PYRUVATE CARBOXYLASE:
STUDIES ON THE REACTION MECHANISM

A THESIS SUBMITTED BY

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CONTENTS

SUMMARY

In view of the uncertainties in the previous interpretation of experimental data associated with the mechanism of ATP hydrolysis coupled to CO_2 fixation as catalysed by pyruvate carboxylases, the kinetic evidence has been re-examined. As a result, a step-wise reaction mechanism has been proposed for the first partial reaction, viz;

$$\text{ENZ-biotin} + \text{ATP} \rightleftharpoons \text{ATP-ENZ-biotin} \qquad \text{(i)}$$

$$\text{ATP-ENZ-biotin} + HCO_3^- \rightleftharpoons \begin{matrix}\text{Pi}\\ \vdots\\ CO_2^-\end{matrix}\!\!>\text{ENZ-biotin} + \text{ADP} \qquad \text{(ii)}$$

$$\begin{matrix}\text{Pi}\\ \vdots\\ CO_2^-\end{matrix}\!\!>\text{ENZ-biotin} \overset{\text{acetyl CoA}}{\rightleftharpoons} \text{ENZ-biotin-}CO_2^- + \text{Pi} \qquad \text{(iii)}$$

Contrary to previous reports, the Mg^{2+}-dependent ATP:ADP exchange reaction was shown to be an integral part of the reaction mechanism and has been incorporated into the reaction pathway for the first partial reaction. The exchange reaction in question was previously considered to be the result of an abortive or kinetically insignificant pathway; however rigorous attempts to remove endogenous levels of bicarbonate have shown that the exchange reaction was influenced by bicarbonate. Similarly acetyl CoA has also been shown to influence the Mg^{2+}-dependent isotopic exchange reaction between ATP and ADP. The probable involvement of an enzyme-bound carboxyphosphate (via eq. ii) is consistent with all of the available kinetic data on the reaction mechanism. Further evidence that the overall pyruvate carboxylase catalysed reaction proceeds via a sequential

pathway, has been obtained by demonstrating a synergistic effect of the second partial reaction substrates on the first partial isotopic exchange reactions involving ATP.

On the basis of the proposed mechanism, experiments were carried out to isolate the postulated $^{Pi}\!\!\diagdown\!\!\diagup_{CO_2}$>ENZ-biotin complex. By reacting the enzyme with [γ-^{32}P]ATP in the absence of acetyl CoA, and subsequent gel filtration at 4°C it was possible to isolate the $^{32Pi}\!\!\diagdown\!\!\diagup_{CO_2}$>ENZ-biotin complex. By its ability to transfer the [^{32}Pi]phosphoryl group to ADP in a back reaction to form ATP, the complex was shown to be kinetically competent. Similarly reacting the enzyme with $H^{14}CO_3^-$, enabled the isolation of a [^{14}C] labelled complex ($^{14}\!\!\diagdown\!\!\diagup_{CO_2}$>ENZ-biotin), by similar procedures. The $^{14}\!\!\diagdown\!\!\diagup_{CO_2}$>ENZ-biotin complex was also shown to be kinetically competent by its ability to transfer the [^{14}C]carboxyl group to pyruvate in the presence of acetyl CoA. Although the carboxyl group on the complex was found to be more labile than the phosphoryl group, the kinetic competency with respect to both the phosphoryl group and the carboxyl group of the complex were found to be similar, indicating that they were part of the same complex.

During attempts to characterize the isolated $^{Pi}\!\!\diagdown\!\!\diagup_{CO_2}$>ENZ-biotin complex, the possibility of an involvement of a phosphorylated biotin derivative was considered. The model involving the formation of o-phosphobiotin as the first step in the mechanistic pathway without the involvement of bicarbonate binding, has been ruled out on the basis of the three criteria viz;

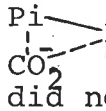
(a) pretreatment of the enzyme with avidin did not reduce the amount of [^{32}Pi] associated with the enzyme, on subsequent gel filtration of the tryptic digest.

(b) the biotin-containing tryptic peptide could be complexed with avidin, without complexing the radioactivity from the main tryptic peptide digest by Sephadex gel filtration.

(c) the pronase digest of the radioactively labelled tryptic peptide did not contain significant levels of biotin.

The stability of both the carboxyl and the phosphoryl group on the isolated 

ENZ-biotin was found to be similar when their kinetic competency was taken as a measure of their stabilities; at 0°C, the complex had a half-life of ≈45 min; at 4°C the half-life was found to be ≈27 min for both the phosphoryl and carboxyl group. Attempts to demonstrate the existence of a free 'carboxyphosphate' was unsuccessful, indicating that the putative 'carboxyphosphate' complex was tightly bound to the enzyme.

To localize the catalytic step in which acetyl CoA manifested its major role, the effect of acetyl CoA on both the formation of ENZ-carboxybiotin as well as the translocation process were tested. The formation of the 

ENZ-biotin complex like the translocation process did not require the participation of acetyl CoA. While the formation of ENZ-carboxybiotin showed an absolute requirement for acetyl CoA. These studies have enabled the locus of acetyl CoA action to be identified with the

catalytic step involving the formation of ENZ-carboxy-biotin from the 

ENZ-biotin complex.

The apparent 'absolute' requirement for acetyl CoA exhibited by pyruvate carboxylase isolated from chicken liver has been re-examined. The enzyme has been shown to catalyse the carboxylation of pyruvate in the absence of acetyl CoA at a rate which was approximately 2% of the catalytic rate in the presence of acetyl CoA. However the acetyl CoA-independent rate showed a marked requirement for K^+.

The 2,3-dialdehyde derivative of ATP (oATP) has been shown to be an affinity label of the $MgATP^{2-}$ binding site of chicken liver pyruvate carboxylase. This property was established previously in the case of the sheep liver enzyme (Easterbrook-Smith *et al.*, 1976b). In this study this affinity label has been utilized to covalently modify and hence facilitate the isolation of the $MgATP^{2-}$-binding site of the chicken liver enzyme. In order to find efficient methods of isolation of the oATP-containing tryptic peptide, two procedures have been investigated.

STATEMENT

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

N.F.B. PHILLIPS

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ABBREVIATIONS

In addition to those accepted for use in the *Biochemical Journal*, the following abbreviations are used in this thesis.

AP_5A	P^1,P^5-Di(adenosine-5')pentaphosphate
DTE	dithioerythritol
DTNB	5,5'-dithio-bis-(2-nitrobenzoic acid)
oATP	2',3-dialdehyde oxidation product of ATP
OAA	oxaloacetate
PEI	polyethyleneimine
U	international enzyme unit

CHAPTER I

INTRODUCTION

1.A GENERAL PROPERTIES OF BIOTIN-CONTAINING ENZYMES.

Pyruvate carboxylase [pyruvate:CO_2 ligase (ADP) E.C.6.4.1.1] is a member of the group of enzymes which contain a covalently bound biotin prosthetic group. The detailed studies of several of these enzymes have revealed strong similarities among them, for example, in relation to their minimal reaction mechanism, their sensitivity to avidin, the biotin binding protein from egg white, their high molecular weights and their complex quaternary structures. Comprehensive reviews on the biotin-containing enzymes have appeared (Moss & Lane, 1971; Wood & Barden, 1977). A review specifically on pyruvate carboxylase has also been published (Utter, Barden & Taylor, 1975).

1.A.1 Reaction Mechanism

The overall reaction catalyzed by the biotin-dependent enzymes,

$$ATP + HCO_3^- + Acceptor \rightleftharpoons Acceptor\text{-}CO_2^- + ADP + Pi \qquad (1:1)$$

can be partitioned into two discrete steps as follows:-

$$ENZ\text{-}biotin + ATP + HCO_3^- \rightleftharpoons ENZ\text{-}biotin\text{-}CO_2^- + ADP + Pi \qquad (1:2)$$

$$ENZ\text{-}biotin\text{-}CO_2^- + Acceptor \rightleftharpoons ENZ\text{-}biotin + Acceptor\text{-}CO_2^- \qquad (1:3)$$

The current view of the active site is that it consists of two spatially distinct subsites. At the first sub-site, the fixation of CO_2 is coupled to the hydrolysis of ATP with the concurrent formation of a carboxy-biotin-enzyme complex (Reaction 1:2). Once formed, the carboxy-biotin functions as a mobile carboxyl group carrier and transports the activated

carboxyl group to the second sub-site, where the bound acceptor is carboxylated (Reaction 1:3). With transcarboxylase, a carboxyl-donor (methyl malonyl-CoA) replaces ATP and HCO_3^- in partial reaction (1:2), while with the decarboxylases, partial reaction (1:3) proceeds right to left, followed by the decomposition of carboxy-biotin to give CO_2.

The first piece of evidence for a 'two-site' active centre for biotin-dependent enzymes come from kinetic studies on transcarboxylase by Northrop (1969). Subsequently, proof for catalysis of each partial reaction at separate sub-sites was obtained with acetyl CoA carboxylase from *Escherichia coli* (Alberts & Vagelos, 1972; Polakis *et al.*, 1974). This enzyme exists as three different subunits, viz., the biotin carboxyl carrier, the biotin carboxylase and the carboxyl transferase subunits. The biotin carboxylase subunit was shown to catalyse the carboxylation of free biotin in the absence of the other two subunits, thus establishing the domain for the ATP and HCO_3^- binding sites. Similarly the carboxyl transferase subunit was found to catalyze the carboxylation of biotin using malonyl CoA as the carboxyl donor; thereby localizing the CoA-ester binding site on this subunit. Further evidence for a 'two-site' mechanism has been obtained from product inhibition data and on the observations that isotopic exchange reactions can occur in the absence of the components of the other partial reaction. These have been demonstrated with pyruvate carboxylases from chicken liver (Barden *et al.*, 1972), rat liver (McClure *et al.*, 1971b, 1971c) and sheep kidney (Ashman & Keech, 1975).

Finally Wood and his co-workers have demonstrated that each partial reaction (Reactions 1:2 and 1:3) is catalysed by a separate subunit of the transcarboxylase (Chuang *et al.*, 1975).

The data obtained from steady state kinetic analyses has been previously interpreted to suggest that the reaction catalysed by the biotin-containing enzymes proceeded by a 'two-site' or non-classical Ping-Pong-Bi-Bi-Uni-Uni mechanism (Northrop, 1969; Barden *et al.*, 1972; McClure *et al.*, 1971a, 1971b; Ashman & Keech, 1975). More recently, Warren & Tipton (1974a, 1974b) have proposed a sequential mechanism for pyruvate carboxylase isolated from pig liver. These conflicting views are discussed in detail in Section 1.C in the light of more recent findings.

1.B. CHEMISTRY OF PYRUVATE CARBOXYLATION

1.B.1 Carboxylation of Biotin

The detection of ε-N-biotinyl-L-lysine ('biocytin') in yeast extract (Wright *et al.*, 1952) suggested that biotin is attached to its apoenzyme through the ε-amino group of a lysyl residue. This was confirmed for propionyl CoA carboxylase from rat liver by the isolation of [^{14}C]biocytin from pronase digests of the enzyme containing [^{14}C]biotin (Kosow & Lane, 1962). The attachment of biotin through a lysyl residue was subsequently demonstrated for several other biotin enzymes and is probably a common feature in all biotin enzymes.

The elucidation of the site of carboxylation on biotin

has been helped by the observation that some carboxylases catalyse the carboxylation of free D-(+)-biotin, a property first demonstrated with the bacterial β-methylcrotonyl CoA carboxylase (Lynen *et al.*, 1959). The carboxylated labile product was stabilized by treatment with diazomethane which allowed its isolation and identification as the dimethyl ester of 1'-N-carboxybiotin. It was concluded that the 1'-N-, rather than the 3'-N position of biotin was the site of carboxylation, since the X-ray crystallographic studies of Traub (1956, 1959) indicated steric hinderance of the 3'-N position by C-6 of the aliphatic side chain. Subsequently the site of carboxylation on the covalently bound prosthetic group was also shown to be at the 1'-N position. This was first shown by the isolation and identification of the stabilized intermediate as 1'-N-carbomethoxy-biocytin, following proteolytic digestion of β-methylcrotonyl CoA carboxylase (Knappe *et al.*, 1963). Since then similar results have been obtained with propionyl CoA carboxylase (Lane & Lynen, 1963) acetyl CoA carboxylase (Numa *et al.*, 1965) and transcarboxylase (Wood *et al.*, 1963).

However, the model studies of Bruice & Hegarty (1970), indicated that the ureido oxygen was the most nucleophilic site in the imidazolidone ring of biotin. They suggested that the ureido oxygen was the site of carboxylation and that the 1'-N derivative is formed as a result of a rearrangement that transferred the carboxyl group to the 1'-N position during the diazomethane trapping experiments. However, Lane and his collaborators (Guchhait *et al.*, 1972) showed that chemically synthesized 1'-N-carboxybiotinol was biologically

reactive and could serve as a carboxyl group donor for carboxylation of acetyl CoA in the presence of the carboxyl transferase component of acetyl CoA carboxylase from *E. coli*. Hence it is apparent that there are difficulties of using model reactions to establish the role of biotin in catalysis by enzymes and as Wood (1976) points out, simple model compounds are not always reliable indicators of reactivity in the environment of an enzyme.

It is unclear why the 1'-N is preferred over the ureido oxygen in carboxylation reactions. From model compound studies (Bruice & Hegarty, 1970) it is evident that compounds with lower basicity gave a greater O attack than an N attack, and as the basicity increased, N attack increased. Moss & Lane (1971) noted that the basicities of the leaving groups would be expected to be very high and thus could favour N attack rather than an O attack.

1.B.2 Specificity for Bicarbonate

The substrate for biotin carboxylases is HCO_3^- rather than CO_2. Kaziro *et al.* (1962) using $HC^{18}O_3^-$ with propionyl CoA carboxylase showed the equivalent of one atom of ^{18}O in the orthophosphate and two in the carboxyl of the methyl malonyl-CoA. If CO_2 had been the reactant only two atoms of ^{18}O would be expected to be utilized. Later Cooper & Wood (1971) confirmed that HCO_3^- was the reactant for pyruvate carboxylase from chicken liver and yeast. It is not clear why HCO_3^-, which is relatively unreactive toward nucleophilic attack, is utilized in preference to the chemically much more reactive CO_2. It has been suggested that the anionic nature

of HCO_3^- renders it a more attractive ligand (Scrutton & Lane, 1969). Resonance stabilization in HCO_3^- makes it less prone to nucleophilic attack and hence might require enzyme-mediated electrophilic activation. Electrophilic activation has been obtained with magnesium methyl carbonate, an analogue of HCO_3^- (Stiles, 1960). It has also been proposed that an ATP-mediated activation of HCO_3^- forming carboxy phosphate, would deliver reactive molecules of CO_2 at high concentrations to the active site for reaction with 1'-N of biotin (Sauers *et al.*, 1975).

1.B.3 Effect of Monovalent Cations

Activation of enzymic activity by a monovalent cation e.g., K^+, is observed for pyruvate carboxylases isolated from a variety of sources, including the enzymes from chicken liver (Barden & Scrutton, 1974), rat liver (McClure *et al.*, 1971a), human liver (Scrutton & White, 1974), sheep kidney (Ashman *et al.*, 1972) *Saccharomyces cervisiae* (Cazzulo *et al.*, 1969), *Aspergillus niger* (Feir & Suzuki, 1969) and *Azotobacter vinelandii* (Scrutton & Taylor, 1974).

A study of specificity of activation reveals marked differences for the various pyruvate carboxylases (Scrutton & Young, 1972). Thus, K^+, NH_4^+, Rb^+ and Cs^+ are all effective activators of the chicken liver (Barden & Scrutton, 1974) and sheep kidney (Ashman *et al.*, 1972) enzymes. In the chicken liver enzyme, $Tris^+$ is a weak, and Na^+ a very weak, activator, whereas in the rat liver pyruvate carboxylase, Na^+ is a weak activator and $Tris^+$ is ineffective (McClure *et al.*, 1971b). Both $Tris^+$ and Na^+ are ineffective for the enzyme from human

liver (Scrutton & White, 1974). There is evidence suggesting that acetyl CoA alters the affinity of the sheep kidney enzyme for the activating monovalent cations (Ashman *et al.*, 1972). Hence in the absence of acetyl CoA the enzyme was weakly activated by Na^+ but not by $Tris^+$, while in the presence of acetyl CoA, the enzyme was activated by $Tris^+$ but not by Na^+.

The site of monovalent cation activation was thought to be predominantly located in the first partial reaction sub-site (1:2) for the enzyme from rat liver (McClure *et al.*, 1971b). However, Barden & Scrutton (1974) suggests that monovalent cations participate in catalysis at both sub-sites since K^+ activates both the exchange of [^{14}C]ADP (or [^{32}Pi]) with ATP as well as the exchange of [^{14}C]pyruvate with oxaloacetate in the chicken and rat liver enzymes.

1.B.4 Activation by Acetyl CoA

There is considerable species variation in the degree of dependence on acetyl CoA for catalytic activity. The requirement varies from an apparently absolute dependence by the enzymes isolated from avian sources, (Scrutton & Utter, 1967; Dugal & Louis, 1972) *Arthrobacter globiformis* (Bridgeland & Jones, 1967) and *Bacillus stearothermophilis* (Cazzulo *et al.*, 1970), to no response as found in the enzyme isolated from *Pseudomonas citronellolis*, (Soubert & Remberger, 1961) and *Azotobacter vinelandii* (Scrutton & Taylor, (1974). The enzyme from mammalian sources catalyses acetyl CoA-independent carboxylation at a rate which, under optimal conditions, is approximately 25% of the maximal rate

observed in the presence of a saturating concentration of this effector (Scrutton & White, 1972; Ashman *et al.*, 1972; Scrutton & White, 1974), while the enzyme from yeast is stimulated only two to three fold (Cazzulo & Stoppani, 1967; Cooper & Benedict, 1966).

It is not clear why there is a great variation in the degree of requirement for acetyl CoA by pyruvate carboxylases from different sources. Despite their common minimal mechanism, it is unlikely that this compound forms any covalent intermediate in catalysis (Utter & Keech, 1963; Scrutton & Utter, 1967).

In attempting to elucidate the mechanism of activation of pyruvate carboxylase by acetyl CoA; Ashman *et al.* (1972) showed that for the sheep kidney enzyme the exchange reaction between orthophosphate and $MgATP^{2-}$ was absolutely dependent upon the presence of acetyl CoA, thus confirming its requirement at the first partial sub-site. In addition, these workers showed that acetyl CoA affects the biotin carboxylation step by lowering the apparent K_m value for HCO_3^-. Subsequently Ashman *et al.* (1973), showed that acetyl CoA did influence events during the transcarboxylation process at the second sub-site since the effector stimulated the pyruvate:oxaloacetate exchange reaction and lowered the apparent K_m value for pyruvate.

1.B.4a Enzyme inactivation upon dilution and protection by acetyl CoA

Working with sheep kidney pyruvate carboxylase, Ashman *et al.* (1972) found that when the enzyme was diluted to

concentrations below 4 E.U.ml^{-1}, the enzyme underwent inactivation. No inactivation was observed when the enzyme was diluted in the presence of acetyl CoA. Although other explanations are possible (Dixon & Webb, 1964), the simplest explanation for the concentration dependent inactivation is that the enzyme dissociates into less active or inactive forms. However, the ability of Nakashima *et al.* (1975) to prepare active monomers from rat liver enzymes, indicates that at least for rat liver pyruvate carboxylase, dissociation does not result in inactivation of the enzyme. Further work carried out in this laboratory (Clements, *et al.*, 1978) demonstrated that the inactivation process appeared to affect the ATP binding site since the inactivation of the ATP:Pi exchange paralleled the loss of overall activity, while pyruvate:oxaloacetate exchange reaction was unaffected. In an attempt to define the influence of various components of acetyl CoA molecule on the enzyme, Clements *et al.* (1978) concluded that the adenosine moiety of acetyl CoA was essential for the activation of the first partial reaction (eq. 1:2) while the acetyl moiety influenced the second partial reaction (eq. 1:3) (see Fig. 1.1). It has been speculated that acetyl CoA in some way, spans across the active site so that it can exert its effects on both sub-sites at the same time (Keech, 1980).

A recent electron microscopic study (Mayer *et al.*, 1980) has indicated that each subunit of the enzyme consists of two 'domains' separated from each other by a 'cleft'. When the enzyme was mounted and fixed in the presence of acetyl

CoA, the 'cleft' was no longer visible. Keech (1980) points out that it appeared as though acetyl CoA prevented the enzyme opening out on dilution. Supporting evidence for this proposal comes from the demonstration that avidin and antibiotin antibodies, when bound to the biotin in pyruvate carboxylases, appeared to be located in a specific area (Cohen *et al.*, 1979a) and the 'cleft' observed by Mayer *et al.* (1980), was in this very same area.

1.B.4b Effect of acetyl CoA on catalytic activity

With the first reports of a sigmoidal velocity response to increasing concentrations of acetyl CoA (Barrit *et al.*, 1966; Scrutton & Utter, 1967) it was assumed that the activator bound to the enzyme in a homotropic co-operative manner. The only direct evidence for co-operativity of binding of acetyl CoA to pyruvate carboxylase came from the work of Frey & Utter (1977). However, unequivocal interpretation of this data was difficult due to the fact that purified preparations of pyruvate carboxylase catalyse the deacylation of acetyl CoA (Scrutton & Utter, 1967). Although this deacylase activity is of little consequence in kinetic studies, if becomes significant in binding studies which require high enzyme concentrations and longer time intervals. As pointed out by Easterbrook-Smith *et al.* (1979), it is possible in using the modified flow dialysis method of Colowick & Womack (1969), that if significant deacylation of acetyl CoA occurred during an experiment, then not only would the [^{14}C]acetyl CoA concentration change but the [^{14}C]-acetate formed would be expected to diffuse across the binding

cell membrane many times faster than the rate of the [1-^{14}C]acetyl CoA diffusion. This in turn would lead to an under-estimate of the binding of acetyl CoA by pyruvate carboxylase.

The kinetic study of the deacylation reaction by Easterbrook-Smith *et al.* (1979) using acetyl CoA as the substrate, revealed a linear double reciprocal plot, suggesting that acetyl CoA bound to the enzyme in a classical Michaelis-Menten manner, thus questioning the origin of the sigmoidal velocity profile in the carboxylation reaction. The observation that the active monomers of rat liver pyruvate carboxylase exhibited a sigmoidal velocity profile (Nakashima *et al.*, 1975), and that only 3 to 4 molecules of acetyl CoA bound to the tetrameric form of chicken liver pyruvate carboxylase (Frey & Utter, 1977), indicated that homotropic co-operativity was not a likely explanation for the observed non-classical acetyl CoA kinetic behaviour.

The findings of Easterbrook-Smith *et al.* (1979) provided a valid explanation for this atypical kinetic behaviour. These authors considered two properties of acetyl CoA in their explanation. Firstly, they reasoned that since acetyl CoA increased the V_{max} and reduced the apparent K_m value for pyruvate (Ashman *et al.*, 1972) then when acetyl CoA is the variable ligand, the fixed concentration of pyruvate, although saturating at high concentrations of acetyl CoA, will become non-saturating as the concentration of acetyl CoA is decreased. This would then result in an under-estimate of the reaction velocity.

Secondly, the phenomenon of irreversible inactivation

of the enzyme on dilution (Ashman *et al.*, 1972) was also regarded by Easterbrook-Smith *et al.* (1979) to be another contributing factor to the sigmoidicity. Although acetyl CoA protects the enzyme against this inactivation process, a rapid loss of enzymatic activity occurs when the enzyme is diluted in solutions where the concentration of acetyl CoA is below 40 μM. The effect of this rapid inactivation process on the velocity profile would be to yield a falsely reduced rate at low concentrations of acetyl CoA.

Hence when the rate of enzymic activity at varying acetyl CoA concentrations was measured under conditions where neither of the above two factors were operating (i.e., at saturating pyruvate concentrations at all times and at high enzyme concentrations), the sigmoidicity of the acetyl CoA velocity profile was completely eliminated (Easterbrook-Smith *et al.*, 1979).

Since the development of the concept of co-operativity (Monod *et al.* 1965; Koshland *et al.*, 1966), non-classical saturation kinetic plots have usually been explained as co-operative processes and very rarely have alternative explanations been sought. For example, the multiple-reaction-pathway model (Sweeney & Fisher, 1968; Wells *et al.*, 1976), the polymerizing enzyme schemes (Nichol *et al.*, 1967) and the mnemonical enzyme concept (Rabin, 1967; Ricard *et al.*, 1974). In fact Keech & Wallace (1981), stress the excessive importance attached to the co-operative models and that too little attention is given to other less aesthetically pleasing explanations. They point out that apart from a few enzymes exhibiting sigmoidal binding or velocity

profiles conforming to one or the other schemes of co-operativity, it was difficult to find other rigorously established examples, and that in many cases, the sigmoidicity arises as a result of artefacts of the assay procedure (as in the case of pyruvate carboxylase) or a failure to appreciate the complexities of the system.

The mechanism of activation by acetyl CoA is further discussed in the light of the experimental findings of this thesis in Chapter VI.

1.C. PYRUVATE CARBOXYLASE REACTION SEQUENCE

The overall reaction catalyzed by pyruvate carboxylase is relatively complex since it involves three substrates: $MgATP^{2-}$, HCO_3^- and pyruvate; and three products: MgADP, orthophosphate and oxaloacetate. In addition there is a requirement for a divalent cation, and the enzyme is also activated by a monovalent cation and an acyl CoA effector. As in other biotin dependent enzymes, the overall reaction catalyzed by pyruvate carboxylase can be partitioned into two partial reactions (1:2 and 1:3) each occurring at catalytically distinct sub-sites.

Pyruvate carboxylases from all sources examined so far have been shown to catalyse isotopic exchange reactions between ATP and ^{32}Pi, ATP and [^{14}C]ADP, oxaloacetate and [^{14}C]pyruvate, and oxaloacetate and $H^{14}CO_3^-$ (for a review see Utter *et al.*, 1975). The isotopic exchange reaction between ATP and ^{32}Pi requires the presence of the first partial reaction (1:2) components but neither of the two

components of the second partial reaction (1:3), pyruvate and oxaloacetate. Similarly, the exchange reaction between oxaloacetate and [^{14}C]pyruvate proceeds in the absence of the first partial reaction components. The above observations are taken as further confirmation that the overall reaction proceeds via the two partial reactions and that the exchanges involving ATP occur at the first subsite while the pyruvate:oxaloacetate exchange reaction occurs at the second subsite.

Kinetic studies of the pyruvate carboxylase mechanism have been undertaken using enzyme isolated from *Aspergillus niger* (Feir & Suzuki, 1969), rat liver (McClure *et al.*, 1971a, b, c), chicken liver (Barden *et al.*, 1972), pig liver (Warren & Tipton, 1974a, b, c) and sheep kidney (Ashman & Keech, 1975). On the basis of the data obtained, it becomes apparent that the enzyme from these different sources catalyses the reaction using the same basic mechanism. However, the various interpretations of the kinetic results have not been consistent.

Until the work of Warren & Tipton (1974a, b, c) it was generally accepted that the reaction (1:1) proceeded via a non-classical bi-bi-uni-uni-ping-pong reaction sequence in the terminology of Cleland (1963). Warren & Tipton (1974a, b, c) suggested that the overall reaction may be described by a sequential reaction in which no products of the reaction are released from the enzyme until all the substrates have bound. Easterbrook-Smith *et al.* (1978) have provided additional evidence for a sequential mechanism using the enzyme from sheep liver.

In view of the controversy concerning the nature of the reaction sequence, the experimental evidence produced in favour of each type of reaction sequence will be discussed.

1.C.1 The Non-classical Ping-Pong Reaction Pathway

On the basis of the isotopic exchange reactions and the isolation of the ENZ-biotin-CO_2^- intermediate, the reaction sequence was described as bi-bi-uni-uni-ping-pong for pyruvate carboxylases isolated from rat liver (McClure *et al.*, 1971a, b, c), chicken liver (Barden *et al.*, 1972) and sheep kidney (Ashman & Keech, 1975). The initial velocity and product inhibition data of McClure *et al.* (1971a) were interpreted as indicative of a two sub-site ping-pong mechanism, Similar to that proposed by Northrop (1969) for methylmalonyl CoA transcarboxylase. For such a mechanism, double reciprocal initial velocity plots, where either ATP or HCO_3^- is varied at different fixed levels of pyruvate, should yield a family of parallel lines. However the patterns obtained were unusual in that at low levels of pyruvate, the lines appeared to be parallel, and as the level of pyruvate was increased, the lines became intersecting. McClure and co-workers rationalized this unusual result by suggesting that the binding of pyruvate at the second sub-site, influenced the affinity of the enzyme for bicarbonate. They did not however, explain how this occurred at high levels of pyruvate but not at low levels. A similar type of pattern was observed with initial velocity studies of the sheep kidney enzyme (Ashman & Keech, 1975).

The data obtained from kinetic analysis of the chicken

liver enzyme (Barden *et al.*, 1972) have also been interpreted to support a non-classical ping-pong mechanism. By restricting their study to a low and narrow concentration range of pyruvate, these authors were able to observe families of parallel lines in double reciprocal plots with either ATP or HCO_3^- as the variable substrates and pyruvate as the fixed variable. However, the narrow range of pyruvate concentrations used was in the region where McClure *et al.* (1971b) and Ashman & Keech (1975) obtained the parallel lines.

The only discrepancy in the predicted product inhibition patterns for the non-sequential pathway, once again involves pyruvate. For the non-sequential pathway, a family of parallel lines in double reciprocal plots, is predicted when either MgADP or Pi is the product inhibitor and pyruvate is the variable substrate. The data obtained by Ashman & Keech (1975) showed unusual patterns of orthophosphate inhibition. At low concentrations of pyruvate, uncompetitive inhibition was observed, but at higher pyruvate concentrations non-competitive inhibition occurred. Barden *et al.* (1972) limited their pyruvate levels to a low concentration range and hence observed only the uncompetitive patterns indicative of a non-sequential reaction. The possible reasons for this atypical kinetic response to various concentrations of pyruvate is discussed in Section 1.F.

Although steady state kinetic analysis have indicated some inconsistencies with respect to a non-sequential mechanism, the fact that the exchange reactions proceed in the absence of the other partial reaction components has been the key evidence in favour of the non-sequential

mechanism. However Warren & Tipton (1974b) point out that the ATP:Pi exchange rate is only 1% of the overall pyruvate carboxylation rate, and this, they proposed, was because significant release of ADP and Pi only occurred after the binding of pyruvate. When considering the rationale behind slow partial exchange reactions, the concept of substrate synergism (Bridger *et al.*, 1968) has to be borne in mind. As pointed out by Lueck & Fromm (1973), partial exchange reactions in a ping pong mechanism are often slow relative to the initial velocity. Bridger *et al.* (1968) suggests that net reaction rates (i.e., steady state conditions) are measured in the absence of product required for the partial reaction and thus a comparison between the two different rates is difficult to make unambiguously. One of the criteria used in evaluating substrate synergism involves comparison of the partial exchange reactions in the presence and absence of the substrate-product pair not involved in the isotopic exchange reaction. Lueck & Fromm (1973) suggests that while it is quite valid to compare partial exchange reactions in the presence of the other substrate-product components of the ping-pong system, it is not possible to relate partial exchanges to initial velocities in an attempt to explain so called slow exchange reactions, a correlation which is too often made.

1.C.2 The Sequential Mechanism

The first proposal of a sequential mechanism by Warren & Tipton (1974, a, b, c) was a major landmark in the study of pyruvate carboxylases. Using pyruvate carboxylase isolated from pig liver, they obtained initial velocity and

product inhibition data which were not very different to those reported for the enzymes from *Aspergillus niger*, rat liver, chicken liver or sheep kidney, however they did point out inconsistensies in the interpretations. In the case of rat and chicken liver enzymes, the proposed non-classical mechanism allows the formation of ternary complexes without affecting the parallel line plots which characterize ping pong mechanisms. Warren & Tipton point out that to achieve this situation the binding sites for the two partial reaction components must bind their substrates at equilibrium and must be totally independent of each other. This was not the case with the rat liver enzyme as bound pyruvate is believed to affect the binding of HCO_3^- (McClure *et al.*, 1971b).

The reaction pathway proposed by Warren & Tipton (1974b) on the basis of their results obtained with pyruvate carboxylase from pig liver is shown in Fig. 1.2. In this scheme free Mg^{2+} binds to the enzyme in an equilibrium fashion and remains bound during all further catalytic cycles. $MgATP^{2-}$ binds next, followed by HCO_3^- and then pyruvate. Oxaloacetate is released before the random release at equilibrium of Pi and MgADP.

Supporting evidence for a sequential mechanism came from two independent experimental approaches carried out by Easterbrook-Smith *et al.* (1978). In the first instance, to avoid the variable stoichiometry associated with oxaloacetate formation (see Section 1.F), the reaction rate was measured by following the release of Pi. Initial velocities, when plotted as a function of varying concentrations of

either ATP or HCO_3^-, at fixed concentrations of pyruvate gave families of straight, intersecting lines, in double reciprocal plots. Secondly when the reaction velocity was determined as a function of varying ATP concentration by using pyruvate and two alternate carboxyl-acceptor substrates, 3-fluoropyruvate and 2-oxobutyrate, the slopes of the double reciprocal plots were significantly different. Both results supported a sequential reaction pathway.

Any proposition supporting a sequential pathway must also provide some explanation for the isotopic exchange reaction catalysed by the enzyme in the absence of components of the other partial reaction. Easterbrook-Smith *et al.* (1978) points out that isotopic exchange reactions independent of the components of the other partial reaction, are not inconsistent with a sequential pathway if the active site is considered to consist of two spatially distinct sub-sites, as is suggested from product-inhibition studies (McClure *et al.*, 1971b; Barden *et al.*, 1972; Ashman & Keech, 1975). Although in the overall reaction measured under steady state conditions, release of MgADP and Pi occurs after pyruvate binds, the separation of the sites implies that there is no mechanistic (in the sense of chemical interaction between the substrates and the active site amino acid residues) requirement for this order of release to be maintained under differing conditions. These recent findings hence indicate that the overall reaction sequence catalysed by pyruvate carboxylase is of a sequential nature.

1.D MECHANISM OF THE FIRST PARTIAL REACTION

In the first partial reaction (reaction 1:2), the hydrolysis of $MgATP^{2-}$ is coupled to the fixation of CO_2 to form a carboxybiotin-enzyme complex. A number of possible mechanisms for the coupled reaction have been postulated by various investigators but there is still a lack of general agreement on the sequence of events leading to the formation of the carboxybiotin-enzyme intermediate. The difficulties in elucidating the reaction mechanism becomes apparent when the complexities of the nature of the reaction is considered. Studies using model compounds revealed that the ureido group of biotin is a weak nucleophile (Caplow, 1965), while Hegarty *et al.* (1969) showed that the nucleophilicity of the ureido group was increased 10^{10}-fold when the tautomer was in the enol form. These authors concluded that although the proportion of the enol form appears to be extremely low (Glasel, 1966), this tautomer may be enolized by interaction with the apo-enzyme.

Another factor contributing to the complexity of reaction 1:2 is the weak electrophilic nature of HCO_3^- (see Section 1.B.2). Hence most attention has been directed towards either the process of activation of the biotin moiety or the electrophilic activation of HCO_3^-.

The coupling mechanisms, involved in the formation of carboxybiotin-enzyme, which have been put forward are:

(a) a concerted reaction which proceeds without the formation of a kinetically significant or detectable intermediate,

(b) activation of the enzyme (or the biotin prosthetic

group) by ATP to form an activated P~Enz-biotin, ADP~Enz-biotin (or P~biotin-Enz) complex, which is now reactive towards HCO_3^-

(c) activation of HCO_3^- by ATP to form a phosphoryl or adenyl species.

1.D.1a The concerted mechanism

The first report suggesting that the first partial reaction proceeded via a concerted mechanism came from Kaziro *et al.* (1962) while investigating the carboxylation of propionyl CoA by propionyl CoA carboxylase. They showed that in experiments with $HC^{18}O_3^-$, one bicarbonate oxygen was incorporated into orthophosphate for every two bicarbonate oxygen atoms incorporated into the carboxyl group of methyl-malonyl CoA. This finding implied the direct participation of phosphoryl bond cleavage in the bicarbonate activation process and a concerted reaction was proposed whereby, the HCO_3^- anion attacks the γ-phosphoryl phosphorous atom of ATP displacing ADP in concert with the nucleophilic attack by the 1'-Nitrogen of biotin on HCO_3^- (Fig. 1.3a).

Pre-requisites for this mechanism are (a) that the products MgADP and Pi be released from the enzyme after the formation of carboxybiotin-enzyme; (b) that there is an absolute requirement for biotin in the isotopic exchange reactions between ATP and either ^{32}Pi or [^{14}C]ADP, and (c) that the exchange reaction between HCO_3^- and oxaloacetate should exhibit an absolute requirement for ATP. The finding that the rat liver enzyme catalysed the ATP:Pi and ATP:ADP exchange reactions at the same rates, when identical assay

conditions were used, gave support to the concerted mechanism (McClure *et al.*, 1971c). However Lane *et al.* (1960) demonstrated that bovine liver propionyl CoA carboxylase catalysed a ATP:ADP exchange reaction dependent only on the presence of Mg^{2+} and was insensitive to inhibition by avidin, suggesting that this exchange was not dependent on biotin. Similar results have been reported for pyruvate carboxylase isolated from chicken liver (Scrutton & Utter, 1966) and sheep liver (Goss & Hudson, unpublished results; and the results presented in Chapter III). In addition Scrutton & Utter (1965) demonstrated that the exchange reaction between HCO_3^- and oxaloacetate proceeded in the absence of ATP. A similar lack of dependence on ATP has been reported for the enzymes isolated from rat liver (McClure *et al.*, 1971c) and sheep kidney (Ashman & Keech, 1975). These findings are inconsistent with a concerted mechanism and the only definite conclusion at this stage is that there is direct participation of HCO_3^- in the hydrolysis of ATP.

1.D.1b The enzyme activation mechanism

The possibility of an activated P~Enz-biotin intermediate was initially postulated by Lynen (1959) as a general mechanism for biotin carboxylases and later Scrutton & Utter (1965) introduced the idea into the mechanism for pyruvate carboxylase on the basis of the ATP:ADP exchange reaction which required only the presence of Mg^{2+}. One prediction of this mechanism (Reaction 1:4 and 1:5) is that pyruvate carboxylase should catalyse a

biotin-independent ATP:ADP isotopic exchange reaction (i.e., via Reaction 1:4).

$$MgATP^{2-} + ENZ\text{-}Bio \rightleftharpoons P\sim ENZ\text{-}Bio + ADP \qquad (1:4)$$

$$P\sim ENZ\text{-}Bio + HCO_3^- \rightleftharpoons ENZ\text{-}Bio\text{-}CO_2^- + Pi \qquad (1:5)$$

As discussed above, this has been satisfied experimentally. However the authors discarded this mechanism on the grounds that the HCO_3^-:oxaloacetate exchange reaction was dependent on ADP. In addition they were unable to isolate the predicted Pi~ENZ-Biotin complex. Scrutton & Utter (1965) considered the ATP:ADP exchange reaction, which was dependent only on Mg^{2+}, to be an abortive reaction and not on the main pathway [Fig. 1.3b(i)]. However the level of endogenous HCO_3^- in the reaction solution is relatively high (Ashman, 1973) and it is conceivable that HCO_3^- could have bound before ADP was released. This could account for the requirement for ADP in the HCO_3^-:oxaloacetate exchange reaction.

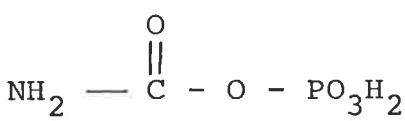
A variation of the enzyme-activation model is the activation of the biotin prosthetic group by phosphorylation, proposed by Retey & Lynen (1965). More recently Kluger & Adawadkar (1976) and Kluger *et al.* (1979) studied models for the reaction by which biotin is carboxylated. On the basis of their findings, they proposed that the urea moiety of biotin is nucleophilic toward phosphate derivatives and proposed a reaction sequence as shown in Fig. 1.3b(ii).

However, as with the enzyme activation model [Fig. 1.3b(i)] the o-phospho-biotin-enzyme model is inconsistent with the requirement of ADP for the HCO_3^-:oxaloacetate exchange reaction. Additional inconsistencies have been obtained from the findings of Ashman & Keech (1975). The

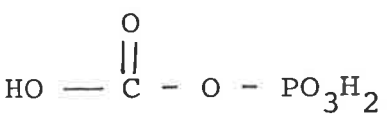
mechanism in Fig. 1.3b(ii) predicts that although MgADP may be bound to the enzyme for carboxylation of biotin and hence for the occurrence of the HCO_3^-:oxaloacetate exchange reaction, phosphorylation of MgADP should not be necessary. Ashman & Keech (1975) demonstrated that two ADP analogues (adenosine-5'-phosphosulphate, and α-β-methylene adenosine diphosphate) both of which bind to the enzyme, but which were not phosphorylated, also could not support the HCO_3^-:oxaloacetate isotopic exchange reaction. This implied that ATP hydrolysis independent of HCO_3^- binding was not the initial event in the overall pyruvate carboxylation reaction. These findings are therefore inconsistent with both types of enzyme activation models.

1.D.1c The substrate activation model

Instead of 'enzyme activation' as a means of facilitating biotin carboxylation, there could be an 'activation' of the substrate, HCO_3^- by ATP to form an activated phosphoryl or adenyl complex, which then interacts with the enzyme. This mechanism was proposed by Polakis *et al.* (1972), who showed that acetyl CoA carboxylase from *E. coli* could catalyse the formation of ATP from carbamyl phosphate and ADP at a rate which was 40% of the overall forward reaction. This reaction indicated that a carboxyphosphate derivative might be an intermediate in the ATP-dependent carboxylation of biotin.



carbamyl phosphate



carboxyphosphate

Supporting evidence was provided by Ashman & Keech (1975) who observed a similar conversion catalysed by pyruvate carboxylase from sheep kidney. In addition they showed that phosphnoacetate was a potent inhibitor of pyruvate carboxylase. The fact that the ionic groups of carboxyphosphate ($^{-}OOC - O - PO_3^{2-}$) and phosphonoacetate ($^{-}OOC - CH_2 - PO_3^{2-}$) were identical, led Ashman & Keech to consider this as additional evidence for the possibility of carboxyphosphate being a transitory intermediate (Fig. 1.3c).

The above mechanism indicates that biotin does not participate in the hydrolysis of ATP. Further evidence for this was provided by Polakis *et al.* (1974) who showed that biotin carboxylase could catalyse the formation of ATP from carbamyl phosphate and ADP at a slow rate in the absence of biotin.

The substrate activation model as outlined by Polakis *et al.* (1972), (Fig. 1.3c) does not account for the isotopic exchange reaction between ATP and ADP which is dependent only on Mg^{2+}; a reaction which Scrutton & Utter (1965) considered to be abortive. Although Moss & Lane (1971) point out that most of the kinetic data seemed in agreement with the substrate activation model, they conclude that ADP release could not precede the carboxylation of biotin. This conclusion is however inconsistent with the ATP:ADP exchange reaction mentioned above.

It becomes apparent that the complexities involved in the coupling of ATP hydrolysis to CO_2 fixation, have contributed to the general disagreement between investigators. The results of a further investigation into the mechanism of ATP

hydrolysis coupled to CO_2 fixation, are presented in Chapters III and IV.

1.E MECHANISM OF TRANSCARBOXYLATION

The attempts to elucidate the mechanism of the carboxylation of pyruvate (Reaction 1:3) have been based on NMR studies of the enzyme bound metal atom and pyruvate (Mildvan *et al.*, 1966; Mildvan & Scrutton, 1967; Reed *et al.*, 1972) and on sterochemical studies (Rose, 1970). In general, a concerted mechanism has been favoured for some time (Fig. 1.4a). An important feature of the concerted mechanism is that it does not require a base at the active site to facilitate proton transfer.

Mildvan & Scrutton (1967) proposed that the enzyme bound metal ion facilitated pyruvate carboxylation because of its electron withdrawing properties. However subsequent findings suggested a tightly co-ordinated water molecule between the metal and pyruvate (Fung *et al.*, 1973) and it was proposed that it was water and not the metal atom which is the electrophile that activated pyruvate. This differs from the mechanism proposed by Retey & Lynen (1965) whereby carboxybiotin is the base that activates pyruvate. Both the findings are consistent with the sterochemical studies of Rose (1970), who showed that carboxylation of pyruvate catalysed by chicken liver pyruvate carboxylase proceeds with the retention of configuration at the C-3 position of pyruvate. Similar results have been observed with propionyl-CoA carboxylase (Arigioni *et al.*, 1966; Prescott & Rubinowitz, 1968) and transcarboxylase (Cheung *et al.*, 1975). In

addition, Rose *et al.* (1976) found that when transcarboxylase acts on [^{3}H]pyruvate and methylmalonyl CoA, tritium is found in the C-2 position of the product propionyl CoA.

More recently, Stubbe *et al.* (1977, 1980) have proposed a mechanism involving the formation of a carbanion as an intermediate (Fig. 1.4b) based on their finding that transcarboxylase and propionyl CoA carboxylase catalyzed the elimination of HF from β-fluoropropionyl-CoA without concomitant carboxylation of the substrate-analogue. This indicated that the abstraction of the substrate α-proton occurs without concomitant carboxylation of the substrate. In addition, Stubbe *et al.* (1980) have questioned the validity of the interpretations advanced in support of the concerted mechanism. They argue that there is no reason why an enzymic carbanion mechanism could not proceed with retention of configuration. They also point out that the tritium transfer observed with transcarboxylase (Rose *et al.*, 1976) could also be consistent with a carbanion mechanism.

A recent study on pyruvate carboxylase carried out in this laboratory (Goodall *et al.*, 1981) has provided evidence that were inconsistent with both the concerted and carbanion mechanisms. These investigators examined a number of pyruvate analogues, which could not be carboxylated but which could bind to the enzyme, and showed that glyoxylate among others, labilizes the carboxybiotin-enzyme at the second sub-site, resulting in a rapid decomposition of carboxybiotin. They point out that glyoxylate is unable to supply a proton and since a concerted mechanism required a proton to be transferred from the substrate to the carbonyl oxygen of biotin, the concerted mechanism could not operate

here. Neither can a carbanion mechanism be involved since glyoxylate cannot form a carbanion.

In view of these recent observations, alternate mechanisms will have to be sought for the transcarboxylation mechanism.

1.F VARIABLE STOICHIOMETRY OF PYRUVATE CARBOXYLATION

A consistent feature of the kinetic data obtained at various concentrations of pyruvate, when using the rate of oxaloacetate synthesis as a measure of reaction velocity, is the occurrence of downward-concave double-reciprocal plots (Taylor *et al.*, 1966; Cazzulo *et al.*, 1970; McClure *et al.*, 1971b; Ashman & Keech, 1975). The original explanations for this 'substrate activation' was not adequately supported by experimental evidences.

In questioning the stoichiometry of the overall reaction, Easterbrook-Smith *et al.* (1976a) measured the reaction velocity both in terms of oxaloacetate produced and orthophosphate liberated. The double-reciprocal plots obtained using oxaloacetate, as a measure of reaction velocity with varying concentrations of pyruvate, was non-linear with downward curvature. However the plot obtained using the amount of orthophosphate released as a measure of the reaction velocity was a straight line. The conclusion these investigators reached, was that oxaloacetate synthesis reflected only the rate of pyruvate carboxylation, which was not stoichiometric with the rate of ATP hydrolysis at non-saturating concentrations of pyruvate.

To account for this lack of stoichiometry, Easterbrook-

Smith *et al.* (1976a) suggested that the binding of pyruvate at the second sub-site labilized the carboxybiotin enzyme intermediate presumably by attracting the complex into the pyruvate binding sub-site. They reasoned that if pyruvate dissociated before carboxylation occurred then the carboxybiotin enzyme hydrolysed to ENZ-biotin and CO_2. These conclusions were supported by two findings, viz:

(a) That pyruvate moves in and out of the second partial reaction sub-site at a rate which is two orders of magnitude greater than the overall reaction (Mildvan & Scrutton, 1967) and

(b) that after pyruvate bound to the enzyme, it was only 50% committed to catalysis (Cheung & Walsh, 1976).

Hence at high levels of pyruvate, carboxylation predominates, while at low levels, a greater proportion of the carboxybiotin breaks down.

