots (10 ml.) were collected on an automatic fraction collector. Absorbance at 280 mμ was determined on every third fraction and assays were performed on every tenth fraction. Absorbance at 280 mμ (———), propionyl-CoA carboxylase activity (■—■). Activity was expressed as c.p.m. which represents the propionyl-CoA dependent $^{14}C\text{-}CO_2$ fixation by the diluted enzyme.

A gradient elution was performed using 250ml. of 0.05M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione in the mixing chamber and 0.2M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione in the reservoir. The fractions of 5ml. were collected with an automatic fraction collector and assayed for protein concentration and enzymic activity. The resulting elution pattern is described in Fig. (3.2). The fractions of highest specific activity were pooled and precipitated with $(NH_4)_2SO_4$ (398g. per litre). The protein precipitate was collected by centrifuging at 22,500 x g. for 30 min. and dissolved in 0.02M-phosphate buffer, pH 6.5, containing 1 x 10^{-3}M-EDTA and 5 x 10^{-4}M-glutathione and stored at -15°. The specific activity = 7.4.

A summary of the purification steps is outlined in Table 3.1. The overall yield of enzyme is of specific activity which is lower than the published reports of Tietz & Ochoa (1959). However, the preferential selection of fractions obtained from the DEAE-cellulose chromatography, meant that discarded enzyme of quite good specific activity could be stored and added to further preparations.

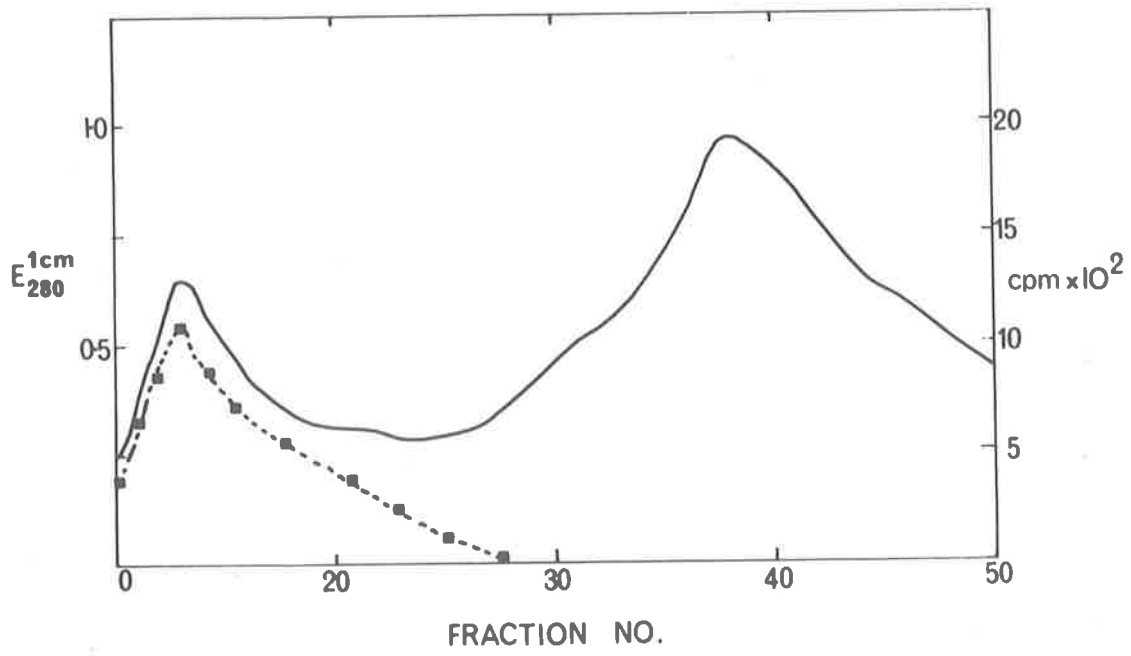

Fig. 3.2. DEAE cellulose chromatography (pH 6.5).

Fig. 3.2 represents the protein and activity profiles resulting from the chromatography of propionyl-CoA carboxylase on DEAE-cellulose, pH 6.5. Aliquots (10 ml.) were collected on an automatic fraction collector. Absorbance at 280 mμ was determined on alternate fractions and assays were conducted on every third fraction. Absorbance at 280 mμ (———), propionyl-CoA carboxylase activity (■·—·■).

TABLE 3.1

Purification of propionyl carboxylase from pig heart

Step	Volume	Units	Protein	Specific activity	Yield
	ml.		mg.	units/mg. protein	%
1. Extract.....	2,900	584	7.2	0.028	100
2. Protamine sulphate and $(NH_4)_2SO_4$ fractionation (after dialysis)..	85	455	21.4	0.25	78
3. DEAE-cellulose chromatography (pH 7.5) (After dialysis).....	10	204	9.27	2.2	35
4. Calcium phosphate gel supernatant...	10	140	4.0	3.5	24
5. DEAE-cellulose chromatography (pH 6.5).....	1.5	47	4.2	7.4	8

One unit of enzyme was defined as the amount catalysing the fixation of one μmole of $^{14}C\text{-}CO_2$ per minute under the condition of the assay and the specific activity is expressed in units per mg. of protein.

The enzymic behaviour of the purified enzyme was briefly examined to ensure that this sample was indeed identical to the propionyl-CoA carboxylase isolated by Tietz & Ochoa (1959).

(1) Identification of methyl malonyl-CoA as the product of the reaction.

A reaction mixture was incubated with 0.05 units of propionyl-CoA carboxylase for 1 hr. at 30°. The reaction was stopped and treated as described in Materials & Methods. A sample of the reaction mixture was taken and chromatographed in isobutyric acid-water-ammonia solvent (66:33:1), pH 3.8, for 5 hr. The radio-active and U.V. absorbing band was eluted, concentrated and hydrolysed at pH 10.0 for 30 min. at 85°. The solution was acidified to pH 2.0 and extracted twice with ether. The ether phases were combined, taken to dryness and a sample co-chromatographed with cold methyl malonic acid in iso-amyl alcohol-formic acid solvent (Flavin & Ochoa, 1957). The R_f value = 0.78 of the radio-active material compared to the authentic methyl malonic acid (R_f = 0.8) would suggest that this compound is methyl malonic acid and that the product of propionyl-CoA carboxylase, prepared in this laboratory, is methyl malonyl-CoA.

(2) Characterization of propionyl-CoA carboxylase.

The time interval and the enzyme concentration required to give a linear relationship were investigated. The ^{14}C-CO_2 assay gave a linear response to time (up to 20 min.) using 0.015

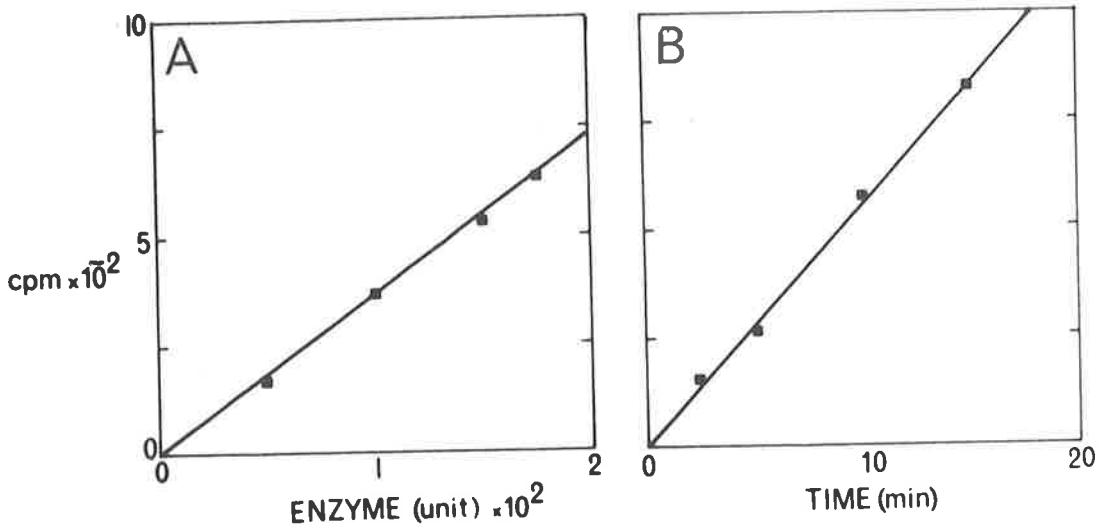

Fig. 3.3 Variation of activity with time The velocity of the propionyl-CoA carboxylase reaction was plotted as a function of time using 0.015 units of enzyme (Graph B).

Fig. 3.4 Variation of activity with enzyme concentration. The velocity of the propionyl-CoA carboxylase reaction was plotted as a function of enzyme concentration over a 10 min. incubation period (Graph A). The velocity of the reaction (in both diagrams) is expressed as c.p.m. and represents the propionyl-CoA dependent fixed radio-activity in the deproteinized supernatant. The incubation temperature was 30° and the details of the assay are outlined in Materials & Methods.

units of enzyme (Fig. 3.3). Furthermore, the fixation of $^{14}C\text{-}CO_2$ was proportional to enzyme concentration (up to 0.02 units of enzyme) during a 10 min. assay period (Fig. 3.4). In the subsequent chapters, kinetic measurements will be derived and it will be essential that initial velocity kinetics are observed, thus the need for this data.

(3) Electron microscopy of propionyl-CoA carboxylase

Investigations into the structures of acetyl-CoA carboxylase (Gregolin et al., 1966) and pyruvate carboxylase (Valentine et al., 1966) have involved the use of electron microscopy. Propionyl-CoA carboxylase was also examined by this technique. Certain structures in the electron micrographs were consistently observed and these could be described as follows:

(a) A number of long "cylinders" (polymers) which were composed of repeating units. These monomeric units (M) can be observed in the dissociated form (Fig. 3.5).

(b) The longer polymeric forms were not observed in recently isolated preparations but only after standing at 4^o for at least 14 days (Fig. 3.6).

(c) K^+, Mg^{2+} and $MgATP^{2-}$ which have been shown by kinetic analyses to cause activation of the enzyme, did not appear to cause any structural changes.

From this information, it was proposed that the

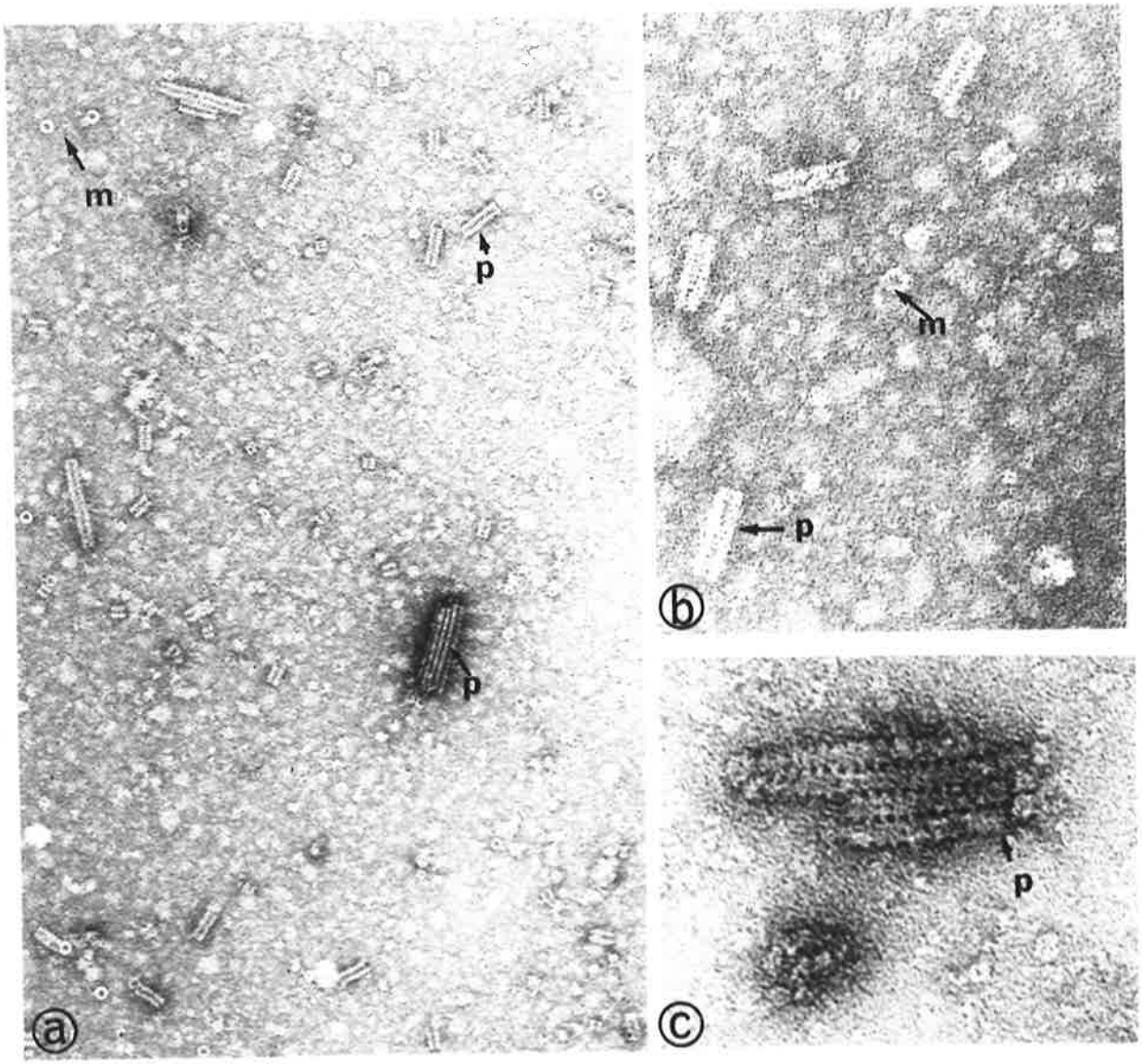

Fig. 3.5. Examination of propionyl-CoA carboxylase in the electron microscope. Specimens of propionyl-CoA carboxylase were prepared as outlined in Materials & Methods. Samples a, b and c were fixed preparations:

(a) Magnification 120,000 x

(b) Magnification 240,000 x

(c) Magnification 504,000 x

Note: the monomeric forms (m) and the polymeric forms (p).

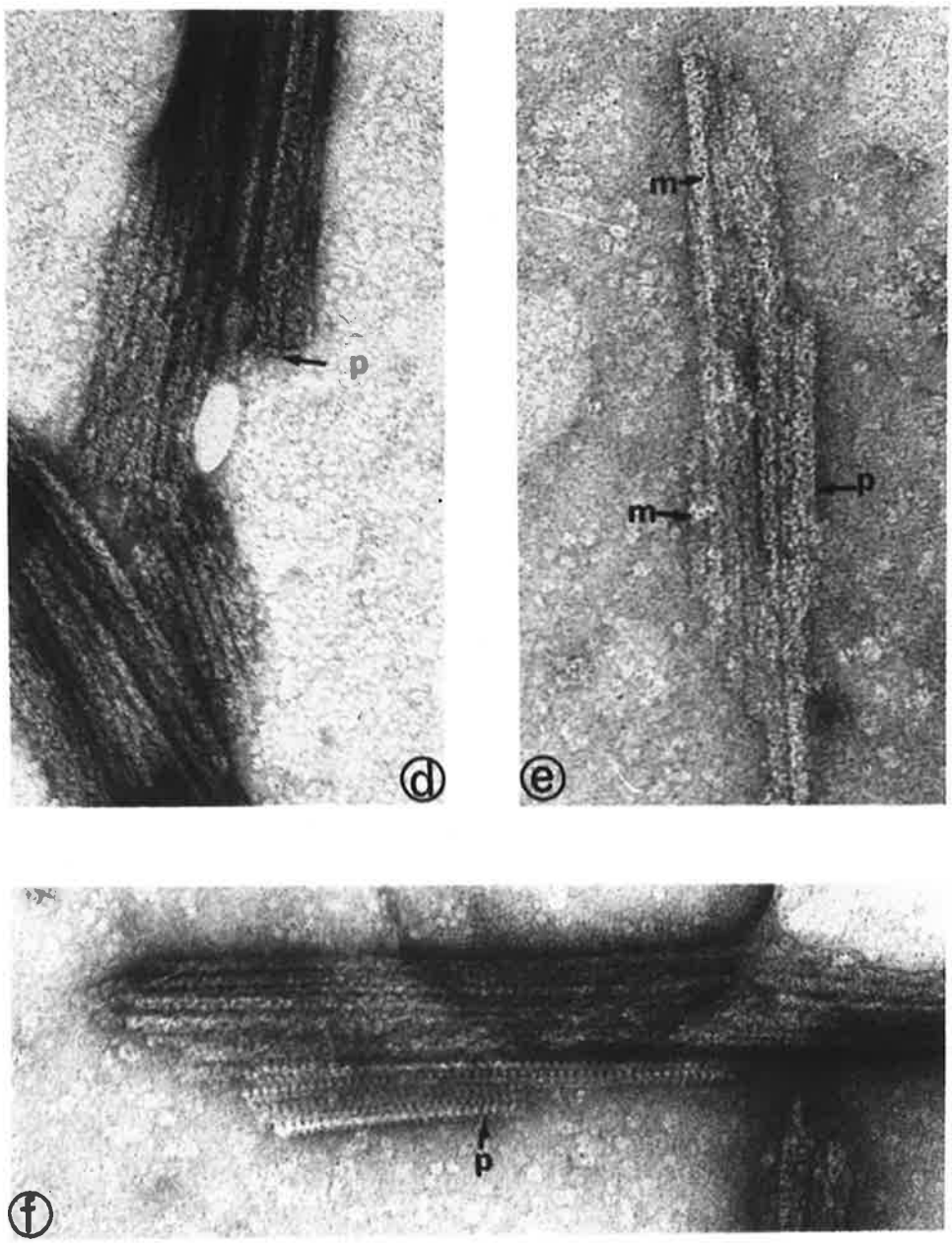

Fig. 3.6. Examination of propionyl-CoA carboxylase in the electron microscope. Samples d, e and f were unfixed preparations:

(d) Magnification 240,000 x

(e) Magnification 240,000 x

(f) Magnification 240,000 x

Note: the polymeric forms (p) which are made up of monomeric units (m).

monomeric units could be the normal form of the enzyme. Furthermore, the enzyme associates on standing to form the long cylinders which, it has been speculated, may be related to the crystalline form of the enzyme (Kaziro *et al.*, 1961). In other words, there appears to be no physiological reason for this polymerization but is due in fact, to a slow crystallization of propionyl-CoA carboxylase. There are also indications that the monomeric units are not tetrads, as in the pyruvate carboxylase situation (Valentine *et al.*, 1966) but appear to contain five or more component parts. However, it should be emphasised that these interpretations are only speculative at this juncture, mainly because it has not been positively demonstrated that these structures are propionyl-CoA carboxylase.

C H A P T E R F O U R

THE ESSENTIAL THIOL GROUP OF PROPIONYL-CoA CARBOXYLASE

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INTRODUCTION:

Previous studies carried out with pig heart propionyl-CoA carboxylase have indicated that the enzyme is dependent on the presence of at least one free sulphydryl group for enzymic activity (Tietz & Ochoa, 1959). Glutathione protected the enzyme against inactivation and also enhanced the reaction rate, suggesting the involvement of thiol groups. Kaziro et al. (1960) investigated the effect of sulphydryl group reagents such as iodoacetamide and p-hydroxy-mercuri-benzoate on this enzyme. Incubation of the enzyme in the presence of propionyl-CoA or to a lesser extent, reduced glutathione, afforded some protection against these inhibitors whereas, ATP had no effect. These results indicated the presence of cysteine residues and also suggested that these groups may be proximal to the propionyl-CoA site on the enzyme.

The ATP-Pi and ATP-ADP exchange reactions were also subject to inhibition by p-hydroxy-mercuri-benzoate (Kaziro et al., 1960). Since these exchange reactions were inhibited only slightly compared with the inhibition of the overall reaction, one can assume that the inhibited groups are not related to the nucleotide sites, but as suggested before, may be involved with the binding of propionyl-CoA. However, this evidence provides no indication of the number of essential

susceptible groups and their function in the enzymic process.

Hegre (1964) demonstrated that the beef liver propionyl-CoA carboxylase was inhibited by a variety of thiol group reagents. The protection against inhibition by propionyl-CoA was similar to the results observed with the pig heart enzyme. In contrast to the protection by propionyl-CoA, the combination of Mg^{2+} and ATP^{4-} appeared to facilitate inhibition. The author speculated that since HCO_3^- ions were also present the CO_2-enzyme complex may be more susceptible to the thiol group reagents than the native enzyme. Propionyl-pantetheine, like propionyl-CoA protected the enzyme against p-hydroxy-mercuri-benzoate inhibition, suggesting that this compound is binding at, or sterically hindering the inhibition of the same thiol residue. Hegre (1964) also demonstrated that, although 3'AMP did not protect against inhibition, it was an effective competitive inhibitor of propionyl-CoA, indicating that the 3'AMP moiety of propionyl-CoA must be binding at a site distant from the thiol residue.

(1) <u>The choice of inhibitor</u>

In practice, no thiol group reagent will be entirely specific under all conditions. The mechanism by which a reagent reacts with thiol groups applies also to amino, aliphatic hydroxyl and several other groups (Leach, 1965). The reagents of choice are those which react much more rapidly

with thiol groups than with any other residue. The temperature, pH and time of reaction must be selected in such a way as to preserve this differential rate of reaction. In particular, the concentration of thiol group reagent must be kept to a minimum. The advantage of this differential rate is lost when procedures involving the use of a large excess of thiol group reagent and prolonged reaction times are used. A variety of thiol group reagents are available, viz:

(a) Those containing reactive metal ions, such as the mercury containing compounds;

(b) Alkylating agents such as the substituted halogenated acetic acid derivatives (e.g. iodo-acetamide and iodo-acetic acid) and the ethylenic compounds (e.g. N-ethyl maleimide).

(c) Oxidizing agents (e.g. iodoso-benzoic acid).

All of these compounds have been used with some success in implicating thiol groups in enzymic reactions.

N-ethyl maleimide (NEM) has been used for the chemical modification of proteins, and it is usually assumed that the reaction occurs specifically at the thiol group of cysteinyl-residues (Morell, Ayers, Greenwalt & Hoffman, 1964). The thiol groups of native proteins are frequently unreactive to N-ethyl maleimide, thus this reagent is not reliable for the titration of total protein thiol groups. However, certain accessible thiol groups react, inferring that N-ethyl maleimide has

specificity, whereas other reagents do not. The advantages of this reagent are:

(a) Its relatively high selectivity for thiol groups

(b) Its reaction with only certain accessible residues on the enzyme.

However, N-ethyl maleimide has also been shown to react with the amino groups of peptides and with the imidazole group of histidine (Smyth, Nagamatsu & Fruton, 1960) and with the amino groups of certain amino acids (Smyth et al., 1960; Riggs, 1961). Although the reaction of N-ethyl maleimide with some proteins is much slower than its reaction with the thiol group of cysteine (Gregory, 1955), the possibility that N-ethyl maleimide could react with proteins, at groups other than cysteine residues, must be considered. In this investigation, N-ethyl maleimide was used at low concentrations, with short incubation periods, in an endeavour to maintain a specific inhibitor attack on the susceptible residue.

RESULTS:

(1) Order of inactivation with respect to time and N-ethyl maleimide concentration

Fig. 4.1 shows the percentage of propionyl-CoA carboxylase activity plotted as a function of time on a semi-log scale for various N-ethyl maleimide concentrations. The linearity of the plot down to 20% of the initial enzymic activity indicates that the inactivation process approximates first order kinetics with respect to time, at any fixed concentration of this inhibitor. The same data replotted and presented in Fig. 4.2 showed that the inactivation process is first order with respect to the N-ethyl maleimide concentrations used in this investigation. Furthermore, if one assumes the inactivation process as:

$$E + n\,I = EI_n \qquad \ldots (4.1)$$

$$\text{then} \quad K = \frac{(EI_n)}{(E)\,(I)^n} \qquad \ldots (4.2)$$

Multiplying both sides of the equation by $(I)^n$ and taking logs, then equation (4.2) becomes:

$$\log \frac{(EI_n)}{(E)} = \log K + n \log (I) \qquad \ldots (4.3)$$

where E, EI_n, n and K are active enzyme, inactive enzyme-inhibitor complex, apparent number of inhibitor molecules reacting to form an inactive complex, and apparent overall association constant respectively. Therefore, when $\log (EI_n)/(E)$ is plotted against

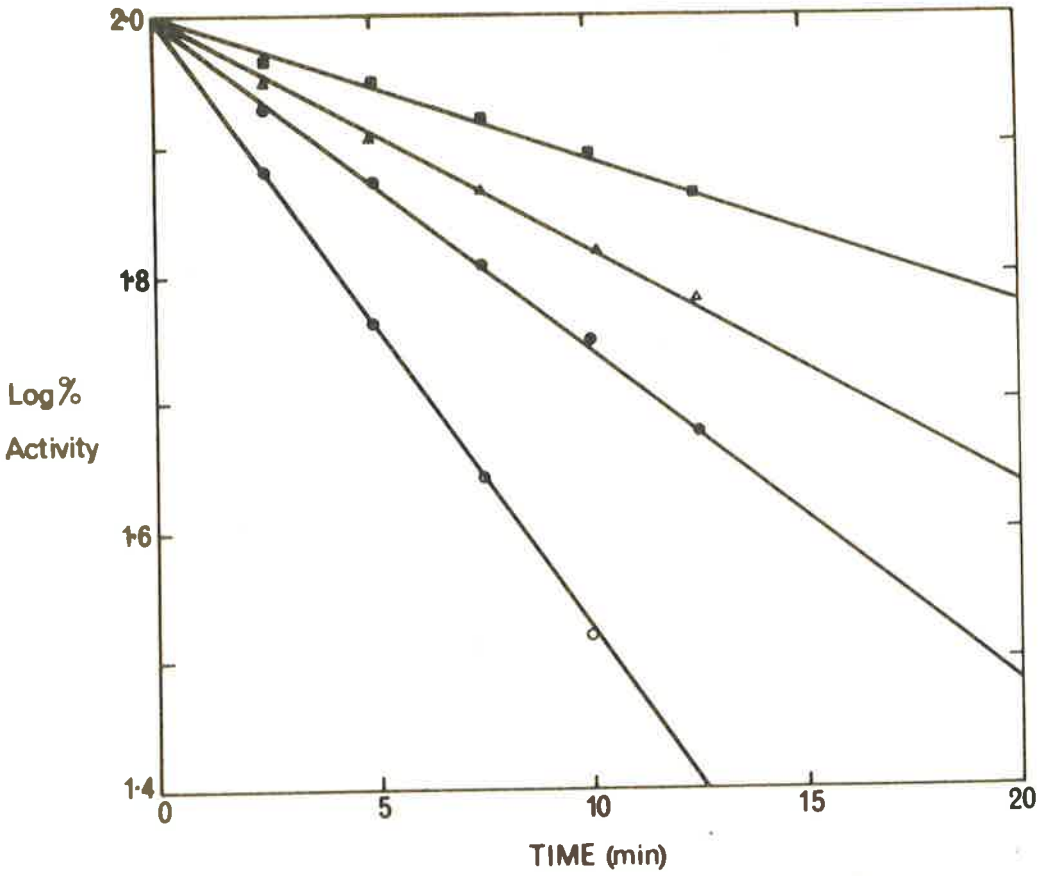

Fig. 4.1. The rate of inhibition of propionyl-CoA carboxylase with varying concentrations of N-ethyl maleimide. Enzyme (200 µg) was incubated with N-ethyl maleimide contained in 1 ml. of 0.05 M tris-HCl buffer, pH 8.0 at 22°. Aliquots of 0.05 ml. were taken at various time intervals and assayed. The reaction mixture also contained 1% BSA and reduced glutathione 1 x 10^{-2} M. The concentrations of N-ethyl maleimide used were 2 x 10^{-3} M (o—o), 1 x 10^{-3} M (●—●), 0.75 x 10^{-3} M (△——△) and 0.5 x 10^{-3} (■—■). Log % activity was plotted against time. Appropriate controls were necessary since enzymic activity decreased slightly under these conditions.

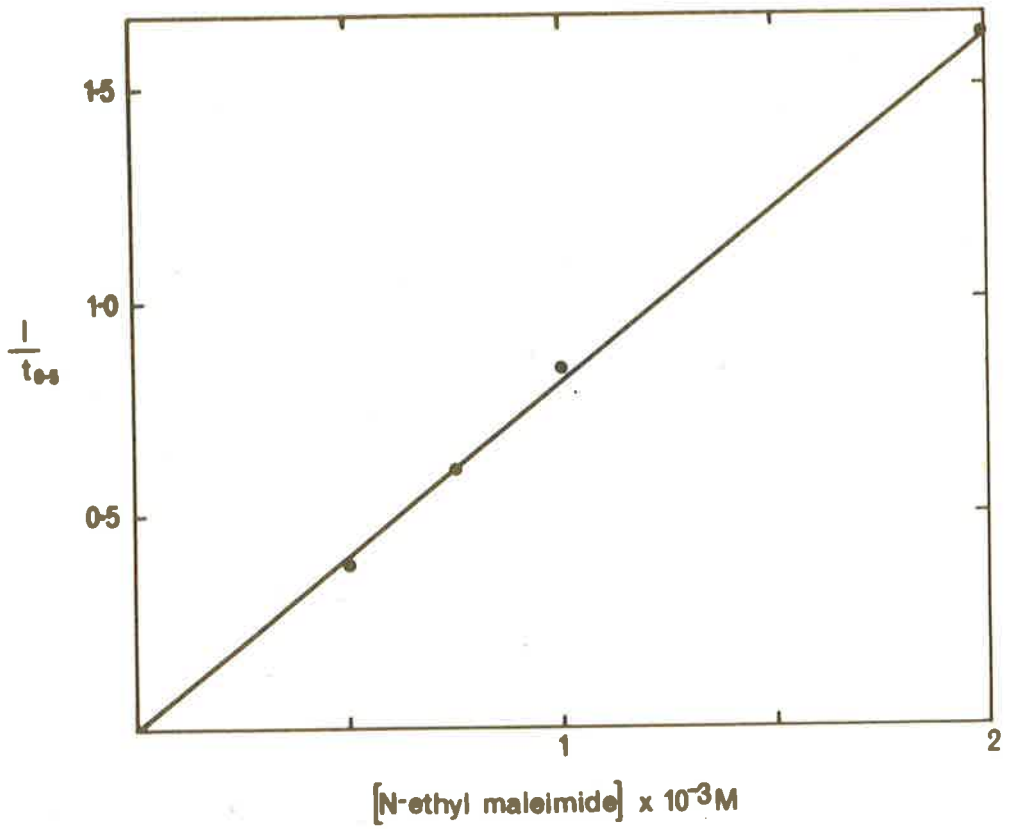

Fig. 4.2. The pseudo first order kinetics of the inactivation with respect to N-ethyl maleimide. The half-time ($t_{0.5}$) was obtained from the data shown in Fig. 4.1. The reciprocal of the half-time was plotted against N-ethyl maleimide concentration.

log (I), a straight line is obtained with slope n, and log K, the intercept on the ordinate when log (I) equals 0.

Fig. 4.3 shows the values obtained in Fig. 4.1 plotted in this manner. The reciprocal of the half-time for inactivation has been used rather than rate of inactivation. The experimental data fit a straight line with a slope (n = 1.05). This is taken to indicate that when inactivation occurs, only one molecule of N-ethyl maleimide reacts with one catalytic site of propionyl-CoA carboxylase. Using a similar plot, Levy, Leber & Ryan (1963) concluded that 3 moles of 2,4-dinitro-phenol bind to one molecule of myosin causing inactivation. Scrutton & Utter (1965) obtained a value of 1.4 for the slope of a similar plot when they studied the inactivation of pyruvate carboxylase by avidin. They concluded that at least two molecules of avidin were involved in the process.

(2) Effect of pH on the apparent K_m and V_{max} values

Since the pK values of various amino acid residues differ appreciably, a tentative identification of an amino acid residue involved in substrate binding or in attaining maximum reaction rates can be made by investigating the variation of V_{max} or K_m with pH. Since propionyl-CoA had been shown to protect the enzyme against thiol group inhibitors, this substrate was used for the following study.

