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THE REACTION MECHANISM OF PYRUVATE CARBOXYLASE

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SUMMARY

1. The 2',3'-dialdehyde derivative of ATP (oATP) was prepared by periodate oxidation, and on the following criteria was considered to be an affinity label of the  $\text{MgATP}^{2-}$  binding site of sheep liver pyruvate carboxylase. The magnesium complex of this inhibitor ( $\text{Mg-oATP}^{2-}$ ) was shown to be a linear competitive inhibitor with respect to  $\text{MgATP}^{2-}$  in both the acetyl CoA-dependent and -independent activities of the enzyme but was a non-competitive inhibitor with respect to bicarbonate and an un-competitive inhibitor with respect to pyruvate.  $\text{Mg-oATP}^{2-}$  was covalently bound to pyruvate carboxylase by reduction using sodium borohydride with concurrent irreversible inactivation of the enzyme. Although pyruvate, oxaloacetate, and bicarbonate were ineffective, both acetyl CoA and  $\text{MgATP}^{2-}$  protected the enzyme against this chemical modification.  $\text{Mg}^{2+}$  enhanced the extent of chemical modification. At 100% inactivation,  $1.1 \pm 0.1$  moles of  $\text{Mg-oATP}^{2-}$  were bound to the enzyme per mole of biotin. The presence or absence of acetyl CoA had no effect on this stoichiometry. Chromatography of samples of an enzymic digest of  $\text{Mg-o}[^{14}\text{C}]\text{ATP}^{2-}$ -labelled enzyme revealed one major band of radioactivity which co-chromatographed with authentic lys-oATP markers.

2. In order to further characterise the lysyl residue(s) in the biotin carboxylation site, the use of cyanate as a potential affinity label of the bicarbonate binding site was investigated. Cyanate was shown to chemically modify sheep liver pyruvate carboxylase, with concurrent inactivation of the enzyme. The rate of loss of acetyl CoA-independent catalytic activity was not pseudo first-order with respect to time. When the inactivation data were analysed assuming that modified



enzyme retains some residual acetyl CoA-independent activity, it was found that only one kinetically significant residue per active site was modified, but cyanate appeared to form a reversible complex with the enzyme prior to modifying this residue.

Acetyl CoA, pyruvate and oxaloacetate were found to have no effect on the rate of modification of the enzyme. Bicarbonate, MgADP, and MgATP<sup>2-</sup> all afforded protection against inactivation of the enzyme. MgATP<sup>2-</sup> afforded almost complete protection against loss of both the acetyl CoA-dependent and -independent catalytic activities. Analysis of the concentration dependence of protection by bicarbonate and MgATP<sup>2-</sup> suggested that cyanate could not inactivate the enzyme-MgATP<sup>2-</sup> and enzyme-bicarbonate complexes. The enzyme MgADP was found to be inactivated at about 30% of the rate of free enzyme.

Modification of the enzyme with cyanate was found to inactivate the ATP/P<sub>i</sub> isotopic exchange reaction, but to enhance the pyruvate/oxaloacetate isotopic exchange reaction. Inactivation of pyruvate carboxylase with [<sup>14</sup>C]cyanate was shown to be associated with modification of at least six amino acid residues per mole of biotin. Chromatography of samples of an enzyme digest of [<sup>14</sup>C]cyanate-labelled enzyme revealed that only amino groups in the enzyme had been modified. A major band of radioactivity was shown to co-chromatograph with authentic homocitrulline.

3. The stoichiometry of the products of the pyruvate carboxylase reaction was shown to vary as the concentration of pyruvate was altered. At high concentrations of pyruvate, the ratio of orthophosphate liberated to oxaloacetate produced approached one, but, as the pyruvate concentration decreased, the ratio increased. On the basis of this evidence, a

model for the reaction pathway was proposed in which the carboxybiotin-enzyme complex could react either with pyruvate to produce oxaloacetate, or water to regenerate enzyme-biotin and bicarbonate. The non-productive breakdown of the enzyme-substrate complex provides an explanation for the non-linear double reciprocal plots obtained for both the overall reaction and the pyruvate/oxaloacetate exchange reaction. Since neither the rate of breakdown of the isolated carboxybiotin-enzyme complex, nor the rate of decarboxylation of oxaloacetate in the absence of pyruvate could account for the difference in the amounts of the two products formed in the overall reaction, it was postulated that the presence of pyruvate was necessary for hydrolysis to occur. Rate equations were derived describing the dependence of the initial velocity release of oxaloacetate in the overall reaction, and the rate of the pyruvate/oxaloacetate exchange reaction, on pyruvate concentration. By assigning appropriate values to the various rate constants, theoretical curves were obtained and fitted to the experimental data.

The reaction pathway of the pyruvate carboxylase catalysed reaction was re-examined using two independent experimental approaches not previously applied to this enzyme. To avoid the variable stoichiometry associated with oxaloacetate formation, the reaction rate was measured by following orthophosphate release. Initial velocities, when plotted as a function of varying concentrations of either  $\text{MgATP}^{2-}$  or bicarbonate, at fixed levels of pyruvate, gave, in double reciprocal form, families of straight, intersecting lines. Furthermore, when the reaction was determined as a function of varying  $\text{MgATP}^{2-}$  concentration, using pyruvate, 2-ketobutyrate, and 3-fluoro-pyruvate as alternative keto-acid substrates, the slopes of the double reciprocal plots were significantly different. Both results suggest that the pyruvate

carboxylase reaction has a sequential pathway, at least at high keto-acid substrate concentrations. However, an analysis of the slope replots of the initial velocity orthophosphate release data suggested that the reaction via a non-sequential pathway at low keto-acid substrate concentrations. Rate equations describing the dependence of initial velocity release of oxaloacetate and orthophosphate on the concentrations of substrates were derived and were shown to be consistent with the initial velocity kinetic data presently available. Possible extensions of the concept of hydrolytic breakdown of an intermediate enzyme complex to other enzymes were discussed.

4. The interaction of acetyl CoA with sheep liver pyruvate carboxylase was investigated using two independent experimental approaches. Evidence consistent with lack of co-operativity of acetyl CoA binding under initial velocity assay conditions was obtained from an analysis of the protection afforded by acetyl CoA against TNBS modification of the enzyme. Furthermore, the dependence of the rate of acetyl CoA deacylation on acetyl CoA concentration was found to be consistent with lack of co-operativity of binding of acetyl CoA.

Evidence was obtained which suggested that the observed non-classical initial-velocity acetyl CoA profile arose because of acetyl CoA-dependent changes in the saturation of the enzyme with pyruvate and bicarbonate, and in the rate of dilution inactivation of the enzyme. The  $n_H$  value obtained from an analysis of the dependence of initial velocity oxaloacetate synthesis on acetyl CoA concentration using the Hill equation was shown to depend on the fixed concentrations of pyruvate and bicarbonate in the assay solutions. The fixed concentrations of  $MgATP^{2-}$  and  $K^+$  were found to have no effect on the  $n_H$  value. The time period over which the reaction rate was linear with time was found to

depend on the acetyl CoA concentration in the assay solution. At high concentrations of acetyl CoA, the process of dilution inactivation was prevented, and hence the reaction was linear with time for a longer period than at low concentrations of acetyl CoA. Dilution inactivation was shown not to involve formation of catalytically active pyruvate carboxylase dimers or monomers.

When an experiment was designed in which all substrates and activators were present at saturating levels regardless of the acetyl CoA concentration, and the enzyme concentration was raised to a level where dilution inactivation did not occur, the dependence of the rate of oxaloacetate synthesis on acetyl CoA concentration was found to be consistent with lack of co-operativity of binding of acetyl CoA to pyruvate carboxylase.

STATEMENT

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

S.B. Easterbrook-Smith

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ABBREVIATIONS

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

DTE	dithioerythritol
FDNB	1-fluoro-2,4-dinitrobenzene
$n_H$	Hill n coefficient
oATP	2',3' dialdehyde oxidation product of ATP.
OAA	oxaloacetate
pyr	pyruvate
TNBS	2,4,6-trinitrobenzene sulphonic acid
U	international enzyme unit

CHAPTER I

INTRODUCTION



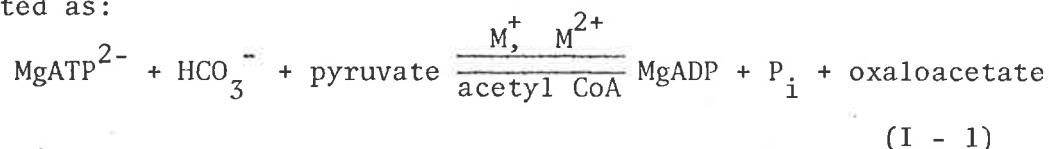
## I. INTRODUCTION

Pyruvate carboxylase activity was first observed in extracts of chicken liver, during the course of a study of the initial reactions of gluconeogenesis from pyruvate (Utter and Keech, 1960). Since that time, the enzyme has been found in a variety of tissues isolated from many other eukaryote species (Utter and Scrutton (1969), Scrutton and Young (1972)), as well as in a number of procaryotes. The results of a study of various aspects of pyruvate carboxylase isolated from sheep liver, are presented in this thesis.

Since a number of recent reviews have been published concerning the structure and catalytic mechanism of this enzyme (Moss and Lane, (1971), Scrutton and Young (1972), Utter, Barden and Taylor (1975)), only those sections of the literature directly relevant to results presented in this thesis will be reviewed herein.

### I.A. PYRUVATE CARBOXYLASE: THE REACTION SEQUENCE

The overall reaction catalysed by pyruvate carboxylase may be represented as:



where  $\text{M}^+$  and  $\text{M}^{2+}$  represent monovalent and divalent cations respectively.

It is generally accepted that the overall reaction proceeds via a  $\text{MgATP}^{2-}$ -dependent carboxylation of the enzyme-bound biotin prosthetic group, at the 1'-N position of the ureido ring. The carboxyl group is then transferred from N-carboxy-biotin to pyruvate, forming oxaloacetate and regenerating the enzyme-biotin species for further catalysis. Thus the carboxyl-carrier role of biotin appears to be the same as that in other biotin enzymes. This has been confirmed by recent work in this

laboratory (Rylatt, 1976). (cf. recent reviews on the biotin-enzymes by Knappe (1970) and Moss and Lane (1971)).

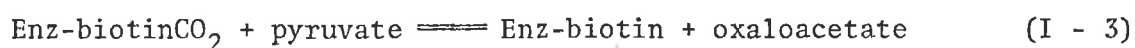
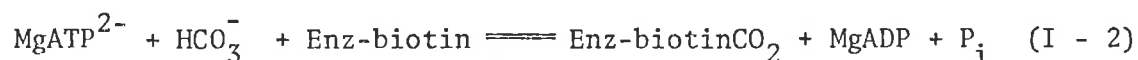
Kinetic studies of the pyruvate carboxylase mechanism have been undertaken using enzyme isolated from Aspergillus niger (Feir and Suzuki, 1969), rat liver (McLure et al., 1971a,b,c), chicken liver (Barden et al., 1972), pig liver (Warren and Tipton, 1974a,b,c) and sheep kidney (Ashman and Keech, 1975).

Feir and Suzuki (1969) applied the technique of King and Altman (1956) to a reaction model with  $\text{MgATP}^{2-}$ ,  $\text{HCO}_3^-$ , and pyruvate binding at independent sites. They then combined the results obtained for each site into one rate equation, which correctly predicted the unusual primary plots of the kinetic data obtained for the Aspergillus enzyme. However, as pointed out by Warren and Tipton (1974b), it is quite invalid to predict steady state rates in isolation and then assert that the reaction sum is the effective sum of these individual components. In view of this theoretical inadequacy, the following discussion will be restricted to data obtained using pyruvate carboxylase isolated from vertebrate sources.

Until recently it was believed that reaction (I - 1) proceeded via a non-classical bi-bi-uni-uni ping-pong reaction sequence (McLure et al., 1971a,b,c; Barden et al., 1972; Ashman and Keech, 1975). However, Warren and Tipton (1974a, b, c), using pyruvate carboxylase isolated from pig liver, have suggested that reaction (I - 1) may be described by a sequential reaction sequence in which pyruvate and oxaloacetate interact with the enzyme in a Theorell-Chance manner, and in which no products of the reaction are released from the enzyme until all the substrates have bound.

### I.A.1. The ping-pong sequence.

For pyruvate carboxylase isolated from rat liver (McLure et al., 1971a,b,c), chicken liver (Barden et al., 1972) and sheep kidney (Ashman and Keech, 1975), it has been proposed that reaction (I - 1) proceeds via two independent partial reactions:



This reaction pathway is analogous to the "non-classical" non-sequential reaction sequence proposed for the bacterial biotin-dependent enzyme, transcarboxylase (Northrop, 1969). The non-classical nature of the sequence arises from the requirement that the binding sites for the two partial reactions must bind their substrates at equilibrium and must be totally independent of each other.

### I.A.2. The sequential reaction sequence.

On the basis of initial velocity and product inhibition data, Warren and Tipton (1974a,b,c) have proposed that the reaction catalysed by pyruvate carboxylase isolated from pig liver proceeds by the sequential pathway shown in Fig. I.1. Binding of the activator,  $\text{Mg}^{2+}$ , and release of the products MgADP and orthophosphate occurs in a rapid equilibrium manner, while the binding of  $\text{MgATP}^{2-}$ ,  $\text{HCO}_3^-$  and pyruvate is an ordered process.

### I.A.3. Discrimination between the models.

The evidence that has been produced in favour of the reaction catalysed by pyruvate carboxylase following either of the proposed sequences may be classified into three types:

- i) initial velocity data
- ii) product inhibition studies
- iii) isotope exchange reaction data.

In view of the controversy concerning the nature of the pyruvate carboxylase reaction sequence, the evidence produced in favour of each proposed pathway will be assessed.

#### I.A.3.a. Initial velocity studies

Initial velocity studies provide a useful tool for distinguishing between sequential and non-sequential enzyme-catalysed reaction sequences. For a reaction sequence of the non-sequential type, a family of parallel lines is obtained when reciprocal initial velocity is plotted as a function of the reciprocal concentration of a substrate of one partial reaction, at fixed concentrations of a substrate of the other partial reaction. Conversely, an intersecting pattern of lines is observed in double reciprocal plots for any varied substrate pair when the reaction pathway is of the sequential type (Cleland, 1963).

Thus, if the pyruvate carboxylase catalysed reaction has the non-classical non-sequential sequence, (I - 2, I - 3) a family of parallel double reciprocal lines should be obtained when initial velocity based on the rate of oxaloacetate synthesis is determined as a function of the concentration of a substrate of the first partial reaction (e.g.,  $\text{MgATP}^{2-}$  or  $\text{HCO}_3^-$ ), at fixed concentrations of a substrate of the second partial reaction (i.e., pyruvate).

However, an unusual pattern of lines was obtained when such studies were undertaken using pyruvate carboxylase isolated from rat or sheep mitochondria (McLure et al., 1971a,b; Ashman and Keech, 1975). At low concentrations of pyruvate, the lines appeared to be parallel, but

became intersecting as the fixed concentration of pyruvate was increased. Moreover, secondary plots of the slope of the lines as a function of the fixed pyruvate concentrations showed that the slope decreased as the pyruvate concentration increased, although the decrease in slope was far more pronounced at high pyruvate concentrations.

McLure and co-workers proposed that the intersecting lines arose because pyruvate, a substrate of the second partial reaction (I - 3) of the non-sequential model influences the binding of bicarbonate, a first partial reaction substrate. This proposal is inconsistent with the non-classical reaction sequence which requires that the two partial reaction sites be completely independent. Furthermore, McLure et al., (1971b) overlooked the fact that the pyruvate/oxaloacetate isotopic exchange reaction shows an equivalent dependence on pyruvate concentration, even though this reaction proceeds in the absence of added bicarbonate. Scrutton et al., (1965). Thus it may be concluded that the initial velocity kinetic studies of pyruvate carboxylase from sheep and rat mitochondria are not completely consistent with the proposed non-sequential reaction sequence.

Barden et al., (1972), using pyruvate carboxylase isolated from chicken liver mitochondria, have also reported families of parallel double reciprocal lines when the varied pair of substrates includes one component of each of the partial reactions, I - 2 and I - 3. However, the narrow range of pyruvate concentrations used was in the region where McLure et al., (1971) and Ashman and Keech (1975) obtained the least dependence of the slopes of the double reciprocal lines on pyruvate concentration. Also the auxiliary data supplied by Barden et al., (1972) does not include the secondary slope replots equivalent to those described above. Therefore, in the absence of data for the chicken pyruvate carboxylase catalysed reaction obtained over a wider range of pyruvate concentration, it would

be premature to conclude that the initial velocity data obtained using the chicken enzyme is uniquely consistent with the non-sequential reaction sequence.

The initial velocity data obtained by Warren and Tipton (1974b,c), using pyruvate carboxylase isolated from pig liver, is consistent with the reaction sequence shown in Fig. I.1. The enzyme catalysed reaction did not follow the non-sequential reaction sequence since all interactions between substrates and effectors yielded intersecting reciprocal plots.

#### I.A.3.b. Product inhibition studies.

The results of the product inhibition studies of the pyruvate carboxylase reaction, assayed in the direction of oxaloacetate synthesis, obtained by Barden et al., (1972) and Warren and Tipton (1974b) are shown in Table I.1. It should be noted that Warren and Tipton (1974b) distinguished between non-competitive and mixed product inhibition. However, as pointed out by Cleland (1970), there is no good theoretical reason for doing this, and thus any inhibition in which both slopes and intercepts of reciprocal plots are affected is described herein as non-competitive. The pattern of product inhibition obtained by McLure et al., (1971b) was the same as that shown for chicken liver pyruvate carboxylase.

It is clear that the only difference between the predicted product inhibition patterns for the sequential and non-sequential reaction pathways is the nature of MgADP and orthophosphate inhibition with pyruvate as variable substrate. From the non-sequential pathway, a family of parallel double reciprocal lines is predicted, whereas the sequential model requires an intersecting, non-competitive pattern.

This discrepancy may be resolved by considering the data obtained by Ashman and Keech (1975) using sheep kidney pyruvate carboxylase. These

workers found an unusual pattern of orthophosphate inhibition, with pyruvate as variable substrate. At low concentrations of pyruvate, uncompetitive inhibition was observed, but at higher pyruvate concentrations non-competitive inhibition occurred. This data suggests that the conflicting results obtained by Barden et al., (1972) and Warren and Tipton (1974b) may be due to the different pyruvate concentration ranges used by the respective workers. As noted above, Barden et al., (1972) deliberately limited their pyruvate levels to a low concentration range. Under these conditions, it is possible that there will be significant dissociation of the products MgADP and orthophosphate from the enzyme before pyruvate has bound. Thus at low pyruvate saturation, the pyruvate carboxylase catalysed reaction may proceed via the proposed non-sequential reaction sequence. However, at high levels of pyruvate saturation, MgADP and orthophosphate may not dissociate from the enzyme until after pyruvate has bound. Under these conditions the reaction would follow a sequential pathway. Such a reaction sequence is consistent with the data obtained, at high concentrations of pyruvate, by McLure et al., (1971b) and by Ashman and Keech (1975), as well as agreeing with the findings of Warren and Tipton (1974b,c).

#### I.A.3.c. Exchange reaction studies.

Pyruvate carboxylase isolated from chicken, rat, and sheep mitochondria, catalyses isotopic exchange reactions between ATP and [ $^{32}\text{P}_i$ ], and between [ $^{14}\text{C}$ ]pyruvate and oxaloacetate (Scrutton et al., 1965, McLure et al., 1971a, Ashman and Keech (1975)). The fact that the exchange reactions proceed in the absence of second and first partial reaction substrates respectively, is key evidence in favour of the reaction sequence being non-sequential.

A major criticism of the work of Warren and Tipton (1974a,b,c) is that they did not attempt to characterise the isotopic exchange reactions catalysed by pig liver pyruvate carboxylase. If the pig liver enzyme catalysed reaction proceeds via the proposed sequential pathway, no isotopic exchange reactions would occur unless all substrates and products of the reaction were present.

Warren and Tipton (1974b) argued that because the ATP/P<sub>i</sub> and ATP/ADP exchange reaction rates are only approximately 1% of the rate of the overall pyruvate carboxylase, then these exchange reactions do not reflect kinetically significant events in the overall reaction process.