1.G CHEMISTRY OF THE ACTIVE SITE

The understanding of the catalytic events occurring during the reaction catalyzed by pyruvate carboxylase has, till recently, relied largely upon kinetic analysis of the enzyme. To describe the mechanism of an enzymically catalysed reaction, the first pre-requisite is to determine the order of addition of substrates and release of products. This information is obtained mainly from steady state kinetic analysis. Isotopic exchange reactions have provided evidence of intermediates and possible transition states. Elucidating the role of various amino acid residues in the binding and

catalytic process is essential in providing a precise understanding of the catalytic mechanism. Since the micro-environment of these essential amino acid residues is of major importance in the process, an understanding of the primary structure and geometry of the polypeptide chain or chains comprising the active site is essential. The characterization of these active sites has increasingly become one of the major goals of protein chemistry. There are a number of approaches to such characterization. X-ray crystallography has been the method of choice in determining the complete three dimensional structure of protein molecules in its free and ligand-bound form (Dickerson, 1972). However, with complex high molecular weight proteins such as pyruvate carboxylase or where the proteins have not been crystallised or sequenced such studies are not yet possible.

Structural information from magnetic resonance studies has contributed to the analysis of the dynamics of enzyme-ligand interactions and in measuring perturbations in structure. In some cases, important information has been obtained that has a direct bearing on the structures of active sites and of enzyme-ligand complexes (Jardetzky & Wade-Jardetzky, 1971; Wüthrich, K., 1976)

However chemical modification of the active site has provided some of the best available evidence for defining the structure of essential intermediates or identifying catalytically important residues.

1.G.1 Chemical Modification

There are several different types of chemical modificatior experiments that have revealed the presence and/or function

of reactive groups at the active sites of enzymes. They include the use of group-specific reagents, affinity labels, pseudo-substrates, suicide substrates, and the trapping of reactive intermediates (Sigman & Mooser, 1975). The most widely used reagents, however are group specific reagents (Stark, 1970; Glazer, 1976) and affinity labels (Singer, 1967; Singer; 1970, Shaw, 1970; Yount, 1975; Glazer, 1976).

Affinity labels differ from 'group specific' reagents in possessing a structural resemblance to a substrate of the enzyme and so enable a more selective modification of a given ligand binding site. The extent to which this specificity is realized depends largely on the nature of the ligand binding site and the functional group incorporated into the affinity label.

Chemical modification studies on pyruvate carboxylase have led to the identification of one to two lysine residues at the binding site of acetyl CoA by the use of 2,4,6-trinitrobenzene sulphonic acid (TNBS) modification (Ashman *et al.* 1973). Further work in this laboratory has identified a lysine residue at the ATP binding site by covalent modification of pyruvate carboxylase with oxidized ATP (Easterbrook-Smith *et al.*, 1976b). The use of bromo-pyruvate to modify the sheep liver enzyme has shown the presence of a cysteine residue for the pyruvate binding site (Hudson *et al.*, 1975).

The application of affinity labels in the investigation of active sites of pyruvate carboxylase is further discussed in Chapter VII.

1.G.2 Protein Sequence Determination

The data from amino acid sequences of proteins has been used for the reconstruction of genetic events occurring during the evolution of species, leading to the understanding of evolutionary development, and for the elucidation of structure-function relationship in proteins. The definition of a structure-function relationship for a protein is only possible when the precise arrangement of essential amino acid residues in the three dimensional structure is known. Since the full complexity of a protein's structure in space is inherent in its amino acid sequence, it becomes evident that protein sequence determination is the first step towards understanding structure-function relations.

Hence one of the long term aims in the study of pyruvate carboxylases is to understand the precise nature of the active site with reference to its catalytic function. This would involve the characterisation of peptides in and around the active site. The information gathered from these studies would not only reveal mechanistic information but would also contribute to the understanding of evolutionary relationship with other biotin containing enzymes. It has become apparent that amino acid sequences which are highly conserved through evolution are generally involved in some important biological function of that protein (Dayhoff *et al.*, 1972).

The only comparative primary sequence data available for biotin carboxylases, is that around the biotin prosthetic group. Rylatt *et al.* (1977) have isolated and sequenced the tryptic peptides containing the covalently bound biotin prosthetic group of pyruvate carboxylase from sheep, chicken and turkey liver. There is almost complete conservation of

the sequence in these three peptides and when compared with the sequence of the related peptides from acetyl CoA carboxylase isolated from *E. coli* (Sutton *et al.*, 1977), and transcarboxylase from *P. shermanii* (Maloy *et al.*, 1979) strong similarities are apparent.

It has been hypothesized that biotin enzymes have evolved from a common ancestor (Lynen, 1975; Obermayer & Lynen, 1976), and the high degree of conservation of sequence in the biotinyl peptides between bacteria and eukaryotes which diverged about 3000 million years ago (Dayhoff, 1972), is usually cited as supportive evidence for this hypothesis. However, this might be misleading as the conservation around the biotin moiety might just be indicative of the sequence specificity required for holo-carboxylase synthetase, the enzyme catalysing the attachment of the biotin prosthetic group to the apo-enzyme. McAllister & Coon (1966) have shown that purified holo-carboxylase synthetases have broad specificity toward different apo-carboxylases from diverse sources. For instance the holoenzyme synthetases from rabbit liver, yeast and *Propionibacterium shermanii* catalysed the formation of active enzyme with apo-propionyl-CoA carboxylase of rat liver, apo-3-methylcrotonyl CoA carboxylase from *Achromobacter*, and apo-transcarboxylase from *P. shermanii*. McAllister & Coon (1966) concluded that this broad specificity of the various synthetases suggested that the different apo-carboxylases from diverse sources may have similar amino acid sequence in the regions surrounding those specific lysine residues which are acylated by biotin.

Hence the information from amino acid sequences in and

around the substrate binding sites maybe of greater value in tracing any evolutionary relationship. The use of bromo-pyruvic acid (Hudson *et al.*, 1975) and oxidized ATP (Easterbrook-Smith *et al.*, 1976b) in covalently modifying the pyruvate and ATP binding sites respectively, have provided opportunities to isolate and sequence peptides at the two catalytic sub-sites of pyruvate carboxylase.

The isolation and characterization of the tryptic peptide comprising the ATP binding site is presented in Chapter VII of this thesis.

1.H THE AIMS OF THE PROJECT

The long-term aim of the project on pyruvate carboxylase in this laboratory is to understand the detailed catalytic mechanism of the enzyme. The over-all programme involves kinetic analysis of the enzyme, elucidating the role of various amino acid residues in the binding and catalytic processes and the characterization of the primary structure and geometry of the polypeptide chain/chains comprising the active site.

The study undertaken in this thesis concerns molecular events occurring at the first partial reaction sub-site. In attempts to elucidate the precise nature of the mechanism of ATP hydrolysis coupled to carbon dioxide fixation, the initial appraoch adopted was to re-examine the pathway by kinetic analysis. Since there is an extensive array of kinetic data on the chicken liver pyruvate carboxylase, only the areas in which ambigious experimental results and interpretation are prevalent have been examined. In addition,

direct physical methods have been utilized to demonstrate and chemically stabilize enzyme reactant intermediates.

Since one of the long term aims is to understand the precise nature of the active site, the characterization of the MgATP binding site has been the final thrust of this thesis. The initial approach was to investigate various methods of isolating the tryptic peptide labelled with oxidized ATP, so as to develop the most convenient and efficient methods of isolation. Finally, the characterization of the isolated peptide has been undertaken.

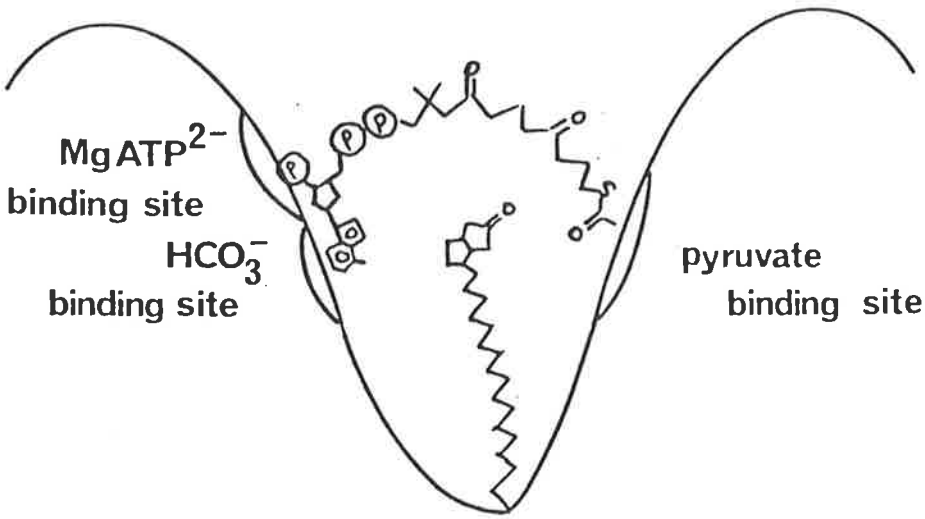


FIGURE 1:1

Schematic representation of the active site.

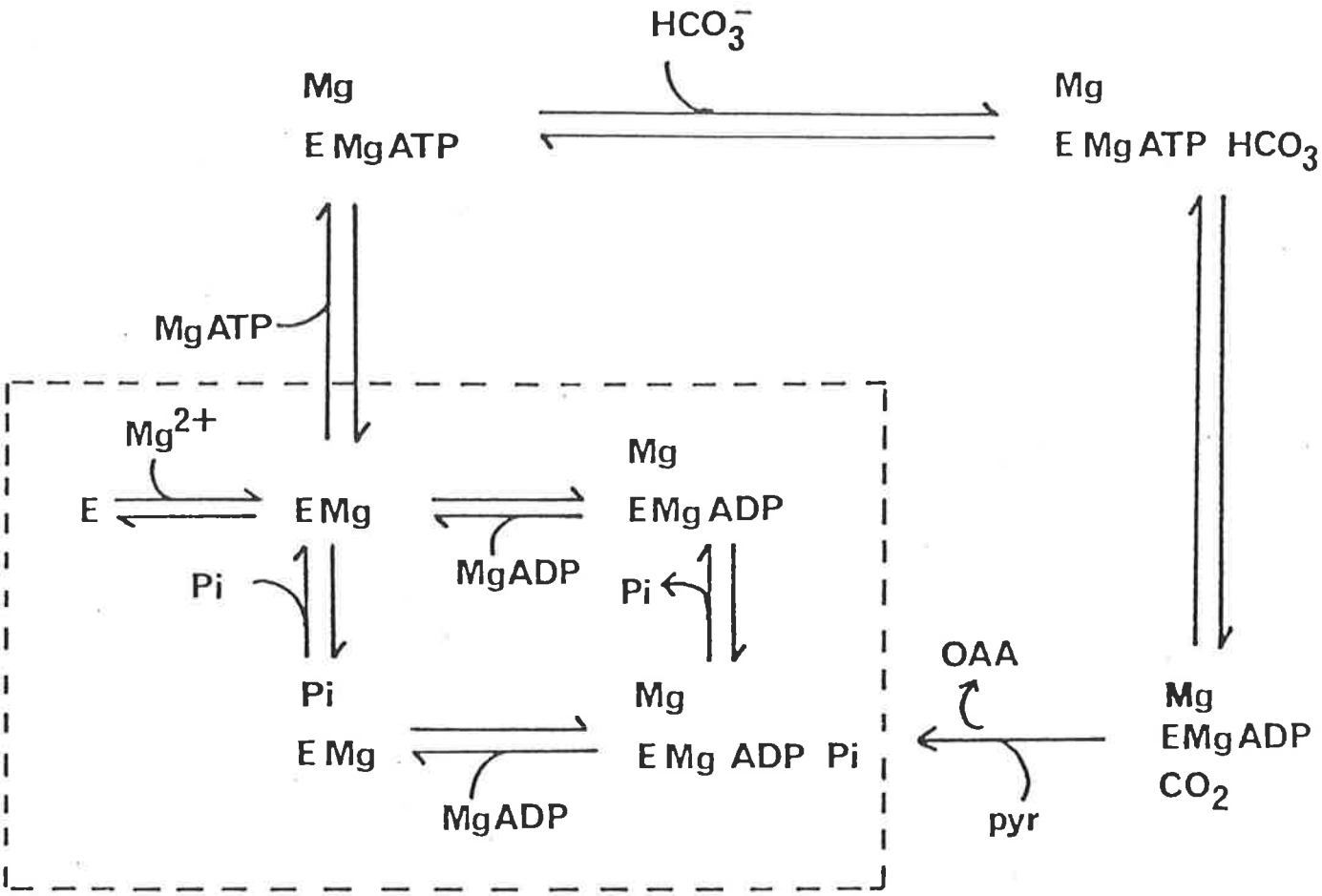


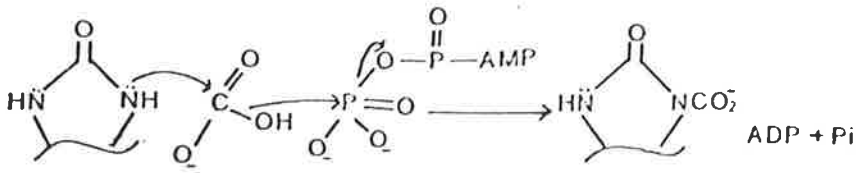
FIGURE 1:2

The Sequential Pyruvate Carboxylase Reaction Pathway

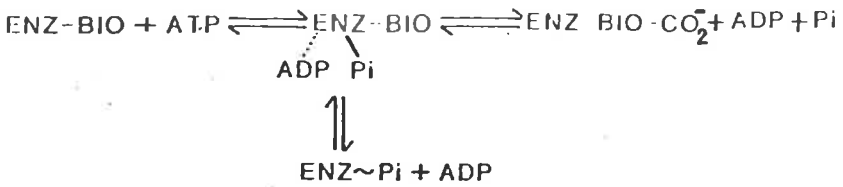
(Adapted from Warrent & Tipton, 1974c).

FIGURE 1:3 Alternate Mechanisms for Biotin Carboxylation

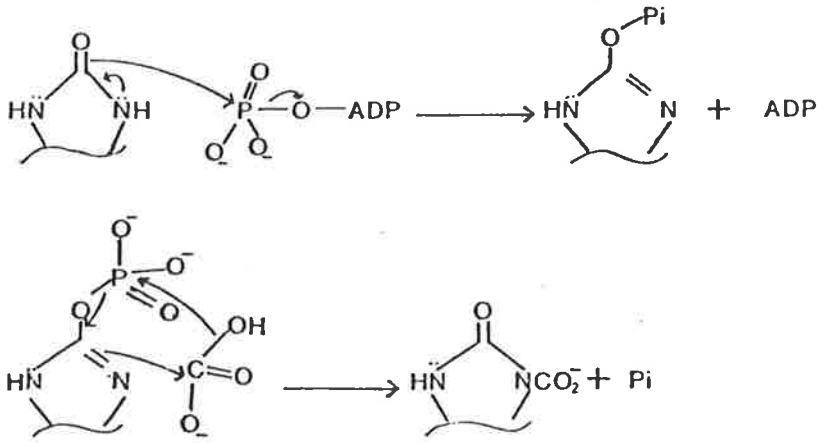
a) Concerted



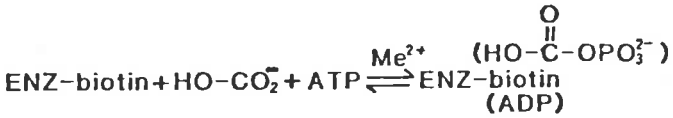
b(i) Enzyme Activation



b(ii) Activation of Biotin



c. Substrate Activation



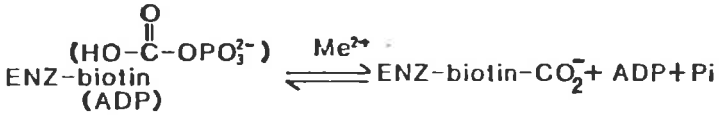
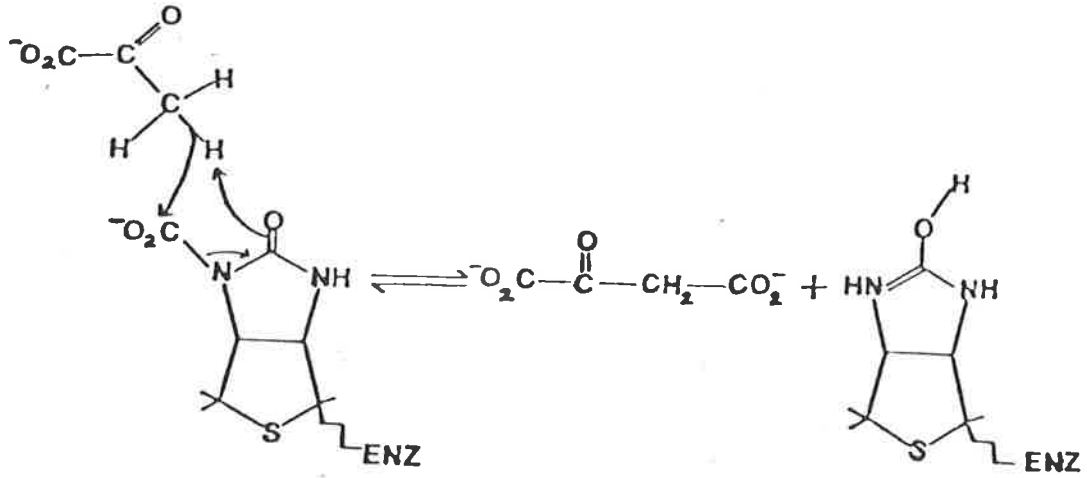
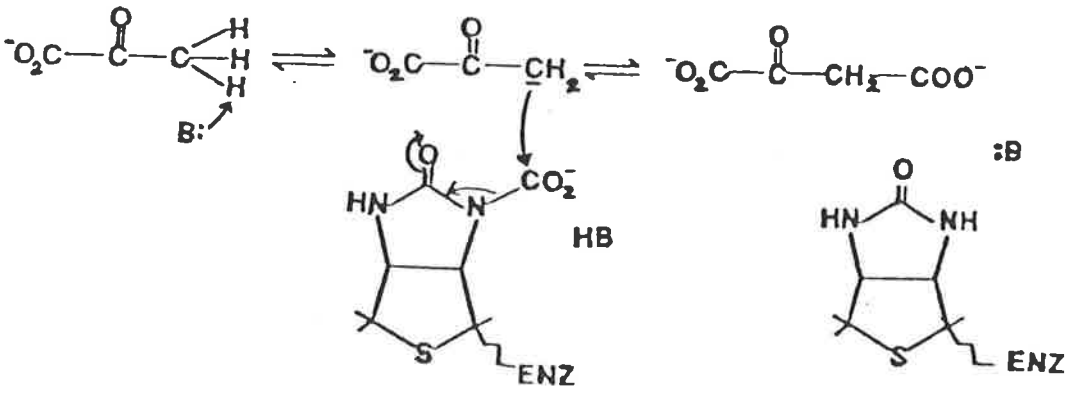


FIGURE 1:4 Mechanisms of Transcarboxylation

A. The Concerted Mechanism.



B. The Carbanion Mechanism.



CHAPTER II

MATERIALS & METHODS

II.A MATERIALS

II.A.1 General Chemicals

ATP (disodium salt, grade I), ADP (disodium salt, grade I), NADH, NADP, NADPH, oxaloacetic acid, 2-oxo-butyrate, sodium pyruvate (type II, dimer free), pyridoxal phosphate, sodium dodecylsulphate, Trizma base, N-ethyl-morpholine, EDTA (disodium salt), 2-mercaptoethanol, dansyl chloride, coenzyme A (grade I), o-phospho-serine, o-phospho-threonine, and acrylamide were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lithium hydroxypyruvate, phenyl-pyruvate (sodium salt) and dansyl-amino acid markers were supplied by Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. PPO (2,5-diphenyloxazole), POPOP (1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene, and iodoacetic acid were obtained from Koch-Light Laboratories Ltd., Bucks, England. p-Toluenesulphonyl-N-methyl-N-nitrosamide was from TCl., Tokyo, Japan. Coomassie brilliant blue was from Schwartz/Mann, Orangeburg, N.Y., U.S.A. Polyethylene glycol was from Union Carbide Corporation. Fluorescamine (4-phenyl-spiro[furan-2(3H),1'-phthalan]-3,3'-dione was obtained from Roche Products, Dee Why, N.S.W., Australia. P^1,P^5-Di-(adenosine-5')pentaphosphate was obtained from Boehringer Mannheim, GmbH, W. Germany. Sodium glutamate and semi-carbazide HCl (Anala R) were obtained from British Drug Houses, Poole, England. $MgCl_2$ was prepared from spectro-scopically pure Mg as described by Bais & Keech (1972) and standardized by atomic absorption spectrophotometry Ammonium sulphate (special enzyme grade) was obtained from Mann Research Laboratories, N.Y., U.S.A.

N,N-methylenebisacrylamide, Phenylisothiocyanate (sequential grade), trifluoracetic acid (sequential grade), thin layer cellulose and silica gel plates were obtained from Eastman Kodak, Rochester, N.Y., U.S.A. Polyamide plates were from Chen-Ching Trading Co., Taipei, Taiwan. Polyethyleneimine thin layers were from Machery-Nagel & Co., Duren, Germany.

SP-(sulphopropyl) sephadex, DEAE-sephadex and all other grades of sephadex were obtained from Pharmacia, Uppsala, Sweden. DEAE-cellulose was obtained from Whatman Chromedia (W. & R. Balston, Ltd., England).

II.A.2 Radioactive Chemicals

[U-^{14}C]ATP (ammonium salt), [U-^{14}C]ADP (ammonium salt), d[carbonyl-^{14}C]biotin, sodium [^{14}C]bicarbonate and sodium [2-^{14}C]pyruvate were obtained from the Radiochemical Centre, Amersham, England. [^{32}P]orthophosphate was supplied by The Australian Atomic Energy Commission, Lucas Heights, Australia.

II.A.3 Enzymes and Proteins

Albumin, bovine serum; malate dehydrogenase (E.C.1.1.1.37) L-alanine:2-oxoglutarate aminotransferase (E.C.2.6.1.2), L-aspartate:2-oxoglutarate aminotransferase (E.C.2.6.1.1), hexokinase (ATP:D-hexose 6-phosphotransferase, E.C.2.7.1.1) type IV from yeast, lactic dehydrogenase (L-lactate:NAD oxidoreductase, E.C.1.1.1.27) type I from rabbit muscle, were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose 6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C.1.1.1.49) from Boehringer Corporation (London) Ltd., London. Avidin from egg white, DFP (Diisopropylphosphofluoridate) treated chymotrypsin, bovine

pancrease; (E.C.3.4.4.5), and TPCK (L-[1-Tosylamido-2-phenyl]ethyl chloromethyl ketone) treated trypsin, bovine pancrease; (E.C.3.4.4.4) were obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Pronase (B grade) was obtained from Calbiochem, (Aust.) Pty. Ltd., Sydney, Australia. Thermolysin (crystalline) was purchased from Daiwa Kasei K.K., Osaka, Japan.

II.B. METHODS

II.B.1 Preparation and Purification of Nucleotides

II.B.1a Acetyl CoA

Acetyl CoA was prepared by a method similar to that of Simon & Shemin (1953), and purified by ascending chromatography on Whatman 3 MM paper. The developing solvent used was isobutyric acid, H_2O, NH_3 (66; 33; 1) adjusted to pH 3.5 using tri-n-ethylamine and a final concentration of 1×10^{-4} M EDTA was added. The acetylated CoA band (R_f 0.45 - 0.54) was cut out and eluted with 1×10^{-4} M EDTA and concentrated by freeze-drying. ($\varepsilon^{mM}_{260\ nm}$ = 16.4; Dawson *et al.*, 1969). The product obtained had a ratio of extinction 232/260 nm (thioester/adenine moiety) of $\simeq$ 0.54 compared with the value of 0.53 from Dawson *et al.* (1969).

II.B.1b [γ-^{32}P]ATP

[γ-^{32}P]ATP was prepared by the method of Schendel & Wells (1973), and purified by the method of Symons (1977). The reaction mixture was diluted with 4 ml of water and chromatographed on a 0.85 x 3 cm column of DEAE-cellulose (HCO_3^- form). The column was washed with 20 ml of 0.07 M

NH_4HCO_3 to elute ^{32}Pi, and the [γ-^{32}P]ATP is eluted with 10 ml 0.25 M NH_4HCO_3, after which 1.5 ml triethylamine and 3 ml ethanol were added to the eluate and the contents evaporated to dryness using a rotary evaporator. The purity of the [γ-^{32}P]ATP was 95 - 99% as determined by thin-layer chromatography on PEI-cellulose using 0.8 M NH_4HCO_3 as the developing solvent.

II.B.2 Purification of Pyruvate Carboxylase

Pyruvate carboxylase was purified from freeze-dried chicken liver mitochondria by the method of Goss *et al.* (1979). The mitochondria (120 g) were suspended in 1686 ml of 25 mM Tris-acetate, pH 6.7, containing 3.5 mM $MgCl_2$, and 1.7 mM ATP. The pH was maintained between 6.5 and 6.7 during addition of the mitochondria. The suspension was stirred for 20 min and the particulate material removed by centrifuging (23,000 g for 20 min at 15°C). The supernatant was adjusted to pH 6.9 - 7.2 and ammonium sulphate was added to give a final saturation of 33% (19.6 g per 100 ml solution). The pH was maintained between 6.9 - 7.2 during the addition of the ammonium sulphate. The suspension was stirred for 20 min and precipitated protein collected by centrifuging (23,000 g for 20 min at 15°C). The precipitate was dissolved in Buffer A (25 mM potassium phosphate buffer, pH 7.2, containing 20 mM ammonium sulphate, 1 mM EDTA and 0.1 mM DTE to a final protein concentration of 10 mg of protein/ml. The suspension was desalted by the addition of finely ground polyethylene glycol (Mr = 6,000) (14.5 g/100 ml).

This suspension was stirred for 40 min while maintaining the pH between 6.8 and 6.9 with acetic acid. The precipitated pyruvate carboxylase was recovered by centrifuging at 23,000 g for 20 min at 15°C. The precipitate was dissolved in a minimum volume (15 ml/10g of mitochondria extracted) of Buffer A described above, and undissolved protein was removed by centrifuging at 23,000 g for 15 min at 15°C. The superantant was loaded on to a column of DEAE-Sephadex A-50 (14 x 5 cm) previously equilibrated with Buffer A. The column was eluted with a linear gradient of 20 mM to 200 mM ammonium sulphate in the above Buffer A. Pyruvate carboxylase of specific activity 25 - 32 units/mg protein was routinely obtained using this procedure.

II.B.3 Storage of Pyruvate Carboxylase

The enzymes could be stored at -80°C in storage buffer (0.1 M NEM acetate, pH 7, containing 1.6 M sucrose and 1% (v/v) saturated $[NH_4]_2SO_4$) for several months with negligible loss of activity.

II.B.4 Protein Estimation

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

protein concentration (mg/ml) = $1.55\ A_{280\,nm}^{1\,cm} - 0.76\ A_{260}^{1\,cm}$

or by the method of Lowry (Lowry *et al.*, 1951).

II.B.5 Determination of Radioactivity

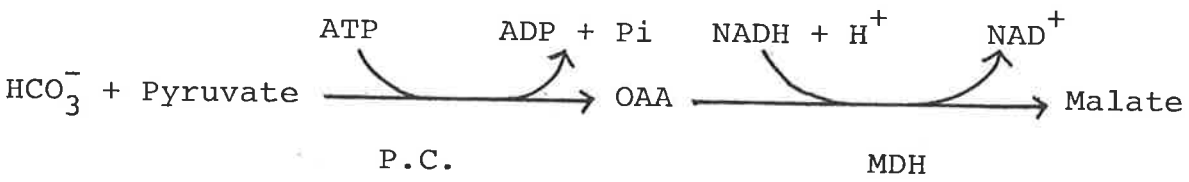
Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid containing Triton X-100,

(7:3 v/v) and counted in a Packard or Beckman Scintillation Spectrometer. When required, samples were dried onto solid supports (2 cm x 2 cm squares of Whatman 3 MM paper) and placed in vials containing 2 ml of toluene scintillation fluid (0.3% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 1,4-bis-2[4-methyl-5-phenoxazolyl]-benzene, in sulphur-free toluene; Bousquet & Christian, 1960) and counted in a Packard or Beckman scintillation spectrometer.

II.B.6 Measurement of Pyruvate Carboxylase Activity

II.B.6a The Coupled Spectrophotometric Assay System

This continuous assay, based on that described by Utter & Keech (1963), involves reduction of the oxaloacetate produced by the pyruvate carboxylase reaction using excess malic dehydrogenase with concommitant oxidation of NADH to NAD^+



The reaction was followed by measuring the absorbance at 340 nm, using either a unicam SP800 spectrophotometer, or a Varian-Techtron 635-O spectrophotometer. The cell block was thermostatted at 30°C. The rate of oxaloacetate synthesized for a 1 cm light-path and 1 ml reaction volume is given by

$$\frac{\Delta A_{340}/\text{min}}{6.22} = \mu\text{moles/min}$$

Where 6.22 is the millimolar extinction coefficient at 340 nm for NADH (Dawson *et al.*, 1969). Assay solutions contained

(in μmoles) in a final volume of 1.0 ml; Tris-Cl, pH 7.8, (or pH 8.4 for sheep liver enzyme), (100); ATP (2.5), $MgCl_2$, (5); HCO_3^-; sodium salt (20); pyruvate, sodium salt, (10); acetyl CoA, (0.25); NADH, (0.125); malate dehydrogenase, (5 units); and pyruvate carboxylase (0.025 - 0.1 units).

II.B.6b The Radiochemical Assay System

In this direct assay, $H^{14}CO_3^-$ fixed in an acid stable form is measured, while unreacted $H^{14}CO_3^-$ is driven off on acidification and subsequent drying on paper squares (cf., Gailiusis *et al.*, 1964). Assay solutions contained, (in μmoles), in a final volume of 0.25 ml, Tris-Cl, (pH 8.4, for sheep liver enzyme or pH 7.8, for chicken liver enzyme), (25); ATP, (0.625); $MgCl_2$, (1.25); pyruvate, sodium salt, (2.5); acetyl CoA, (0.0625), and up to 0.06 units of pyruvate carboxylase*. The reaction was initiated by the addition of enzyme and allowed to proceed for 5 min at 30°C in a fume hood before being quenched by addition of 50 μl of 2 M semicarbazide in 2 M HCl. Besides terminating the reaction, this reagent also drives off unreacted $H^{14}CO_3^-$ and stabilises the oxaloacetate formed as the carbazone derivative. Samples (0.05 ml) were applied in triplicate on to 2 cm squares of Whatman 3 MM paper, dried at 90°C for 5 min and counted as described in Section II.B.5. Quench correction by the channels ratio method was not necessary as the semicarbazone was colourless, and therefore did not cause any quenching. The radiochemical assay was found to be more convenient than the spectrophotometric assay for experiments involving a large number of assays. Moreover, use of the radiochemical assay removed the need to measure

*as described by Taylor *et al.* (1969)

the reaction rate in a coupled system in the presence of a ligand (NADH) which was not required for pyruvate carboxylase activity.

The acetyl CoA-independent assay system used for sheep liver enzyme was that developed by Ashman *et al.* (1972). The assay solutions contained, (in μmoles), in a final volume of 0.5 ml; Tris-Cl, pH 7.8, (50); ATP, (1.25); $MgCl_2$, (4); $H^{14}CO_3^-$, sodium salt, 5 x 10^5 cpm/μmole, (20); pyruvate, sodium salt, (20); NH_4Cl, (50); and 2 units of pyruvate carboxylase. The amount of radioactivity fixed into [^{14}C]oxaloacetate was determined by the procedure described above for the acetyl CoA-dependent assay.

II.B.7 Separation of Radioactive Nucleotides and Inorganic Phosphate

Polyethyleneimine (PEI) ion exchange thin layer plates (polygram CEL 300 PEI/UV_{254}) stored at 4°C were used. Radioactive samples (5 or 10 μl) were applied on to 20 x 10 cm PEI thin layers together with 2 μl of cold carrier (10 mM in each of ATP, ADP, AMP). Chromatograms were washed in distilled water for 5 min, dried without heating and devleoped in freshly prepared 0.8 M NH_4HCO_3 in an open tank. Nucleotide spots were located by their absorption of ultra violet light, and together with the region where orthophosphate was located, 1 cm strips were cut out and counted as described in Section II.B.5.

II.B.8 Isotopic Exchange Reaction Assays

II.B.8a ATP:Pi Isotopic Exchange Reaction Assay

Reaction mixtures contained, (in μmoles), in a

final volume of 0.25 ml: N-ethyl morpholine (Cl^-, pH 7.8), (25); ATP, (0.5); ADP, (0.5); $MgCl_2$, (2); HCO_3^-, sodium salt, (0.5); acetyl CoA, (0.0625); phosphate, sodium salt, (2.5), and pyruvate carboxylase, 1 - 2 units. After incubating the reaction mixtures at 30°C for 10 min, the reaction was initiated by addition of carrier-free [^{32}P]-orthophosphate (1 - 2 x 10^6 cpm). Samples (0.05 ml) were withdrawn at various time intervals and reacted in 0.01 ml of 26 N formic acid, at 0°C. The mixture was centrifuged to remove the denatured protein and the ATP and orthophosphate in 10 µl of the supernatant are separated and counted as described in Section II.B.7. The rate of incorporation of radioactive isotope into ATP was linear for 40 min using 0.88 enzyme units per assay.

II.B.8b ATP:ADP Isotopic Exchange Assay

The basic assay system contained, (in µmoles), in a final volume of 0.25 ml: N-ethyl morpholine (Cl^-, pH 7.8), (25); ATP, (0.5); ADP, (0.5); $MgCl_2$, (2); and 1 - 2 units of enzyme. Enzyme was omitted from the controls. After equilibration of 30°C for 10 min, assays were initiated by the addition of 5 µl of [8-^{14}C]ADP (Ca 0.005 µ-moles; 2 x 10^8 cpm/µmole). Samples (0.05 ml) were taken at various time intervals and reacted with 0.01 ml of 26 N formic acid at 0°C, then centrifuged to remove denatured protein. The nucleotides in the supernatant were separated for counting as described in Section II.B.7. The incorporation of isotope into ATP was linear with time over 20 min with up to 3.6 enzyme units per assay.

II.B.8c HCO_3^-: oxaloacetate Isotopic Exchange Reaction Assay

The test system for isotopic exchange between HCO_3^- and oxaloacetate contained, (in μmoles), in a final volume of 0.5 ml: N-ethyl morpholine (Cl^-, pH 7.8), (50); $MgCl_2$ (2.5); oxaloacetate, (1); pyruvate, sodium salt, (1); HCO_3^-, sodium salt, (1); ADP, (1); orthophosphate, (5); acetyl CoA, (0.125); and enzyme, 0.2 unit. After equilibrating at 30°C for 10 min, 0.005 ml of $NaH^{14}CO_3$ (0.02 μmoles; 2 x 10^8 cpm/μmole) was added to initiate the isotopic exchange reaction. Samples (0.2 ml) were withdrawn at various time intervals and reacted with 0.05 ml of a 2 M solution of semicarbazide in 2 M HCl, and aliquots (0.05 ml) were applied in triplicate onto 2 cm squares of Whatman 3 MM paper and processed as described in Section II.B.5. Incorporation of ^{14}C into oxaloacetate was linear with time for at least 20 min.

II.B.8d Pyruvate/oxaloacetate Isotopic Exchange Reaction Assay

Assays contained, (in μmole), in a final volume of 0.5 ml: Tris-Cl, pH 7.8, (50); oxaloacetate, (1); pyruvate, sodium salt, (1); acetyl CoA, (0.125); and pyruvate carboxylase, 0.1 units. The reaction was initiated, after a 5 min incubation period at 30°C, by adding sodium[2-^{14}C]pyruvate (0.005 μmole, 4 x 10^7 cpm/μmole), dissolved in 0.02 M HCl. At various time intervals, aliquots (0.1 ml) were withdrawn, and the reaction quenched with 0.01 ml avidin (10 mg/ml in 0.02 M sodium phosphate,

pH 7.4). After 2 min, pyruvate and oxaloacetate were converted to alanine and aspartate, respectively, by the addition of 0.05 ml of 0.5 M sodium glutamate, 0.02 ml of 2 mM pyridoxal phosphate, and 0.02 ml of 0.1 M sodium phosphate, pH 7.4, containing one unit each of L-alanine aminotransferase, (E.C.2.6.1.2) and L-aspartate aminotransferase (E.C.2.6.1.1). The transamination reaction was allowed to proceed for 15 min at 30°C and then stopped with 0.025 ml of 5 M formic acid. Alanine and aspartate were separated by high voltage electrophoresis on a Michl apparatus with white spirit as the cooling liquid using a pH 6.5 buffer (pyridine/acetic acid/water in the ratio of 25:1:225). Samples (20 µl) were applied to Whatman 3 MM papers which included lanes for marker alanine and aspartate samples. Electrophoretograms were developed for 45 min at 2,600 V, 60 mA [40 V/cm, 2 mA/cm]. After drying the electrophoretogram, the aspartate and alanine markers were located by spraying with 0.5% ninhydrin in acetone. Sample lanes were cut into 1 cm pieces and radioactivity determined as described in Section II.B.5. Since the contaminant para-pyruvate moved very close to aspartate on the electrophoretograms, correction was made for it by substracting control values. The rate of exchange was constant for 15 min, and was linear with enzyme concentrations up to at least 0.25 units/assay.

II.B.8e Calculation of Rates of Isotopic Exchange

The rate of exchange reaction can be calculated at any point in the exchange reaction using the formula

(Wahl & Bonner, 1951; Frost & Pearson, 1961)

$$R = - \frac{(A)\ (B)}{(A) + (B)} \cdot \frac{\ln\ (1 - F)}{t}$$

Where A and B are the concentrations of the exchanging species, t is the exchange time, and F is the fractional equilibrium at time t. The fractional equilibrium,

$$F = \frac{\text{specific activity of B at time t}}{\text{specific activity of B at isotopic equilibrium}}$$

$$\text{which is } \frac{\text{\% of isotope in B at time t}}{\text{\% of isotope in B at equilibrium}}$$

Since, at isotopic equilibrium, the specific activities of the exchanging species are identical, the per cent of isotope in B at equilirbium =

$$\frac{(B)}{(A) + (B)} \times 100$$

Then at any time t,

$$Ft = \frac{\text{\% of isotope in B}}{\left[\frac{(B)}{(A) + (B)} \times 100\right]}$$

The rates of all isotopic exchange reactions studied in this thesis have been calculated by using the above formula.

II.B.9 Polyacrylamide Gel Electrophoresis

Separation of proteins was carried out using polyacrylamide gel electrophoresis in the presence of SDS as

described by Laemmli (1970) using 10% acrylamide gels. For separation of oligo peptides, the method of Swank & Munkres (1971) was followed.

II.B.10 S-Carboxymethylation of Cysteinyl Residues

Modification of cysteinyl residues of pyruvate carboxylase with iodoacetic acid was routinely carried out in all peptide isolation procedures, by the method described by Beyreuther *et al.* (1975) except that iodoacetamide was replaced by iodoacetic acid. The protein was dissolved in 0.1 M Tris-Cl, pH 8; containing 6 M recrystallized guanidine-HCl to a concentration of 10 mg per ml. A ten-fold molar excess of DTE over total protein thiol was added and the solution incubated at 37°C for 1 h, under nitrogen, to ensure complete reduction. Following reduction of the thiol groups, recrystallized iodoacetic acid was added in equimolar amounts to the DTE and the solution incubated in the dark at 37°C for 1 h under nitrogen. The progress of the reaction was followed by the disappearance of sulphydryl groups using the DTNB test of Ellman (1959). Unreacted iodoacetic acid was quenched by the addition of a large excess of DTE, after which the samples were dialyzed against 0.1 M ammonium bicarbonate and the precipitate collected by lyophilization.

II.B.11 Peptide Hydrolysis and Sequencing Procedure

II.B.11a Amino Acid Analyses

Peptides were prepared for amino acid analyses by hydrolyzing 10 to 15 nmol of peptide under nitrogen in 6 M HCl which contained 1 drop of 1% aqueous phenol, for

24 h at 105°C. The amino acids were analyzed by the procedure of Piez & Morris (1960) using a Beckman 120 C analyzer modified as described by Harding & Rogers (1971).

II.B.11b Analytical Peptide Maps

The development of analytical peptide maps were carried out on thin-layer cellulose sheets using the procedure described by Van de Kevckhone & Van Montagu (1974) and peptides, where applicable were located by autoradiography. When required, the peptides were scraped from the thin-layer chromatograms and eluted from the matrix with 1 mM NH_4OH and 50% pyridine.

II.B.11c N-terminal Sequence Determination

Sequence determinations were carried out using the dansylation procedure described by Bruton & Hartley (1970) except that the dansyl peptides were hydrolyzed for 7 h at 105°C. The method of Hartley (1970) was used to identify the dansyl amino acids except that toluene: acetic acid (9:1 v/v) was used as the second developing solvent.

II.B.12 Determination of Biotin Concentration

Biotin concentration was determined by the radiochemical assay developed by Rylatt *et al.* (1977). The assay solution contained in a final volume of 0.25 ml; potassium phosphate, pH 7.2 (50 µmole), avidin (0.028 units) and biotin sample (10 - 100 pmole biotin). After incubating for 10 min at room temperature 0.05 ml [^{14}C]biotin (0.13 nmole, 1.1 x 10^5 cpm/nmole) was added and 10 min later the

avidin-biotin complex was precipitated by adding 0.1 ml 4% zinc sulphate, followed by 0.1 ml 0.2 M sodium hydroxide. The precipitate was collected by centrifuging and samples of the supernatant were determined for their radioactivity using the Triton based scintillant (Section II.B.5). The correlation between the radioactivity of the supernatant and the biotin content of the samples was established by constructing a standard curve using known amounts of non-radioactive biotin as reference samples. There was a linear relationship between biotin content and radioactivity over the range 10 - 70 pmoles of biotin.

The biotin content of the native enzyme or of a peptide, were determined after hydrolyses. This was accomplished by either acid hydrolysis or enzymic digestion. Acid hydrolysis was carried out by placing the protein in a hydrolysis tube (0.5 cm x 4 cm) in 6 M HCl containing 1% β-mercaptoethanol. The contents of the tube were frozen and evacuated by allowing it to thaw out under vacuum, after which the tube is sealed and placed in an oven at 100°C for 18 h. After hydrolysis, the HCl was removed by lyophilizing. When enzymic hydrolysis was employed, the protein (10 mg) was suspended in 1 ml of 0.2 M NEM-acetate, pH 8, and the protein digested with 10 µl of 2% trypsin solution. After 2 h at 37°C, 10 µl of 5% pronase solution was added and incubated for an additional 2 h at 37°C.

CHAPTER III

MECHANISM OF THE FIRST PARTIAL REACTION.

III.A INTRODUCTION

As a consequence of the active site of pyruvate carboxylase consisting of two spatially distinct subsites, the classical approaches for evaluating the mechanism of action of an enzyme viz: initial velocity, product inhibition and isotope exchange studies, are complicated by difficulties in interpretation of experimental data. Although initial velocity and product inhibition studies do define the order of substrate binding and product release, they do not provide information about the composition of the enzyme-reactant complexes nor their interconversions. This information can however be obtained, to a certain extent, from isotopic equilibrium studies. In attempting to relate the coupling of ATP hydrolysis to CO_2 fixation in the first partial reaction catlaysed by pyruvate carboxylase, investigators have in most cases, utilized the information obtained from both approaches namely the overall steady state kinetic analysis and isotope equilibrium studies. However the results obtained by these two approaches have not been entirely consistent.

As discussed in detail in Section I.D one can envisage three possible coupling mechanisms:

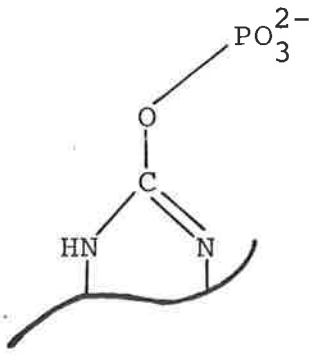
(i) a concerted reaction which proceeds without the formation of a kinetically significant or detectable intermediate.

(ii) activation of the enzyme (or the biotin prosthetic group) by ATP to form either an activated Pi~ENZ-biotin, (or Pi~biotin-ENZ) or an ADP~ENZ-biotin complex, which can then react with HCO_3^-.

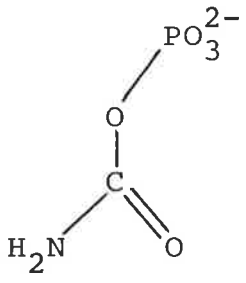
(iii) activation of HCO_3^- by formation of a phosphorylated or an adenosylated species.

From the various investigations on the coupling mechanisms, experimental evidence consistent with each type of mechanism has been obtained. However, these kinetic studies have also revealed numerous inconsistencies in their interpretations as discussed in Section I.D. The evidence put forward in support of a concerted mechanism by Kaziro *et al.* (1962), viz, that part of the ^{18}O from $HC^{18}O_3^-$ appeared in orthophosphate during the course of the reaction, is also consistent with mechanisms (ii) and (iii), provided that the activated intermediates of (ii) and (iii) contain the orthophosphate rather than the ADP moiety.

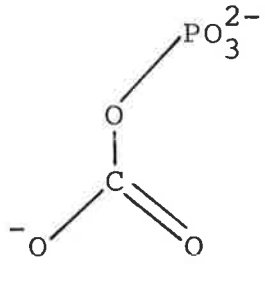
The elegant experiments of Polakis *et al.* (1972) who showed that acetyl CoA carboxylase from *E. coli* could catalyse the formation of ATP from carbamyl phosphate and ADP, were the basis for the proposed substrate activation model (mechanism iii). As pointed out by Kluger *et al.* (1979), this transferase reaction could occur through O-phosphobiotin and hence is also consistent with the enzyme activation model (mechanism ii). These authors in addition argue that carbamyl phosphate serves as an analogue of O-phosphobiotin and not of carboxyphosphate, since the latter differs by having an extra negative charge at neutral pH.



phosphobiotin



carbamyl phosphate



carboxyphosphate

The other major area of general disagreement concerns the isotope exchange studies at the first partial reaction subsite, in particular the isotopic exchange reaction between ATP and ADP which is apparently dependent only on Mg^{2+}. Pyruvate carboxylase from chicken liver (Scrutton & Utter, 1965), sheep kidney (Ashman & Keech, 1975), sheep liver (this thesis, Section III.C.1) and bovine liver propionyl CoA carboxylase (Lane *et al.*, 1960) catalyse an isotopic exchange between ADP and ATP which is dependent only on Mg^{2+}. This exchange reaction was shown to be a property of the chicken liver pyruvate carboxylase (Scrutton & Utter, 1965). However these authors considered this exchange reaction to be the result of a kinetically insignificant, alternative or abortive pathway. In the case of the sheep kidney enzyme, the reaction was apparently caused, at least in part, by the presence of contaminating adenylate kinase. The results obtained with propionyl CoA carboxylase have not been consistent. Contrary to the findings of Lane *et al.* (1960), the ATP:ADP exchange reaction was found to be dependent on orthophosphate (Kaziro *et al.*, 1960). The results obtained with the chicken liver enzyme led Scrutton & Utter (1965) to consider the possibility of a phosphoenzyme intermediate. However they discounted this mechanism on the basis that the enzyme failed to catalyse an ADP-independent isotope exchange between $H^{14}CO_3^-$ and oxaloacetate. However these authors assumed, without any evidence, that ADP must be released before HCO_3^- bound to the enzyme. Strangely the Mg^{2+}-dependent ATP:ADP exchange has not been rigorously shown to be independent of HCO_3^- even though solutions at mildly alkaline pH always contain a considerable

amount of HCO_3^- in equilibrium with atmospheric CO_2.

A mechanism involving carboxyphosphate (mechanism iii) could account for the requirement for ADP in the HCO_3^-: oxaloacetate exchange reaction, as well as the requirement for Mg^{2+} and HCO_3^-, but not orthophosphate, for the ATP:ADP isotopic exchange reaction.

It is apparent that any attempt to understand the precise nature of the mechanism of CO_2 fixation coupled to ATP hydrolysis, has to account for the properties of the isotopic exchange reactions. Although it is established that the overall reaction catalysed by pyruvate carboxylases occurs via a sequential mechanism (Warren & Tipton, 1974a, b, c; Easterbrook-Smith *et al.*, 1978), isotopic exchange reactions catalysed by the enzyme in the absence of the components of the other partial reactions are possible due to the spatial separation of the subsites (Easterbook-Smith *et al.*, 1978).

In an attempt to distinguish between the possible mechanism of coupling of ATP hydrolysis to CO_2 fixation in this investigation, the initial approach adopted was to re-examine those areas in which ambiguities exist and interpretations were suspect. The Mg^{2-}-dependent ATP:ADP isotopic exchange reaction in particular has been examined to determine if the reaction was part of the productive pathway. The second approach adopted here has been to examine the effect of various components, analogues and inhibitors on the first partial isotopic exchange reactions, in an effort to characterize the nature of the enzyme-reactant complexes as well as attempting to provide possible explanations for the slow isotopic exchange reactions catalyzed by pyruvate carboxylase.