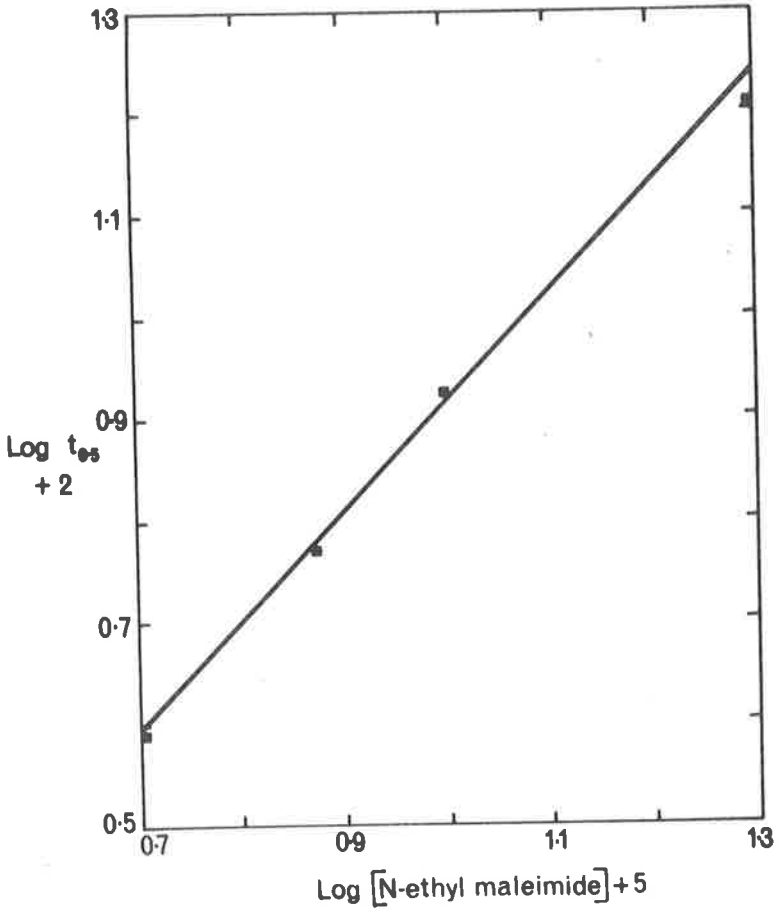

Fig. 4.3. Determination of the order of the reaction between propionyl-CoA carboxylase with respect to N-ethyl maleimide.

The data were those of Fig. 4.1 plotted as $\log_{10}$ of the reciprocal of the half-time of inactivation against $\log_{10}$ concentration of N-ethyl maleimide.

Apparent Michaelis constants were calculated from a series of double reciprocal plots obtained at various pH values using propionyl-CoA as the variable substrate. Fig. 4.4 shows a plot of $\log(1/K_m)$ against pH. The important feature of the pH profile is the diminution of the pK_m in the pH range where the enzyme exhibits maximum activity. This indicates a pH dependence of K_m when V_{max} or velocity (V) at high substrate concentration is independent of pH. From the theory of Dixon (1953), the inflection at pH 8.2 is due to the change in ionization of the enzyme-substrate complex.

This interpretation is applicable if the enzyme forms a single enzyme-substrate intermediate. If, on the other hand, a number of enzyme-substrate complexes are formed, the experimentally derived value for the apparent K_m then represents a complex kinetic function and the change in K_m with pH may instead reflect a change in the rate controlling step.

(3) Effect of pH on the rate of loss of activity

Although previous workers have indicated that a reactive thiol group is required for propionyl-CoA carboxylase activity, the pK_a value of 8.2 obtained in Fig. 4.4 could not be taken as positive proof that the ionization was due to the thiol group. The pK_a value of a thiol group is markedly dependent on the ionic environment present, that is, on the vicinal ionic groups. The pK_a value for the thiol group may

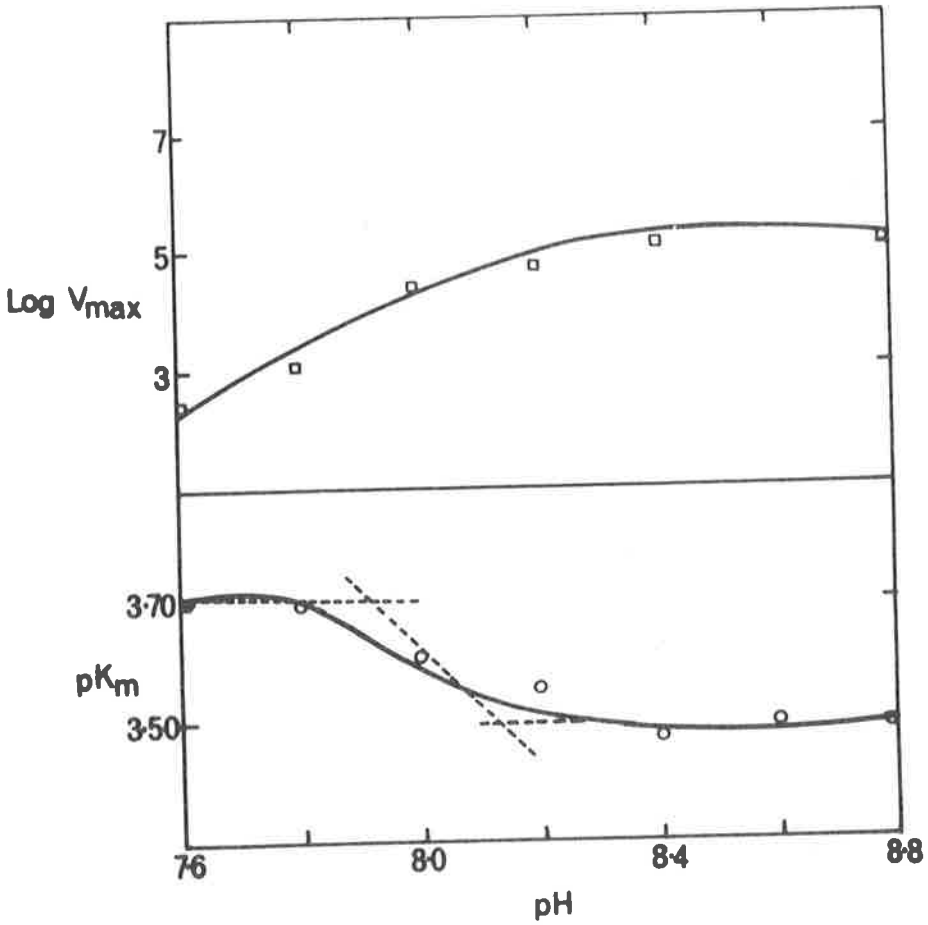

Fig. 4.4. Apparent K_m for propionyl-CoA calculated at different pH values. The reaction mixture was as described in general methods and tris-HCl buffer was used throughout. The reaction mixtures were incubated with 0.015 units of enzyme for 10 min. at 30°. The K_m and V_{max} values for propionyl-CoA were calculated from Lineweaver-Burk plots.

vary between 7.2 and 8.5 when it is situated near a positively charged group. The value may vary between 8.5 and 9.2 if there is no electric field in the vicinity of the group, while, if the group is in close proximity to a negatively charged group, the pK_a value may vary between 9.2 and 10.2 (Webb, 1963). Therefore, to positively identify the ionization at pH 8.2 as being due to a thiol group, a series of experiments were carried out to determine the rate at which the thiol group reagent (i.e. N-ethyl maleimide) would react with the enzyme at varying pH values. The rationale used here is that the rate at which N-ethyl maleimide reacts with the thiol group will depend on the state of ionization of this group.

Alkylation of the enzyme followed first order kinetics with respect to inhibitor concentration (Fig. 4.1). Therefore, the apparent rate constant for the loss of enzymic activity was determined over the pH range from 7.2 to 8.8 (Fig. 4.5). It can be seen that when the rate constant (or $1/t_{0.5}$) of inactivation of propionyl-CoA carboxylase by N-ethyl maleimide was plotted as a function of pH, the rate of inactivation remained constant as the pH increased to pH 8.0. At higher pH values the rate of inactivation increased rapidly. The intersection of the two linear sections of the graph (Fig. 4.5) at pH 8.2 is similar to the pK_a value of the enzyme-substrate complex obtained in Fig. 4.4. The increase in the rate of

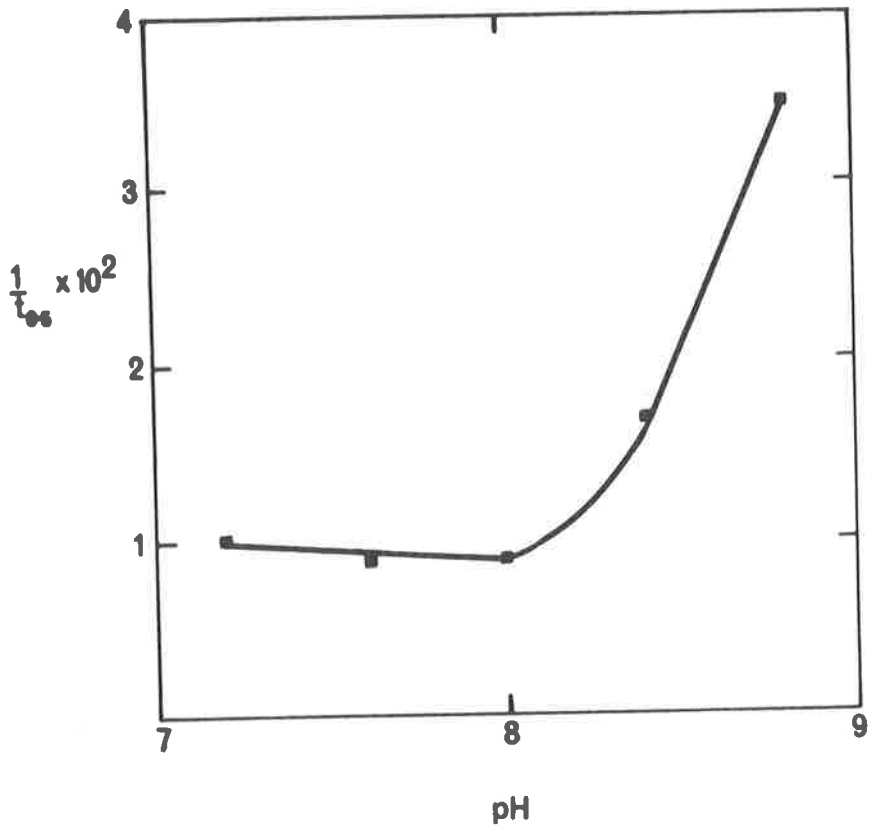

Fig. 4.5. Rate of inhibition of propionyl-CoA carboxylase with N-ethyl maleimide at varying pH values. The inhibitor procedure was as described in Fig. 4.1 using a concentration of N-ethyl maleimide 1 x 10^{-4} M. Tris-HCl buffer were used throughout. The reciprocal of half-time of inactivation was plotted against pH.

inactivation at pH values above 8.0 indicates that the reactive amino acid residue is undergoing an ionization change and that the inhibitor reacts more readily with the ionized form. Virden & Watts (1966) obtained a similar result when they inhibited arginine kinase with iodoacetamide. Furthermore, Hollaway, Mathias & Rabin (1964) found that the free thiol group in ficin reacts with an equimolar concentration of iodoacetamide at a rate too fast to measure above pH 8.2.

(4) Effect of chemical modification of the active site

Most experiments involving chemical modification of amino acid residues of enzymes result in the production of catalytically inactive protein. If an enzyme can be minimally modified by a chemical reagent so as to decrease the activity (but not completely destroy it), studies on the residual activity may define the function of the modified residue, as distinct from simply defining its existence at the active site.

The investigation to be carried out was based on the fact that amino acids may be one of four types in a protein.

(a) Binding - concerned with the formation of enzyme-substrate complexes.

(b) Catalytic - involved in the activation step of enzymic catalysis.

(c) Structural - required to give the correct spatial relationship between (a) and (b).

(d) Non-contributing - non essential and hence may be modified without affecting the catalytic activity.

Enzymic reactions may be considered in terms of two distinct steps:

(i) Reaction of the substrate with the enzyme to form an enzyme-substrate complex.

(ii) Activation of the enzyme-substrate complex which leads to the formation and release of products.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products} \qquad \ldots (4.4)$$

If a "binding" amino acid is modified by chemical means then one could observe a change in apparent K_m value i.e. either k_1 and k_2 or both are changed, i.e. apparent K_m is a reflection of the ability of the enzyme to combine with the substrate. If a catalytic amino acid is modified by chemical means then one should observe a change in V_{max} i.e. a change in k_3. Zerner & Bender (1964); Knowles (1965) and Koshland, Strumeyer & Ray (1962) have used this technique to investigate certain modified enzymes. Using this approach, an attempt has been made to determine the function of the susceptible residue in propionyl-CoA carboxylase.

The apparent K_m and V_{max} values of propionyl-CoA carboxylase were determined for HCO_3^-, ATP and propionyl-CoA with the enzyme in the native state and again, after various

stages of inactivation using N-ethyl maleimide as the blocking reagent.

From the data shown in Figs. 4.6, 4.7 and 4.8 respectively, it can be seen that the apparent K_m values for HCO_3^- and ATP are unchanged in the modified enzyme but the apparent K_m value for propionyl-CoA increases as more of the enzyme is modified. This later observation implies that although the velocity of the reaction decreased at fixed propionyl-CoA concentrations, the V_{max} does not change, i.e., the substrate concentration required to saturate the enzyme increases with increasing modification. It is concluded from this series of experiments that the modification alters a binding amino acid for propionyl-CoA.

It could be argued that the changes observed in the V_{max} for ATP and HCO_3^- (Figs. 4.6 & 4.7) reflect a modification of catalytic residues involved in the breakdown of enzyme-substrate complex involving these substrates. However, the fact that only one molecule of N-ethyl maleimide reacts with the enzyme and that ATP stimulates the rate of inactivation due to p-hydroxy-mercuri-benzoate in the liver enzyme tends to lessen this possibility. The most likely explanation for the decrease in V_{max} when either ATP or HCO_3^- is the variable substrate, is that the fixed propionyl-CoA concentration used

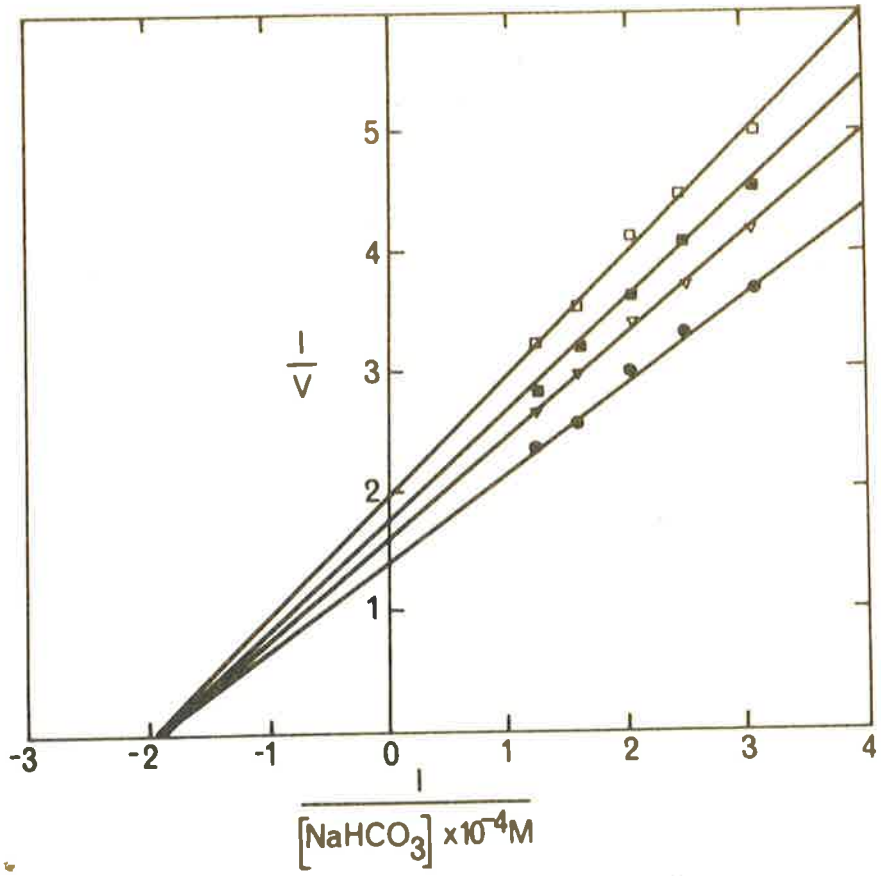

Fig. 4.6. Kinetic constants for $NaHCO_3$ in the chemically modified enzyme. Enzyme (0.6 units) was incubated in 1 ml. containing 1 x 10^{-4}M N-ethyl maleimide in 0.05M tris-HCl pH 8.0 at 22°. Aliquots of the enzyme (0.2 ml.) were removed at 0, 5, 10, 15 min. and the modification stopped by dilution in 1% BSA and 1 x 10^{-2}M reduced glutathione. The modified enzyme was then assayed using varying amounts of $NaHCO_3$. Double reciprocal plots of data obtained after modification for time 0, (●—●); 5 min. (▽—▽); 10 min. (■—■); and, 15 min. (□—□).

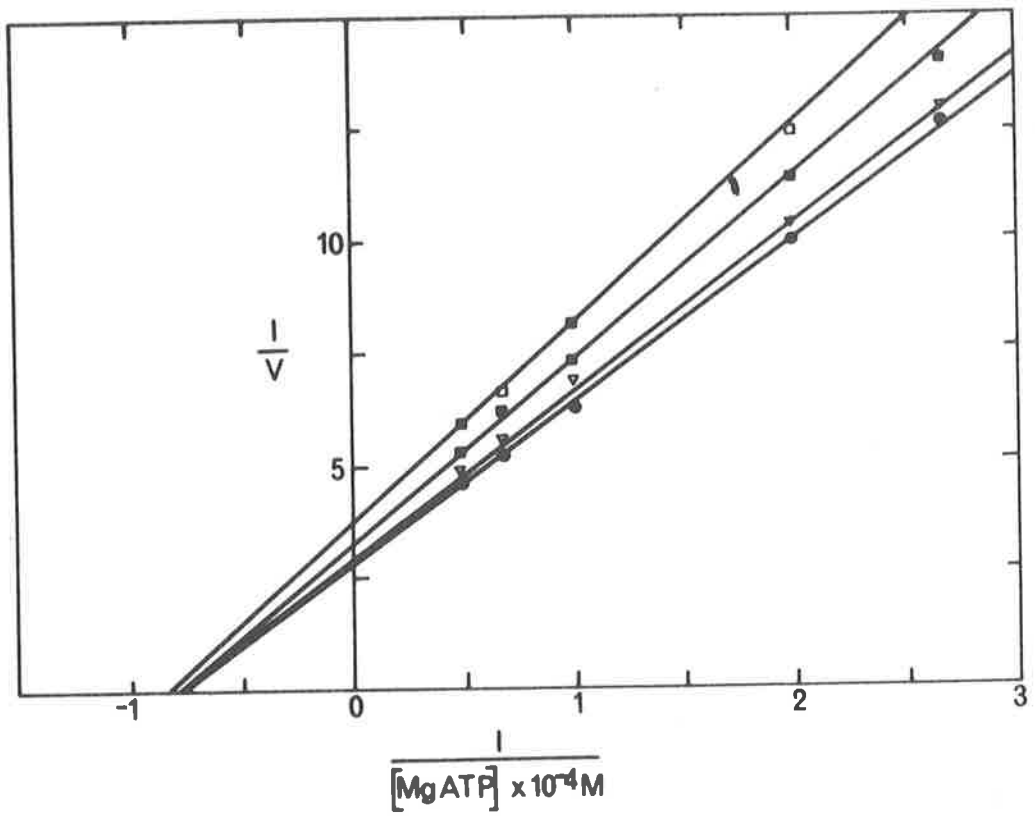

Fig. 4.7. Kinetic constants for ATP in the chemically modified enzyme. The enzyme was modified as described in Fig. 4.6. The enzyme was assayed using varying amounts of ATP. Double reciprocal plots of data obtained after modification for time 0 min. (●—●); 5 min. (▽—▽); 10 min. (■—■); and, 15 min. (□—□).

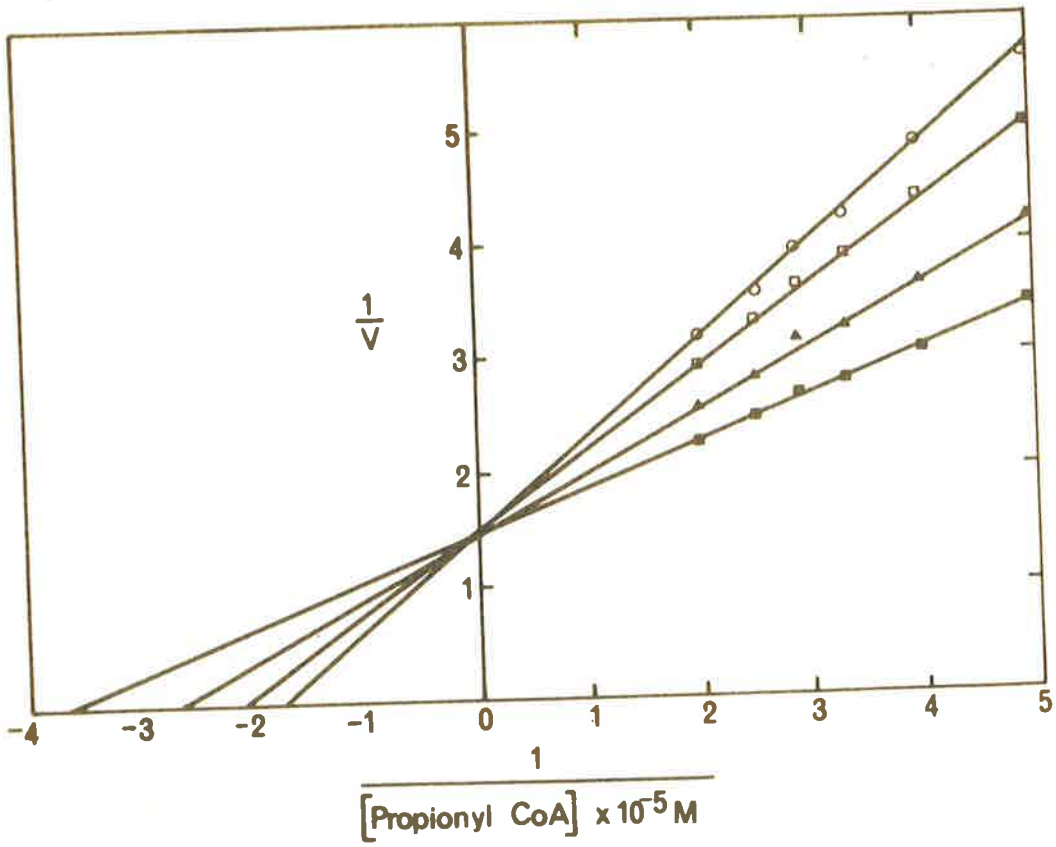

Fig. 4.8. Kinetic constants for propionyl-CoA in the chemically modified enzyme. Modification of the enzyme was as described in Fig. 4.6. The enzyme was assayed using varying amounts of propionyl-CoA. Double reciprocal plots of data obtained after modification for time 0 min. (■—■); 5 min. (▲—▲); 10 min. (□—□); and, 15 min. (o—o).

in the assay system is no longer saturating the chemically modified enzyme. A further characteristic of the inhibition by covalently bound N-ethyl-maleimide is that it introduces a new side chain into the active site and the change in V_{max} may be due to the presence of this side chain. This explanation could very well apply to the observed reduction in the rate of the propionyl-CoA carboxylase catalysed ATP : Pi exchange in the presence of p-hydroxy-mercuri-benzoate.

(5) Propionyl-CoA protection against N-ethyl maleimide inhibition

Previous reports of protection by propionyl-CoA against thiol group reagents indicated that propionyl-CoA may be bound to a cysteine residue, (Hegre & Lane, 1966; Kaziro et al., 1960). In this investigation, additional evidence both from the pK_m/pH profile and the change in the apparent K_m value for propionyl-CoA in the chemically modified enzyme implicates a cysteine residue at the CoA-ester binding site. Further kinetic proof was obtained by determining the ratio of the rate constants for the inactivation of the free enzyme and the enzyme-propionyl-CoA complex. This was accomplished by measuring the inactivation rate constant in the presence of varying concentrations of propionyl-CoA (Fig. 4.9).

Scrutton & Utter (1965a) have used this technique to demonstrate that avidin cannot react with pyruvate carboxylase-ATP

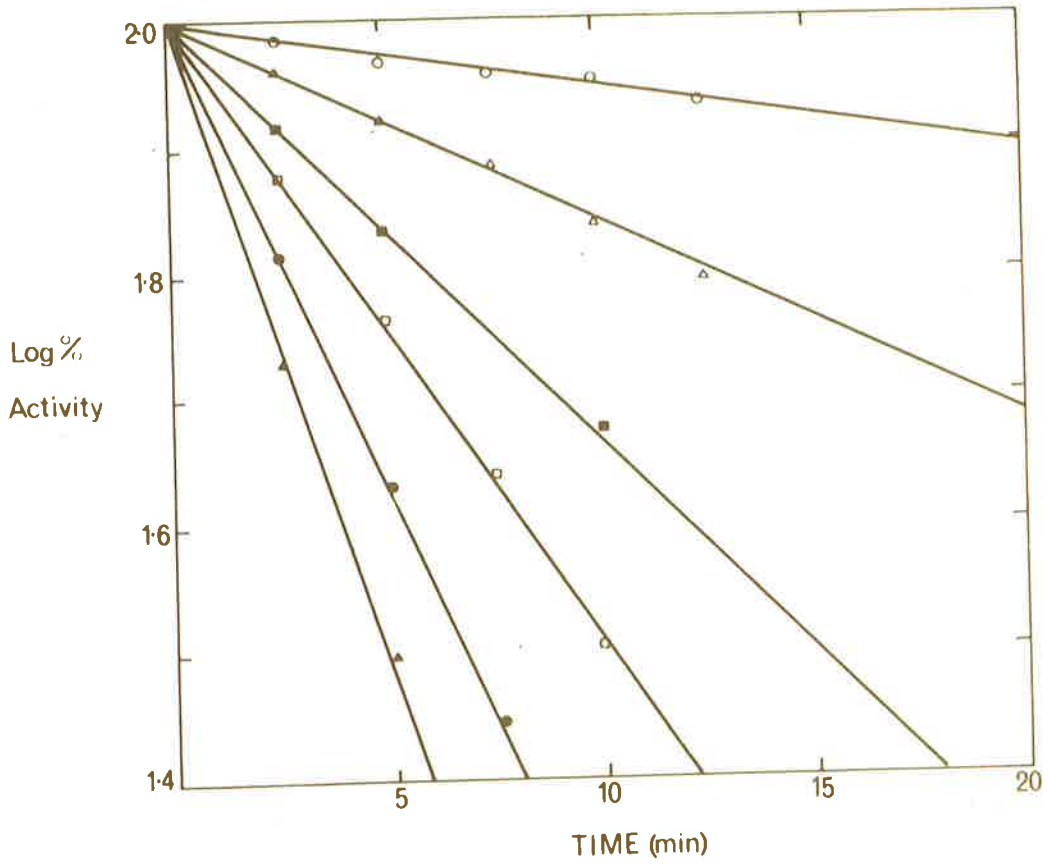

Fig. 4.9. Protection of propionyl-CoA carboxylase by propionyl-CoA against N-ethyl maleimide inhibition. The enzyme was modified with N-ethyl maleimide, 1×10^{-3} M as described in legend for Fig. 4.1 in the presence of varying concentrations of propionyl-CoA. The concentrations of propionyl-CoA used were 0 min. (▲——▲); 2.5×10^{-4}M, (●——●); 5×10^{-4} M, (□——□); 10×10^{-4} M, (■——■); 15×10^{-4} M, (△——△); and, 20×10^{-4} M, (○——○).

complex. They derived the equation:

$$\frac{V_a}{V_o} - \frac{k_2}{k_1} + k_d \frac{(1 - V_a/V_o)}{A} \quad \ldots (4.4)$$

where V_a and V_o represent, respectively, the pseudo first order rate constants for inactivation in the presence and absence of A, the protecting agent, i.e. propionyl-CoA; k_1 and k_2 are the fractional order rate constants for inactivation of free enzyme (equation 4.5) and the enzyme-propionyl-CoA complex (equation 4.6) respectively; and k_d, the dissociation constant for EA (equation 4.7).

$$E + nI \xrightarrow{k_1} EI_n \quad \ldots (4.5)$$

$$EA + nI \xrightarrow{k_2} EAI_n \quad \ldots (4.6)$$

$$E + A \underset{k_d}{\rightleftharpoons} EA \quad \ldots (4.7)$$

When the ratio of the pseudo first order constants for inactivation in the presence and absence of propionyl-CoA, i.e. V_a/V_o, is plotted against $\frac{1 - V_a/V_o}{A}$ the intercept represents k_2/k_1 i.e. the ratio of the fractional order rate constants for the reaction between free enzyme and enzyme propionyl-CoA complex with inhibitor. The slope of the graph is k_d. Such a plot derived from the data obtained in Fig. 4.9 is presented in Fig. 4.10.

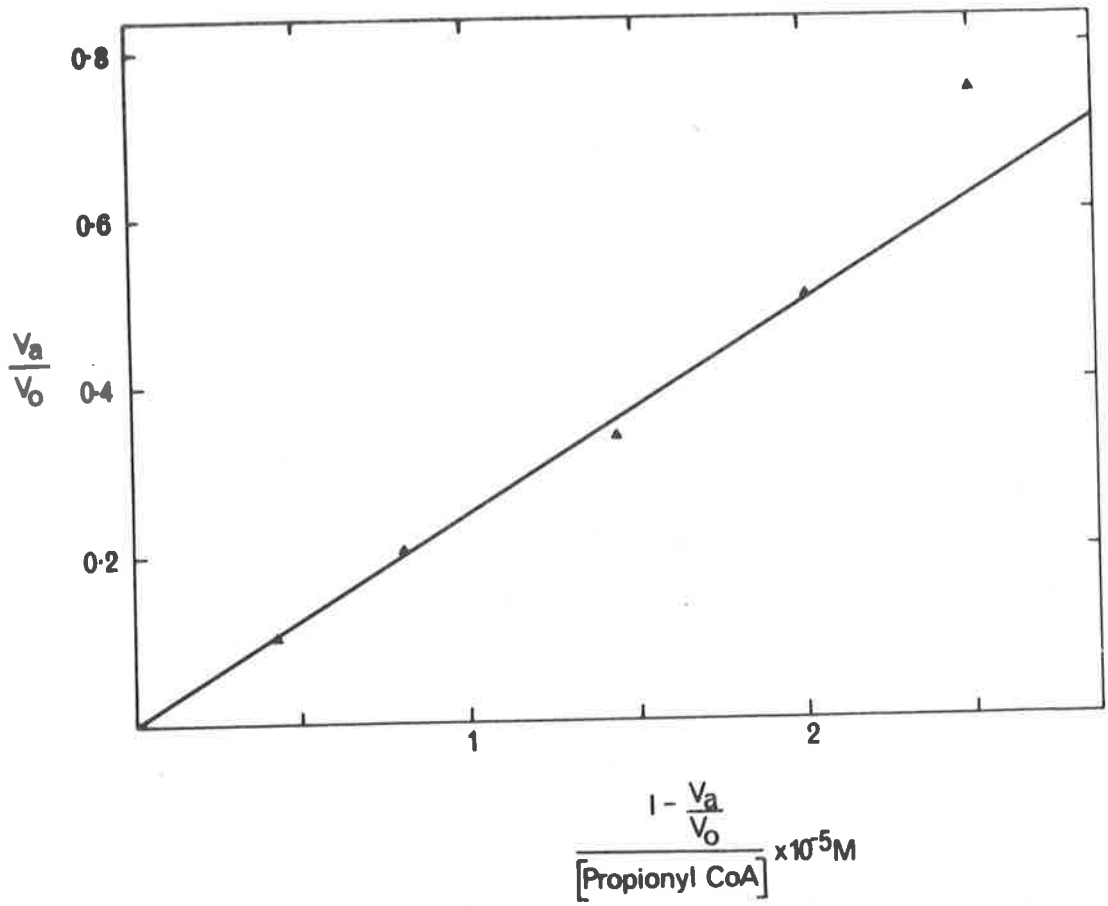

Fig. 4.10. Determination of the K_a (dissociation constant of propionyl-CoA). The $t_{0.5}$ for protected (V_a) and unprotected (V_o) enzyme inhibited as described in Fig. 4.9 were replotted

$$\frac{V_a}{V_o} \quad \text{against} \quad \frac{(1 - V_a/V_o)}{\text{Prop-CoA}}$$

From the fact that the line in Fig. 4.10 passes through the origin it is concluded that N-ethyl maleimide cannot combine with the enzyme-propionyl-CoA complex i.e., k_2 is very small compared with k_1. The dissociation constant, k_d is calculated to be 2.5×10^{-4}M.