There are at least two possible explanations for the observed low rates of exchange. First, Barden et al., (1972) have postulated the formation of the abortive complexes: enzyme-HCO<sub>3</sub><sup>-</sup> - MgADP and enzyme-HCO<sub>3</sub><sup>-</sup> - P<sub>i</sub> during the overall reaction sequence. The presence of these complexes under ATP/P<sub>i</sub> isotopic exchange reaction conditions would clearly lead to a lower rate of exchange than would otherwise be expected.

In addition, the ATP/P<sub>i</sub> and ATP/ADP exchange reaction rates may be limited by the slow rate of decarboxylation of N-carboxybiotin-enzyme. A simplified view of this system is shown below, in which cations are omitted, and only reactive enzyme forms are shown (after McLure et al., 1971b)



The interconversions of each of the pairs of enzyme species shown above must occur at equal rates because, under isotopic exchange conditions, the system is at chemical equilibrium. Now, results reported by Scrutton et al., (1965), and data presented in this thesis (Section V.C.5) show that decarboxylation of enzyme bound N-carboxy-biotin occurs at a very low rate, relative to the rate of the overall pyruvate carboxylation reaction,



unless pyruvate is present. Thus the conversion of  $E.CO_2.ADP.P_i$  to  $E.ATP.HCO_3$  will proceed at a slow rate in the absence of pyruvate. Hence, because the system is at chemical equilibrium, the overall exchange reaction will proceed at a slow rate. This explanation is consistent with the observation that the pyruvate analogue, 2-ketobutyrate, stimulates the ATP/ADP exchange rate in rat liver pyruvate carboxylase (McLure *et al.*, 1971b).

In conclusion, therefore, it would appear that Warren and Tipton (1974b) may have incorrectly dismissed the evidence provided by isotopic exchange studies, and pending an investigation of the isotopic exchange reactions catalysed by pig liver pyruvate carboxylase, the evidence in favour of the proposed sequential pathway should be regarded as incomplete. The results of a further investigation of the reaction pathway are presented in Chapter V of this thesis.

## I.B. CHEMISTRY OF PYRUVATE CARBOXYLATION

Although overall kinetic studies may define the order of substrate binding and product release, they alone do not elucidate the details of bond making and breaking that occur during an enzyme catalysed reaction. Other approaches are necessary to define the chemical mechanism of the catalysed reaction, including the roles the amino acid residues of the enzymes' active site have in the catalytic process. In particular, kinetic studies alone have shed little light on the coupling of biotin carboxylation and  $MgATP^{2-}$  hydrolysis in the reaction catalysed by pyruvate carboxylase.

### I.B.1. Mechanism of Biotin Carboxylation.

#### I.B.1.a. Substrate specificity.

The biotin carboxylation reaction catalysed by all pyruvate

carboxylases examined to date has been shown to depend on the presence of both a monovalent and divalent metal cation. The monovalent cation specificity depends on the source of the enzyme, (Scrutton and Young, 1972), and it has been suggested that this specificity is determined by the size of the associated anion and its anionic field strength, (McLure et al., 1971a).

The divalent cation appears to have a dual function. Pyruvate carboxylase isolated from a number of sources, (Keech and Barritt, 1967; Cazzulo and Stoppani, 1967, 1969; Feir and Suzuki, 1969; Cazzulo et al., 1970; McLure et al., 1971a; Bais and Keech, 1972; Warren and Tipton, 1974b), has been shown to utilize  $\text{MgATP}^{2-}$  rather than  $\text{ATP}^{4-}$  as a substrate. In addition, the optimal divalent metal ion concentration is several fold higher than the concentration of nucleotide required for maximal activity.

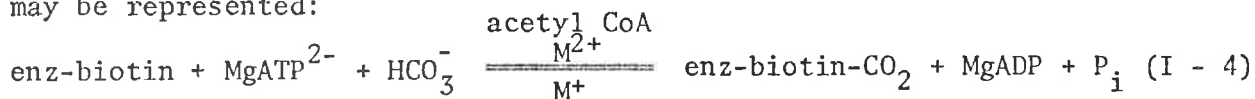
Some workers (e.g., Cazzulo and Stoppani, 1969) have suggested that the excess divalent metal ion does not activate pyruvate carboxylase directly, but acts by removing inhibitory  $\text{ATP}^{4-}$ . However, this interpretation is inconsistent with the initial velocity kinetics of the mammalian pyruvate carboxylases (Warren and Tipton, 1974b).

The active species of carbon dioxide has been shown to be the bicarbonate anion for the chicken liver pyruvate carboxylase reaction (Cooper et al., 1968) and also in the reactions catalysed by the related biotin-containing enzymes, propionyl CoA carboxylase (Kaziro et al., 1962). Thus it seems that a requirement for bicarbonate, rather than carbon dioxide, is typical of the biotin carboxylation reaction catalysed by this class of enzymes.

#### I.B.1.b. Coupling mechanisms.

The biotin carboxylation reaction catalysed by pyruvate carboxylase

may be represented:



One can envisage three possible mechanisms for this coupling of  $\text{MgATP}^{2-}$  hydrolysis and biotin carboxylation: (Fig. I.2).

- a) a concerted reaction without formation of any detectable or kinetically significant intermediates,
- b) activation of the enzyme or its biotin prosthetic group by  $\text{MgATP}^{2-}$  to form a phosphoryl or adenyl complex which then reacts with bicarbonate.
- c) activation of bicarbonate by  $\text{MgATP}^{2-}$  to form a phosphoryl or adenyl species, which then interacts with the enzyme.

#### I.B.1.c. The concerted mechanism.

This mechanism (Fig. I.2a) requires that the products  $\text{MgADP}$  and orthophosphate are released from the enzymes after formation of  $^{14}\text{C}$  carboxybiotin-enzyme, and that there is an absolute requirement for biotin in the isotopic exchange reactions between orthophosphate and  $\text{MgATP}$ , and between  $\text{MgATP}$  and  $\text{MgADP}$ . This mechanism is inconsistent with the avidin insensitive  $[^{14}\text{C}]\text{-MgADP} / \text{MgATP}^{2-}$  exchange reaction catalysed by some biotin containing enzymes. Lane *et al.*, (1960) demonstrated that bovine liver propionyl CoA carboxylase catalysed an  $\text{MgADP} / \text{MgATP}$  exchange reaction dependent only on the presence of  $\text{Mg}^{2+}$  and insensitive to avidin inhibition. Similar results have been reported for pyruvate carboxylase isolated from chicken and sheep liver (Scrutton and Utter, 1965; Goss and Hudson, unpublished results). These data clearly show that not only is biotin not absolutely required for the  $\text{MgADP} / \text{MgATP}^{2-}$  exchange reaction, but that at least one product ( $\text{MgADP}$ ) of reaction (I - 4) is released before formation

of N-carboxybiotin-enzyme. Thus the available evidence clearly disproves the concerted model.

I.B.1.d. The stepwise models.

In the remaining two models it is suggested that discrete enzyme complexes form during the conversion of biotin-enzyme to N-carboxybiotin-enzyme.

I.B.1.d.i The enzyme-activation mechanism.

This mechanism (Fig. I.2b) was proposed by Scrutton and Utter (1965b), and has recently been re-examined by Klugar and Adawadkhar (1976). One prediction of this mechanism is that pyruvate carboxylase should catalyse a biotin-independent  $\text{MgADP} / \text{MgATP}^{2-}$  isotopic exchange reaction. As discussed above, this prediction has been satisfied experimentally. However, one of the critical predictions of this scheme, the bicarbonate independence of the  $\text{MgADP} / \text{MgATP}$  isotopic exchange reaction, has not been sufficiently investigated. Scrutton and Utter (1965) made no attempts to reduce the levels of endogenous bicarbonate in their reaction mixture. At the mildly alkaline pH used, the bicarbonate concentration resulting from equilibrium with atmospheric carbon dioxide can reach 1-2 mM (Rylatt, 1976), and it is possible that this concentration alone could satisfy the bicarbonate dependence of the exchange reaction. In fact, recent results obtained in this laboratory showed that when elaborate measures were taken to remove endogenous bicarbonate, the  $\text{MgADP}/\text{MgATP}$  isotopic exchange was dependent on the presence of bicarbonate (Goss and Hudson, unpublished results).

In addition, the recent study of Ashman and Keech (1975) appears to be inconsistent with the enzyme activation model. These workers examined

the effects of the ADP analogues adenosine-5'-phosphosulphate, and  $\alpha,\beta$  methylene adenosine diphosphate on the isotopic exchange reaction between [ $^{14}\text{C}$ ]-bicarbonate and oxaloacetate catalysed by sheep kidney pyruvate carboxylase. Mechanism b of Fig. I.2 predicts that although MgADP must be bound to the enzyme for carboxylation of biotin, and hence occurrence of the [ $^{14}\text{C}$ ]-bicarbonate/oxaloacetate isotopic exchange reaction, phosphorylation of MgADP should not be necessary. Ashman and Keech (1975) showed that the MgADP analogues could not be phosphorylated by the enzyme, and also could not support the [ $^{14}\text{C}$ ]-bicarbonate/oxaloacetate isotopic exchange reaction. This indicated that phosphorylation of MgADP was necessary for this exchange reaction to proceed, and hence that MgATP<sup>2-</sup> hydrolysis, independent of bicarbonate binding is not the first step in the overall pyruvate carboxylation reaction; therefore the enzyme activation model is not applicable to the pyruvate carboxylase catalysed reaction.

#### I.B.1.d.ii The substrate activation model.

A second stepwise mechanism, involving the existence of enzyme-bound carbonylphosphate, has been proposed by Polakis et al., (1972) for acetyl CoA carboxylase isolated from E. coli. A similar type of activation has been demonstrated in the glutamine synthesis catalysed reaction in which a  $\gamma$ -glutamylphosphate intermediate was formed (Meister, 1968).

Direct evidence for the formation of carbonylphosphate during the biotin carboxylation process has come from the studies of Polakis et al., (1972) on acetyl CoA carboxylase, and Ashman and Keech (1975), on sheep kidney pyruvate carboxylase. Both groups were able to demonstrate net enzyme-catalysed synthesis of MgATP<sup>2-</sup> from MgADP dependent on the carbonylphosphate analogue, carbamylphosphate. Furthermore, carbamylphosphate was

shown to be a competitive inhibitor with respect to  $\text{MgATP}^{2-}$  in the overall reaction catalysed by pyruvate carboxylase.

Further evidence that biotin may not be involved in carbonyl-phosphate formation comes from studies of the acetyl CoA carboxylase isolated from E. coli. Polakis et al., (1974) showed that biotin was not involved catalytically by demonstrating that biotin analogues modified at the I-N position of the ureido ring, and hence incapable of being carboxylated, could support phosphoryl transfer from carbamylphosphate to MgADP. This result suggests that biotin may have a structural rather than catalytic role in carbonylphosphate formation.

Using the substrate activation model (Fig. I.2c) it is possible to make a number of predictions concerning the dependence of various biotin carboxylation reaction processes on substrates and cofactors.

Firstly, the isotopic exchange reaction between [ $^{14}\text{C}$ ]-MgADP and MgATP should be dependent on bicarbonate, but not completely dependent on biotin. As described above, Scrutton and Utter (1965) and Goss and Hudson (unpublished results) have shown that pyruvate carboxylase, isolated from chicken and sheep liver respectively, catalyses an  $\text{MgADP}/\text{MgATP}^{2-}$  isotopic exchange reaction which satisfies the above dependence requirements.

In addition, the substrate activation model predicts that the  $\text{MgATP}/\text{P}_i$  isotopic exchange reaction should be completely dependent on biotin. This is evident from Fig. I.2c, where it can be seen that biotin carboxylation must occur in order for orthophosphate to be released from the enzyme surface. The  $\text{MgATP}/\text{P}_i$  isotopic exchange reactions catalysed by pyruvate carboxylase isolated from chicken liver (Scrutton and Utter, 1965), and sheep kidney (Ashman and Keech, 1975) have been shown to be completely sensitive to avidin inactivation, and hence are completely

dependent on biotin.

Thus, the available evidence suggests biotin need have no direct catalytic role in formation of a carbonylphosphate moiety from  $\text{MgATP}^{2-}$  and bicarbonate. However, it is unclear whether carbonylphosphate is ever present as a discrete intermediate, or only exists transiently during the biotin carboxylation process. Regardless of this uncertainty, the next step in elucidating the details of the mechanism of biotin carboxylation is to discover which amino acid residues at the active site participate in the phosphoryl transfer process.

In a number of phosphoryl group transferring enzymes, positively charged arginyl and/or lysyl residues at the active site have been implicated in the catalytic process. For example, the nuclear magnetic resonance studies of James and Cohn (1974), on creatine kinase, have led to the suggestion that the  $\epsilon\text{-NH}_3^{\oplus}$  group of an active site lysyl residue is involved in binding of the transferrable phosphoryl group in the transition state complex. It was proposed that besides helping to maintain the proper alignment of substrates on the enzyme surface for catalysis to occur, the  $\epsilon\text{-NH}_3^{\oplus}$  group acted to withdraw electrons from the terminal phosphate group of  $\text{MgATP}^{2-}$ , thereby facilitating nucleophilic attack by the nitrogen atom of the guanido group of creatine during the catalytic process. When this proposed mechanism is compared for that suggested for biotin carboxylation in the pyruvate carboxylase catalysed reaction, it can be seen that a close analogy exists between phosphoryl transfer to creatine, in the creatine kinase reaction, and phosphoryl transfer to bicarbonate in the substrate activation model of biotin carboxylation.

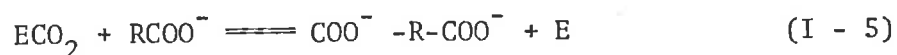
Thus, it is a reasonable hypothesis that the biotin carboxylation active site of pyruvate carboxylase contains lysyl and/or arginyl residues

which participate in phosphoryl transfer of the terminal phosphate of  $\text{MgATP}^{2-}$  to bicarbonate. The results of experimental tests of this hypothesis are reported in Chapters III and IV.

### I.B.2 Mechanism of Transcarboxylation.

#### I.B.2.a Substrate specificity.

The transcarboxylation reaction catalysed by pyruvate carboxylase may be represented as:



where  $\text{RCOO}^-$ ,  $\text{ECO}_2$ , E, and  $\text{COO}^- - \text{R-COO}^-$  represented the keto-acid substrate, carboxy-biotin-enzyme, biotin-enzyme, and the keto-acid product respectively. Keech and Utter (1963) reported that 2-keto-butyrate and 2-ketovalerate could substitute for pyruvate as the keto-acid substrate of reaction I - 5. More recently, Cheung and Walsh (1976) showed that fluoropyruvate could be carboxylated by chicken liver pyruvate carboxylase. However, there have not been extensive studies of the substrate specificity of reaction I - 5.

#### I.B.2.b Activator requirements.

Reaction I - 5 can be studied in an isolated fashion as the isotopic exchange reaction between [ $^{14}\text{C}$ ]-pyruvate and oxaloacetate. The pyruvate/oxaloacetate isotopic exchange reaction catalysed by chicken liver pyruvate carboxylase has been shown to be relatively independent of the activators; acetyl CoA,  $\text{Mg}^{2+}$  and  $\text{K}^+$ , (Scrutton, Keech and Utter, 1965). However, acetyl CoA and  $\text{K}^+$  have been shown to stimulate the exchange reaction catalysed by pyruvate carboxylase isolated from rat liver (McLure et al., 1971a), and from sheep kidney (Ashman, 1973). Moreover, Barden and Scrutton (1974) have demonstrated



an interaction between  $K^+$  and pyruvate in the chicken liver pyruvate carboxylase catalysed reaction. A detailed investigation of the effect of acetyl CoA on the interaction of pyruvate with the enzyme isolated from sheep liver is reported in Chapter VI of this thesis.

#### I.B.2.c Chemistry of Transcarboxylation.

Attempts to describe the chemistry of the enzyme catalysed carboxylation of pyruvate have been based on the idea that pyruvate must be electrophilically activated in order for transfer of the carboxyl group from biotin to occur. Two distinct models for pyruvate activation have been developed, one based on nuclear magnetic resonance studies of the enzyme-bound metal atom and pyruvate, and one on stereochemical studies of pyruvate carboxylation.

##### I.B.2.c.i The Role of the Bound Metal Ion.

Since pyruvate carboxylase isolated from chicken liver mitochondria was shown to be a manganese metalloprotein (Scrutton et al., 1966), a number of investigators have shown that the enzyme isolated from a variety of other sources is also a metalloprotein, with manganese, magnesium, zinc, or cobalt as a tightly bound constituent, (for review see Scrutton and Young, 1972). A catalytic role for this bound metal ion has been proposed for the reactions catalysed by the enzyme isolated from chicken liver (Scrutton et al., 1966), yeast (Scrutton et al., 1970), and calf and turkey liver (Scrutton et al., 1972).

The observation that chicken liver pyruvate carboxylase was a manganese metalloprotein, coupled with extensive nuclear magnetic resonance studies of the enzyme, prompted the suggestion that the bound metal

participated in the pyruvate carboxylation process by forming a bridge between pyruvate and the enzyme (Mildvan et al., 1966, Mildvan and Scrutton, 1967). Furthermore, it was proposed that the metal ion facilitated pyruvate transcarboxylation because of its electron withdrawing properties (Mildvan et al., 1966). This proposed mechanism is illustrated in Fig. 1.3a.

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

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

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

There is, however, strong evidence that the bound metal atom has no direct role in the pyruvate carboxylase catalysed reaction. It has been shown that pyruvate carboxylase isolated from some sources does not

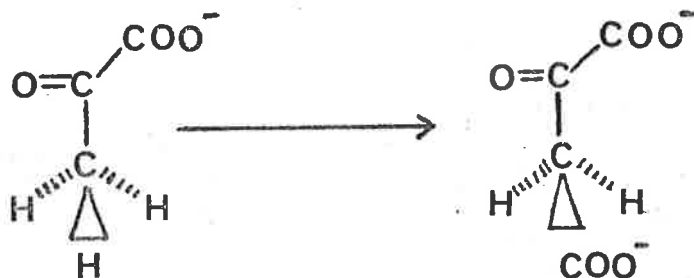
have bound metal in the correct stoichiometry of one metal ion per biotin required by the metal activation mechanism. The enzyme isolated from rat liver (McLure *et al.*, 1971a) and sheep kidney or liver (Bais, 1974) contains only one metal atom per two biotin molecules, and the enzyme isolated from *Pseudomonas citronellolis* does not even appear to be a metalloprotein (Taylor B., personal communication).

In addition, Bais (1974) repeated the stoichiometry determination for chicken liver pyruvate carboxylase and was able to establish a stoichiometry of one metal atom per two molecules of biotin. Finally, in terms of the metal activation mechanism, it is surprising that substitution of different metal ions results in only small changes in the catalytic properties of the enzyme isolated from yeast ( $\text{Co}^{2+}$  for  $\text{Zn}^{2+}$ , Scrutton *et al.*, 1970) and chicken liver ( $\text{Mg}^{2+}$  for  $\text{Mn}^{2+}$ , Scrutton *et al.*, 1972).

In view of this evidence, although it is clear that the divalent metal binding site is close to the transcarboxylation site, it would appear that a defined catalytic role for the metal has yet to be unequivocally established.

#### I.B.2.c.ii Stereochemistry of Pyruvate Carboxylation.

It has been shown that the carboxylation of pyruvate catalysed by chicken liver pyruvate carboxylase proceeds with retention of configuration at the C-3 position of pyruvate (Rose, 1970);



Similarly, both the carboxylation of propionyl CoA by propionyl CoA carboxylase (Arigioni et al., 1966; Prescott and Rabinowitz, 1968) and the carboxylation of pyruvate and propionyl CoA by transcarboxylase (Cheung et al., 1975) proceed with retention of configuration around the substituted carbon atom. Retey and Lynen (1965) have suggested that biotin in an enol form is the active species in the carboxylation of propionyl CoA by transcarboxylase, and this scheme allows both retention of configuration on carboxylation, and the proton transfer from pyruvate to propionyl CoA observed by Rose et al., (1976), as is shown in Fig. 1.3b.