III.B. MATERIALS AND METHODS

III.B.1 General Methods

Purification of nucleotides and pyruvate carboxylase was performed as described in Section II.B.1. The procedure for the determination of radioactivity was described in Section II.B.5. The procedures for separating radioactive nucleotides and orthophosphate are detailed in Section II.B.7.

III.B.2 Assay Methods

III.B.2a Overall Reaction Assay Procedures

The rate of the overall reaction catalysed by pyruvate carboxylase was measured by the coupled spectrophotometric assay system as described in Section II.B.6a. For experiments involving a large number of assays, the overall rate of pyruvate carboxylation was measured by the radiochemical assay as described in Section II.B.6b. In experiments involving HCO_3^- as a varied substrate, measures were taken to reduce the concentration of endogenous HCO_3^-. All reagents were prepared from freshly boiled, double distilled water and kept stoppered under N_2 over a saturated solution of NaOH. Assays were conducted in a nitrogen filled polyethylene lined chamber with gloved inlets to exclude atmospheric CO_2. The levels of endogenous HCO_3^- in the assay solutions were determined by incubating assay mixtures without added HCO_3^-, with sufficient pyruvate carboxylase to take the reaction rapidly to completion. Since the equilibrium for the reaction strongly favours product formation, almost quantitative fixation of HCO_3^-

to oxaloacetate is expected if the concentration of HCO_3^- is low relative to that of the other substrates. The amount of oxaloacetate formed was determined by the coupled spectrophotometric assay described in Section II.B.6a. The endogenous HCO_3^- levels were found to be in the range of 0.08 to 0.18 mM in these experiments. In the radiochemical assays, the specific radioactivity of the $H^{14}CO_3^-$ was corrected for endogenous HCO_3^-.

III.B.2b Isotopic Exchange Reaction Assays

The conditions used for measuring the rates of the ATP:Pi, the ATP:ADP, HCO_3^-:oxaloacetate and the pyruvate: oxaloacetate isotopic exchange reactions have been described in Section II.B.8.

III.B.2c Measurement of the Rate of $MgATP^{2-}$ Hydrolysis

The assay system for measuring the rate of $MgATP^{2-}$ hydrolysis in the absence of pyruvate contained, (in µmoles), in a final volume of 0.25 ml; N-ethylmorpholine (Cl^-, pH 7.8), (25); $MgCl_2$, (1.75); HCO_3^-, sodium salt, (0.5); acetyl CoA, (0.0625); [γ-^{32}P]ATP, (0.52×10^6 cpm/µmole) or [u-^{14}C]ATP, (2×10^6cpm/µmole), (0.5) and 4 units of pyruvate carboxylase. The reaction was carried out at 30°C and stopped at specified time intervals by removing 50 µl of the assay solution into 10 µl of ice-cold 26 N formic acid. Either the [^{32}Pi] or [^{14}C]ADP formed was separated by chromatography and radioactivity determined as described in Section II.B.7.

III.B.2d Assay of the Enzymic Reaction in the Reverse Direction

The overall reaction in the direction of ATP synthesis was followed by coupling with hexokinase and glucose-6-phosphate dehydrogenase (Lamprecht & Trantschold, 1963). Assays contained, in μmoles in a final volume of 1 ml, N-ethyl-morpholine (Cl^-, pH 7.8), (100); $MgCl_2$, (8); acetyl CoA, (0.25); oxaloacetate, (2); phosphate (2 Na^+, pH 7.8), (10); ADP, (2.5); NADP, (0.5); glucose, (10); hexokinase, (10 units), and glucose-6-phosphate dehydrogenase, 2 units. The reaction was initiated by the addition of 0.1 unit of pyruvate carboxylase and the reaction was measured by following the increase in absorbance at 340 nm due to the reduction of $NADP^+$. In the controls where acetyl CoA was omitted, no change in absorbance was observed under the conditions used. Calculations on the doubly coupled system as described by McClure (1969) and using literature K_m values for the coupling enzymes predict attainment of 99% of the steady-state rate within 10 sec.

III.B.3 Data Processing

Where appropriate, data was fitted to a straight line using a Fortran linear regression computer program. Initial velocity data were analysed by computing the hyperbolae of best fit, using the Fortran program HYPER (Cleland, 1963). Lines shown in the double-reciprocal plots were drawn from the computed kinetic constants and bars shown indicate the computed standard errors.

III.C RESULTS

III.C.1 The Mg^{2+} Dependent ATP:ADP Isotopic Exchange Reaction catalysed by Chicken Liver Pyruvate Carboxylase

The effect of the various components of the first partial reaction (eq. 1:2) on the exchange of ADP with ATP is detailed in Table III.1. As was found previously (Scrutton & Utter, 1965), the exchange reaction proceeds in the absence of added acetyl-CoA, HCO_3^- or inorganic phosphate. However contrary to the previous findings, the exchange reaction is stimulated 2.2-fold by the addition of acetyl-CoA and inorganic phosphate together. Inclusion of 2 mM HCO_3^- with acetyl CoA and inorganic phosphate decreased the stimulation to 1.9-fold relative to the basic exchange reaction rate. The rate of the ATP:ADP exchange reaction was insensitive to inhibition by avidin which is a specific inhibitor of the overall reaction, the ATP:Pi and pyruvate:oxaloacetate isotopic exchange reactions (Scrutton *et al.*, 1965).

Preparations of sheep liver pyruvate carboxylase also catalyses an ATP:ADP isotopic exchange reaction dependent only on the addition of Mg^{2+}. The effect of reaction components on this exchange reaction is shown in Table III.2. It is apparent that the effect of the reaction components on the rate of the exchange reaction is similar to the pattern observed for the chicken liver enzyme. The results detailed in Tables III.1 and III.2 are in agreement with the findings with the sheep kidney enzyme (Ashman, 1973). However, in that case it was found that the Mg^{2+}-dependent

ATP:ADP isotopic exchange reaction was catalysed, at least in part, by a contaminating adenylate kinase (Ashman & Keech, 1975).

Although the Mg^{2+} dependent ATP:ADP exchange reaction persists in chicken liver pyruvate carboxylases with a high specific activity (32 units/mg protein) as was used in this study, it was necessary to rule out the possibility of any contaminating adenylate kinases. When the enzyme (at a concentration similar to that used in the ATP:ADP exchange reaction assays) was incubated with reaction components as specified for the ATP:ADP isotopic exchange in Section II.B.8b, and samples of the reaction mixture, analysed as described in Section II.B.7, there was no incorporation of radioactivity into AMP. However, the incorporation of radioactivity into ATP increased with time (Fig. III.1). In addition, the Mg^{2+} dependent ATP:ADP exchange reaction is found to persist in chicken liver pyruvate carboxylase which had been preincubated with an excess of a adenylate kinase inhibitor, P^1, P^5-Di(adenosine-5')pentaphosphate (Ap_5A) described by Lienhard & Secemski (1973), (Fig. III.2). These results indicate that the Mg^{2+}-dependent ATP:ADP exchange reaction catalysed by chicken liver pyruvate carboxylase preparations is not due to a contaminating adenylate kinase.

The Mg^{2+}-dependent ATP:ADP exchange catalysed by chicken liver pyruvate carboxylase has been extensively characterized and shown to be a property of the enzyme (Scrutton & Utter, 1965) and hence no attempt was made here to confirm this finding. In attempting to incorporate the Mg^{2+} dependent

ATP:ADP isotope exchange reaction in the reaction pathway, these investigators, considered the possible existence of an 'activated Pi~ENZ-biotin' complex in a mechanism as depicted in reaction 1:4 and 1:5 (Section I.D.1b). However Scrutton & Utter (1965) did not consider the Pi~ENZ-biotin intermediate or the Mg^{2+} dependent ATP:ADP exchange reaction, to be kinetically significant, on the following evidence; -

(a) the exchange reaction between HCO_3^- and oxaloacetate required the presence of both ADP and Pi. According to the reaction pathway (reaction 1:4 and 1:5) postulated on the basis of the Mg^{2+} dependent ATP:ADP isotopic exchange reaction, $H^{14}CO_3^-$ should be incorporated into oxaloacetate in the absence of ADP,

(b) they were unable to isolate the predicted Pi~ENZ-biotin complex from their reaction solutions.

The two pieces of evidence cited above are equivocal. Firstly, the attempt to isolate a phosphoryl-enzyme intermediate involved precipitation of the enzyme by trichloroacetic acid and thus assumed that the complex was acid stable. Secondly, since the ATP:ADP exchange reaction only required the addition of Mg^{2+}, it was assumed that ADP must be released before HCO_3^- binds. However, as pointed out earlier the level of endogenous HCO_3^- in the reaction solution is relatively high (1 - 2 mM, at mildly alkaline pH). (Rylatt, 1976) and it is conceivable that HCO_3^- could have bound before ADP was released. This could account for the requirement for ADP in the HCO_3^-:oxaloacetate exchange reaction. Hence the effect of HCO_3^- on the isotopic exchange reactions

was re-examined.

III.C.2 Effect of HCO_3^- on Isotopic Exchange Reactions Involving ATP

The results detailed in Table III.1, indicate that the Mg^{2+}-dependent ATP:ADP exchange reaction, lacks the requirement for HCO_3^-. However it is possible that the endogenous levels of HCO_3^- were at saturating levels and therefore could have masked any effect of the added HCO_3^-. Hence measures were taken to remove endogenous HCO_3^- from the reaction solutions as described in Section III.B.2a. The effect of varying the HCO_3^- concentration on the ATP:ADP isotopic exchange reaction is shown in Fig. III.3. Contrary to previous reports (Scrutton & Utter, 1965), the rate of ATP:ADP exchange reaction is influenced by the presence of HCO_3^-. Despite the rigorous precautions taken to exclude endogenous HCO_3^- from the reagents, it was not possible to demonstrate an absolute dependence for the presence of HCO_3^-. The rate observed in the absence of any added HCO_3^- probably represents the failure to completely remove the carbon dioxide. However the velocity profile did pass through an optimum activity range and then as the concentration of HCO_3^- was increased, a strong substrate inhibition pattern was obtained. A similar result was obtained when the velocity of the ATP:Pi exchange reaction was plotted as a function of HCO_3^- concentration (Fig. III.4) although the substrate inhibition pattern was less pronounced. Substrate inhibition by HCO_3^- has been reported for the ATP:ADP and the ATP:Pi isotopic exchange reactions catalysed by rat liver pyruvate carboxylase (McClure *et al.*, 1971c) although, these invest-

igators measured both the exchange reactions under similar conditions, while in the present investigation, the ATP: ADP isotopic exchange reaction was measured in the absence of Pi and acetyl-CoA.

The resulting velocities of the two exchange reactions were used to calculate $K_{HCO_3^-}$ and V values. The apparent K_m value for HCO_3^- in the ATP:ADP exchange reaction was calculated to be 0.1 mM, while in the ATP:Pi exchange reaction a calculated value of 0.17 mM was obtained. The kinetic constants are summarised in Table III.3.

Although it was not possible to demonstrate an absolute dependence on HCO_3^- for the ATP:ADP or the ATP:Pi isotopic exchange reactions, the observation that HCO_3^- influences the Mg^{2+}-dependent ATP:ADP exchange reaction invalidates one of the criteria used for dismissing the reaction as an abortive one, (Scrutton & Utter, 1965). The difficulties in reducing endogenous levels of CO_2, together with the observed low K_m value for HCO_3^- in the exchange reaction in question, has contributed to the inability to show an absolute dependence on HCO_3^-. The low K_m value implies that endogenous CO_2 might be saturating for the exchange reaction and this could account for the failure by earlier investigators to demonstrate any dependence on added HCO_3^-.

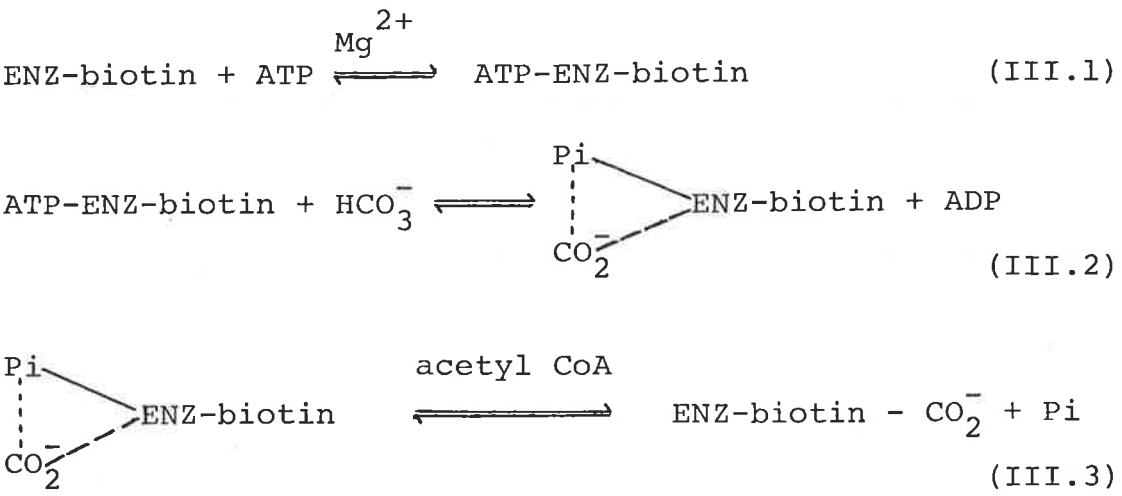
III.C.3 Effect of Acetyl CoA on Isotopic Exchange Reactions Involving ATP

In agreement with previous findings (Scrutton *et al.*, 1965; Scrutton & Utter, 1965), the ATP:Pi isotopic exchange reaction as well as the overall activity showed an absolute requirement for acetyl CoA, while the ATP:ADP exchange

reaction occurred in the absence of this effector (Table III.4 The results indicate a slight stimulation of the ATP:ADP exchange reaction rate when acetyl CoA (0.16 mM) was included. However the results presented in Table III.1 reveal no such stimulation with 0.25 mM acetyl CoA concentration which is 8-fold greater than the K_m value for acetyl CoA in the overall forward reaction (Scrutton & Utter, 1976). When the rate of the ATP:ADP exchange reaction was measured at varying concentrations of acetyl CoA, it is seen from Fig. III.5a, that above 0.1 mM, acetyl CoA becomes inhibitory. A similar inhibition above 0.1 mM acetyl CoA concentrations was observed for the ATP:Pi exchange reaction (Fig. III.6a). The overall forward reaction does not however show a substrate inhibition pattern at this concentration (Fig. III.7).

The lack of requirement of acetyl CoA for the ATP:ADP exchange reaction suggests that the release of ADP is not mediated by acetyl CoA, while the release of Pi is mediated by this effector as implied by the dependence of the ATP:Pi exchange reaction on its presence. The above findings indicate that under exchange reaction conditions the release of ADP and Pi might be a stepwise process with ADP being released before the addition of acetyl CoA.

The involvement of HCO_3^- in both the ATP:ADP and ATP:Pi exchange reactions as shown in Fig. III.3 and Fig. III.4, suggest that the sequence of events occurring in the first partial reaction may proceed as indicated in the scheme as shown



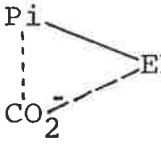
Scheme I.

In this scheme, it is proposed that ATP binds first to the enzyme (eq. III.1) with the subsequent binding of HCO_3^- to displace ADP (eq. III.2). Unlike the substrate activation model depicted in Figure I.3c, it is proposed that ADP is released prior to the carboxylation of biotin, to be consistent with the ATP:ADP isotopic exchange reaction. HCO_3^- in displacing ADP reacts either with the enzyme itself, or the bound γ-phosphoryl phosphorous atom of ATP or both to form a carboxyphosphate derivative. The third step in the sequence (eq. III.3) requires the participitation of acetyl CoA which allows the release of phosphate and facilitates the transfer of the activated carboxyl group from the carboxyphosphate derivative to the 1'-Nitrogen of biotin.

The various pieces of experimental evidence supporting this mechanism are:

(1) the ATP:ADP exchange reaction does not require the presence of either orthophosphate or acetyl CoA and is only partially inhibited by avidin (Scrutton & Utter, 1965; Ashman & Keech, 1975), while the findings in this thesis

show (Table III.1) that the exchange reaction is not inhibited by avidin. The inhibition of the exchange reaction at high concentrations of HCO_3^- supports the proposed mechanism by suggesting that increased concentrations of HCO_3^- tend to increase the concentration of the



species thus making it more difficult for ATP to dissociate and associate.

(2) The ATP:Pi exchange reaction which requires the presence of acetyl CoA is completely inhibited by avidin (Scrutton *et al.*, 1965; Ashman & Keech, 1975) indicating that the exchange reaction requires the involvement of biotin. Again the substrate inhibition pattern exhibited by HCO_3^- is similar to that shown for the ATP:ADP exchange reaction, although in this case, the inhibition is less pronounced suggesting additional complications in the mechanism of the ATP:Pi exchange reaction.

(3) The isotope exchange between HCO_3^- and oxaloacetate, which involves both the first and second partial reactions, has been shown to be dependent on the presence of both ADP and Pi. This has been demonstrated with the enzyme from chicken liver (Scrutton & Utter, 1965), rat liver (McClure *et al.*, 1971c) and from Sheep kidney (Ashman & Keech, 1975). This requirement is accommodated by the proposed mechanism.

(4) The participation of bicarbonate in phosphoryl bond cleavage implied from the studies of Kaziro *et al.* (1962) and the suggestion that a carboxy-phosphate derivative may be involved in the activation of HCO_3^- by biotin-dependent

enzymes (Polakis *et al.*, 1972; Ashman & Keech, 1975) are all compatible with the proposed mechanism.

(5) Although the ATP:ADP exchange reaction functioned in the absence of acetyl CoA, the ATP:Pi exchange reaction exhibited complete dependence on the presence of the activator (Table III.4 and Fig. 6a). However, acetyl CoA was inhibitory at higher concentrations for both the exchange reactions. These observations tend to suggest that the presence of acetyl CoA facilitates the formation of, and shifts the equilibrium toward the carboxybiotin intermediate (eq. III.3). This mechanistic role of acetyl CoA has been further investigated in Chapter VI and is discussed in some detail there.

III.C.4 Slow Isotopic Exchange Reactions and Substrate Synergism

The results presented in Sections III.C.2 and III.C.3 indicate that the Mg^{2+} dependent ATP:ADP exchange reaction is on the main pathway and is not a result of an abortive reaction as was suggested previously. The ATP:ADP as well as the ATP:Pi exchange reactions are usually thought to be slow when compared to the overall reaction. However a comparison of the two rates of reaction is difficult to make unambigously, (Leuck & Fromm, 1973; Boyer, 1978). While it is not possible to relate the rates of partial exchange reactions to initial velocity rates, it is quite valid to compare partial exchange reactions in the presence of the other substrate to test the possibility of substrate synergism (Bridger *et al.*, 1968; Purich & Allison, 1980), where one substrate at a catalytic site promotes the react-

ivity of another substrate although the two substrates do not react directly with each other.

The steady state kinetic analysis of the pig liver enzyme indicated that MgADP and Pi are released only after the binding of pyruvate (Warren & Tipton, 1974b). Therefore pyruvate would be expected to have a synergistic effect on the first partial exchange reactions measured under equilibrium conditions. To test this hypothesis both the ATP:ADP and the ATP:Pi isotopic exchange reactions were measured in the presence of pyruvate and oxaloacetate. The results detailed in Table III.5, show that the ATP:Pi exchange reaction is stimulated 1.6 fold when measured under an overall equilibrium condition, while a 1.3 fold stimulation is observed for the ATP:ADP exchange. This synergistic effect of the second partial reaction components on the first partial exchange reactions, is in agreement with the findings of McClure *et al.* (1971c), who showed that α-oxobutyrate stimulated the ATP:ADP exchange reaction approximately 1.3 fold.

In contrast to the first partial exchange reactions, the pyruvate:oxaloacetate exchange rate is not affected by the presence of the first partial reaction components, (Table III.5) i.e., under conditions where the entire reaction is allowed to run backwards and forwards. This can be explained if it is assumed that the presence of pyruvate tends to keep the carboxybiotin-enzyme at the keto acid subsite long enough for maximal pyruvate:oxaloacetate exchange velocities to be reached. The evidence supporting this view are:

(a) low concentrations of pyruvate induced the abortive hydrolysis of carboxybiotin-enzyme, while in the absence of pyruvate this hydrolytic leak was not observed (Easterbrook-Smith *et al.*, 1976a). The authors' conclusion was that the binding of pyruvate shifts the carboxybiotin enzyme to the pyruvate binding site in readiness for the carboxylation,

(b) Goodall *et al.* (1981) have shown that the analogue of pyruvate, 2-oxobutyrate had a similar effect on the carboxybiotin-enzyme. In addition these investigators demonstrated that in the presence of Mg^{2+}, very little carboxybiotin-enzyme leaves the nucleotide binding subsite (Site I) until after pyruvate binds. At Site I, the half-life of carboxybiotin-enzyme was two fold greater than when it was at theketo acid subsite (Site II).

(c) that excess Mg^{2+} was a strong inhibitor of the pyruvate:oxaloacetate exchange reaction (Ashman, 1973).

Hence the synergism observed with the first partial exchanges may be due to the influence of pyruvate on the movement of carboxybiotin-enzyme to the keto acid subsite, if it is assumed that the carboxybiotin-enzyme somehow 'traps' the ADP and Pi at their respective binding sites (Warren & Tipton, 1974b), and that the movement of carboxybiotin to Site II enhances the release of ADP and Pi, resulting in an increased isotopic exchange reaction. The substrate synergism shown by pyruvate on the first partial exchange reactions, is consistent with the sequential model proposed by Warren & Tipton (1974b), since for a ping-pong mechanism the rate of a partial exchange reaction must

exceed the rate of the same exchange reaction in the presence of all the other substrates if the same catalytic steps and efficiencies are involved (Lueck & Fromm, 1973).

The data shown in Table III.5 were compiled under standard conditions to enable direct comparison of the rates of the exchanges and the overall reaction in the forward and reverse directions. Comparison of the rates of the ATP:Pi (or ATP:ADP) and the pyruvate:oxaloacetate exchange reactions indicates that the first partial reaction is rate-limiting. Comparable rates were observed for the ATP:Pi and the Mg^{2+}-dependent ATP:ADP exchange reactions, while the HCO_3^-:oxaloacetate exchange reaction is approximately 60% of the first partial exchange rates. The rates of these exchanges, although low relative to that of the forward reaction, were approximately 1/3 of that of the reverse reaction. This is in agreement with the findings with the sheep kidney enzyme (Ashman & Keech, 1975). A lower exchange rate of 7% of the reverse velocity has been observed with the rat liver enzyme (McClure *et al.*, 1971c). The findings of Table III.5 clearly demonstrate that one of the steps in the reverse reaction is slow. It could be argued that exchange reactions are measured in the presence of products, and hence would be expected to yield slower rates compared to overall forward velocities. However the observation that the reverse reaction velocity, measured in the absence of products in the reverse reaction, is slow, implicates a slow step in the reverse reaction and not just the presence of products as the cause of 'slow' exchange reactions. An obvious extension of this study would be to

determine the rate limiting steps particularly in the reverse reaction. There are various methods for obtaining kinetic constants although for discrete steps in the reaction these kinetic constants are not easily obtained. As pointed out by Wimmer & Rose (1978), such expressions as K_{cat} and V_{max}/E_t only represent lower limits to the real reaction rate constants and that only a small portion of E_t may participate in a rate limiting step if the equilibria prior to the slow step are unfavourable. The observed rate (i.e. steady state rates) is the product of concentration and rate constant while for reversible steps, the observed rate is only a difference between the forward and reverse reaction rates. Nevertheless some recent techniques have become sufficiently sophisticated to enable the accurate determination of rate-limiting steps, among them the isotope partition method of Rose *et al.* (1974) and the analysis of isotope effects (see review by Northrop, 1981), are of great value. The application of these methods on the analysis of rate-limiting steps in the pyruvate carboxylase catalysed reactions are discussed in detail in Chapter VIII.

III.C.5 Variability of the Mg^{2+}-dependent ATP:ADP Isotopic Exchange Reaction Rate

A consistent feature observed with the Mg^{2+}-dependent ATP:ADP exchange reaction has been the variability of the rate with respect to the specific activity of the enzyme. This inconsistency in the rate persists in enzymes with high specific activity (≈ 30 units/mg protein) and varies

with different batches of enzyme as well as with the same enzyme measured after varying peroids of time. To examine this variability, the enzyme was diluted in a Tris buffer and allowed to inactivate at 30°C. The overall forward velocity, the ATP:Pi and the Mg^{2+}-dependent ATP:ADP exchange reactions were followed. The results presented in Fig. III.8 show that while the ATP:Pi exchange reaction is inactivated at the same rate as the overall forward reaction, the ATP:ADP exchange is only slightly affected. Hence the amount of enzyme units (based on overall reaction velocity), used for the ATP:ADP exchange reaction studies will vary. The enzyme is known to lose overall activity on storage, therefore more 'inactive' enzyme will be used to initiate the ATP:ADP exchange reaction which would account for the variability. This finding also gives support to the mechanism proposed in Fig. III.9 where the release of ADP occurs prior to the carboxylation step, while Pi is released after or in conjunction with biotin carboxylation, when measured under equilibrium conditions.

III.D. DISCUSSION

In attempting to elucidate the mechanism of the ATP hydrolysis coupled to CO_2 fixation, most of the studies undertaken in this Chapter were based on equilibrium exchange measurements. Nevertheless the scheme for the first partial reaction depicted in Fig. III.9 is based on the available kinetic data in addition to the findings of

this section. Until recently equilibrium exchange studies were employed almost exclusively as an adjunct to initial-rate studies for the ordering of substrate binding and product release. Now the approach has been considerably extended to involve rate measurement of exchange of essentially all possible substrate atoms or functional groups of atoms, the determination of kinetic isotopic effects and the examination of the interference of regulatory activitors, inhibitor, and interconverting enzymes with the detailed chemical steps in the catalytic process (Purich & Allison, 1980). However extensive studies have not been done on multisite enzymes such as those of the biotin-containing carboxylases.

The results presented in Section III.C.2 indicate substrate inhibition by HCO_3^- on both the ATP:ADP and ATP:Pi exchange reactions. A similar substrate inhibition pattern observed with the rat liver enzyme (McClure *et al.*, 1971c) was found to be uncompetitive with respect to ATP. This finding suggested that there was ordered addition of the two reactants, with HCO_3^- adding after ATP. These authors however concluded that the addition was random, since carboxybiotin was free to move to Site II, and hence could account for the uncompetitive inhibitory patterns observed with ATP. This assumption and conclusions are in error since the recent findings of Goodall *et al.* (1981) indicated that in the presence of Mg^{2+}, very little carboxybiotin leaves Site I until after the binding of pyruvate.

Therefore, contrary to the conclusions of McClure *et al.* (1971c) and Cleland, (1977), the uncompetitive patterns

observed indicate a ordered addition of ATP and HCO_3^-. This is consistent with the proposed mechanism in Fig. III.9.

In the sequence of events depicted in Fig. III.9 the release of ADP is followed by the release of orthophosphate, where-as product inhibition data indicate random release of ADP and orthophosphate. As discussed in detail in Section I.C.2 although MgADP and orthophosphate are released only after the binding of pyruvate under steady state conditions (as in Initial Velocity and Product Inhibition Measurement) the spatial distinction of the subsites implies that there is no mechanistic requirement for this order of release to be maintained. Hence under conditions where the partial reactions are studied in isolation as in equilibrium exchange studies, the release of products can occur without the substrate(s) of the other partial reaction being present.

TABLE III.1

EFFECT OF REACTION COMPONENTS ON THE ATP:ADP ISOTOPIC EXCHANGE REACTION CATALYSED BY CHICKEN LIVER PYRUVATE CARBOXYLASE

The assay procedure was as described in Section II.B. 8b. The exchange rates were calculated as described in Section II.B.8e. The values shown are the mean and standard errors of mean of three measurements.

Assay System	Exchange rate (μmoles min^{-1} $E.U.^{-1}$)
Basic system[1]	0.004 ± 0.0005
+ 0.25 mM acetyl CoA	0.0041 ± 0.0003
+ 2 mM HCO_3^-	0.004 ± 0.0004
+ 10 mM Pi	0.0036 ± 0.0003
+ 2 mM HCO_3^- + 10 mM Pi	0.0044 ± 0.0007
+ 0.25 mM acetyl CoA + 2 mM HCO_3^-	0.0035 ± 0.00005
+ 0.25 mM acetyl CoA + 10 mM Pi	0.0093 ± 0.001
+ 0.25 mM acetyl CoA + 10 mM Pi + 2 mM HCO_3^-	0.0075 ± 0.001
Basic system (untreated enzyme)	0.0062 ± 0.0001
Basic system (avidin inactivated enzyme)	0.007 ± 0.0001

[1] The basic system contained 100 mM N-ethylmorpholine-Cl, pH 7.8; 8 mM, $MgCl_2$; 2 mM ATP; 2 mM, ADP and endogenous bicarbonate.

TABLE III.2

EFFECT OF REACTION COMPONENTS ON THE ATP:ADP ISOTOPIC EXCHANGE REACTION CATALYSED BY SHEEP LIVER PYRUVATE CARBOXYLASE

The assay procedure was as described in Section II.B.8b. The values shown are the mean and standard errors of mean of three values.

Assay System	Exchange rate (μmoles min^{-1} $E.U.^{-1}$)
Basic System[1]	0.019 ± 0.001
+ 0.25 mM Acetyl CoA	0.02 ± 0.002
+ 2 mM HCO_3^-	0.019 ± 0.0004
+ 10 mM Pi	0.019 ± 0.0009
+ 2 mM HCO_3^- + 10 mM Pi	0.02 ± 0.0002
+ 0.25 mM acetyl CoA + 2 mM HCO_3^-	0.019 ± 0.002
+ 0.25 mM acetyl CoA + 10 mM Pi	0.029 ± 0.004
+ 0.25 mM acetyl CoA + 10 mM Pi + 2 mM HCO_3^-	0.025 ± 0.003

[1] The basic system contained 100 mM, N-ethylmorpholine-Cl, pH 8.4; 8 mM, $MgCl_2$; 2 mM, ATP; 2 mM, ADP and endogenous bicarbonate.

FIGURE III.1

INCORPORATION OF RADIOACTIVITY FROM [U-^{14}C]ADP INTO ATP AND AMP UNDER THE Mg^{2+}-DEPENDENT ATP: ADP EXCHANGE ASSAY CONDITIONS

The assay conditions and methods were as described in Section III.B.2b. Each data point is a mean of three values. Radioactivity in ATP (■) and AMP (□) is expressed in terms of the percent of the total radioactivity.

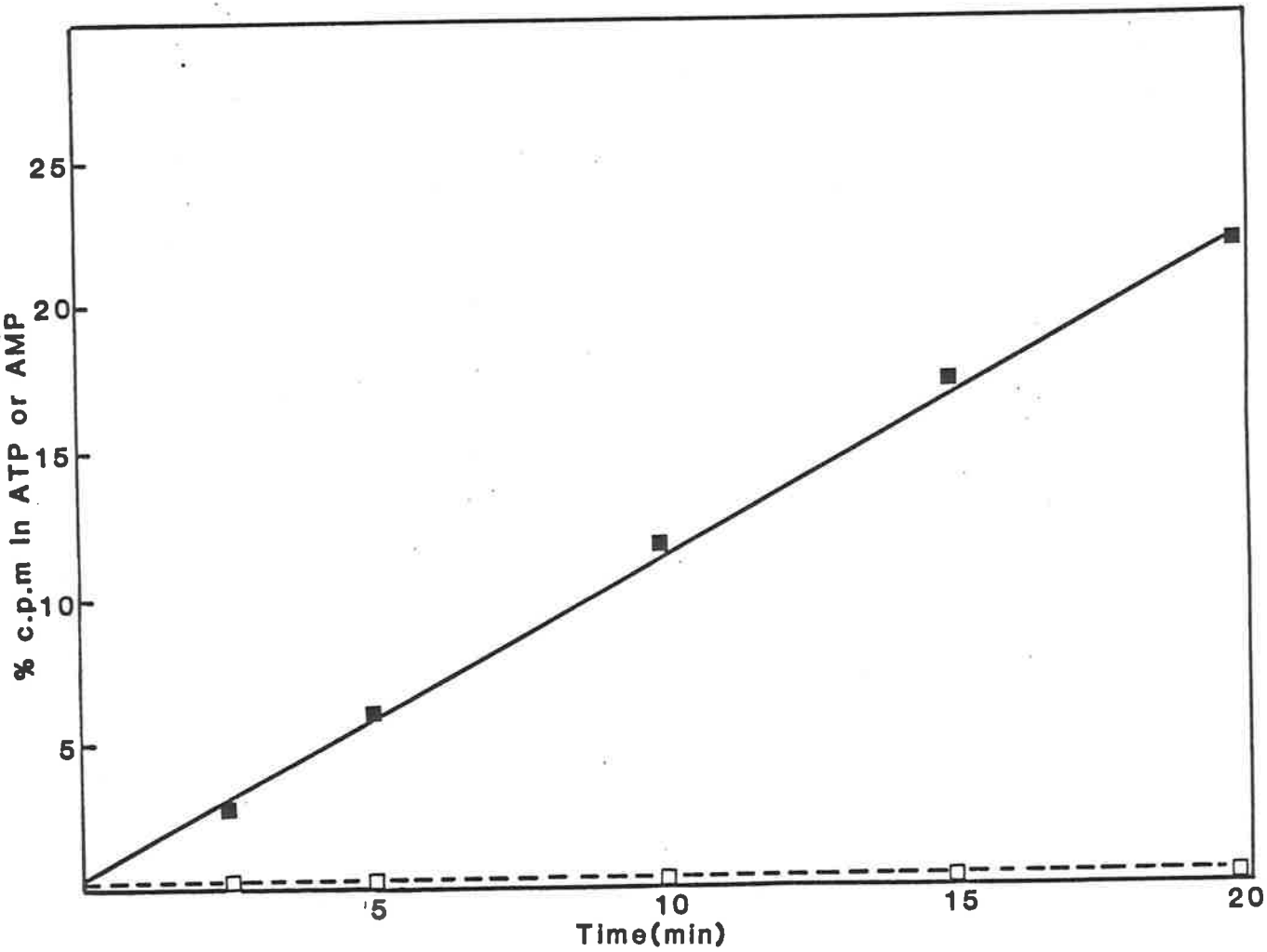

25
20
15
10
5
% c.p.m In ATP or AMP
5
10
15
20
Time(min)

FIGURE III.2

THE EFFECT OF THE ADENYLATE KINASE INHIBITIOR (AP_5A) ON THE RATE OF THE Mg^{2+}-DEPENDENT ATP:ADP EXCHANGE REACTION RATE

The assay procedures were as described in Section III.C.1. The enzyme was incubated with an excess of AP_5A and at the times indicated aliquots were removed to initiate the ATP:ADP exchange reaction. The exchange rate is a mean of three values (■). In the control (□), untreated enzyme was used to initiate the exchange reaction.

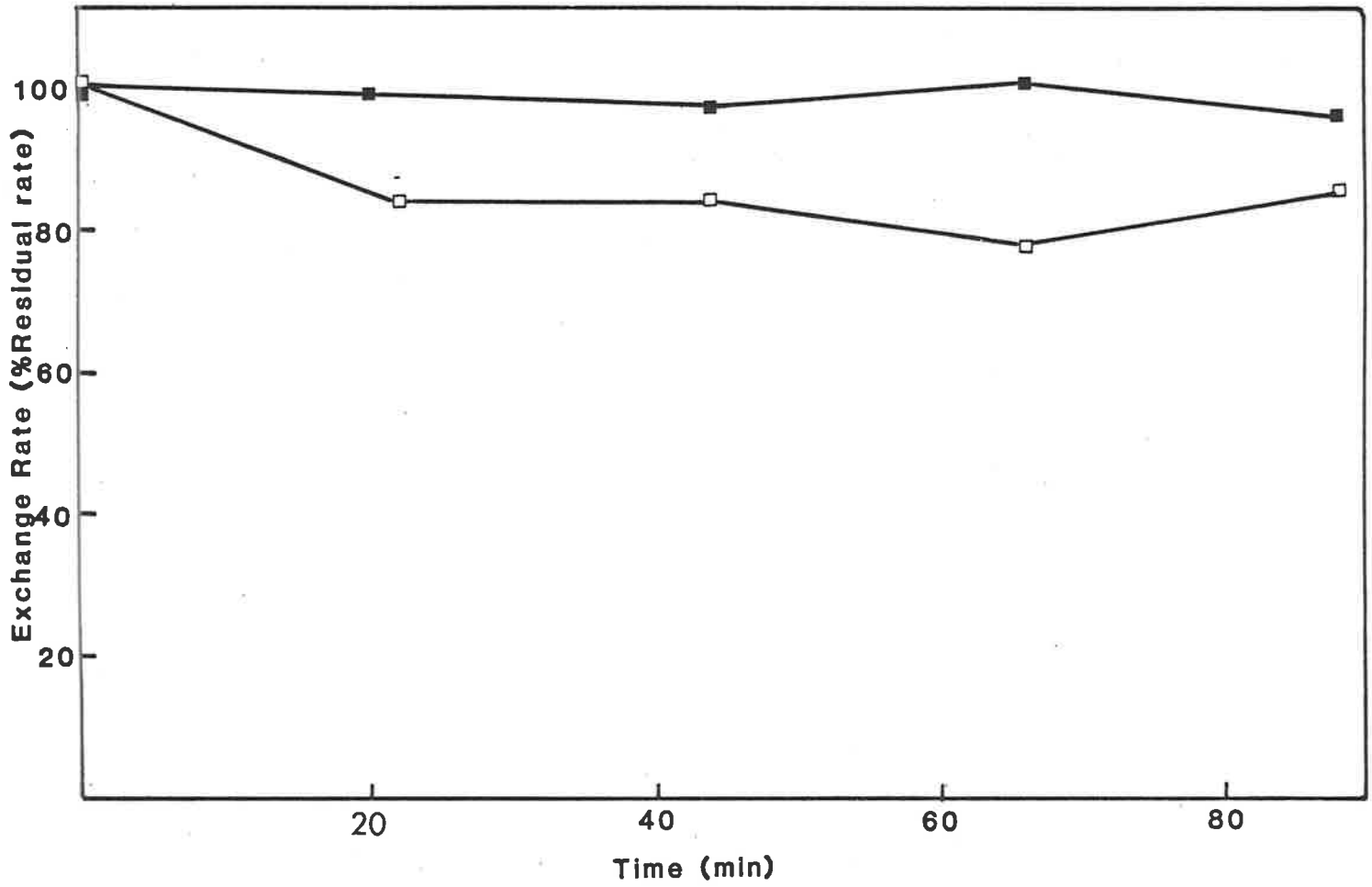

100
80
60
40
20
Exchange Rate (%Residual rate)
20
40
60
80
Time (min)

FIGURE III.3

THE EFFECT OF HCO_3^- ON THE Mg^{2+}-DEPENDENT ATP:ADP EXCHANGE REACTION

The assay procedure for measuring the rate of the Mg^{2+}-dependent ATP:ADP exchange reaction was as described in Section II.B.8b. The rate of the ATP:ADP exchange reaction is plotted against varying HCO_3^- concentrations as indicated. Each data point is a mean of three values. NaCl was added to keep total Na^+ = 5 mM.

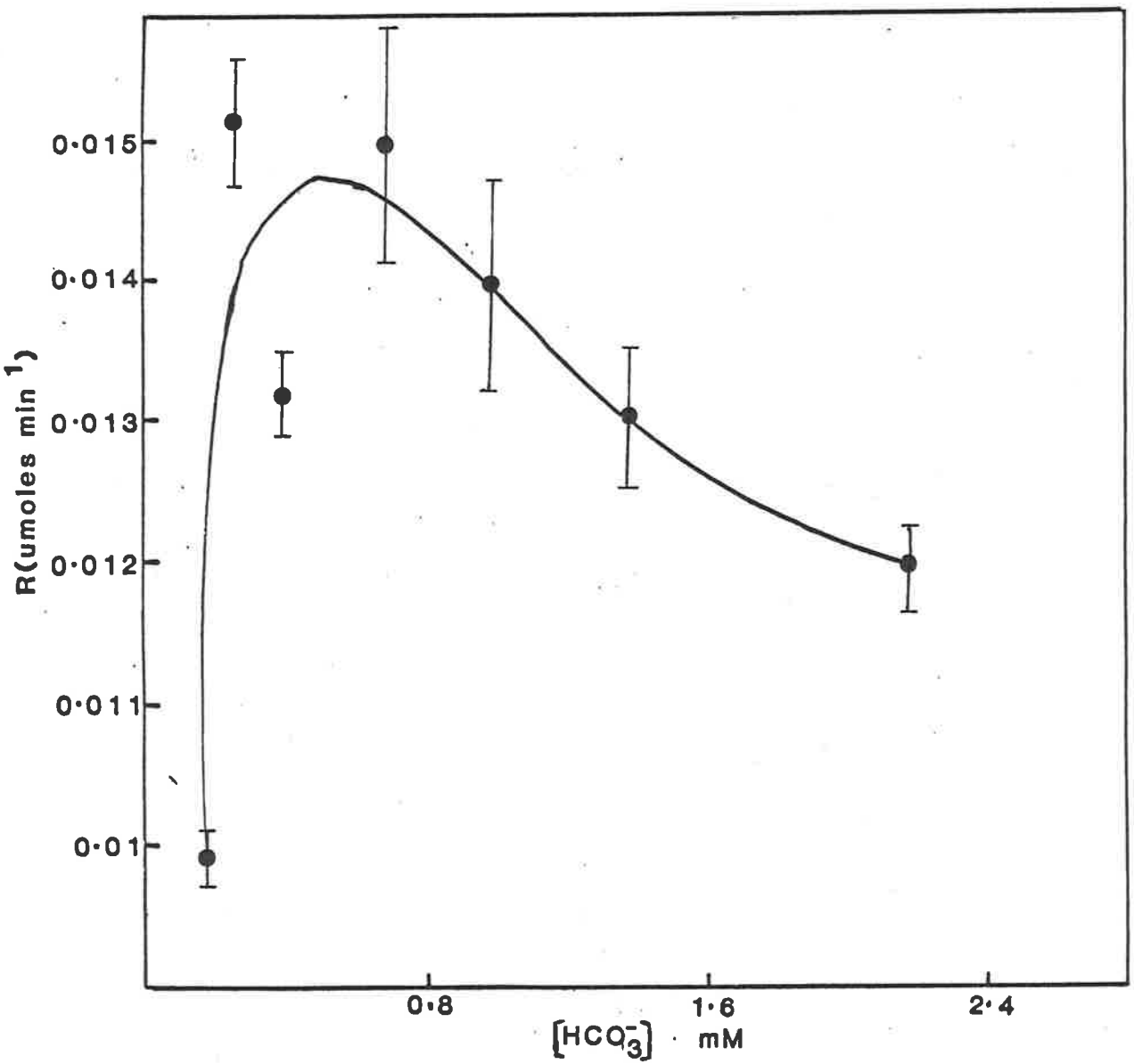

0·015
0·014
0·013
0·012
0·011
0·01
R(umoles min 1)
0·8
1·6
2·4
[HCO3-] mM

FIGURE III.4

THE EFFECT OF HCO_3^- ON THE ATP:Pi ISOTOPIC EXCHANGE REACTION

The rate ATP:Pi exchange reaction was measured as described in Section II.B.8a. A mean of three values for the measured rate in the presence (■) and absence (□) of acetyl CoA are plotted against varying indicated concentrations of HCO_3^-. NaCl was added to keep total Na^+ = 5 mM.

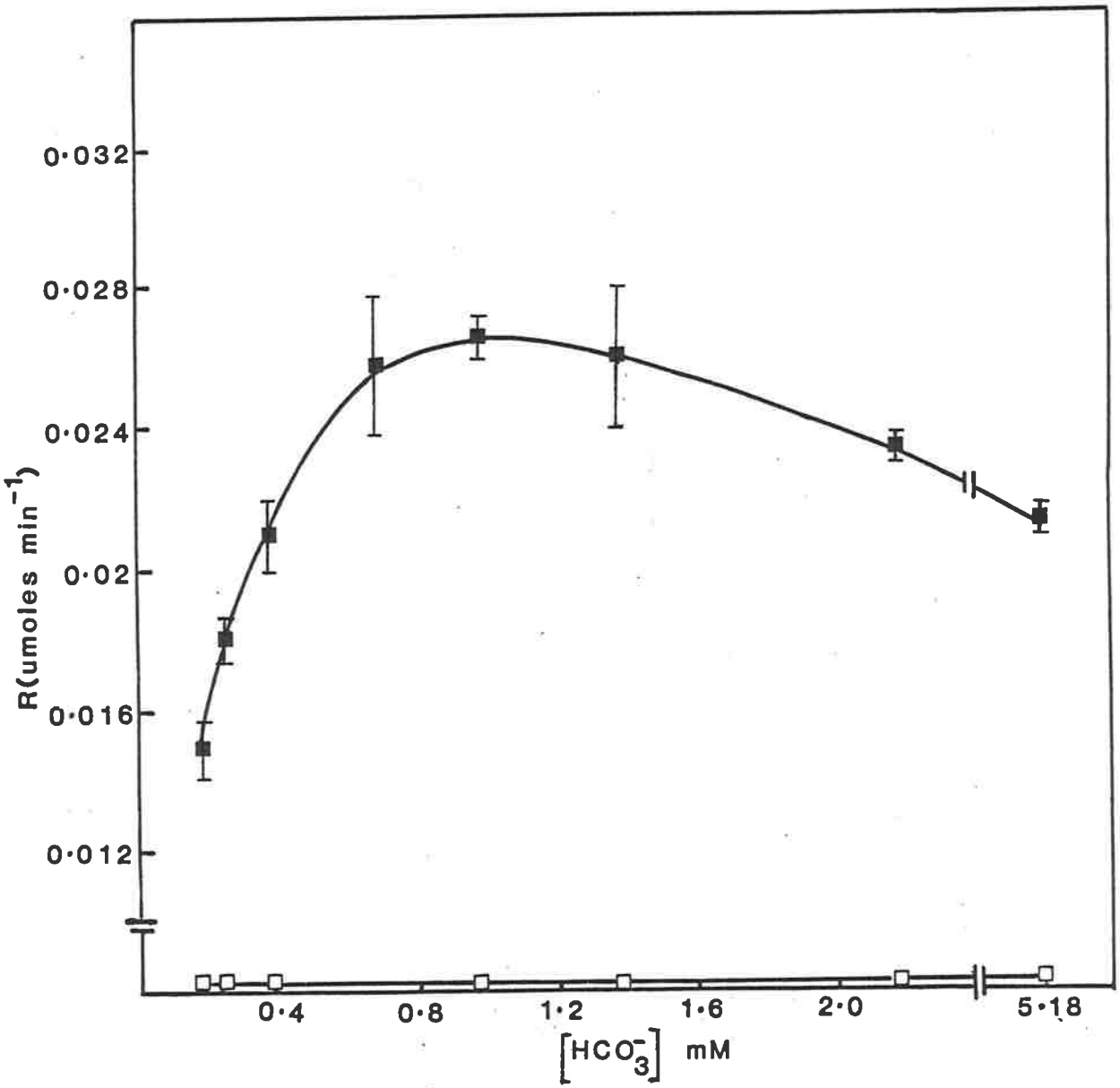

0·032
0·028
0·024
0·02
0·016
0·012
R(umoles min^{-1})
0·4
0·8
1·2
1·6
2·0
5·18
[HCO$_3^-$] mM

TABLE III.3

APPARENT KINETIC CONSTANTS FOR HCO_3^- DETERMINED BY ISOTOPIC EXCHANGE REACTIONS

Kinetic constants were calculated from the data points at non-inhibitory concentrations of HCO_3^- in Fig. III.3 and Fig. III.4.

Isotopic Exchange Reaction	Apparent K_m (mM)	Apparent V_{max} (μmoles min^{-1} E.U.$^{-1}$)
ATP:ADP	0.1	0.017
ATP:Pi	0.17	0.031

TABLE III.4

REQUIREMENT OF ACETYL CoA FOR OVERALL ACTIVITY AND THE ATP:Pi EXCHANGE REACTION CATALYSED BY CHICKEN LIVER PYRUVATE CARBOXYLASE

The overall forward reaction was measured by the radiochemical assay procedure as described in Section II.B. 6b. The ATP:Pi and the ATP:ADP exchange reactions rates were measured as described in Section II.B.8a and II.B.8b respectively.