This kinetic data in conjunction with the substantial evidence presented above confirms that a cysteine residue provides a point of attachment for propionyl-CoA at the active centre of propionyl-CoA carboxylase.

(6) Isolation of the reactive of cysteine residue in propionyl-CoA carboxylase

From the circumstantial evidence presented by other workers, it was suggested that an amino acid residue, presumably cysteine, was involved in binding propionyl-CoA to the enzyme surface. In order to verify the assumption that the inhibitor, N-ethyl maleimide was specifically reacting with a thiol residue, an experiment was carried out with a view to positively identifying this amino acid. This investigation involves the use of ^{14}C-labelled N-ethyl maleimide which should allow a radioactive inhibitor-amino acid complex to be isolated after degradation of the modified protein.

The labelled enzyme was modified with ^{14}C-N-ethyl maleimide as described in Materials & Methods. After

degradation with Pronase and subsequent chromatography on Sephadex G-10, the radioactive material was subjected to paper electrophoresis. The radioactive areas on the electrophoretogram were located and the migration rate compared with authentic N-ethyl maleimide-cysteine. A diagramatic representation of the electrophoretogram is presented in Fig. 4.11. It should be noted that the major portion of the radioactivity has similar electrophoretic characteristics to the chemically synthesized N-ethyl maleimide-cysteine derivative. A feature of N-ethyl maleimide derivatives is their modification under strong acid conditions causing an opening of the imido ring. This process results in the appearance of two carboxylic acid residues which significantly alter the electrophoretic mobility of this derivative. This characteristic was used to confirm the previous identification, since it could be suggested that radio-active peptides could be contaminating the slowly migrating ^{14}C-N-ethyl maleimide-cysteine complex.

The radio-active material from the previous electrophoretogram was eluted and hydrolysed using 6N-HCl for 72 hr. and the hydrolysate (after removal of the acid by repeated vacuum distillation) was subjected to an electrophoretic analysis as previously described. Fig. 4.12 is a diagram which depicts the radio-active pattern obtained from this electrophoretogram.

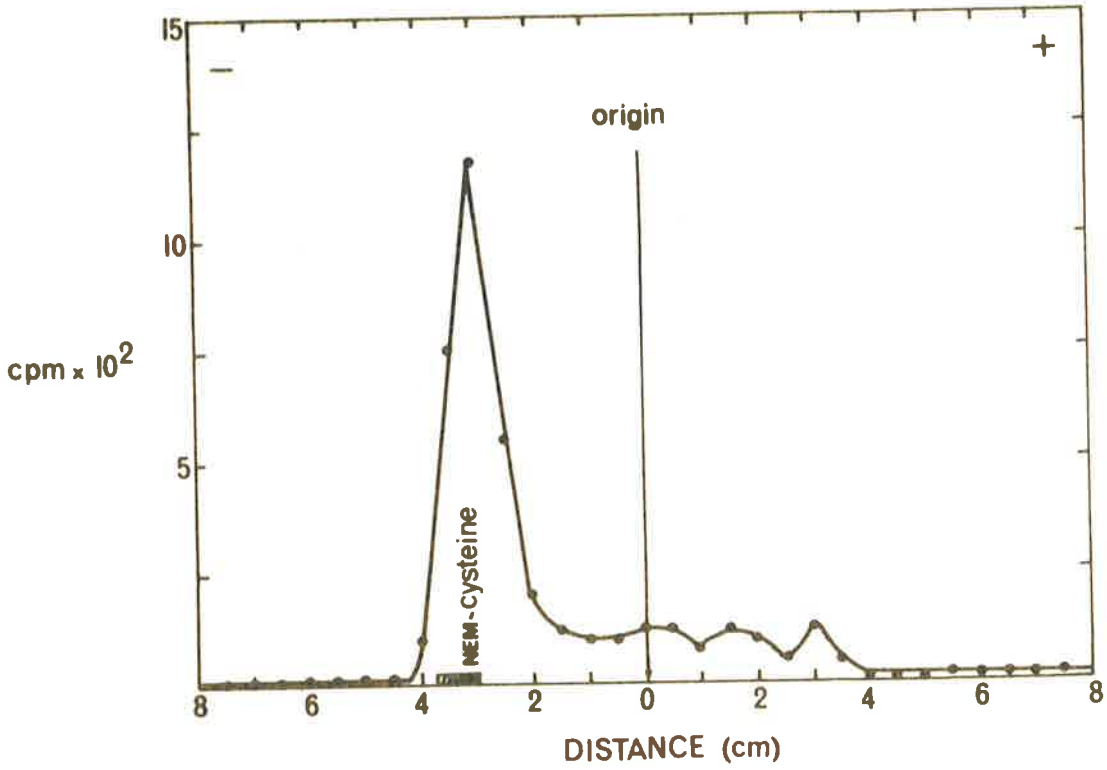

Fig. 4.11. Electrophoresis of pronase digest of ^{14}C-N-ethyl maleimide-enzyme. The labelled enzyme after pronase digestion was subjected to electrophoresis at pH 6.5 as described in Materials & Methods. Fig 4.11 represents the radio-active profile obtained after 0.5 c m strips of the electrophoretogram were counted by scintillation counting. The N-ethyl maleimide-cysteine complex was detected by the ninhydrin spray (Materials & Methods).

The acid degradation product of the N-ethyl maleimide-cysteine complex (i.e. S-(1,2-dicarboxy-ethyl)-L-cysteine) was chemically synthesized from maleic acid and cysteine (see Materials & Methods). The migration of the biologically prepared radio-active compound was compared with the chemically synthesized substance. Fig. 4.12 shows that the radio-active compound has similar electrophoretic characteristics to the chemically synthesized S-(1,2-dicarboxy-ethyl)-L-cysteine.

This evidence in conjunction with substantial kinetic evidence presented above confirms the original tentative suggestion that a cysteine residue is involved in binding propionyl-CoA to the enzyme surface.

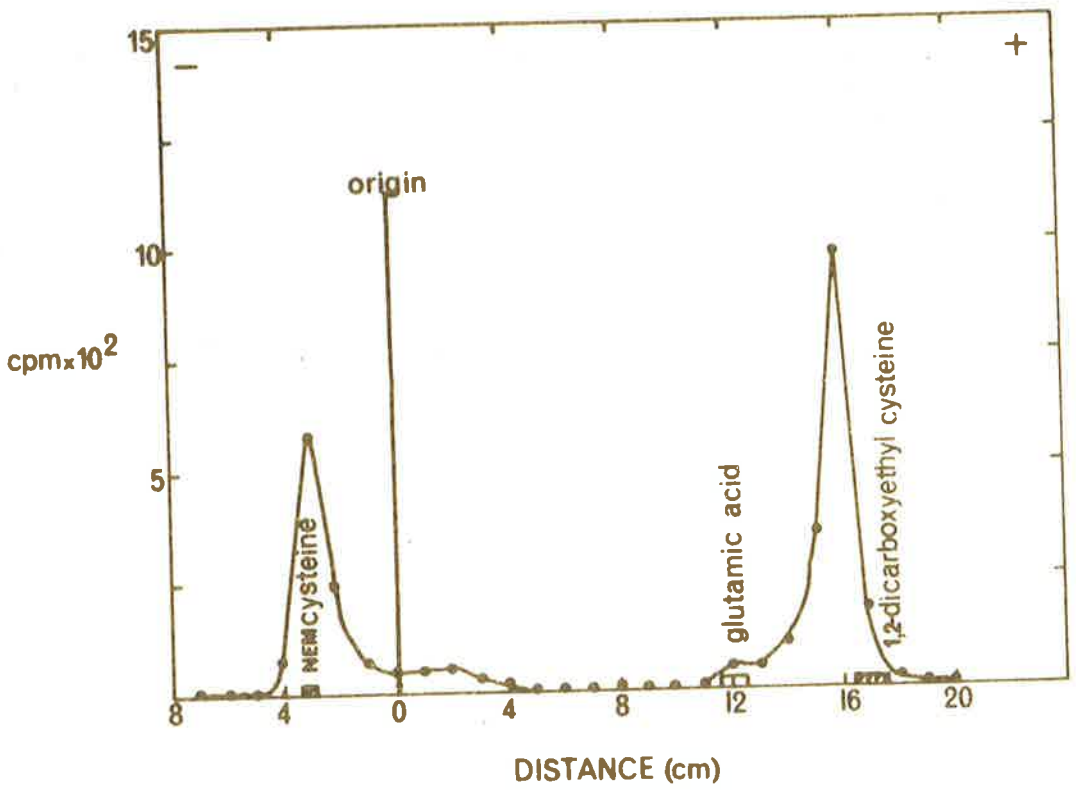

Fig. 4.12 Electrophoresis of the acid hydrolysate of the ^{14}C-N-ethyl maleimide-cysteine complex. The radio-active N-ethyl maleimide-cysteine complex obtained in Fig. 4.11 was hydrolysed for 72 hr. at 110° in 6N-hydrochloric acid. The hydrolysate was analysed by electrophoresis and the radio-active profile examined as described in Fig 4.11 except that 1 c.m. strips were examined. The standard amino acid derivatives were detected by the ninhydrin spray

DISCUSSION:

The important aspects of the investigation described in this section were:

(a) The positive identification of a reactive cysteine residue in propionyl-CoA carboxylase

(b) Assigning a function for cysteine in the reaction mechanism, and

(c) The use of kinetic techniques, particularly on the chemically modified enzyme, as a tool for investigating the chemistry of the active site of the enzyme.

Using both liver and heart as the source of propionyl-CoA carboxylase, Kaziro *et al*. 1960 and Hegre & Lane (1966) first reported that the enzymic activity was susceptible to thiol group reagents. In the present study, the reactivity of the essential thiol group was investigated; it was labelled covalently with ^{14}C-N-ethyl maleimide, isolated from a pronase digest and identified by paper electrophoresis. Thus the kinetic evidence and actual isolation of the reactive residue provided conclusive proof of the cysteinyl group.

The thiol groups of many enzymes are known to react with thiol group reagents yielding catalytically inactive products. In most cases it is not clear whether the thiol group at the active site has been blocked or whether the reaction with a group elsewhere in the protein has caused conformational changes

that have led to an inactive enzyme. Webb (1966) has pointed out that it is difficult to distinguish whether the exposed reaction group,

(a) Is at the active site and functional in the reaction mechanism,

(b) Is at the active site but non-functional,

(c) Is vicinal to the active site, or

(d) Reacts and alters the enzyme structure causing non-specific denaturation.

Proof of the functional role of reactive groups must then come from evidence additional to the inhibition and protection experiments carried out by Kaziro et al. (1960) and Hegre & Lane (1966), since the demonstration of inhibition by thiol group reagents and protection by a substrate indicates at best that one or more cysteine residues are sufficiently near the active site to influence the catalysis. In the present investigation several kinetic approaches have been used to implicate and assign a function for the essential thiol residue in the reaction mechanism of propionyl-CoA carboxylase.

The protection experiments of Hegre (1964) and Kaziro et al. (1960) indicated that propionyl-CoA rather than either ATP or HCO_3^- was involved in reacting with the cysteine residue. In the experiments reported here, kinetic evidence was obtained to show that the thiol group reagent, N-ethyl maleimide, could

not react with the propionyl-CoA-enzyme complex thus confirming the previous tentative evidence. Support for this conclusion was obtained from the pK_m/pH profiles (using propionyl-CoA as the variable substrate) which showed that the propionyl-CoA-enzyme complex has a pK_a at pH 8.2. This value coincided with a change in the rate of inactivation of the enzymic activity by N-ethyl maleimide at varying pH values. Final proof was obtained by showing that the chemical modification of the enzyme increased the apparent K_m value for propionyl-CoA but not for the other two substrates.

Cecil (1963) has pointed out that the high reactivity of the thiol groups in enzymes has led investigators to postulate their participation at the active site. In very few instances, however, has it been shown that the thiol group forms part of the active site and becomes involved in the catalytic sequence. Some of these examples are described in the following paragraphs.

The reaction of acetyl-CoA with the fatty acid synthetase resulted in the formation of an acetyl-enzyme (Lynen, 1962). Furthermore, he suggested that the acetyl group is transferred from CoA to a thiol group bound to the enzyme. However, recently it has been shown that two thiol residues have roles in the multi-enzyme complex. Firstly, a central thiol group which seems to be due to 4'-phospho-pantetheine and secondly, a peripheral thiol group which seems to be a cysteinyl residue (Lynen, 1967). Apparently the acetyl-moiety is transferred

from the central to the peripheral group in the course of the reaction.

Glyceraldehyde-3-phosphate dehydrogenase can react with acetyl phosphate to form an acetyl-enzyme (Krimsky & Racker, 1955). By measurement of the number of thiol groups reacting with p-hydroxy-mercuri-benzoate before and after the acylation, it was inferred that the acetylation site was a thiol residue (Koeppe, Boyer & Stalberg, 1956).

Smith (1958) proposed that the reaction mechanism of papain involved an acylation of an essential thiol group. However, his scheme involved an internal thiol ester which was not consistent with the ease of inhibition by thiol group inhibitors. The original scheme was then modified to include a free thiol group and aspartic acid residues which provide a change distribution favourable for this reaction (Hill & Smith, 1960).

These three examples would indicate that cysteine residues are functional for the formation of acyl-enzyme complexes. Indeed, Cecil (1963) has suggested that in those enzymes where the thiol group is operating at the active centre and not involved in maintaining the configuration, an acyl-enzyme complex should be suspected. However, Hegre (1964) tried unsuccessfully to show that an acyl-propionyl-CoA carboxylase was

formed. Also Virden & Watts (1966) concluded that at least one of the five thiol groups of arginine kinase is essential for activity and it probably forms part of the catalytic mechanism. However, the involvement of an acyl-enzyme was not reported. It, therefore, seems that cysteine residues can operate at the active centres of enzymes without being required for acyl-enzyme formation. At present, the group on the propionyl-CoA molecule interacting with the cysteine residue has not yet been identified. Preliminary evidence by Hegre (1964) would suggest that the functional group resides in the propionyl-pantetheine moiety of propionyl-CoA.

In this study, evidence was presented to indicate that an essential thiol group was required to bind propionyl-CoA to the enzyme. The data required to define such a role came from a study of the kinetic constants of both the native and the chemically modified enzyme. This rationale can be illustrated, in what is obviously an oversimplification, by considering a simple one substrate enzymic reaction:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{products} \qquad \ldots (4.8)$$

$$\text{The apparent } K_m = \frac{k_2 + k_3}{k_1} \qquad \ldots (4.9)$$

$$\text{and the apparent } V_{max} = k_3 [E] \qquad \ldots (4.10)$$

If a binding amino acid, i.e. an amino acid involved in forming ES, is modified so that the rate constant, k_1, is reduced, then K_m will increase. That is, the affinity for the substrate will be decreased. The V_{max} will not be altered. The present studies showed that the affinity of the enzyme for propionyl-CoA decreased as the modification of the enzyme increased (Fig. 4.8).

However, if a catalytic residue, i.e. an amino acid involved in breaking down the ES complex, is modified so that the rate constant, k_3, is reduced, then both K_m and V_{max} will be reduced since both expressions contain the term, k_3. This situation could yield parallel double reciprocal plots for the native and modified enzymes. Since propionyl-CoA carboxylase did not yield plots of this kind, theoretical plots were prepared by assigning values to k_1, k_2 and [E] (which were kept constant) while k_3 was varied. It can be seen from Fig. 4.13 that, under certain conditions, pseudo-parallel lines could be obtained, thus supporting this hypothesis.

The change in slope when either HCO_3^- or MgATP was the variable substrate (Figs. 4.6 & 4.7) is not explained by changes in k_1 or k_3. Therefore, it was assumed that the observed decrease in the V_{max} values was due to the fact that the propionyl-CoA concentration in the assay solution was no longer at saturating levels with respect to the modified enzyme. A similar result would be obtained if the inhibition denatured

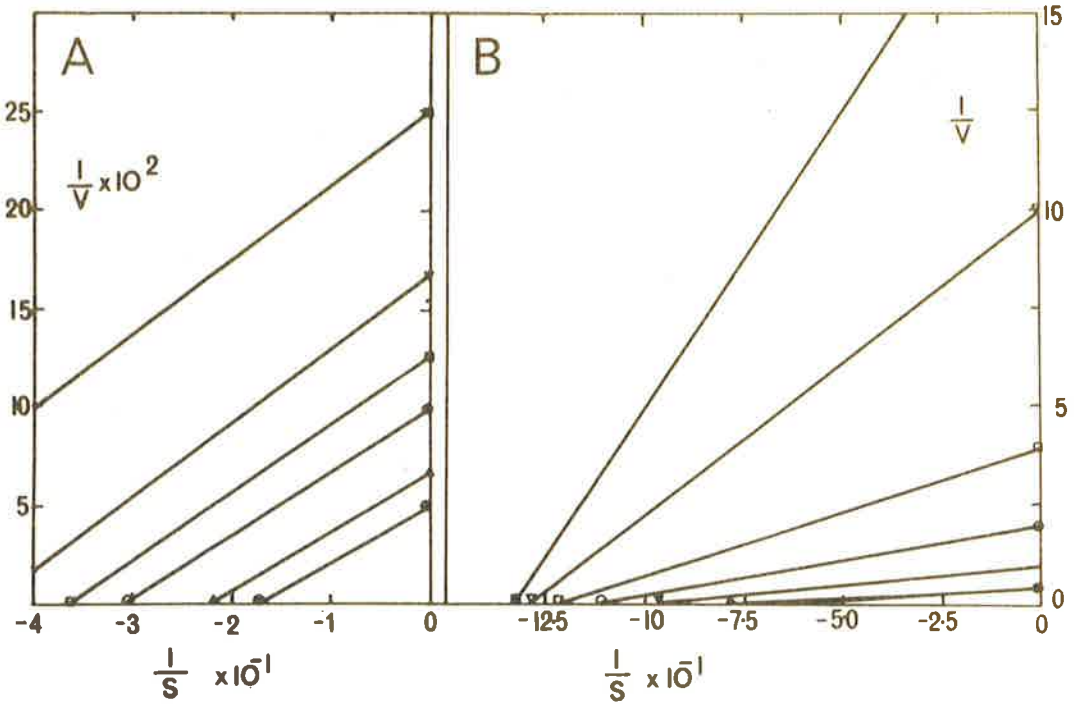

Fig. 4.13. Theoretical Lineweaver-Burk plots for various values of "k_3". The K_m and V_{max} values were determined for various values of k_3 whilst the other rate constants, k_2 and k_1 together with [E] were kept constant. The values of k_1 and k_2 were 3 and 4 respectively and the values for k_3 were 20 (●—●); 10 (o—o); 8 (□—□); 6 (▼—▼); 4 (■—■) in graph A and k_3 = 2.0 (■—■); 1.0 (▽—▽); 0.5 (□—□); 0.25 (o—o); 0.1 (▼—▼); and 0.05 (●—●) in graph B.

the enzyme non-specifically. In this case the total active enzyme concentration would decrease, thus decreasing V_{max} without affecting K_m. However, in the case presented in Fig. 4.8 the V_{max} is dependent on propionyl-CoA concentration and therefore non-specific denaturation is not considered as a likely explanation for the decreased V_{max} with HCO_3^- and MgATP as the variable substrates.

Although experimental support for the above concepts is not as yet well developed, some data from this laboratory supporting these hypotheses is worthy of comment. Keech & Farrant (1967) using sheep kidney pyruvate carboxylase have shown that a lysine residue is essential for binding the allosteric activator, acetyl-CoA. It would be expected that modification of this residue with dinitrofluoro-benzene would effect only the V_{max} but not the binding of the other substrates. This was the case when HCO_3^- and pyruvate were the variable substrates but not when MgATP was the variable substrate. Variation of this substrate resulted in a series of parallel lines as modification increased indicating a change in k_3 for the breakdown of the E-MgATP complex. This was interpreted to indicate that the binding of acetyl-CoA to pyruvate carboxylase induced changes in the enzyme which reduced the rate of E-MgATP hydrolysis.

C H A P T E R F I V E

THE ROLE OF POTASSIUM IN THE PROPIONYL-CoA CARBOXYLASE REACTION

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INTRODUCTION:

In a recent review concerning the effect of univalent cations on the various enzyme systems, Evans & Sorger (1966) emphasise that the monovalent cations may either activate or stimulate a fixed reaction rate. For example, pyruvate kinase exhibits an absolute requirement for K^+ ions (Boyer, Lardy & Phillips, 1942) while the malic enzyme isolated from Lactobacillus arabinosus is active in the absence of K^+ but is stimulated by its presence (Nossal, 1952). Almost without exception, where K^+ ions stimulate or activate enzyme systems, they can be replaced by either Rb^+ or NH_4^+ ions. The molar concentrations of the cations required for maximal activity in most cases is high compared with the corresponding molar concentration of the substrate for the particular enzyme. For example, acetaldehyde dehydrogenase requires for maximal activity; 0.05M-K^+ salt, 0.00017M-acetaldehyde, and 0.0005M-NAD (Black, 1951). These generalisations, and the fact that the reactions involving univalent cations are chemically unrelated makes it difficult to conceive a general mechanism of action involving activated complexes of substrates or cofactors or both, and univalent cations.

Nevertheless, some proposals have been advanced to explain the activation processes induced by the various monovalent cations. Chemical models of Na-ATP and K-ATP complexes

show that the Na^+ ion is completely buried in the complex molecule but, in contrast, K^+ is almost completely exposed (Melchior, 1954). This author concluded that certain enzymes may catalyse reactions involving the K-ATP complex but fail to catalyse reactions involving Na-ATP because of the steric considerations. Lowenstein (1960) proposed a trimer relationship between the nucleotide, divalent and monovalent cations. In agreement with Melchior (1954), Lowenstein proposed that differences in the shape of the univalent cation-nucleotide-divalent cation complex might affect the rate of the reactions catalysed by enzymes. However, these proposals have not received general acceptance because, (a) not all K^+ ion activated systems have nucleotide substrates, and (b) for maximum activation, the concentration of the K^+ ions is much greater than the other substrates.

Recently, Neujahr & Mistry (1963) and Neujahr (1963) showed that the enzyme activity of propionyl-CoA carboxylase was stimulated by monovalent ions. Cs^+ and Rb^+ ions were the most effective univalent cation activators although NH_4^+ and K^+ ions were also able to stimulate enzymic activity. However, since all assay solutions used by previous investigators including those of Neujahr & Mistry (1963) were carried out in the presence of either Na^+ or K^+, it has not been possible to ascertain from the literature whether the enzyme possesses an absolute requirement for monovalent cations or whether the cation simply

stimulates a basal enzymic activity.

The investigation presented in this chapter was carried out to ascertain;

(a) Whether the enzyme possessed an absolute requirement for monovalent cations or whether the cation simply stimulated a basal activity,

(b) the locus of action of the cation in the reaction mechanism, and

(c) The mechanism by which the cation exerts its effect.

To ensure that the level of univalent cation contamination of the enzyme preparation and the reagents used in the assay mixture was at a very low level, purification of all reagents was carried out as outlined in Materials & Methods.

RESULTS:

(1) K^+ ion stimulation.

Although every effort was made to ensure that alkali metal-free reagents were used in the preparation of the enzyme and the assay solutions, it was not possible to demonstrate a univalent cation requirement. However, a 1.6-fold stimulation of activity was consistently observed. To exclude the possibility that the anion of the K^+ salt i.e., the chloride ion, was responsible for the stimulation, the effect of various anions of potassium salts was examined. The results presented in Table 5.1 indicate that there was no difference in enzymic activity whatever univalent anion was added with the K^+ although SO_4^{2-}, a divalent anion, caused a 50% loss of activity.

In order to demonstrate that the observed stimulation was not confined to K^+ ions, various univalent cations were used to replace the K^+ ion. Fig. (5.1) represents the data obtained in the form of double reciprocal plots, where the reciprocal of the reaction velocity is plotted as a function of the reciprocal of the cation concentration. It can be seen that the apparent V_{max} for each cation was identical. Therefore, a comparison of various cations based on a single velocity measurement at a single metal ion concentration is not a valid procedure. The apparent K_m values and affinity constants i.e., the reciprocal of the apparent K_m value for each of the cations was determined and the results are summarised in Table 5.2. The order of

TABLE 5.1

Effect of anions on the propionyl-CoA carboxylase reaction.

ANION	ACTIVITY %
Chloride	100
Iodide	99.7
Chlorate	99.0
Acetate	98.9
Sulphate	54.0

The reaction mixture was as described in Fig. 5.1. The concentration of anion was 8×10^{-3}M.

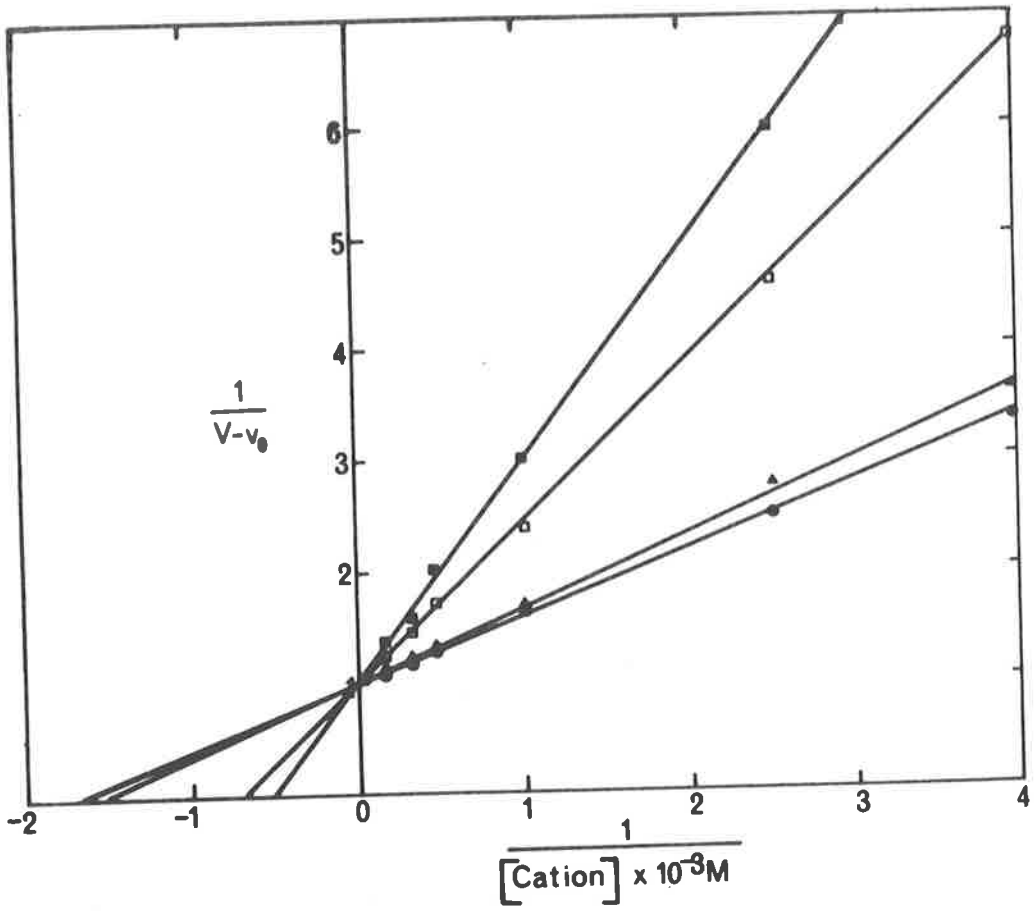

Fig. 5.1. Determination of the apparent K_m and V_{max} values for Rb^+, K^+, NH_4^+ and Cs^+. The velocity of the reaction in the absence and presence of the activating cations (V_o) and (V) respectively was calculated. The reciprocal of $1/V-V_o$ was plotted against the reciprocal of the cation concentration. Rb^+ (●——●); K^+ (▲——▲); NH_4^+ (□——□) and Cs^+ (■——■). The reaction mixture (final volume 0.5 ml.) contained in μmoles: N-ethyl morpholine HCl buffer, pH 8.0, 100; ^{14}C-N-ethyl morpholine bicarbonate (2.2×10^5 c.p.m. per μmole), 5.0; propionyl-CoA, 0.5; N-ethyl morpholine glutathione, 0.25; Mg^{2+}, 3.0 and N-ethyl morpholine-ATP^{4-}, 1.5.

TABLE 5.2

Kinetic constants of activating cations.

Metal	Apparent K_m ($\times 10^{-4}$M)	Affinity constant
Rb^+	5.9	1,695
K^+	6.6	1,515
NH_4^+	14.6	685
Cs^+	19.6	510

The data summarised in this table were obtained from Fig. 5.1. Experimental conditions were the same as those described in Fig. 5.1.

affinity for the binding of the cations was Rb^+, K^+, NH_4^+ and Cs^+. Na^+ ions did not stimulate the enzyme in the experiments where it was used.

(2) Time course of activation by K^+ ions.

The activation of some enzymes by metal ions has been shown to be time dependent. For maximum activation of threonine dehydrogenase, preincubation for 24 hr. with K^+ ions was required (Green, 1964). The K^+ ion stimulation of acetaldehyde dehydrogenase (Black, 1951) showed a 10-30 sec. lag period. Apart from the univalent cations, the slow activation of certain peptidases by Mn^{2+} (Smith, 1951), and of arginase by Mn^{2+} and Co^{2+} (Mohammed & Greenberg, 1945) have been demonstrated. Since any postulated mechanism of K^+ ion stimulation of propionyl-CoA carboxylase would require a knowledge of the time course of the process, this aspect was investigated. The enzyme was placed in two separate reaction mixtures containing high specific activity $^{14}C\text{-}HCO_3^-$, one solution containing K^+ ions and the other without this cation. At 15 sec. intervals, aliquots were removed using a Hamilton press-button syringe. The enzymic reaction was stopped by addition of the standard aliquot to measured volumes of 10% cold aqueous TCA, and the fixed $^{14}C\text{-}CO_2$ was determined.