This mechanism is quite distinct from those proposed incorporating metal ion activation. In this mechanism, carboxy-biotin is the base that activates pyruvate, and enol-biotin is the species that decarboxylates oxaloacetate. The relative merits of the two mechanisms have been reviewed by Wood and Zwolinski (1976) and at this time it seems likely that the carboxylation of pyruvate catalysed by pyruvate carboxylase proceeds by a mechanism similar to that proposed by Retey and Lynen (1965). The mechanism of carboxyl transfer from 1-N<sup>1</sup>-carboxybiotin to pyruvate is further discussed in the light of experimental data presented in this thesis in Section V.D.4.

#### I.C. ACTIVATION OF PYRUVATE CARBOXYLASE BY ACETYL CoA.

Although the available evidence suggests that the basic catalytic mechanism of the various species of pyruvate carboxylase is very similar if not identical, there is considerable species variation in the degree of dependence on an acetyl CoA for catalytic activity. In some species a nearly absolute dependence on an acetyl CoA species has been reported. The enzymes isolated from chick liver (Scrutton and Utter, 1967), turkey

liver (Wallace, J.C. and Utter, M.F. unpublished results), pigeon liver (Dugal and Louis, 1972), Arthrobaeter globiformis (Bridgeland and Jones, 1967), and Bacillus stenrothermophilis (Cazzulo et al., 1970), fall into this class.

The pyruvate carboxylases of mammalian origin are less dependent on acetyl CoA for catalytic activity (Ashman et al., 1972, Scrutton and White, 1972). Under appropriate conditions, the acetyl CoA independent activities of these enzymes may approach 25% of the activity in the presense of acetyl CoA. The enzyme from yeast shows a further decrease in dependence on an acetyl CoA activator: under appropriate conditions the stimulation achieved by addition of an acetyl CoA is only two to three-fold (Cazzulo and Stoppani, 1967; Cooper and Benedict, 1968). The enzyme isolated from Pseudamonas citronellolis represents yet another class of pyruvate carboxylase that has apparently no dependence on an acetyl CoA activator (Soubert and Remberger, 1961).

#### I.C.1. The Acetyl CoA Binding Site.

Although it has been postulated from initial velocity kinetic studies, that acetyl CoA acts as an allosteric modifier of pyruvate carboxylase, it has only been possible to demonstrate the existence of discrete activator and catalytic sites for the enzymes of the second class discussed above. Ashman et al., (1972) and Scrutton and White (1972) have shown that the sheep and rat enzymes respectively can be desensitised to acetyl CoA activation by treatment with the chemical modifier, trinitrobenzene sulphonic acid (TNBS). In the case of sheep liver pyruvate carboxylase, the acetyl CoA independent activity was enhanced by TNBS modification, while acetyl CoA dependent activity was

lost. TNBS modification had no significant effect on the acetyl CoA independent activity of rat liver pyruvate carboxylase, although once again the dependent activity was lost during modification. Pyruvate carboxylase from yeast shows a similar response to TNBS modification, although the acetyl CoA independent activity is gradually lost at a rate far slower than the acetyl CoA dependent activity (Scrutton and White, 1972).

#### I.C.2. Kinetic Studies of Acetyl CoA Activation.

The initial velocity saturation profiles for acetyl CoA, and the effects of acetyl CoA on the kinetic constants of the substrates of pyruvate carboxylase have been the subjects of many investigations (for references see Utter et al., 1976).

Considerable stress has been placed on the fact that the saturation profile obtained for chicken liver pyruvate carboxylase is sigmoidal, with an  $n_H$  value approaching three, whereas that for the mammalian enzymes is less sigmoidal, with an  $n_H$  value closer to two. It has been claimed that the  $n_H$  values greater than unity arise because binding of acetyl CoA to pyruvate carboxylase is a homotropic co-operative process, and that the differences in  $n_H$  value between the avian, and the mammalian enzymes reflect differences in the co-operativity of binding of acetyl CoA to the enzyme's allosteric sites (Utter et al., 1976).

These claims are questionable as they depend on two dubious assumptions: that an  $n_H$  value greater than one necessarily implies the ligand binds to the enzyme in a co-operative fashion, and that initial velocity saturation profiles are necessarily equivalent to binding saturation profiles. The following discussion will attempt to show the

doubtful rigour of these assumptions.

### I.C.3. Alternatives to Co-operativity.

In the last decade, since the allosteric models of Monod et al., (1965), and Koshland et al., (1966), were developed, it has become popular to interpret any non-hyperbolic initial velocity substrate saturation profile in terms of a model in which ligand binding to the enzyme is either positively or negatively co-operative. Usually this interpretation is made without first considering alternative explanations of the data. Such alternative models include: the multiple reaction pathway model (Sweeney and Fisher, 1968; Wells et al., 1976), the polymerising enzyme schemes (Nicol, et al., 1967), and the mnemonical enzyme concept (Rabin, 1967; Ricard et al., 1974). One explanation of sigmoid substrate saturation curves of particular relevance to the mechanism of acetyl CoA activation of the pyruvate carboxylase catalysed reaction is that developed by Fischer and Keleti (1975). These workers showed that if an enzyme is partially inactivated during the course of reaction velocity determination, and if the substrate alters the rate of inactivation, then a sigmoid substrate saturation curve will be obtained. The sigmoid threonine saturation curve of the threonine deaminase catalysed reaction has been shown to arise from a mechanism of this type (Harding, 1969). A similar conclusion was reached by Hemphill et al., (1971) from their studies of the AMP saturation profile of  $K^+$  activated AMP aminohydrolase.

In the case of pyruvate carboxylase, Ashman et al., (1972) have suggested that the acetyl CoA activation is due, in part, to stabilisation of the enzyme against inactivation. This suggestion has since been investigated in more detail (Section VI.C.6).

In summary therefore, none of the alternative theories mentioned above has been seriously considered as possible explanations for the non-classical acetyl CoA saturation profile observed in the pyruvate carboxylase catalysed reaction. Indeed, it has been generally assumed, in the absence of any supporting evidence that an explanation based on cooperativity of binding of acetyl CoA is necessarily valid.

#### I.C.4. Binding and Initial Velocity Saturation Profiles.

An important point which is often overlooked by workers in the field of allosteric enzymes is that the theories developed by Monod et al., (1965), and Koshland et al., (1966) are concerned with binding saturation profiles, rather than initial velocity saturation profiles. Usually the tacit assumption is made that the shape of a saturation curve will be the same, whether initial reaction velocity or ligand binding is determined as a function of ligand concentration. This assumption is generally not valid.

For example, it is well known that the "concerted" allosteric model developed by Monod et al., (1967) does not allow negative homotropic co-operativity. While this is clearly true for binding isotherms, it is far from clear whether it also holds for initial velocity saturation curves (Goldbetter, 1974; Paulus and De Riel, 1975).

Another experimental situation which is inconsistent with the above tacit assumption is that of a "half-site reactive" enzyme. Such oligomeric enzymes behave as if only half their apparently identical active sites were catalytically competent. It appears that this phenomenon may be due to strong negative co-operativity of binding or catalysis by one or more enzymes substrates (for review, see Seydoux et al., 1974).



However, the important point is that many of these enzymes exhibit classical Michaelis-Menten initial velocity kinetic behaviour, even though negative intersubunit co-operativity is known to be involved in the catalytic process.

In the light of the above arguments, it was clear that the nature of the acetyl CoA activation of the pyruvate carboxylase reaction warranted further examination. The results of such a study are presented in Chapter VI of this thesis.

Fig. I.1      The sequential pyruvate carboxylase reaction pathway.

(Adapted from Warren and Tipton, 1974c). The portion of the pathway enclosed within dotted lines is assumed to be at rapid equilibrium.

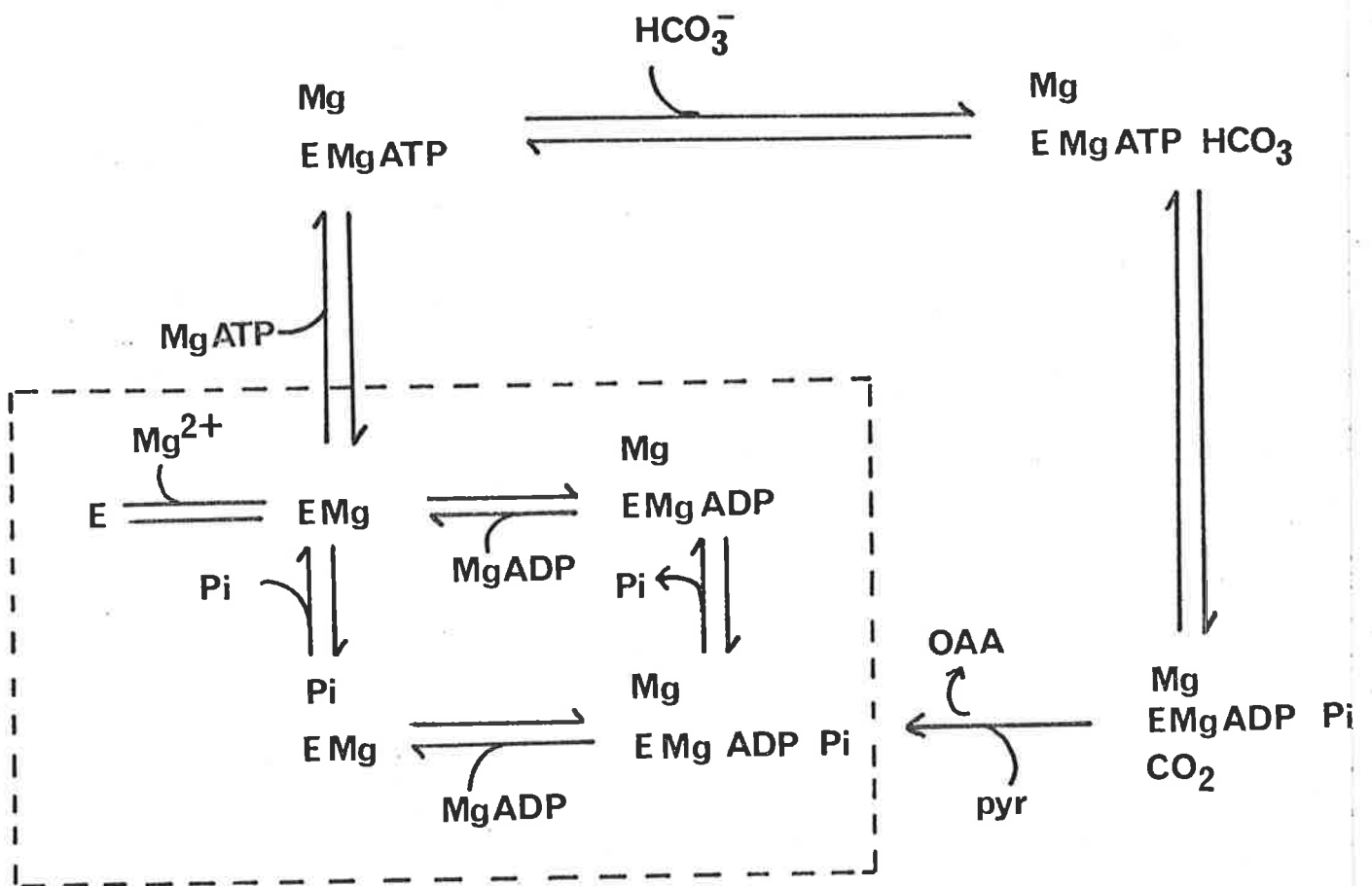


Table I.1 Product inhibition patterns for pyruvate carboxylase.

The abbreviations used are: CLPC; chicken liver pyruvate carboxylase, PLPC; pig liver pyruvate carboxylase, C; competitive inhibition, NC; non-competitive inhibition, UC; uncompetitive inhibition. The data for chicken liver carboxylase are those reported by Barden et al., (1972), and the pig liver enzyme data is that of Warren and Tipton, (1974b).

Inhibitor	Substrate	Inhibition Pattern	
		PLPC	CLPC
MgADP	MgATP <sup>2-</sup>	C	C
	HCO <sub>3</sub> <sup>-</sup>	NC	NC
	pyruvate	NC	UC
phosphate	MgATP <sup>2-</sup>	C	C
	HCO <sub>3</sub> <sup>-</sup>	NC	NC
	pyruvate	NC	UC
oxaloacetate	MgATP <sup>2-</sup>	NC	NC
	HCO <sub>3</sub> <sup>-</sup>	NC	NC
	pyruvate	C	C

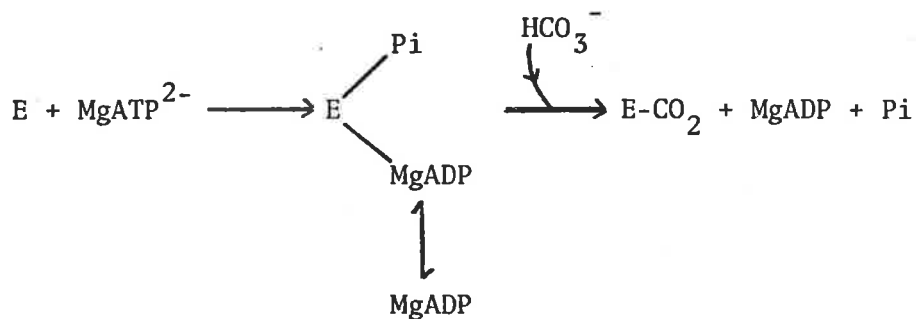
Fig. I.2 Alternative mechanisms for biotin carboxylation

(Adapted from Ashman and Keech, 1975).

a. concerted



b. enzyme activation



c. substrate activation

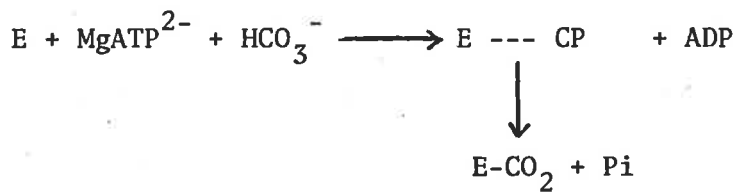
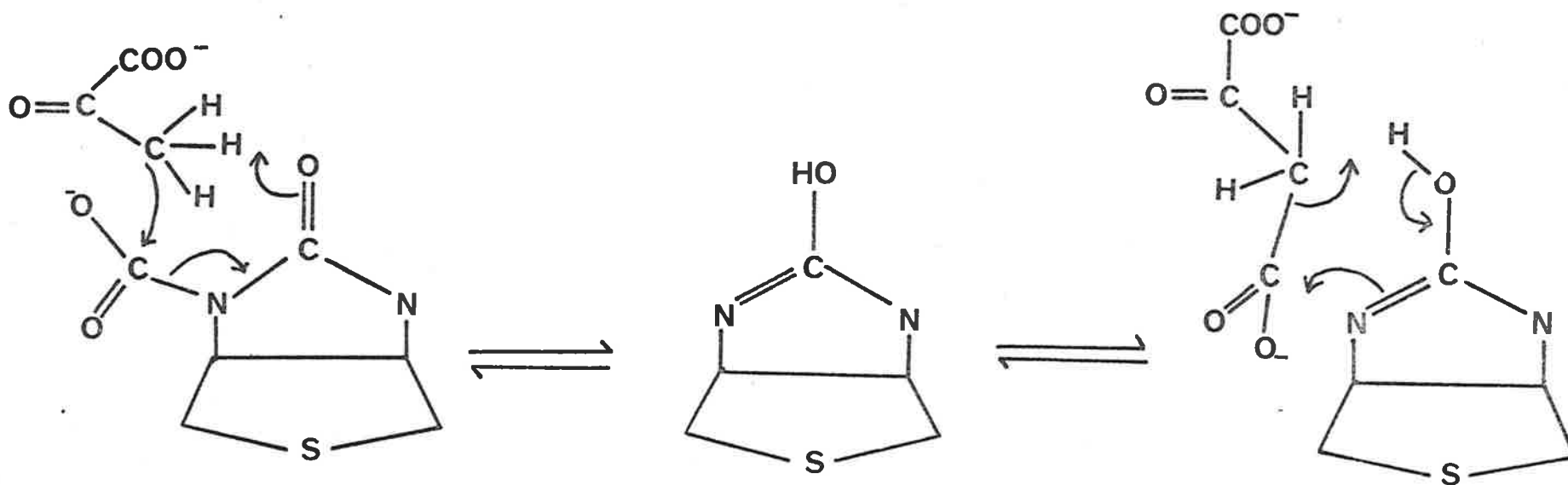
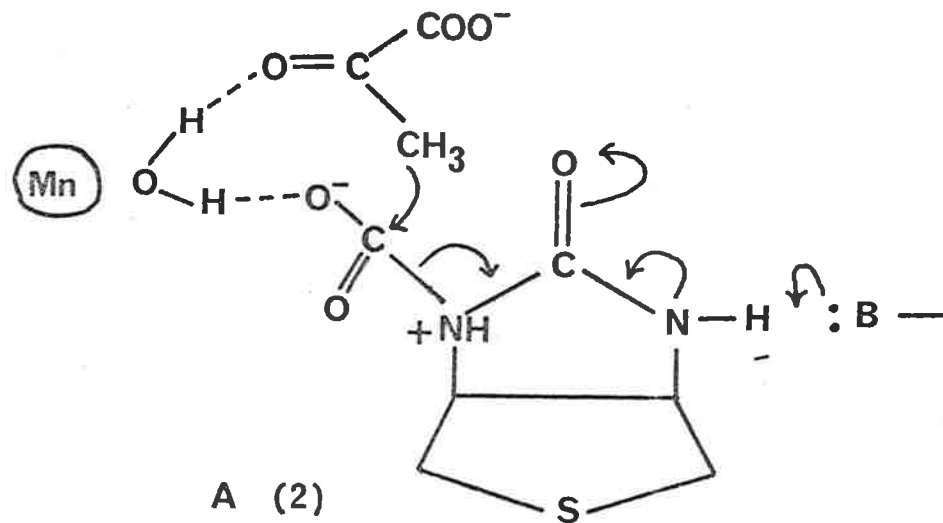
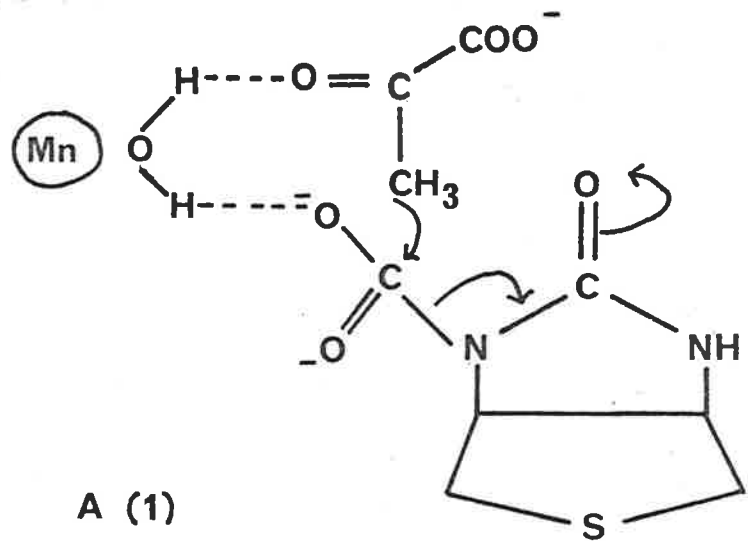


Fig. I.3. Alternative mechanisms for pyruvate carboxylation

- A. Alternative models involving metal ion activation (after Fung et al., 1976).
1. Carboxyl transfer parallel to the plane of the biotin ring. This mechanism requires oscillation of the biotin between the biotin carboxylation and transcarboxylation sites.
  2. Carboxyl transfer perpendicular to the plane of the biotin. This mechanism does not necessitate oscillation.
- B. Mechanism based on the stereochemistry of pyruvate carboxylation (Retey and Lynen, 1965). Some mechanism (not shown) is required to stabilise the enol form of biotin.