Reaction	Addition	Rate (μmoles min^{-1} $E.U.^{-1}$)
ATP:ADP exchange	- Acetyl CoA	$0.009 \pm 8 \times 10^{-5}$
	+ 0.16 mM Acetyl CoA	$0.0128 \pm 5 \times 10^{-4}$
ATP:Pi exchange	- Acetyl CoA	0
	+ 0.16 mM Acetyl CoA	$0.024 \pm 8 \times 10^{-4}$
Overall reaction	- Acetyl CoA	0
	+ 0.16 mM Acetyl CoA	0.54 ± 0.02

FIGURE III.5a

EFFECT OF ACETYL CoA ON THE Mg^{2+}-DEPENDENT ATP:ADP EXCHANGE REACTION

The assay procedure was the same as that described in Section II.B.8b except that the concentration of acetyl CoA was varied. The data points are a mean of the rates obtained at three different time intervals of the exchange reaction.

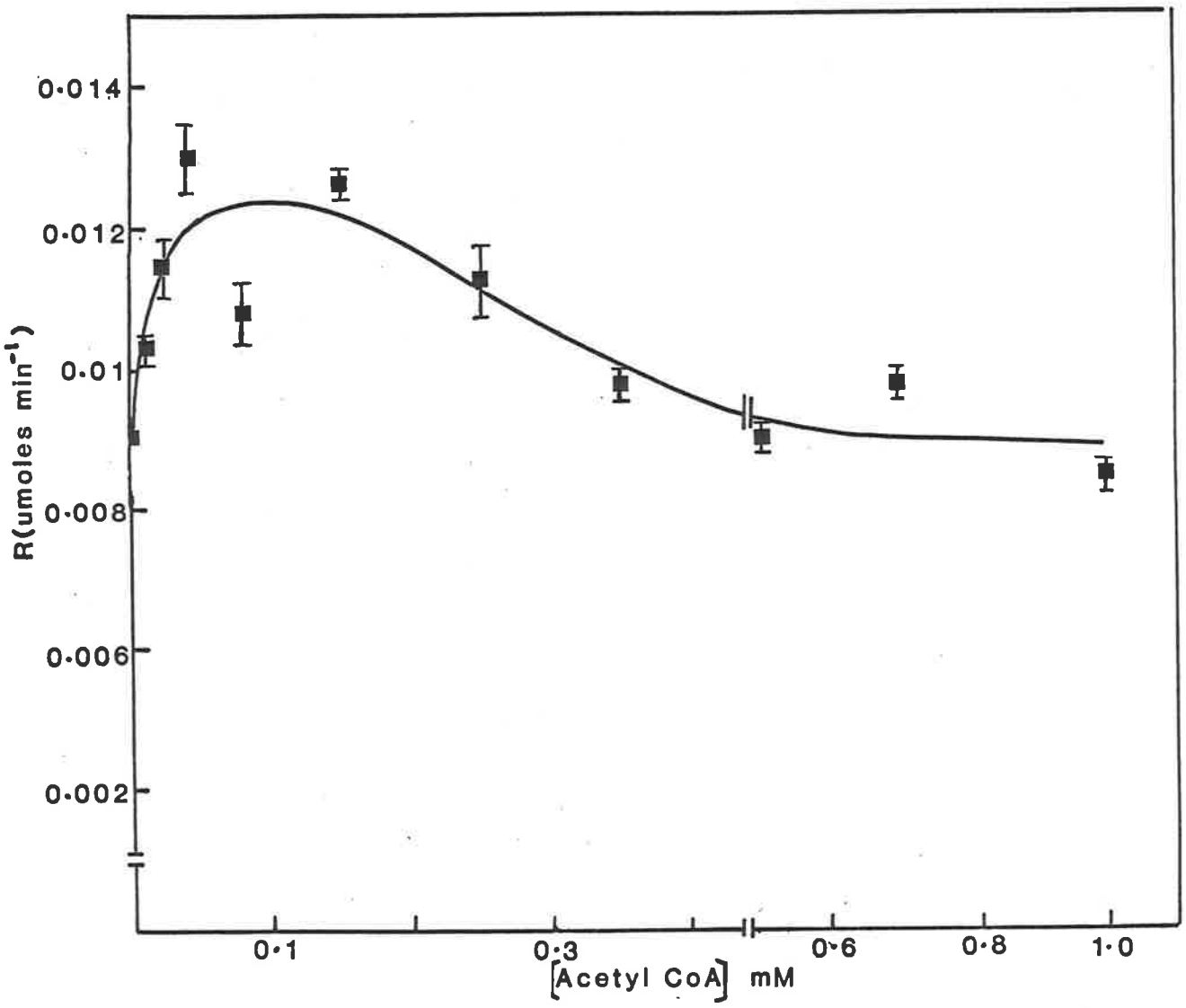

0·014
0·012
0·01
0·008
0·006
0·002
R(umoles min⁻¹)
0·1
0·3
0·6
0·8
1·0
[Acetyl CoA] mM

FIGURE III.5b

DOUBLE RECIPROCAL PLOT OF THE DATA OBTAINED FROM FIGURE III.5a.

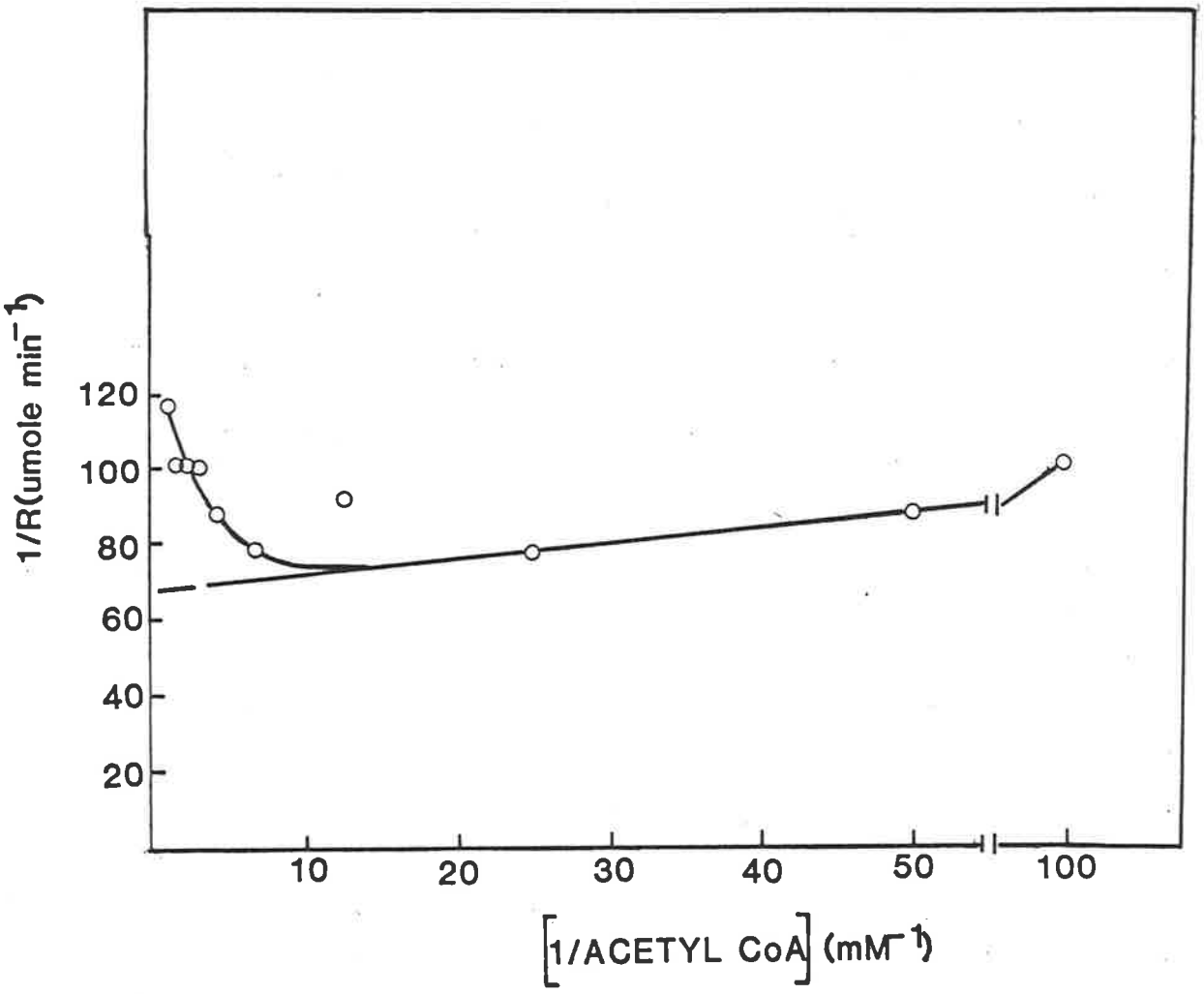

1/R(umole min⁻¹)
120
100
80
60
40
20
10
20
30
40
50
100
[1/ACETYL CoA] (mM⁻¹)

FIGURE III.6a

EFFECT OF ACETYL CoA ON THE ATP:Pi EXCHANGE REACTION

The assay procedure for measuring the rate of the ATP:Pi exchange reaction was similar to that described in Section II.B.8a except that the concentration of acetyl CoA was varied. The data points are a mean of the rates obtained at three different time points of the exchange reaction.

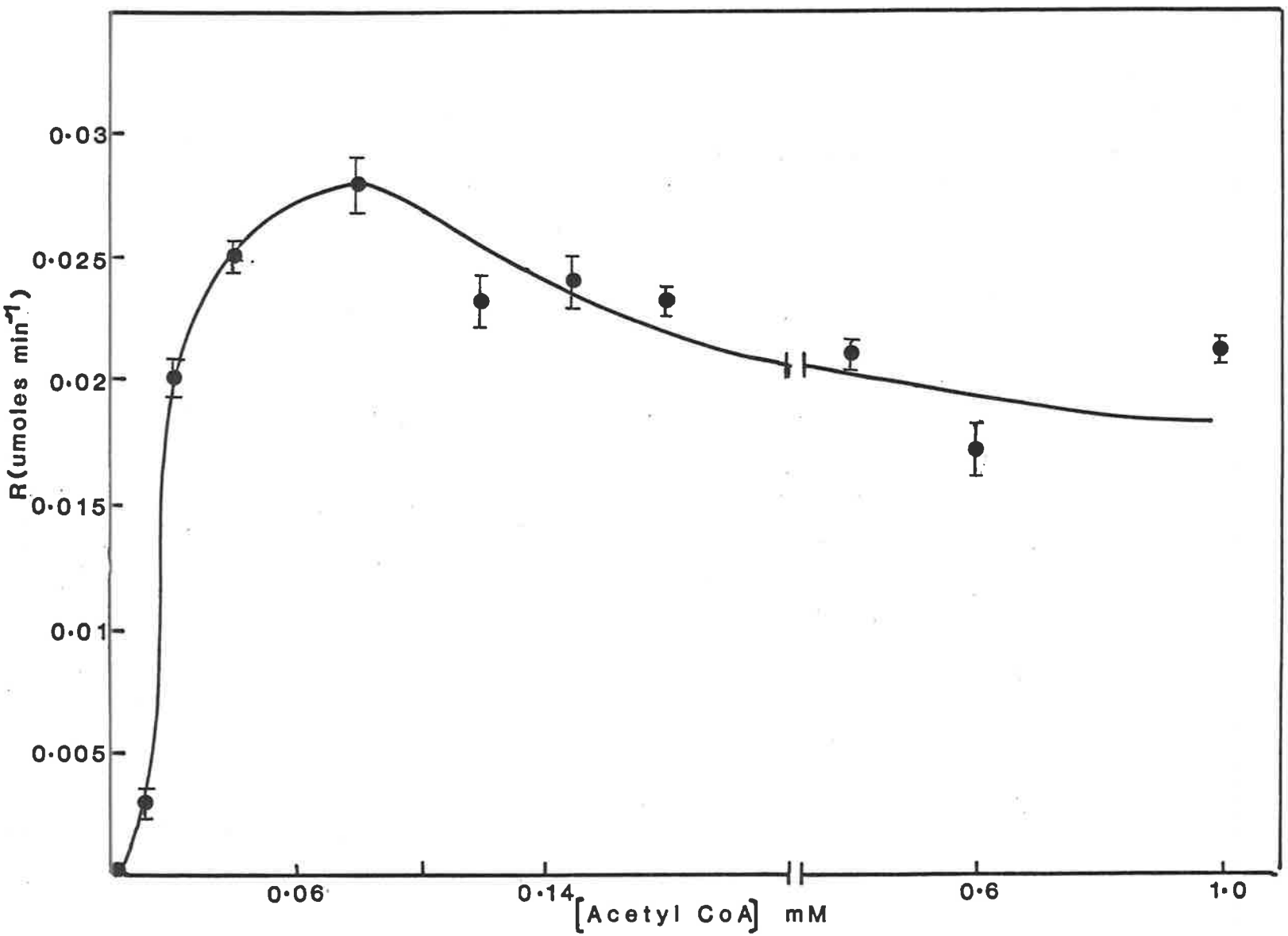

0·03
0·025
0·02
0·015
0·01
0·005
R(umoles min⁻¹)
0·06
0·14
0·6
1·0
[Acetyl CoA] mM

FIGURE III.6b

DOUBLE RECIPROCAL PLOT OF THE DATA FROM FIGURE III.6a

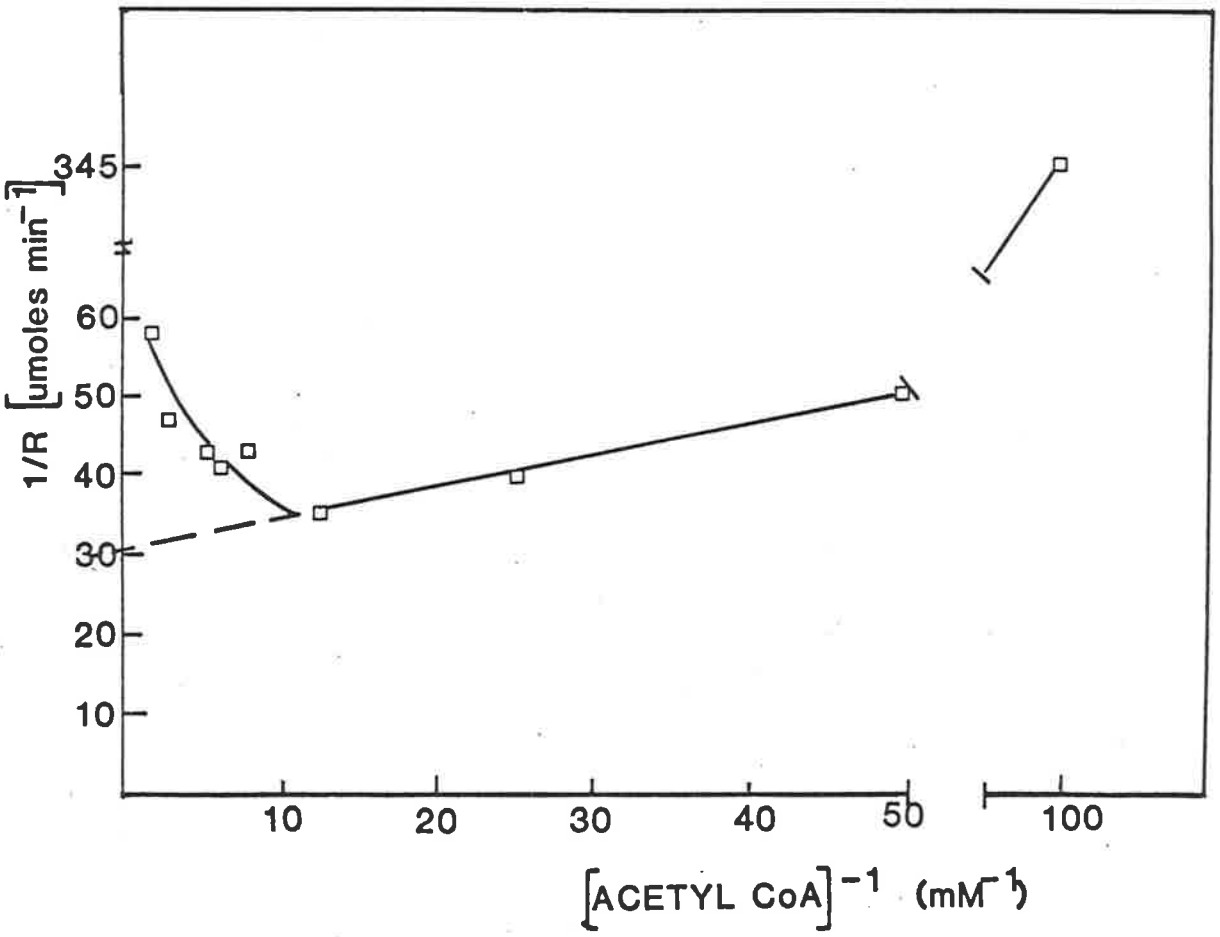

1/R [umoles min⁻¹]
345
60
50
40
30
20
10
10
20
30
40
50
100
[ACETYL CoA]⁻¹ (mM⁻¹)

FIGURE III.7

EFFECT OF ACETYL CoA ON THE OVERALL FORWARD REACTION VELOCITY CATALYSED BY PYRUVATE CARBOXYLASE FROM CHICKEN LIVER

The overall reaction velocity was measured by the radiochemical assay procedure described in Section II.B.6b except that the concentration of acetyl CoA was varied as indicated.

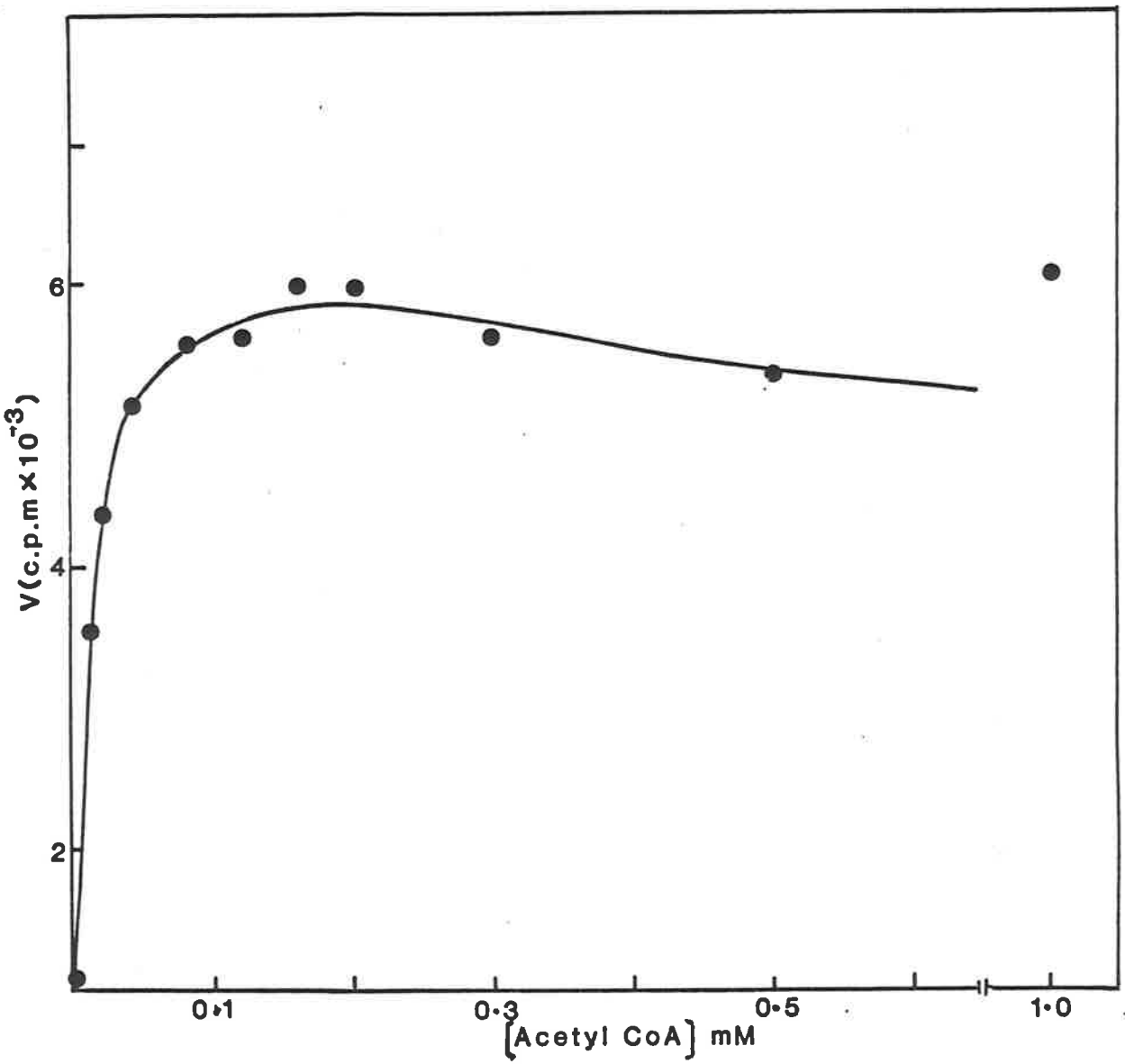

6
4
2
V(c.p.m×10⁻³)
0·1
0·3
0·5
1·0
[Acetyl CoA] mM

TABLE III.5

COMPARISON OF THE EXCHANGE REACTIONS UNDER PARTIAL EXCHANGE REACTION CONDITIONS AND UNDER AN OVERALL EQUILIBRIUM CONDITION

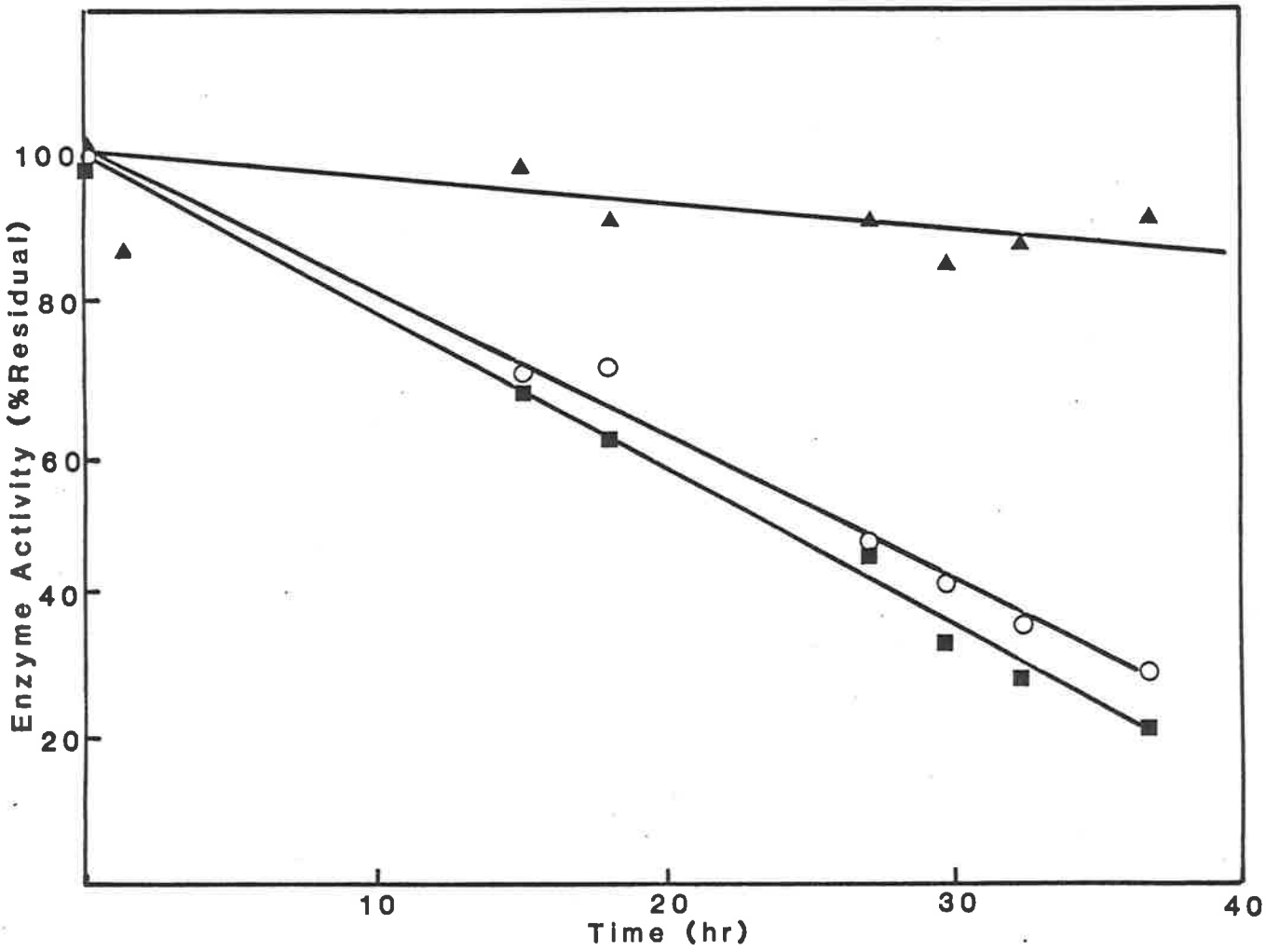
The assay procedures for measuring the rates of the ATP:ADP, ATP:Pi, HCO_3^-:OAA and the pyruvate:OAA are described in Section II.B.8. The reverse reaction was assayed by the method described in Section III.B.2d. When the ATP:ADP exchange reaction was measured with all components of the first partial reaction, the assay system was the same as that used in the ATP:Pi exchange reaction assay system. When the first partial exchange reactions were measured with all components of the first and second partial reactions, the assay system contained in addition, 2 mM pyruvate and 2 mM OAA. When the pyruvate:OAA exchange reaction was measured with all components, the assay system in addition contained 2 mM, ATP; 2 mM, ADP; 8 mM, $MgCl_2$; 2 mM, HCO_3^-; 10 mM, Pi; 0.25 mM, acetyl CoA.

Reaction	Rate (μmoles min^{-1} $E.U.^{-1}$)	% Forward rate
ATP:ADP exchange Mg^{2+} only added	0.012 ± 0.0001	1.2
ATP:ADP exchange all compts. of first partial reaction	0.023 ± 0.0003	2.3
ATP:ADP exchange all compts. of first and second partial reactions	0.016 ± 0.002	1.6
ATP:Pi exchange all compts. of first partial reaction	0.014 ± 0.0001	1.4
ATP:Pi exchange all compts. of first and second partial reactions	0.021 ± 0.002	2.1
HCO_3:OAA exchange	0.008 ± 0.0006	0.8
Pyruvate:OAA exchange all compts of second partial exchange	0.77 ± 0.028	77
Pyruvate:OAA exchange all compts. of first and second partial exchange	0.72 ± 0.07	72
Reverse reaction	0.048 ± 0.0001	4.8

FIGURE III.8

INACTIVATION OF THE OVERALL FORWARD REACTION AND THE ATP:Pi EXCHANGE REACTION.

Chicken liver pyruvate carboxylase was diluted to a concentration of 20 units ml^{-1} in Tris-Cl, pH 7.8 and was allowed to stand at 30 °C. At the indicated time intervals, aliquots were removed to initiate the overall forward reaction (■) by the coupled spectrophotometric assay system (Section II.B.6a); the ATP:Pi exchange reaction (○) (Section II.B.8a); and the Mg^{2+}-dependent ATP:ADP exchange reaction (▲) (Section II.B.6b). The rates were mean of two measurements.


Enzyme Activity (%Residual)
100
80
60
40
20
10
20
30
40
Time (hr)

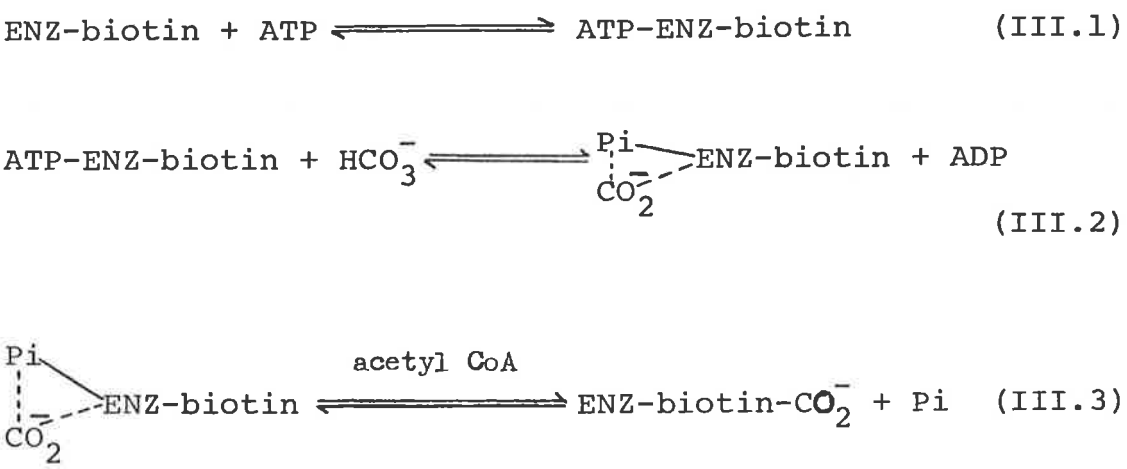


FIGURE III.9

PROPOSED REACTION MECHANISM FOR THE FIRST PARTIAL REACTION

CHAPTER IV

ISOLATION OF THE PUTATIVE

ENZ-BIOTIN COMPLEX

IV.A. INTRODUCTION

The results presented in the preceding chapter provide evidence that the first partial reaction occurs via a stepwise, rather than a concerted mechanism. As pointed out by Spector (1973); 'proof' for the concerted mechanism usually relies on the failure to find positive evidence for other types of mechanism although such 'proof' is not adequate criteria to establish a particular mechanism. In the absence of direct proof, for the intermediacy of enzyme-substrate complexes, indirect criteria are generally used as suggestive evidence for their formation (Jencks, 1969).

One of the most common procedures used to demonstrate a mechanism occurring via an enzyme-substrate intermediate is to study isotope exchange reactions. However, as pointed out by Wimmer & Rose (1978), many mechanisms that have been 'established' with the aid of the isotope exchange technique, were not conclusive due to the slow rates of exchange reactions. Evidence for an intermediate obtained by the use of isotope exchange technique may be masked if product dissociation is slow or does not occur in the absence of one or more components of the overall reaction.

Since the proposed mechanism depicted in Fig. III.9 was based entirely on kinetic data, more direct evidence in support of the mechanism was required. Since the most direct approach is the actual physical isolation of the enzyme-substrate intermediate in the chemically competent state, this has been attempted here, in an effort to give support to the proposed mechanism for the first partial reaction (Fig. III.9).

IV.B. MATERIALS AND METHODS

IV.B.1 General Methods

Purification of acetyl CoA and [γ-^{32}P]ATP was carried out as described in Section II.B.1. Radioactivity was determined as outlined in Section II.B.5.

IV.B.2 Assay Methods

The rate of pyruvate carboxylation was determined by the procedure described in Section II.B.6a. Protein concentrations were determined by the spectrophotometric method of Layne (1957). The biotin content of the native enzyme was determined after proteolytic digestion of the enzyme as described in Section II.B.12.

IV.B.3 [^{14}C]carboxyl Group Transfer to Pyruvate

The transfer solution contained, (in µmoles) in a final volume of 0.65 ml, N-ethyl morpholine (Cl^-, pH 7.8), (65); pyruvate, (6.5); acetyl CoA, (0.16); and the $^{14}CO_2^-$-labelled enzyme isolated as described in Section IV.C.1a. After 5 min at 25°C, the reaction was stopped by the addition of 50 µl of a saturated solution of semicarbazide in 6 N HCl. Aliquots (100 µl) were counted on Whatman filter paper squares as described in Section II.B.5, to determine the amount of radioactivity stabilized as the carbazone derivative of oxaloacetate.

IV.B.4 Transfer of [^{32}Pi] from [^{32}Pi]-Enzyme-biotin Complex to ADP

The 'ADP transfer' solution contained, (in µmoles) in a final volume of 0.65 ml, N-ethyl morpholine (Cl^-, pH 7.8),

(65); $MgCl_2$, (5.2); ADP, (1.3); and the isolated [^{32}Pi]-enzyme-biotin complex. After 15 min at 30°C, the reaction was stopped by the addition of 50 µl of 26 N, HCOOH at 0°C. The reaction solution was centrifuged to remove denatured protein and the supernatant was concentrated by lyophylization. The resuspended material was analysed for the amount of radioactivity incorporated into ATP by the method described in Section II.B.7.

IV.C RESULTS

IV.C.1a Isolation of Carboxy-enzyme Complexes

The mechanism of the first partial reaction depicted in Fig. III.C.9 predicts the formation of two enzyme intermediate complexes, ENZ-carboxybiotin and an Pi, CO_2^- ENZ-biotin. The ENZ-carboxybiotin complex has been isolated from a number of sources including pyruvate carboxylase from chicken liver, and has been shown to be able to transfer its activated carboxyl group to pyruvate (Scrutton *et al.*, 1965). The procedure used by these investigators was to incubate the enzyme with Mg^{2+}, ATP, $H^{14}CO_3^-$ and acetyl CoA and then separate the ENZ-biotin-CO_2^- complex by Sephadex gel filtration for subsequent transfer of the carboxyl group to pyruvate. The mechanistic scheme shown in Fig. III.9 indicates the formation of Pi, CO_2^- ENZ-biotin complex prior to the formation of the ENZ-carboxybiotin intermediate, a step which is indicated as requiring the presence of acetyl CoA. Hence if the scheme is correct, it should be possible to isolate the Pi, CO_2^- ENZ-biotin complex by using the same procedure as Scrutton *et al.* (1965) but omitting acetyl CoA

from the reaction medium. The preparation of the $^{14}CO_2$(Pi)–Enzyme-biotin complex was carried out by incubating pyruvate carboxylase (30 - 50 units) with 1 mM $NaH^{14}CO_3$ in 0.5 M N-ethyl morpholine-Cl buffer, pH 7.8, containing 8 mM $MgCl_2$ amd 2 mM ATP. The reaction was allowed to proceed for 15 min at 4°C, after which the incubation solution was chromatographed on a column of Sephadex G-25 (45 x 1.4 cm), previously equilibrated with 0.1 N N-ethyl morpholine-acetate, pH 7.2 at 4°C. The results of the chromatographic elution profiles are shown in Fig. IV.1, where it can be seen that [^{14}C]labelled material eluted with the pyruvate carboxylase activity peak. The labelled enzyme was clearly separated from unreacted $H^{14}CO_3^-$.

IV.C.1b Transfer of [^{14}C]carboxyl group to Pyruvate

In order to demonstrate that the radioactivity eluting with the enzyme was associated in a kinetically competent form, it was necessary to show that the [^{14}C]labelled material could be transferred to pyruvate. However in view of the probable lability of the $^{14}CO_2$(Pi)–ENZ-biotin complex, the protein-containing fractions from the Sephadex-G25 column were eluted directly into tubes containing acetyl CoA. The presence of acetyl CoA would have the effect of converting the labile complex to ENZ-^{14}carboxybiotin which was assumed to be relatively more stable. Aliquots of this radioactive material were then treated with pyruvate as described in Section IV.B.3. As seen from the profile shown in Fig. IV.1 the [^{14}C]carboxyl group could be incorporated into pyruvate and stabilized as the acid stable carbazone derivative of oxaloacetate. However when the enzyme-bound

radioactive material was not 'stabilized' by eluting into acetyl CoA, the [^{14}C]carboxyl group could not be incorporated into pyruvate although the pyruvate solution contained acetyl CoA (Fig. IV.2). Hence it was apparent from these results that although the isolated complex $\overset{Pi}{\underset{CO_2^-}{\diagdown\!\!>}}$ENZ-biotin, was kinetically active, it was extremely labile.

A summary of the data obtained is presented in Table IV.1 where it can be seen that the amount of radioactivity associated with the enzyme eluted from the column and transferred to pyruvate was 25% of the theoretical amount. The amount of radioactivity associated with the enzyme could be calculated on the basis of the specific activity of the $H^{14}CO_3^-$ used, while the amount of enzyme in these fractions was measured by the overall coupled spectrophotometric assay. Although these experiments were repeated a number of times the efficiency of the transfer rarely exceed 25%.

This observation is not surprising if the postulated complex, viz., $\overset{Pi}{\underset{CO_2}{\diagdown\!\!>}}$ENZ-biotin is considered to be more labile than the ENZ-carboxybiotin species. Furthermore these findings indicate that the [^{14}C]carboxyl group eluting with the enzyme fractions on gel filtration, is not an ENZ-carboxybiotin species, since it has been previously shown that acetyl CoA is required for the formation of ENZ-carboxybiotin (Scrutton *et al.*, 1965; and results presented in Chapter VI).

IV.C.2a Isolation of the $^{32}\overset{Pi}{\underset{CO_2^-}{\diagdown\!\!>}}$ENZ-biotin Complex

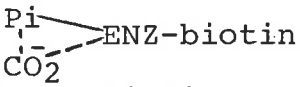
A further prediction from the scheme proposed (Fig. III.9) is that a phosphoryl moiety should be associated

with the enzyme provided that the activation of the enzyme was carried out in the absence of acetyl CoA. A previous attempt at the isolation of an enzyme-phosphate intermediate (Scrutton & Utter, 1965) involved incubating the chicken liver enzyme with Mg^{2+} and [γ-^{32}P]ATP followed by either precipitation of the protein with trichloroacetic acid or gel filtration at pH 8 and 4°C. These procedures however failed to reveal any association of the radioactivity with the protein, which is not surprising in view of the fact that any acid labile phosphoryl compounds would have been hydrolysed by the acid precipitation step involved. In the case of the gel filtration procedures, the details of the procedures used were not supplied.

The method employed here was to react the enzyme with [γ-^{32}P]ATP and Mg^{2+} then separate the enzyme from unbound [γ-^{32}P]ATP under neutral conditions. The preparation of the $^{32}Pi \xrightarrow[CO_2]{}$ Enzyme-biotin complex was carried out using reaction conditions identical to those used in the preparation of the $^{32}Pi \xrightarrow[CO_2]{}$ Enzyme-biotin complex except that $NaH^{14}CO_3$ was replaced with $NaHCO_3$ and ATP was replaced with [γ-^{32}P]ATP. The isolation of the labelled enzyme was performed in a manner similar to that described in Section IV.C.1a. The elution profile is shown in Fig. IV.3 where it can be seen that a peak of radioactivity was associated with those fractions containing protein.

In order to ascertain whether the radioactivity associated with the protein peak was either [γ-^{32}P]ATP or ^{32}Pi, control experiment was performed using identical experimental conditions except that [γ-^{32}P]ATP was replaced by [U-^{14}C]ATP. The elution profile of the Sephadex G-25 filtration process is shown in Fig. IV.4, and indicates the absence of a peak of

radioactivity eluting with the enzyme fractions. Although there seems to be some radioactivity showing a 'tailing effect' with the protein peak, this was calculated to be less than 10% of the enzyme content of these fractions. This minor 'contamination' of the enzyme fractions with radioactivity could be attributed to tightly bound [U-^{14}C]-ADP which was dissociating during the gel filtration procedure. This slow dissociation of [^{14}C]ADP could account for the second radioactive peak (Peak II) which eluted just after the protein peak. That Peak II was largely [U-^{14}C]ADP was confirmed by analysing aliquots of the radioactive fractions on PEI thin layer chromatographic plates as described in Section II.B.7. The radioactive content of the fractions containing enzyme were however too low for accurate analysis.

In the mechanism proposed for the first partial reaction (Fig. III.9), the formation of the 

complex occurs prior to the carboxylation of the biotin prosthetic group. The scheme further predicts that in the presence of acetyl CoA, carboxylation of biotin occurs with concurrent release of orthophosphate. Hence, reacting the enzyme with [γ-^{32}P]ATP in the presence of acetyl CoA, and subsequent gel filtration would have the effect of eliminating the radioactivity associated with the protein peak. The elution profile of such an experiment is shown in Fig. IV.5. The details of the experimental conditions were similar to the one described in Section IV.C.2a except that 0.25 mM acetyl CoA was included in the reaction mixture. From the chromatographic elution profile, the absence of a

distinct radioactive peak associated with the enzyme fraction is evident, although complete separation of the released [^{32}P]orthophosphate was not achieved. To ascertain that the radioactivity was due to [^{32}P]orthophosphate and not [γ-^{32}P]ATP, aliquots from the radioactive fractions were applied to PEI thin layer chromatographic plates and the nucleotides as well as the orthophosphate were separated and analysed as described above. It is seen from Fig. IV.5 that the radioactivity in Peak I is largely ^{32}Pi while that of Peak II is the unreacted [γ^{32}P]ATP. The observation that there are two radioactive peaks as compared to the three peaks observed on gel filtration of the enzyme reacted with [γ-^{32}P]ATP in the absence of acetyl CoA (Fig. IV.3), provides additional evidence that acetyl CoA does displace Pi from the enzyme.

From the results obtained with the [^{14}C]carboxyl group transfer from the $\overset{Pi}{\underset{CO_2}{\diagdown\!\!\diagup}}$ENZ-biotin complex, to pyruvate via the ENZ-carboxybiotin intermediate, it is apparent that the complex is relatively unstable, even at low temperatures. Hence it is expected that at higher temperatures of incubation of the enzyme with [γ-^{32}P]ATP, and HCO_3^- the resulting $\overset{Pi}{\underset{CO_2}{\diagdown\!\!\diagup}}$ENZ-biotin complex would be rapidly broken down, before initiating the isolation procedure. As a consequence of this the second radioactive peak, attributed to the slow dissociation of [^{32}P]-orthophosphate (Fig. IV.3), during the filtration process, should be completely eliminated. To test this possibility, the enzyme was incubated with [γ-^{32}P]ATP as described in Section IV.C.2a except that an incubation temperature of 25°C for 10 min was used. The

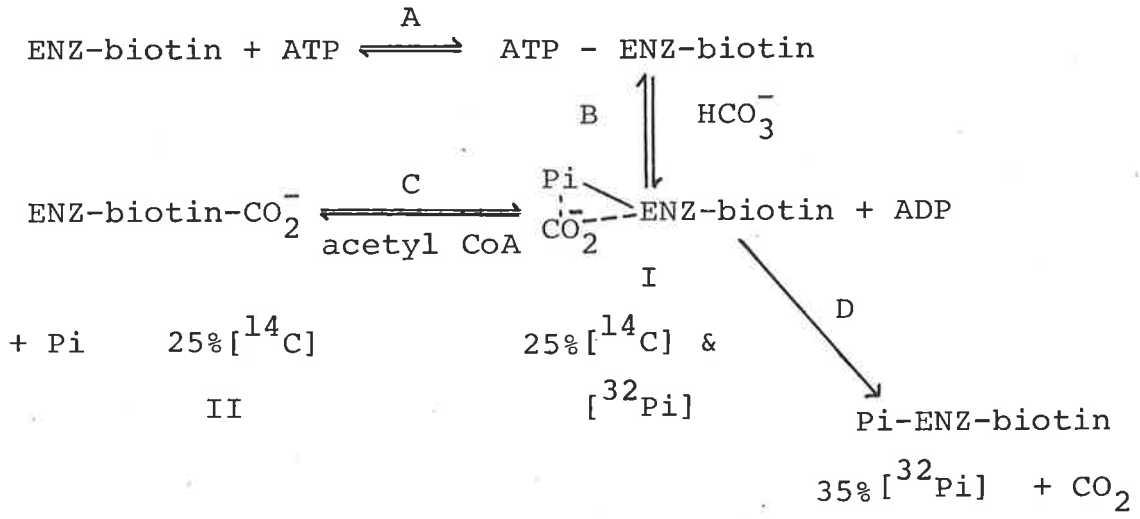
reaction mixture was chromatographed by gel filtration on Sephadex G-25 (27 x 1.4 cm) at 4°C. The resulting elution profile shown in Fig. IV.6 show a lack of radioactivity eluting with the enzyme fractions. Furthermore the radioactive peak (Peak II), observed in Fig. IV.3 and Fig. IV.5 is also absent under these experimental conditions.

IV.C.2b Transfer of [^{32}Pi] from $^{32}Pi\!\!>\!\!CO_2$-ENZ-biotin Complex to ADP

Although the results presented above indicate that the radioactivity associated with the protein is not the result of non-specifically bound [γ-^{32}P]ATP, it was necessary to show that the $^{32}Pi\!\!>\!\!CO_2$-ENZ-biotin complex is kinetically competent. In order to demonstrate this property, aliquots of fractions containing radioactivity associated with the enzyme (fractions 38 - 46, of Fig. IV.3) were reacted with ADP in an 'ADP transfer solution' as described in Section IV.B.4. The 'ADP transfer Solution' was devised to test the ability of $^{32}Pi\!\!>\!\!CO_2$-ENZ-biotin complex to transfer the radioactivity to ADP in a reverse reaction to form ATP. The results of the radioactivity incorporated into ATP is shown in Table IV.2. It is evident that the isolated $^{32}Pi\!\!>\!\!CO_2$-ENZ-biotin complex could catalyse the formation of radioactive ATP from ADP and this can occur through a reversal of the reactions III.2 and III.1 (of Fig. III.9).

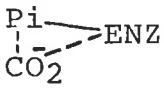
A summary of the data obtained from the chromatographic procedures (Fig. IV.3) are described in Table IV.2. Although the radioactivity associated with the enzyme fractions was found to be 60% of the theoretical amount based on the

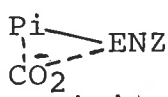
biotin content of the enzyme (i.e., on the basis of one mole of phosphoryl group per mole of available monomer), only half of this radioactivity could be transferred back to ADP. This apparently conflicting observation can be rationalized if the degradation of the $\overset{Pi}{\underset{CO_2^-}{\diagdown\!\diagup}}$>ENZ-biotin complex in the absence of acetyl CoA, is non-synchronous, i.e., if the rate of the dissociation of the phosphoryl group and the carboxyl group are not the same. This can be readily visualized by incorporating this abortive hydrolytic step into the first partial reaction pathway of Fig. III.9:-



Scheme II

Hence if it is considered that prior to the isolation procedure, there was 60% association of the phosphoryl as well as the carboxyl group on the $\overset{Pi}{\underset{CO_2^-}{\diagdown\!\diagup}}$>ENZ-biotin complex and after the isolation procedure, complex I and complex III are formed as a result of the carboxyl group being more labile. Thus complex I would have 25% of its sites saturated with $[^{32}Pi]$ as well as $[^{14}C]$ while complex III would have 35% of its sites saturated with only $[^{32}Pi]$, (with reference to the available sites based on their biotin content). This

would account for the higher yields of total [^{32}Pi] radioactivity (60%) in the isolated enzyme complexes as compared to the [^{14}C] radioactive content (25%). Although the total [^{32}Pi] radioactivity was 60% of the available sites on the enzyme, only about 25% of this could be transferred back to ADP. It is possible then that ADP will not be able to react with isolated ^{32}P-ENZ-biotin complex (complex III). On the other hand all of the associated [^{14}C] radioactivity could be transferred to pyruvate if complex I is immediately converted to ENZ-biotin-$^{14}CO_2^-$ by treatment with acetyl CoA (Fig. IV.1 and Table IV.1). Hence the observation that the extent of transfer of either the [^{32}P]phosphoryl group to ADP or the [^{14}C]carboxyl group to pyruvate are similar (i.e., 25% of the active enzyme) although there is 60% association of the [^{32}Pi] with the available sites in the enzyme, is consistent with the suggestion that the [^{14}C]-carboxyl group on the 

ENZ-biotin complex is more labile than the [^{32}P]phosphoryl group. In addition ADP binding in the reverse reaction (Step B) requires the presence of the carboxyl group on the enzyme. This conclusion is also consistent with the requirement of ADP for the HCO_3^-:oxaloacetate isotopic exchange reaction, where the release of HCO_3^- from the 

ENZ-biotin in the reverse direction requires the participation of ADP (see Scheme II).

This kinetic competency of 25% observed with both the phosphoryl and carboxyl groups associated with the enzyme then reflects the inherent instability of the isolated enzyme complex with reference to the carboxyl group.