The results (Fig. 5.2) indicate that the activation process proceeded at a rate too fast to be measured by this

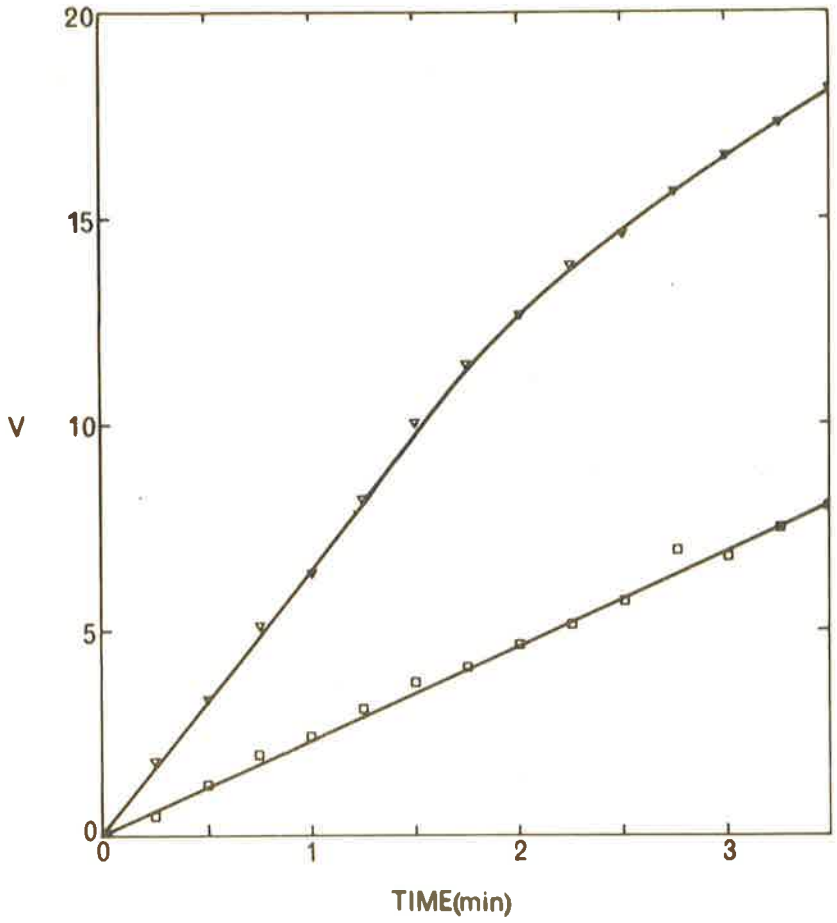

Fig. 5.2. Time of activation. The velocity of the propionyl-CoA carboxylase reaction in the presence (▽——▽) and absence of 8×10^{-3} M-K^+ (□——□) plotted as a function of time. Aliquots of the assay mixture were taken at 15 sec. intervals using a Hamilton press button syringe and the reaction stopped with 0.5 volumes of 5% trichloro-acetic acid. The assay mixture as described in Fig. 5.1 was used including N-ethyl morpholine bicarbonate (2.0×10^6 c.p.m per µmole) and 0.2 units of enzyme.

particular type of experiment, i.e., the K^+ activated enzyme reaction rate curve when extrapolated to zero time intersected the time scale at the same point as the rate curve for the K^+-free system. Furthermore, even the earliest time sample showed a 1.6-fold stimulation of activity indicating that the stimulation had reached a maximum within 15 sec. The increase in activity remained constant throughout the time of the experiment.

(3) Effect of K^+ ions on the kinetic constants.

The addition of K^+ ions to various enzyme systems has resulted in a number of different effects. In some cases the V_{max} value was effected while in others, the cation caused an alteration in the apparent K_m value for one or more of the substrates (Evans & Sorger, 1966). Therefore, the investigation into the mode of K^+ ion stimulation on propionyl-CoA carboxylase was extended to include the effects of the activation on the kinetic constants of the substrates in the presence and absence of K^+ ions.

Double reciprocal plots were obtained using propionyl-CoA, $MgATP^{2-}$ and HCO_3^- as the variable substrates respectively, in the presence and absence of K^+ ions (Figs. 5.3, 5.4 & 5.5). The apparent K_m and V_{max} values were calculated from a computer analysis of the hyperbolic curves and the values obtained are summarised in Table 5.3.

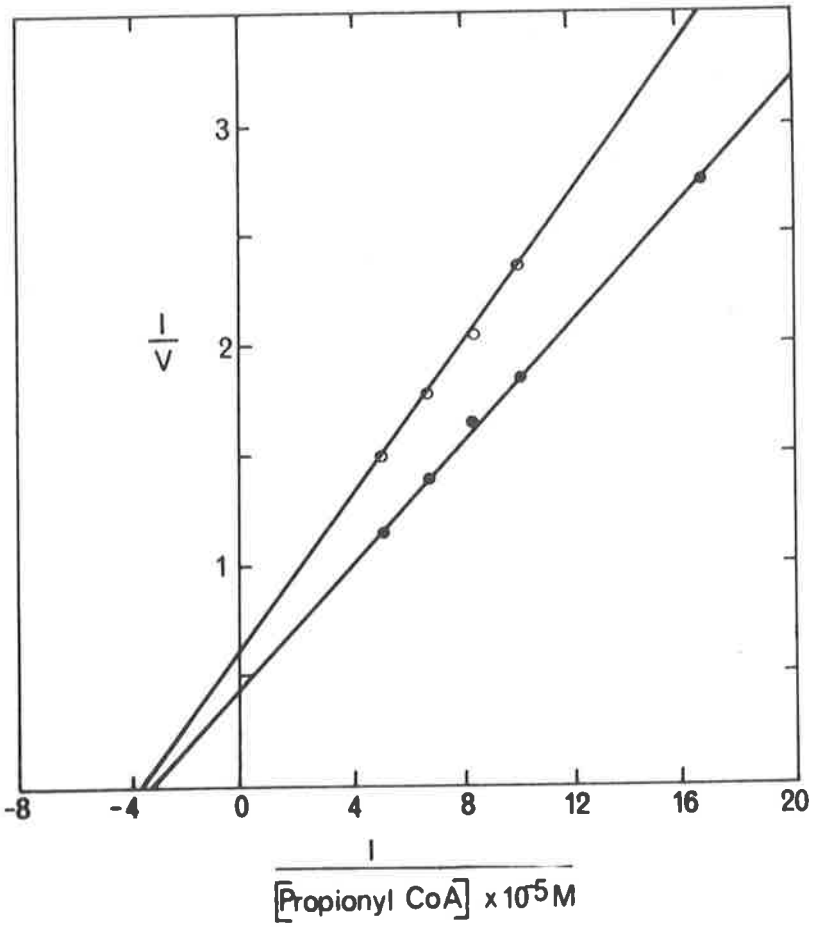

Fig. 5.3. The determination of the apparent K_m and V_{max} for propionyl-CoA in the presence and absence of K^+.

The reciprocal of the velocity was plotted against the reciprocal of the propionyl-CoA concentrations in the absence (o—o) and presence of 8 x 10^{-3}M-K^+ (●—●). The reaction mixture was as described in Fig. 5.1.

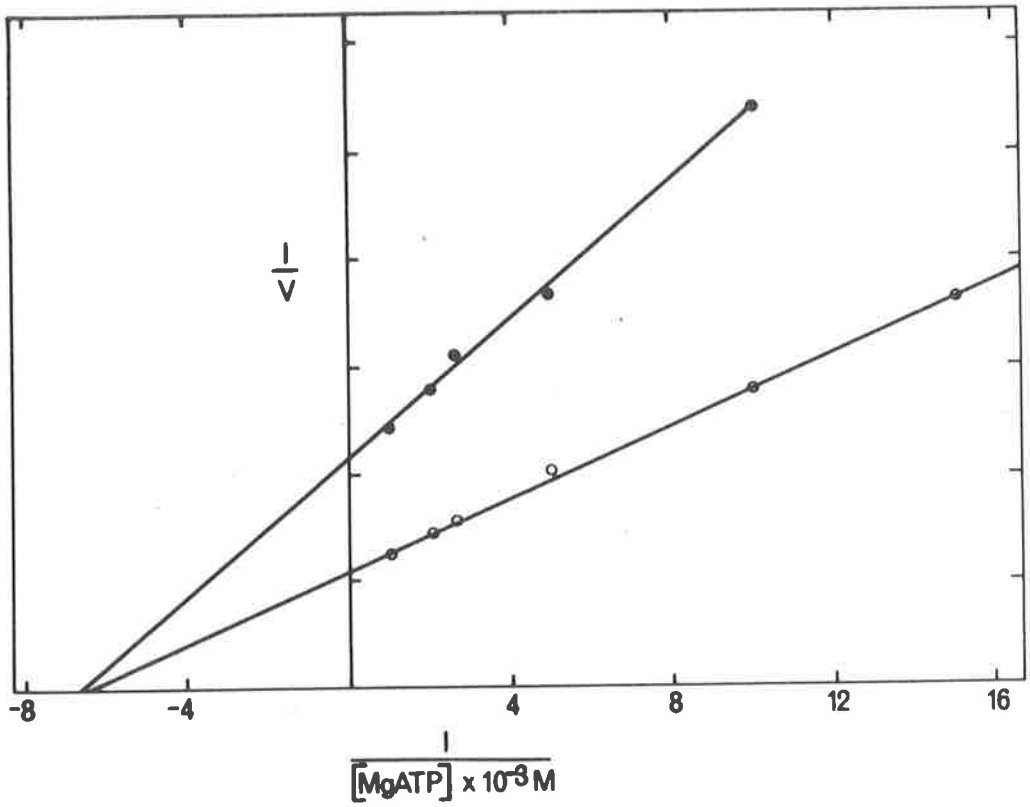

Fig. 5.4. Determination of the apparent K_m and V_{max} for $MgATP^{2-}$ in the presence and absence of K^+. The reciprocal of the velocity was plotted against the reciprocal of the $MgATP^{2-}$ concentration in the absence (●—●) and presence of 8×10^{-3}M-K^+ (o—o). The reaction mixture was as described in Fig. 5.1.

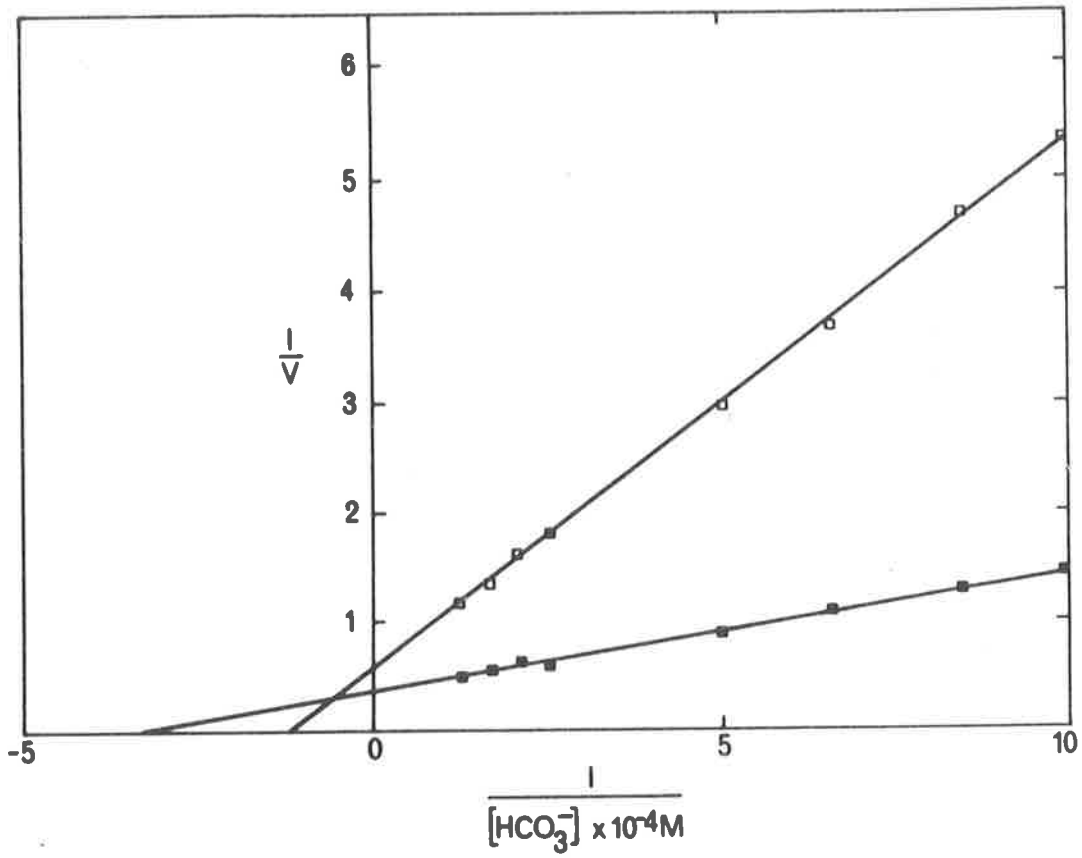

Fig. 5.5. Determination of the apparent K_m and V_{max} for HCO_3^- in the presence and absence of K^+. The reciprocal of the velocity was plotted against the reciprocal of the HCO_3^- concentration in the absence (□—□) and presence of 8×10^{-3} M-K^+ (■—■). The reaction mixture was as described in Fig. 5.1.

TABLE 5.3

Effect of K^+ ions on the kinetic constants of the reaction components

Substrate	With K^+		Without K^+	
	app. K_m	app. V_{max}	app. K_m	app. V_{max}
$MgATP^{2-}$	1.5×10^{-4}M	9,000	1.5×10^{-4}M	4,655
Propionyl-CoA	2.7×10^{-4}M	2,200	2.48×10^{-4}M	1,612
HCO_3^-	3.0×10^{-3}M	2,855	8.0×10^{-3}M	1,667

Data summarised in this table was obtained from Figs. 5.3, 5.4 and 5.5. Experimental conditions were the same as those described in Fig. 5.1.

The apparent K_m value for propionyl-CoA was similar to the value obtained by Tietz & Ochoa (1959) using the spectrophotometric assay method and of similar magnitude to that obtained by Halenz et al. (1962) using the enzyme isolated from beef liver. The stimulation observed in the presence of K^+ ions did not result in a significant change in the apparent K_m value. The same type of result was obtained when $MgATP^{2-}$ was the variable substrate (Fig. 5.4) i.e., the apparent K_m value was not altered while the apparent V_{max} almost doubled. It should be noted that the apparent K_m value for $MgATP^{2-}$ is different to that obtained by Tietz & Ochoa (1959). The difference could be due to the fact that these investigators plotted ATP concentrations (using excess free Mg^{2+} in the reaction mixture), whereas in this experiment the $MgATP^{2-}$ complex was used as the variable substrate.

In contrast to the other substrates, the presence of K^+ ions had a marked effect on the apparent K_m value for HCO_3^-. This value changed from 8.0×10^{-3}M to 3.0×10^{-3}M. The site of action of allosteric acetyl-CoA on the yeast pyruvate carboxylase (Cooper & Benedict, 1966) is also at the HCO_3^- binding site since the K_m value for HCO_3^- decreased 7-fold in the presence of acetyl-CoA.

(4) Effect of K^+ ions on the tertiary structure of the enzyme.

The data presented in the previous section showed that at least one of the effects of the K^+ ion on propionyl-CoA

carboxylase was to reduce the concentration of HCO_3^- required to half saturate the enzyme. A change of this magnitude in the kinetic constant for one of the substrates has been taken to indicate a protein conformational change. For example, Cooper & Benedict (1966) using yeast pyruvate carboxylase showed that acetyl-CoA lowered the apparent K_m value for HCO_3^- and concluded that the allosteric activator caused a change in the tertiary structure of the protein. However, kinetic evidence for conformational changes can only be circumstantial. More definitive evidence can be obtained from thermodynamic data and therefore, an attempt was made to measure the entropy change due to the presence of univalent cations.

Hill plots for K^+ over the temperature range from 20^o to 40^o are presented in Fig. 5.6. Since the slopes of the lines over the whole temperature range never exceeded a value of 1, it is assumed that there is only one binding site for K^+ at the active centre. Support for this conclusion comes from the fact that the enzyme exhibits classical Michaelis-Menten kinetics. Therefore, the reaction between the enzyme and cation can be written as:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_3} E + \text{products} \qquad \ldots (5.1)$$

In systems exhibiting Michaelis-Menten kinetics, the apparent K_m value is equal to $k_2 + k_3/k_1$. However, since the enzyme-potassium complex cannot break down to enzyme and products, then

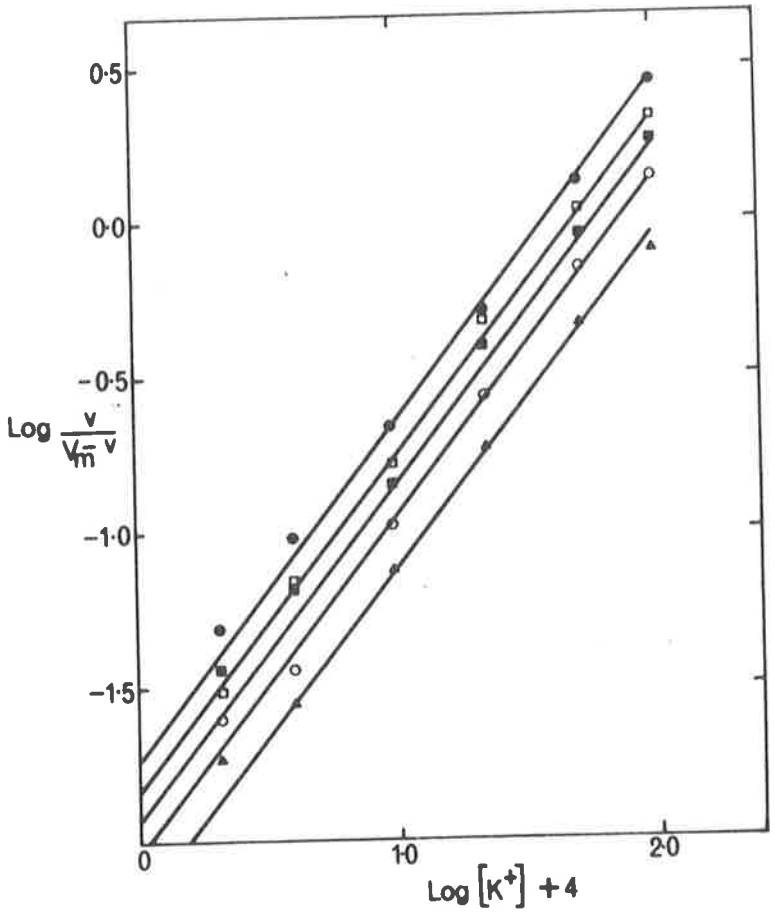

Fig. 5.6. Calculation of the $\bar{n}$ value for K^+ at various temperatures. Log v/V_m-v was plotted against the K^+ concentration at various temperatures. V_{max} was determined from Lineweaver-Burk plots similar to those described in Fig. 5.1. Temperatures were: 40° (●—●); 35° (□—□); 30° (■—■); 25° (○—○); and 20° (▲—▲).

$k_3 = 0$. That is, the apparent K_m value for potassium is k_2/k_1, which is also the dissociation constant for the enzyme-potassium complex.

As can be seen from Table 5.4., the apparent K_m value for potassium decreases with increasing temperature. From this data, other thermodynamic properties of the K^+ stimulation can be determined.

The use of enthalpy (ΔH) and entropy changes (ΔS) as aids in the understanding of biochemical reactions is illustrated by a consideration of temperature dependence on the denaturation of proteins e.g. for chymotrypsin the ΔS is 438 e.u. per mole (entropic units); trypsin, ΔS is 180 e.u. per mole and for trypsin inhibitor, ΔS is 213 e.u. per mole (Mahler & Cordes, 1966). These entropy changes are exceedingly large - the ΔS values for most chemical reactions falling in the range +10 to -30 e.u. per mole, and thus emphasises in a dramatic fashion, the degree of structural organisation of these proteins in the native state and the loss of this organisation in the thermal denaturation process.

The standard entropy change;

$$\Delta S = \frac{\Delta H - \Delta F}{T} \qquad \ldots (5.2)$$

where ΔH is the standard enthalpy change and ΔF is the change in free energy. ΔH can be evaluated from measurements of the

TABLE 5.4

Thermodynamic properties of the K^+ activation.

H = 10,067 cals. per mole

Temp.	App. K_m	ΔF	ΔS
	(x 10^{-4}M)	(cals. per mole)	(e.u.)
20°	11.5	- 3,927	47.76
25°	9.2	- 4,305	48.23
30°	6.6	- 4,394	47.73
35°	4.9	- 4,648	47.78
40°	4.5	- 4,777	47.42

Experimental conditions as described in Fig. 5.1.

dissociation constant as a function of temperature, i.e.

$$\ln K = -\frac{\Delta H}{RT} + \text{constant} \qquad \ldots (5.3)$$

A plot of log K (Fig. 5.7) against the reciprocal of the absolute temperature yielded a straight line with slope, $-\Delta H/2.3R$, from which H was calculated to be 10.1 kcal. per mole. The change in free energy, ΔF, is derived from the following equation:

$$\Delta F = -RT \ln K \qquad \ldots (5.4)$$

This value, ΔF, together with H can be substituted in equation 5.2 to derive ΔS. Table 5.5 summarises the data obtained from a series of experiments. In particular it should be noted that the standard entropy change, ΔS was 47.8 e.u. per mole. Since the range of values for most chemical reactions is much less than this figure, the values presented in Table 5.5 predict a significant conformational change in the protein.

Two other examples are known where thermodynamic evidence has been used to support kinetic evidence for conformational changes. For example, Taketa and Pogell (1965) studied the inhibition of fructose-1-6, diphosphatase by AMP and obtained a ΔS value of -99 e.u. per mole. Worcel (1966) investigated the activation of NADH dehydrogenase by AMP and showed that the ΔS value was +57 e.u. per mole. In each case the authors concluded that the allosteric ligand produced a significant change in the tertiary structure.

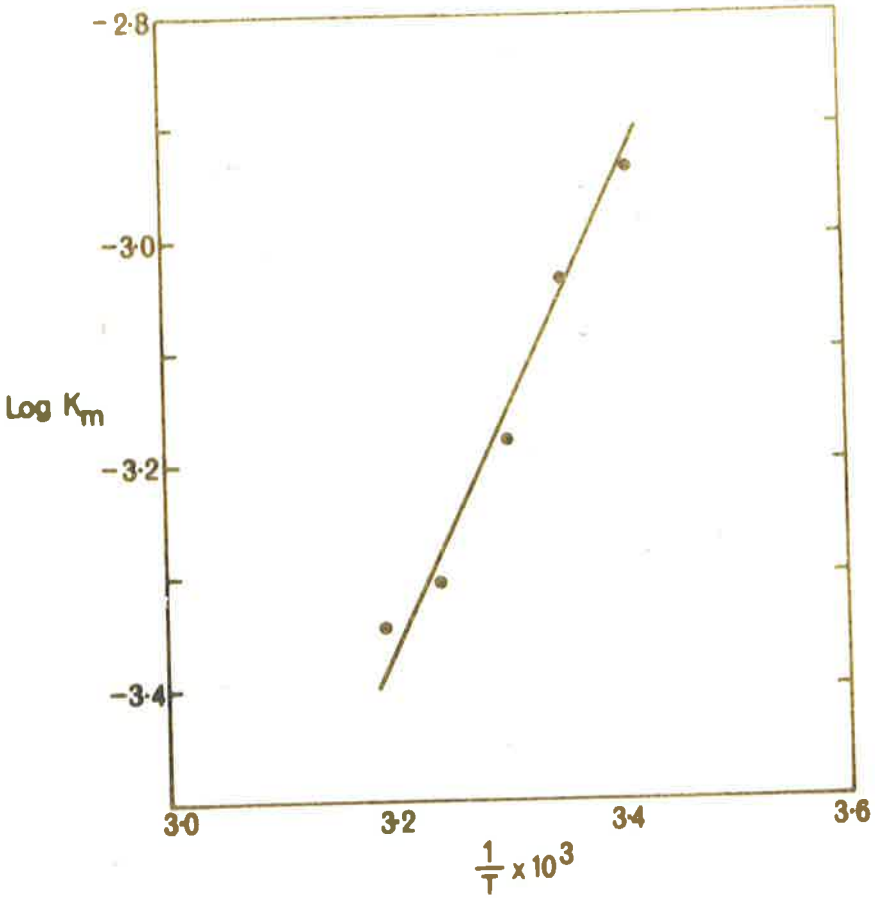

Fig. 5.7. Determination of the ΔH of the activation of propionyl-CoA carboxylase by K^+ Log K_m was plotted against the reciprocal of the absolute temperature. The apparent K_m for K^+ was determined as described in Fig. 5.1 except that the temperature was varied as described. The slope of the line was calculated from the method of least squares.

(5) Supporting evidence for a K^+ induced conformational change.

Conformational changes in proteins induced by different perturbations can be investigated by a number of techniques. Kayne & Seulter (1965) observed a difference in the absorbancy in the 280 to 300 mμ region between pyruvate kinase incubated with and without a combination of activating univalent and divalent cations. They concluded that although different conformational states of the enzyme existed and depended on the cationic environment, the spectral changes in the enzyme resulting from the addition of cations may not be related to the active forms of the enzyme.

Immuno-electrophoretic behaviour of pyruvate kinase is also markedly changed by manipulating the cationic environment (Sorger, Ford & Evans, 1966). Richards & Rutter (1961) and Rothstein & Enns (1964) have reported that similar conditions exist with the two mammalian aldolases, one of which requires univalent cations for activity while the other does not. Deuterium exchange is another technique which has been used to indicate changes in the tertiary structure of proteins but like the other physical methods it has the disadvantage in that, although changes may occur, they often cannot be related to biological activity (Stracher, 1960).

In the case of propionyl-CoA carboxylase, spectral changes in the ultraviolet region in the presence and absence

of K^+ ions were not observed. Several explanations are possible to account for this lack of a change i.e.

(a) Spectral changes can only be expected if the environment around aromatic residues is altered, and

(b) Since the molecular weight of the enzyme is so high, it is possible that changes affecting one or two aromatic residues would represent a very small percentage of the total so that a change would not be expected.

With these difficulties in mind, it was decided that confirmatory evidence for a change in tertiary structure due to K^+ ions should be obtained by utilising some other property of the enzyme. Therefore, the rate of inactivation of the enzyme by N-ethyl-maleimide, in the presence and absence of K^+, was investigated. The data obtained is presented in Fig. 5.8 where it can be seen that the presence of K^+ ion increased the rate of inactivation of the enzyme. It should be pointed out that the difference in the two inhibition rates is not very great but this may be expected when it is considered that this inhibition is, in the main, an expression of the behaviour at the propionyl-CoA site.

To confirm this result, the experiment was repeated using ^{14}C-N-ethyl maleimide as the thiol blocking reagent. The radio-activity fixed to the protein was determined by isolation of the enzyme-^{14}C-inhibitor complex as described in

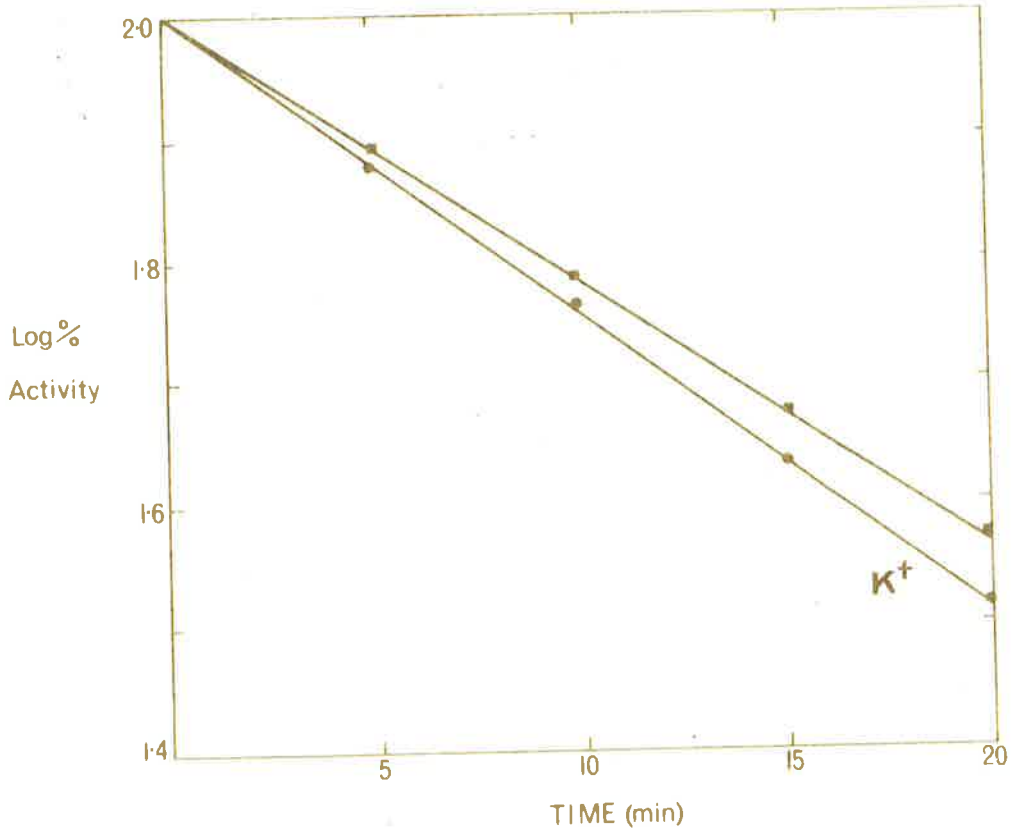

Fig. 5.8 Effect of K^+ on the rate of inhibition of propionyl-CoA carboxylase by N-ethyl maleimide. Propionyl-CoA carboxylase (0.2 units) was incubated at 22° with 2×10^{-4}M-N-ethyl maleimide (final volume 0.5 ml.) in 0.04M-tris-HCl buffer, pH 8.2 with and without 8×10^{-3}M-K^+. Aliquots of the enzyme (0.05 ml.) were removed at the various time intervals and assayed using the normal assay method except that 1×10^{-2}M reduced glutathione and 1% (W/V) bovine serum albumen were also present. Log % activity was plotted against time.

Materials & Methods. However, in this case the membrane filters were dried and the radio-activity determined by scintillation counting. Fig. 5.9 depicts the results obtained from this experiment. The presence of K^+ increased the amount of radio-active inhibitor fixed to the enzyme. Since in this situation, the fixed radio-activity could be due to binding at essential and non essential residues, the effect of K^+ may be expected to be greater compared to the result from the previous experiment (Fig. 5.8) where the reactivity of an essential residue was being observed. This particular approach i.e. inhibition studies, was used to provide support for the hypothesis that a conformational change is experienced by acetaldehyde dehydrogenase in the presence of univalent cations (Stoppani & Milstein, 1957).

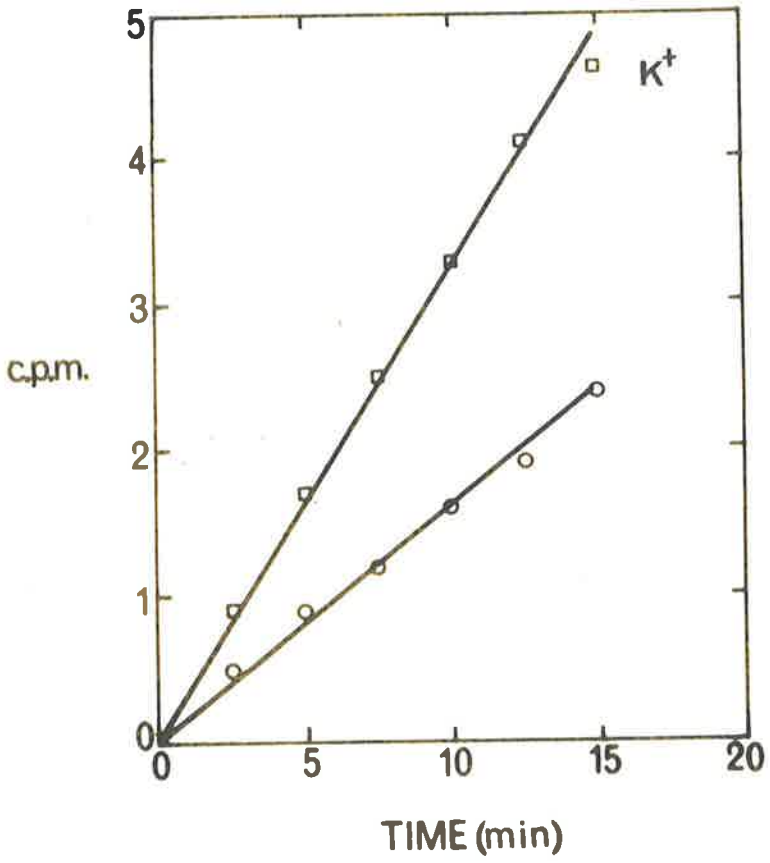

Fig. 5.9. Effect of K^+ on the uptake of ^{14}C-N-ethyl maleimide by propionyl-CoA carboxylase. Propionyl-CoA carboxylase (0.5 units) was incubated with 2×10^{-4}M ^{14}C-N-ethyl maleimide contained in 0.04M tris-HCl buffer, pH 8.2, at 22° with and without 8×10^{-3}M K^+. Aliquots of the incubation mixture were taken at various time intervals and the reaction stopped quickly by dilution into 10% TCA containing 1×10^{-2}M N-ethyl maleimide. The denatured protein was isolated by filtration on a membrane filter apparatus, washed thoroughly with N-ethyl maleimide and finally with 1% acetic acid. The membrances were dried at 100° for 30 min. and the radio-activity determined by scintillation counting.