B

CHAPTER II

MATERIALS AND METHODS

## II.A. MATERIALS.

### II.A.1. Enzymes and proteins

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

### II.A.2. Radioactive chemicals

Sodium [2-<sup>14</sup>C]pyruvate, [8-<sup>14</sup>C]ATP, sodium [<sup>14</sup>C]bicarbonate, potassium [<sup>14</sup>C]cyanide, potassium [<sup>14</sup>C]cyanate, [1-<sup>14</sup>C]acetyl CoA and [U-<sup>3</sup>H]lysine were obtained from The Radiochemical Centre, Amersham, England. [<sup>32</sup>P]ortho phosphate was supplied by The Australian Atomic Energy Commission, Lucas Heights, Australia.

### II.A.3. General chemicals

ATP (disodium salt, Grade I), ADP (disodium salt, Grade I, AMP, NADH, CoA (Grade I), oxaloacetic acid, pyridoxal phosphate, 2-oxoglutarate, fluoropyruvate, sodium pyruvate (type II, dimer free), 3-phosphoglycerate, 2-mercaptoethanol, TNBS, DTE, and Trizma base were supplied by Sigma Chemical Co., St. Louis, Mo., USA. N-ethylmorpholine was obtained from Eastman Organic Chemicals, and polyethylene glycol (molecular weight 20000) from

Union Carbide Corporation. Sodium glutamate and L-lysine hydrochloride were obtained from British Drug Houses. Homocitrulline was supplied by Calbiochem. 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzine and 2,5-diphenyl-oxazole were supplied by Koch-Light Laboratories Ltd., Bucks., England.  $MgCl_2$  was prepared from Spec-pure magnesium (Hilger-Watts Ltd., London) and redistilled HCl, and was standardised by titration against EDTA, using Eriochrome Black as an indicator (Vogel, 1961). Triton X-100 was supplied by ICI (Australia) Ltd., Melbourne.

## II.B. METHODS

### II.B.1. Preparation and purification of nucleotides

Acetyl CoA was prepared by a method similar to that of Simon and Shemin (1953), and purified as described by Ashman (1973).  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was prepared by the method of Glynn and Chappell (1964), and purified by ion exchange chromatography using either a DEAE-cellulose column (10x1 cm) eluted with a linear 0-0.5M  $\text{NH}_4\text{HCO}_3$  gradient, or a Dowex-1 (formate form) column (1x5 cm) eluted with 25 ml 0.2M ammonium formate, (pH 4.0), followed by 25 ml 0.2M ammonium formate (pH 3.45), 25 ml 0.4M ammonium formate (pH 3.45), and finally 15 ml 1M HCl. The eluate from the last wash was neutralised with NaOH, concentrated by freeze-drying, and remaining contaminating  $[\text{}^{32}\text{P}]\text{orthophosphate}$  removed by gel filtration using Sephadex G-10.

### II.B.2 Purification of pyruvate carboxylase

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

twenty minutes, and undissolved material removed by centrifuging (23000g, for ten minutes, at 4°C).

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

The precipitated protein was collected by centrifuging (23000g, for ten minutes, at 4°C) and suspended in 25 mM potassium phosphate, pH 7.2, containing 1 mM EDTA, 0.1 mM DTE and 0.5% saturated with ammonium sulphate, to give a final protein concentration of 10 mg/ml. This suspension was desalted by addition of 14.5g polyethylene glycol/100 mls. The suspension was stirred for thirty minutes and precipitated protein was then collected by centrifuging (23000g, for twenty minutes, at 4°C). The protein was then suspended in 5-6 ml of the phosphate buffer described above, per 10g of mitochondria, and undissolved protein removed by centrifuging (23000g, for ten minutes, at 4°C). The supernatant, containing essentially all the pyruvate carboxylase was loaded on to a DEAE-Sephadex A-50 column (14x5 cm) previously equilibrated with 25 mM potassium phosphate, pH 7.2, containing 1 mM EDTA, 0.1 mM DTE, and 1% saturated with ammonium sulphate. The column was eluted with a linear gradient of 1-5% saturated ammonium sulphate, in the above buffer. Pyruvate carboxylase of specific activity 15-20 units/mg protein was routinely obtained using this procedure. The major contaminant of this enzyme preparation was glutamate dehydrogenase. If necessary this

contaminant was removed by affinity chromatography, using a  $\text{NAD}^+$ -Agarose column (1x5 cm) previously equilibrated with 50 mM tris Cl pH 8.4, containing 0.1 mM DTE. Pyruvate carboxylase dissolved in 0.1M N-ethylmorpholine-Cl, pH 7.2, containing 1.6M sucrose, was applied to the column, which was then washed with 50 ml of 50 mM tris Cl, pH 8.4, containing 0.1 mM DTE. The enzyme was eluted using a linear gradient of 0.05-0.5M tris Cl, containing 0.1 mM DTE. There was usually a 60-70% recovery of enzyme, with a three fold increase in specific activity.

### II.B.3 Determination of radioactivity

Samples dried on to solid supports (1"x1" squares of Whatmans 3MM paper) were placed in vials containing 2 ml scintillation fluid (0.3% (w/v) 2,5-diphenyloxazole, 0.03% (w/v) 1,4-bis-2 (4-methyl-5-phenoxazolyl)-benzene, in sulphur-free toluene; Bosquet and Christian, 1960) and counted in a Packard Scintillation Spectrometer. When the samples contained coloured material, (as was the case in the radiochemical pyruvate carboxylase assay) correction was made for colour quenching using the channels ratio method (Baille, 1960). Liquid samples were placed in vials containing a ten-fold volume excess of Triton X-100 scintillation fluid (toluene scintillation fluid, as above, containing Triton X-100, 7:3 v/v), and counted in a Packard Scintillation Spectrometer.

### II.B.4. Measurement of pyruvate carboxylase activity

#### II.B.4.a. The spectrophotometric assay system.

This continuous assay procedure, based on that described by Utter and Keech (1963) involves reduction of the oxaloacetate produced by the pyruvate carboxylase reaction, using malate dehydrogenase, with concomitant oxidation of NADH to  $\text{NAD}^+$ . Assay solutions contained, (in

umoles) in a final volume of 1.0 ml; tris Cl, pH 8.4, (100); ATP, (2.5);  $\text{MgCl}_2$ , (5);  $\text{HCO}_3^-$ , sodium salt, (20); pyruvate, sodium salt, (10); acetyl CoA, (0.25); NADH, (0.125); malate dehydrogenase, (5 units); and pyruvate carboxylase (0.025-0.1 units).

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

#### II.B.4.b The radiochemical assay system

In this procedure [ $^{14}\text{C}$ ]bicarbonate fixed in an acid stable form is measured, while unreacted bicarbonate is driven off by acidification and subsequent drying on paper squares. Assay solutions contained, (in umoles), in a final volume of 0.5 ml; tris Cl, pH 8.4, (50); ATP, (1.25);  $\text{MgCl}_2$ , (2.5); [ $^{14}\text{C}$ ] $\text{HCO}_3^-$ , sodium salt,  $5 \times 10^5$  cpm/ $\mu\text{mole}$ , (5); pyruvate, sodium salt, (5); acetyl CoA, (0.125), and up to 0.06 units of pyruvate carboxylase. The reaction was initiated by addition of enzyme and allowed to proceed for a time up to five minutes at 30°C before being quenched by addition of 50  $\mu\text{l}$  of a saturated solution of 2,4-dinitrophenylhydrazine in 6M HCl. As well as terminating the reaction, this reagent drives off unreacted [ $^{14}\text{C}$ ]bicarbonate and stabilises the oxaloacetate formed as the dinitrophenylhydrazone. Triplicate samples (0.05 ml) were applied to one inch squares of Whatmans 3MM paper, dried at 90°C for five minutes, and the remaining radioactivity determined as described in Section II.B.3. This procedure was found to be more convenient than the spectrophotometric assay for experiments involving a large number of assays. Moreover, use of the radiochemical assay obviated

the need to measure the reaction rate in the presence of a ligand (NADH) which was not essential for pyruvate carboxylase activity.

The acetyl CoA-independent assay system used was that developed by Ashman (1973). The assay solutions contained, (in  $\mu$ moles), in a final volume of 0.5 ml; tris Cl, pH 8.4, (50); ATP, (1.25);  $MgCl_2$ , (4); [ $^{14}C$ ]HCO $_3^-$ , sodium salt,  $5 \times 10^5$  cpm/ $\mu$ mole, (20); pyruvate, sodium salt, (20); NH $_4$ Cl, (50), and 2 units of pyruvate carboxylase. [ $^{14}C$ ]oxaloacetate was determined by the procedure described above for the acetyl CoA-dependent assay.

#### II.B.5 ATP/P $_i$ isotopic exchange reaction assay

Reaction mixtures contained, (in  $\mu$ moles), in a final volume of 0.5 ml: tris Cl, pH 8.4, (50), ATP, (1); ADP, (1);  $MgCl_2$ , (4); HCO $_3^-$ , sodium salt, (1); acetyl CoA, (0.125); phosphate, sodium salt, (5), and pyruvate carboxylase, 0.5-1 units. In the controls, pyruvate carboxylase that had been specifically inactivated by previously treating with avidin was used. The reaction was initiated by addition of carrier-free [ $^{32}P$ ]ortho phosphate ( $1-2 \times 10^6$  cpm) after incubating the reaction mixtures at 30°C for ten minutes. Samples (0.2 ml) were taken at various time intervals and quenched with 0.1 ml 5M formic acid, at 0°C.

Separation of ATP and ortho phosphate was achieved by either chromatography on polyethyleneimine ion exchange paper (prepared by the method of Gilliland et al., 1966) using 0.2M NH $_4$ HCO $_3$  as the developing solvent, or by an activated charcoal separation procedure.

When the latter technique was used, a 0.05 ml sample of the formic acid quenched sample was diluted with 1 ml sodium phosphate (0.1M, pH 4.4). 0.2 ml of an activated charcoal suspension in 6M HCl (250 mg/ml) was then added and the mixture applied to a glass-fibre disk. After six



washes with 0.1M HCl, and one with water, the disk was dried, and its radioactivity determined using the procedure described in Section II.B.3.

#### II.B.6 Pyruvate/oxaloacetate isotopic exchange reaction assay

Assays contained, (in  $\mu\text{mole}$ ), in a final volume of 0.5 ml: tris Cl, pH 8.4, (50); oxaloacetate, (1); pyruvate, (unless otherwise stated), (1), acetyl CoA, (0.125); and pyruvate carboxylase, 0.1 units. Usually the reaction was initiated, after five minutes incubation at 30°C, by addition of sodium [2- $^{14}\text{C}$ ]pyruvate (0.005  $\mu\text{mole}$ ,  $4 \times 10^7$  cpm/ $\mu\text{mole}$ ), dissolved in 0.02 M HCl.

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

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

in acetone. Sample lanes were cut into 1 cm pieces and radioactivity determined as described in Section II.B.3. Since contaminant parapyruvate moved very close to aspartate on the electrophoretograms, correction was made for it by subtracting controls from which enzyme was omitted. The rate of isotope exchange was calculated using the formula: (Wahl and Bonner, 1951)

$$\text{rate} = - \frac{[\text{pyr}] \cdot [\text{OAA}]}{[\text{pyr}] + [\text{OAA}]} \cdot \frac{\ln(1-F)}{t}$$

where  $t$  is time of exchange, and  $F$ , the fractional equilibrium, is given by the expression:

$$F = \frac{\% \text{ label in oxaloacetate}}{\left( \frac{[\text{oxaloacetate}]}{[\text{pyruvate}] + [\text{oxaloacetate}]} \times 100 \right)}$$

#### II.B.7 Determination of biotin concentration

Biotin concentration was determined using the radiochemical assay developed by Rylatt et al., (1977). The assay solution contained, in a final volume of 0.5 ml; potassium phosphate, pH 7.2 (50  $\mu$ mole), avidin (0.028 units) and biotin sample (10-100 pmole biotin). After incubating for ten minutes at room temperature, 0.05 ml [ $^{14}$ C]biotin (2.2 nmole,  $10^5$  cpm/nmole) was added, and ten minutes later the avidin-biotin complex was precipitated by adding 0.2 ml 0.1M zinc sulphate, followed by 0.2 ml 0.1M sodium hydroxide. The precipitate was collected by centrifuging, and samples of the supernatant taken and their radioactivity determined using the Triton based scintillant (Section II.B.3). The correlation between the radioactivity of the supernatant and the biotin content of the sample was established by constructing a standard curve using known amounts of unlabelled biotin as reference samples. There was a linear relationship between biotin content and radioactivity for the range 10-70 pmoles of biotin.

CHAPTER III

AFFINITY LABELLING OF THE MgATP<sup>2-</sup>

BINDING SITE

### III.A. INTRODUCTION

To describe the mechanism of an enzymically catalysed reaction at the molecular level, the first pre-requisite is to determine the order of addition of substrates and release of products, and then to elucidate the role various amino acid residues play in the binding and catalytic processes. Clearly, the micro-environment of these residues is of paramount importance in the process, and therefore an understanding of the primary structure and geometry of the polypeptide chain(s) comprising the active site is essential. However, with complex high molecular weight proteins like pyruvate carboxylase this information is not always readily available. For example, X-ray diffraction studies are not possible when the protein has not been crystallised, and primary sequences cannot be unequivocally localised without a method of specifically labelling the active site. In these cases affinity labelling provides a useful alternative technique to use in studies of the chemistry of the active site.

#### III.A.1. Affinity Labelling.

Affinity labelling is a method for covalently modifying one or more amino acid residues in or near the ligand site(s) of a protein molecule. For this purpose a reagent (usually an analogue of the ligand of interest) is designed so that it first combines specifically and reversibly at the ligand binding site in question, and then, by virtue of a reactive group on the reagent, reacts to form a covalent bond with some juxtaposed amino acid residue in or near the ligand binding site. Shaw (1970), and Singer (1970) have reviewed the application of both chemically active compounds, and photo-reactive compounds, in which the

reactive group is generated by photolysis once reversible binding has occurred.

Experimental criteria are needed to establish that a presumptive affinity label is attached to a contact residue in the ligand binding site under investigation. These criteria have been discussed in detail by Singer (1967). Affinity labelling of a ligand binding site of a pure soluble protein should result in stoichiometric inactivation of the reversible binding activity and/or catalytic activity of the site; that is the number of moles of label bound per mole of original active sites should equal the fraction of binding sites modified, and should not exceed 1.0. If a specific ligand of the binding site in question fails to afford protection against inactivation, then the putative affinity label is unlikely to be acting solely at the ligand binding site. In designing experiments to test for specific protection it is important to realise that the protection effect is a rate phenomenon, because labelling is an irreversible process; thus, given a long enough reaction time, even a protected sample may be completely inactivated by the affinity labelling reagent.

Another criterion is affinity inactivation in which the effect of reaction with an affinity labelling reagent is compared with that of another reagent with the same reactive group, but having no specific affinity for the ligand binding site in question. However, if the two reagents have radically different intrinsic reactivities, requiring different reaction conditions to achieve comparable rates of inactivation, then this criterion will be at best ambiguous.

### III.A.2 ATP Affinity Labels

The use of ATP analogues as affinity labels has been recently

reviewed by Yount (1975). Given that ATP is listed as a substrate for over 120 enzymes (Stadtman, 1970), many of which are of central importance in metabolism, it is clear that the use of ATP analogues is a rapidly expanding area of biochemical research. Probably the most successful affinity label has been thioinsine triphosphate, and its disulphide. These compounds have been used to label the myosin ATPase (Murphy and Morales, 1970), the aspartokinase activity of threonine-sensitive aspartokinase-homosevine dehydrogenase (Truffa-Bachi and Heck, 1971), and phosphofructokinase (Bloxham et al., 1973). Analogues modified at the 5' phosphate position (Berghauser and Geller, 1974), and in the ribose ring (Pal et al., 1975), have also been employed as affinity labels.

### III.A.3. Selection of an affinity label for the pyruvate carboxylase MgATP binding site.

Although a number of adenine nucleotide binding enzymes have been shown to contain lysyl residues at their nucleotide binding sites (e.g. Kubo et al., 1960; Hollenburg et al., 1971; Nixon et al., 1972; James and Cohn, 1974) direct chemical modification of such groups in pyruvate carboxylase, did not seem feasible. The difficulty stems from the fact that the lysine-specific reagent TNBS, reacts with a lysyl residue at the allosteric site, and desensitises the enzyme against the action of the adenine nucleotide allosteric effector, acetyl CoA (Keech and Farrant, 1968; Ashman et al., 1972). Accordingly, we have attempted to design MgATP<sup>2-</sup>-based affinity labels which would be reactive with respect to the putative lysyl residues in the MgATP<sup>2-</sup> binding site.

One group of nucleotide analogues which are reactive with lysyl residues are the 2', 3' dialdehyde derivatives, prepared by periodate cleavage of the ribose ring. The dialdehyde derivatives of adenosine and

AMP have been shown to inhibit nucleic acid synthesis in Erlich tumor cells, both in vivo and in vitro (Cory et al., 1974). The ATP derivative inhibits ribonucleotide reductase, and the labelled derivative co-chromatographs with the protein on Sephadex G-25 gel filtration (Cory and George, 1973). The dialdehyde derivative of UDP has been used as an affinity label of the UDP-galactose binding site of bovine colostrum galactosyl transferase (Powell and Brew, 1976). The dialdehyde oxidation product of ribosyl-6-methylthiopurine has been employed as an affinity label of RNA polymerase (Nixon et al., 1972), RNase A, (Spoor et al., 1973), and DNA-dependent DNA polymerase I (Salvo et al., 1976).

In this chapter the use of the 2', 3'-dialdehyde derivative of ATP as an affinity label of the  $\text{MgATP}^{2-}$  binding site of pyruvate carboxylase is reported.

### III.B. MATERIALS AND METHODS

#### III.B.1. Assay Methods

The rate of pyruvate carboxylation was measured by the procedure described in Section II.B.4. The pyruvate/oxaloacetate isotopic exchange reaction, and the  $\text{ATP}/\text{P}_i$  isotopic exchange reaction were measured by the procedure described in Section II.B.6, and II.B.5. Biotin determinations were carried out as described in Section II.B.7.

#### III.B.2. Synthesis of oATP.

ATP (0.1 mmole) was dissolved in water, and the solution adjusted to pH 7.0 at 0°C. Sodium periodate (0.11 mmole) was added, and the solution allowed to stand at 4°C in the dark for one hour, by which time reaction was complete as judged by chromatography of the

reaction mixture on polyethylene imine thin layer chromatograms, developed with 0.8 M ammonium bicarbonate. The reaction was stopped by addition of ethylene diol (0.05 mmole) and the reaction mixture loaded on to a Sephadex G10 column (25x2 cm) previously equilibrated with glass-distilled water at 4°C. The column was eluted with distilled water, 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 an extinction coefficient of  $14900 \text{ cm}^2\text{mol}^{-1}$  (Hansske et al., 1974). The purity of the oATP was confirmed by chromatography in two different solvent systems. Thin layer chromatography was performed on polyethylene-imine sheets, using 0.8 M ammonium bicarbonate as the developing solvent, and ultraviolet light (254 nm) to locate the position of the nucleotide. Chromatography of the purified sample revealed only one compound with an  $R_f$  value of 0.02. No ATP ( $R_f$  0.24) could be detected. Ascending paper chromatography was performed on Whatmann 3MM paper, using 1-butanol: acetic acid:water (4:1:5 v/v) as the developing solvent, and again only one compound was detected ( $R_f$  0.10).

Although 2', 3'-dialdehyde nucleotide derivatives undergo  $\beta$ -elimination (Khyn and Cohn, 1961) it was not possible to detect the products of this reaction in the purified preparation. The  $\beta$ -elimination product, an  $\alpha$ - $\beta$  unsaturated aldehyde, would be expected to have an ultraviolet absorption peak at 220-230 nm (Williams and Fleming, 1966) but no such absorbance was observed. [ $^{14}\text{C}$ ]oATP was prepared from [ $^{14}\text{C}$ ]ATP, by the same procedure.

### III.B.3. Synthesis of [ $^3\text{H}$ ]lysyl-oATP.

The reaction mixture contained in  $\mu\text{mole}$ , in a final volume of 0.45 ml; oATP, 1.8; [ $^3\text{H}$ ]lysine,  $5(4.4 \times 10^4 \text{ cpm}/\mu\text{mole})$ ;  $\text{MgCl}_2$ , 2.5, and



sodium borohydride, 5. The mixture was adjusted to pH 11 with KOH. The mixture was allowed to stand at room temperature for 40 min after addition of the sodium borohydride. The adduct was purified by ascending chromatography on Whatmann 3MM paper, using 1-butanol: acetic acid:water (4:1:5 v/v) as the developing solvent. It was not possible to detect oATP adducts of cysteine, histidine, or arginine by this procedure.