IV.D DISCUSSION

Ever since the first demonstration of the direct participation of phosphoryl bond cleavage in the bicarbonate activation process (Kaziro *et al.*, 1962), various models for this activation process have been considered, (Section I.D). One such model was the involvement of carboxyphosphate (Polakis *et al.*, 1972; Ashman & Keech, 1975). Although the results presented in this Chapter indicate the formation of kinetically competent phosphoryl and carboxylated enzyme complexes, the precise structure and properties of these complexes are at present not known. In addition to the evidence presented here, the available kinetic data seems to suggest that the phosphoryl and carboxyl groups on the enzyme are in close proximity if not linked together. In view of the fact that one bicarbonate oxygen is found in Pi for every $-CO_2^-$ transferred (Kaziro *et al.*, 1962; Cooper *et al.*, 1968), and that the degree of kinetic competency of both the phosphoryl and the carboxyl groups are similar, it seems likely that the isolated phosphorylated and carboxylated enzyme intermediates are part of the same complex. Although the involvement of ATP cleavage in the bicarbonate activation process is also consistent with a concerted mechanism, in the light of the findings of this Chapter and the available kinetic data, a concerted process can be ruled out. On the other hand the findings of this Chapter seem to indicate that the isolated complex may be enzyme-bound carboxyphosphate, the possibility of which was first considered by Kaziro *et al.* (1962). The extreme lability of this inter-

mediate may have been chiefly responsible for the inability of various investigators to demonstrate its existence earlier. Nevertheless Powers & Meister (1976) have reported trapping of a carboxyphosphate intermediate in the *E. coli* carbamyl phosphate synthetase system and showed that the stoichiometry of the carboxyphosphate was about 33% in relation to the enzyme (assuming monomer form). However, in a later publication, Powers & Meister (1978) reported a stoichiometry of 72%. As pointed out by these investigators the binding of carboxyphosphate on the enzyme could confer considerable stability on carboxyphosphate. Sauers *et al.* (1975), on the basis of an extrapolation from a structure-reactivity study of alkyl monocarbonates, estimated that free carboxyphosphate would have a half-life of less than 0.1 s, while the studies by Powers & Meister (1978) indicated a half-life of about 2.5 min for the enzyme-bound carboxyphosphate at 37°C. Notwithstanding the labile nature of carboxyphosphate, Powers & Meister (1976b) were able to stabilize this intermediate as the trimethyl ester with diazomethane, or as formate with borohydride.

In the case of the carboxylases, however, the existence of carboxyphosphate has been inferred through indirect evidence and although the findings of this chapter, strongly suggest that the isolated enzyme-substrate complex may be enzyme-bound carboxyphosphate, characterization of the isolated enzyme-complex was necessary for confirming its identity. The results of such an investigation are presented in Chapter V.

FIGURE IV.1

SEPHADEX G-25 CHROMATOGRAPHY OF [^{14}C] LABELLED CHICKEN LIVER PYRUVATE CARBOXYLASE

The reaction mixture contained (in μmoles) in a final volume of 0.5 ml; N-ethylmorpholine (Cl^-, pH 7.8), (50); ATP, (1); $MgCl_2$, (4); $NaH^{14}CO_3$, (0.5) (7.6×10^7 cpm/μmole), and 30 units of pyruvate carboxylase. After 15 min at 4°C, the incubation solution was loaded onto the column. The labelled enzyme was eluted into fractions containing 0.25 mM (final volume) acetyl CoA.

The enzyme activity (●) was measured by the spectrophotometric assay described in Section II.B.6a. While the radioactivity (▲) in the fractions was determined by counting aliquots (50 μl) from the fractions in triton scintillation fluid. The amount of radioactivity incorporated into pyruvate (■) was measured as described in Section IV.B.3. The column flow-rate was 0.63 ml min^{-1}.

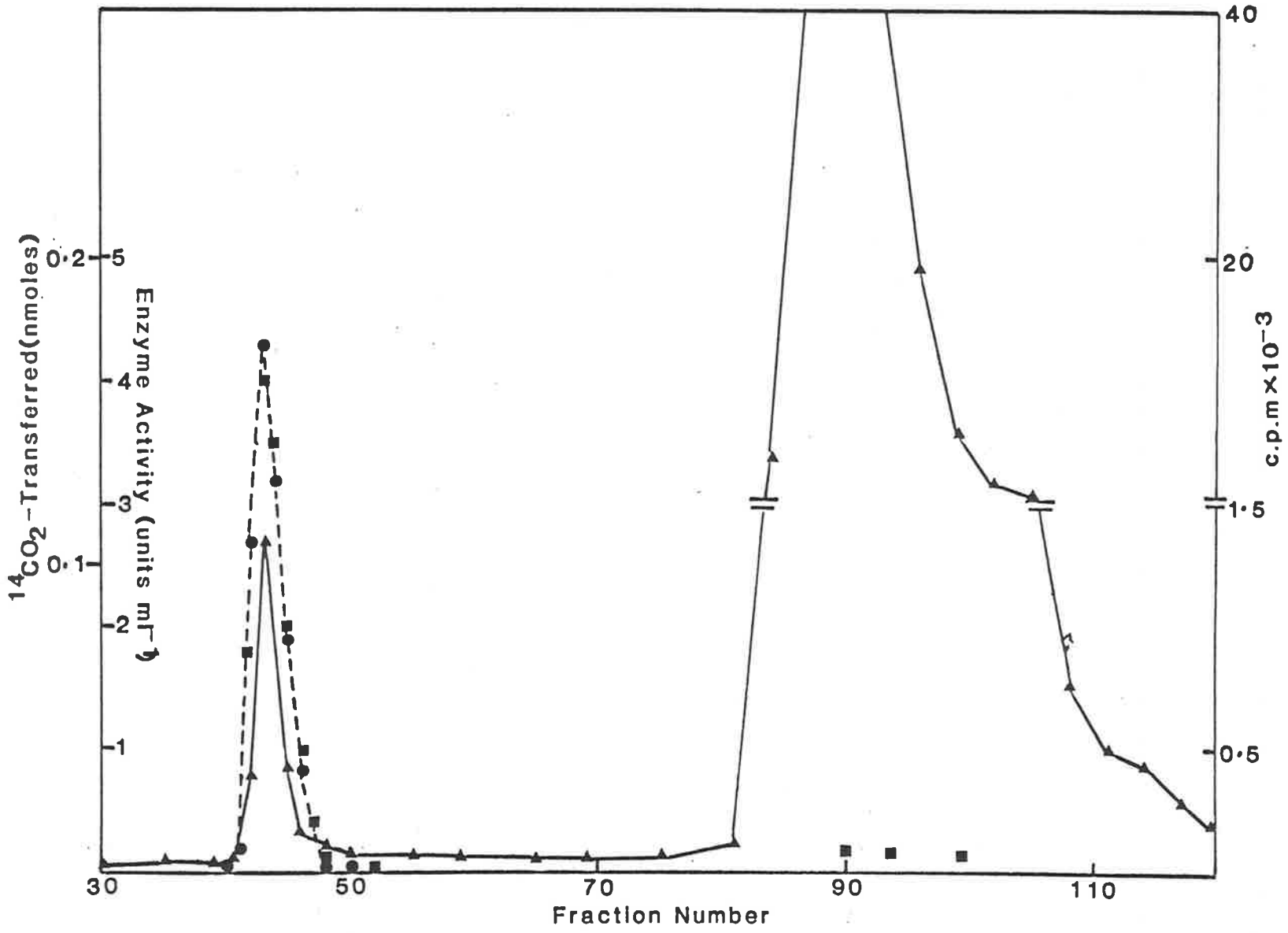

$^{14}CO_2$-Transferred(nmoles)
0·2
0·1
Enzyme Activity (units ml^{-1})
5
4
3
2
1
40
20
1·5
0·5
c.p.m×10^{-3}
30
50
70
90
110
Fraction Number

TABLE IV.1

SUMMARY OF THE DATA OBTAINED

FROM FIGURE IV.1

Fraction number	Radioactivity (total c.p.m.)	Radioactivity transferred to pyruvate (total c.p.m.)	$\frac{-^{14}CO_2}{\text{active enzyme (monomer)}}$	$\frac{-^{14}CO_2^-}{\text{biotin}}$
42	5027	5385	0.21	0.17
43	16846	12086	0.29	0.22
44	N.D.[1]	10992	0.35	0.23
45	5254	6279	0.34	0.24
46	2356	3096	0.39	0.27
47	1967	1355	0.28	0.18

[1] Not determined

FIGURE IV:2

SEPHADEX G-25 CHROMATOGRAPHY OF [^{14}C] LABELLED CHICKEN LIVER PYRUVATE CARBOXYLASE

The reaction conditions were similar to that described in the legends of Figure IV.1 except that the labelled enzyme was eluted into fraction tubes without acetyl CoA. The enzyme activity (O), the radioactivity (■) and the amount of radioactivity incorporated into pyruvate (▲) were determined by similar procedures as described in the legends of Figure IV.1.

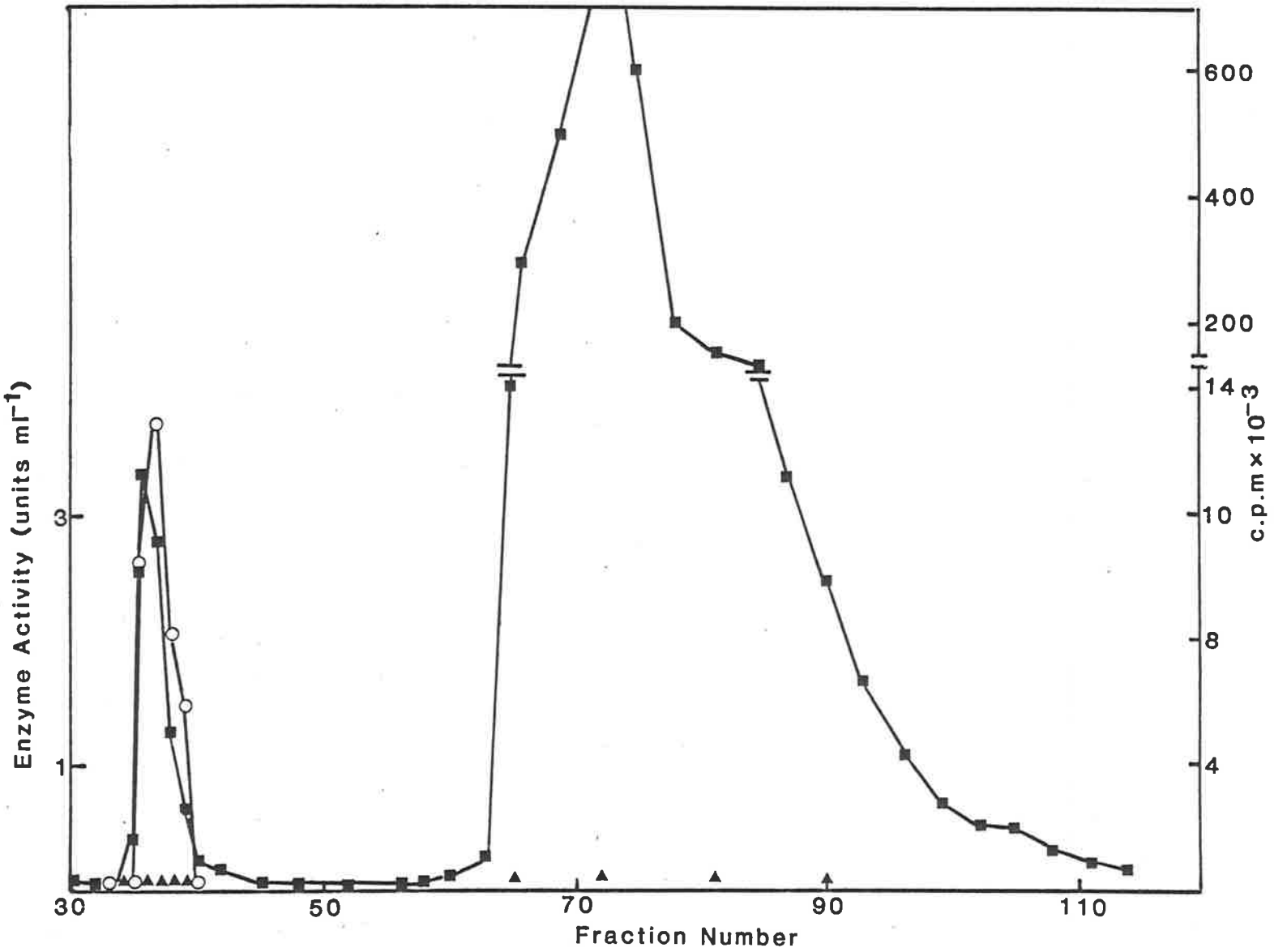

Enzyme Activity (units ml⁻¹)
3
1
600
400
200
14
10
8
4
c.p.m × 10⁻³
30
50
70
90
110
Fraction Number

FIGURE IV.3

SEPHADEX G-25 CHROMATOGRAPHY OF [^{32}Pi] LABELLED CHICKEN LIVER PYRUVATE CARBOXYLASE

The reaction solution contained (in μmoles) in a final volume of 0.5 ml, N-ethylmorpholine (Cl^-, pH 7.8), (50); $MgCl_2$, (4); $NaHCO_3$, (0.5); [γ-^{32}P]ATP, (1) (4.9 x 10^7 cpm/μmole) and 50 units of pyruvate carboxylase. After 20 min at 4°C, the incubation solution was loaded on the column, and the [^{32}Pi] labelled enzyme was eluted with 0.1 M N-ethylmorpholine-acetate pH 7.2.

The enzyme activity (■) was measured by the spectrophotometric assay (Section II.B.6a) while the radioactivity in the fractions (O) was determined by counting aliquots (0.1 ml) in triton scintillation fluid. The abosrbance at 260 nm (●) was also determined. The column flow-rate was 0.67 ml min^{-1}.

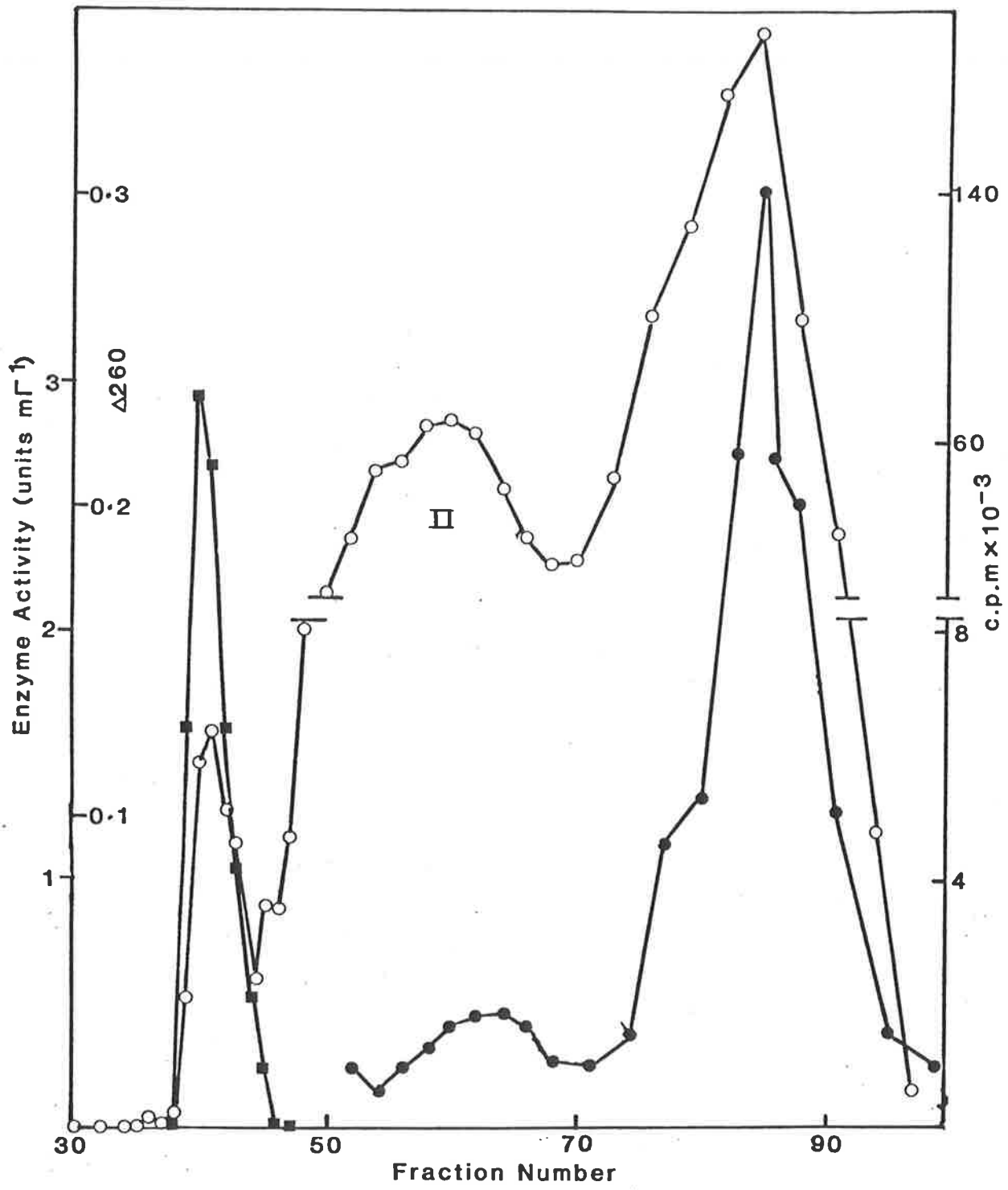

Enzyme Activity (units mΓ¹)
Δ260
c.p.m x10⁻³
Fraction Number
II
0·3
0·2
0·1
3
2
1
140
60
8
4
30
50
70
90

FIGURE IV.4

SEPHADEX G-25 CHROMATOGRAPHY OF PYRUVATE CARBOXYLASE REACTED WITH [U-^{14}C]ATP

The incubation solution and reaction conditions were similar to that described in the legends of Figure IV.3 except that the [γ-^{32}P]ATP was replaced with [U-^{14}C]ATP (1.34 x 10^7 cpm/ μmole). The enzyme activity (□), radioactivity (O) and the absorbance at 260 nm (■) were determined as described in the legends of Figure IV.3.

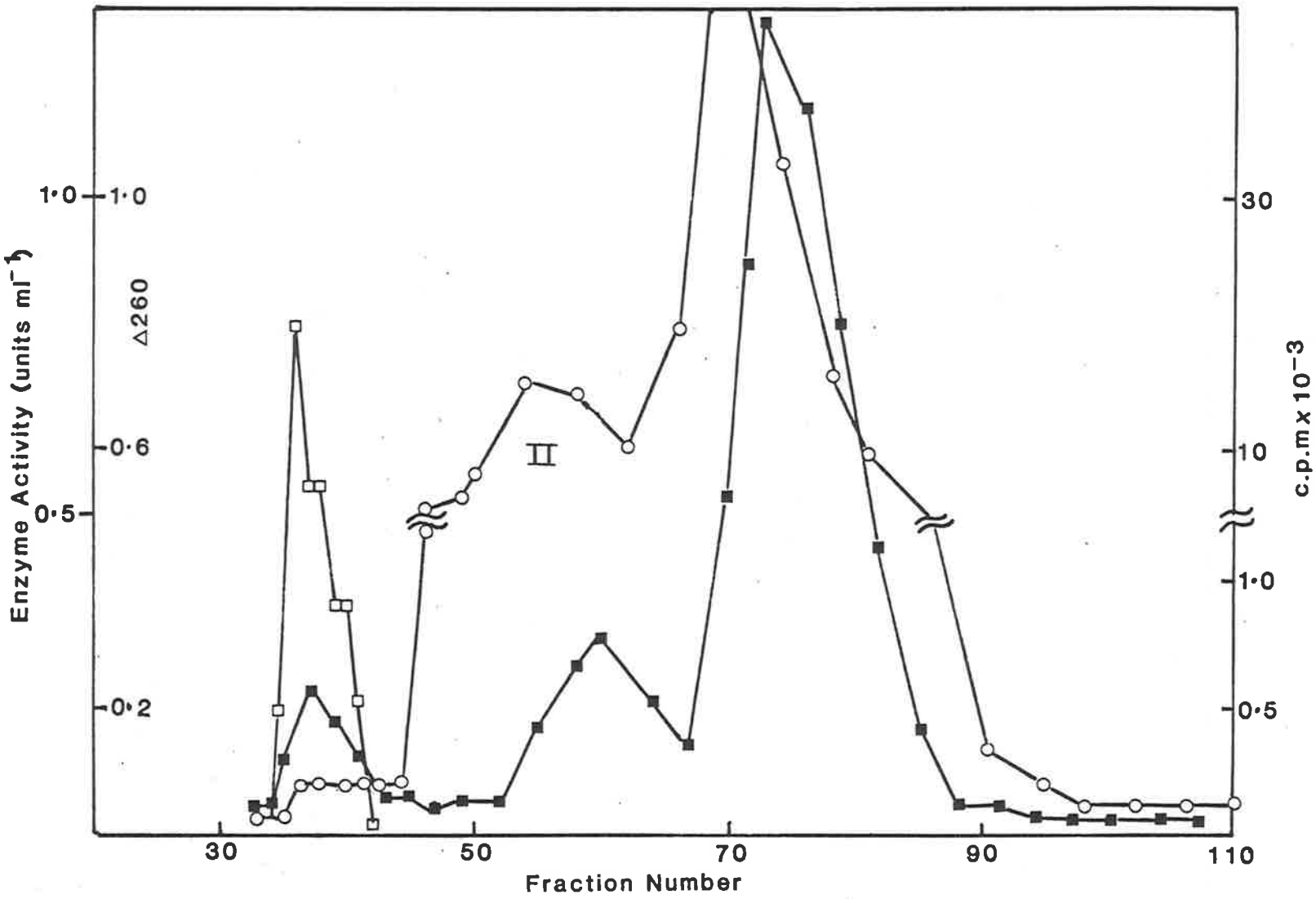

Enzyme Activity (units ml⁻¹)
Δ260
c.p.m x 10⁻³
1·0
0·6
0·5
0·2
30
10
1·0
0·5
II
30
50
70
90
110
Fraction Number

FIGURE IV.5

SEPHADEX G-25 CHROMATOGRAPHY OF PYRUVATE CARBOXYLASE REACTED WITH [γ-^{32}P]ATP IN THE PRESENCE OF ACETYL CoA

The reaction conditions were similar to that described in the legends of Figure IV.3 except 0.25 mM acetyl CoA was included in the reaction solution. The enzyme activity (●) radioactivity (□) and the absorbance at 260 nm (▲) were determined as described in the legends of Figure IV.3.

Fraction Number	Counts in ^{32}Pi (% total counts)
40	94
44	88
51	73
55	78
60	69
65	51

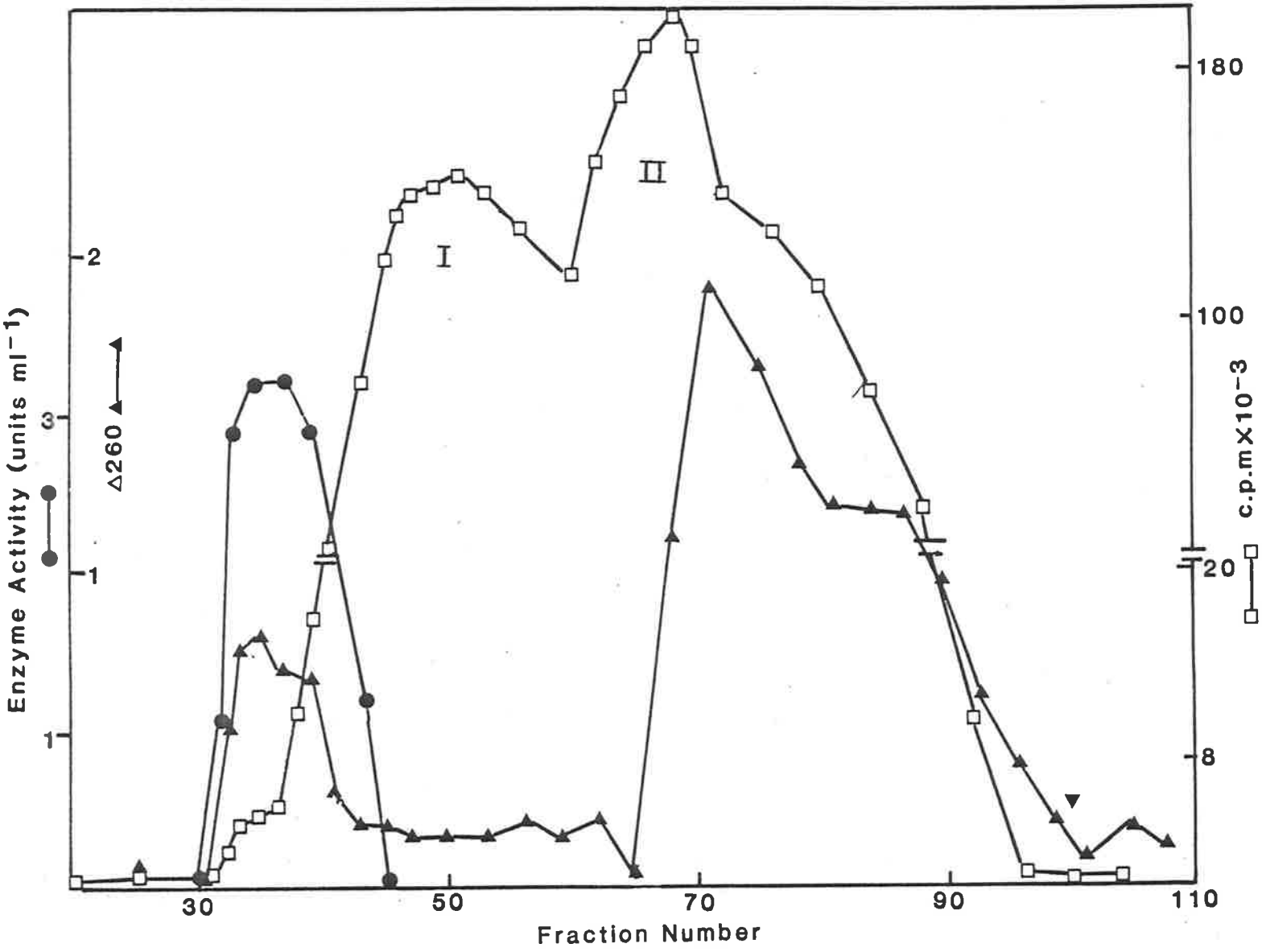

Enzyme Activity (units ml⁻¹)
Δ260
c.p.m X10⁻³
I
II
1
2
3
20
8
100
180
30
50
70
90
110
Fraction Number

FIGURE IV.6

SEPHADEX G-25 CHROMATOGRAPHY OF PYRUVATE CARBOXYLASE REACTED WITH [γ-^{32}P]ATP

The incubation solution and reaction conditions were similar to that described in the legends of Figure IV.3 except that the incubation temperature was 25°C. The solution was loaded onto the column (27 x 1.4 cm) and the enzyme eluted with 0.1 M N-ethyl morpholine acetate, pH 7.2 at 4°C.

- ● Enzyme activity
- O Radioactivity
- ■ Δ_{260}

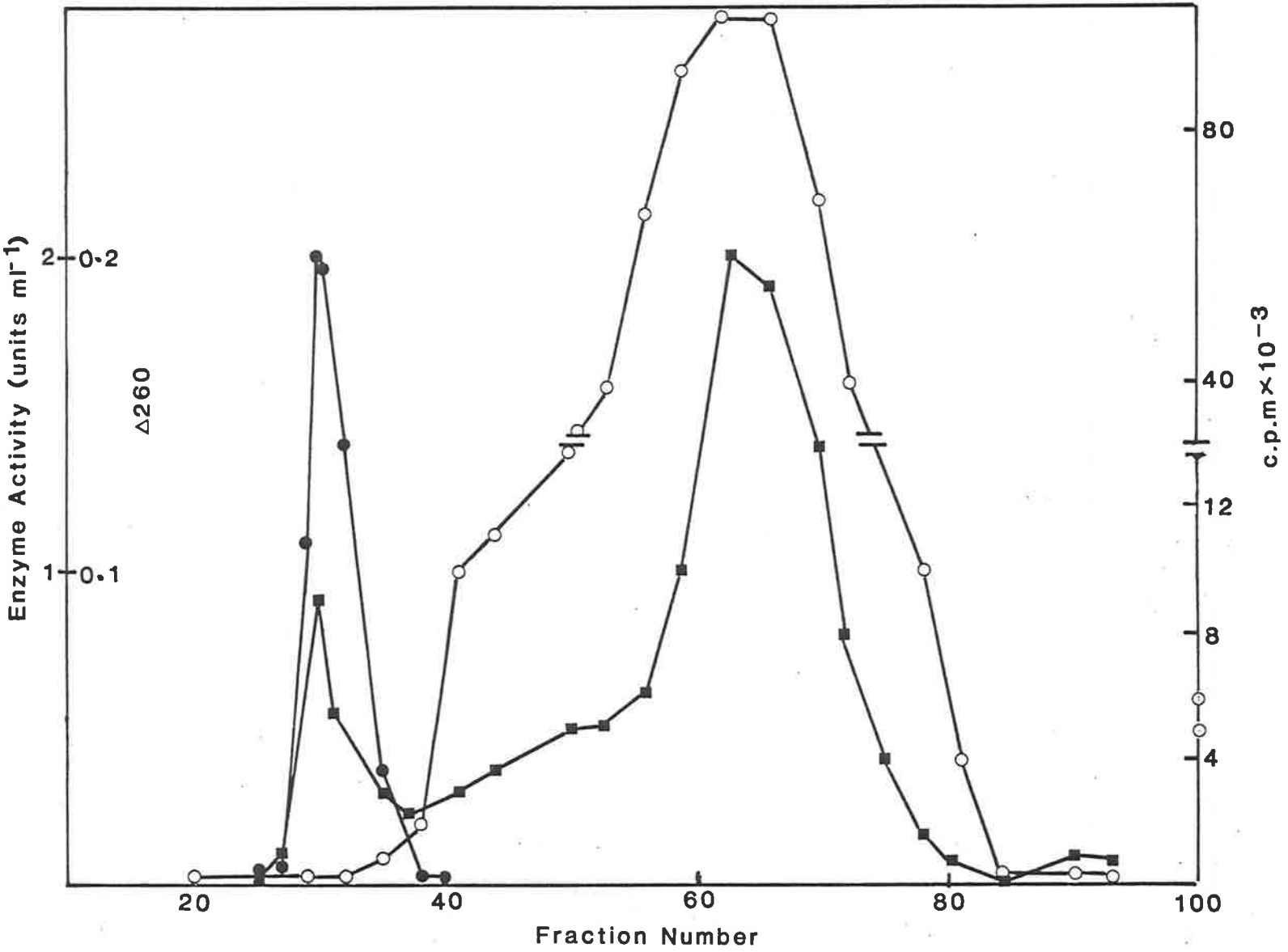

Enzyme Activity (units ml⁻¹)
Δ260
c.p.m×10⁻³
Fraction Number
2
1
0·2
0·1
80
40
12
8
4
20
40
60
80
100

TABLE IV.2

SUMMARY OF THE DATA OBTAINED FROM FIGURE IV.3

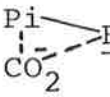
Fr.No.	Radio activity (Total cpm)	(nmoles)	Biotin (nmoles)	[^{32}Pi] transferred (total cpm)	to ADP (nmoles)	% of total ^{32}Pi transferred
39	13614	0.28	1.52	1903	0.04	14.3
40	39316	0.8	1.42	5400	0.12	15
41	42940	0.88	1.35	10693	0.23	26
42	34660	0.71	1.43	10358	0.22	31
43	31300	0.64	0.77	8549	0.18	28
44	16402	0.33	0.5	-	-	-

Pi/Biotin = 0.58 ± 0.09

% of total Pi transferred = 23 ± 3

CHAPTER V

CHARACTERIZATION OF THE ISOLATED



ENZ-BIOTIN COMPLEX

V.A INTRODUCTION

The evidence presented in the preceding two chapters suggests that the mechanism of the first partial reaction catalysed by pyruvate carboxylase proceeds via a stepwise process, with the possible involvement of an enzyme-bound carboxyphosphate complex. Evidence for the involvement of carboxyphosphates as the means of HCO_3^- activation by ATP seems to be accumulating. With the carbamyl phosphate synthetases from ureolytic vertebrates, an early study by Jones & Spector (1960) established the presence of one oxygen atom derived from bicarbonate in the Pi formed, in the overall reaction, and thereby implicated the direct involvement of ATP in the activation process. In the case of the glutamine-dependent enzyme from *E. coli*, the pulse-labelling experiments conducted by Anderson & Meister (1965) suggested that the ATP-dependent formation of activated carbon dioxide was associated with cleavage of ATP to ADP and Pi. The enzyme from both sources catalyzes a HCO_3^--dependent cleavage of ATP (Metzenberg *et al.*, 1958; Anderson & Meister, 1966), while the *E. coli* enzyme was reported to catalyze a HCO_3^--dependent ATP:ADP exchange reaction (Duffield *et al.*, 1969). However it was the studies of Powers & Meister (1976a, 1976b) that provided the first direct evidence for the existence of this compound in the glutamine-dependent carbamyl phosphate synthetase catalyzed system. This was achieved by trapping the carboxyphosphate intermediate either by reduction with KBH_4 in dimethylsulphoxide or by methylation with diazomethane. Finally Powers & Meister in collaboration with

Wimmer & Rose (1979) have shown that carboxyphosphate was kinetically competent and was formed on the main catalytic pathway of carbamyl phosphate synthesis.

A similar situation appears to apply in the probable existence of carboxyphosphate in the reaction mechanism of biotin-dependent carboxylases. As early as 1962, Kaziro *et al.* (1962) suggested that their observation in the propionyl CoA carboxylase reaction of one oxygen atom of HCO_3^- appearing in the Pi released did not rule out the involvement of carboxyphosphate as an enzyme-intermediate. They nevertheless argued in favour of a concerted mechanism for the carboxylation of biotin. Additional indirect evidence came from Polakis *et al.* (1972), who showed that acetyl CoA carboxylase from *E. coli* could catalyse the formation of ATP from ADP and carbamyl phosphate, a structural analogue of carboxyphosphate. Support for the intermediacy of carboxyphosphate was also provided by Ashman & Keech (1975) who observed a similar conversion of carbamyl phosphate and ADP to ATP catalyzed by pyruvate carboxylase from sheep kidney. In addition they showed that phosphonoacetate was a potent inhibitor of pyruvate carboxylase. This provided further evidence for the intermediacy of carboxyphosphate.

However Kluger & Adawadkar (1976) pointed out that all previous kinetic findings were also consistent with an involvement of a O-phosphobiotin intermediate, a possibility which was first suggested by Retey & Lynen (1965). The finding by Polakis *et al.* (1974) that chemical substitution at 1'-N position of biotin (the carboxylation site) reduced but did not abolish the ability of this derivative to support

phosphoryl transfer from carbamyl phosphate to ADP in the acetyl CoA carboxylase system was cited by Wimmer & Rose (1978) as evidence against the involvement of o-phosphobiotin. Kluger *et al.* (1979) nevertheless point out that the above finding could be consistent with phosphorylation of biotin at the carbonyl oxygen of the ureido ring of biotin and hence the substitution at the 1'-N could support the phosphoryl transfer as observed. In addition these authors suggested that since carboxyphosphate differs from carbamyl phosphate by having an extra negative charge at neutral pH, carbamyl phosphate is a closer analogue of o-phosphobiotin than of carboxyphosphate. As discussed in more detail in Section I.D.1b, Kluger & Adawadkar (1976) proposed the involvement of o-phosphobiotin in the carboxylation of biotin, based on their findings with model compounds [Fig. I.3b (ii)]. In an extension of this work, Kluger *et al.* (1979) considered two stereochemically distinct pathways for carboxylation of phosphobiotin to form carboxybiotin namely an 'in-line' pathway (Fig. V.1, Scheme I) and a 'adjacent' pathway (Fig. V.1, Scheme II), by applying the Westheimer rules (Westheimer, 1968) for reaction intermediate stereochemistry of phosphorus. They concluded that both mechanisms were possible, however the 'in-line' mechanism required the formation of free carboxyphosphate as shown in Scheme I (Fig. V.1). Nevertheless, they point out, that either stereochemical mechanism would not rule out the involvement of o-phosphobtion.

Although the results presented in Chapters III and IV are consistent with the participation of carboxyphosphate in

the pyruvate carboxylase catalysed reaction, in view of the findings and conclusions of Kluger *et al.* (1979), involvement of o-phosphobiotin in the carboxylation of biotin is a possibility which cannot be ruled out merely on kinetic evidence. Hence the approach adopted in attempting to characterize the isolated complexes (of Chapter IV) has been to devise methods and conditions which could also enable the demonstration of the presence or absence of o-phosphobiotin, in addition to methods specific for other possibilities.

V.B METHODS

V.B.1 General Methods

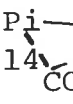
Purification of acetyl CoA and [γ-^{32}P]ATP was carried out as described in II.B.1. All gel filtration procedures were carried out using Super-fine Sephadex gels and conducted at 4°C to minimise loss of any labile compounds by hydrolysis.

V.B.2 Assay Methods

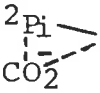
The rate of the overall reaction catalyzed by pyruvate carboxylase was measured by the assay system described in Section II.B.6a. Biotin determinations were carried out as described in Section II.B.13. In addition modifications to the procedures, when required are described in the individual results section. When the biotin samples contained [^{32}Pi] as was the case in estimating the biotin content of [^{32}P]ENZ-biotin complexes, a dual channel counting method was optimized for the radioactive determination of [^{32}P]ENZ-biotin and

[^{14}C]biotin with minimal overlap of the radioisotopes.

V.B.3 Preparation and Isolation of Radioactively Labelled  ENZ-biotin Complexes

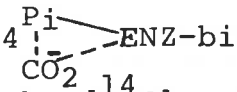
Pyruvate carboxylase was reacted either with [γ-^{32}P]-ATP or $H^{14}CO_3^-$ as described in Section IV.C.2a and IV.C.1a to prepare the  ENZ-biotin or  ENZ-biotin complexes respectively.

V.B.4 Determination of half-life of  ENZ-biotin Complex

The stability of the  ENZ-biotin complex was determined by measuring the half-life of the complex at various temperatures. The  ENZ-biotin complex was prepared as described above and was isolated at 4°C by the rapid microcentrifuge desalting technique as described by Helmerhorst & Stokes (1980). The isolated enzyme complex was incubated at different temperatures for different time intervals and the [^{32}Pi] remaining in the  ENZ-biotin complex was determined by transfer of [^{32}Pi] to ADP. This was achieved by removing aliquots of the enzyme complex to a 'ADP Transfer Solution' as described in Section IV.B.4.

V.B.5 Determination of half-life of  ENZ-biotin Complex

The stability of the  ENZ-biotin complex with reference to the [^{14}C] on the enzyme complex was determined by similar procedures as described above for determining the stability of the [^{32}P] on the enzyme. The isolated complex was incubated at different temperatures as indicated

in the figure legends, for different time intervals, and the [$^{14}CO_2^-$] remaining on the  was determined by transfer of the [^{14}C]carboxyl group to pyruvate in the presence of acetyl CoA as described in Section IV.B.3. The radioactivity incorporated into oxaloacetate was stabilized as the semicarbazone derivative and its radioactive content determined as described in Section II.B.6a.

V.B.6 Preparation of Diazomethane

Diazomethane was prepared by the procedure described by Vogel (1967). p-Toluenesulfonyl-N-methyl-N-nitrosamide (2.14 g) was dissolved in a solution of ether (30 ml) and cooled on ice. Potassium hydroxide (0.4 g) in redistilled, 96% ethanol (10 ml) was then added. More ethanol was added if a precipitate formed, until the solution turned clear. After 5 min at 4°C, the ethereal diazomethane solution was distilled off from a water bath (60°C), and collected in a smooth glass flask cooled on ice. The concentration of diazomethane in the distillate was determined by reacting an aliquot of the ethereal solution with benzoic acid in anhydrous ether. When the solution was completely decolourized, indicating an excess of benzoic acid, the solution was diluted with water and the excess benzoic acid was titrated with 0.1 N NaOH using phenolphthalein as indicator. The recovery of diazomethane was found to be 75% using this method.

V.B.7 Preparation of Tryptic Peptides

Tryptic peptides were prepared from radioactively labelled enzyme complexes which were stabilized as described in the results section for the individual experments.

V.C RESULTS

V.C.1 The Possibility of o-phosphobiotin-ENZ

Attempts were made to isolate a possible o-phosphobiotin species using suitable experimental criteria which could enable the demonstration of its existence or its absence. In view of the probable lability of such an intermediate, the method employed was to stabilize any intermediates prior to the chromatographic isolation procedures. Although ENZ-carboxybiotin is known to be relatively unstable, Lynen (1967) was able to isolate this species in the stable methyl ester form by methylation with diazomethane. In addition Powers & Meister (1976b) have also successfully used this technique in stabilizing carboxyphosphate. Hence if an o-phosphobiotin enzyme species did exist, it could probably be stabilized as the methyl phosphoester form. In attempting to detect a phospho-biotin enzyme intermediate, three criteria have been used, viz;

(a) that in an avidin inactivated enzyme, [^{32}Pi] radioactivity will not be associated with the enzyme, on the assumption that biotin which has been complexed with avidin will not be phosphorylated,

(b) that the [^{32}Pi] radioactivity co-purifies with the biotin containing peptide on chromatographic isolation

of peptides obtained by trypsin digestion of the reacted enzyme,

(c) that there should be a stoichiometric relationship between the enzyme-bound radioactivity and the biotin content of the material after purification.

V.C.1a Trapping of enzyme-bound intermediate(s) with diazomethane

Chicken liver pyruvate carboxylase was reacted with [γ-^{32}P]ATP using the experimental conditions detailed in the specific figure legends. After 2 min at 30°C, 5 ml of diazomethane solution was added and the esterification reaction was allowed to proceed for 1 h at room temperature in the dark. The ethereal solution was removed and the precipitate was washed with 0.1 M KPi (pH 7.2) and was resuspended in 0.5% NH_4HCO_3. The protein was then digested with trypsin as described in Section II.B.11. The tryptic digest was reacted with an excess of avidin to complex the biotin-containing peptide and then fractionated using a Sephadex G-50 column (3 cm x 130 cm). Rylatt *et al.* (1977) have successfully utilized this gel filtration technique to purify biotin-containing tryptic peptides. Hence if the [^{32}P] radioactivity was linked to the biotin residue, then it is possible that avidin would complex the radioactivity and be eluted at the exclusion volume of the column. As seen from Fig. V.2, the [^{32}P] radioactivity eluted with the bulk of the tryptic pool, while avidin and presumably the avidin-biotin-peptide complex eluted at the exclusion volume without removing any radioactivity. This suggests that either

the radioactivity associated with the tryptic pool is not linked to the biotin peptide or that [^{32}Pi]phosphorylated biotin residue on the peptide can no longer be complexed by avidin.

To test the second possibility, the enzyme was reacted with [γ-^{32}P]ATP and diazomethylated as described above. The unbound radioactivity was removed by washing the precipitate with 0.1 M KPi (pH 7.2), and the precipitated protein was digested with pronase (1:10 w/w) for 4 h at 37°C. Aliquots of this digest were acid hydrolysed in 6 M HCl, at different time intervals and the biotin content of the hydrolysed samples were estimated by the avidin titration method as described in Section II.B.12. The acid hydrolysis procedure would be expected to hydrolyse the phospho ester bond linked via the carbonyl group of biotin. Hence if a phospho-biotin complex, stabilized as the phospho ester-biotin derivative, did exist, then on removal of the phosphate, the biotin should now complex with avidin. The results shown in Table V.1 indicate that approximately 96% of the biotin is complexed with avidin by the avidin titration biotin assay, even without acid hydrolysis. Hence it is evident that avidin could complex the biotin residue even if it was in the methylated phospho ester form. Thus in Fig. V.2, radioactivity should have been expected to elute with the avidin-biotin-peptide fractions, if biotin was phosphorylated. These findings suggest that the radioactivity associated with the tryptic pool was not associated with biotin.

To further support the conclusion that the [^{32}P] radio-

activity associated with the protein was on a residue other than biotin, an experiment was conducted similar to that described in the legends of Fig. V.2, except that the enzyme used was previously inactivated with avidin. After digestion with trypsin, a further excess of avidin was added and the tryptic digest was purified by gel-filtration on Sephadex G-50 as described. In using avidin inactivated enzyme in the reaction mixture, it was expected to achieve two objectives. Firstly, inactivation of the enzyme with avidin, should also eliminate any phosphorylation of the biotin residue. Secondly, it is possible that although avidin inhibits the carboxylation of biotin at the 1'-N position, it may not hinder phosphorylation at the carbonyl oxygen. In either case a phosphorylated biotin-peptide should elute at the exclusion volume of the Sephadex column, since the enzyme was complexed with avidin prior to the phosphorylation and methylation reactions, and furthermore avidin is resistant to digestion by trypsin (Green, 1975). The results of the experiment are shown in Fig. V.3 where it can be seen that in the first, [^{32}P] radioactivity eluting with the tryptic peptide pool was not eliminated by using avidin inactivated enzyme.

Secondly, no radioactivity was observed with the avidin-containing fractions in the void volume. Hence, these findings eliminate the possibility that the radioactivity associated with the protein is due to a phosphorylated biotin enzyme intermediate.

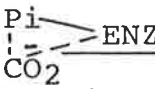
Although the above findings rule out the possibility of a o-phosphobiotin enzyme intermediate on the basis of the

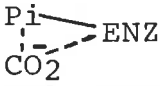
first criterion, described above, it was necessary to test the other two criteria, namely that [^{32}P] radioactivity co-purifies with the biotin containing peptide and that there is a stoichiometric relationship between the purified enzyme-bound radioactivity and the biotin content of the purified residue. To accomplish this, the radioactive fractions from the Sephadex G-50 column (Fig. V.2) were pooled; dried by lyophilization, resuspended in 0.5% NH_4HCO_3, and the tryptic peptides were subjected to pronase digestion (1:10 w/w) for 24 h at 37°C. The digest was chromatographed on a Sephadex G-10 column (2.5 cm x 80 cm) with 0.5% NH_4HCO_3 as the elution buffer. The chromatographic profile shown in Fig. V.4 reveals the presence of two radioactive peaks eluting with the amino acid residues. The fractions containing radioactivity were pooled separately (i.e., Fractions 28 - 34, Peak I; 35 - 40, Peak II), lyophilized and their biotin content determined after acid hydrolysis. The results detailed in Table V.2 indicate that most of the biotin-peptide was complexed by avidin and was recovered with the non-radioactive fractions at the exclusion volume of the Sephadex G-50 column. The biotin content of the fractions containing radioactivity (Peak I and Peak II of Fig. V.4) was found to be less than 5% of the original amount of biotin used in the experiment. These results clearly indicate that the [^{32}P] radioactivity associated with the protein was not linked to the biotin residue and hence the model proposed by Kluger & Adawadkar (1976) [Fig. 1.3.b(i)] involving activation of the biotin prosthetic group by phosphorylation at the carbonyl oxygen as the first

step in the first partial reaction can be ruled out. However an involvement of a phosphorylated biotin species at a latter stage in the reaction mechanism would not have been detected under the experimental conditions used, although there is no evidence to suggest such a possibility. On the other hand the above data are consistent with an involvement of the postulated enzyme-bound carboxyphosphate.

V.C.1b Stability of Biotin to Acid Hydrolysis

During the course of the experiments conducted in this Chapter, it was realized that biotin was modified (i.e., in terms of its capacity to bind avidin) during the hydrolysis with 6 M HCl. Hence methods were optimized to obtain maximum yields of biotin after hydrolysis. A summary of the protective methods used as well as yield of biotin is detailed in Table V.3. The hydrolysis performed under vacuum in the presence of 1% mercaptoethanol gave the maximum yields of biotin and this condition was employed in the hydrolysis of the biotin samples in all of the preceding experiments.

V.C.2 Stability of  ENZ-biotin complex

The results presented in Section IV.C had indicated that although the carboxyl group on the  ENZ-biotin complex was more labile than the phosphoryl group, the kinetic competency of the phosphoryl group was dependent on the presence of the carboxyl group on the complex. As a consequence of this it was anticipated that both the carboxyl and phosphoryl groups would have similar half-lives when their kinetic competency was taken as a measurement of

their stability. The results of the stability experiments are presented in Fig. V.5 and Fig. V.6. It is apparent from the stability profiles that the half-lives of the ^{32}Pi (CO$_2$)=ENZ-biotin complex (46.1 min) and the ^{14}Pi (CO$_2$)=ENZ-biotin complex (44.7 min) at 0°C are quite similar. The results shown in Fig. V.7 and Fig. V.8 are the decay curves of the corresponding complex measured at 4°C, where the half-life of 25.7 min was obtained for the phosphoryl group stability while the stability of the carboxyl group on the complex had a half-life of 28 min. These findings suggest strongly that the carboxyl and the phosphoryl group on the complex are part of the same species, and provides additional support for the conclusion that the isolated complex is enzyme-bound carboxyphosphate. Although the experimental errors within individual experiments described above, were small, variations were observed in the results obtained from different experiments. This was due to the differing background levels of trace amounts of free [γ-^{32}P]ATP which eluted with the enzyme during the microcentrifuge desalting procedure. In addition the difficulty of maintaining the complex at a constant low temperature also contributed to this variability. Hence emphasis was placed not on the absolute values of the half-lives but rather on the similarity of the half-lives of the phosphoryl group and the carboxyl group on the Pi (CO$_2$)=ENZ-biotin complex.