DISCUSSION:

The original aims of this investigation were;

(a) To determine whether the enzyme exhibited an absolute requirement for monovalent cations or whether the cation simply stimulated a basal activity.

(b) To locate the point in the reaction sequence where K^+ ions influenced the rate of the reaction, and,

(c) If possible, to obtain some information which could elucidate the mechanism by which the cation exerts its effect.

With regard to the first objective, it was not possible to demonstrate an absolute K^+ ion requirement even after complete removal of alkali metals from both the enzyme preparation and the reagents used in the assay mixture.

The locus of K^+ ion stimulation, as deduced from a kinetic analysis in the presence and absence of the modifier, suggests that the point of attack is at or near the HCO_3^- binding site. Studies by Giorgio & Plaut (1967) using bovine liver propionyl-CoA carboxylase, which is stimulated 7-9 fold in the presence of K^+ ions, showed that the ATP-Pi, ADP-ATP exchange reactions and the transcarboxylase activity catalysed by the enzyme were stimulated by K^+. Two possible explanations could be advanced to account for the apparent difference between the data of Giorgio & Plaut (1967) and the evidence

presented here. First of all it is possible that the liver enzyme and the heart enzyme have different reaction mechanisms. Alternatively, the increase in the ATP-Pi exchange rate observed by these workers could be due to the presence of K^+ ions at or near the HCO_3^- binding site since HCO_3^- is essential for this exchange reaction. The ATP and HCO_3^- binding sites may be in close proximity and therefore, it is not unreasonable to expect that a changed environment induced at one site would not be without some effect at the other site. The fact remains that K^+ ions caused a significant increase in the affinity of the enzyme for HCO_3^-.

It would be expected that to be most effective, potassium would have to exert its activity at the slowest step in the reaction sequence. Tentative evidence was obtained from the profiles of pK_m versus pH plots to suggest that the formation of the $E\text{-}CO_2$ complex is the slow step in the reaction pathway. This type of plot has been used to determine the rate-determining step in the glyceraldehyde-3-phosphate dehydrogenase reaction (Keleti & Batke, 1967). The interpretation of the data relies on the theory that the velocity of the reaction depends upon the concentration of the Michaelis complexes. If, however, one of the elementary steps is rate-determining the relationship between the apparent V_{max} and apparent K_m is altered i.e. the apparent K_m for that substrate

involved in the limiting step will be high when the apparent V_{max} reaches a maximum. In Fig. 5.10 the pK_m for HCO_3^- was plotted as a function of pH. It can be seen that the apparent K_m for HCO_3^- is high at pH 8.0 to 8.2 which is the pH optimum for the enzyme (Tietz & Ochoa, 1959). This information would suggest that the rate limiting step in the propionyl-CoA carboxylase reaction is the formation of the CO_2-enzyme complex, a step which could be accelerated by the presence of univalent cations.

At this stage, any postulated mechanism of K^+ action must remain speculative. That a substantial conformational change is induced by potassium is supported by several different lines of evidence, viz.,

(a) A significant shift in the apparent K_m value for HCO_3^- in the presence of K^+ ions,

(b) a ΔS of 48 e.u. induced by K^+ ions, and

(c) An increased rate of inactivation by sulphydryl group reagents in the presence of K^+.

The stimulation process requires a mechanism whereby the activator changes the conformation of the protein instantaneously so that the modified protein is fully stimulated as soon as the cation reacts with the enzyme. It is difficult to visualise how K^+ ions could induce a conformational change except by influencing the ionic environment at the active site. Therefore a detailed

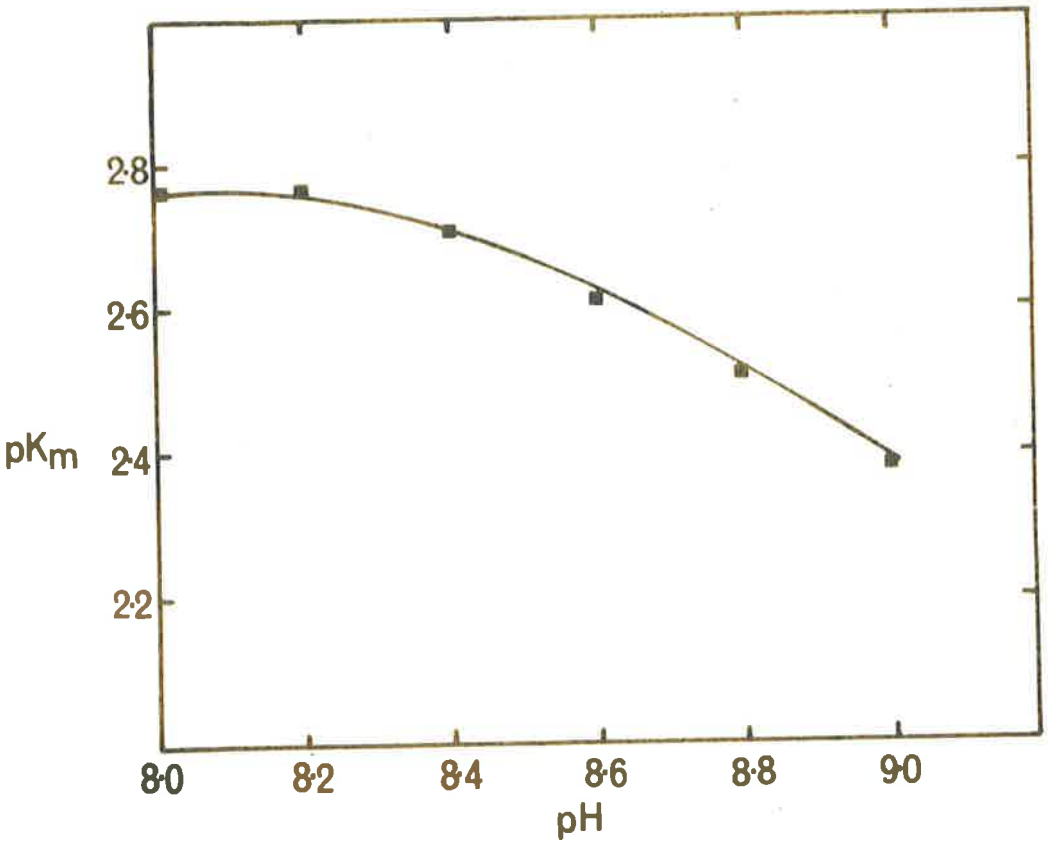

Fig. 5.10. Apparent K_m for HCO_3^- calculated at different pH values. The reaction mixture was as described in Materials & Methods and tris-HCl buffer was used throughout. The reaction mixtures were incubated with 0.015 units of enzyme for 10 min. at 30°. The K_m and V_{max} values for HCO_3^- were calculated from Lineweaver-Burk plots.

description of the stimulation process will have to wait until more information about the chemical environment around the biotin molecule is available.

C H A P T E R S I X

THE ALLOSTERIC ACTIVATION OF PROPIONYL-CoA CARBOXYLASE BY MAGNESIUM (Mg^{2+}) AND MAGNESIUM ADENOSINE TRIPHOSPHATE ($MgATP^{2-}$)

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INTRODUCTION:

Various theories have been advanced to explain the function of the metal ion in metal-ion catalysed reactions. It has been proposed for example that the metal ion;

(a) May participate in binding either the substrate, co-factor or both to the enzyme surface.

(b) Could activate the enzyme-substrate complex, or

(c) May participate in both (a) and (b).

(d) May help to maintain the secondary, tertiary and quaternary structure or to interact with side-chain such that the ensuing co-ordination complex can function catalytically.

(e) May undergo oxidation-reduction during the enzymic reaction and thus are considered to transfer electrons in the oxidative processes, e.g. Fe^{2+} in cytochrome oxidase.

In the propionyl-CoA carboxylase reaction, there is a requirement for both ATP and Mg^{2+} (Tietz & Ochoa, 1959). Since the apparent stability constant for $MgATP^{2-}$ is $73,000M^{-1}$, this would suggest that in the presence of excess Mg^{2+}, the amount of free ATP^{4-} is extremely small (O'Sullivan & Perrin, 1964). The interaction between metal ion and labile phosphate ion has always been a source of interest and speculation. There are two theories which have been proposed. Firstly, Koshland (1952) suggested that chelation i.e. the formation of the cyclic intermediate by the metal ion and labile phosphate

is of importance. Secondly, Kosower (1962) proposed charge neutralization i.e. the reduction of the ionic charge on the phosphate anion by the interaction of a metal ion of opposite charge. In this case, chelation is not required. Infra-red studies of ATP^{4-} and Mg^{2+} complex by Feldman & Keel (1965) have indicated that Mg^{2+} does not interact with the adenine moiety. The same conclusion was reached by Happe & Morales (1966) using ^{15}N-N.M.R.

Conley & Martin (1965) suggested that chelation to a divalent metal cation makes a species more susceptible to attack by hydroxide ion and (presumably also by water) than does protonation. Oestreich & Jones (1967) concluded from their experiments that the role of the metal ion is to neutralize the charge. Therefore, although the nature of the interaction is not fully understood, there is no doubt that a metal-nucleotide complex exists.

In the previous chapter evidence was presented which suggested that monovalent cations can stimulate the activity of propionyl-CoA carboxylase. The present chapter is concerned mainly with the role of the divalent cation in the propionyl-CoA carboxylase reaction. The reaction mechanism proposed for the biotin-group of enzymes has been derived from isotope exchange reactions (Lynen *et al.*, 1959; Kaziro *et al.*, 1960; Scrutton *et al.*, 1965; and Scrutton *et al.*, 1965(a)). As a

result of these investigations the following two-step reaction sequence has been postulated for these enzymes:

$$\text{Biotin-enzyme} + \text{ATP} + HCO_3^- \rightleftharpoons CO_2\text{-biotin-enzyme} + \text{ADP} + \text{Pi} \quad \ldots (6.1)$$

$$CO_2\text{-biotin-enzyme} + \text{acceptor} \rightleftharpoons CO_2\text{-acceptor} + \text{biotin-enzyme} \quad \ldots (6.2)$$

Magnesium is known to be required for reaction (6.1) but until recently no role had been assigned for this metal in the reaction mechanism. The investigations of Keech & Barritt (1967) using pyruvate carboxylase isolated from sheep kidney have disclosed that magnesium has two roles in the reaction sequence. Firstly, it complexed with ATP^{4-} to form the true substrate, MgATP, and secondly, it formed a complex with the enzyme to activate the enzyme. Furthermore, the addition of $MgATP^{2-}$ to the enzyme deviated from normal Michaelis-Menten kinetics, in such a way as to indicate a homotropic co-operative effect. The addition of free Mg^{2+} caused a threefold change in the enzymic activity at saturating level of $MgATP^{2-}$ and also changed the apparent K_m value and the slope of the Hill plot, both with respect to $MgATP^{2-}$. It was concluded that Mg^{2+} exhibited a heterotropic co-operative effect with respect to $MgATP^{2-}$. With this information in mind, the authors proposed two possible mechanisms for the binding of Mg^{2+} and $MgATP^{2-}$ to the enzyme surface. They suggested that Mg^{2+}

may bind either at a specific effector site or alternatively, at one of the binding sites for $MgATP^{2-}$ which is capable of functioning as an effector site. Before these proposals can be accepted as representative of all the biotin carboxylating enzymes, further confirmatory evidence must be forthcoming from other members of the group.

Propionyl-CoA carboxylase from pig heart (Kaziro *et al.*, 1960; Kaziro *et al.*, 1961; Tietz & Ochoa, 1959) exhibits maximum activity when the ratio of Mg^{2+} to ATP^{4-} is at least 2:1 or greater. This suggests that $MgATP^{2-}$ may be the true substrate in the reaction and also that free Mg^{2+} can also react with the enzyme, thus acting in a similar fashion to pyruvate carboxylase.

The interpretation of any information resulting from a study of the role of Mg^{2+} and ATP^{4-} in the propionyl-CoA carboxylase reaction is complicated by the possible involvement of the univalent cation, K^{+}. The previous chapter, which described experiments involving the univalent cation, provided evidence for suggesting that the site of action of this cation was in the formation of the CO_2-enzyme complex. Although both Mg^{2+} and ATP^{4-} are also required for the formation of the CO_2-biotin-enzyme complex (Equation 6.1), it will be shown in this chapter that the site of action of the divalent cation is different to that proposed for the K^{+} ion. An alternate mechanism for the formation of the active enzyme-substrate complex

will be proposed. Tentative evidence will also be produced to suggest that the rates of breakdown of these complexes are identical and furthermore, the Mg^{2+} and $MgATP^{2-}$ ions are binding at the same modifier site, thus supporting the second mechanism proposed by Keech & Barritt (1967).

RESULTS:

The preparation of special substrates as outlined in Materials & Methods was necessary to ensure that the kinetic observations resulted from specific and uncontaminated reagents. This was especially pertinent for the magnesium chloride which was contaminated with potassium chloride. N-ethyl morpholine was used as the buffer since the complex formation between this compound and Mg^{2+} ions is much less than with tris (Morrison, O'Sullivan & Ogston, 1961). Constant boiling hydrochloric acid was used to adjust the pH of the N-ethyl morpholine buffer, since the association between Mg^{2+} and Cl^{-} is considerably less than between other anions and Mg^{2+} (Cannan & Kibrick, 1938). The conditions of the assay were not changed from the ^{14}C-CO_2 fixation method of Tietz & Ochoa (1959) since these were ideal for studying the reaction sequence using the dissociation constant calculated by O'Sullivan & Perrin (1964), which was also determined at pH 8.0.

(1) Homotropic co-operative effect of $MgATP^{2-}$

When the velocity of the propionyl-CoA carboxylase reaction was plotted as a function of $MgATP^{2-}$ concentration a sigmoid-shaped curve was obtained (Fig. 6.1). This type of curve is indicative of a homotropic co-operative effect (Monod, Wyman & Changeux, 1965) and is very similar to the data published for several other enzymes (Sanwall & Cook, 1966; Okazaki &

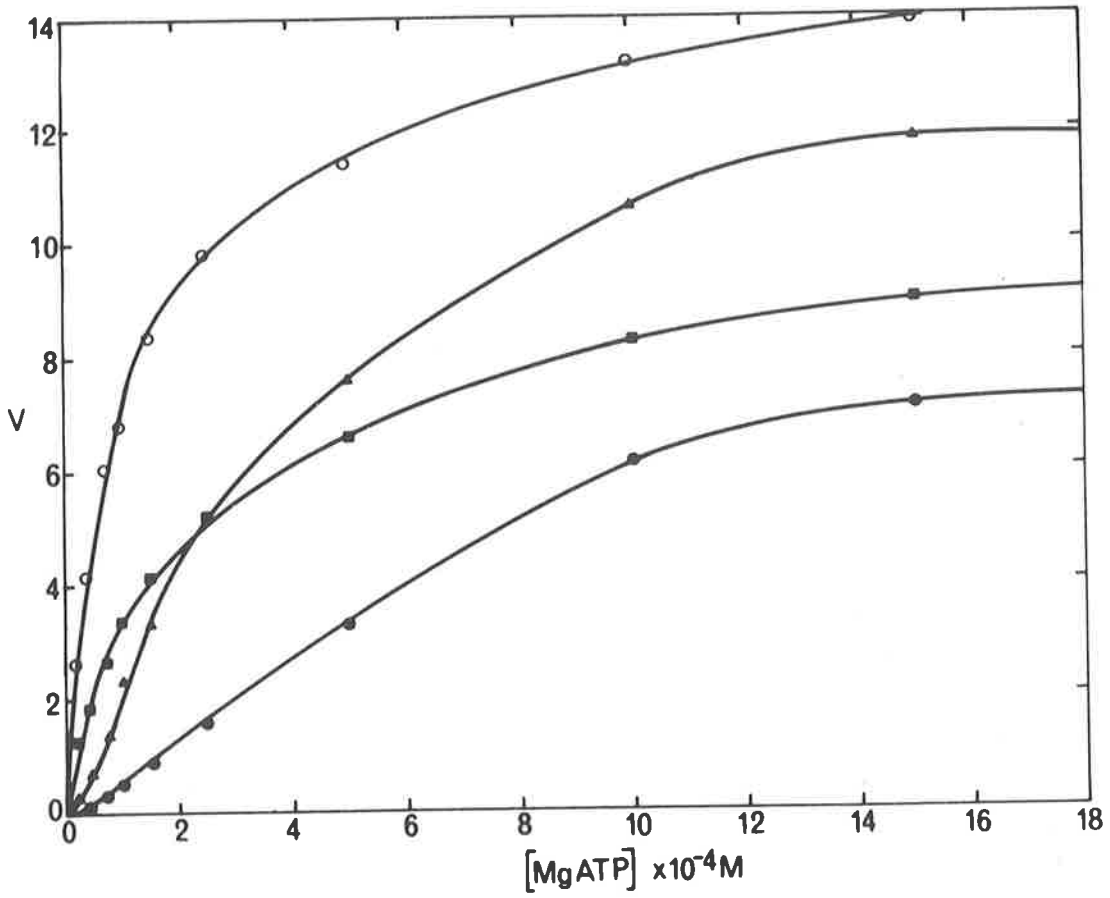


Fig. 6.1. Allosteric activation by $MgATP^{2-}$. The plot of velocity against $MgATP^{2-}$ concentrations in the presence of 0 (●——●); 1 x 10^{-3} M-Mg^{2+} (■——■); 8 x 10^{-3} M-K^{+} (▲——▲) and 1 x 10^{-3} M-Mg^{2+} and 8 x 10^{-3} M-K^{+} (o—o). The reaction mixture (final volume 0.5 ml.) contained in µmoles: N-ethyl morpholine-HCl buffer, pH 8.0, 100; ^{14}C-N-ethyl morpholine bicarbonate (3.2 x 10^{5} cpm per µmole), 5.0; propionyl-CoA, 0.5; N-ethyl morpholine glutathione, 0.25: and the indicated concentration of $MgATP^{2-}$.

Kornberg, 1964; Changeux, 1963 and Atkinson & Walton, 1965). The same data, when plotted in the double reciprocal form, showed a non-linear relationship (Fig. 6.2). From this data the V_{max} was calculated by extrapolation of the double reciprocal plot to infinite substrate concentration. Subsequently the K_m value was determined from Fig. (6.1) as the concentration of $MgATP^{2-}$ required to give half V_{max}. These constants were tabulated in Table (6.1).

The same data was also fitted to the Hill equation:

$$\text{Log } \frac{v}{(V-v)} = \bar{n} \log S + \log K \qquad \ldots (6.3)$$

where V, v, S, $\bar{n}$ and K are maximal velocity, initial reaction velocity, $MgATP^{2-}$ concentration, a complex function of an interaction coefficient and number of binding sites (Atkinson, Hathaway & Smith, 1965) and a complex equilibrium constant respectively. The curved double reciprocal plots observed for $MgATP^{2-}$ as the variable substrate and the value of 1.6 for the Hill slope of the Hill plot (Fig. 6.3) suggested that at least two molecules of $MgATP^{2-}$ were involved in the reaction and that these bind at different sites and exhibit a homotropic co-operative effect.

It can also be seen that the sigmoidal curve obtained with $MgATP^{2-}$ as the variable substrate has a positive initial slope, even at very low substrate concentrations, suggesting that a partial allosteric effect is operating i.e. the reaction

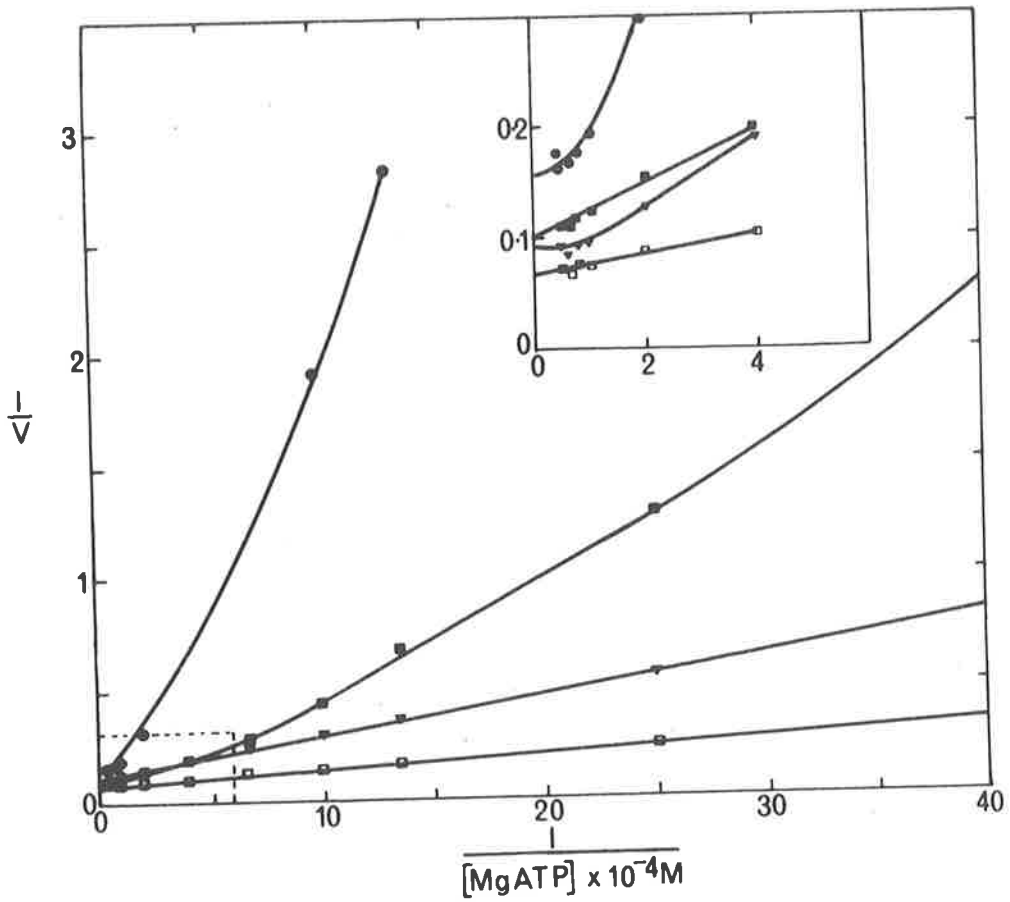


Fig. 6.2. Allosteric activation by $MgATP^{2-}$. The plot of the reciprocal of velocity against the reciprocal of $MgATP^{2-}$ concentration in the presence of 0 (●——●); Mg^{2+} (▼——▼); K^+ (■——■) and (Mg^{2+} + K^+) (□——□) as described in Fig. 6.1. The insert shows the data plotted at high concentrations of $MgATP^{2-}$.

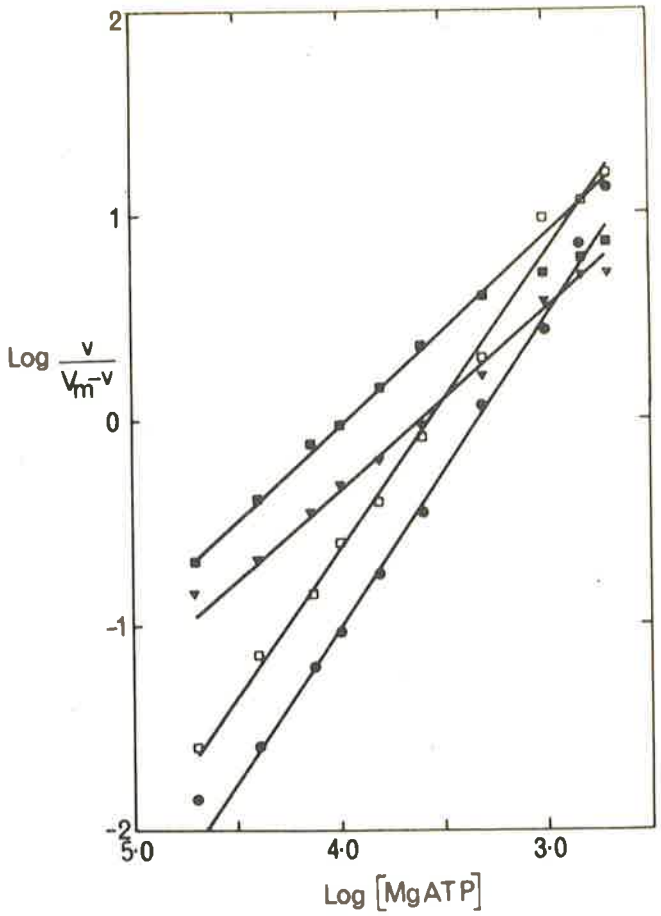


Fig. 6.4. Calculation of the $\bar{n}$ value for $MgATP^{2-}$ in the presence of Mg^{2+}, K^+ and (Mg^{2+} + K^+). The velocity of the propionyl-CoA carboxylase reaction as a function of $MgATP^{2-}$ fitted to the Hill equation in the absence (●—●) and presence of free Mg^{2+} (▼—▼); K^+ (□—□) and (Mg^{2+} + K^+) (■—■). The data was derived from Fig. 6.1. Log v/V_m-v was plotted against Log [$MgATP^{2-}$]. V_{max} was calculated from Fig. 6.2 and v = reaction velocity.

occurs with the addition of one molecule of $MgATP^{2-}$ but is greatly enhanced when the two molecules are added. This is similar to the allosteric effect reported for aspartate trans-carboxylase (Gerhart & Pardee, 1964).

(2) Activation by magnesium.

A plot of initial velocity as a function of $MgATP^{2-}$ concentration was made in the presence of excess Mg^{2+} (Fig. 6.1). It can be seen that the enzyme exhibited classical Michaelis-Menten kinetics in the presence of excess Mg^{2+} ions (i.e. a hyperbolic curve as seen in Fig. 6.1 resulted). The same data when replotted in the double reciprocal form now showed a straight line relationship (Fig. 6.2). Furthermore, the addition of Mg^{2+} to the reaction mixture induced changes in the apparent K_m value for $MgATP^{2-}$, the apparent V_{max} and the slope of the Hill plot (Fig. 6.3). A change in the Hill plot from 1.6 to 1.0 either indicated that the interactions between the binding sites are markedly decreased or that the number of binding sites is altered from 2.0 to 1.0. This information is summarized in Table (6.1). It should also be noted from Table 6.1 that the K_m value for $MgATP^{2-}$ in the presence of Mg^{2+} is half the value in the absence of Mg^{2+}; a result which again suggests that only one site is operating thus requiring only half the previous $MgATP^{2-}$ concentration to saturate the enzyme.

(3) Effect of K^+

The effect of K^+ ions on the binding of $MgATP^{2-}$ to

TABLE 6.1.

The effect of Mg^{2+} and K^+ on the K_m, V_{max} and $\bar{n}$ values for $MgATP^{2-}$

Addition	K_m	V_{max}	$\bar{n}$
Nil	4.8×10^{-4}M	6.25	1.52
Mg^{2+} (1×10^{-3}M)	2.2×10^{-4}M	9.0	0.87
K^+ 8×10^{-3}M	2.8×10^{-4}M	11.2	1.47
Mg^{2+} + K^+ (1×10^{-3}M + 8×10^{-3}M)	1×10^{-4}M	13.8	0.90

the protein was also examined (Fig. 6.1). The cation did not alter the sigmoidal nature of the curve, although a small activation did occur. The apparent V_{max} values obtained by extrapolation of the double reciprocal plots indicated that the univalent cations caused a small stimulation of activity. Moreover, the apparent K_m values obtained by calculating the substrate concentration required to give half V_{max} indicated that K^+ caused a change in this kinetic constant (Table 6.1). This value is different to the value obtained in the previous chapter where the differences in the apparent K_m values in the presence and absence of K^+ were insignificant. In that particular case, the concentrations of $MgATP^{2-}$ were kept high in order to derive straight lines in the double reciprocal plot. Furthermore, the presence of K^+ did not cause a change in the slope of the Hill plot indicating that this cation did not alter either the strength of the interaction or the number of binding sites for $MgATP^{2-}$ - a result quite different to that observed with Mg^{2+}.

The combined effect of Mg^{2+} and K^+ on the binding of $MgATP^{2-}$ to the enzyme was also examined and is depicted in Fig. (6.1). The stimulation induced by both of these cations was additive; the hyperbolic curve was still apparent, with the K^+ causing only a small change in the apparent K_m value for $MgATP^{2-}$ (calculated in the presence of free Mg^{2+}). There was no change in the slope of the Hill plot. This data indicated

that the most significant kinetic changes with respect to $MgATP^{2-}$ are brought about by Mg^{2+} and not K^{+} (Table 6.1).

It can be observed that both Mg^{2+} and K^{+} stimulate the enzymic activity, but the greatest stimulation was observed with Mg^{2+} at the lower $MgATP^{2-}$ concentrations. When the activation function i.e. V-Vo/Vo was plotted as a function of $MgATP^{2-}$ concentration, a 14-fold stimulation was obtained and with increasing concentration the effect graudally decreased until only a basal stimulation was noted (Fig. 6.3). This pronounced stimulation was induced mainly by Mg^{2+} whereas K^{+} had little effect. Furthermore, Fig. (6.3) shows that the activation by (K^{+} + Mg^{2+}) was additive.

(4) <u>Activation by $MgATP^{2-}$</u>

When the reciprocal of the velocity was plotted as a function of the reciprocal of the Mg^{2+} concentration, at various fixed concentrations of $MgATP^{2-}$, a set of lines which intersected on the velocity axis resulted (Fig. 6.4). This type of pattern is similar to the plots obtained when studying competitive inhibition, however, in this case increasing $MgATP^{2-}$ concentrations caused activation instead of inhibition. Furthermore, when the slopes were plotted as a function of $MgATP^{2-}$ concentration, a parabola resulted (Fig. 6.5). Modifiers which reduce the combination of the substrate with the enzyme in the absence of any complicating effects can give a competitive type plot, but the replot of the slopes of the lines

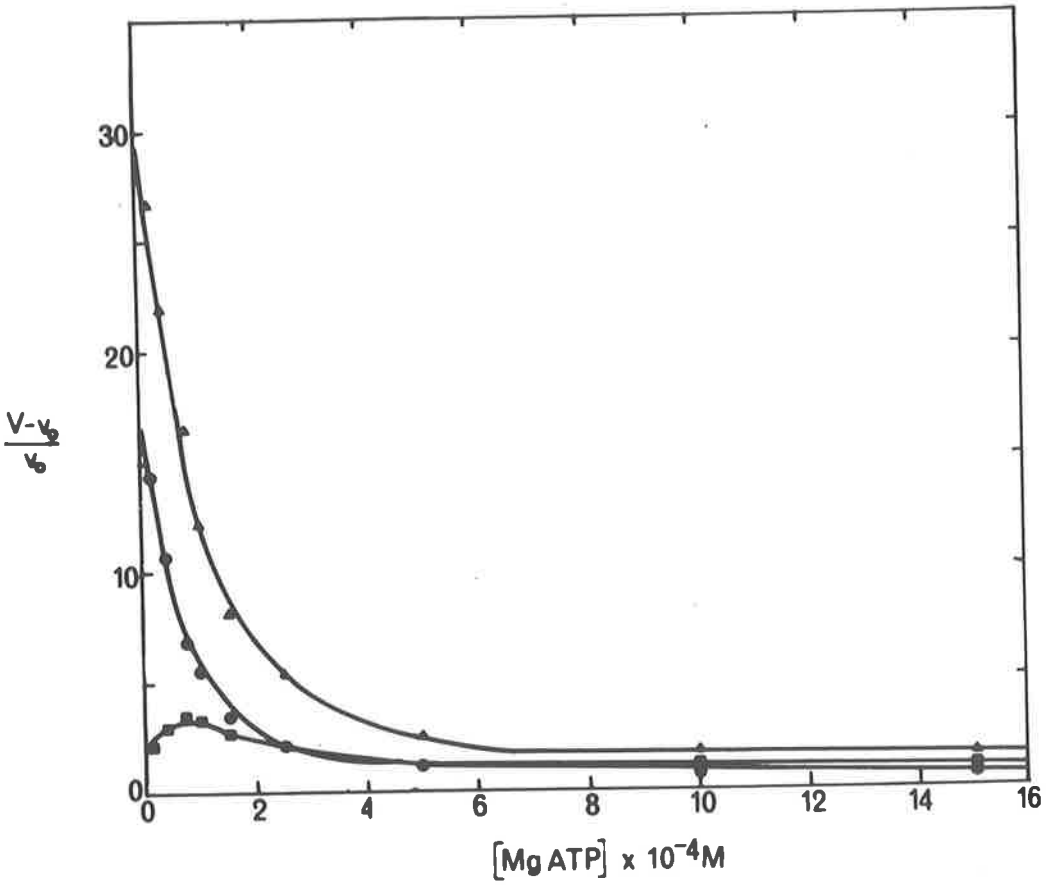


Fig. 6.3. Activation by Mg^{2+}, K^+ and (Mg^{2+} + K^+). The plot of $V-V_o/V_o$ against $MgATP^{2-}$ concentrations in the presence of Mg^{2+} (●—●); K^+ (■—■) and (Mg^{2+} + K^+) (▲——▲) as described in Fig. 6.1. (V = velocity obtained in the presence of K^+ and Mg^{2+} or both, whereas V_o = velocity in the absence of these cations).