#### III.B.4. Covalent modification of the enzyme.

Pyruvate carboxylase (10-20 units/ml) was incubated with Mg-oATP<sup>2-</sup> in 0.125 M N-ethyl morpholine acetate buffer, pH 8.4, containing 1 mM EDTA and 7.5 mM MgCl<sub>2</sub>. After five minutes sodium borohydride was added in ten-fold molar excess over the Mg-oATP<sup>2-</sup> used and the solution allowed to stand for 35 minutes. After this time enzyme activity remained constant, as is shown in Fig. III-1.

#### III.B.5. Data Processing

Kinetic data were plotted graphically to determine the inhibition patterns and then fitted to the appropriate rate equations by using the method of least squares, with the Fortran computer programmes of Cleland (1967). Where appropriate other data were fitted to a straight line using a Fortran least mean squares programme.

### III.C. RESULTS

#### III.C.1. Irreversible inhibition by reduction of the enzyme.

##### Mg-oATP<sup>2-</sup> complex.

In order to establish if reduction of the enzyme-Mg-oATP<sup>2-</sup> complex resulted in irreversible loss of catalytic activity, the effect of incubating pyruvate carboxylase with varying concentrations of Mg-oATP<sup>2-</sup>

was investigated. The data presented in Fig. III.2 establishes the fact that reduction of the enzyme-Mg-oATP<sup>2-</sup> complex using sodium borohydride inactivated the enzyme. The inactivation process was dependent on the presence of Mg-oATP<sup>2-</sup> since the control system, in which Mg-oATP<sup>2-</sup> was replaced by MgATP<sup>2-</sup>, did not lose enzyme activity. Furthermore, the extent of inactivation was a function of the Mg-oATP<sup>2-</sup> concentration. The irreversible nature of the inactivation was demonstrated by freezing the reduced enzyme-Mg-oATP<sup>2-</sup> complex of low molecular weight molecules by Sephadex G-25 gel filtration. After this treatment there was no significant restoration of enzymic activity. In order to establish that Mg-oATP<sup>2-</sup> was acting as an affinity label of the MgATP<sup>2-</sup> binding site it was necessary to satisfy the experimental criteria for an affinity label discussed in Section III.A.1. One of these criteria is that MgATP<sup>2-</sup> should specifically protect the enzyme against the inactivation process. Accordingly, the effects of the substrates and activators of pyruvate carboxylase on the inactivation process were investigated. It was found that when pyruvate, oxaloacetate, or bicarbonate were present, even at concentrations twenty-five times their apparent  $K_m$  values, none gave significant protection.

The data shown in Figs. III.3 and 4 show that acetyl CoA and MgATP<sup>2-</sup> both protected the enzyme against the inactivation process. The profile of the protection curve obtained with increasing concentrations of acetyl CoA could be interpreted in two not necessarily exclusive ways. First of all it is possible that oATP binds to both the acetyl CoA activator site, and the MgATP<sup>2-</sup> binding site, and that acetyl CoA protects against modification at the former site. However, evidence presented below shows that only one molecule of Mg-oATP<sup>2-</sup> binds per pyruvate carboxylase monomer. Also, attempts to reduce oxidised dephospho-acetyl CoA (i.e. prepared by the same procedure as used to synthesise oATP) on to the enzyme using sodium

borohydride have been unsuccessful (P.R. Clements, unpublished results). This result implies that there is no residue in the acetyl CoA binding site of sheep pyruvate carboxylase that is accessible to modification by periodate-oxidised nucleotides.

Secondly, the more likely explanation for the protection observed in the presence of acetyl CoA is that the allosteric effector, even at low concentrations, changes the environment of the  $\text{MgATP}^{2-}$  binding site, inducing a partial protection of the enzyme which does not increase with increasing concentrations of acetyl CoA. This explanation is consistent with the observation that the  $K_m$  value for bicarbonate is lower in the presence of saturating acetyl CoA levels than in the absence of acetyl CoA (Ashman et al., 1972). The simplest interpretation of this observation is that acetyl CoA binding induces a change in the structure of the biotin carboxylation site (which includes the  $\text{MgATP}^{2-}$  and bicarbonate binding sites).

It has been reported that  $\text{Mg}^{2+}$  increases the affinity of sheep pyruvate carboxylase for  $\text{MgATP}^{2-}$  (Keech and Barritt, 1967). Thus, if  $\text{Mg-oATP}^{2-}$  binds at the  $\text{MgATP}^{2-}$  binding site,  $\text{Mg}^{2+}$  would be expected to increase the affinity of the enzyme for  $\text{Mg-oATP}^{2-}$ . In order to test this hypothesis, the effect of varying the  $\text{Mg}^{2+}$  concentration on the extent of inactivation by  $\text{Mg-oATP}^{2-}$  was investigated. The results shown in Fig. III-5 show that the extent of inactivation is increased at higher concentrations of  $\text{Mg}^{2+}$ . This observation is therefore consistent with  $\text{Mg-oATP}^{2-}$  binding at the  $\text{MgATP}^{2-}$  binding site.

### III.C.2 Reversible inhibition by $\text{Mg-oATP}^{2-}$ .

In the absence of sodium borohydride,  $\text{Mg-oATP}^{2-}$  behaved as a reversible inhibitor with respect to  $\text{MgATP}^{2-}$  in both the acetyl CoA

dependent and -independent reactions. It was a non-competitive inhibitor with respect to bicarbonate and an uncompetitive inhibitor with respect to pyruvate.

This inhibition pattern is identical with that reported for MgADP acting as a product inhibitor of the enzyme reaction (Ashman and Keech, 1975). This equivalence implies that Mg-oATP<sup>2-</sup> binds at the MgATP<sup>2-</sup> binding site. Secondary plots of the reversible inhibition data were linear, indicating that only one molecule of Mg-oATP<sup>2-</sup> was binding at the active site. These results are summarised in Table III-1.

### III.C.3 Effect of oATP modification on the isotopic exchange reactions

Further evidence to support the hypothesis that Mg-oATP<sup>2-</sup> was acting only at the MgATP<sup>2-</sup> binding site was obtained by studying the effect of covalent modification on the exchange reactions catalysed by pyruvate carboxylase. These data are presented in Table III.2 where it can be seen that the ATP/<sup>32</sup>P<sub>i</sub> isotopic exchange reaction was inhibited to the same extent as the overall catalytic reaction, while the [<sup>14</sup>C]pyruvate/oxaloacetate exchange reaction was not significantly affected by the chemical modification. If Mg-oATP<sup>2-</sup> acted as a modifier of the acetyl CoA activation site, then the extent of acetyl CoA activation of the [<sup>14</sup>C]pyruvate/oxaloacetate exchange reaction would be reduced, resulting in a lower rate of exchange catalysed by modified enzyme relative to the control.

### III.C.4 The stoichiometry of the inactivation process.

The data presented above provide evidence that Mg-oATP<sup>2-</sup> was binding to the enzyme at the MgATP<sup>2-</sup> binding site. However, probably

the most important criterion for affinity labelling is that of stoichiometric inactivation of the enzyme. For this purpose the enzyme was modified using variable concentrations of [ $^{14}\text{C}$ ]oATP ( $1.32 \times 10^7$  cpm/ $\mu\text{mole}$ ) by the procedure described in Section III.B.4. Forty-five minutes after addition of the sodium borohydride enzymic activity was determined and the remainder of the enzyme was precipitated with 30% trichloroacetic acid ( $^w/v$ ) after 8 mg of bovine serum albumin had been added to act as a carrier protein. The precipitate was collected by centrifuging and then washed exhaustively in 10% trichloroacetic acid ( $^w/v$ ) until the radioactivity in the supernatant had been reduced to background levels. The denatured protein was then dissolved in formic acid, aliquots applied to 4 x 4 cm paper squares, dried and counted using the toluene scintillant.

Sheep liver pyruvate carboxylase, like all pyruvate carboxylases isolated from vertebrate sources, is a tetramer containing four molecules of biotin, and is therefore assumed to have four catalytic sites. Furthermore, since there is no definite procedure of sufficient sensitivity for measuring absolute protein concentrations it seemed more precise to express the stoichiometric relationship in terms of biotin content. Results of a typical experiment are shown in Fig. III.6 where it can be seen that at 100% inactivation the extrapolated line indicates that there are  $1.09 \pm 0.11$  molecules of [ $^{14}\text{C}$ ]oATP bound per molecule of biotin. Table III.3 summarises the results of a number of experiments and shows that over a wide range of specific activities this ratio does not significantly alter, nor does the presence of acetyl CoA have any influence on the value of the ratio.

Two conclusions may be drawn from this data. Firstly, the observation of one molecule of oATP bound per active site at 100% inactivation supports the conclusion that  $\text{Mg-oATP}^{2-}$  is an affinity label of the

MgATP<sup>2-</sup> binding site. Also, the observation that the stoichiometry of labelling is independent of the presence of acetyl CoA provides further evidence that the protection afforded by acetyl CoA against inactivation is not due to protection against modification at the acetyl CoA activator site.

### III.C.5 Identification of the modified amino acid

To identify the residue labelled by covalently bonding oATP to the enzyme, 60 units of pyruvate carboxylase (specific activity 30 units/mg of protein) were modified with [<sup>14</sup>C]oATP (1.1 x 10<sup>7</sup>cpm/μmole) by the procedure described previously. After reducing with sodium borohydride, the solution was dialysed against glass-distilled water. The dialysate was then lyophilised to dryness, redissolved in 0.5ml 0.1 M N-ethyl morpholine acetate, pH 8.0, and enzymically digested in the following way. The protein was first treated for two hours with 0.2 mg trypsin, followed by a fifteen hour incubation with 0.2 mg of α-chymotrypsin, and two successive treatments twenty-four hours apart with 2 mg of pronase, and finally a twenty-four hour treatment with 0.2 mg of α-amino-peptidase. Aliquots of the resulting digest were resolved by ascending chromatography on Whatman 3MM paper using n-butanol/acetic acid/water (4:1:5 v/v) as the developing solvent. After drying the developed chromatogram, it was cut into one cm sections and the radioactivity in each section determined. The results of a typical experiment are presented in Fig. III.7 where it can be seen that there was present in the protein digest a single radioactive compound which had the same R<sub>f</sub> value as the [<sup>3</sup>H]lysyl-oATP marker.

Thus it may be concluded that a lysyl residue is modified by oATP during inactivation of pyruvate carboxylase. The observation that a lysyl residue is in fact modified is not unexpected in view of the specificity

of periodate oxidised nucleotides for such groups reported by a number of investigators (Nixon et al., 1972; Powell and Brew, 1975).

### III.D DISCUSSION

#### III.D.1 The reactive form of oATP

The organic chemistry of the periodate oxidation products of nucleotides and nucleosides has been the subject of several studies. In an investigation of the periodate oxidation products of adenosine and AMP, Hansske et al., (1973) were able to show, using the techniques of infra-red spectroscopy and nuclear magnetic resonance, that in aqueous solution, there was a complex equilibrium between the dialdehyde form, the hydrate form, and the cyclic diether. Jones et al., (1976) have shown that the oxidised nucleotides exist in polymeric form in the solid state, and in concentrated aqueous solution. In dilute aqueous solution they were able to detect the hydrate form and the cyclic diether. We have not tried to distinguish which form is involved in the irreversible modification of pyruvate carboxylase although it is difficult to visualise how anything but the dialdehyde form could be reactive with respect to the  $\epsilon$ -amino of lysine. For the purpose of investigating the mechanism of pyruvate carboxylase, the important point was to establish whether the oxidation product of ATP is an affinity label of the  $\text{MgATP}^{2-}$  binding site of the enzyme.

Because of the uncertainty of the precise levels of the various forms of oATP present in solution, a quantitative investigation of the dependence of the rate of enzyme inactivation on nucleotide concentration was not carried out. This meant it was not possible to establish if oATP exhibited "saturation kinetics" of inactivation (Meloche, 1967), although the observation that  $\text{Mg-oATP}^{2-}$ , in the absence of borohydride, acted as a

reversible inhibitor of the reaction implies that it does form a complex with the enzyme.

### III.D.2 The role of the lysyl residue

It is not clear whether the lysyl residue identified in the  $\text{MgATP}^{2-}$  binding site plays a significant role in the catalytic mechanism of pyruvate carboxylase. The fact that deoxy-ATP can replace ATP in the  $\text{CO}_2$ -fixation reaction without a significant change in the apparent  $K_m$  and  $V$  values for both the avian and rat liver enzymes (Scrutton and Utter, 1965; McClure *et al.*, 1971) suggests that the lysyl residue is not involved in binding the ribose moiety. This does not necessarily imply that the lysyl residue is not in close proximity to the pentose ring when  $\text{MgATP}^{2-}$  is bound in the catalytically active stereochemical position.

However, given that the enzyme utilises ATP and deoxyATP with equal facility, it is perhaps surprising that modification of the ribose ring by periodate oxidation results in a derivative which is unable to support  $\text{CO}_2$  fixation. This suggests that  $\text{Mg-oATP}^{2-}$  does not bind in the catalytically active stereochemical position. An alternative possibility is that the lysyl residue is involved in binding the 5'-phosphates of  $\text{MgATP}^{2-}$  as illustrated in Fig. III.8. As was discussed in Section I.B.1.d.ii besides maintaining the proper alignment of substrates on the enzyme surface for catalysis to occur, the  $\epsilon\text{-NH}_3^{\oplus}$  group could withdraw electrons from the terminal phosphate group of  $\text{MgATP}^{2-}$ , thus facilitating nucleotide attack by an oxygen atom of bicarbonate during the formation of carbonyl phosphate. This hypothetical positioning of the lysyl group near the interface of the  $\text{MgATP}^{2-}$  and bicarbonate binding sites has been further investigated using the lysine-modifying chemical reagent: cyanate. The results of this investigation are presented in Chapter IV.



### III.D.3 Extensions of the oATP affinity labelling studies

Although the identification of a lysyl group in the  $\text{MgATP}^{2-}$  binding site has allowed the above speculation as to its role in the catalytic process, the data obtained to date does not allow a direct assignment of a role for this residue. Clearly, the next step in the oATP affinity labelling studies will be the preparation and purification of peptides containing the oATP modified lysyl residue. When the sequence of these peptides has been determined, more information concerning the identity and role(s) of the amino acid residues comprising the ATP binding site will become available. Preparation and purification of the oATP labelled tryptic peptide(s) has been hindered by seasonal variation in the yields and purification of sheep liver pyruvate carboxylase. The procedure to be used in purifying the tryptic peptide is designed to exploit the attachment of a hydrophobic purine ring to the peptide of interest. The procedure involves adsorption of the labelled peptide to a charcoal/celite column, followed by extensive washing with aqueous solvent to remove unbound peptide material. The bound peptide will then be expected to be specifically eluted with 3% ammonia in 50% acetone. A procedure of this type has been already used to purify nucleotide-labelled peptides (Anderson et al., 1973).

In order to build up a picture of the three dimensional orientation of all the residues comprising the ATP binding site, it will be necessary to purify and sequence other peptides labelled with different ATP affinity labels. The potential photo-affinity labels, 8-azido ATP and 6-azido ATP, as well as thioinosine-triphosphate are presently under investigation in this laboratory (Hayward, unpublished results).

Fig. III.1 Irreversible inactivation by reduction of the enzyme-  
Mg-oATP<sup>2-</sup> complex.

Pyruvate carboxylase (13 U/mg, 10 U/ml) was modified with 1mM Mg-oATP<sup>2-</sup> ( ○ ), for the times indicated, by the procedure described in Section III.B.4. In the control ( ● ) Mg-oATP<sup>2-</sup> was replaced by MgATP<sup>2-</sup>.

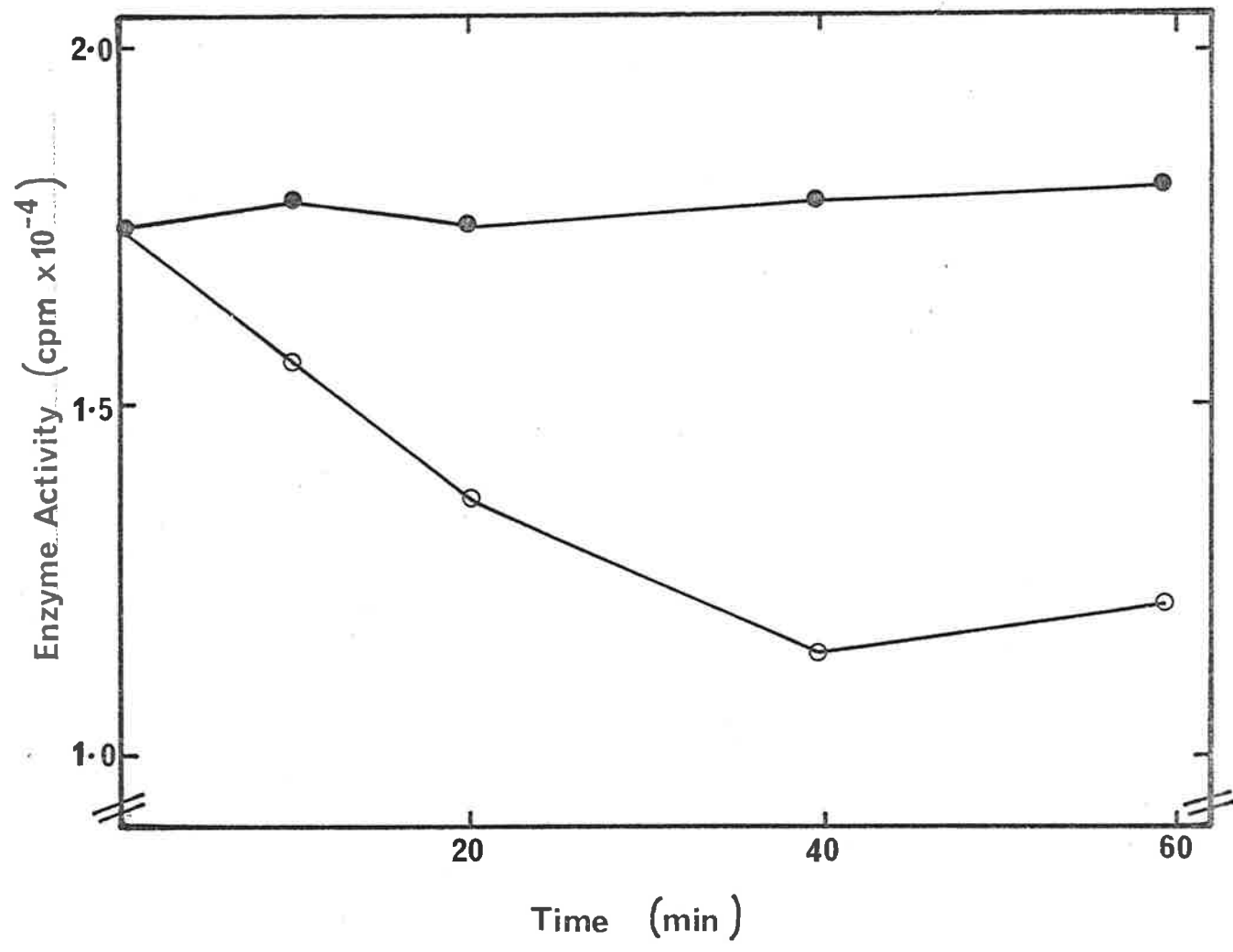


Fig. III.2 Irreversible inactivation by reduction of the enzyme-  
Mg-oATP<sup>2-</sup> complex.

Pyruvate carboxylase (13.6 U/mg), 12 U/ml) was modified with variable concentrations of Mg-oATP<sup>2-</sup> ( □ ), by the procedure described in Section III.B.4. In the control, ( ■ ) Mg-oATP<sup>2-</sup> was replaced by MgATP.