V.C.3 Attempts to trap and demonstrate carboxyphosphate In the Reaction Solution

Despite the fact that carboxyphosphate was bound to the enzyme in the carbamyl phosphate synthetase catalysed reaction, Powers & Meister (1976b) demonstrated its presence in the reaction solution after stabilizing carboxyphosphate as the trimethyl ester form with diazomethane. The appearance of trimethyl carboxyphosphate in the solution was presumably due to the breaking of the enzyme-linked bonds during esterification. Hence attempts were made to observe if a similar situation existed in the case of pyruvate carboxylase.

Pyruvate carboxylase from chicken liver was reacted with either [γ-^{32}P]ATP or NaH$^{14}CO_3$ as detailed in the legends of Fig. V.9 and Fig. V.10 to prepare radioactively labelled 'carboxyphosphate' with the label either in the phosphoryl or the carboxyl group. After a 5 min incubation period at 30°C, 10 ml of diazomethane was added and the reaction solution was kept on ice for 15 min. The ethereal solution of diazomethane was removed by passing a stream of nitrogen through the solution, and aliquots of the esterified solution were analysed by ascending chromatography on silica gel with chloroform-methanol (90:10) as the developing solvent. Radioactive compounds were located by counting 1 cm strips of the silica gel in toluene. The results shown in Fig. V.9 and Fig. V.10 indicate the absence of a carboxyphosphate methyl ester derivative separating from the origin. Using similar developing procedures, Powers & Meister (1976b) showed that the trimethyl carboxyphosphate

ester migrated with an RF value of 0.62. The radioactive material at the origin of the chromatogram was found to be HCO_3^- in Fig. V.10 and mono methyl ester of [γ-^{32}P]ATP as well as [γ-^{32}P]ATP in Fig. V.9. Similarly the radioactivity migrating with the solvent front were found to be by comparison with appropriate standards, dimethyl carbonate (Fig. V10) and the trimethyl phosphate (Fig. V.9). From these results it is apparent that the phosphoryl and carboxyl group is associated with the enzyme and that a 'free' carboxy phosphate does not occur in the pyruvate carboxylase catalyzed reaction.

V.D. DISCUSSION

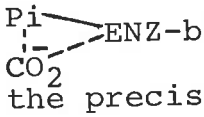
The evidence presented in this Chapter argues against the involvement of a phosphorylated biotin intermediate in the pyruvate carboxylase catalyzed reaction although the occurrence of such a species is chemically feasible, as found by studies with model compounds. Despite the fact that studies with model compounds have contributed favourably to the understanding of enzyme catalyzed reactions, very often, the findings from such studies can be misleading. For instance from model studies, Bruice and Hegarty (1970) suggested that carboxylation of biotin occurs on the 2'-oxygen position rather than the 1'-N position since the carbonyl oxygen was the most nucleophilic site, on the biotin moiety. However Guchhait and others (1974) provided very convincing evidence that in fact 1'-N-carboxybiotin was the true biochemical intermediate. Similarly the strongest argument for an involvement of o-phosphobiotin is based on

its chemical feasibility in attempting to provide a means for the activation of biotin for subsequent carboxylation at the 1'-N position. In addition to the evidence presented in this Chapter, other kinetic findings which are inconsistent with an involvement of o-phosphobiotin intermediate are:

(a) that chemical substitution at 1'-N position of biotin reduces but does not abolish the ability of the derivative to support the biotin carboxylase catalysed phosphoryl transfer from carbamyl phosphate to ADP (Polakis *et al.*, 1974). These investigators have also demonstrated that this phosphoryl-transfer reaction proceeds at a slow but finite rate in the absence of biotin.

(b) the requirement of ADP for the HCO_3^-:oxaloacetate isotopic exchange reaction (Scrutton & Utter, 1965; Ashman & Keech, 1975). The involvement of the o-phosphobiotin model in the reaction scheme [Fig. 1.D.1b(ii)] proposed by Kluger & Adawadkar (1976) indicates hydrolysis of ATP with the formation of o-phosphobiotin as the first step in the carboxylation reaction. If this was the situation the enzyme should catalyse a HCO_3^-:oxaloacetate isotopic exchange reaction in the absence of ADP. A requirement for ADP could arise however if this nucleotide had to remain bound for subsequent carboxylation of biotin to occur, and hence for the occurrence of an isotopic exchange reaction between HCO_3^- and oxaloacetate. Nevertheless, the phosphorylation of ADP should not be necessary in this situation. However, Ashman & Keech (1975) demonstrated that two analogues of ADP, α, β-methylene adenosine diphosphate and

adenosine 5'-phosphosulphate both of which bind to the enzyme but neither of which could be phosphorylated, also could not support the HCO_3^-:oxaloacetate isotopic exchange. Hence phosphorylation of ADP was required for this exchange to take place. This implied that ATP hydrolysis, independent of HCO_3^- binding, was not the first step in the reaction. This finding is also inconsistent with the o-phosphobiotin model.

There seems to be compelling evidence from the results presented in the preceding Chapters that the isolated complex,  , is enzyme-bound carboxyphosphate, although the precise nature of the amino acid residues involved in this association is not known. However a reactive lysyl residue has been implicated near the interface of the $MgATP^{2-}$ and HCO_3^- binding sites (Easterbrook-Smith, 1978) and as suggested, besides maintaining the proper alignment of substrates on the enzyme surface for catalysis to occur, the ε-NH_3^+ group could withdraw electrons from the terminal phosphate group of $MgATP^{2-}$, thus facilitating nucleophilic attack by an oxygen atom of HCO_3^- during the formation of carboxyphosphate. The involvement of carboxyphosphate is attractive in that it overcomes the barriers involved in the enzymatic carboxylation of biotin, namely, the low nucleophilicity of the 1'-N of biotin and the poor electrophilic nature of HCO_3^-. It has been suggested that the activation of HCO_3^- by ATP to form carboxyphosphate localizes molecules of CO_2 at high concentrations at the active site for reaction with the amine acceptor. The high local concentration of activated CO_2 is thought to

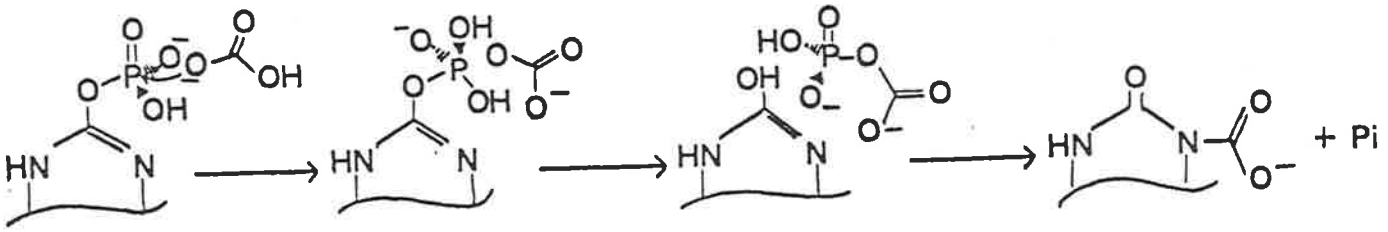
provide an effective driving force for its reaction with the 1'-N of biotin (Sauers *et al.*, 1975).

FIGURE V.1

PATHWAYS FOR CARBOXYLATION OF PHOSPHOBIOTIN TO FORM CARBOXYBIOTIN.

(adapted from Kluger *et al.*, 1979).

SCHEME I "IN LINE"



SCHEME II "ADJACENT"

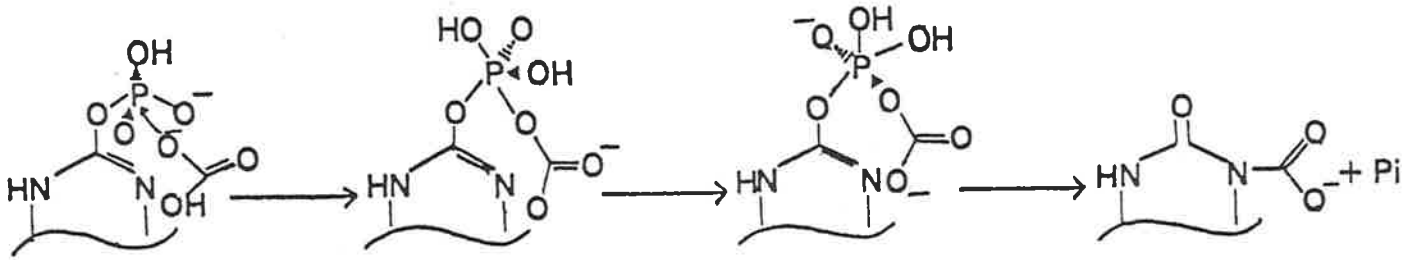


FIGURE V.2

SEPHADEX G-50 CHROMATOGRAPHY OF A TRYPTIC DIGEST OF CHICKEN LIVER PYRUVATE CARBOXYLASE REACTED WITH [γ-^{32}P]ATP AND ESTERIFIED

The incubation solution contained in (μmoles) in a final volume of 0.5 ml, N-ethylmorpholine-acetate pH 7.8, (50); $MgCl_2$, (4); [γ-^{32}P]ATP, (5.3×10^6 cpm/μmole), (1); $NaHCO_3$, (1); and 65 nmoles of chicken liver pyruvate carboxylases. The incubation solution was processed as described in Section V.C.1a. The peptides were eluted with 0.5% NH_4HCO_3 at 4°C. Peptides were detected by their absorption at 220 nm (□), while the radioactivity (■) was determined by counting aliquots of the column fractions in triton scintillation fluid.

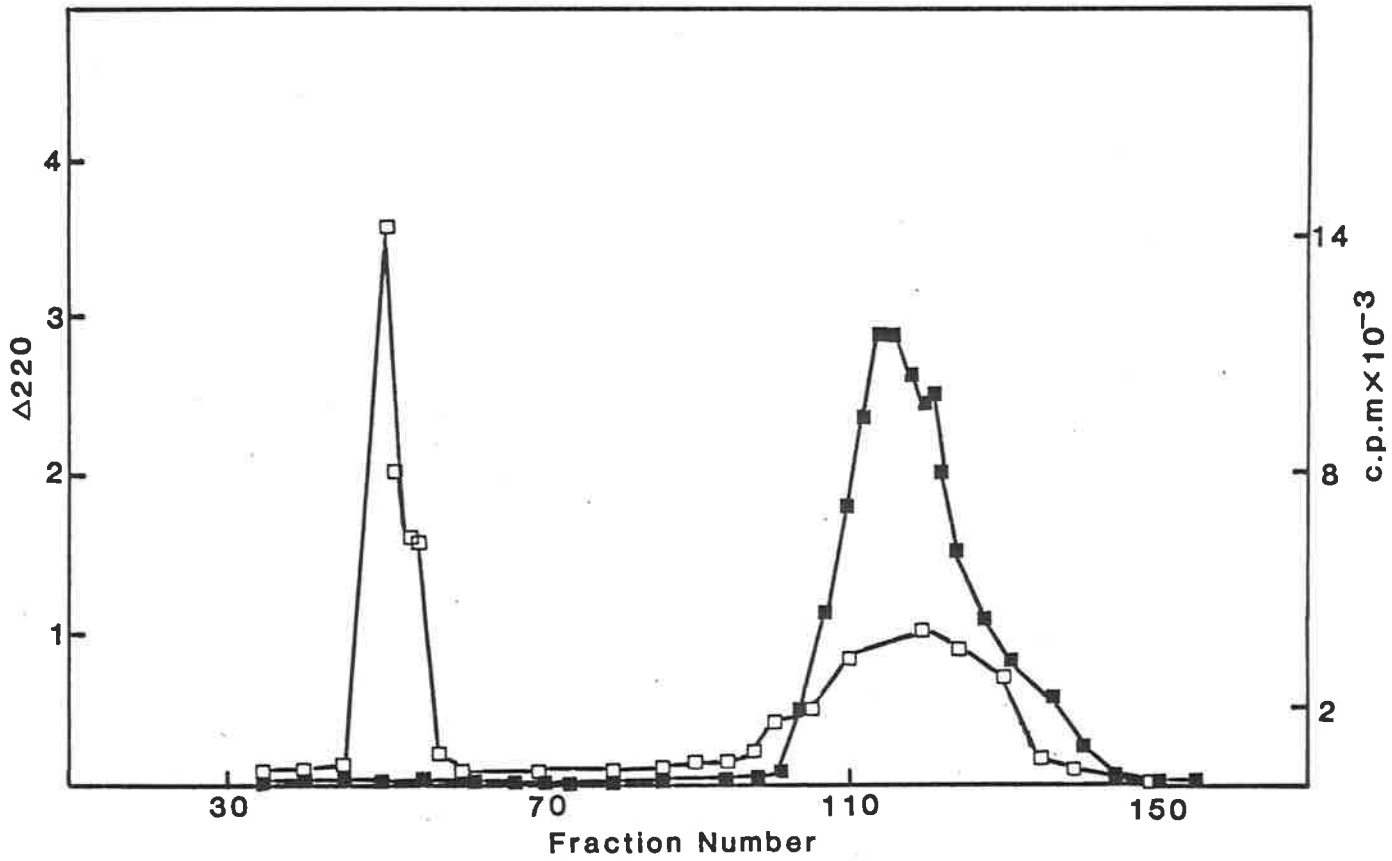

Δ220
4
3
2
1
14
8
2
c.p.m×10⁻³
30
70
110
150
Fraction Number

TABLE V.1

BIOTIN DETERMINATION OF THE PRONASE DIGEST OF THE ESTERIFIED [^{32}Pi]LABLLED ENZYME AFTER ACID HYDROLYSIS

The [^{32}Pi]labelled enzyme was prepared by the method described in the text. Aliquots of the pronase digest were acid hydrolysed in 6 M HCl containing 1% β-mercaptoethanol, in an evacuated and sealed tube. Biotin was determined by the method described in Section II.B.12.

Hydrolysis Time (h)	Biotin (pmoles)	
	Determined Value	Expected Value[1]
0	46.8 ± 4	49
0.5	31.9 5	"
1	33 4.5	"
4	39 3	"
6	41 4	"
12	35.8 4	"
18	34 2	"
24	37.3 3	"

[1] The expected value is the determined value of the biotin content of the unmodified enzyme.

FIGURE V.3

SEPHADEX G-50 CHROMATOGRAPHY OF A TRYPTIC DIGEST OF AVIDIN INACTIVATED PYRUVATE CARBOXYLASE REACTED WITH [γ-^{32}P]ATP AND ESTERIFIED

The reaction conditions were the similar to that described in the legends of Figure V.2 except that the enzyme was previously inactivated with an excess of avidin.

- □ Δ_{220}
- ■ [^{32}P] radioactivity

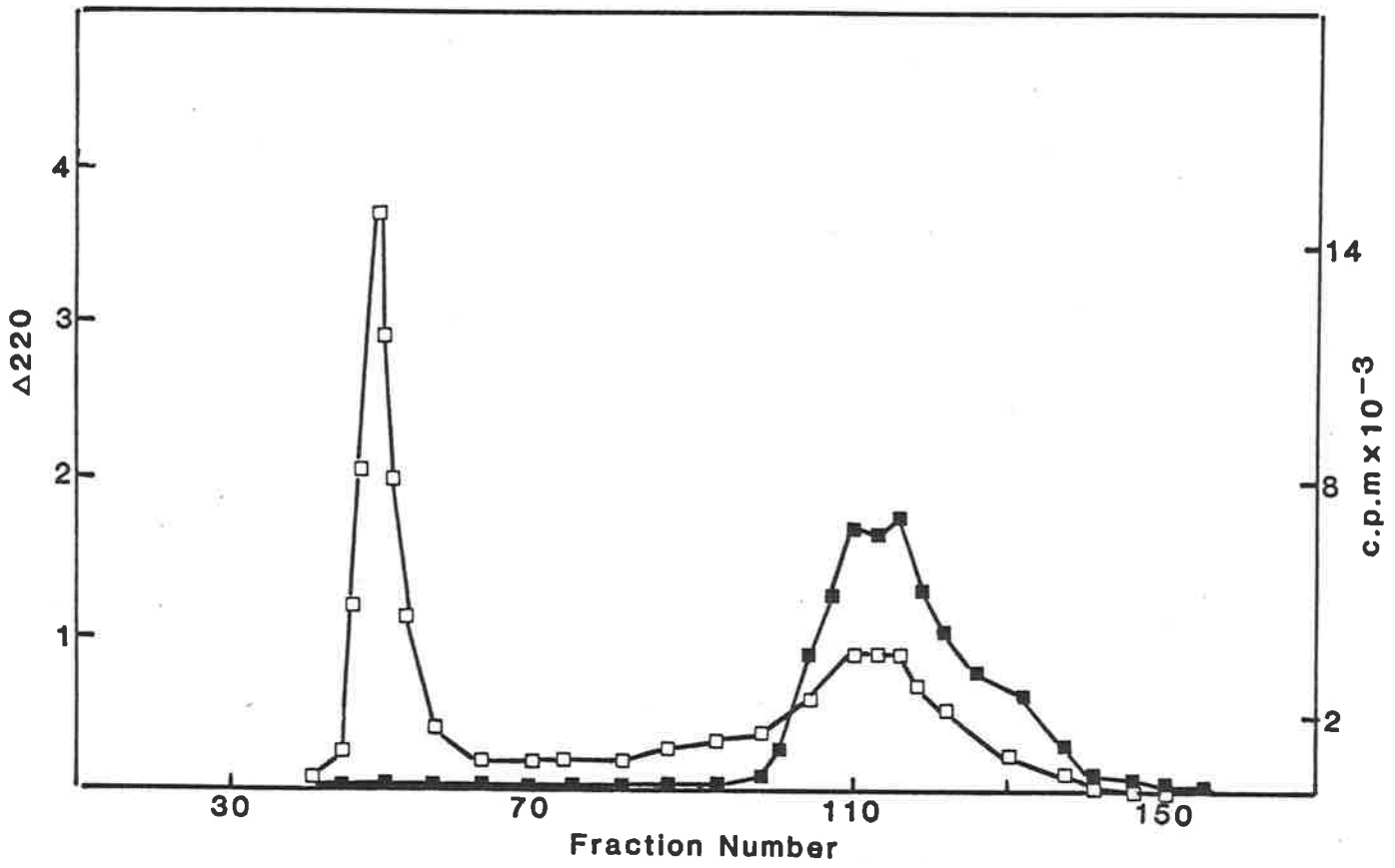

Δ220
4
3
2
1
14
8
2
c.p.m×10⁻³
30
70
110
150
Fraction Number

FIGURE V.4

SEPHADEX G-10 CHROMATOGRAPHY OF THE PRONASE DIGEST OF THE RADIOACTIVELY LABELLED TRYPTIC PEPTIDES (FROM FIGURE V.2)

The pronase digest of the tryptic peptides were prepared as described in the text. The column was eluted with 0.5% NH_4HCO_3 at 4°C.

o Δ_{260}

● $[^{32}P]$ radioactivity

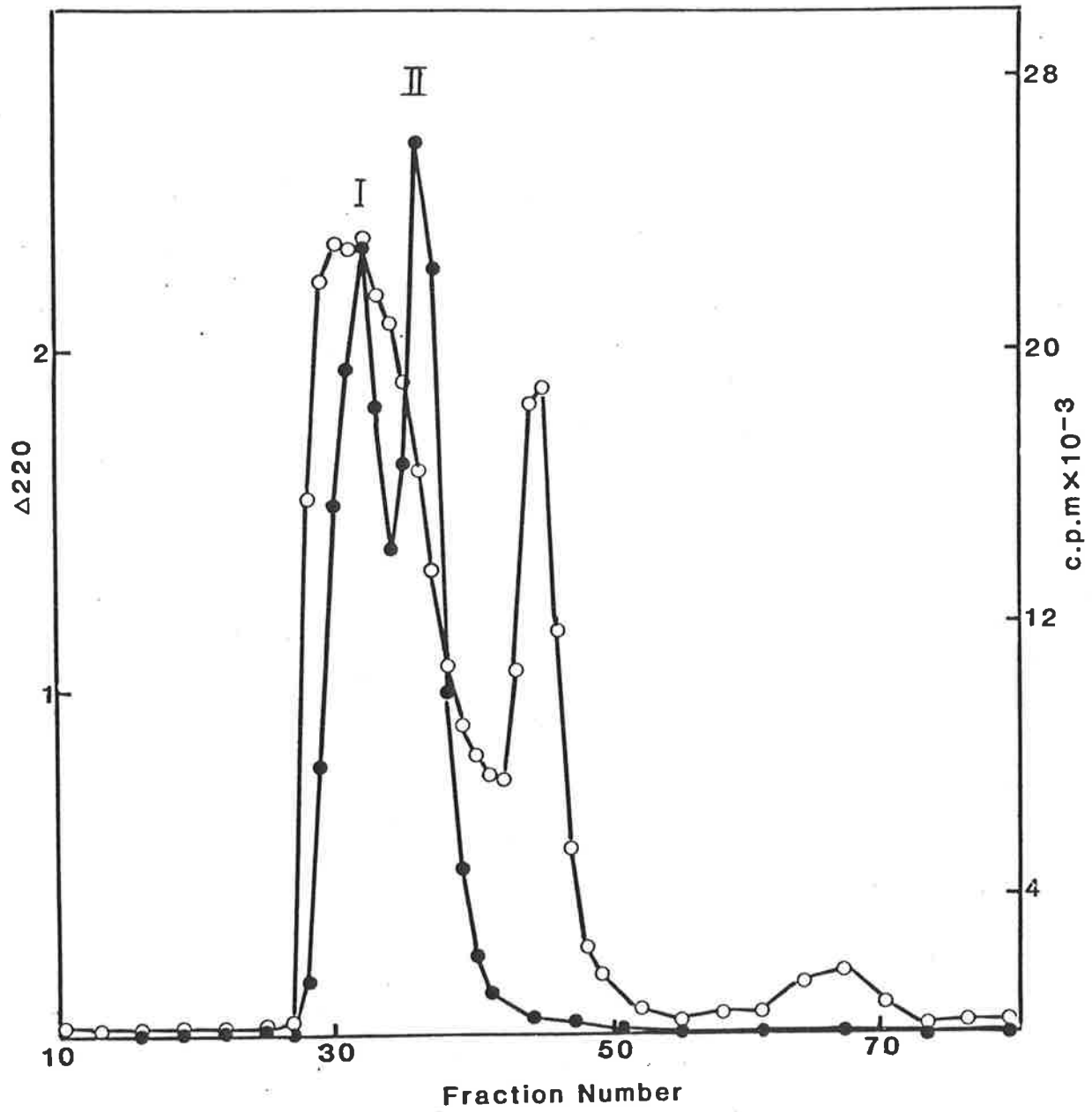

Ⅱ
Ⅰ
28
20
12
4
2
1
Δ220
c.p.m×10⁻³
10
30
50
70
Fraction Number

TABLE V.2

BIOTIN CONTENT OF THE RADIOACTIVE TRYPTIC (FIGURE V.2) AND AMINO ACID (FIGURE V.4) FRACTIONS

Sample	Total Biotin (nmoles)
Incubation solution	51
Avidin-biotin peptide fractions (45 - 55, Figure V.1)	42
G-50 radioactive fractions (Figure V.1)	not detected
G-10 radioactive Peak I (fractions, 28 - 34)	1.92
G-10 radioactive Peak II (fractions, 35 - 40)	0.2

TABLE V.3

STABILITY OF BIOTIN TO ACID HYDROLYSIS

Biotin (200 pmoles) was hydrolysed with 6 M HCl (200 µl) in a sealed tube for 24 h at 105°C. After hydrolysis, tubes were opened and samples freeze-dried over NaOH pellets. Samples were redissolved in 0.2 M KPi buffer (200 µl) and aliquots were taken for biotin determination as described in Section II.B.13. When the hydrolysis was done under vacuum (3) the samples were initially frozen and evacuated by allowing the samples to thaw out; after 5 min, the tubes were sealed while still attached to the vacuum line.

	Conditions	Biotin (pmoles)		
		Expected value	Determined value	% Recovery
1.	Aerobic	50	0.3	0.6
	" + 1% mercapto-ethanol	"	41.9	83.8
2.	Flushed with nitrogen	"	15.1	30
	" + 1% mercapto-ethanol	"	40.2	80.4
3.	Under vacuum	"	18.4	36.8
	" + 1% mercapto-ethanol	"	>50	≈100

FIGURE V.5

STABILITY OF $^{32}Pi \rightarrow CO_2$ ENZ-BIOTIN COMPLEX AT 0°C

The complex was prepared by the procedure described in Section IV.C.2a. The isolation and the determination of its half-life were carried out as described in Section V.B.4.

$t_{\frac{1}{2}}$ = 46.1 min.

FIGURE V.5 STABILITY OF ^{32}Pi ⟩ ENZ-BIOTIN COMPLEX AT 0°C.
CO_2^-

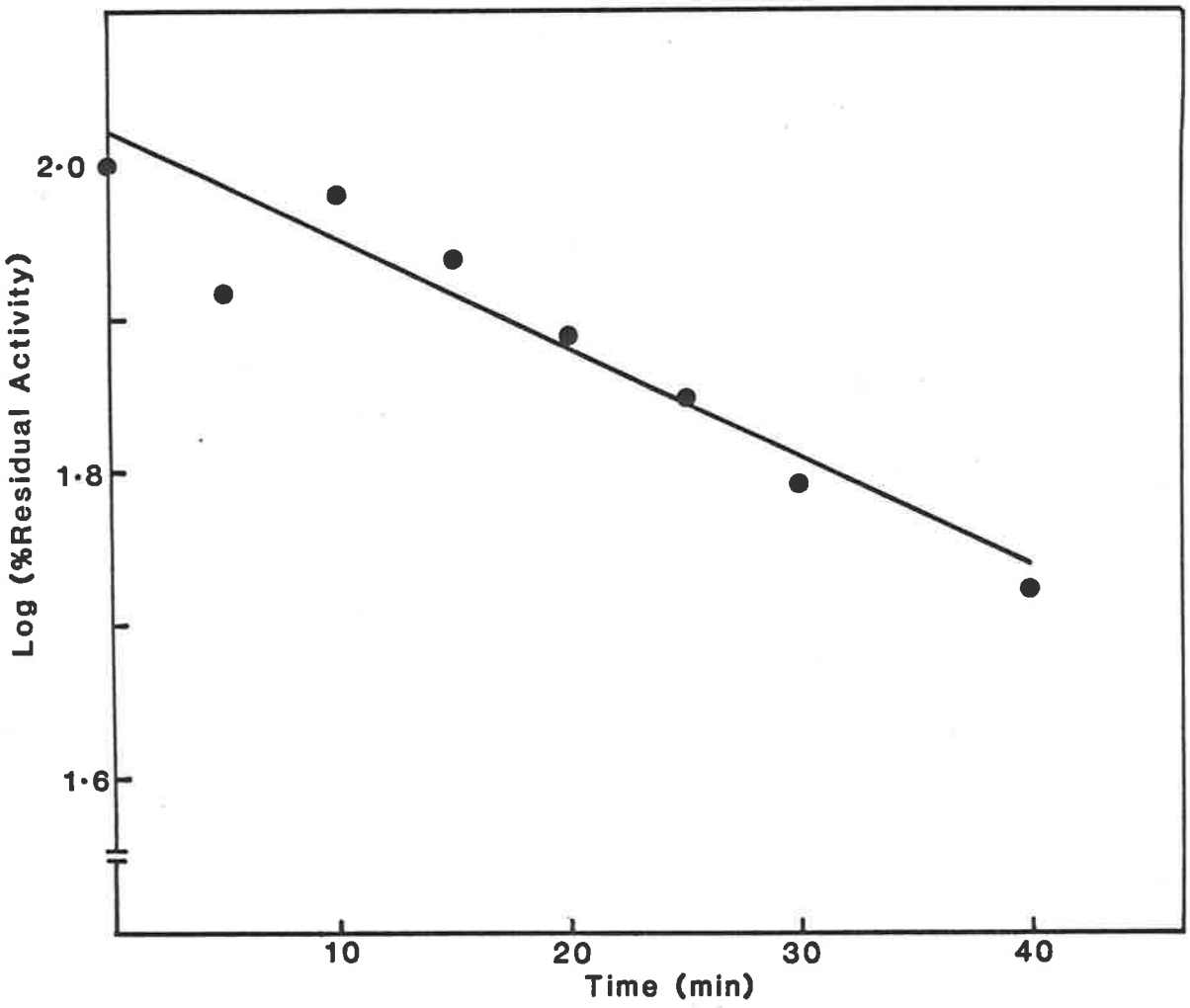


FIGURE V.6

STABILITY OF $^{14}CO_2$ (Pi) ENZ-BIOTIN COMPLEX AT 0°C

The complex was prepared by the procedure described in Section IV.C.1a. The isolation and the determination of its half-life were carried out as described in Section V.B.5.

$t_{1/2}$ = 44.7 min.

Values are the means ± SEM for triplicate assays.

FIGURE V. 6 STABILITY OF $^{14}\overset{Pi}{\underset{CO_2}{}}$ ENZ-BIOTIN COMPLEX AT 0 °C.

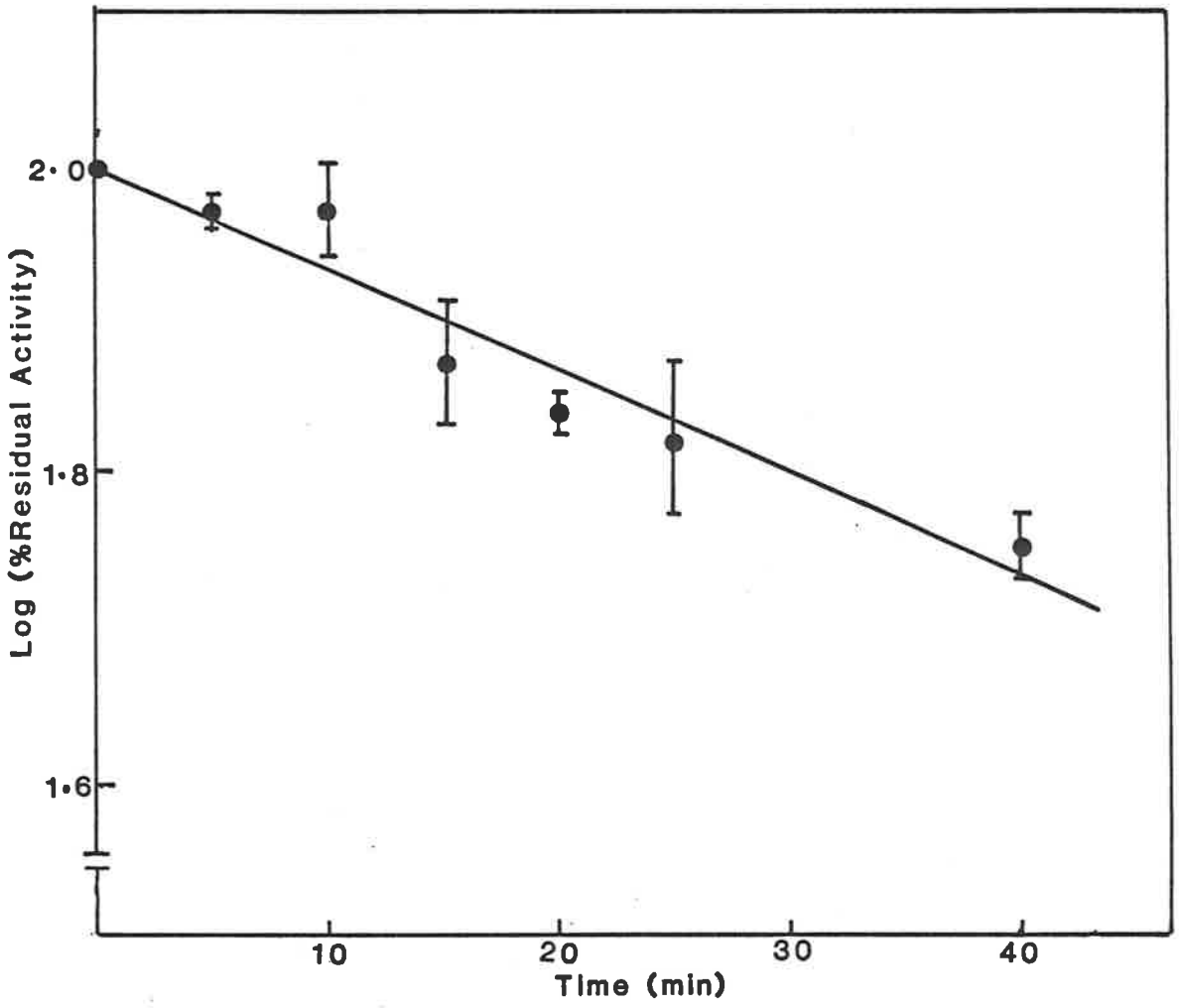


FIG. V.7 STABILITY OF ^{32}Pi ⋮ CO_2^- -ENZ-BIOTIN COMPLEX AT 4° C

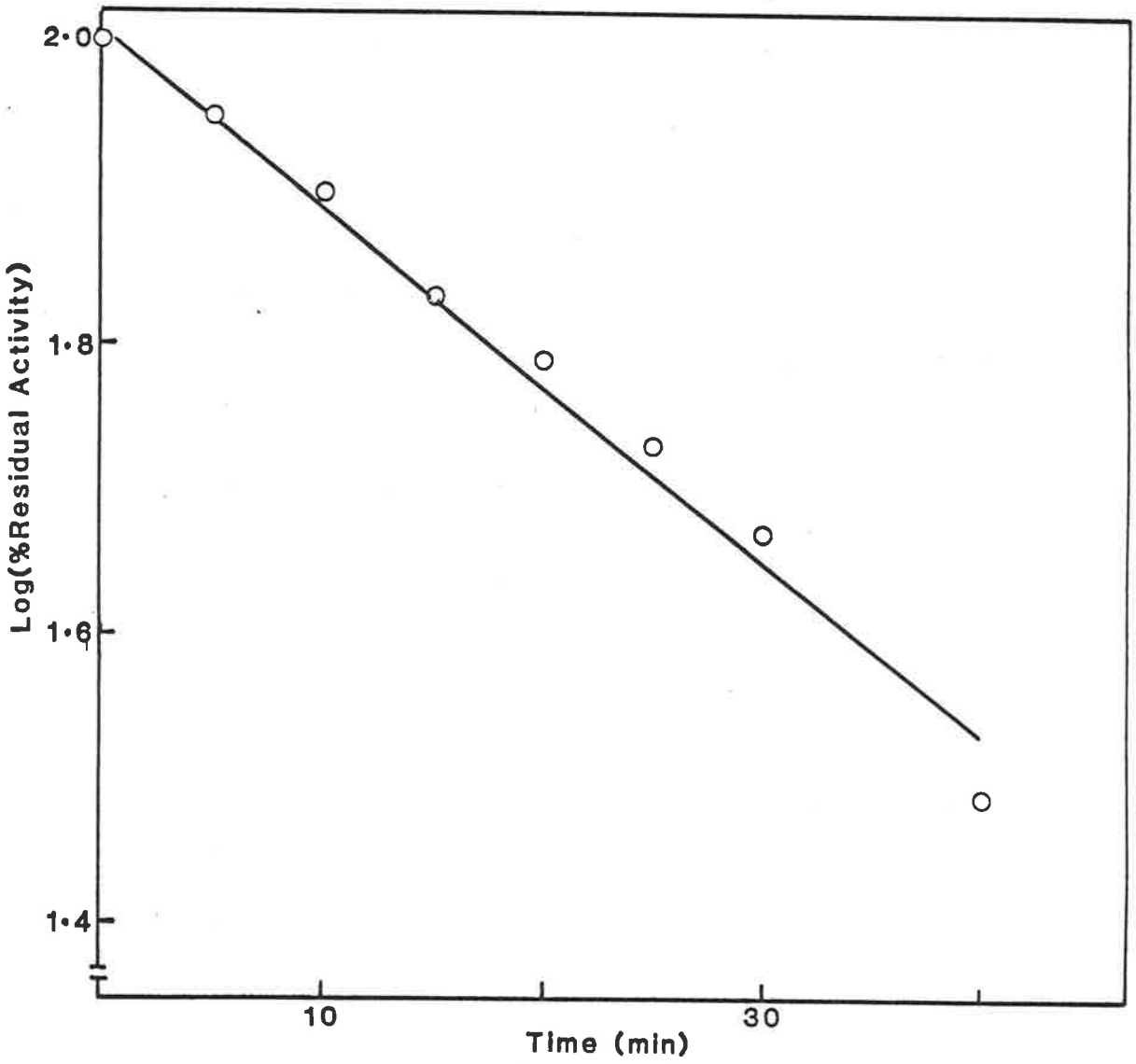


FIG. V.8 STABILITY OF $^{14}CO_2^-$ (Pi) ENZ-BIOTIN COMPLEX AT 4° C

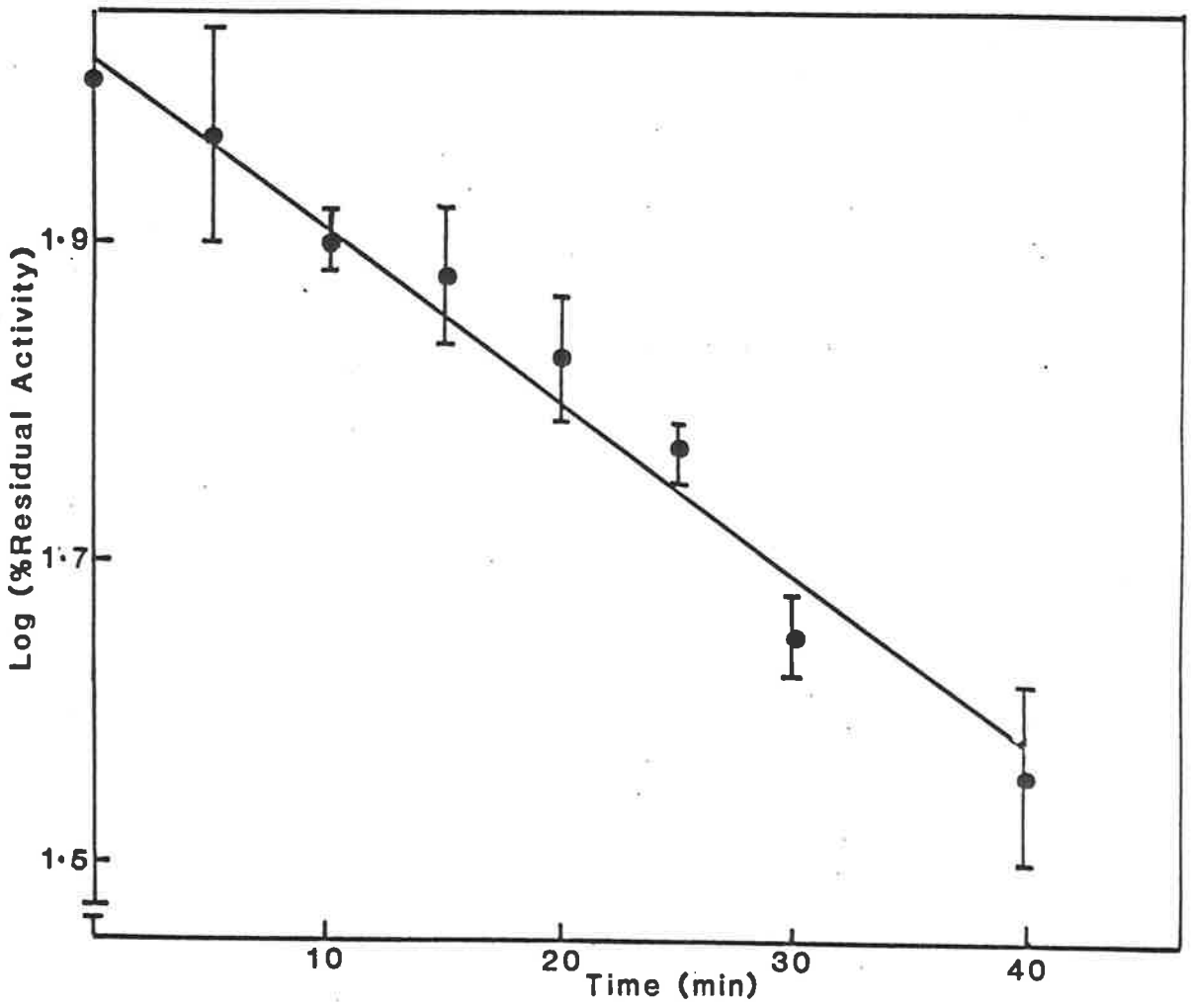


FIGURE V.9

ANALYSIS OF METHYLATED REACTION SOLUTION (REACTED WITH [γ-^{32}P]ATP) BY THIN-LAYER CHROMATOGRAPHY

The reaction solution contained (in μmoles) in a final volume of 0.25 ml, sodium barbital buffer, pH 7.6, (5); $NaHCO_3$, (0.5), [γ-^{32}P]ATP, (0.5) (4.7×10^7 cpm/μmole); $MgSO_4$, (1); and 35 units of chicken liver pyruvate carboxylase. After 5 min at 30°C, 10 ml of diazomethane was added and the esterification reaction was allowed to proceed for 15 min, and processed as described in Section V.C.3.

△ - enzyme

▲ + enzyme

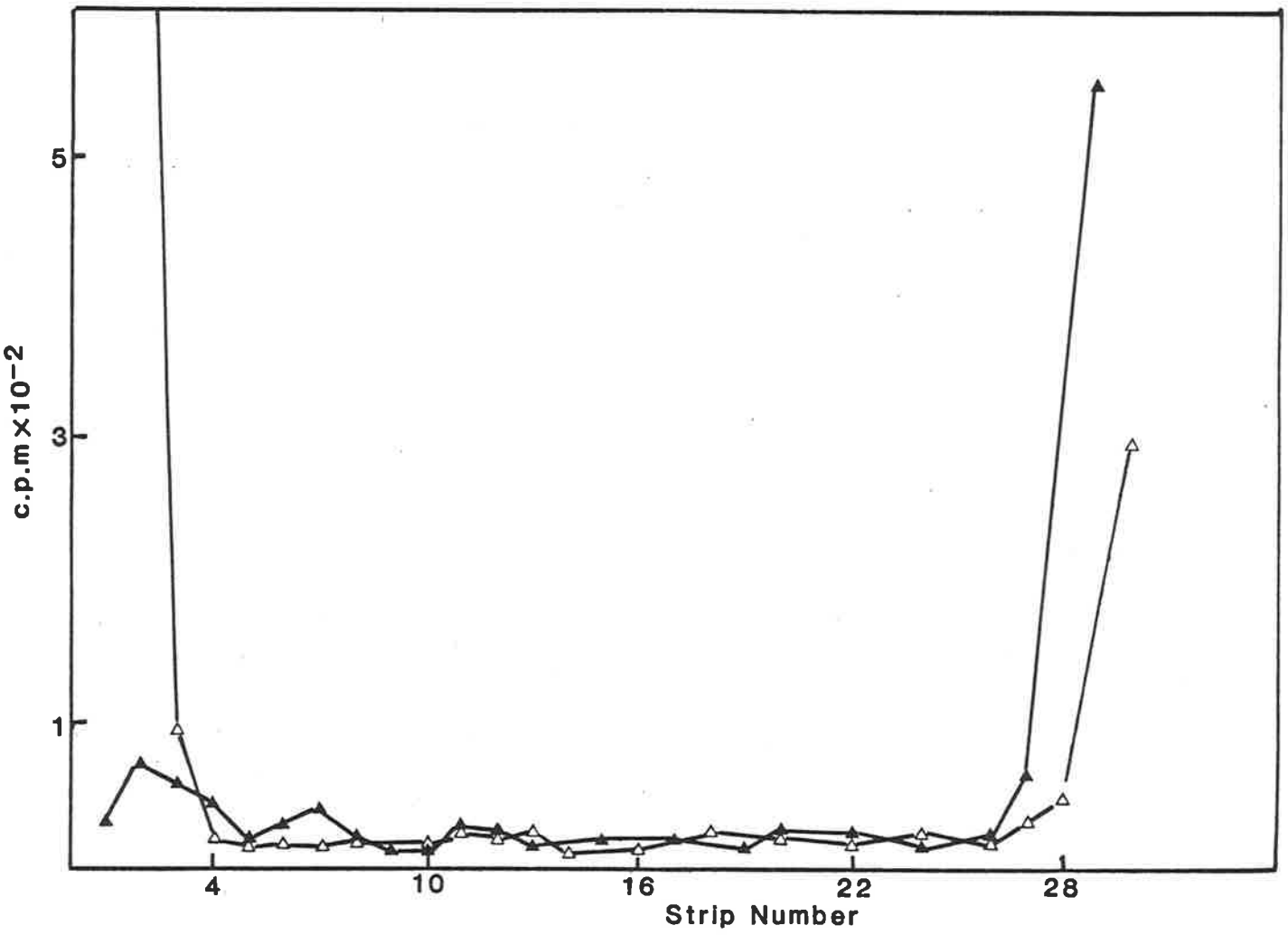

c.p.m×10⁻²
5
3
1
4
10
16
22
28
Strip Number

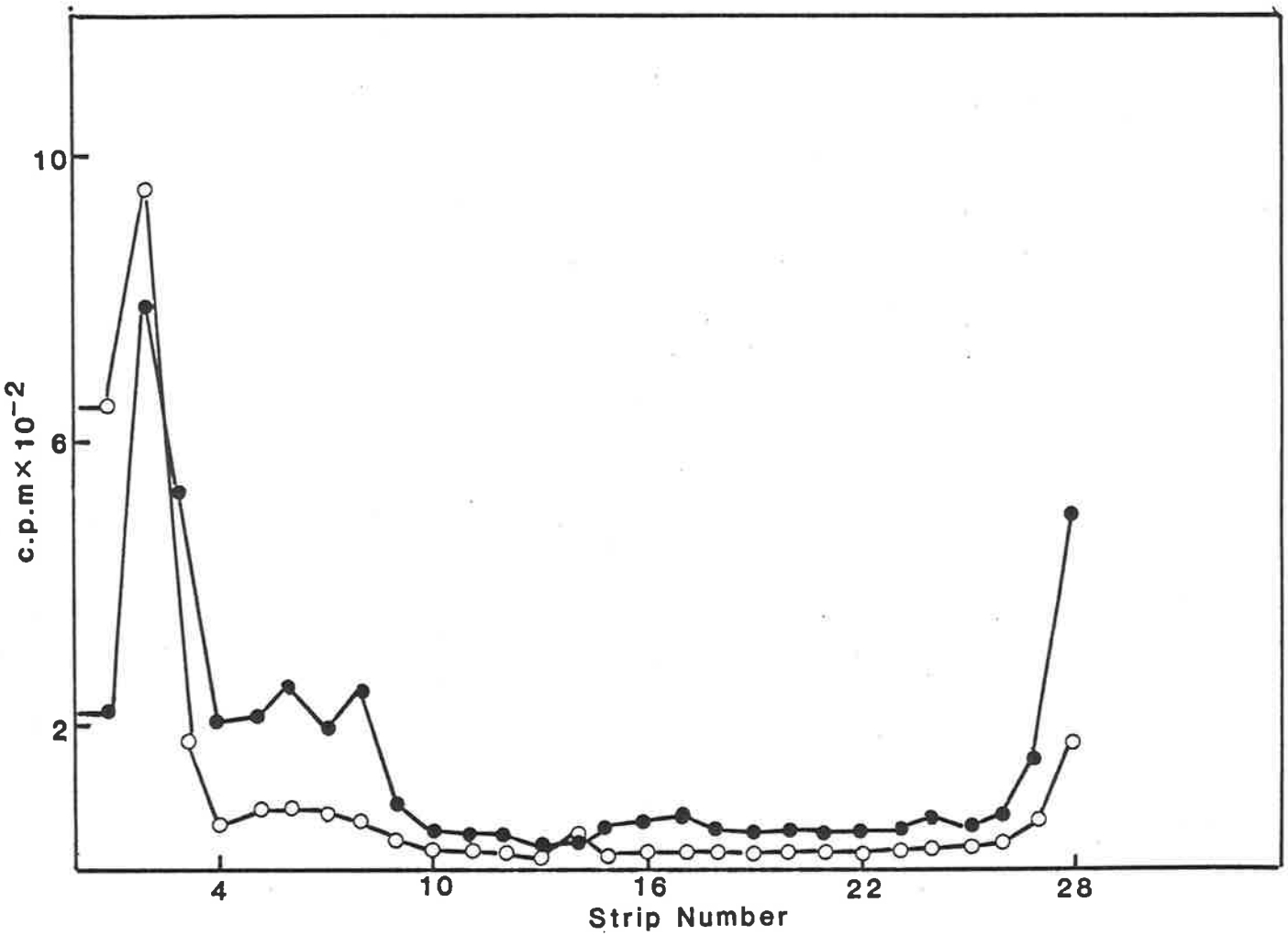
FIGURE V.10

ANALYSIS OF METHYLATED REACTION SOLUTION (REACTED WITH $NaH^{14}CO_3$) BY THIN-LAYER CHROMATOGRAPHY

The reaction solution was similar to that described in Figure V.9 except that [γ-^{32}P]ATP was replaced with ATP and $NaHCO_3$ with $NaH^{14}CO_3$ (1.5×10^7 cpm/μmole).