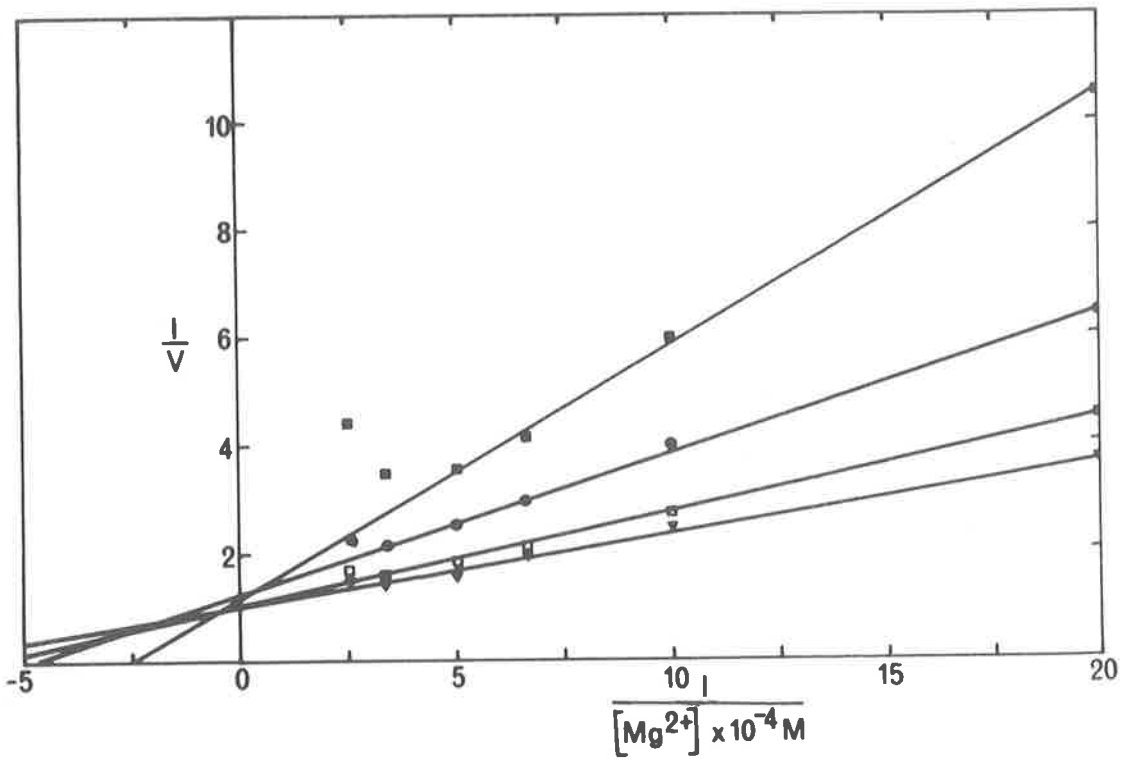


Fig. 6.7. Activation by Mg^{2+} at various concentrations of $MgATP^{2-}$. The reciprocal of the velocity was plotted against the reciprocal of Mg^{2+} concentration at various $MgATP^{2-}$ levels. The concentrations of $MgATP^{2-}$ were: 2.5×10^{-4}M (■——■); 5×10^{-4}M (●——●) 1×10^{-3}M (□——□); 2×10^{-3}M (▼——▼). The reaction mixtures were as described in Fig. 6.1.

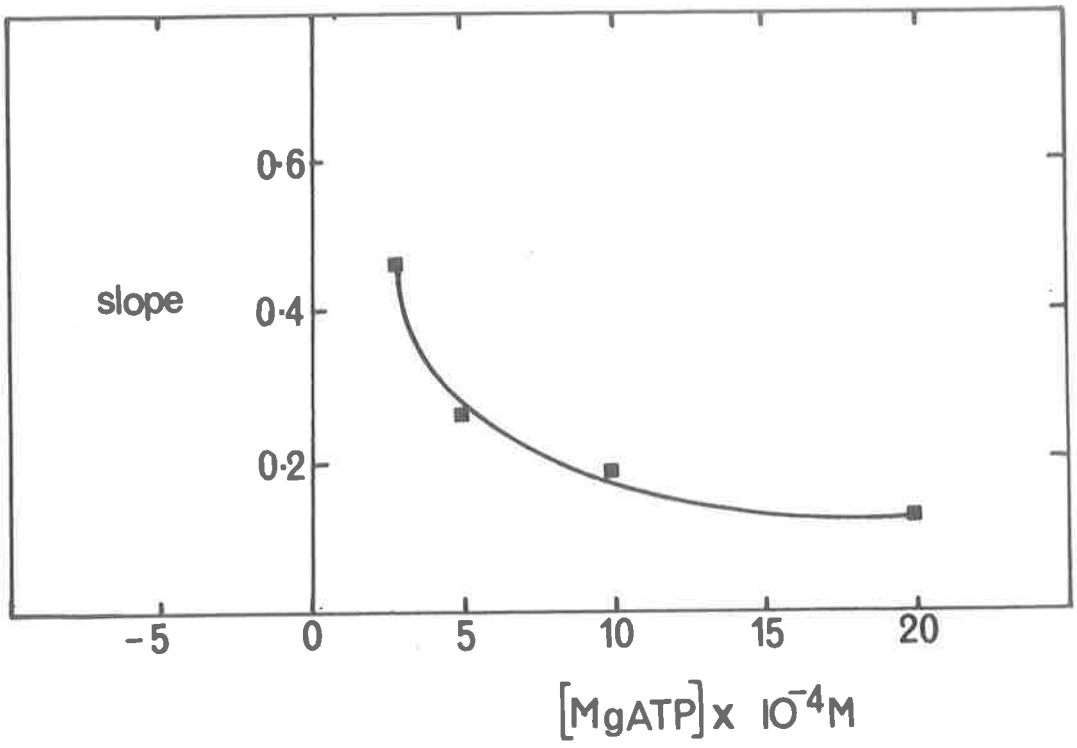


Fig. 6.8. Secondary plots of the slopes from Fig. 6.7 against $[MgATP^{2-}]$. The slopes of the lines from Fig. 6.7 were plotted against $MgATP^{2-}$ concentrations.

against inhibitor concentration yields a hyperbola. This is known as Hyperbolic Competitive or Partial Competitive inhibition and occurs when both the enzyme-substrate and enzyme-modifier-substrate complexes break down at the same rate (Morrison, 1965). Although the effects of "activating" modifiers have not been studied as comprehensively, a similar conclusion may be reached concerning the effect of the modifier. In this case, the activation was competitive i.e. $MgATP^{2-}$ and Mg^{2+} may be operating at the same site. If however the reciprocal of the slope is plotted against the $MgATP^{2-}$ concentration, a hyperbolic curve is observed. This activation pattern is opposite to that obtained for a classical inhibited system, but if the same principles apply, then this would infer that enzyme-substrate and the enzyme-modifier-substrate are breaking down at the same rate.

(5) Inhibition by ATP^{4-}

An inhibition pattern can be observed in Fig. 6.5 when the reciprocal of velocity is plotted as a function of the reciprocal of $MgATP^{2-}$ concentration at various concentrations of ATP^{4-}. However, since the graphs are non-linear, no real conclusions can be reached as to the type of inhibition. If the same data were plotted to an equation of the form:

$$\frac{1}{v} = a\frac{1}{S^2} + \frac{1}{V} \qquad \ldots (6.4)$$

where v, V, a and S are the initial velocity, maximum velocity,

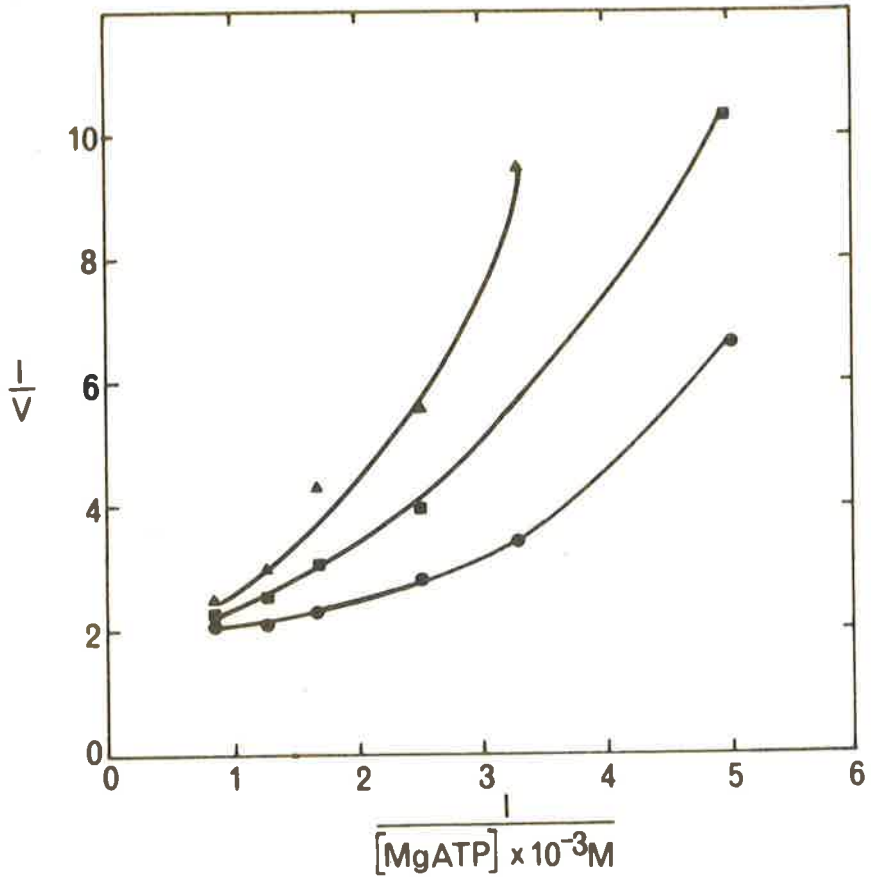


Fig. 6.5. Inhibition of $MgATP^{2-}$ with ATP^{4-}. The reciprocal of the velocity was plotted as a function of the reciprocal of $MgATP^{2-}$ concentration at varying ATP^{4-} levels. The concentrations of ATP^{4-} were: 0 (●—●); 2 x 10^{-4}M (■—■) and 4 x 10^{-4}M (▲—▲).

a constant and $MgATP^{2-}$ concentration respectively, then some predictions may be made (Fig. 6.6).

A number of workers have used a plot of the reciprocal of reaction rate against the reciprocal of the square of the substrate concentration and have shown a pseudo-Michaelis-Menten kinetic relationship (Okazaki & Kornberg, 1964; Umbarger & Brown, 1958 & Sanwal et al., 1966). However, in using an equation of this type, it should be realised that this does not completely describe the data and hence has not been used to derive any kinetic constant. From Fig. 6.6 it can be seen that ATP^{4-} inhibited the reaction in a partial competitive manner. The inhibition by ATP^{4-} indicated that ATP^{4-} could combine with the same form of the enzyme as $MgATP^{2-}$ to form a dead end complex and further suggested, that in this case, the nucleotide moiety of $MgATP^{2-}$ was responsible for binding this complex to the enzyme.

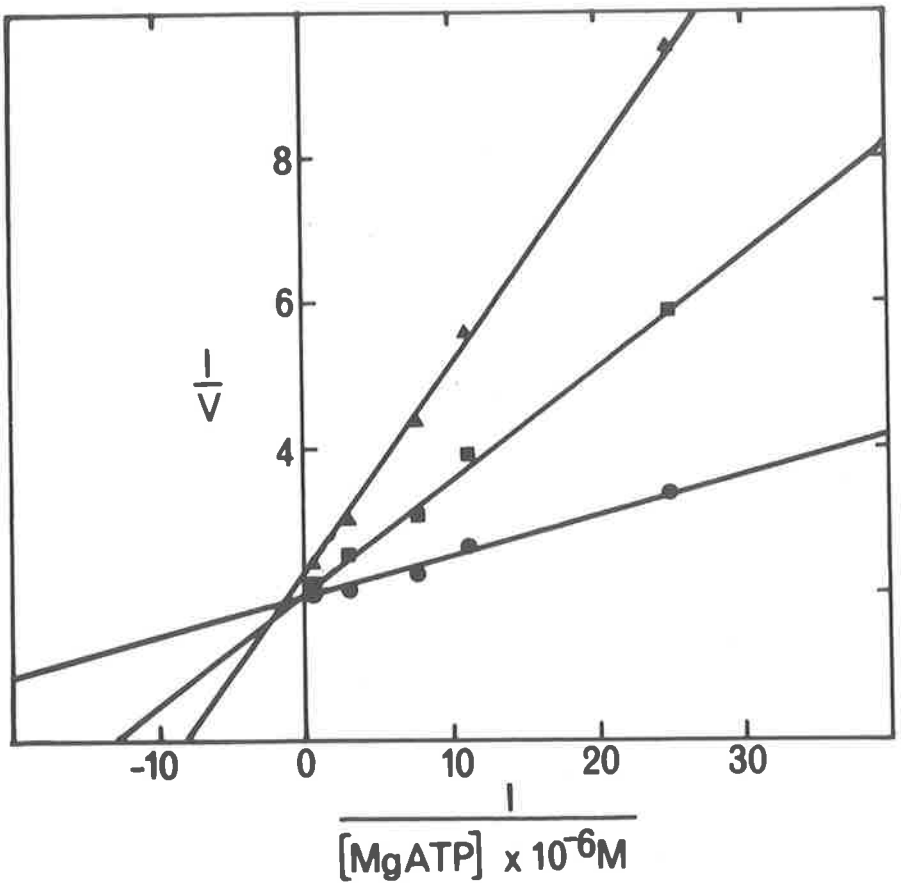


Fig. 6.6. Inhibition of $MgATP^{2-}$ by ATP^{4-}. The velocities obtained in Fig. 6.5 were replotted as the reciprocal of the velocity against the reciprocal of the square of the $MgATP^{2-}$ concentration. The concentrations of ATP^{4-} were: 0 (●——●); 2×10^{-4}M (■——■) and 4×10^{-4}M (▲——▲).

DISCUSSION:

The evidence presently available indicates that propionyl-CoA carboxylase requires a divalent cation for the first partial reaction (Equation 6.1). The subsequent studies of Keech & Barritt (1967), working with sheep kidney pyruvate carboxylase, revealed that Mg^{2+} could have two roles in the reaction mechanism and they proposed two theories which could account for their findings. They suggested that Mg^{2+} reacted with ATP^{4-} to form $MgATP^{2-}$ and also as the free metal to activate the enzyme. They further indicated that Mg^{2+} could bind at one of the $MgATP^{2-}$ sites or alternatively at a specific activator site.

The evidence presented in this chapter indicates that propionyl-CoA carboxylase behaves in a similar fashion to pyruvate carboxylase with respect to $MgATP^{2-}$ and Mg^{2+}. However, there are some differences between the two enzymes. In the pyruvate carboxylase system, free Mg^{2+} did not alter the slope of the Hill plot for $MgATP^{2-}$ appreciably and the authors suggested that this was consistent with the two site hypothesis, (Atkinson et al., 1965). However, in this present investigation, free Mg^{2+} caused the slope of the Hill plot to be changed to $\bar{n} = 1.0$, the apparent K_m value for $MgATP^{2-}$ was halved and normal Michaelis-Menten kinetics were observed - all indicating that in the presence of free Mg^{2+} only one site exists for

$MgATP^{2-}$ at the active centre.

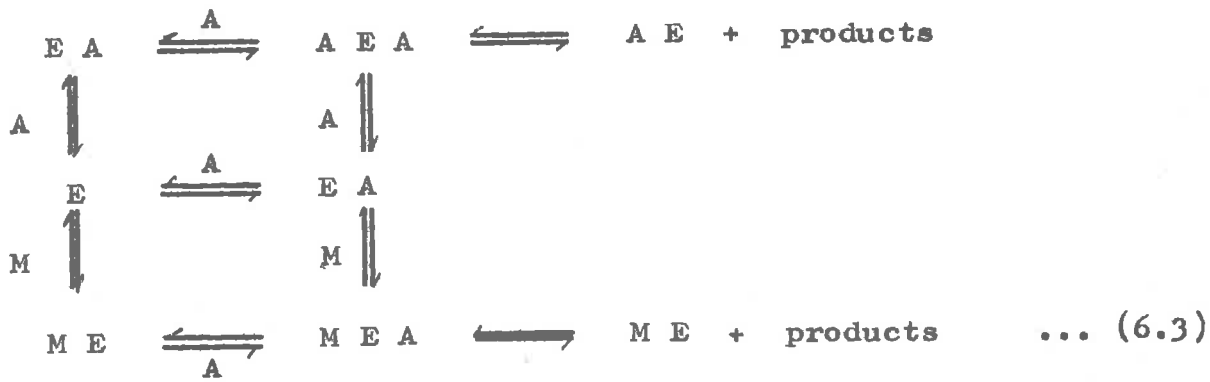
To simplify any further discussion, a theoretical model which could account for some of the behaviour exhibited by propionyl-CoA carboxylase will be outlined. It is proposed that propionyl-CoA carboxylase possesses two distinct sites:

(a) An active site at which the substrate, $MgATP^{2-}$ combines and undergoes reaction to yield products.

(b) A modifier site at which another molecule of $MgATP^{2-}$ or Mg^{2+} can react so as to influence the reaction of the substrate at the active site.

The modifier site would possess no catalytic activity and the modifier, i.e. Mg^{2+}, in this case, is an activator because it aids the combination of the substrate at the active site.

This can be diagrammatically represented as:



... (6.3)

where A = $MgATP^{2-}$, M = Mg^{2+} and E = enzyme. When the modifier is present at non saturating conditions, the rate equation predicts that a parabola will be evident when the reciprocal of the velocity is plotted as a function of substrate

concentration. On the other hand, in the presence of saturating concentrations of the modifier, the rate equation is simplified and predicts a straight line for the double reciprocal plot. Investigations into the propionyl-CoA carboxylase reaction indicated that this enzyme possesses most of the properties outlined above. For example, in the absence of free Mg^{2+}, the reciprocal of the velocity versus the reciprocal of the $MgATP^{2-}$ concentration resulted in a parabolic curve. Furthermore, in the presence of the modifier, a straight line was observed in the double reciprocal plot. A scrutiny of the data from the Hill plots indicated a definite alteration in the order of the reaction with respect to $MgATP^{2-}$ due to the presence of the free Mg^{2+}. The change from second order to first order with respect to $MgATP^{2-}$ by the modifier strongly suggests that only one $MgATP^{2-}$ site is now available for reaction. This could mean that most of the enzyme is forced into the enzyme-metal form so that the reaction proceeds mainly via the lowest pathway as described in Equation 6.3.

Although the main emphasis of this discussion has been centred on the modifier site or sites, evidence was also forthcoming which may help elucidate the nature of the substrate site. The demonstration of the competitive inhibition of $MgATP^{2-}$ by free ATP^{4-} could indicate that $MgATP^{2-}$ may be bound to the enzyme surface via the nucleotide moiety. Since

there is evidence to suggest the common binding of Mg^{2+} and $MgATP^{2-}$ at the modifier site, possibly by means of the metal, the substrate site may be the site specifically inhibited by the free ATP^{4-}. In other words, there is a discrimination in the attachment of $MgATP^{2-}$ to either the substrate or modifier sites due to the fact that $MgATP^{2-}$ binds to these sites in different ways. It could even be speculated that the $MgATP^{2-}$ is only hydrolysed to ADP + Pi at the substrate site even when another molecule of $MgATP^{2-}$ is occupying the modifier site, since the different means of binding the same molecule to the respective sites must indicate different amino acid sequences.

Several investigators have demonstrated that other divalent cations can be substituted for Mg^{2+} in the reactions carried out by the biotin-containing enzymes. Giorgio & Plaut (1967), using liver propionyl-CoA carboxylase, observed that Mn^{2+} could function in the ATP-Pi and ADP-ATP exchange reactions. They further demonstrated that when both Mg^{2+} and Mn^{2+} were both included in the assay mixture, the previous exchange rate was significantly decreased. Nielsen & Keech (1967) also substituted Mn^{2+} for Mg^{2+} in the reaction catalysed by sheep liver pyruvate carboxylase and showed that $MnATP^{2-}$ could function adequately, whereas free Mn^{2+} did not stimulate this activity. They assumed that both Mn^{2+} and Mg^{2+} could be operating similarly when bound to ATP^{4-}

but as the free metal, behave differently.

Collectively these two pieces of information could suggest that Mn^{2+} could bind with ATP^{4-} to form a substrate $MnATP^{2-}$ which can bind at the catalytic site. However, in the presence of free Mn^{2+}, a stimulation is not observed because this cation cannot bind at the activator site. When Mg^{2+} is used as the divalent cation, free Mg^{2+} causes a stimulation because it binds at the activator site. It could also be speculated that in the presence of free Mg^{2+} the reaction is uni-directional, i.e. the activation by Mg^{2+} is in the direction of carboxylation. Thus the exchange reactions which rely on a reversible reacting mechanism will not operate optimally in the presence of free Mg^{2+}, whereas free Mn^{2+} has no effect because it does not bind at the activator site. This could be the reason why the exchange reactions previously conducted on this enzyme have not resulted in the expected exchange rates (Kaziro et al., 1960).

Whilst these interpretations are only speculative at this juncture, they provide some basis from which a more precise mechanism may be elucidated and also aid in devising experiments to prove some of these hypotheses. At present, the sequence of events involving both $MgATP^{2-}$ and Mg^{2+} are assumed to be as follows:

(a) $MgATP^{2-}$ binds at the substrate site through the

nucleotide portion of this substrate and here it is hydrolysed to yield ADP + Pi.

(b) $MgATP^{2-}$ by binding at the modifier site by means of the metal ligand, can facilitate the binding of $MgATP^{2-}$ to the substrate site.

(c) Mg^{2+} may be either binding at a specific modifier or at the same modifier site occupied by $MgATP^{2-}$.

An alternative pathway for the formation of a nucleotide-enzyme complex is proposed involving in the first instance, two molecules of $MgATP^{2-}$ and in the second, one molecule of $MgATP^{2-}$ and one molecule of Mg^{2+}. It is suggested that in the presence of free Mg^{2+}, the formation of the $E\text{-}Mg^{2+}\text{-}MgATP^{2-}$ is the preferred pathway, thus resulting in the changes in the kinetic characteristics of the enzyme with respect to $MgATP^{2-}$. Furthermore, it was suggested that these two complexes break down at the same rate. Although the investigations using Mn^{2+} are only preliminary, certain findings suggest that both Mn^{2+} and Mg^{2+} can react with ATP^{4-}, but excess free Mn^{2+} does not activate the enzyme. The activation by free Mg^{2+} is assumed to be uni-directional and the consequences of this finding on the exchange reaction has been speculated.

C H A P T E R S E V E N

INITIAL VELOCITY STUDIES

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INTRODUCTION:

Information about enzymic reaction sequences may be gained from initial velocity studies. Together with product inhibition and isotope exchange investigations, it has enabled investigators to postulate the order of addition of substrates and removal of products from the enzyme surface.

Cleland (1963; 1963(a) and 1963(b)) has simplified the kinetic equations for enzymes involving two or more substrates or products by assuming that irrespective of the number of central inter-conversions, the forms of the velocity equations do not change. The possible mechanisms for these enzymes can be divided into two main classes:

(a) Sequential, where both substrates must add to the enzyme before either products are released.

(b) Non sequential, where a product is released before the second substrate adds to the enzyme.

Initial velocity equations for these mechanisms have been derived. For example,

$$v = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB}$$ for the sequential mechanism ... (7.1)

and $$v = \frac{VAB}{K_aB + K_bA + AB}$$ for the non-sequential mechanism ... (7.2)

respectively where v, V, A, B, K_a, K_b and K_{ia} are velocity,

maximum velocity, substrates A and B, Michaelis constants for A and B and the dissociation constant between enzyme and substrate A.

For sequential mechanisms, plots of 1/v against 1/A at different fixed concentrations of B will yield a family of straight lines whose slopes and intercepts are a function of the concentration of B, i.e.

$$\frac{1}{v} = \frac{K_a}{V}\left(\frac{K_{ia}K_b}{K_aB} + 1\right)\frac{1}{A} + \frac{1}{V}\left(\frac{K_b}{B} + 1\right) \qquad \ldots (7.3)$$

Thus the lines will intersect at a point to the left of the ordinate and this point may be above, below or on the abscissa, depending on the ratio of K_{ia}/K_a.

For the non-sequential mechanism, the slopes of the lines are independent of the concentration of B, i.e.

$$\frac{1}{v} = \frac{K_a}{V}\cdot\frac{1}{A} + \frac{1}{V}\left(\frac{K_b}{B} + 1\right) \qquad \ldots (7.4)$$

while the vertical intercepts will vary with the concentration of B. The result is that a series of parallel lines is obtained.

With this background it was considered that confirmation of the sequence of events in the propionyl-CoA carboxylase reaction sequence may be confirmed using initial velocity studies. This was considered particularly important since

the order of events as deduced from isotope exchange studies provided no clues to indicate when either ADP or Pi were released from the surface of the enzyme.

RESULTS:

In this section the initial velocity patterns will be analysed as outlined by Cleland 1963(b), i.e. the mechanisms will be predicted by inspection. This was considered necessary since the complexity of the reaction together with the complications arising from allosteric effectors could make a systematic study very tedious.

(1) The Influence of propionyl-CoA on the HCO_3^- kinetics.

Fig. 7.1 shows that the Lineweaver-Burk plots in terms of the concentration of HCO_3^- at different propionyl-CoA levels gives a set of lines with a common intercept on the HCO_3^- axis. This indicates that the Michaelis constant for HCO_3^- is independent of the propionyl-CoA concentration. Replots of the slopes and intercepts versus the propionyl-CoA concentration were linear (Fig. 7.3).

(2) The influence of HCO_3^- on the propionyl-CoA kinetics.

Lineweaver-Burk plots in terms of the propionyl-CoA concentration at different HCO_3^- levels gave intersecting lines (Fig. 7.2). The replots of slopes and intercepts were again linear (Fig. 7.4).

From the theory of Cleland (1963(b), 1967), the intersecting patterns and the linear replots of both slopes and intercepts obtained from Figs. 7.1, 7.2, 7.3 and 7.4 indicate that a reversible connection exists between the

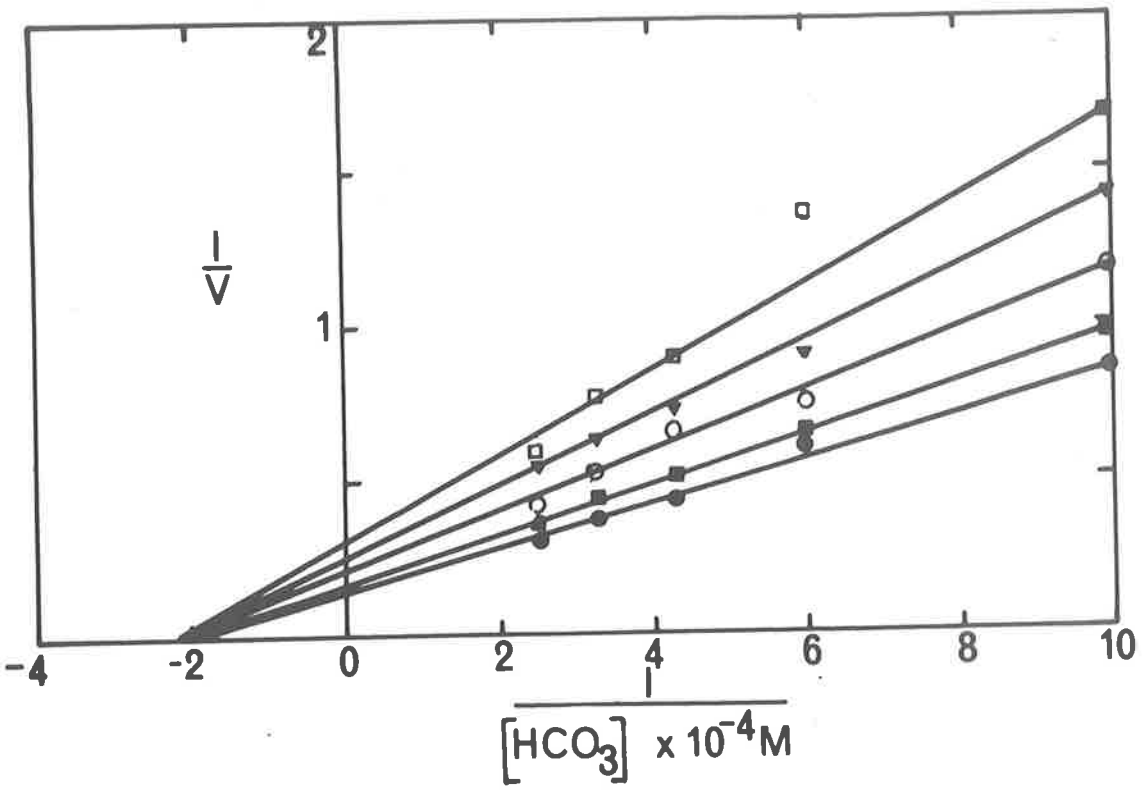


Fig. 7.1 The influence of propionyl-CoA on the HCO_3^- kinetics. The reciprocal of the velocity was plotted as a function of the reciprocal of the HCO_3^- concentration at various propionyl-CoA concentrations. The concentrations of propionyl-CoA were: 1.38×10^{-4}M (□——□); 2.08×10^{-4}M (▼——▼); 2.78×10^{-4}M (○——○); 4.15×10^{-4}M (■——■); 5.55×10^{-4}M (●——●). The reaction mixture (final volume 0.5 ml.) contained in μmoles: N-ethyl morpholine buffer, pH 8.0, 100; Mg^{2+}, 3; N-ethyl morpholine-ATP^{4-}, 1.5; K^+, 4; N-ethyl morpholine bicarbonate, 5; N-ethyl morpholine glutathione, 0.25; propionyl-CoA, 0.5. These were the concentrations used except when the particular substrate was the variable or fixed variable substrate.