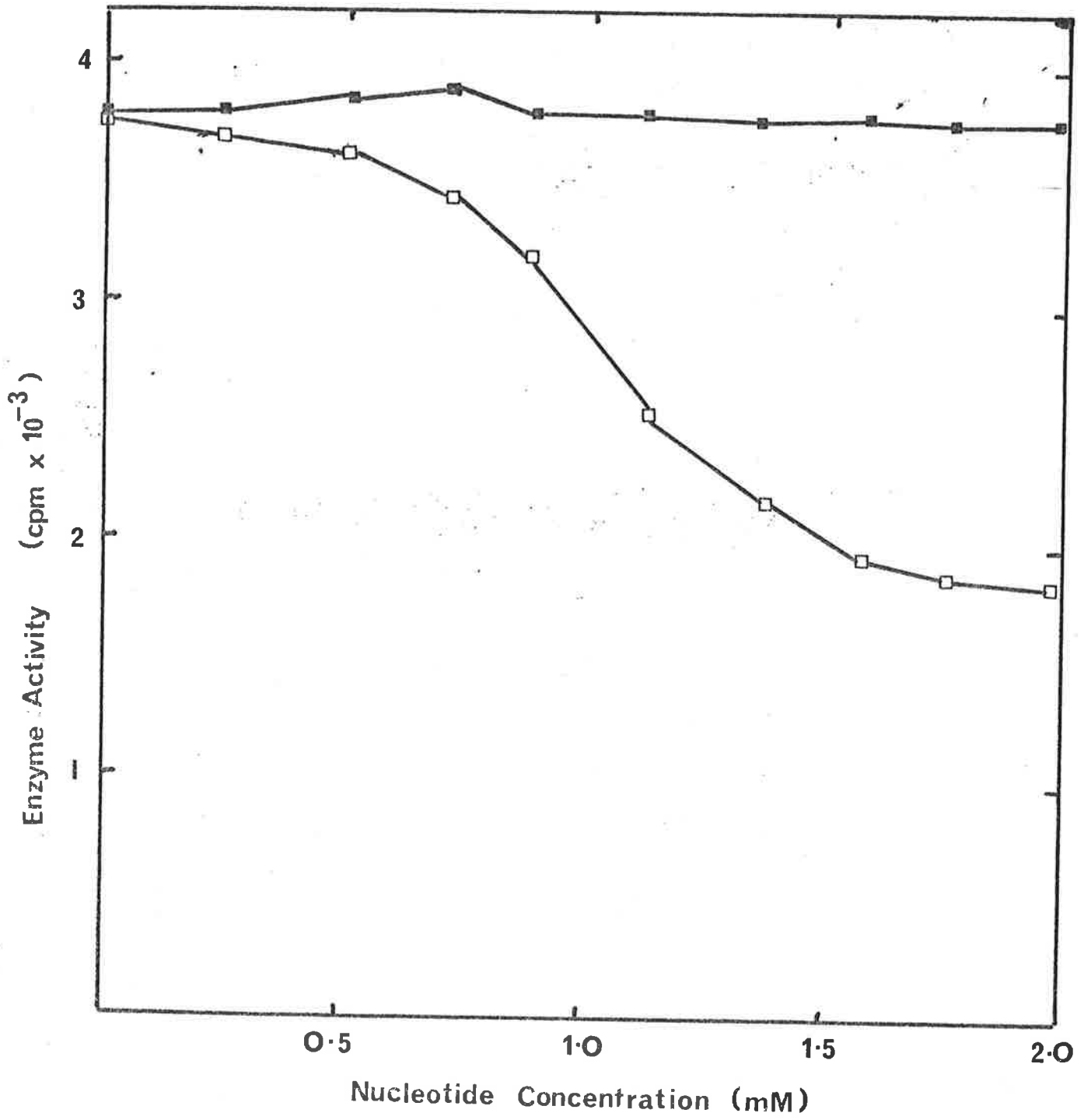


Fig. III.3 Protection of pyruvate carboxylase against irreversible  
Mg-oATP<sup>2-</sup> modification by MgATP<sup>2-</sup>.

Modification using 0.5 mM Mg-oATP<sup>2-</sup> ( □ ), in the presence of variable concentrations of MgATP<sup>2-</sup> was carried out using the procedure described in Section III.B.4. In the control ( ■ ), Mg-oATP<sup>2-</sup> was replaced by MgATP<sup>2-</sup>.

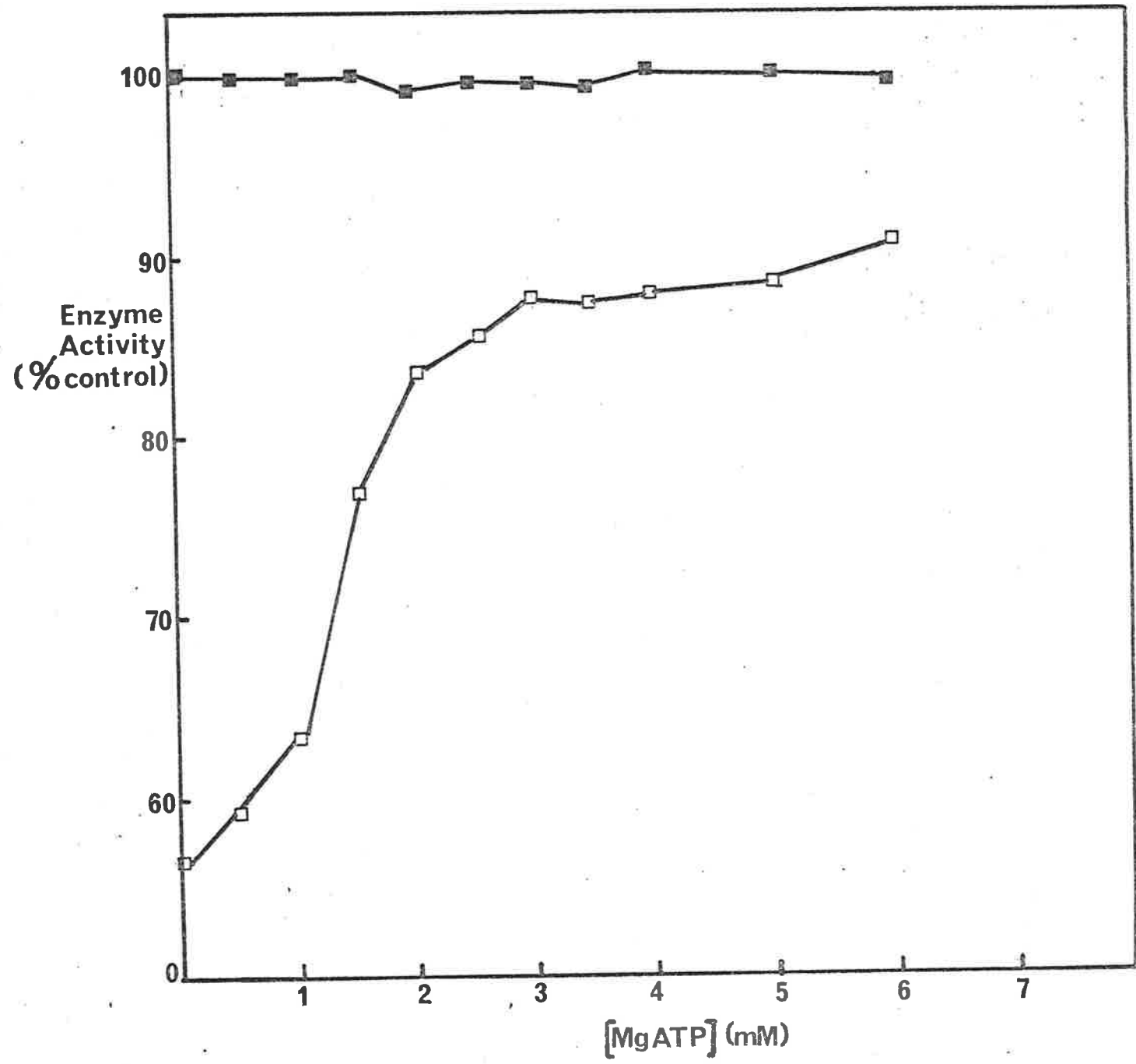


Fig. III.4 Protection of pyruvate carboxylase against irreversible modification by acetyl CoA.

Modification using 1.8 mM Mg-oATP<sup>2-</sup> ( O ), in the presence of variable concentrations of acetyl CoA was carried out using the procedure described in Section III.B.4. In the control ( ● ) Mg-oATP<sup>2-</sup> was replaced by MgATP.



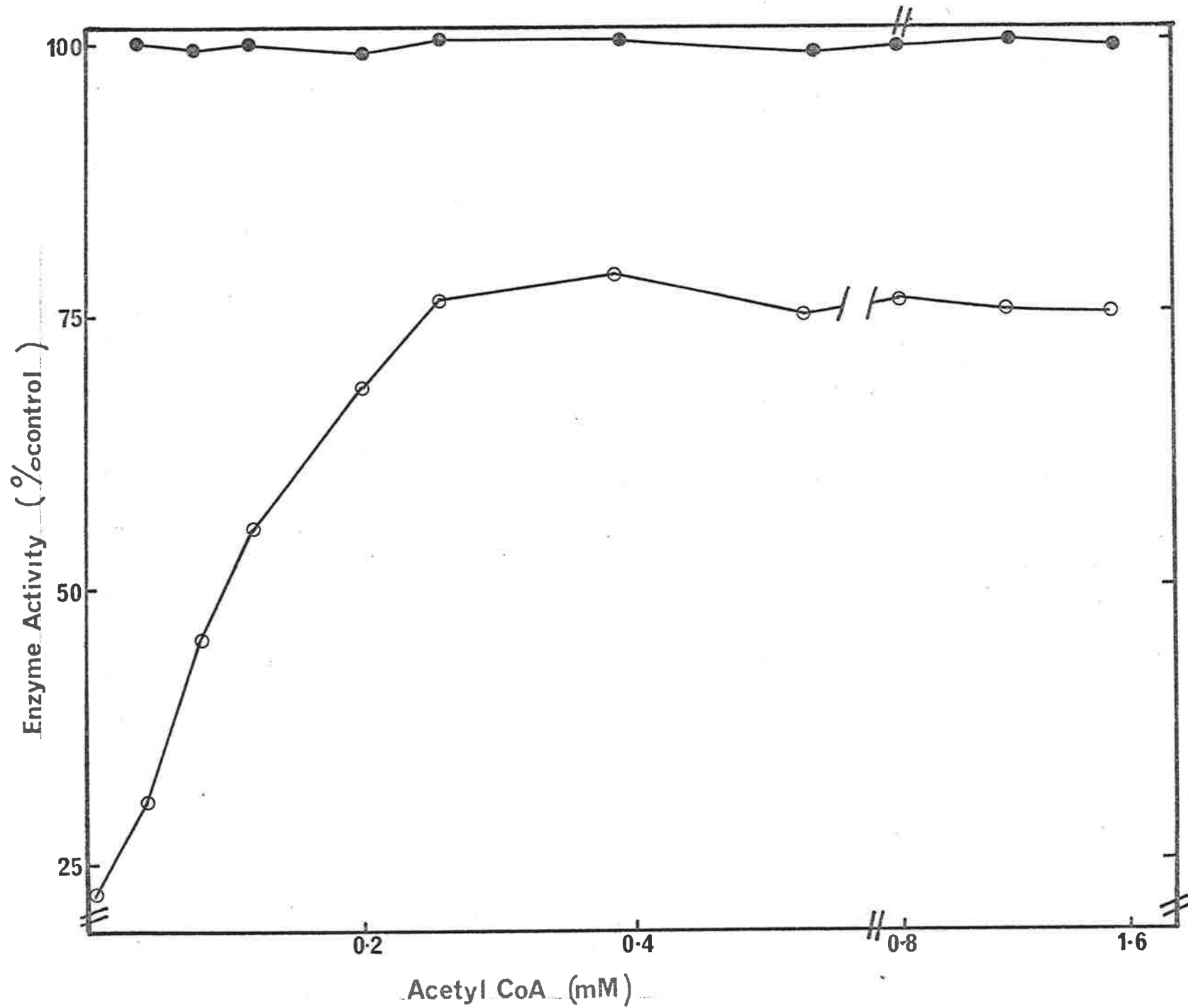


Fig. III.5 Effect of  $Mg^{2+}$  on irreversible inactivation of pyruvate carboxylase by  $Mg\text{-oATP}^{2-}$ .

Pyruvate carboxylase (12 U/mg) was modified with 0.8 mM  $Mg\text{-oATP}^{2-}$  in the presence of variable concentrations of  $Mg^{2+}$ , using the procedure described in Section III.B.4.

In the control  $Mg\text{-oATP}^{2-}$  was replaced by  $MgATP^{2-}$ . The modification was carried out either in the absence of free  $Mg^{2+}$  (○), or at the free  $Mg^{2+}$  concentrations indicated (●).

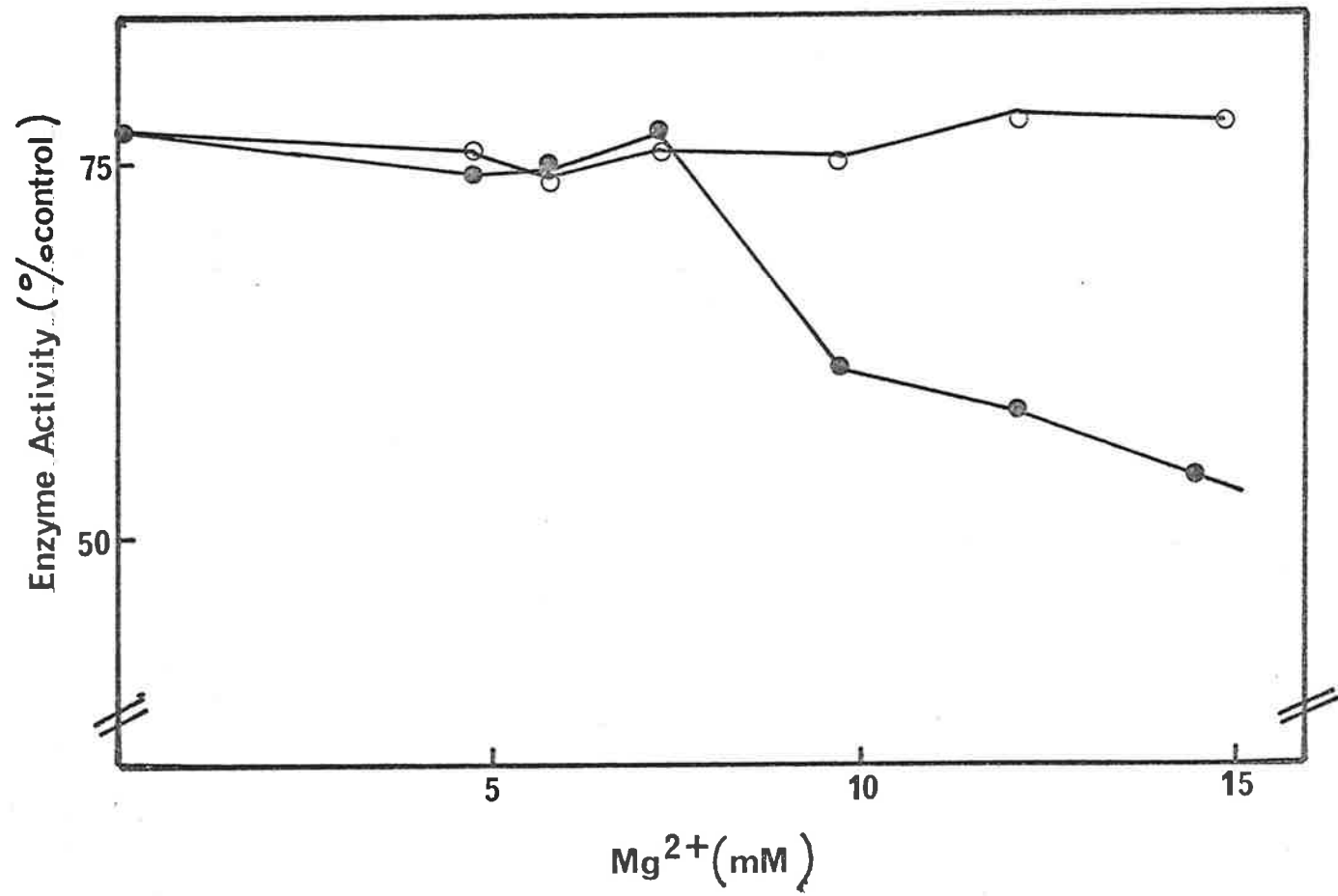


Table III.1 Reversible inhibition of pyruvate carboxylase by Mg-oATP<sup>2-</sup>.

The abbreviations used are: C, competitive; NC, non-competitive; UC, uncompetitive.  $K_{is}$  and  $K_{ii}$  are inhibition constants derived from replots of slopes and intercepts respectively.

Varied substrate	Acetyl CoA	Type of inhibition	Apparent kinetic constants
	(mM)		(mM)
MgATP <sup>2-</sup>	0.25	C	$K_{is} = 0.25 \pm 0.02$
MgATP <sup>2-</sup>	0.00	C	$K_{is} = 0.33 \pm 0.03$
HCO <sub>3</sub> <sup>-</sup>	0.25	NC	$K_{ii} = 0.38 \pm 0.04$ $K_{is} = 0.91 \pm 0.05$
Pyruvate	0.25	UC	$K_{ii} = 0.66 \pm 0.06$

Table III.2 Effect of modification on partial exchange reactions.

Pyruvate carboxylase (13.3 U/mg) was modified with 2 mM Mg-oATP<sup>2-</sup> as described in Section III.B.4. The modified enzyme was purified by Sephadex G25 chromatography and used to initiate the ATP/orthophosphate and pyruvate/oxaloacetate exchange reactions using the procedures described in Sections II.B.5 and II.B.6 respectively. In the control, MgATP<sup>2-</sup> was substituted for Mg-oATP<sup>2-</sup> in the modification procedure.

	Overall reaction	ATP/orthophosphate exchange reaction	Pyruvate/oxalo- acetate exchange reaction
	(%)	(%)	(%)
Control	100	100	100
Experimental	62 ± 3	58 ± 2	98 ± 2

Fig. III.6 Stoichiometry of irreversible inactivation of pyruvate carboxylase by Mg-oATP<sup>2-</sup>.

Pyruvate carboxylase (23 U/mg) was modified with varying concentrations of Mg-o-[<sup>14</sup>C]ATP<sup>2-</sup> using the procedure described in Section III.C.4. The data shown were fitted to a straight line using a least mean squares computer programme.