○ - enzyme

● + enzyme


10
6
2
c.p.m × 10⁻²
4
10
16
22
28
Strip Number

CHAPTER VI

MECHANISM OF ACETYL CoA ACTIVATION

VI.A INTRODUCTION

In previous Chapters of this thesis it has been implied from isotopic exchange studies that the role of acetyl CoA in the reaction mechanism is to facilitate the interconversions of two of the enzyme-substrate species viz. $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ ENZ-biotin and ENZ-carboxybiotin. However the evidence so far has been equivocal. In this Chapter data are presented from experiments designed to provide direct proof of its involvement in this step. Since the enzyme from avian liver apparently exhibits an absolute requirement for acetyl CoA (Keech & Utter, 1963), identification of the step with an absolute requirement would provide direct evidence for the proposed role of acetyl CoA.

VI.B MATERIALS AND METHODS

VI.B.1 Assay Methods

The rate of pyruvate carboxylation, the ATP:Pi and the ATP:ADP isotopic exchange reactions were determined by the procedures described in Sections II.B.6b; II.B.8a and II.B.8b respectively.

VI.B.2 [^{14}C]carboxyl group transfer reactions

[^{14}C]carboxyl group transfer reactions were carried out to test the ability of the isolated $^{14}\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ ENZ-biotin and the ENZ-biotin-$^{14}CO_2^-$ complexes to transfer the carboxyl group to pyruvate. The complexes were prepared by the procedure described in IV.B.3, except that ENZ-biotin-$^{14}CO_2^-$

was prepared by the inclusion of 0.25 mM acetyl CoA. The isolation of the above complexes were carried out by Sephadex gel permeation using the microcentrifuge desalting technique described by Helmerhorst & Stokes (1980) and added to pyruvate under the conditions described in the Fig. legends. The radioactive oxaloacetate produced was stabilized as its carbazone derivative.

VI.C. RESULTS

VI.C.1 Effect of acetyl CoA on the Formation of ENZ-carboxybiotin and the Translocation Process

Direct evidence that the formation of ENZ-carboxybiotin complex from the $^{Pi}_{CO_2}$>ENZ-biotin complex is the step which requires acetyl CoA, was demonstrated in the following way:- The two forms of the enzyme viz., $^{Pi}_{^{14}CO_2}$>ENZ-biotin and ENZ-^{14}carboxybiotin were prepared in the absence and presence of acetyl CoA respectively. Both of the complexes were isolated by the procedures as described in Section V.B.5 and added to a buffered solution containing 2.5 μmoles of pyruvate, and the oxaloacetate formed was stabilized by the addition of semi-carbazide in HCl. It can be seen from Table VI.1 that in the case of the complex activated in the presence of acetyl CoA (presumably the ENZ-carboxybiotin complex), the addition of acetyl CoA in the pyruvate solution had no effect on the amount of radioactivity appearing in the acid stable product, confirming that acetyl CoA had little or no effect on the efficiency of either the translocation process (i.e., the shifting of the biotin moiety from the first to the second sub-site) or the transfer phase (i.e.,

the movement of the carboxyl group from carboxybiotin to pyruvate) of the reaction mechanism. This finding is in agreement with the observation of Goodall *et al.* (1981) who showed that acetyl CoA did not influence the rate of translocation.

However the $^{14}\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ENZ-biotin complex formed in the absence of acetyl CoA was unable to transfer any of its radioactivity to pyruvate unless acetyl CoA was present in the pyruvate solution (Table VI.1). Hence the conclusion to be drawn from these data is that acetyl CoA must be involved in the conversion of the $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ENZ-biotin complex to the ENZ-carboxybiotin species, which can then undergo translocation to the second sub-site. It is suggested that acetyl CoA in facilitating the transfer of the carboxyl group from the $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ENZ-biotin complex to the biotin moiety, also displaces Pi from the enzyme. This is consistent with the finding that the ATP:Pi isotopic exchange reaction requires the presence of acetyl CoA, while the ATP:ADP exchange reaction does not, (Table III.4).

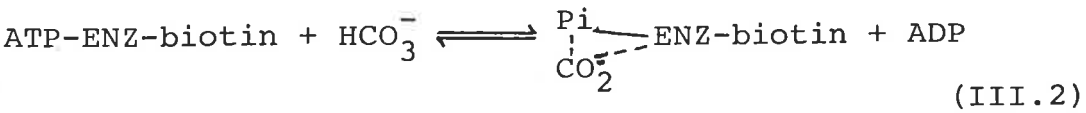
Nevertheless, the above observation still leaves some unanswered questions. First of all, it does not explain how acetyl CoA participates in the interconversion between the putative carboxyphosphate-ENZ-biotin complex and the ENZ-carboxybiotin complex. Secondly, assuming that all biotin-dependent carboxylases possess the same basic first partial reaction mechanism, how do those enzymes which do not require an allosteric activator, carry out the transition between the two complexes.

In the first instance, to gain some insight into the

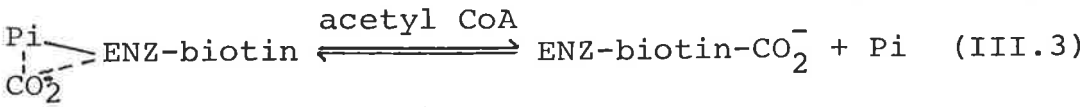
nature of acetyl CoA participation in the interconversion, the property of analogues of pyruvate to induce the movement of the ENZ-carboxybiotin to the keto-acid binding site was exploited to test the resulting effects on the ATP:ADP exchange reaction. Since the ATP:ADP exchange reaction utilizes one of the interconverting species, i.e., $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ENZ-biotin while the pyruvate analogue influences the other, i.e., ENZ-carboxybiotin, the above experiment was designed to test events on either side of the step in which acetyl CoA participates, in the hope that it will reveal the nature of this participation.

The results shown in Fig. VI.1a and Fig. VI.1b reveal that in the absence of acetyl CoA, the two analogues of pyruvate did not influence the rate of the ATP:ADP exchange reaction, while in the presence of the effector, the exchange rate was significantly depressed. Possible explanations for these observations are as follows:-

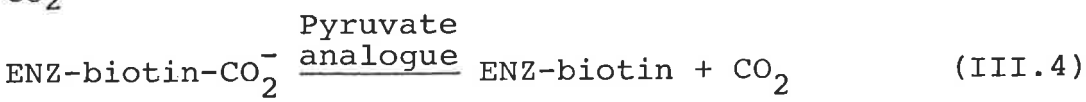
(a) acetyl CoA facilitates the transfer of the carboxyl group to 1'-N of biotin, with the release of Pi, as indicated in eq.(III.3) of Fig. III.9 reproduced below. At the same time, the binding of the pyruvate analogues at the second sub-site induces the ENZ-carboxybiotin to move to the keto acid binding site (Goodall *et al.*, 1981). As a result, the amount of enzyme available for the ATP:ADP exchange reaction, i.e., $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ENZ-biotin would be decreased hence causing a reduction in the exchange rate. Furthermore, once the carboxybiotin is in the second sub-site, hydrolysis occurs (Easterbrook-Smith *et al.*, 1976a) and CO_2 is liberated as indicated in eq. (III.4)



(III.2)

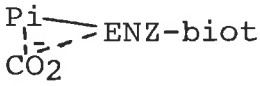


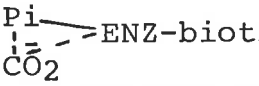
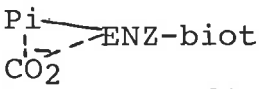
(III.3)



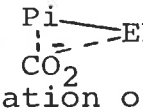
(III.4)

SITE I SITE II

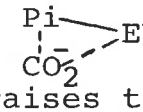
Once this happens, there is no way for the reaction to reverse and for Pi to bind to the enzyme and subsequently form a bond of sufficient energy to form ATP. In the absence of acetyl CoA however, ENZ-carboxybiotin is not formed and although the biotin moiety may be shifted to the keto acid sub-site on the binding of the analogues, the rate of the ATP:ADP exchange reaction would not be affected since the concentration of the  complex would not be altered.

(b) the role of acetyl CoA maybe to labilize the carboxyl group on the  complex rather than to facilitate its actual transfer to 1'-N of biotin; thus in the presence of the analogue if the biotin moiety is shifted to the keto acid site, the carboxyl group on the  complex would be hydrolysed and this would have the effect of inhibiting the ATP:ADP exchange reaction, since the studies presented in Section IV.C.2b had indicated that the Pi-ENZ-biotin complex whose carboxyl group has been hydrolysed, can no longer catalyse the formation of ATP from ADP.

(c) acetyl CoA may labilize and release the phosphoryl

group on the 

ENZ-biotin complex and thus facilitate the carboxylation of biotin. The effect of the analogues in this instance would be similar to that mentioned above. The observation that the rate of the ATP:ADP exchange reaction was reduced in the presence of acetyl CoA without the inclusion of the analogues, is consistent with the various possible roles of acetyl CoA as discussed above.

Thus if acetyl CoA participates in the interconversion between the 

ENZ-biotin complex and ENZ-carboxybiotin, then this raises the second issue queried viz., how do those biotin-dependent enzymes which do not require an allosteric activator carry out the transition between the two complexes.

VI.C.2 The 'Absolute' Requirement for Acetyl CoA

The initial studies on the activation of the chicken liver enzyme by acetyl CoA, had reported that the enzyme had an absolute requirement for this effector (Scrutton *et al.*, 1965; Scrutton & Utter, 1967). However Ashman *et al.* (1972) have reported that the chicken liver enzyme did have measureable activity in the absence of an activator, but the ratio of this rate to the rate in the presence of acetyl CoA was only about 0.06%. This situation is similar to the enzymes of mammalian origin. Table VI.2 (adapted from Ashman *et al.*, 1972) shows the variations in the rates of the catalytic activities both in the presence and absence of acetyl CoA as exhibited by the enzyme from various sources. Under usual assay conditions the activities of enzymes from rat liver (McClure *et al.*, 1971) and sheep kidney (Ashman *et al.*, 1972) in the absence of the activator approaches 2 - 4% of that of the activated enzyme.

However, under optimal conditions, i.e., at very high concentrations of substrates and K^+, the acetyl CoA-independent activities may approach 25% of the activity in the presence of acetyl CoA.

In view of the above findings, conditions were sought to demonstrate on acetyl CoA-independent activity in the chicken liver enzyme. The acetyl CoA-independent activity and the activity in the presence of saturating amounts of acetyl CoA were measured as a function of time. In addition, the acetyl CoA-independent activity in the presence of high concentrations of all substrates including K^+ was also measured. The details of the reaction conditions are described in the figure legends and as seen from Fig. VI.3 the acetyl CoA-independent activity in the presence of high concentrations of substrates increased with time while there was minimal activity in the assay system containing normal concentrations of substrates. To determine whether it was the presence of K^+ or the high concentrations of the other substrates that caused the stimulation of the reaction rate in the absence of acetyl CoA, the effect of K^+ on both the assay systems (i.e., under high substrate concentration and under normal substrate concentration) was tested. The results shown in Fig. VI.4 indicate that there was a 3.5 fold stimulation by K^+ in both systems where acetyl CoA had been omitted. However, the presence of substrates at high concentration had the effect of enhancing the activator-independent activity. In the presence of acetyl CoA, the enzyme exhibited a greater activity even in the absence of K^+. However the inorganic

cation stimulated the acetyl CoA-dependent activity by 2.4 fold. These findings are similar to those observed with the enzyme from sheep kidney (Ashman *et al.*, 1973).

The early studies of Scrutton & Utter (1967) on the effect of various pH values on the activator constant indicated that the n_H value decreased from 3 at pH 6.75 to 2.03 at pH 9. This decrease was associated with a decrease in the activator constant from 170 μM at pH 6.95 to 9.5 μM at pH 9.0. Their results also indicated the involvement of an ionizing group having a pK value of pH 8.6. These findings imply firstly, that at higher pH values, the enzyme exhibits a smaller degree of requirement for the effector, to achieve maximal velocities; secondly an involvement of an ionizing group at high pH may indicate that acetyl CoA may in some way facilitate the ionization of this group which at lower pH values may be only partially ionized. If this proposal is correct, the rate of the catalytic activity of the enzyme in the absence of acetyl CoA, should be enhanced at higher pH values. The effect of varying the pH on the catalytic activity in the absence of acetyl CoA is shown in Fig. VI.5. It is obvious from the results that there is a 4-fold increase in the reaction velocity from pH 7.8 to pH 9.5, measured under optimal conditions of high concentration of substrates as well as the inclusion of K^+. The maximal rate observed under the above conditions approaches 2% of acetyl CoA stimulated rate (measured at optimal conditions). This value is a ≈30 fold increase over the previous value reported for the chicken liver enzyme (Table VI.2). Hence it is apparent

from the results presented above that the enzyme from chicken liver does catalyse pyruvate carboxylation in the absence of acetyl CoA, although at a slow rate.

VI.D DISCUSSION

The evidence presented in this chapter as well as those in Chapter III have made it possible to localize the catalytic step which exhibited an absolute requirement for acetyl CoA. Although various possibilities by which acetyl CoA participates in the interconversion between the $\overset{Pi}{\underset{CO_2}{\rightleftharpoons}}$ ENZ-biotin complex and ENZ-carboxybiotin, were considered, the available evidence was inadequate to define the precise manner of this participation.

The demonstration of a slow rate of acetyl CoA-independent catalytic activity, could provide further insight into the mechanism of activation of acetyl CoA. The apparently 'absolute' requirement for acetyl CoA in the chicken liver enzyme, could have risen due to the sub-optimal conditions used to measure the catalytic activity in the absence of acetyl CoA. For instance in the absence of acetyl CoA, the maximal catalytic activity was observed at pH 9.5, in the presence of K^+ and high substrate concentrations. While the maximal rate of the acetyl CoA-dependent activity was observed at pH 8.5 under optimal assay conditions. In addition the observation of an ionizing group with a pK value of 8.6 (Scrutton & Utter, 1967) may indicate that at lower pH values, acetyl CoA facilitates the ionization of this group, which at lower pH values may not be ionized without the effector. This might account for an 'absolute'

requirement for acetyl CoA. These observations tend to suggest that the residue in question may play a vital role in the formation of ENZ-carboxybiotin from the Pi, CO_2 >ENZ-biotin complex. Although a reactive lysine residue has been implicated in the interaction with the adenosyl nucleotide portion of the acetyl CoA molecule (Clements *et al.*, 1981), it is not known whether this residue plays any role in the catalytic reaction, other than to bind acetyl CoA. Although the maximal rates observed in the absence of acetyl CoA are low, when compared to the acetyl CoA-independent rates catalysed by other enzymes isolated from mammalian sources, it is nevertheless emphasized that extensive attempts were not made to find optimal conditions for the acetyl CoA-independent reactions. However these preliminary findings do indicate that the chicken liver enzyme catalyses the carboxylation of pyruvate in the absence of acetyl CoA.

The observation that K^+ significantly enhanced the catalytic activity in the absence of acetyl CoA, may provide the answer to the query raised at the beginning of this Chapter, viz; how do other biotin-dependent enzymes which do not require an allosteric activator, catalyse the reaction. In the light of the above findings it is tempting to suggest that K^+ might mimic the role of acetyl CoA in facilitating the interconversion of the two complexes viz; Pi, CO_2 >ENZ-biotin and ENZ-carboxybiotin. However, the available evidence is insufficient to enable a suggestion of a possible mechanism.

TABLE VI.1

EFFECT OF ACETYL CoA ON [^{14}C]CARBOXYL GROUP TRANSFER REACTIONS

The carboxyl group transfer reactions were carried out as described in Section VI.B.2.

Incubation[1] solution	Transfer[2] solution	-$^{14}CO_2$-Transferred (nmoles)
+ acetyl CoA	+ acetyl CoA	0.091 ± 0.1×10^{-4}
+ acetyl CoA	- acetyl CoA	0.096 ± 8×10^{-4}
- acetyl CoA	+ acetyl Coa	0.023 ± 7×10^{-4}
- acetyl CoA	- acetyl CoA	0.0015 ± 1.5×10^{-4}

[1] Incubation solution contained (in μmoles) in a final volume of 0.25 ml, N-ethylmorpholine (Cl^-, pH 7.8), (25); $MgCl_2$, (2); ATP, (0.5); $NaH^{14}CO_3$ (7.6×10^4 cpm/μmole), (0.5); acetyl CoA, (0.063) (where indicated) and 15 units of chicken liver pyruvate carboxylase.

[2] Transfer solution contained 100 mM N-ethylmorpholine (Cl^-, pH 7.8), 10 mM pyruvate (Na^+) and where indicated, 0.25 mM acetyl CoA. Reaction was for 5 min at 25°C.

Values are the means ± SEM for triplicate assays.

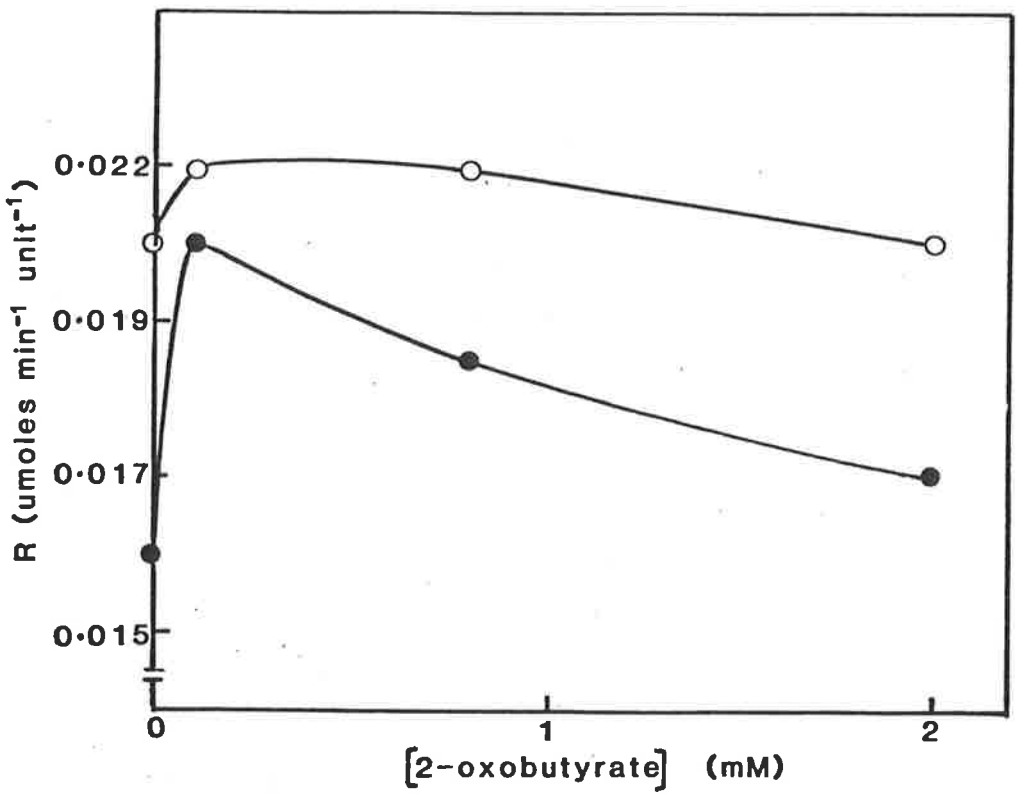


FIGURE VI.1a

EFFECT OF 2-OXOBUTYRATE ON THE Mg^{2+}-DEPENDENT ATP: ADP EXCHANGE REACTION

The exchange reaction was carried out by the procedure described in Section II.B.8b. The rate in the presence of 0.25 mM acetyl CoA (●) or in the absence (O) are the mean of three measurements.

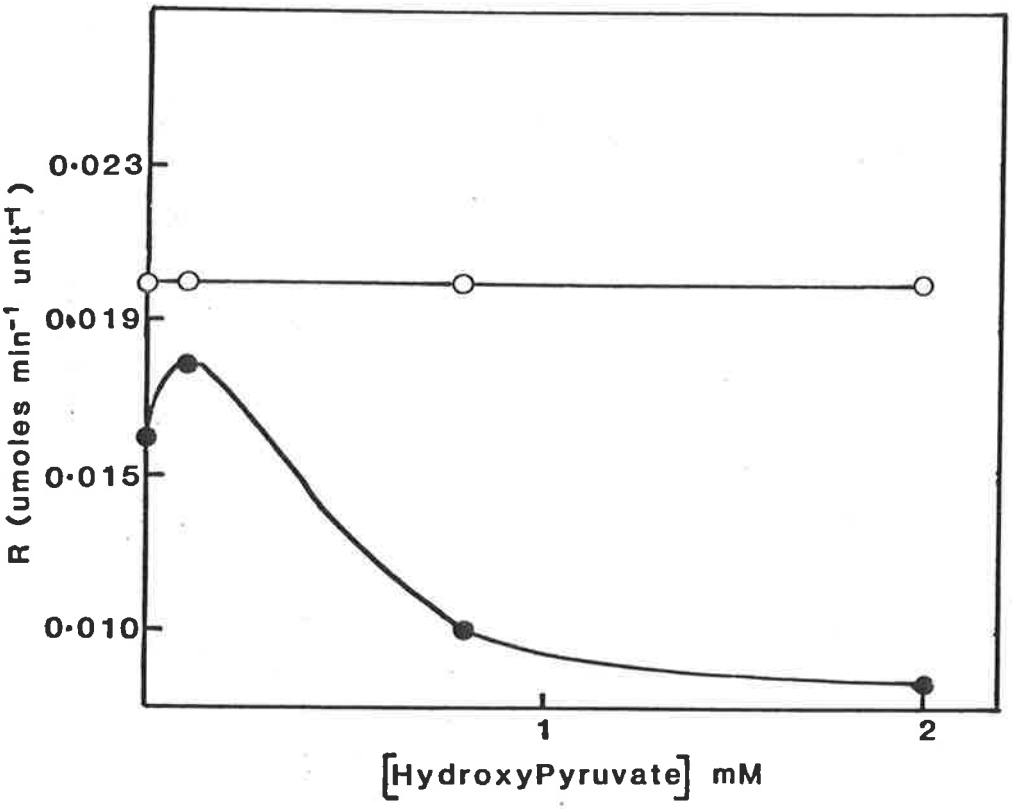


FIGURE VI.1b

EFFECT OF HYDROXYPYRUVATE ON THE Mg^{2+}-DEPENDENT ATP:ADP EXCHANGE REACTION

The rate in the presence of 0.25 mM acetyl CoA (●) and in the absence (O) are the mean of three determinations.

TABLE VI.2

ACETYL CoA-DEPENDENT ACTIVITIES OF PYRUVATE CARBOXYLASES FROM VARIOUS SOURCES

(Adapted from Ashman *et al.*, 1972)

Source	Specific Activity		- acetyl CoA/ + acetyl CoA x 100
	+ acetyl CoA	- acetyl CoA	
Sheep kidney	13	0.62	4.8
Sheep liver	20	0.96	4.8
Chicken liver	20	0.012	0.06
Rat liver	7.0	0.13	1.8

FIGURE VI.3

EFFECT OF HIGH CONCENTRATIONS OF SUBSTRATES ON THE ACETYL CoA-INDEPENDENT ACTIVITY

The assay system for the overall reaction in the presence of acetyl CoA (O) was carried out by the procedure described in Section II.B.6b. The assay system using high substrate concentration (●), contained (in μmoles) in a final volume of 0.25 ml, N-ethylmorpholine (CL^-, pH 7.8), (25); $MgCl_2$, (2.5); ATP, 1.25; $NaH^{14}CO_3$, (10); pyruvate, (10); (all as N-ethylmorpholine salts) and KCl, (12.5). The assay system with normal substrate concentration was (□) as described in Section II.B.6b but without added acetyl CoA.

The acetyl CoA-dependent assays were started by the addition of 0.025 units of enzyme, while the acetyl CoA-independent assays were initiated with 1 unit of enzyme.

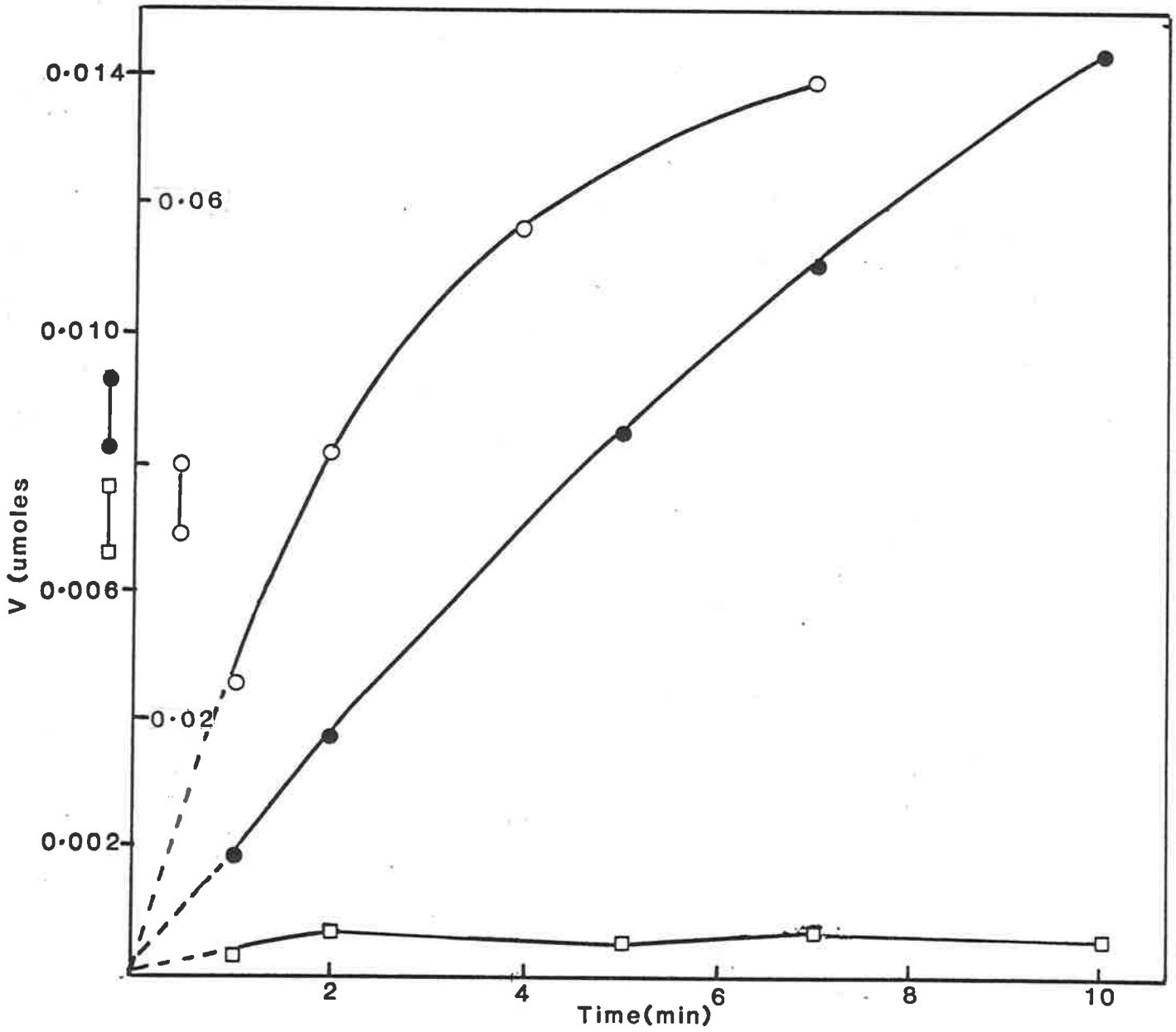

0·014
0·06
0·010
0·006
0·02
0·002
V (umoles
2
4
6
8
10
Time(min)

FIGURE VI.4

EFFECT OF VARYING K^+ CONCENTRATIONS ON THE RATE OF THE ACETYL CoA-INDEPENDENT ACTIVITY

The assay systems in the presence of acetyl CoA (▲), in the absence of acetyl CoA with high concentrations of substrates (■) and in the absence of acetyl CoA with normal concentrations of substrates (□) were as described in Figure VI.3., except that the concentration of KCl was varied, as indicated. The Cl^- ion was maintained at 100 mM with N-ethylmorpholine Cl^-, pH 7.8.

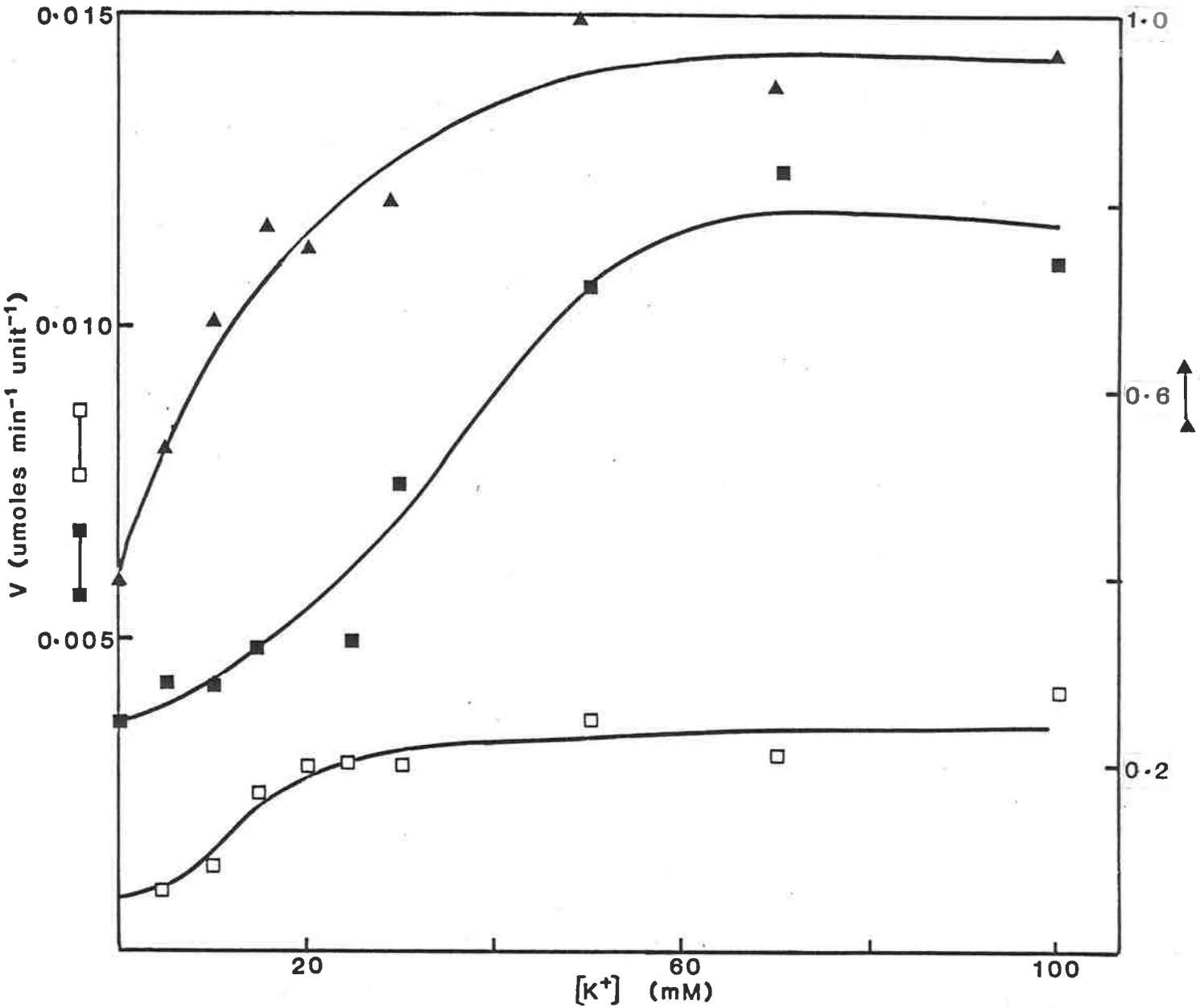
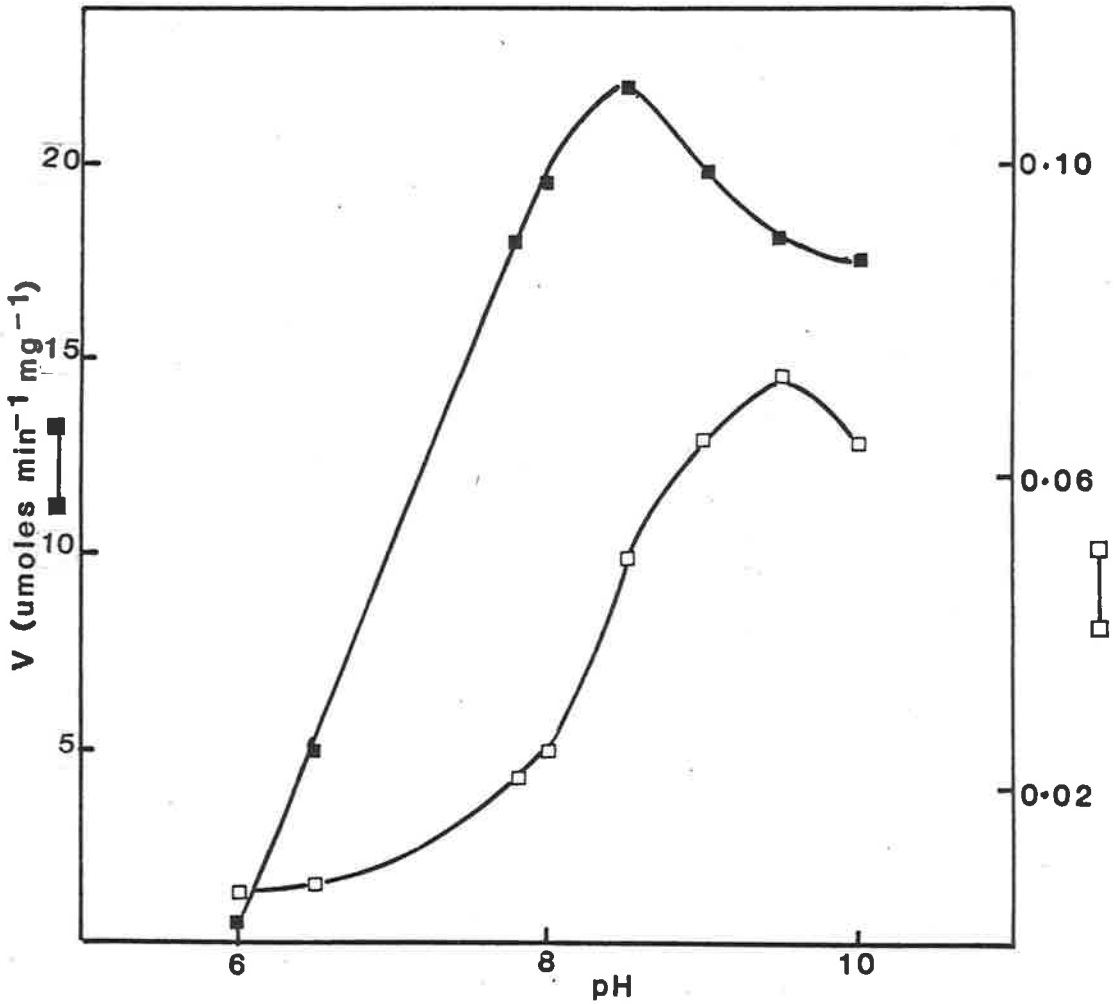

0·015
0·010
0·005
V (umoles min⁻¹ unit⁻¹)
1·0
0·6
0·2
20
60
100
[K⁺] (mM)

FIGURE VI.5

EFFECT OF pH ON THE ACETYL CoA-INDEPENDENT REACTION CATALYSED BY CHICKEN LIVER PYRUVATE CARBOXYLASE

The assay system in the presence of acetyl CoA (■) and in the absence of acetyl CoA (□) were as described in Figure VI.3., except that the pH of the N-ethylmorpholine (Cl^-) buffer was as indicated.


20
15
10
5
V (umoles min⁻¹ mg⁻¹)
0·10
0·06
0·02
6
8
10
pH

CHAPTER VII

AFFINITY LABELLING AND ISOLATION OF THE Mg-o-ATP-PEPTIDE

VII.A INTRODUCTION

The results presented in the preceding chapters described sequence of events occurring at the first partial reaction subsite. For a better understanding of the catalytic events in terms of possible intermediates formed at the first partial subsite, it is important to elucidate the role of various amino acid residues involved in the binding and catalytic processes. As discussed in more detail in Section I.G., an understanding of the amino acid sequence and the arrangement of the essential residues in the three dimensional structure is a pre-requisite for a precise description of the reaction mechanism.

Chemical modification of amino acid residues at the active site has provided invaluable evidence for identifying catalytically important residues, and affinity labelling in particular enables a more specific modification of a ligand at the binding site.

The 2',3'-dialdehyde derivative of ATP (oATP) has been shown to be an affinity label of sheep liver pyruvate carboxylase (Easterbrook-Smith *et al.*, 1976b) and has led to the identification of a lysine residue at the ATP binding site (The use of ATP analogues as affinity labels has been reviewed by Yount, 1975). Owing to the seasonal variation in the yields of the sheep liver pyruvate carboxylase, and in view of the large amounts of protein required for isolation and purification procedures so as to be left with sufficient quantities for subsequent sequence determination, pyruvate carboxylase from chicken liver was chosen for this study. Although it was expected that oATP would function as an

affinity label of ATP for the chicken liver enzyme, nevertheless it was necessary to establish this property in the chicken liver enzyme. In addition, the possibility of the affinity label reacting with reactive lysine residues other than the one at the ATP binding site could lead to complications in the subsequent purification of tryptic peptides covalently labelled with oATP. Hence, the initial approach used in this study was to establish the specificity of oATP as an affinity label of the ATP binding site of the chicken liver enzyme, using the experimental criteria as detailed by Singer (1967).

VII.B. MATERIALS AND METHODS

VII.B.1 Assay Methods

The procedures for measuring the rate of pyruvate carboxylation; the determination of the concentrations of protein and biotin were carried out as described in the preceding Chapters.

VII.B.2 Synthesis of oATP

Synthesis of oATP was carried out by the periodate oxidation method as described by Easterbrook-Smith *et al.* (1976b). o[U-^{14}C]ATP was prepared from [U-^{14}C]ATP. oATP was purified on a Sephadex G-10 column (20 x 2 cm) previously equilibrated with glass distilled water at 4°C. The column was eluted with distilled water, and the leading half of the nucleotide peak pooled, and stored at -80°C. The concentration of oATP was determined by measuring the absorbance at 258 nm using a value of 14900 $cm^{-1}M^{-1}$ for the extinction

coefficient (Hansske *et al.*, 1974).

The purity of the oATP was determined by chromatography on PEI thin layers using 0.8 M NH_4HCO_3 as the developing solvent. The nucleotide was detected by exposure to UV light. Chromatography of the purified sample revealed only one compound with an Rf value of ≈0.02. No ATP (Rf ≈0.24) could be detected. When radioactive starting material was used, the areas corresponding to ATP and oATP were cut out and counted in toluene scintillation fluid to determine the purity of the oATP.

VII.B.3 Synthesis of Lysyl-[U-^{14}C]oATP

This synthesis was carried out by the method described by Easterbrook-Smith *et al.* (1976b). The reaction mixture contained (in μmoles) in a final volume of 0.45 ml; o[U-^{14}C]-ATP, (1.8) (2 x 10^6 cpm/μmole); lysine, (5); $MgCl_2$, (2.5); and sodium borohydride, (5). The solution was adjusted to pH 11 with KOH, and the mixture allowed to stand at room temperature for 40 min after addition of the sodium borohydride. The adduct was purified by ascending chromatography on Whatmann 3 MM paper, using 1-butanol:acetic acid:water (4:1:5 v/v, top phase) as the developing solvent.

VII.B.4 Covalent Modification of the Enzyme

Pyruvate carboxylase (10 - 20 units/ml) was incubated with oATP in 0.1 M N-ethyl morpholine acetate buffer, pH 7.8, containing $MgCl_2$ in two-fold excess over oATP. After 5 min at 30°C, sodium borohydride was added in ten-fold molar excess over the oATP used and the solution allowed to stand for 60 min.

VII.C RESULTS

VII.C.1 Irreversible Inhibition by oATP

Incubation of chicken liver pyruvate carboxylase with Mg-oATP^{2-} at 30°C and on reduction with sodium borohydride, resulted in loss of 50% of the overall activity in 10 min after which a further slow inactivation occurred. Finally the activity remained constant after 60 min (Fig. VII . 1). The effect of incubating the enzyme with varying concentrations of Mg-oATP^{2-} is shown in Fig. VII. 2. The control system, in which Mg-oATP^{2-} was replaced by MgATP^{2-}, did not lose enzyme activity indicating that the inactivation was dependent on the presence of oATP.

VII.C.2 Protection of the Enzyme from Inactivation by oATP

One important criterion to ascertain whether an inhibitor is active site directed is to observe protection by the normal substrate against the inactivation process. The results presented in Fig. VII. 3 show that ATP protected the enzyme against the inactivation by oATP. Protection was also afforded by acetyl CoA (Fig. VII. 4), although the extent of the protection was less in this case. The protection obtained by increasing concentrations of acetyl CoA could be interpreted to suggest that oATP binds at the acetyl CoA binding site. Although a lysine residue has been implicated at the binding site of the adenine moiety of acetyl CoA (Ashman *et al.*, 1973), the evidence presented below shows that only one molecule of oATP binds per enzyme monomer. It is possible then, that the binding sites for acetyl CoA and ATP are close and/or that the binding of

acetyl CoA influences the microenvironment of the ATP binding site.

The data presented above provides evidence that oATP was binding at the ATP binding site of the enzyme. Another criterion for affinity labelling is that the interaction between the number of moles of label and the number of active sites should have a one-to-one stoichiometric relationship. To establish this criterion, the enzyme was modified using o[U-^{14}C]ATP as described in Section VII.B.4 and after addition of sodium borohydride the residual enzymic activity was determined. The amount of radioactivity covalently attached to the enzyme was also determined as described by Easterbrook-Smith *et al.* (1976b). The results of several experiments carried out are shown in Table VII.1. This summary shows that at 100% inactivation one molecule of o[U-^{14}C]ATP is bound per biotin molecule.

These findings are similar to those found in the case of the sheep liver enzyme (Easterbrook-Smith *et al.*, 1976b) and supports the conclusion that oATP is an affinity label. of the chicken liver enzyme.

VII.C.3 Isolation of the oATP-Peptide

VII.C.3.1 Preparation

Chicken liver pyruvate carboxylase (1023 units), specific activity, 20) was covalently modified with o[U-^{14}C]-ATP (1.4×10^6 cpm/μmole) as described in Section VII.B.4. After 60 min when the enzymic activity remained constant, the modified enzyme was dialysed in 0.1 M citrate buffer pH 5.9 (Hemington & Dawson, 1968); under these conditions pyruvate carboxylase precipitates. This acid precipitation

step was included due to the large amounts of other proteins present in enzyme preparations of low specific activity. The precipitated protein was found to be pure as judged by electrophoresis on 10% SDS gels as described in Section II.B.9. The precipitated protein was collected by centrifuging, dissolved in 6 M guanidine hydrochloride, and was subjected to S-carboxymethylation as described in Section II.B.10. The solution was then dialyzed against 2 L, 0.5% NH_4HCO_3 for 24 h with two changes of the NH_4HCO_3 solution. The protein was then digested with 1% (w/w) TPCK-treated trypsin for 2 h at 37°C, after which a further 1% (w/w) was added and digestion continued for another 2 h at 37°C. The solution was then lyophilized.

VII.C.3.2 Identification of the modified Amino Acid

To identify the residue modified, an aliquot of the trypsin digest was dissolved in 0.5 ml of 0.1 M N-ethyl morpholine acetate buffer pH 8.0 and enzymically digested as follows; The tryptic peptides were first digested with two successive treatments of 1% (w/w) chymotrypsin at 37°C, 7.5 h apart. This was then subjected to pronase digestion 10% (w/w) at 37°C in two successive treatments, 24 h apart.

Aliquots of the digest were resolved by ascending chromatography as described in Section VII.B.3. The results of the experiment are shown in Fig. VII.5 where it can be seen that the radioactive compound from the protein digest co-chromatographed with the lysyl-o[U-^{14}C]ATP marker. The radioactive material which appeared as a streak at the

origin of the chromatogram may be due to overloading of the paper with the material.

VII.C.3.3 Fractionation of Peptides

The rest of the tryptic digest from Section VII. C.3.1 was dissolved in 0.5% NH_4HCO_3, applied to a Sephadex G-50 column (3 x 130 cm), and eluted with the same buffer at 4°C. Peptide containing fractions were detected by measuring the absorbance at 220 nm. Those peptide-containing fractions which were radioactive were pooled, lyophilized, dissolved in 200 µl of 50% pyridine, and applied to Whatman 3 MM paper. Electrophoresis was carried out at 3 Kv for 40 min using pH 1.9 buffer (Acetic acid/formic acid/water, 8:2:90). Peptides were detected by staining a 1 cm strip with Ninhydrin-acetone (0.1% ninhydrin) reagent. The location of the ^{14}C-labelled peptide which remained at the origin was determined by autoradiography. This ^{14}C-labelled material (Fig. VII.6) was eluted with 50% pyridine, lyophilized and dissolved in 50% pyridine, and further purification was carried out by electrophoresis on thin layer cellulose sheets, at 300 V for 3.5 h using pH 3.5 buffer (pyridine/acetic acid/H_2O, 1:10:189). The peptides were detected by staining a edge strip of 1 cm with ninhydrin-acetone while the radioactivity was detected by strip counting of the thin layer. Fig. VII.7 shows the resulting profile of the thin layer electrophoretogram. The major radioactive and ninhydrin-positive band which migrated 4 - 6 cm towards the anode was scrapped off the thin layer chromatogram, resuspended in 50% pyridine and transferred to a 5 ml syringe fitted with cotton-wool plugs. The radio-

active material was eluted by centrifuging (2000 rpm for 5 min). The centrifugation was repeated after adding more eluting buffer (50% pyridine). The peptide solution, filtered through the cotton-wool plug was collected in a 10 ml centrifuge tube and freeze-dried.

The dried material was dissolved in 50% pyridine and a small aliquot (1 nmole) was subjected to N-terminal analysis by dansylation as described in Section II.B.11c. The dansyl amino acids which appeared at this stage of the purification were o-dansyl-tyrosine dansyl-isoleucine and a faint dansyl amino acid spot appearing in the region where α-dansyl-lysine appears.

In view of the possibility of a minor contaminating peptide, a further purification step was included. The material from above was subjected to electrophoresis on a thin layer cellulose sheet at 300 V for 4 h using a pH 4.5 buffer (pyridine/acetic acid/acetone/H_2O, 2:4:15:73). The radioactivity and the peptides were detected as described above and the results are shown in Fig. VII.8, where the major ninhydrin positive band was detected with the radioactivity (3 - 5 cm towards the anode). In addition two faint ninhydrin bands migrating towards the cathode were detected as well as a small amount of material which remained at the origin. The radioactive material was recovered by the procedures described above, freeze-dried and subjected to N-terminal analysis by dansylation. The dansyl amino acids which were detected were o-dansyl-tyrosine and dansyl-isoleucine. These results suggested that the isolated peptide sample contains a single peptide with isoleucine as the N-terminal amino acid. Although

the above procedures achieved purification of the oATP-peptide, the amount of material recovered was not sufficient for sequence determination. A preliminary analysis of the amino acid content of the oATP-peptide indicated a large peptide of approximately 30 residues, however an accurate determination was not possible due to insufficient quantities. Since the above methods involved numerous steps in the purification procedures, great losses were incurred and hence other methods were attempted to achieve purification of the peptide in the least number of steps possible.

VII.C.4 Other Methods of Purification

VII.C.4.1 The Charcoal-adsorption Method.