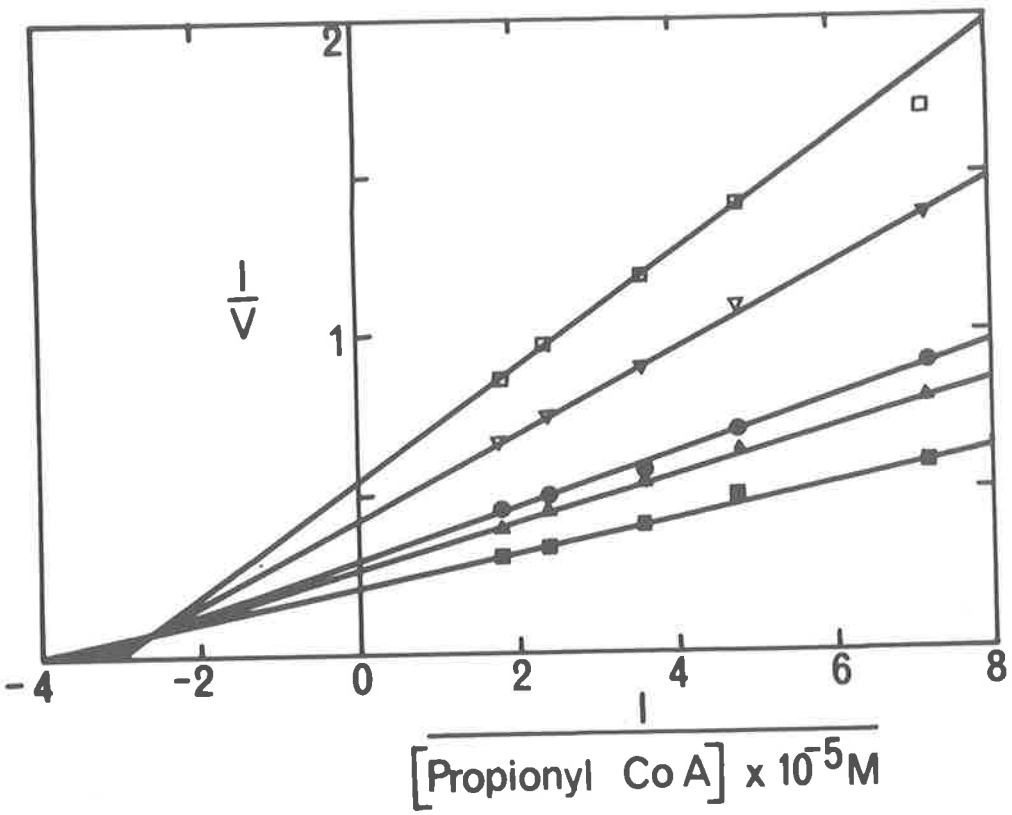


Fig. 7.2. The influence of HCO_3^- on the propionyl-CoA kinetics. The reciprocal of the velocity was plotted as a function of the reciprocal of the propionyl-CoA concentration at various HCO_3^- levels. The concentrations of HCO_3^- were: 1×10^{-3}M (□—□); 1.66×10^{-3}M (▽—▽); 2.32×10^{-3}M (●—●); 3×10^{-3}M (▲—▲); 4×10^{-3}M (■—■).

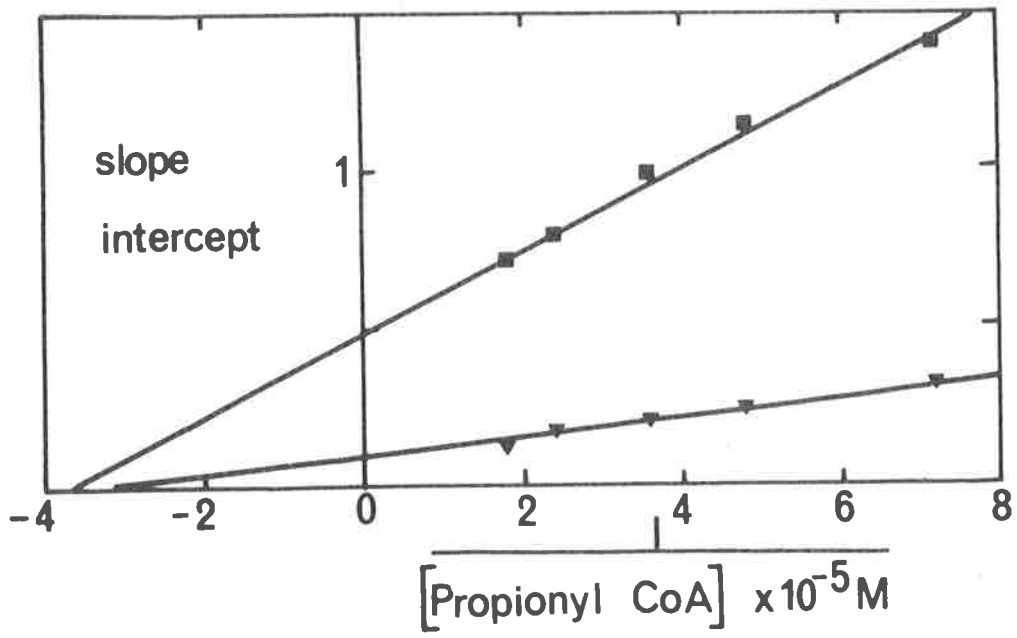


Fig. 7.3. Replots of slopes and intercepts from Fig. 7.1.

The slopes and intercepts from Fig. 7.1 were replotted against the reciprocal of the propionyl-CoA concentration.

Intercept (▼——▼); Slope (■——■).

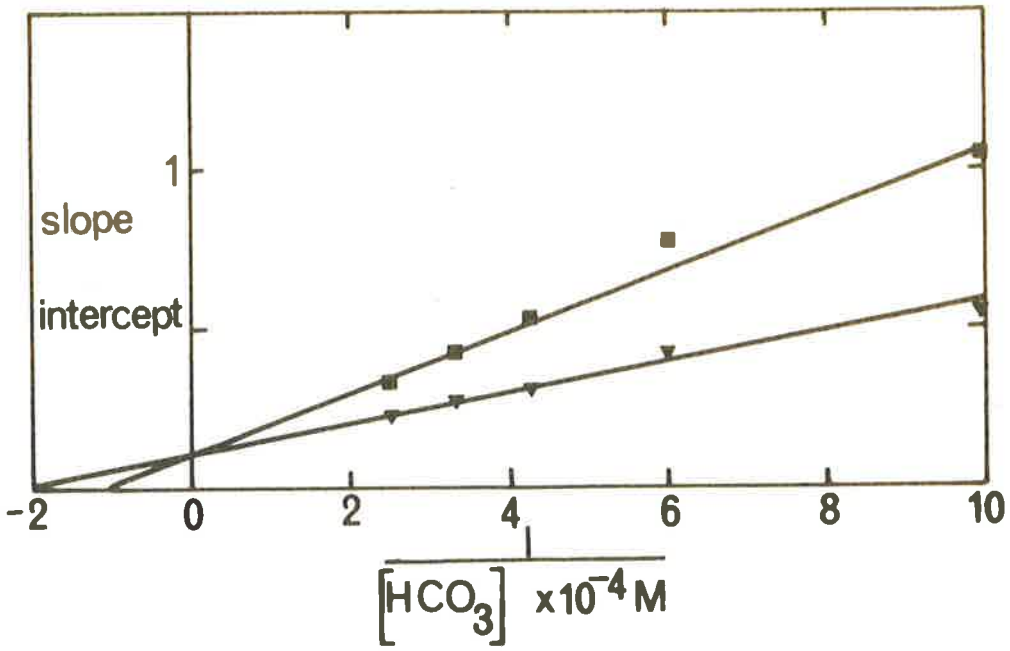


Fig. 7.4 Replots of slopes and intercepts from Fig. 7.2.

The slopes and intercepts from Fig. 7.2 were replotted against the reciprocal of the HCO_3^- concentration. Intercept (■—■), Slope (▼—▼).

addition of propionyl-CoA and HCO_3^- to the enzyme. In other words, a product is not released or a substrate is not added between the addition of these two substrates to the enzyme surface. This result conflicts with previous proposals, especially concerning the removal of the two products, ADP and Pi. It was assumed that ADP and Pi must leave the enzyme after the HCO_3^- was added because the ^{18}O-exchange studies conducted by Kaziro et al. (1962) indicated that the label from the bicarbonate species was incorporated into the orthophosphate. Furthermore, it is also realised that ADP-Pi cannot remain on the enzyme for the duration of the reaction since the propionyl-CoA : methyl malonyl-CoA exchange reaction proceeds without the intervention of either ADP or Pi. One could only suggest then that ADP and Pi come off before the HCO_3^- is added, a scheme involving perhaps a protein relaxation effect.

Atkinson (1966) has pointed out that examples of protein relaxation have been cited, i.e. DPNH oxidase of Mycobacterium tuberculosis is activated by AMP (Worcel, Goldman & Cleland, 1965), but the reversion of the enzyme to the inactivated form required minutes to hours depending upon the conditions. In the propionyl-CoA carboxylase situation this would suggest that time is required for the enzyme to relax back to its inactivated conformation after ADP and Pi have

been released from the enzyme surface. The studies of Gregolin et al. (1966) and Greenspan & Lowenstein (1966) have shown that acetyl-CoA carboxylase can be significantly affected by $MgATP^{2-}$ and Mg^{2+} respectively indicating that these two ligands are both capable of forming "activated" enzymes.

(3) The influence of ($MgATP^{2-}$ + Mg^{2+}) on the propionyl-CoA kinetics.

Fig. 7.5 shows that Lineweaver-Burk plots in terms of the concentration of propionyl-CoA at different ($MgATP^{2-}$ + Mg^{2+}) levels gave a set of parallel lines. The replots of both slopes and intercepts against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration resulted in straight lines (Fig. 7.7).

(4) The influence of propionyl-CoA on the ($MgATP^{2-}$ + Mg^{2+}) kinetics.

Reciprocal plots of velocity versus the reciprocal of ($MgATP^{2-}$ + Mg^{2+}) concentration at varying fixed concentrations of propionyl-CoA resulted in a series of parallel lines (Fig. 7.6). The analysis of slopes and intercepts plotted against the reciprocal of the propionyl-CoA concentration showed linear relationships (Fig. 7.8).

The parallel pattern of lines obtained in Figs. 7.5,

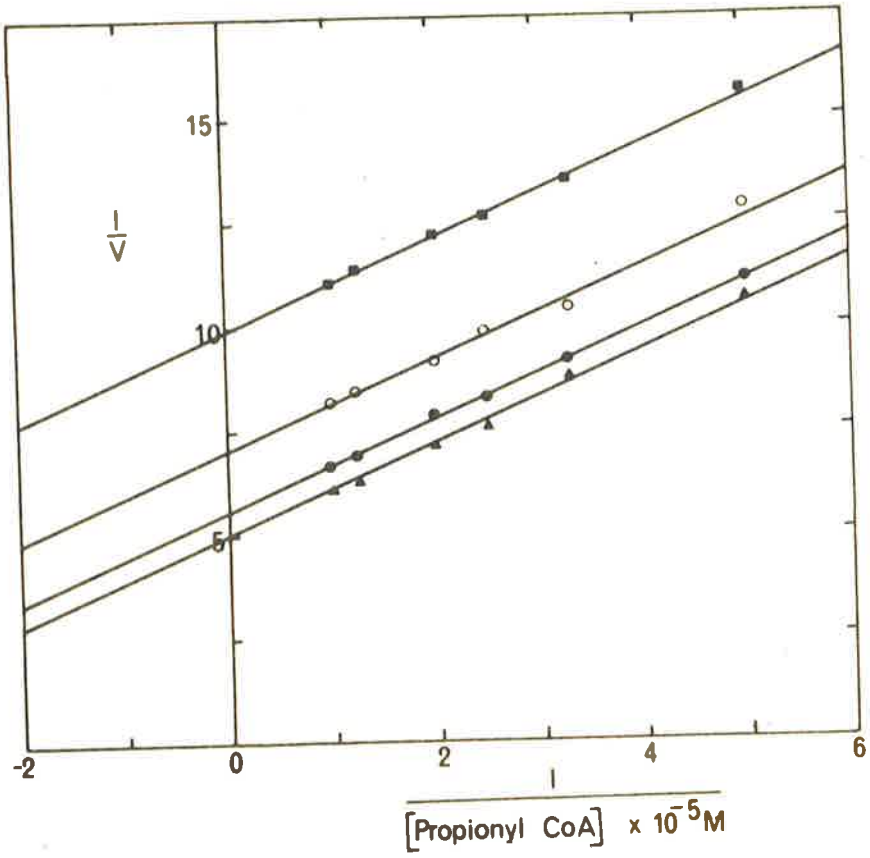


Fig. 7.5 The influence of ($MgATP^{2-}$ + Mg^{2+}) on the propionyl-CoA kinetics. The reciprocal of the velocity was plotted against the reciprocal of the propionyl-CoA concentrations at various ($MgATP^{2-}$ + Mg^{2+}) levels. The concentrations of ($MgATP^{2-}$ + Mg^{2+}) were: 5×10^{-5}M and 5×10^{-5}M (■——■); 1×10^{-4}M and 1×10^{-4}M (o——o); 2×10^{-4}M and 2×10^{-4}M (●——●); 5×10^{-4}M and 5×10^{-4}M (▲——▲)

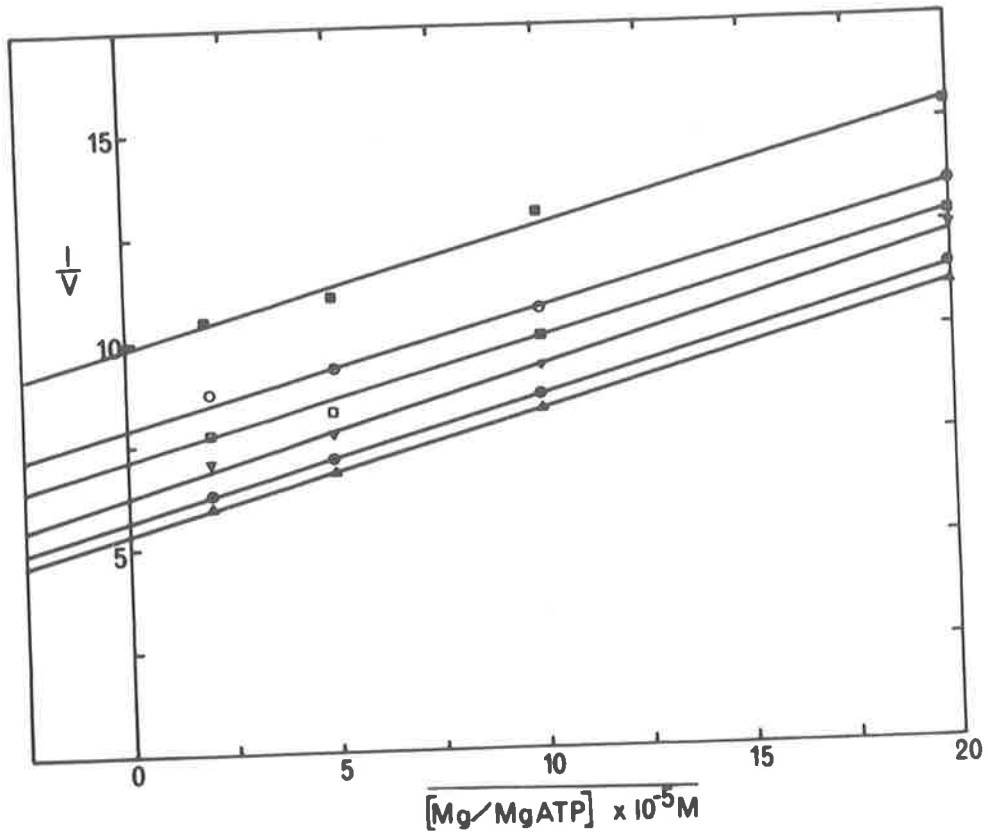


Fig. 7.6. The influence of propionyl-CoA on the ($MgATP^{2-}$ + Mg^{2+}) kinetics. The reciprocal of the velocity was plotted against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration at various propionyl-CoA concentrations. The concentrations of propionyl-CoA were: 2×10^{-4}M (■——■); 3×10^{-4}M (○——○); 4×10^{-4}M (□——□); 5×10^{-4}M (▼——▼); 8×10^{-4}M (●——●); 1×10^{-3}M (▲——▲).

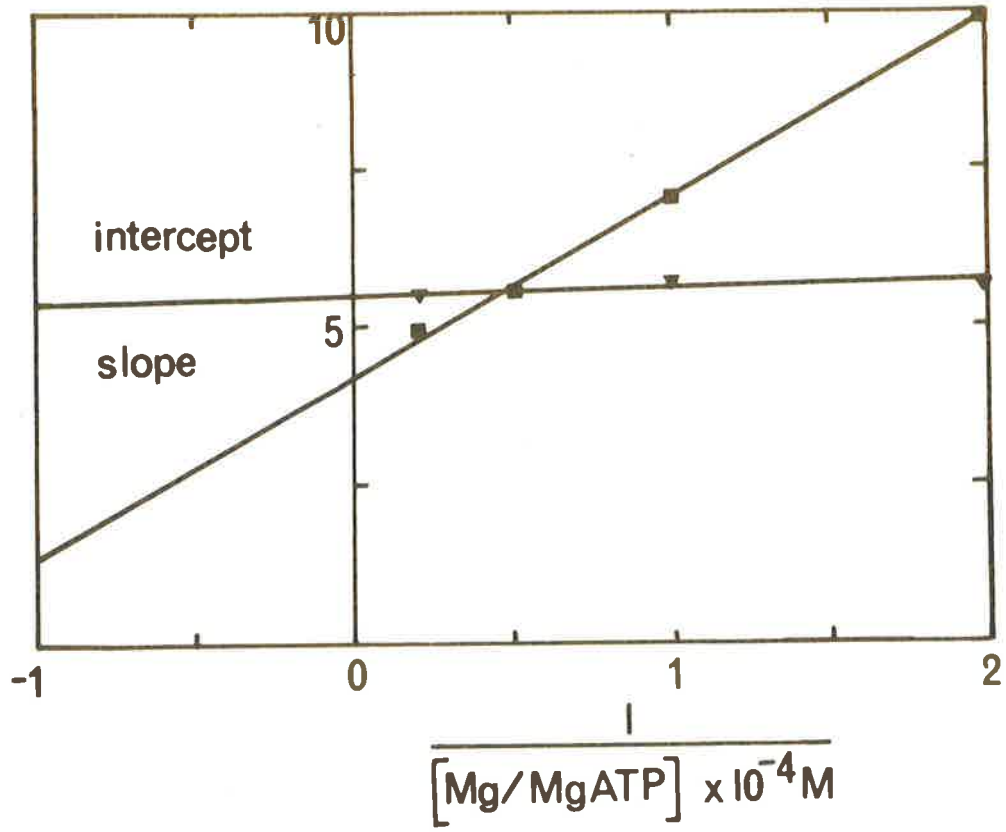


Fig. 7.7 Replots of slopes and intercepts from Fig. 7.5.

The slopes and intercepts from Fig. 7.5 were replotted against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration.

Slope (▲——▲); Intercept (■——■).

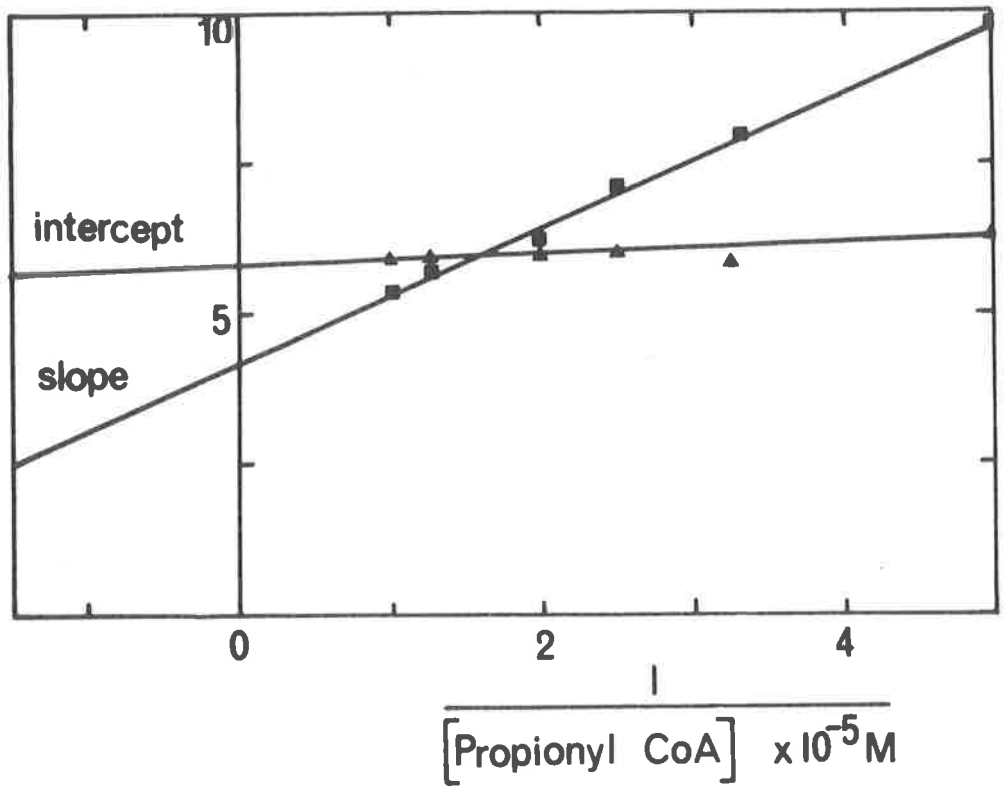


Fig. 7.8 Replots of slopes and intercepts from Fig. 7.6.

The slopes and intercepts from Fig. 7.6 were replotted as a function of the reciprocal of the propionyl-CoA concentration. Slopes (▲—▲); Intercepts (■——■).

7.6 and the linear replots of both intercepts and slopes in Figs. 7.7 and 7.8 indicate that an irreversible connection exists between the combination of these two substrates to the enzyme. In order that linear double reciprocal plots would result, the double "substrate" ($MgATP^{2-}$ + Mg^{2+}) was used instead of $MgATP^{2-}$. The irreversible connection mentioned above can be due to either;

(a) The release of product at zero concentration

(b) The addition of substrate at saturating concentrations. In this case, the addition of HCO_3^- at saturating concentrations to the enzyme will constitute an irreversible step between the binding of ($MgATP^{2-}$ + Mg^{2+}) and propionyl-CoA to the enzyme.

(5) <u>The influence of HCO_3^- on the $MgATP^{2-}$ kinetics</u>.

In Fig. (7.9) the effect of HCO_3^- concentration is observed at various levels of $MgATP^{2-}$ (variable substrate) on the velocity of the propionyl-CoA carboxylase reaction. At the present, a literature survey has not indicated examples of sigmoidal relationships being analysed in two substrate kinetics. Since the theories of Cleland (1963, 1963(b)) are based on differences in V_{max} and K_m values, it seemed possible, by using this approach, to replot this data in order to conform to the more usual linear patterns. The V_{max} values for the curves in Fig. 7.9 were obtained by

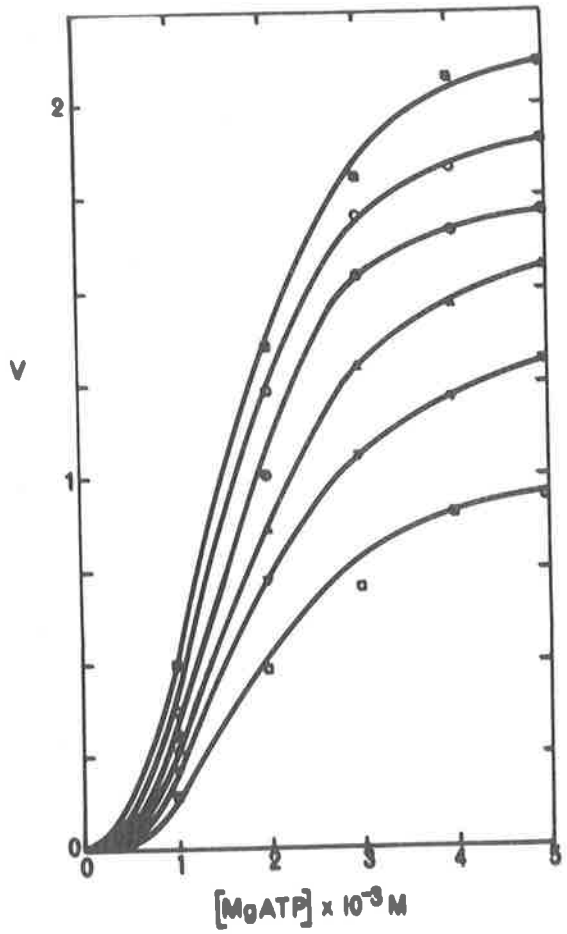


Fig. 7.9 The influence of HCO_3^- on the $MgATP^{2-}$ kinetics.

The velocity of the propionyl-CoA carboxylase reaction was plotted as a function of the $MgATP^{2-}$ concentration at various fixed concentrations of HCO_3^-. The concentration of HCO_3^- were: 1×10^{-3}M (□—□); 2×10^{-3}M (▼—▼); 3×10^{-3}M (▲—▲); 4×10^{-3}M (●—●); 6×10^{-3}M (o—o); 8×10^{-3}M (■—■).

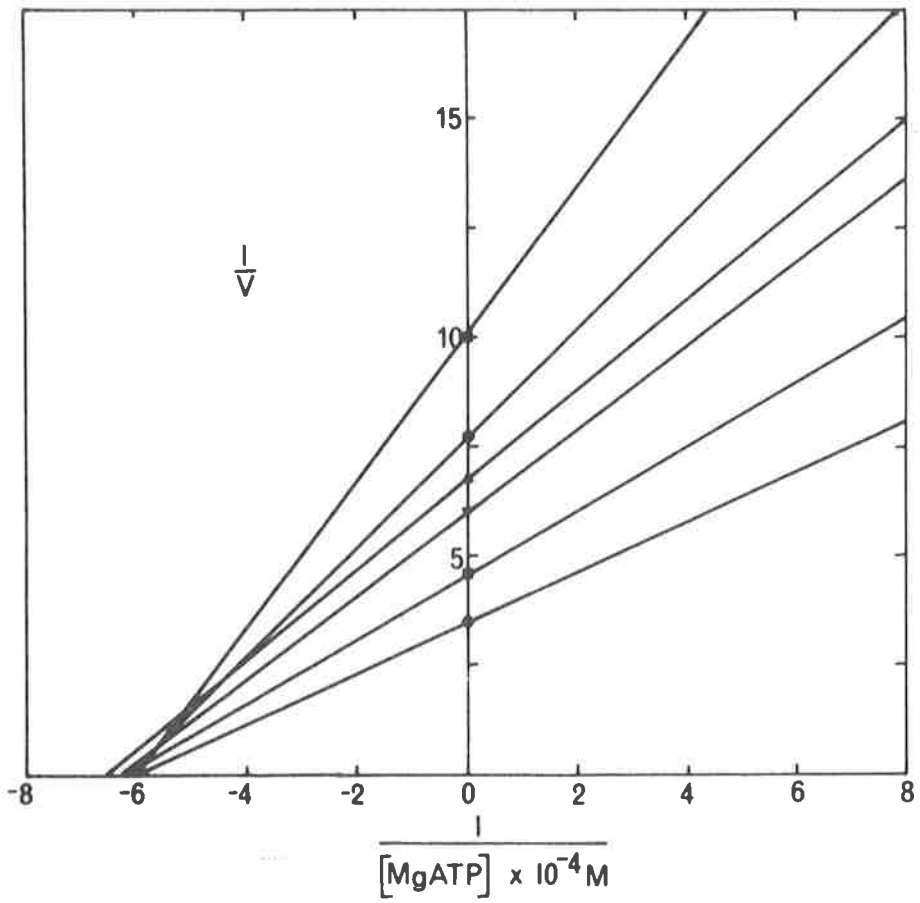


Fig. 7.10. Reciprocal plots from data obtained in Fig. 7.9.

The apparent K_m and apparent V_{max} values for the plots in Fig. 7.9 were calculated. The reciprocals of these two values were derived, plotted and lines were drawn between the two points as observed in Fig. 7.10. The apparent V_{max} value was obtained by extrapolation of the double reciprocal plot and the apparent K_m value determined as the concentration of $MgATP^{2-}$ at half V_{max}.

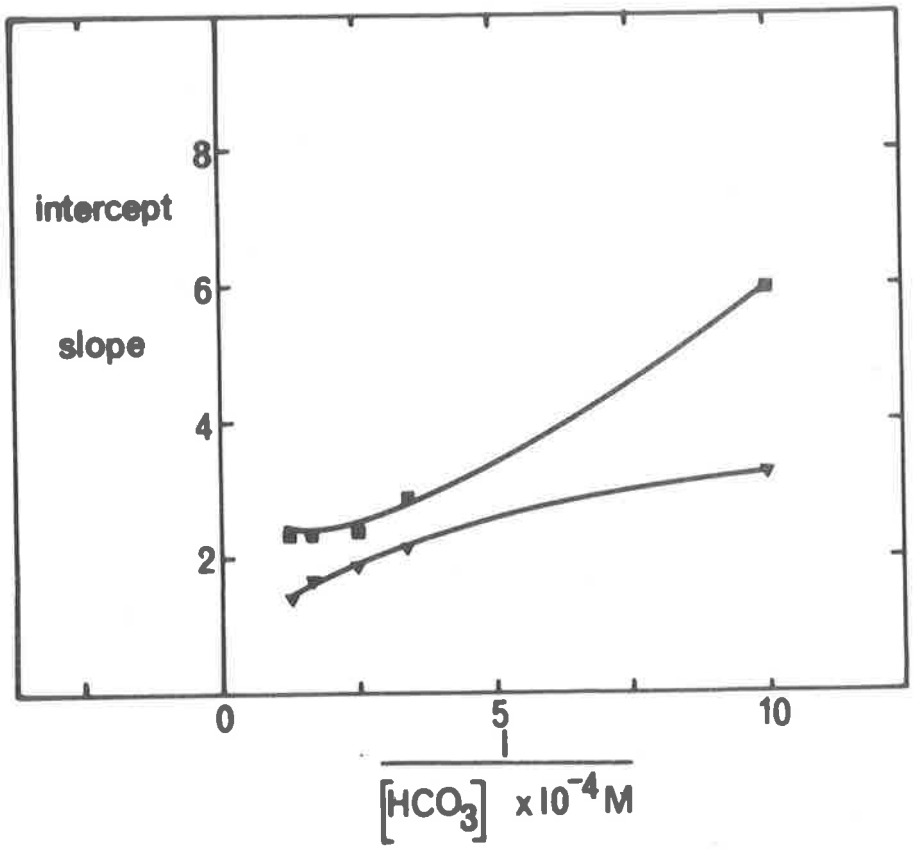


Fig. 7.12. Replots of slopes and intercepts from Fig. 7.10.

The slopes and intercepts from Fig. 7.10 were replotted against the reciprocal of the HCO_3^- concentration. Slopes (■—■); Intercepts (▼—▼).

extrapolation of the parabolic curves by plotting the reciprocal of the velocity versus reciprocal of the $MgATP^{2-}$ concentrations. From these values, the apparent K_m values were obtained by determining the $MgATP^{2-}$ required to give half V_{max} in Fig. 7.9. With the reciprocal of these two kinetic constants, two point lines were drawn in the normal double reciprocal plots (Fig. 7.10).

This intersecting pattern indicates that there is no product being released or substrate added between the addition of these two substrates. However, analysis of the replots of slopes and intercepts appears to be somewhat complicated (Figs. 7.12). It is hoped that a comprehensible result may be forthcoming when the same data is plotted against the reciprocal of the HCO_3^- concentration as outlined in the succeeding section.

(6) <u>The influence of $MgATP^{2-}$ on the HCO_3^- kinetics</u>

When the reciprocal of the velocity is plotted as a function of the reciprocal of HCO_3^- concentration at various fixed concentrations of $MgATP^{2-}$, a set of lines, which meet (with a common intersection) to the left of the velocity axis, resulted (Fig. 7.11). However, when the slopes and intercepts were plotted against the reciprocal of the $MgATP^{2-}$ concentration, parabolic curves were observed (Fig. 7.13). This would indicate that $MgATP^{2-}$ is combining at two places

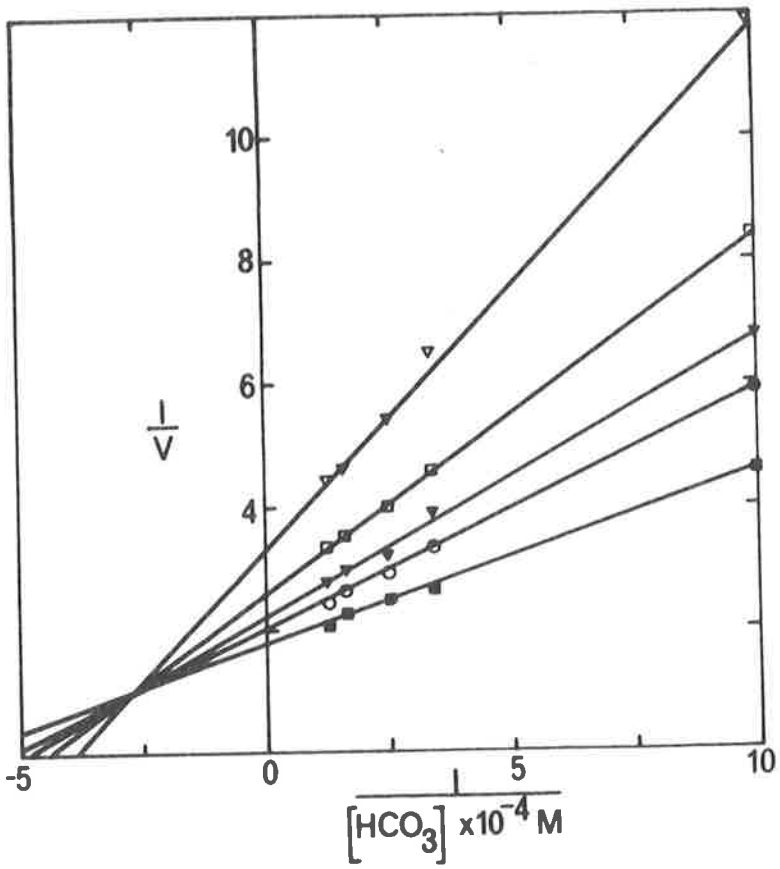


Fig. 7.11. The influence of $MgATP^{2-}$ on the HCO_3^- kinetics.