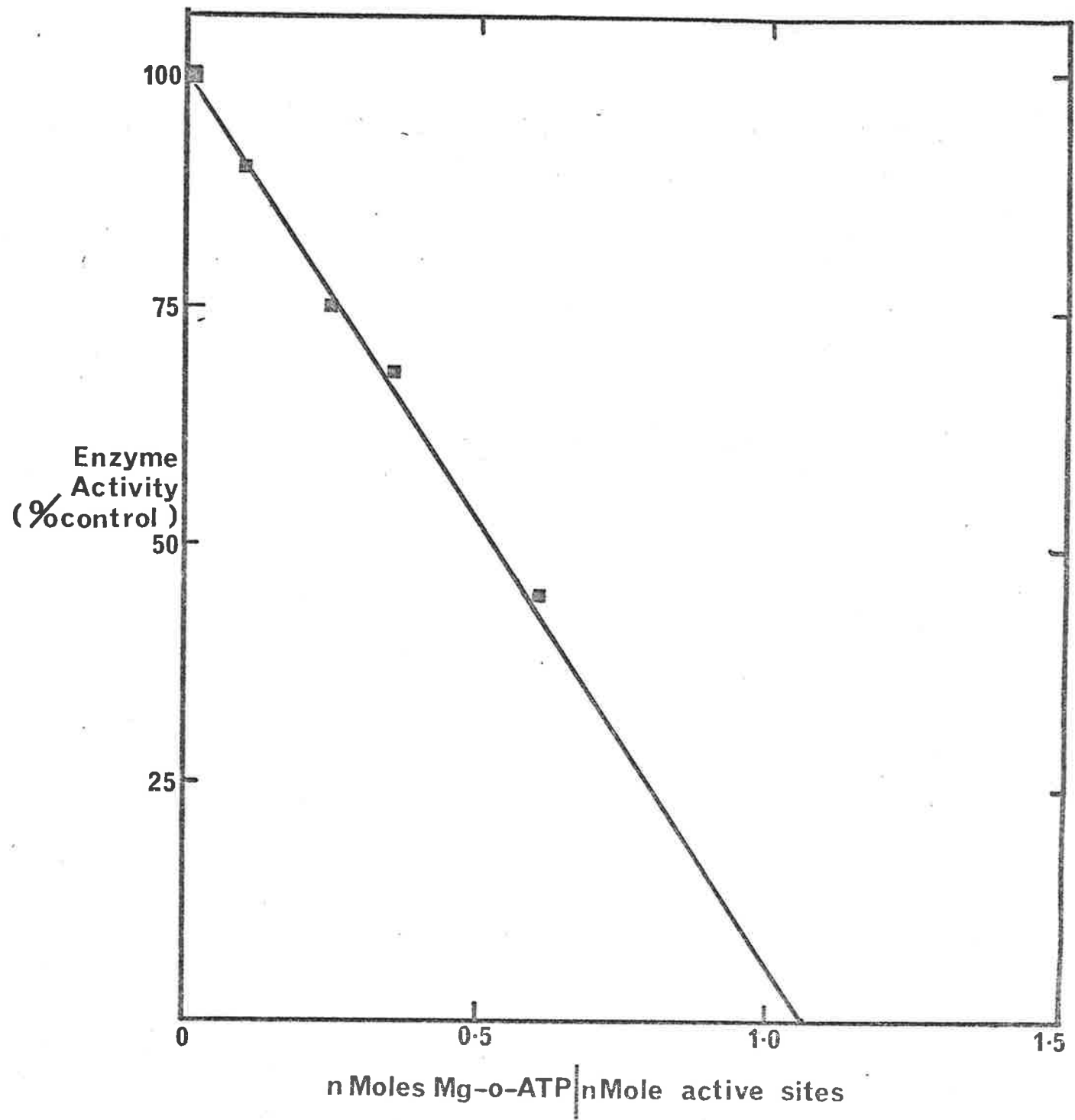


Table III.3 Stoichiometry of the inactivation of pyruvate carboxylase  
by Mg-oATP<sup>2-</sup> in the presence of NaBH<sub>4</sub>.

Enzyme specific activity	Acetyl CoA	$\frac{\text{Moles oATP bound}}{\text{Moles biotin}}$ at 100% inactivation
(units/mg of protein)	(mM)	
30	0.0	1.04 ± 0.03
23	0.0	1.13 ± 0.17
23	1.06	1.07 ± 0.04
29	0.0	1.10 ± 0.09
17	1.50	1.10 ± 0.07

Fig. III.7 Identification of the modified amino acid residue

Aliquots of an enzymatic digest of o[<sup>14</sup>C]ATP modified pyruvate carboxylase (30 U/mg) were subjected to paper chromatography on Whatman 3MM paper, using 1-butanol/acetic acid/water (4:1:5 v/v) as the developing solvent [<sup>3</sup>H]lysyl-oATP was used as a marker. (■) <sup>14</sup>C; (□) <sup>3</sup>H.

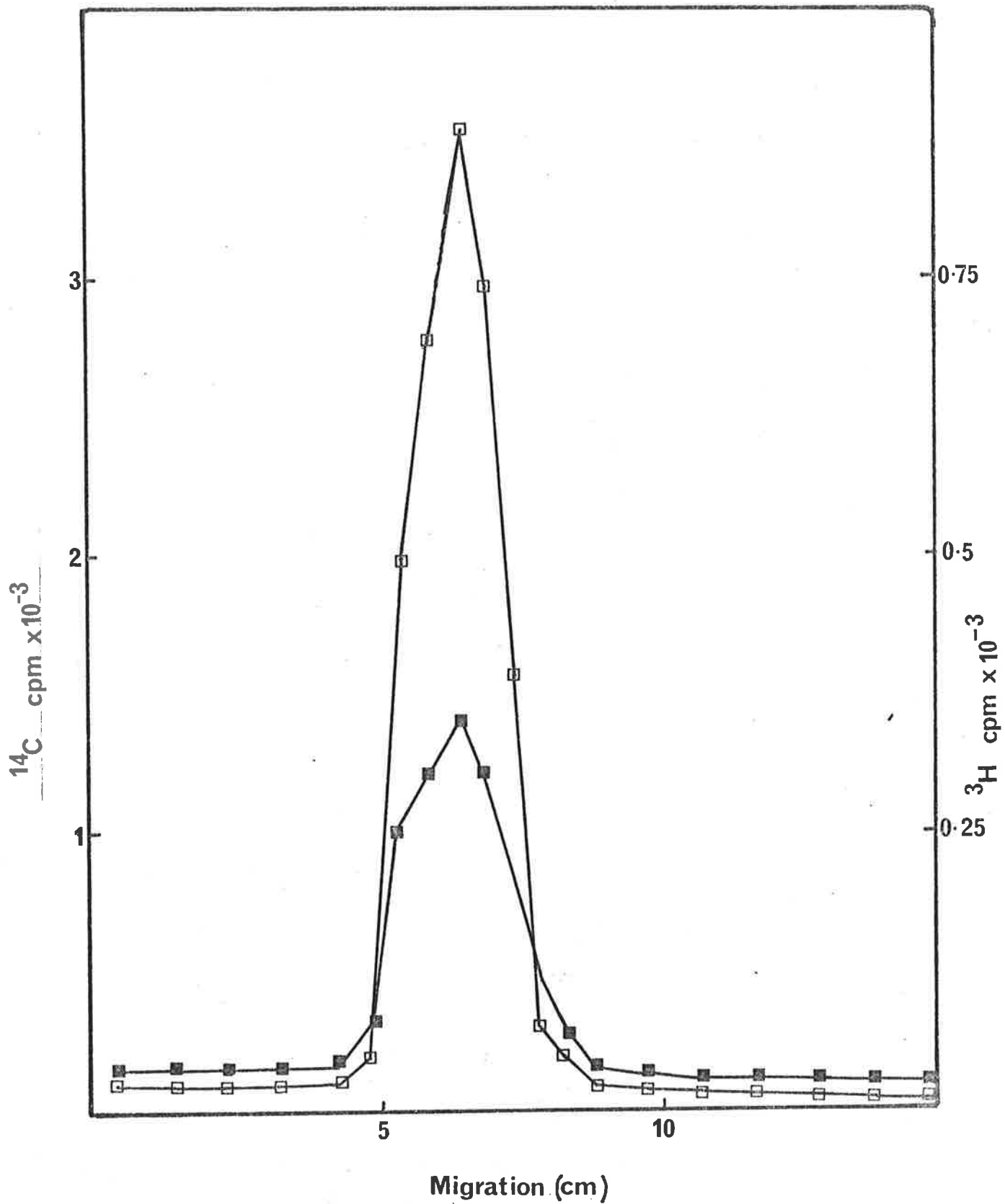
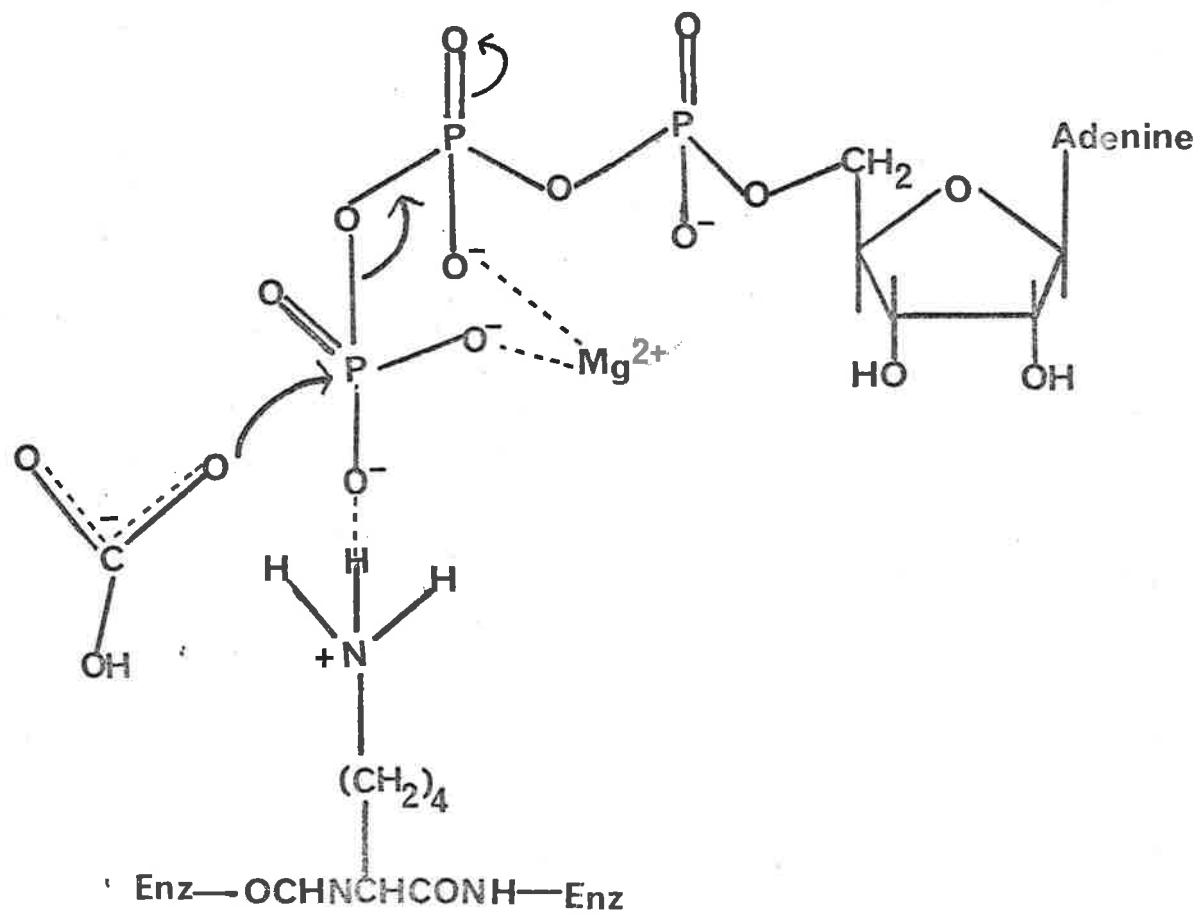


Fig. III.8 Postulated transition state for the biotin carboxylation reaction of pyruvate carboxylase.



CHAPTER IV

CHEMICAL MODIFICATION OF THE BIOTIN

CARBOXYLATION SITE BY CYANATE



#### IV.A INTRODUCTION

The results presented in the previous chapter provide strong evidence that there is a lysyl residue in the biotin carboxylation subsite of sheep liver pyruvate carboxylase. It was suggested that the residue could be located at or near the interface of the binding sites for  $\text{MgATP}^{2-}$  and bicarbonate. In order to test this hypothesis, a lysine-specific reagent which could act as an affinity label of bicarbonate was sought.

##### IV.A.1. Selection of a bicarbonate affinity label.

Keech and Farrant (1968) have reported that modification of pyruvate carboxylase with potassium cyanate resulted in loss of catalytic activity. It was assumed that the residue modified was the lysyl residue in the acetyl CoA binding site that is susceptible to modification by TNBS and FDNB. The susceptibility of this residue to modification by hydrophobic reagents suggests that the acetyl CoA binding site is a hydrophobic pocket on the enzyme surface. This suggestion is compatible with the hydrophobic nature of the adenine nucleotide moiety of acetyl CoA. In view of the probable hydrophobic environment of the lysyl residue, it is not clear whether cyanate, a small hydrophilic molecule, would easily enter the acetyl CoA binding site to modify the lysyl residue.

An alternative site of cyanate modification, in the light of the data presented in the previous chapter, is the biotin carboxylation site. Because the cyanate molecule is similar in size to the bicarbonate molecule, it is possible that the cyanate modification site is at or near the bicarbonate binding site. Experimental tests of this hypothesis are described below.

#### IV.A.2. Cyanate as a chemical modification reagent.

Although cyanate has been shown to modify a number of amino acid residues, only the N-carbamyl derivatives formed by reaction with amino groups are stable at alkaline pH:



It should be noted that the cyanate modification product is rather more similar to the parent amine than the modification products of other amine specific chemical modification reagents such as FDNB and TNBS. The derivatives with other residues (cysteine, tyrosine, aspartate, glutamate, and histidine) are unstable and decompose on dilution (Means and Feeney, 1971; Glazer *et al.*, 1975). The reaction with amino groups has been shown to involve an unprotonated amino group, and neutral cyanic acid (Johncock *et al.*, 1958; Stark, 1965). The reaction rates for the modification of amines with cyanate are dependent on the pH of the medium, and it is possible to predict the rate of reaction of an amino group once its  $\text{pK}_a$  value is known (Stark, 1965).

### IV.B MATERIALS AND METHODS.

#### IV.B.1. Assay methods.

The rate of the pyruvate carboxylase catalysed reaction was determined by the procedure described in Section II.B.4. The pyruvate/oxaloacetate isotopic exchange reaction and the ATP/ $\text{P}_i$  isotopic exchange reaction were measured as described in Section II.B.5 and II.B.6 respectively.

#### IV.B.2 Cyanate inactivation procedure

The reaction mixture, final volume 0.5 ml, contained 50  $\mu\text{moles}$  N-ethylmorpholine-Cl, pH 8.4, and 3-5 units of pyruvate carboxylase. The

inactivation was initiated by the addition of cyanate ( $K^+$  salt) to give the indicated final concentrations. At the time periods indicated, samples were withdrawn and their enzymic activity determined by the procedure described in Section II.B.4.

#### IV.B.3. Data Processing

Where appropriate, data was fitted to a straight line using a Fortran linear regression computer program.

### IV.C RESULTS

#### IV.C.1 Order of pyruvate carboxylase inactivation by cyanate with respect to time.

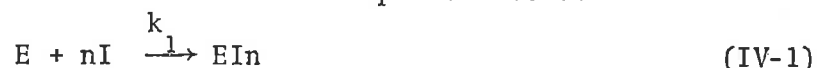
In order to characterise the inactivation process, the time course of inactivation at various cyanate concentrations was investigated. Plots of log percentage initial activity as a function of time of inactivation were linear to loss of at least 75% activity, when the acetyl CoA-dependent assay procedure was used (Fig. IV.1). This indicated that the inactivation process was pseudo first-order with respect to cyanate in the concentration range 50-300 mM.

#### IV.C.2. Order of pyruvate carboxylase inactivation by cyanate with respect to inactivator concentration.

In order to determine if a reversible cyanate-enzyme complex formed prior to inactivation, the steady state kinetic analysis of Meloche (1967) was employed. When the time required for 50% loss of enzymic activity was plotted as a function of reciprocal cyanate concentration, the line shown in Fig. IV.2 was obtained. The vertical intercept

of the line was not significantly different from zero, which, from the analysis of Meloche (1967) implied that the cyanate inactivation process did not involve formation of a reversible complex prior to inactivation. A similar conclusion was reached by Hudson et al., (1975) in their investigation of an affinity label of the pyruvate binding site of sheep pyruvate carboxylase.

If one assumes the inactivation process to be:



where E, I, EIn, n and  $k_1$  represent the free enzyme, the inactivator concentration, the enzyme-inactivator complex, the number of catalytically significant residues modified per active site, and the second order rate constant respectively.

The role of inactivation (v) can be written as:

$$v = k_1 EI^n \quad (IV-2)$$

Although the reaction is a bimolecular process, it exhibited pseudo-first order inactivation kinetics, with an observed inactivation constant  $k'$  defined by:

$$k' = k_1 E \quad (IV-3)$$

Substituting this value into equation IV-2 gives

$$v = k' I^n \quad (IV-4)$$

and hence, if the rate of inactivation is expressed as the reciprocal of the half time ( $1/t_{0.5}$ ), then equation IV-4 becomes:

$$\log (1/t_{0.5}) = \log k' + n \log I \quad (IV-5)$$

Hence, the slope of the line obtained by plotting  $\log(1/t_{0.5})$  as a function of  $\log I$  represents the number of catalytically significant residues modified per active site. Fig. IV-3 shows the data of Fig. IV-2 plotted in this manner. The slope of the line ( $1.01 \pm 0.02$ ) indicates

that there is only one residue essential for catalytic activity that is modified by cyanate. This approach has been used by a number of workers to investigate the number of amino acid residues per active site modified by an inactivating reagent (Levy et al., 1963; Edwards and Keech, 1967; Keech and Farrant, 1968).

#### IV.C.3. Cyanate inactivation measured in the absence of acetyl CoA.

In order to determine if the single residue modified by cyanate was in the acetyl CoA activator site or the catalytic site, the time-dependence of cyanate inactivation was measured by the usual procedure, except that enzyme activity was measured under acetyl CoA independent conditions, in the absence of acetyl CoA. If the modified residue was in the acetyl CoA activation site, then cyanate modification might be expected to have little effect on the acetyl CoA-independent rate of pyruvate carboxylation. On the other hand, if the residue modified is essential for catalysis per se, cyanate modification should result in loss of catalytic activity, whether measured in the absence or in the presence of acetyl CoA. The results shown in Fig. IV-4 show that cyanate modification causes loss of enzymic activity determined in the absence of acetyl CoA. However, the lines shown in this semi-log plot are biphasic, in contrast to the straight lines obtained for the same process monitored using an acetyl CoA-dependent enzyme assay procedure.

One possible explanation for this pattern of cyanate inactivation involves modification of a residue in the acetyl CoA binding site. Such modification would be expected to result in complete loss of enzymic activity, when measured under acetyl CoA-dependent conditions. However, the investigation of protection afforded by the substrates and activators described

below appears to rule out this explanation. On the other hand, if the cyanate modified enzyme retains some catalytic activity when assayed under acetyl CoA-independent conditions, then an inactivation profile of the type shown in Fig. IV.4, would be expected. The theory of this form of enzyme inactivation has been investigated by Ray and Koshland (1960), who were able to show that equation IV-6 describes the dependence of observed enzymic activity as a function of time;

$$\frac{A/A_0 - F}{1 - F} = \exp(-kt) \quad \dots \quad (\text{IV-6})$$

where  $A_0$ ,  $A$ ,  $F$ , and  $K$  represent the initial enzymic activity, the enzymic activity at time  $t$ , the fractional activity of modified enzyme, and the observed inactivation constant respectively. When the data shown in Fig. IV.4 were analysed using equation IV-6, the inactivation pattern shown in Fig. IV-5 was obtained. The fractional activity of cyanate modified enzyme was estimated to be 0.3 from the maximum extent of inactivation shown in Fig. IV.4.

Thus, if it is assumed that cyanate modified enzyme retains some residual activity in the acetyl CoA-independent assay system, then the inactivation process can be shown to be pseudo first-order with respect to cyanate concentration. When the data shown in Fig. IV.5 were analysed using equation IV-5, the slope of the line shown in Fig. IV.6 was  $0.89 \pm 0.03$ , which suggests that only one modified residue per active site is responsible for loss of acetyl CoA-independent enzymic activity. However, when the data shown in Fig. IV.5 were analysed using the method of Meloche (1967) a different pattern to that for the loss of acetyl CoA-dependent activity was observed. Firstly, a comparison of Figs. IV.2 and IV.7 reveals that acetyl CoA-independent activity is lost at a greater rate than is

acetyl CoA-dependent activity, at any given concentration of cyanate. Moreover, the vertical intercept of the line shown in Fig. IV.7, ( $7.8 \pm 2.5$ ), was significantly different from zero, which implies that cyanate forms a reversible complex with the enzyme prior to modifying the residue responsible for loss of acetyl CoA-independent activity. Thus, it may be concluded that cyanate appears to modify at least two residues, one of which is involved in maintaining acetyl CoA-dependent enzymic activity, and one associated with the acetyl CoA-independent catalytic activity. This point is discussed at greater length in Section IV.D.1.