In this method (adapted from Anderson *et al.*, 1973) the adenine moiety of oATP was exploited to selectively adsorb the oATP-peptide on to charcoal and hence an extensive purification was expected in this single step. The method involved adding a suspension of acid-washed charcoal (Norit A) in water at pH 5, to the tryptic digest prepared from the o[U-^{14}C]ATP modified enzyme, (prepared as described in Section VII.C.3.1). The Norit A suspension was added in small increments till all of the ^{14}C-radioactivity was bound and then the suspension was centrifuged. The charcoal was resuspended in water and washed exhaustively to remove nonspecifically bound peptides. The oATP-peptide was then extracted from the Norit A by repeatedly washing the suspension with a solution of 3% ammonia in 50% acetone. The suspension was centrifuged and the oATP-peptide was recovered in the supernatant. The

charcoal was further washed with a solution of 5% ammonia in 50% acetone. This method has been used successfully to purify nucleotide-labelled peptides (Anderson *et al.*, 1973). However in the case of the oATP-peptide from chicken liver pyruvate carboxylase the recovery was poor (≈10%) probably due to the strong adsorption of this large peptide to the charcoal. In view of this result, a purification procedure utilizing cation-exchange resin to adsorb other peptides while not binding the oATP-peptide, was expected to give higher recoveries of the desired peptide. The strategy was to exploit the low pK values of the phosphate groups on the oATP-adduct and to design a purification procedure utilizing a cation exchanger with a anionic buffer of suitably low pH, so as to adsorb all other peptides (with net positive), on to the resin. The oATP-peptide which would have a net negative charge would not be bound and hence could be purified in this manner.

VII.C.4.2 The ion-exchange method

Chicken liver pyruvate carboxylase was covalently modified with o[U-^{14}C]ATP as described in Section VII.B.4. The modified enzyme was dialysed in the citrate buffer as described in Section VII.C.3.1. The precipitate which appeared on dialysis, was recovered by centrifuging. Tryptic peptides were then prepared from the precipitated material by the same procedures as described in Section VII.C.3.1. The tryptic digest was lyophilized, redissolved in 0.5 M sodium acetate buffer, pH 2.5, and added to a slurry of sulpho-propyl-Sephadex C-50 (SP-C-50), previously equilibrated with the same buffer. After stirring for 1 h at

4°C, the slurry was filtered and the oATP-peptide recovered in the filtrate. The filtrate was lyophilized, redissolved in pH 2.5 buffer (10% acetic acid, pH adjusted with pyridine) and was subjected to electrophoresis at 300 V for 1½ h using the pH 2.5 buffer. The results shown in Fig. VII.9 indicate that the major portion of the peptides were removed by the cation resin at this low pH and hence this procedure relying as it does on the low pk's of the phosphate groups on the oATP-adduct is potentially an efficient way of enabling substantial purification of the oATP-peptide in a single step.

VII.D CONCLUSION

The 2',3'-dialdehyde derivative of ATP (oATP) was shown to be a affinity label of the $MgATP^{2-}$ binding site of chicken liver pyruvate carboxylase, using the experimental criteria of Singer (1967). Having established the specificity of oATP in modifying a single lysine residue at the $MgATP^{2-}$ binding site, the tryptic oATP-peptide has been isolated and was shown to be a large peptide with ~30 amino acid residues. In order to isolate sufficient quantities of the oATP-peptide for subsequent determination of the primary structure, different methods have been evaluated and a method using a cation exchange resin as an initial purification procedure followed by electrophoresis on thin layer cellulose sheets, has proved to be the most efficient one.

FIGURE VII.1

IRREVERSIBLE INACTIVATION BY REDUCTION OF THE ENZYME-MgoATP^{2-} COMPLEX

Pyruvate carboxylase (30 u/mg, 10 units/ml) was modified with Mg-oATP^{2-} (0.6 mM) (■), for the times indicated, by the procedure described in Section VII.B.4. In the control (O) Mg-oATP^{2-} was replaced by MgATP^{2-}.

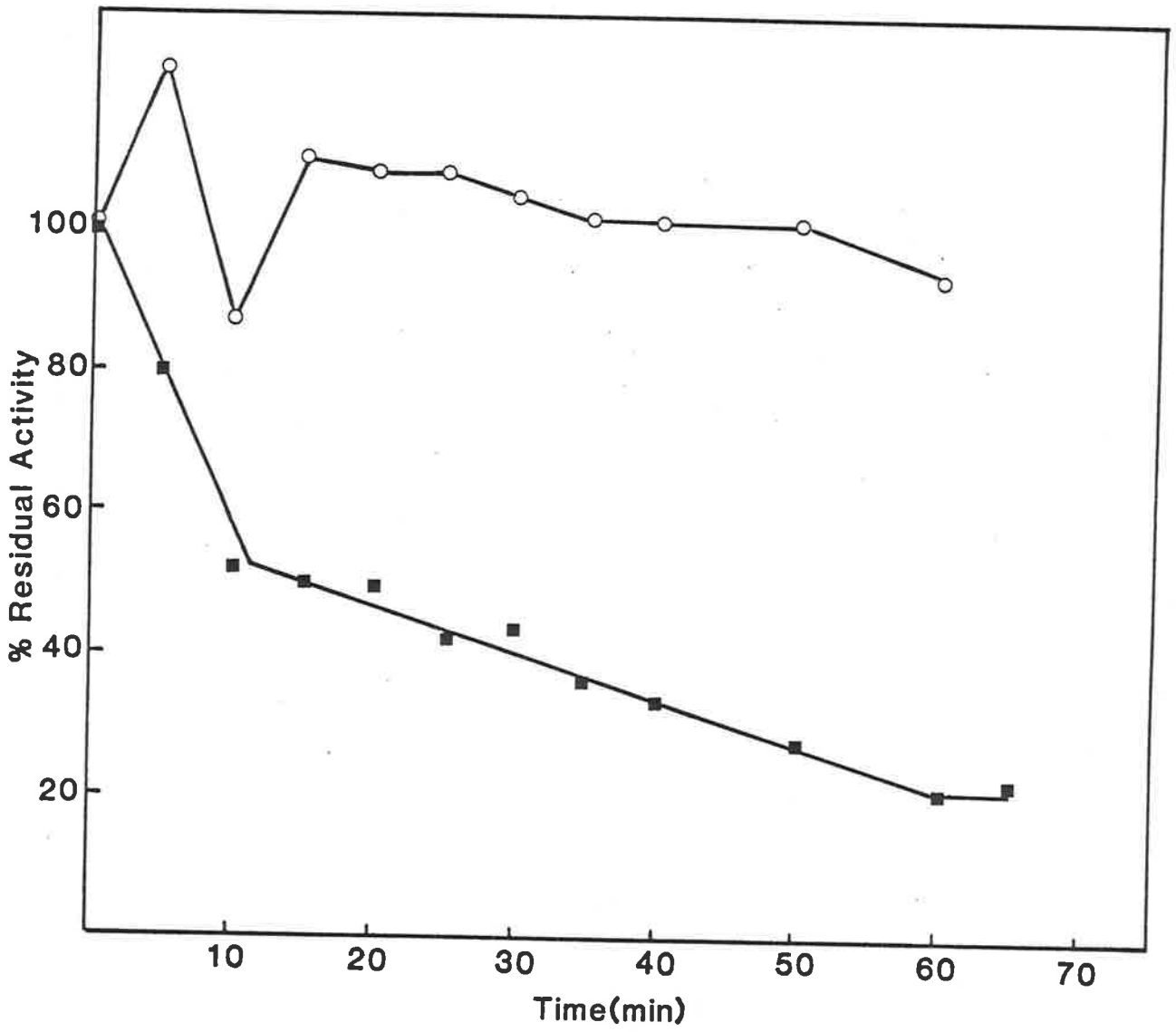

100
80
60
40
20
% Residual Activity
10
20
30
40
50
60
70
Time(min)

FIGURE VII.2

IRREVERSIBLE INACTIVATION BY REDUCTION OF THE ENZYME-MgoATP^{2-} COMPLEX

Pyruvate carboxylase (25 u/mg, 10 units/ml) was modified with variable concentrations of Mg-oATP^{2-} (□), by the procedure described in Section VII.B.4. In the control (▲) Mg-oATP^{2-} was replaced by MgATP^{2-}.

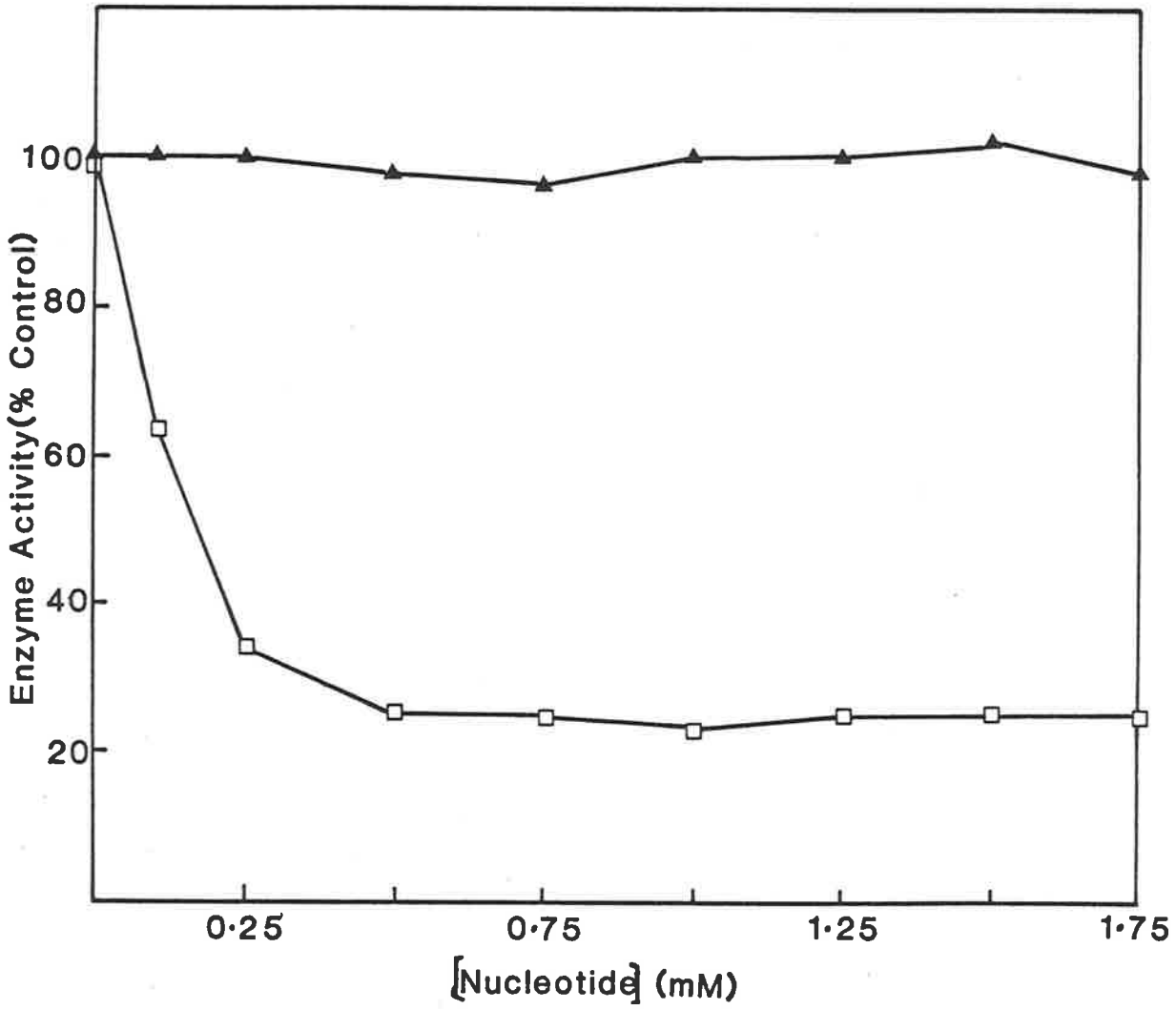

100
80
60
40
20
Enzyme Activity(% Control)
0·25
0·75
1·25
1·75
[Nucleotide] (mM)

FIGURE VII.3

PROTECTION OF PYRUVATE CARBOXYLASE AGAINST IRREVERSIBLE Mg-oATP^{2-} MODIFICATION BY MgATP^{2-}.

Modification of pyruvate carboxylase (30 u/mg, 10 u/ml) using 0.5 mM Mg-oATP^{2-} (□), in the presence of variable concentrations of MgATP^{2-} was carried out by the procedure described in Section VII.B.4. In the control (▲), Mg-oATP^{2-} was replaced by MgATP^{2-}.

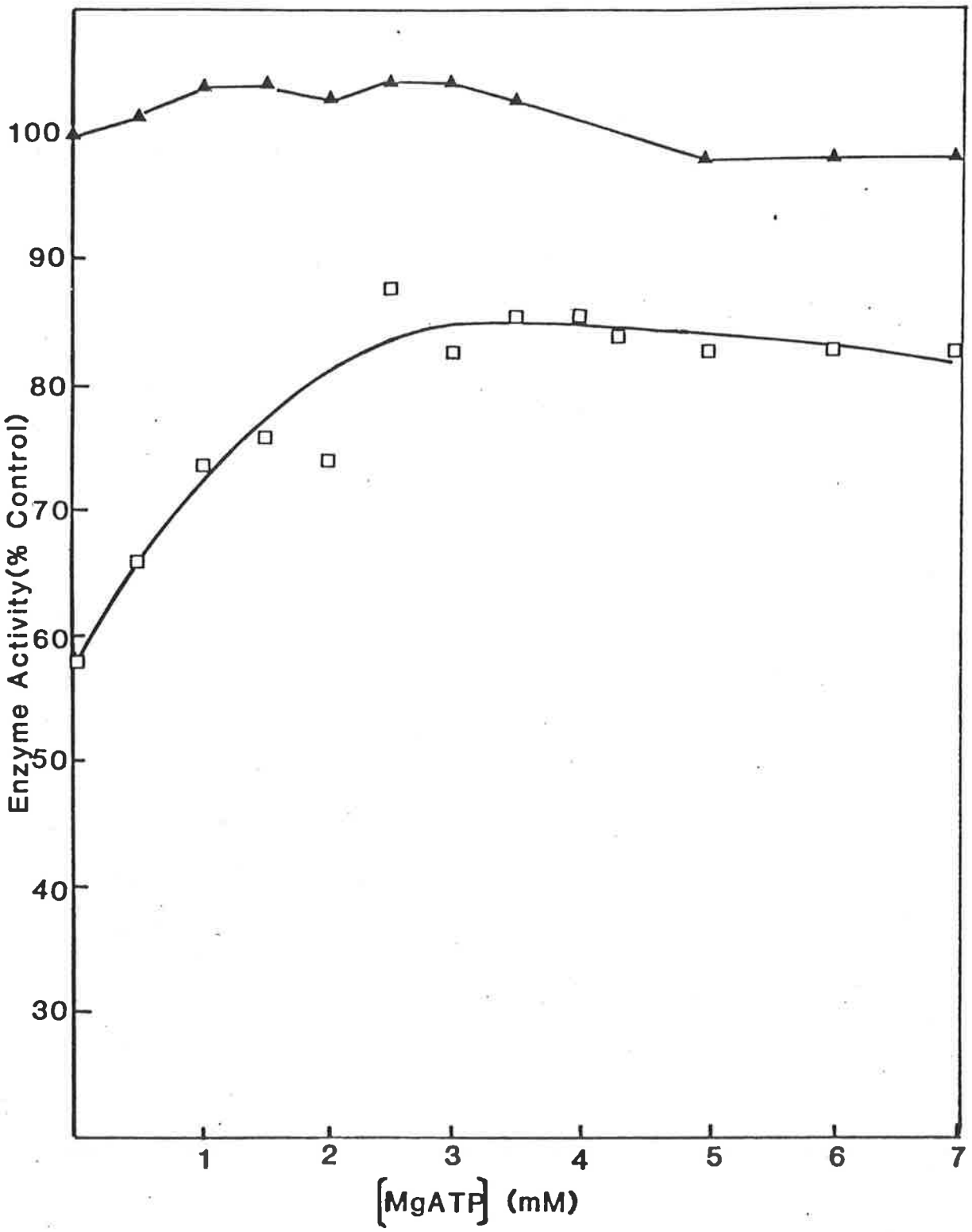

100
90
80
70
60
50
40
30
Enzyme Activity(% Control)
1
2
3
4
5
6
7
[MgATP] (mM)

FIGURE VII.4

PROTECTION OF PYRUVATE CARBOXYLASE BY ACETYL CoA AGAINST IRREVERSIBLE MODIFICATION BY $Mg\text{-}oATP^{2-}$

Modification of pyruvate carboxylase using 1.4 mM Mg-oATP (□) in the presence of variable concentrations of acetyl CoA was carried out using the procedure described in Section VII.B.4. In the control (▲) $Mg\text{-}oATP^{2-}$ was replaced by $MgATP^{2-}$.

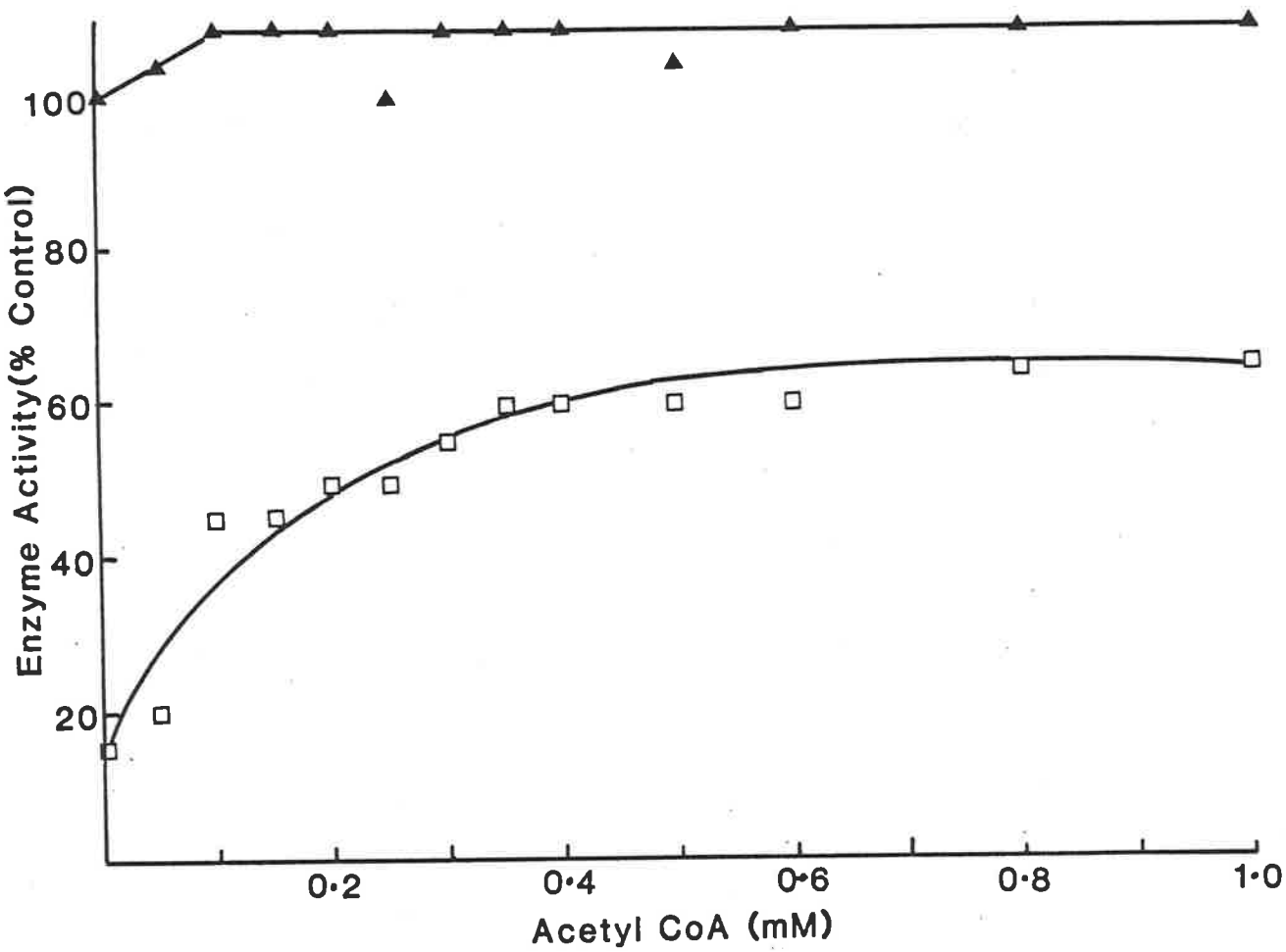

Enzyme Activity(% Control)
100
80
60
40
20
0·2
0·4
0·6
0·8
1·0
Acetyl CoA (mM)

TABLE VII.1

STOICHIOMETRY OF IRREVERSIBLE INACTIVATION OF PYRUVATE CARBOXYLASE BY Mg-oATP^{2-}

Pyruvate carboxylase (16 - 30 u/mg) was modified with varying concentrations of Mg-o[U-^{14}C]ATP using the procedure described in Section VII.C.2.

Enzyme specific Activity (units/mg of protein)	$\frac{\text{Moles of oATP bound}}{\text{Moles of biotin}}$ at 100% inactivation
16	0.91
20	1.05
25	1.01
30	1.09

FIGURE VII.5

IDENTIFICATION OF THE MODIFIED AMINO ACID RESIDUE

Aliquots of the enzyme digest of o[U-^{14}C]-ATP modified pyruvate carboxylase (prepared as described in Section VII.C.3.2) were subjected to paper chromatography on Whatman 3 MM paper, using 1-butanol/acetic acid/water (4:1:5 v/v), (top phase), as the developing solvent lysyl-o[U-^{14}C]ATP was used as the marker (●), digest of the modified enzyme (O).

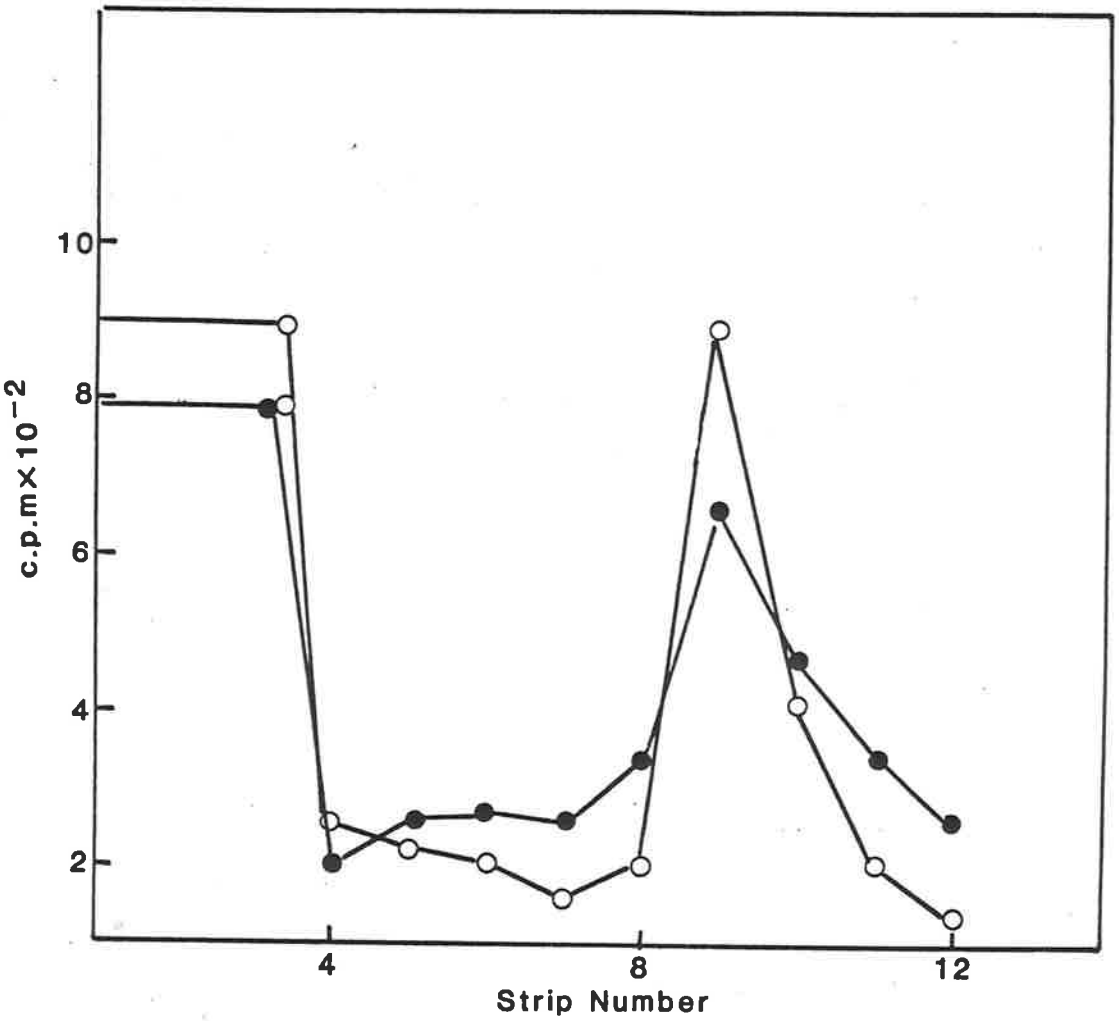

10
8
6
4
2
c.p.m×10⁻²
4
8
12
Strip Number

FIGURE VII.6

PURIFICATION OF oATP-PEPTIDE BY PAPER ELECTROPHORESIS

Electrophoresis was carried out on Whatman 3 MM paper using pH 1.9 buffer (acetic acid/formic acid/water, 8:2:90) for 40 min at 30 kV. 1 cm strip from the edge of the chromatogram was stained with ninhydrin-acetone (0.1 ninhydrin) to detect the peptides (⊢⊣). The location of the ^{14}C-labelled peptide (////) detected by autoradiography.

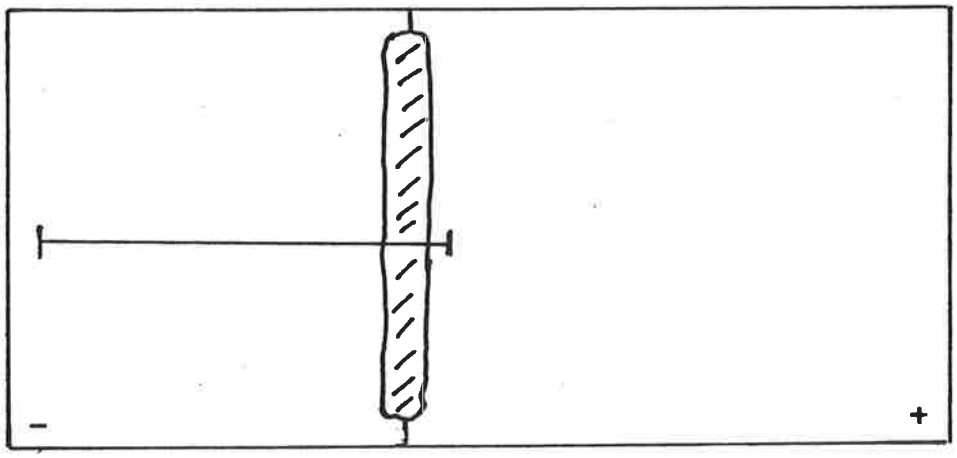


FIGURE VII.7

PURIFICATION OF oATP-PEPTIDE BY THIN-LAYER ELECTROPHORESIS

Electrophoresis was carried out on thin-layer cellulose sheets, using pH 3.5 buffer (pyridine/acetic acid/H_2O, 1:10:189), at 300 V for 3.5 h. Peptides (⊢⊣) were detected by staining an edge strip of 1 cm, with ninhydrin-acetone, while the radioactively labelled peptide (■) was detected by counting 1 cm pieces of a 1 cm edge strip, in toluene scintillation fluid.

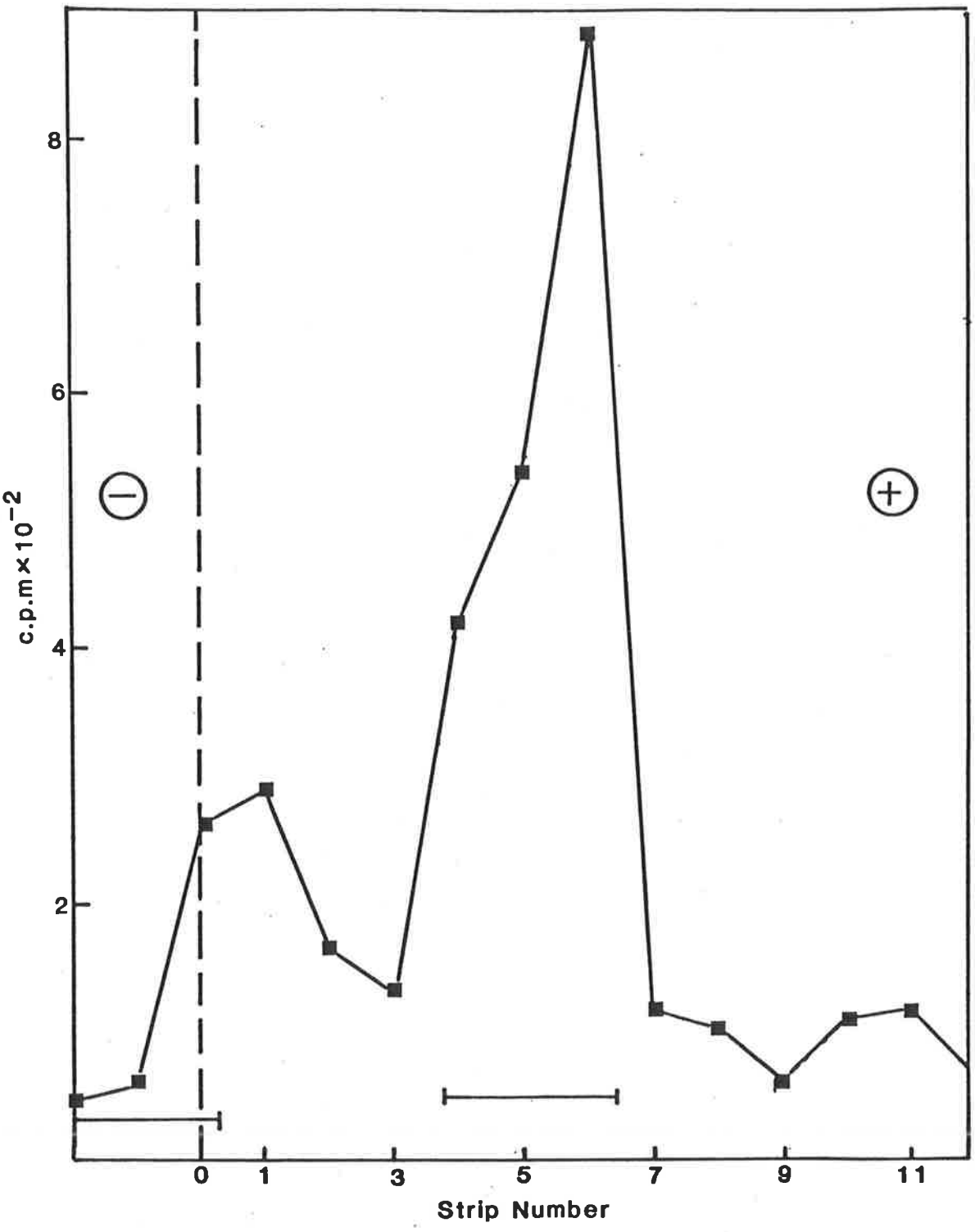

8
6
4
2
c.p.m×10⁻²
⊖
⊕
0
1
3
5
7
9
11
Strip Number

FIGURE VII.8

PURIFICATION OF oATP-PEPTIDE BY THIN-LAYER ELECTROPHORESIS

Electrophoresis was carried out on thin-layer cellulose sheets, using a pH 4.5 buffer (Pyridine/acetic acid/acetone/H_2O, 2:4:15:73), for 4 h at 300 V.

A 1 cm strip from the edge was stained with ninhydrin-acetone (⊢⊣), while the [^{14}C] labelled material (●) was detected as described in Figure VII.7.

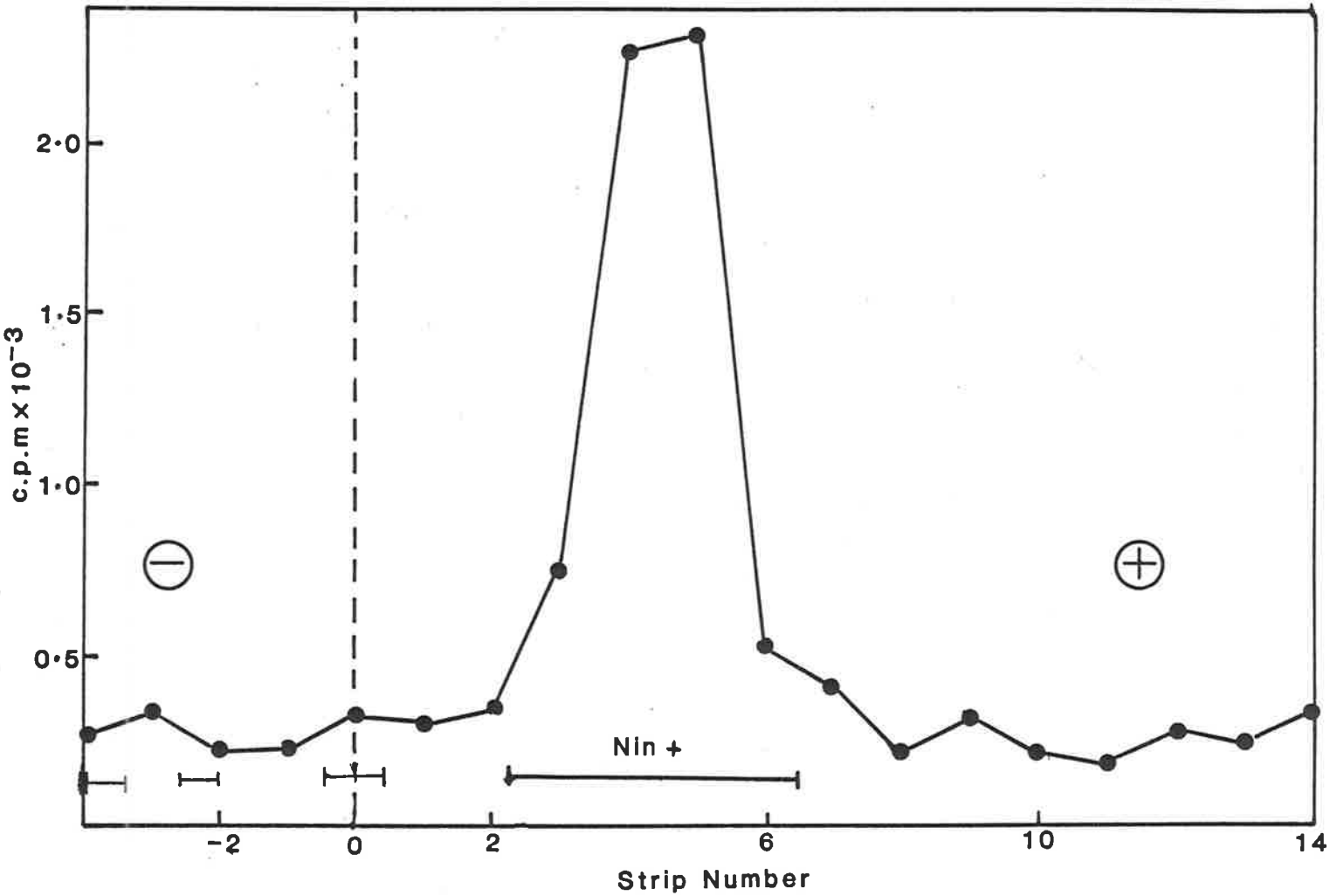
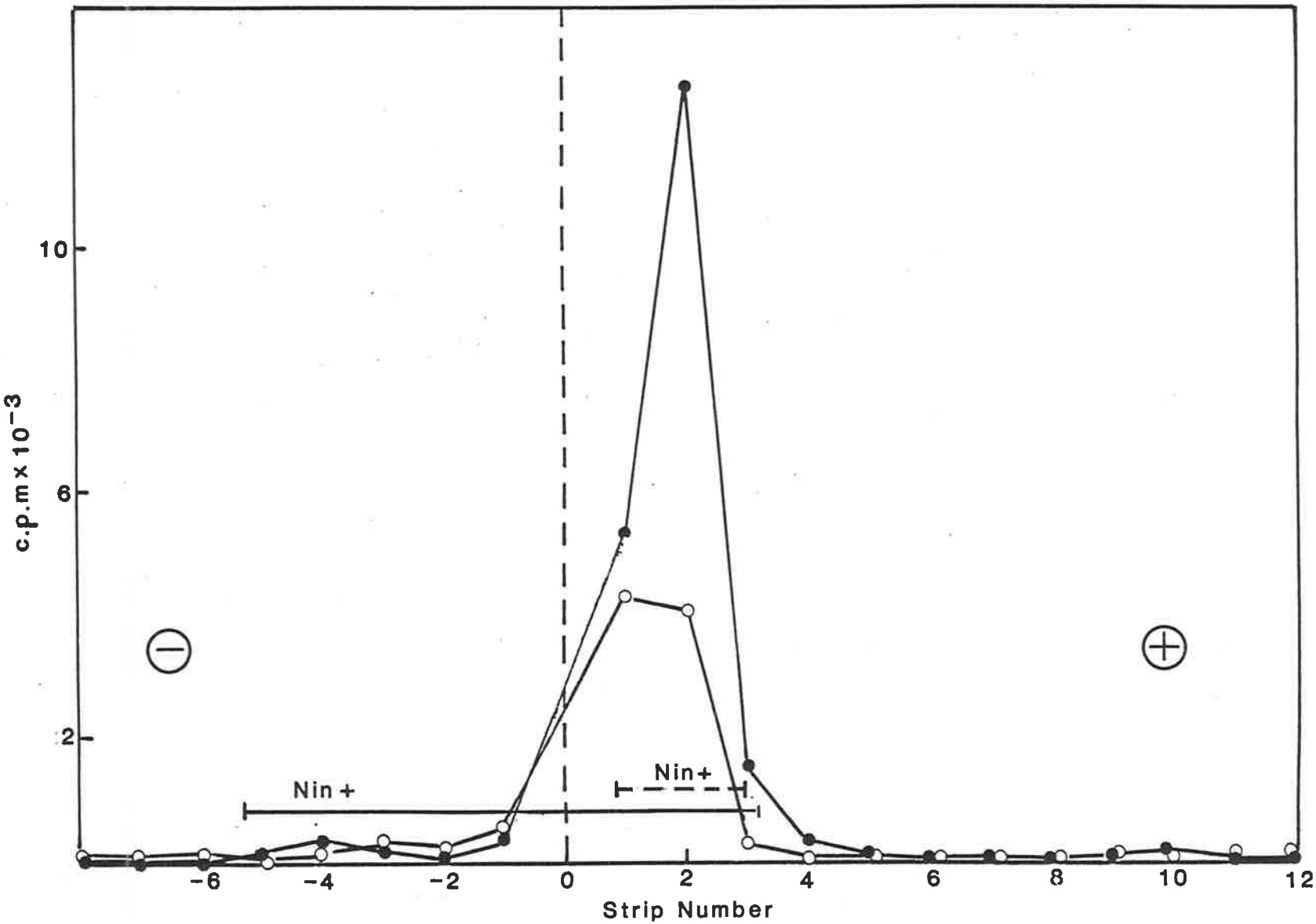

2·0
1·5
1·0
0·5
c.p.m x 10⁻³
⊖
⊕
Nin +
-2
0
2
6
10
14
Strip Number

FIGURE VII.9

THIN-LAYER ELECTROPHORESIS OF A TRYPTIC DIGEST OF o-[U-^{14}C]ATP MODIFIED ENZYME AFTER PURIFICATION BY SP-C-50 CATION EXCHANGE CHROMATOGRAPHY

Electrophoresis was carried out on thin-layer cellulose sheets, using a pH 2.5 buffer (10% acetic acid, pH adjusted with pyridine) at 300 V for 1.5 h. The peptides were detected by staining a 1 cm strip with ninhydrin-acetone (⊢—⊣), before purification by SP-C-50; (⊢-⊣), after purification. The [^{14}C] labelled material before purification by SP-C-50, (●) and after purification (○).


10
6
2
c.p.m x 10⁻³
⊖
⊕
Nin +
Nin+
-6
-4
-2
0
2
4
6
8
10
12
Strip Number

CHAPTER VIII

GENERAL DISCUSSION

VIII. GENERAL DISCUSSION

The studies presented in this thesis have been directed towards elucidation of sequence of events occurring at the first partial reaction subsite of pyruvate carboxylase isolated from chicken liver mitochondria. The data obtained and their significance have been discussed to some extent in the respective chapters and in this section an over-view of the findings as well as some possible extensions to these studies are described.

VIII.1 The Mechanism of the First Partial Reaction

The question of a concerted versus stepwise mechanism for the coupling of ATP hydrolysis to CO_2 fixation in the biotin-dependent enzymes has been the subject of extensive studies which have yielded results consistent with either type of mechanism. The studies described in this thesis have shown that the carboxylation of biotin is not a concerted process and on the basis of all the available data, a step-wise mechanism involving the possible participation of carboxyphosphate has been proposed.

Although the mechanism proposed (Fig. III.9) was not very different from the substrate-activation model (Fig. I.3c), some main differences are

(i) that ATP binds to the enzyme before the binding of HCO_3^-,

(ii) that ADP is released before the release of Pi and

(iii) that acetyl CoA facilitates the formation of ENZ-carboxybiotin from the postulated ENZ-carboxyphosphate with concurrent release of Pi.

The evidence for the proposal of the mechanism has been obtained from studies with the chicken liver enzyme, although as discussed in Section I.D., the data obtained from studies on different biotin-dependent enzymes have shown strong similarities. It is possible then, that in view of the common minimal mechanism of the first partial reaction in the biotin-dependent carboxylases, the mechanism as proposed in Fig.III.9, may well be a common feature of biotin-dependent carboxylases. In those enzymes which do not catalyse an ATP:ADP isotopic exchange reaction which is dependent only on Mg^{2+}, it is possible that ADP is associated strongly with the postulated ENZ-carboxyphosphate and is only released after the formation of ENZ-carboxybiotin, together with Pi. In such cases, investigation of intramolecular positional exchange in the reisolated ATP may reveal the existence of an intermediate such as the postulated carboxyphosphate. The method developed by Midelfort & Rose (1976) could be used to detect reversible cleavage of bound ATP to bound [ADP · $HO\text{-}\overset{O}{\overset{\|}{C}}\text{-}OPO_3^{2-}$]. This reaction does not require that ADP dissociated from the enzyme. The method involves enriching the ATP βγ-bridge oxygen with ^{18}O, and then incubating the enzyme under conditions postulated to produce enzyme-bound [ADP · carboxyphosphate]. [^{18}O]ATP is then reisolated and analyzed for ^{18}O content of the β-nonbridge oxygens. When ATP is cleaved to bound ADP, the isotope becomes scrambled among the β-phosphorous oxygens of ADP. Thus, reformed ATP will exhibit β-nonbridge ^{18}O enrichment due to positional exchange of the βγ-bridge and β-nonbridge oxygens. In addition to demonstrating the

existence of an intermediate, the method can also evaluate the kinetic competency of the enzyme-bound intermediate. The isotope scrambling method has been used successfully to demonstrate the kinetic feasibility of a γ-glutamyl-P intermediate in the glutamine synthetase reaction (Midelfort & Rose, 1976).

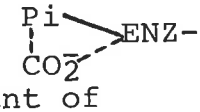
The isotopic exchange studies involving ATP, in particular the Mg^{2+}-dependent ATP:ADP isotope exchange reaction, have been the major source of evidence in support of the proposed step-wise mechanism; however the slow rate of these exchange reactions has often caused doubts about their kinetic significance. The evidence presented here suggests that the slow rate of the first partial exchange reaction, is due to a slow step in the reverse reaction. This slow step in the reverse reaction may be attributable to a rate-limiting step, very often involving a conformational change in the enzyme which in turn permits product release (Cleland, 1975). An extension to the above studies would be to determine the rate limiting step by the method first developed by Rose *et al.* (1974). The use of rapid-quench techniques can greatly enhance the efficiency of the above method and can easily determine the product/s which are dissociated at a slower rate.

VIII.2 The Nature of the Isolated Pi, CO_2^- ENZ-biotin Complex

The isolation of the Pi, CO_2^- ENZ-biotin complex has provided direct evidence for the validity of the step-wise mechanism. Although the precise nature of the complex is not known, it was postulated that the complex is enzyme-bound carboxy-

phosphate. The observation that the complex showed the same degree of kinetic competency with reference to its carboxyl as well as the phosphoryl group, supports the postulate, that the complex is carboxyphosphate derivative. Additional evidence was obtained when it was shown that the complex showed a similar degree of stability with reference to its phosphoryl or carboxyl group.

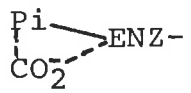
In any case an obvious extension to this area of study would be to determine if the isolated complex was carboxyphosphate derivative. Preliminary attempts to characterize the isolated complex involved eluting the radioactively labelled complex from the Sephadex column, directly into diazomethane. After esterification, the protein was digested with trypsin and the radioactive tryptic peptides were resolved by electrophoresis. However, the results were inconclusive due to breakdown of the complex during the separation procedures, and insufficient quantities of the material did not permit a suitable separation method to be evaluated. It is envisaged that a modification of the above procedures would lead to the identification of the isolated complex.

In attempting to characterize the isolated 

biotin complex, the possibility of an involvement of o-phosphobiotin in the carboxylation of biotin was considered. However the evidence detailed in Chapter V did not indicate its presence and hence the model proposed by Kluger & Adawadkar (1979) involving activation of the biotin prosthetic group by phosphorylation at the carbonyl oxygen as the first step in the first partial reaction has

been eliminated. However the experiments were designed to test the o-phosphobiotin model as outlined by Kluger & Adawadkar, and the involvement of a phosphorylated biotin species at a later stage in the reaction mechanism cannot be ruled out. Similarly the ability of biotin carboxylase to produce ATP from carbamyl phosphate and ADP in the absence of biotin is another piece of evidence against the involvement of biotin in ATP cleavage (Guchait *et al.*, 1974). This observation also eliminates the involvement of o-phosphobiotin. However as mentioned above this evidence would not rule out an involvement of o-phosphobiotin at a later stage of the reaction sequence i.e., after the hydrolysis of ATP. Nevertheless there seems to be no evidence to suggest such an involvement.

VIII.3 The Requirement of Activation by Acetyl CoA

The results presented in this thesis have shown that the 'absolute' requirement for activation by acetyl CoA exhibited by chicken liver pyruvate carboxylase is localized to the step catalysing the interconversion of the 

biotin complex and the ENZ-carboxybiotin complex. However the precise mechanism by which acetyl CoA facilitates this interconversion is not known. That acetyl CoA may exert its effect by labilizing either the phosphoryl or the carboxyl group of the putative enzyme-carboxyphosphate for subsequent carboxylation of the biotin moiety, has been considered as a possibility. Hence there remain a number of aspects of the participation of acetyl CoA which warrant further investigation.

Firstly the identification of that portion of the acetyl CoA moiety which participates in the interconversion might give some indication of the molecular aspect of the reaction. Although it is known that the adenosine moiety of acetyl CoA binds at the ATP binding site (Clements *et al.*, 1981) it is not known if other portions of the acetyl CoA molecule are involved in the participation of the interconversion between the two enzyme species. In view of the flexible molecular framework of acetyl CoA in aqueous solutions (Lee & Sarma, 1975), it is quite possible that the acetyl group, the β-cysteamine moiety or the pantothenic acid portions of the acetyl CoA molecule may be involved.

Secondly the data presented in this thesis indicate that K^+ may mimic the chief effect exerted by acetyl CoA. Hence substituting K^+ for acetyl CoA in the experiments carried out in Section VI.C.1 (where the formation of ENZ-carboxybiotin was shown to exhibit an absolute requirement for acetyl CoA) may reveal more information on the molecular events of acetyl CoA participation, in view of the simplicity of the function of the monovalent cation.

VIII.4 The Mg-ATP Binding Site

The data obtained in Chapter VII have shown that oATP reacts specifically with a lysyl residue at the $MgATP^{2-}$ binding site of the chicken liver enzyme. This affinity label has been exploited to isolate the oATP-labelled tryptic peptide. However the large size of the labelled tryptic peptide has led to some difficulties in its purifi-

cation by published methods. The method involving the use of cation exchange has shown to be potentially, an efficient way of enabling substantial purification of the oATP-labelled tryptic peptide. Once the sequence of this peptide has been determined, and the amino acid residue/s involved in the 

complex determined, this information could be used to build up a picture of the structural orientation of the first partial reaction sub-site. The sequence analysis of the ATP binding site in other biotin dependent carboxylases could yield information about the evolutionary relationship in addition to revealing further mechanistic information of the first partial reaction catalysed by biotin-dependent carboxylases.

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APPENDIX

PAPERS PUBLISHED OR IN PREPARATION

1. Pyruvate carboxylase: Mechanism of the first partial reaction
   (with D.B. Keech & J.C. Wallace)
   Proc. Aust. Biochem. Soc. 14, 12.

2. Pyruvate carboxylase: Mechanism of the first partial reaction
   (with J.C. Wallace & D.B. Keech)
   Manuscript in preparation.