The reciprocal of the velocity was plotted against the reciprocal of the HCO_3^- concentration at various fixed concentrations of $MgATP^{2-}$. The concentrations of $MgATP^{2-}$ were: 1×10^{-3}M (▽—▽); 2×10^{-3}M (□—□); 3×10^{-3}M (▼—▼); 4×10^{-3}M (○—○), and 5×10^{-3}M (■—■).

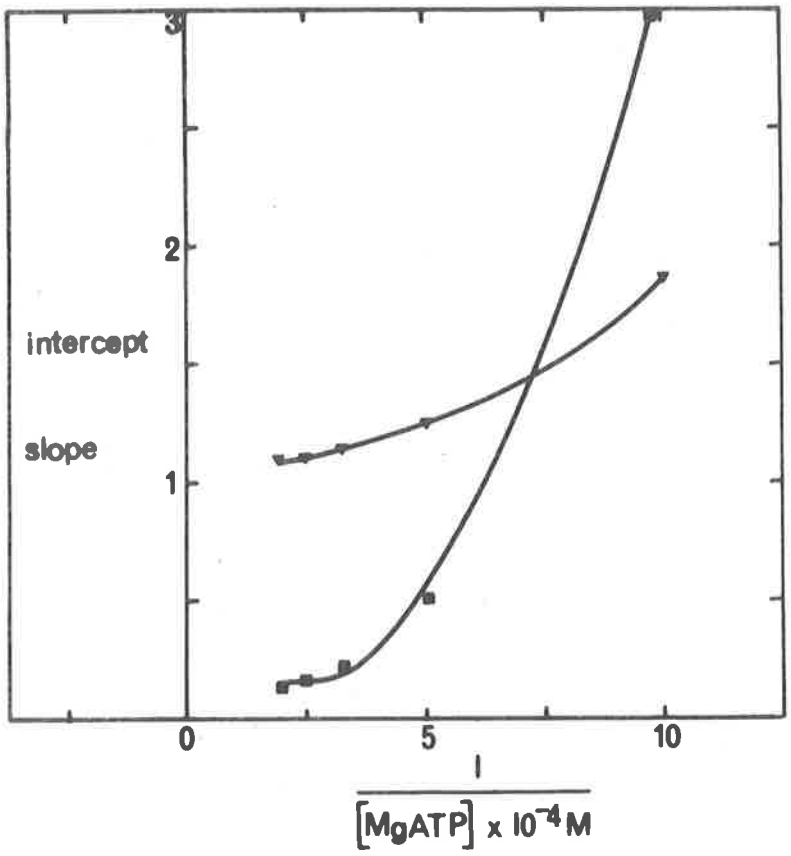


Fig. 7.13. Replots of slopes and intercepts from Fig. 7.11.

The slopes and intercepts from Fig. 7.11 were replotted against the reciprocal of the $MgATP^{2-}$ concentration.

Slopes (■—■); Intercepts (▼—▼).

in the reaction sequence. Moreover, these two points of combination are separated by reversible steps along which interaction may take place so that an increase in the concentration of $MgATP^{2-}$ causes, as a result of this combination, an increase in the steady state concentration of the enzyme form reacting with this compound at the next point of combination.

This idea was proposed in the last chapter, where sigmoidal curves resulted when the velocity of the propionyl-CoA carboxylase reaction was plotted as a function of $MgATP^{2-}$ concentration. This indicated a homotropic co-operative effect, i.e. one molecule of $MgATP^{2-}$ by binding presumably at the activator site, facilitates the binding of another molecule of $MgATP^{2-}$ at the substrate site. Furthermore, the intersecting pattern again indicates that a reversible connection exists between the addition of $MgATP^{2-}$ and HCO_3^- to the enzyme.

(7) <u>The influence of HCO_3^- on ($MgATP^{2-} + Mg^{2+}$) kinetics</u>.

The plots obtained when the reciprocal of the velocity is plotted as a function of ($MgATP^{2-} + Mg^{2+}$) concentration in the presence of varying levels of HCO_3^- are non linear (Fig. 7.14). These lines do not conform exactly to parabolic or hyperbolic curves. It could be suggested that the lines have been erroneously plotted and

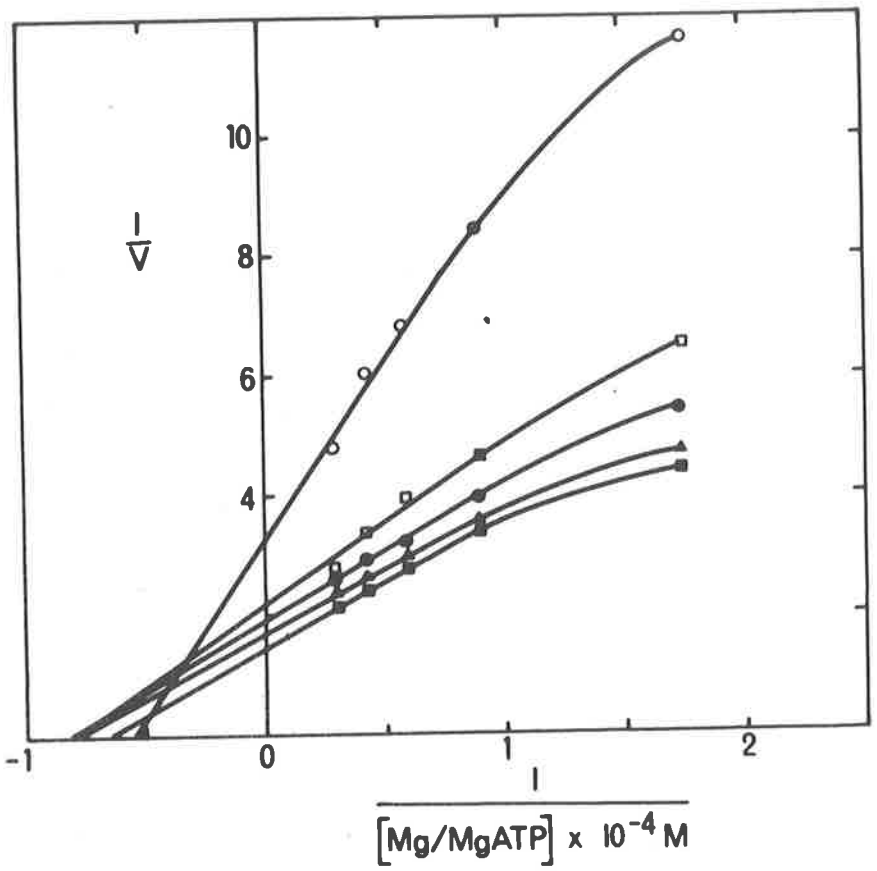


Fig. 7.14. The influence of HCO_3^- on the $(MgATP^{2-} + Mg^{2+})$ kinetics. The reciprocal of the velocity was plotted as a function of the reciprocal of the $(MgATP^{2-} + Mg^{2+})$ concentration at various fixed HCO_3^- levels. The concentrations of HCO_3^- were: 1×10^{-3}M (o—o); 3×10^{-3}M (□—□); 4×10^{-3}M (●—●); 5×10^{-3}M (▲—▲); 8×10^{-3}M (■—■).

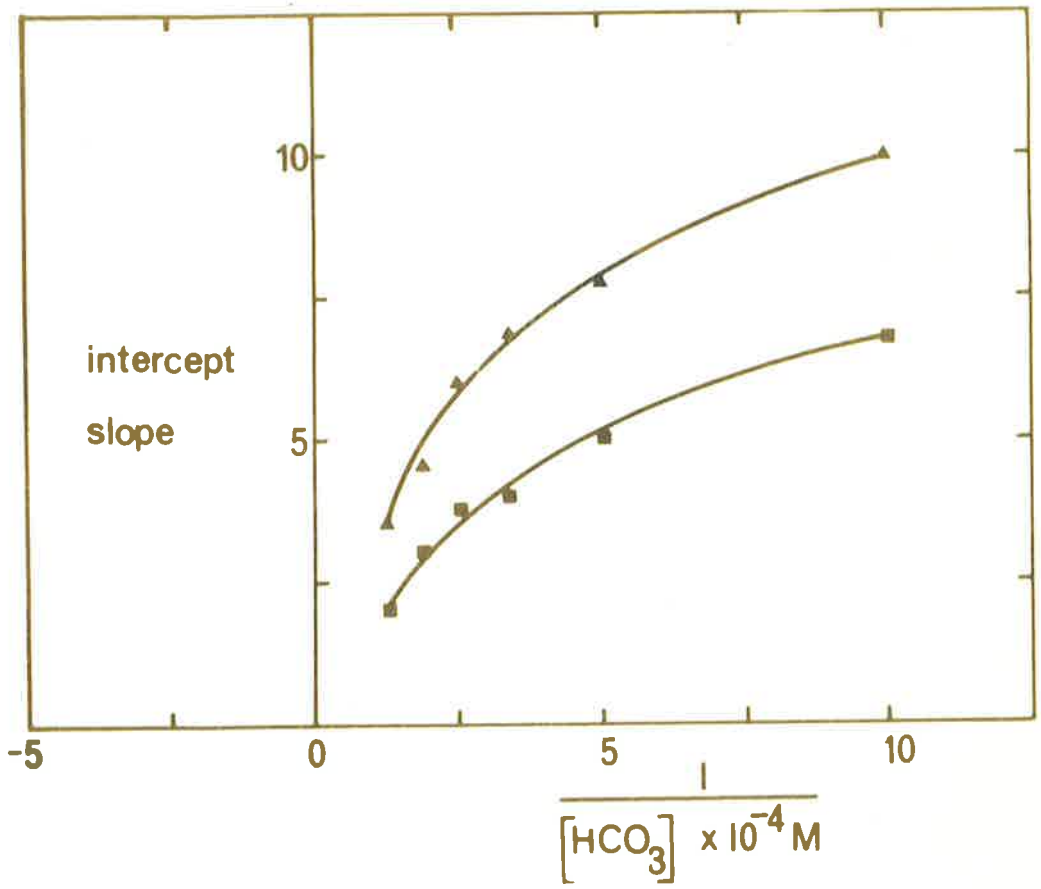


Fig. 7.16. Replots of the intercepts and slopes from Fig. 7.14.

The slopes and intercepts from Fig. 7.14 were replotted against the reciprocal of the HCO_3^- concentration. Slopes (■—■); Intercepts (▼—▼)

should not include those few points which amplify the non linearity. Yet a similar type of plot has been observed with phosphoglycerate kinase (Larsson-Raznikiewicz, 1967) where $MgATP^{2-}$ (in the presence of free Mg^{2+}) was the variable substrate and phosphoglycerate the changing fixed substrate. The interpretation of this phenomenon was that there were two sites on the enzyme molecule for $MgATP^{2-}$. High Mg^{2+} might then change the second centre in such a way that the kinetic parameters for the substrates will change.

This explanation when applied to propionyl-CoA carboxylase could very well be accurate since our previous evidence has indicated that two sites for $MgATP^{2-}$ are available and that Mg^{2+} could be affecting one of these sites. Furthermore, Okazaki & Kornberg (1964) demonstrated that deoxy-thymidine kinase yielded non linear plots similar to those observed in Fig. 7.14. They argued that this behaviour was due to either;

(a) Two enzymes with different kinetic parameters acting on the same substrate, or

(b) The single enzyme present in two different forms each of which has different kinetic parameters.

Moreover, when the slopes and intercepts were replotted against the reciprocal of the HCO_3^- concentration hyperbolic curves resulted (Fig. 7.16). According to

Cleland (1963(b)), hyperbolic replots indicate that a compound reacts with two different enzyme forms to cause inhibition at one point and activation at another. This evidence from both the double reciprocal plots and the replots of both slopes and intercepts indicates that HCO_3^- combines with two different forms of the enzyme in the reaction sequence.

(8) <u>The influence of ($MgATP^{2-}$ + Mg^{2+}) on HCO_3^- kinetics.</u>

When the reciprocal of the velocity is plotted as a function of HCO_3^- concentration at varying fixed levels of ($MgATP^{2-}$ + Mg^{2+}), a set of lines, intersecting above the abscissa, resulted (Fig. 7.15). Furthermore, when the slopes were replotted against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration, a hyperbolic curve was observed (Fig. 7.17). When the intercepts were plotted in the same way, a near linear plot was demonstrated. From these results it was suggested that no substrate was added or product was released between the addition of these two substrates. Moreover, the hyperbolic pattern seen in the replot of the slopes against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration indicates that this double "substrate" is reacting with two different forms of the same enzyme. Analysis of the replot of the intercepts is rather difficult at this juncture because of the non linearity of the replot.

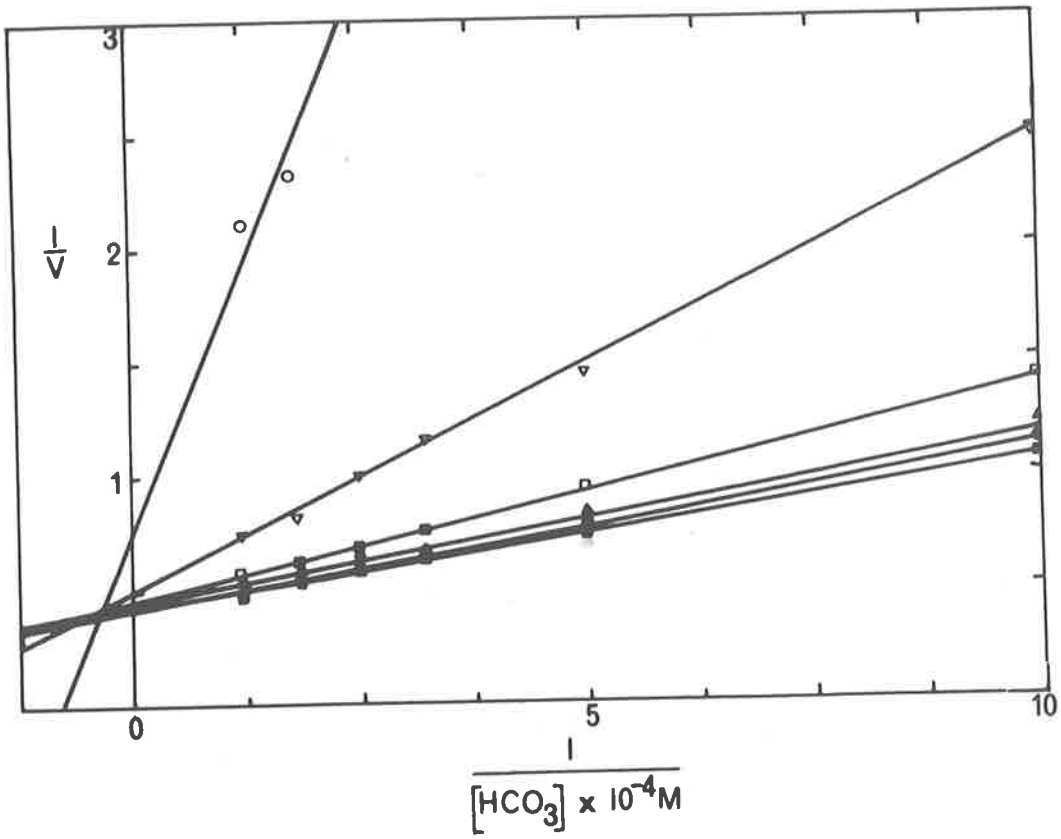


Fig. 7.15. The influence of $(MgATP^{2-} + Mg^{2+})$ on the HCO_3^- kinetics. The reciprocal of the velocity was plotted as a function of the reciprocal of the HCO_3^- concentration at various fixed $(MgATP^{2-} + Mg^{2+})$ concentrations. The concentrations of $(MgATP^{2-} + Mg^{2+})$ were: 3.4×10^{-4}M and 3.4×10^{-4}M (▲——▲); 2.4×10^{-4}M and 2.4×10^{-4}M (□—□); 1.75×10^{-4}M and 1.75×10^{-4}M (▽——▽); 1.2×10^{-4}M and 1.2×10^{-4}M, (○——○).

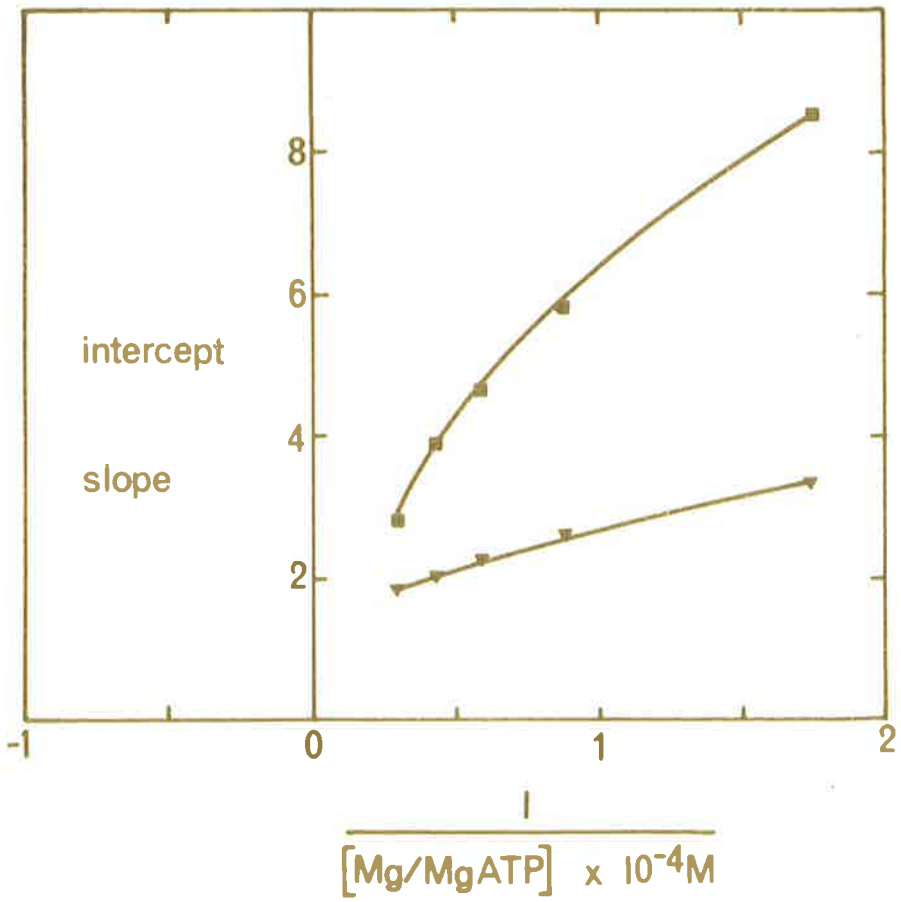


Fig. 7.17. Replots of the slopes and intercepts from Fig. 7.15. The slopes and intercepts from Fig. 7.15 were replotted against the reciprocal of the ($MgATP^{2-}$ + Mg^{2+}) concentration. Slopes (■—■); Intercepts (▼——▼).

This initial velocity study has produced a number of interesting yet confusing results. An attempt was made to interpret these findings using the "inspection" method put forward by Cleland (1963(b)), yet in some situations this approach was not adequate. In spite of this, some of this data will be brought forward in the discussion and it is hoped that these confusing pieces can be collected together to form a feasible and accurate picture of the reaction mechanism.

DISCUSSION:

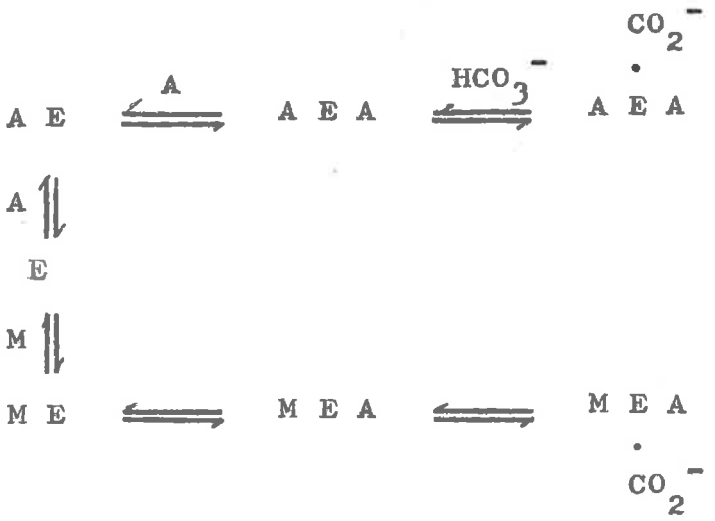
In this section the data will be discussed under two headings:

(1) Evidence for the existence of an alternative pathway in the reaction sequence.

(2) Proposals for the release of the products ADP and Pi from the enzyme.

(1) Evidence for the existence of an alternative pathway in the reaction sequence.

Data presented in this chapter suggested that HCO_3^- could be reacting with either two forms of the one enzyme or two different enzymes (Fig. 7.14). Since it is considered unlikely that a contaminating enzyme could be reacting with HCO_3^-, the possibility of two different forms of propionyl-CoA carboxylase should be considered. In the previous chapter, an alternative mechanism for the formation of the enzyme-nucleotide complex was put forward. It was suggested that both enzyme-$MgATP^{2-}$-$MgATP^{2-}$ and enzyme-Mg^{2+}-$MgATP^{2-}$ could act as the enzyme-nucleotide complex. Therefore, it is proposed that these two species are the two different forms of the enzyme with which HCO_3^- has been reacting. This idea is represented in Equation 7.5.



... (7.5)

In view of this proposal, the hyperbolic curves observed in Figs. 7.16 and 7.17 may not indicate that HCO_3^- is binding with two enzyme forms to cause inhibition at one point and activation at another as previously suggested. This is due to the restriction placed on this assumption by Cleland (1963(b)) who stated "In a mechanism, <u>without alternate reaction sequences</u>, when a compound reacts with two different enzyme forms to cause inhibition at one point and activation at another ..... Replots of slopes and intercepts would be hyperbolas". Therefore, it seems as though the hyperbolic curves obtained in the replots could be due in fact to the existence of an alternative pathway and consequently no conclusion can be made about the inhibition and activation by HCO_3^- at the different points of attachment. However, it should be noted that the idea of activation and inhibition by HCO_3^- could be of consequence since the ATP-Pi exchange reaction catalysed by propionyl-CoA carboxylase is

activated by HCO_3^- but inhibited at high concentrations of HCO_3^-. Of course, it is also possible that the two enzyme-nucleotide complexes could have different affinities for HCO_3^-.

In spite of several contentious points, it is felt that the initial velocity data discussed above supports the alternative reaction scheme proposed in the last chapter. Furthermore, it is possible that due to this alternative pathway, the exchange reaction rates which have caused considerable concern, may have been affected.

(2) Proposals for the release of the products from the enzyme surface.

The initial velocity pattern obtained when ($MgATP^{2-}$ + Mg^{2+}) and propionyl-CoA were the variable substrates, was a set of parallel lines. This was the expected result since the binding of HCO_3^- (at saturating concentrations) to the enzyme surface between the addition of the two variables would lead to an irreversible connection between propionyl-CoA and ($MgATP^{2-}$ + Mg^{2+}).

However, the following data which summarises the findings obtained when other combinations were used did not produce the expected patterns. For example, when HCO_3^- and propionyl-CoA were the varied pair - an intersecting pattern

resulted. A similar pattern occurred when HCO_3^- and $MgATP^{2-}$ or ($MgATP^{2-}$ + Mg^{2+}) and HCO_3^- were the respective combinations. In other words, there was no indication in any of these plots that ADP, Pi or both had left the enzyme surface. It was thought from the ^{18}O-exchange studies that ADP and Pi should come off the enzyme after the addition of the HCO_3^- (Kaziro et al., 1962). Yet the double substrate kinetics using propionyl-CoA and HCO_3^- did not indicate this. In view of this result, the idea of a protein relaxation effect was proposed. This suggestion may have had some merit, if the data from the other substrate combinations had indicated that products had come off before the addition of HCO_3^- to the enzyme. Therefore, for the present, any idea of this type will have to await further investigation.

The intersecting velocity patterns obtained when $MgATP^{2-}$ and HCO_3^- or ($MgATP^{2-}$ + Mg^{2+}) were the varied substrates again indicated that ADP, Pi or both were not released between the addition of the two variables to the enzyme. However, this interpretation as proposed by Cleland (1963(b)) is only valid when a reversible connection exists between the two substrates. In the alternate pathway scheme as proposed in Equation 7.5, even if a product was released between the addition of $MgATP^{2-}$ and HCO_3^-, an irreversible step would not result, because the different

enzyme forms can shuttle back and forth along the bypass. Thus a parallel pattern would not be produced even though the product was released between these particular variables. This argument may apply to the data obtained when ($MgATP^{2-}$ + Mg^{2+}) and HCO_3^- were the varied pair because the presence of free Mg^{2+} would allow the alternate pathway scheme to operate. However, the use of $MgATP^{2-}$ and HCO_3^- as the variable substrates resulted in the same intersecting pattern although in this case, it would be expected that only one pathway would be functional. It could be argued that enough free Mg^{2+} may be present to allow the dual pathway to operate sufficiently and thus prevent any irreversible step from becoming apparent.

At present, this data concerning the release of products is far from satisfactory. This may be due in part to the existence of an alternative pathway, the double binding of $MgATP^{2-}$ or my own lack of understanding of these terreactant systems. It is hoped that the approach adopted here, although preliminary, has demonstrated that certain features of the propionyl-CoA carboxylase reaction are not fully comprehended.

CONCLUDING REMARKS

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CONCLUDING REMARKS:

This thesis reports the investigations into the kinetic properties of propionyl-CoA carboxylase. Several aspects have been studied yet more intensive investigations into some of these areas could enable a more precise mechanism to be established. This information would not only elucidate the steps involved in forming the respective intermediates but could identify the specific amino acids involved in binding and catalysing the particular substrate.

Although preliminary theories concerning modification experiments have been evolved, further substantiating evidence from other enzyme systems must be forthcoming to provide general proof for these concepts. The obvious use of modification experiments requires that a start must be made to interpret results, especially since, as indicated in the pyruvate carboxylase situation, the results could be very informative about the site of action of the allosteric effectors, a question that may not be solved by other kinetic procedures.

A further extension of the modification type of experiment is its use as a safeguard against non specific inhibition. In the case of propionyl-CoA carboxylase, the essential thiol group is reactive towards cther amino acid inhibitors i.e. dinitro-fluoro-benzene, trinitro-benzene

sulphonic acid, diazo-benzene sulphonic acid etc. to such an extent that investigations into other amino acids responsible for some function at the active centre could be thwarted.

It was proposed to modify the thiol group with N-ethyl maleimide to a degree where this residue would be completely protected. Propionyl-CoA would then be added in high concentrations to the assay mixture to provide a measurable assay of activity. Thus, by this procedure, the other amino acid specific reagents could be tested with the knowledge that the inhibition of cysteine residue would not be contributing to any loss of activity. However, there are certain drawbacks to this idea. Firstly, the determination of the 100% loss of activity; a value which is meaningless under these conditions since, even in the presence of low concentrations of propionyl-CoA some activity should be observed. This means that some way of determining this value must be found. Secondly, the extended modification of the thiol group could, as suggested previously, result in an attack on other residues apart from the specific thiol group at the propionyl-CoA site. If these problems could be remedied, it seems theoretically possible to use such an approach to deduce more useful information about active centres.

Another problem which is being attempted in this laboratory, is the elucidation of the sequence of amino acids

around the active thiol group. This is probably an extension of the exercise carried out in chapter 4, when the thiol group was positively identified by the labelling experiments with ^{14}C-N-ethyl maleimide. In this case, however, the ^{14}C-labelled protein will not be degraded completely to the amino acid-inhibitor adduct but a selective enzymic hydrolysis will be employed to allow a peptide of practical length to be isolated. The sequence analysis would then be followed by use of the appropriate techniques.

Another project which could be attempted is the elucidation of the sequence of amino acids around the biotin group of propionyl-CoA carboxylase. In this case, instead of using ^{14}C-biotin labelled enzyme, the biotin-^{14}C-CO_2 complex could be formed. This complex could be treated with diazomethane to stabilize the label and the enzyme degraded as previously suggested. What is more, the fact that the propionyl-CoA site must be proximal to the biotin moiety could suggest that both these sequences may be complementary to each other, that is, of course, provided that both sites are on the same peptide chain. As you can see the investigation into this thiol group has furnished interesting information not only in itself but also because it has opened up other interesting avenues.

In several members of the biotin-containing group

of enzymes, the site of action of their respective activators has been studied. In the pyruvate carboxylase from yeast, acetyl-CoA caused a significant decrease in the K_m for HCO_3^- (Cooper & Benedict, 1966), whilst in the kidney enzyme preliminary evidence has been presented to suggest that the site of action is the breakdown of the enzyme-ATP complex. Furthermore, citrate, the allosteric effector of acetyl-CoA carboxylase, stimulates the breakdown of the CO_2-enzyme complex - an effect which was also observed for the activation of the avian pyruvate carboxylase by acetyl-CoA (Utter et al., 1967). All of these examples seem to imply that the action of the activating compounds is focussed in an area which, to some extent, is common to all these enzymes. This area could be the sequence of reactions in which the enzyme-ATP complex breaks down to form CO_2-enzyme complex. It could be argued that since the reaction mechanisms are very similar, it could be expected that the slowest step in the reaction catalysed by any one enzyme could be the same rate determining event in the others. It could be speculated that the formation of the CO_2-enzyme complex is similar in all the biotin-containing enzymes but the effectiveness of the activating ligand provides the controlling specificity. In this regard, it is fully realised that acetyl-CoA and citrate are metabolically of greater significance than K^+, yet the data and the subsequent interpretations forthcoming in this thesis may help to fully elucidate

the roles of these allosteric modifiers.

The effects of the allosteric effectors, Mg^{2+} and $MgATP^{2-}$, have been studied and various proposals have been put forward to explain their roles in the reaction mechanism. From these investigations, it was speculated that specific modifier and substrate sites exist and therefore the identification of these areas could be of importance in the future. The amino acids which comprise these areas on the protein may be susceptible to attack by amino acid specific reagents. These sites may be protected against this inhibition to some extent by the incubation with Mg^{2+}, ATP^{4-}, Mn^{2+}, $MnATP^{2-}$ etc., all of which have been suggested to have some specific function in the reaction sequence. For example, incubation with Mg^{2+} may cause protection of the modifier site but exposure of the substrate site whereas, ATP^{4-} would have the opposite effect. Furthermore, the comparison of the kinetic behaviours of both Mn^{2+} and Mg^{2+} may also lead to interesting results. Moreover, by this use of Mn^{2+}, which is paramagnetic, such techniques as NMR may be employed to good effect.

The initial velocity studies reported in this thesis were originally undertaken to define the stage at which the products, ADP and Pi, left the enzyme surface. Although this endeavour was not realised, this approach brought forward some interesting yet complex data. These findings did not conform to established predictions and because of this, the results

from this study may be suspect. Indeed, the double addition of $MgATP^{2-}$ to the enzyme and the possible existence of an alternate mechanism may confuse the interpretation of the initial velocity patterns. Yet, the same could be said for the data obtained from isotope exchange which has been the main technique used to propose the mechanisms for the biotin-containing enzymes. For this reason, it is hoped that other approaches will be used to unravel some of the unusual behaviour displayed by this group of enzymes.

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