A comparison of the acetyl CoA-dependent and -independent assay procedures reveals that the concentrations of pyruvate and bicarbonate are elevated in the acetyl CoA-independent assay compared to those used in the acetyl CoA-dependent assay mix. Also, the acetyl CoA-independent assay solution contains ammonium chloride which is not present in the acetyl CoA-dependent assay. In an effort to determine which of these differences is involved in conferring residual activity on cyanate modified enzyme, the inactivation process was followed using the acetyl CoA-dependent assay system in which the pyruvate, bicarbonate and ammonium chloride concentration were raised, either separately or together, to those used in the acetyl CoA-independent assay. However, although all possible combinations of elevated concentrations were investigated, the biphasic inactivation profile was never observed. This observation implies that the absence of acetyl CoA in the assay solution is necessary in order for cyanate modified enzyme to retain residual activity.

#### IV.C.4. Substrate protection against cyanate modification.

In order to determine the locus of cyanate inactivation, the

effects of the various substrates and activators of pyruvate carboxylase on the cyanate inactivation process were examined. It was found that neither oxaloacetate nor pyruvate, even at concentrations twenty times their apparent  $K_m$  values afforded any protection against cyanate modification. Thus it may be concluded that cyanate does not modify any catalytically significant residues in the pyruvate carboxylation subsite.

In view of the difference in the inactivation profiles when enzymic activity was measured under acetyl CoA-dependent compared to -independent conditions, it was important to establish whether acetyl CoA afforded any protection against cyanate modification. The results presented in Table IV.1 show that the presence of acetyl CoA has no significant effect on the rate of cyanate modification. Moreover, the results presented in Fig. IV.8 show that 5 mM  $MgATP^{2-}$  affords essentially complete protection against cyanate inactivation, irrespective of whether enzymic activity is measured under acetyl CoA-dependent, or -independent conditions. These results are consistent with only catalytically significant residues in the biotin carboxylation site being modified by cyanate.

The effect of bicarbonate on the rate of cyanate inactivation is shown in Fig. IV.9, in which the data is plotted according to equation IV-7, which was first developed by Scrutton and Utter (1965),

$$\frac{V_A}{V_0} = \frac{k_2}{k_1} + \frac{K_A(1 - \frac{V_A}{V_0})}{A} \quad \dots \quad (IV-7)$$

where  $V_A$  and  $V_0$  represent, respectively, the pseudo first-order rate constants for inactivation in the presence and absence of A, the protecting agent;  $k_1$  and  $k_2$  are the fractional order rate constants for inactivation of free enzyme (equation IV-8), and the enzyme-A complex (equation IV-9), and  $K_A$ , the dissociation constant for EA (equation IV-11).





$$K_a = \frac{[EA]}{[E][A]} \quad \dots\dots (IV-11)$$

From the fact that the plot reveals a straight line, the extrapolation of which passes through the origin, it may be concluded that cyanate cannot combine with the enzyme-bicarbonate complex, i.e.,  $k_2$  is very small compared to  $k_1$ . Using the same procedure Scrutton and Utter (1965) have reported the complete protection of chicken liver pyruvate carboxylase by ATP against inactivation by avidin, and Keech and Farrant (1968) have shown that acetyl CoA affords complete protection of sheep pyruvate carboxylase against inactivation by FDNB.

The effect of bicarbonate on the rate of inactivation of pyruvate carboxylase by cyanate is consistent with the hypothesis that cyanate inactivates the acetyl CoA-dependent activity of the enzyme by modifying a residue at the interface of the  $MgATP^{2-}$  and bicarbonate binding sites. In order to further test this hypothesis, the effects of  $MgATP^{2-}$  and  $MgADP$  on the rate of inactivation by cyanate were investigated. The results shown in Fig. IV.10 suggest that  $MgATP^{2-}$  affords complete protection against cyanate modification in agreement with the data shown in Fig. IV.8. Indeed, at higher concentrations of  $MgATP^{2-}$  than those shown in Fig. IV.10, the extent of protection increased disproportionately, leading to a deviation from the linear relationship shown in Fig. IV.10. It is possible that this increased protection arises through formation of carboxylated-enzyme from the  $MgATP^{2-}$  and endogenous bicarbonate present in the buffered solution.

The observation that the plot for MgADP protection yields a straight line with a non-zero intercept (Fig. IV.10) suggests that MgADP affords incomplete protection against cyanate modification. Together, the effects of bicarbonate,  $\text{MgATP}^{2-}$ , and MgADP, suggest that the residue modified is situated at the interface of the  $\text{MgATP}^{2-}$  and bicarbonate binding sites.

When the effect of  $\text{Mg}^{2+}$  on the rate of cyanate inactivation was investigated, the unusual protection pattern shown in Fig. IV.11 was obtained. In view of the effect of  $\text{Mg}^{2+}$  on the binding of  $\text{MgATP}^{2-}$  (Keech and Barritt, 1967), the fact that  $\text{Mg}^{2+}$  affects the rate of cyanate modification is consistent with the residue modified being near to the  $\text{MgATP}^{2-}$  binding site. The fact that  $\text{Mg}^{2+}$  is an allosteric activator of pyruvate carboxylase allows a possible explanation for the unusual effect of varying the  $\text{Mg}^{2+}$  concentration on the rate of cyanate inactivation. Warren and Tipton (1974d) have concluded that it is necessary to invoke the sequential allosteric model of Koshland *et al.*, (1966) in order to explain the interaction of  $\text{Mg}^{2+}$  with pyruvate carboxylase. In this model, ligand binding to one subunit leads to a ligand-induced conformational change, such that the affinity of the remaining subunits for the ligand is increased.

Clearly, as the ligand concentration increases, the concentration of ligand-altered binding sites initially increases, passes through a maximum, and approaches zero as the protein becomes completely saturated with the ligand. Thus, the effect of varying the  $\text{Mg}^{2+}$  concentration on the rate of cyanate modification may be explained if it is assumed that the rate of modification of active sites of monomers without bound  $\text{Mg}^{2+}$ , but contained within tetramers with  $\text{Mg}^{2+}$  bound to one or more of the

other subunits, is faster than the rate of modification of monomers within tetramers with no bound  $Mg^{2+}$ , or monomers within tetramers completely saturated with  $Mg^{2+}$ . At low  $Mg^{2+}$  concentrations, most tetramers will contain no bound  $Mg^{2+}$ , and hence a low rate of cyanate modification would be expected. However, as the  $Mg^{2+}$  concentration is increased the concentration of tetramers partially saturated with  $Mg^{2+}$  will increase to a maximum, and then decrease to zero as the  $Mg^{2+}$  concentration approaches a saturating level. Hence, if, as is indicated from the data of Fig. IV.11, the rate of inactivation of enzyme completely saturated with  $Mg^{2+}$  is similar to that of enzyme in the absence of  $Mg^{2+}$ , the rate of cyanate inactivation will increase to a maximum and then decrease as the enzyme becomes completely saturated with  $Mg^{2+}$ . It should be noted that, although the above explanation assumed that the sequential allosterism model applied to the interaction of  $Mg^{2+}$  with pyruvate carboxylase, it would be possible to develop a similar argument using the concerted model of Monod et al., (1965). Therefore, the cyanate inactivation data cannot be easily used to discriminate between the sequential and concerted models.

#### IV.C.5. Effect of cyanate modification on the isotopic exchange reaction.

Further evidence to support the hypothesis that cyanate was acting only at the biotin carboxylation site was obtained by investigating the effect of cyanate modification on the isotopic exchange reactions catalysed by pyruvate carboxylase. The results presented in Table IV.2 show that during cyanate modification the  $ATP/P_i$  isotopic exchange reaction is lost to a greater extent than overall catalytic activity, while the pyruvate/oxaloacetate isotopic exchange reaction is activated relative to the rate for unmodified enzyme. These results are qualitatively

ively consistent with the hypothesis that cyanate modifies a residue in the biotin carboxylation subsite. In order to determine why the rate of the pyruvate/oxaloacetate isotopic exchange reaction catalysed by cyanate modified enzyme is faster than that catalysed by unmodified enzyme, the effect of cyanate modification on the rate of the enzyme catalysed oxaloacetate decarboxylation was investigated. The results shown in Table IV.2 show that cyanate modification decreases the rate of decarboxylation to a small extent. However, the 20% decrease in the rate of oxaloacetate decarboxylation is not sufficient to account for the nearly 50% increase in the rate of the modified enzyme catalysed pyruvate/oxaloacetate isotopic exchange reaction.

The results presented in Table IV.2 may be explained if it is assumed that entry of biotin into the biotin carboxylation site is hindered in cyanate modified enzyme, in the presence of acetyl CoA. Hence, in cyanate modified enzyme, the biotin prosthetic group will reside in the pyruvate carboxylation site to a disproportionate extent, relative to unmodified enzyme, which might be expected to lead to a faster rate of modified enzyme catalysed pyruvate/oxaloacetate isotopic exchange reaction. Moreover, because the biotin group occupies the biotin carboxylation site to a lesser extent in modified relative to unmodified enzyme, cyanate modification would be expected to lead to a loss of ATP/P<sub>i</sub> isotopic exchange reaction activity. The observation of greater loss of ATP/P<sub>i</sub> exchange reaction activity than overall catalytic activity in modified enzyme suggests that the rate limiting step of the ATP/P<sub>i</sub> isotopic exchange reaction is not the same as the rate limiting step of the overall reaction. It is possible that the greater occupancy of the pyruvate carboxylation site by biotin partially compensates for the decreased occupancy of the biotin carboxylation site, leading to a smaller loss of overall catalytic

activity than might have been expected from the effect of cyanate modification on the  $\text{ATP}/\text{P}_i$  isotopic exchange reaction.

#### IV.C.6. Determination of the stoichiometry of cyanate modification.

Although the results described in Sections IV.C.3 and IV.C.4 show that only one kinetically significant residue is modified during cyanate modification, such an analysis of the concentration dependence of cyanate modification cannot give any information on the extent of non-specific labelling of pyruvate carboxylase by cyanate. This information was obtained by modifying pyruvate carboxylase with  $[^{14}\text{C}]$ cyanate and determining the radioactivity associated with the protein.

The use of this approach was complicated by the fact that most preparations of pyruvate carboxylase are contaminated with glutamate dehydrogenase. This contaminant was substantially removed by  $\text{NAD}^+$ -Agarose affinity chromatography. Pyruvate carboxylase, (specific activity 25 U/mg) prepared by this procedure was separated from the salt in the elution buffer by Sephadex gel filtration using a Sephadex G-25 column (1x10 cm) previously equilibrated with 0.1M N-ethylmorpholine-Cl, pH 8.4. In order to identify the modified residues, 25 units of this enzyme were modified with  $[^{14}\text{C}]$ cyanate ( $2.05 \times 10^5$  cpm/ $\mu\text{mole}$ ), at a concentration of 36 mM until 50% of the initial catalytic activity had been lost. In order to determine the extent of non-specific modification, another sample of enzyme was modified under the same conditions, except the solution contained 6 mM  $\text{MgATP}^{2-}$  and 4 mM  $\text{Mg}^{2+}$ . The inactivation was quenched by addition of alanine in a five-fold molar excess over the cyanate. The enzyme was then precipitated by addition of 10% trichloroacetic acid ( $^w/v$ ). The precipitate was collected by centrifuging, and then washed with 5% trichloroacetic acid ( $^w/v$ ) until the radioactivity in the

supernatant had been reduced to background levels. The precipitate was resuspended in 40 mM potassium 3-(N-morpholino)-propane sulphonate buffer, pH 6.5 and enzymically digested with pronase for three hours at 37°C; 1 mg of pronase was added at 0, 60, and 120 min. Aliquots of the resulting digest were taken and their radioactivity and biotin content determined, using the procedures described in Sections II.B.3 and II.B.7 respectively. It was found that the enzyme modified in the presence of  $\text{MgATP}^{2-}$  and  $\text{Mg}^{2+}$  contained  $5.9 \pm 0.2$  molecules of cyanate bound per molecule of biotin, while the enzyme modified in the absence of  $\text{MgATP}^{2-}$  and  $\text{Mg}^{2+}$  contained  $6.1 \pm 0.2$  molecules of cyanate bound per molecule of biotin.

These results indicate that, although only one kinetically significant residue is modified during cyanate inactivation, at least five other residues, removed from the catalytic and activator sites are accessible to cyanate. In order to determine if cyanate could be a useful reagent for labelling specific peptides of pyruvate carboxylase, for subsequent purification and sequence determination, it was necessary to attempt to characterise the amino acid residues modified by cyanate. The point here is that the reaction products of cyanate with either the  $\epsilon\text{-NH}_2$  group of lysine, or the  $\alpha\text{-NH}_2$  group of any amino acid are stable, while the adducts formed by reaction with other residues in a protein are labile under alkaline conditions (Means and Feeney, 1971). Thus, if it could be shown that the non-specific cyanate labelling was due to reaction with cysteine, histidine, tyrosine, aspartate or glutamate residues, then it would be possible to remove the non-specific label by dialysing the modified protein against an alkaline buffer prior to preparation and purification of labelled peptides for sequence determination.

#### IV.C.7. Identification of the cyanate-modified residues.

In order to determine the residues modified by cyanate aliquots of the digested material described in Section IV.C.6 were subjected to ascending chromatography on Whatmans 3MM paper, using 1-butanol:acetic acid:water (12:3:5 <sup>v</sup>/v) as the developing solvent. The developed chromatograms were dried, cut into 0.5 cm strips, and the radioactivity on each strip determined using the toluene scintillant described in Section II.B.3. In both cases over 90% of the radioactive material was localised at the origin, while the remainder co-chromatographed with marker homocitrulline ( $R_f$  0.36). Thus, it may be concluded that modification of lysyl residues occurred during cyanate modification. In order to determine if the radioactive material remaining at the origin represented incompletely hydrolysed protein, samples of the digests from both inactivations were further incubated with 1 mg of trypsin for one hour at 37°C, followed by a three hour incubation at 37°C in the presence of a further 2 mg of pronase. Aliquots of this material were subjected to paper chromatography using the procedure described above. It was found that in chromatography of digested material from the modification in the presence of  $MgATP^{2-}$ , approximately 15% of the radioactive material co-chromatographed with homocitrulline, while the remainder was localised at the origin.

However, analysis of chromatograms of the digested protein from the modification in the absence of  $MgATP^{2-}$  showed that 40% of the radioactive material co-chromatographed with homocitrulline.

In order to determine if modification of amino acid residues other than lysine had occurred during the cyanate inactivation, samples of the digested material were taken and incubated at pH 9.5 for one hour

at 37°C. Under these alkaline conditions hydrolysis of modified amino acids other than lysine would be expected to occur, leading to the regeneration of cyanate. The alkaline hydrolysed samples were neutralised (to pH 7) with acetic acid, and aliquots subjected to paper chromatography using the procedure described above. No radioactive material co-chromatographing with marker [ $^{14}\text{C}$ ]cyanate ( $R_f$  0.59) could be detected.

Thus it may be concluded that the only reaction products of cyanate inactivation of pyruvate carboxylase are N-carbamyl amino acids. Presumably the observation of radioactive material remaining at the origin of the chromatograms reflect resistance of modified pyruvate carboxylase to proteolytic cleavage.

#### IV.D DISCUSSION

##### IV.D.1. The mechanistic significance of the modified residues.

The results presented in Sections IV.C.2 and IV.C.3 suggest that cyanate modification of different residues is responsible for loss of the acetyl CoA-dependent and -independent enzymic activities. The lack of protection afforded by acetyl CoA against cyanate modification, coupled with the observation that 5 mM  $\text{MgATP}^{2-}$  afforded near-complete protection for both the acetyl CoA-dependent and -independent enzymic activities, suggests that the residues modified are situated in the biotin carboxylation site. Furthermore, the dependence of the rate of loss of acetyl CoA-independent activity on cyanate concentration suggested that cyanate modified enzyme retained some residual acetyl CoA-independent catalytic activity.

One possible interpretation of these observations is that the binding of acetyl CoA leads to a change in the three dimensional structure



of the biotin carboxylation site. This proposal is consistent with the effect of acetyl CoA on the  $K_m$  value for bicarbonate (Ashman et al., 1972). The effects of  $MgATP^{2-}$ ,  $MgADP$ , and bicarbonate on the rate of loss of acetyl CoA dependent enzyme activity suggest that the modified residue responsible for loss of this activity forms part of the binding site for the 5'-phosphates of  $MgATP^{2-}$ , and bicarbonate. As has already been discussed in Chapters I and III, a lysyl residue so situated would be well positioned to participate in the reaction mechanism by promoting nucleophilic attack of the bicarbonate on to the terminal phosphate of  $MgATP^{2-}$ . Presumably, when the biotin carboxylation site is in the "acetyl CoA-dependent" configuration the homocitrulline modification product cannot fulfil the role of the normal lysyl residue, leading to complete loss of activity during cyanate modification.

On the other hand, when the biotin carboxylation site is in the "acetyl CoA-independent" configuration, then it is possible that the residue whose modification is responsible for loss of acetyl CoA-dependent activity becomes removed from the bicarbonate binding site. Hence modification of this residue might be expected to have a minimal effect on acetyl CoA-independent catalytic activity.

However, the observation of loss of some acetyl CoA-independent activity on cyanate modification implies that a lysyl residue important for efficient catalysis under these conditions is modified by cyanate. The observation of residual acetyl CoA-independent activity implies that the residue modified in this case is so situated as to permit the homocitrulline modification product to continue to facilitate the reaction, albeit at a reduced rate relative to the native lysyl residue. This situation may arise because the residue responsible for facilitating the

acetyl CoA-independent activity is more distant from the bicarbonate binding site than is the residue responsible for facilitating the acetyl CoA-dependent activity. The greater distance may be the reason that the maximum velocity of the acetyl CoA-independent reaction is only 25% that of the acetyl CoA-dependent reaction. On the other hand, the greater distance would be compatible with insertion of the  $-\overset{\text{O}}{\parallel}{\text{C}}-\text{NH}_2$ , formed by reaction of the  $\epsilon$ -amino group with cyanate, without causing such steric hindrance as to make the catalytic processes of the biotin carboxylation reaction impossible, as appears to be the case during modification of the "acetyl CoA dependent" lysyl residue.

#### IV.D.2. Extensions of the cyanate labelling studies.

There is one obvious question which arises from the labelling studies reported in this and the previous chapter: are the same lysyl residues in the biotin carboxylation site modified by cyanate and oATP? A number of experimental approaches could be used to resolve this question. One approach would be to determine what effect modification with one reagent has on the rate or extent of modification with the other reagent. Clearly, if the same residue was modified there would be an inverse relationship between the extent of modification with the first reagent, and the extent of modification obtainable using the second reagent.

However, such an inverse relationship would also be expected if two different lysyl residues were modified, but modification of one resulted in hindered access of the second modifying reagent to the second residue. It would be difficult to distinguish between this steric hindrance case, and the case of a unique lysyl residue, using the data from an experiment of the type discussed above. In view of this ambiguity

of interpretation, such an experimental approach was not attempted.

Another approach to the problem, which is free of the ambiguities discussed above, would be to isolate and sequence tryptic peptides labelled with radioactive oATP and cyanate. If peptides of identical sequence were obtained from enzyme labelled with the two reagents, then it would be very probable that the same lysyl residue was modified by oATP and cyanate. In the case of the oATP-labelled peptide, it would be possible to utilise the presence of the adenine ring in designing a purification procedure, as was discussed in Section III.D.2. However, there appears to be no such obvious procedure available for purifying the cyanate labelled peptide. Moreover, the high concentrations of cyanate necessary to obtain significant modification would have an adverse effect on the specific activity of the [ $^{14}\text{C}$ ]cyanate used in such a procedure. In addition, the observation of at least six cyanate-modified amino groups (Section IV.C.7), shows that it would be necessary to purify each one before sequencing studies could commence.

In view of all these problems, it is clear that obtaining the sequence of the peptide containing the cyanate-modified biotin carboxylation site lysyl residue would be a major undertaking. However, to be able to correlate the studies of the pyruvate carboxylase reaction sequence with the chemistry of catalysis of the reaction, it will be necessary to determine the sequences of as many active site peptides as possible, in order to build up a model of the three dimensional orientation of the amino acid residues comprising the active site of the enzyme.

Another extension of the results presented above relates to the observation that cyanate-modified pyruvate carboxylase appeared to retain some residual acetyl CoA independent catalytic activity. It may be

possible to elucidate the role the modified lysyl residue(s) play in the catalytic reaction by examining the initial velocity kinetics of cyanate modified enzyme. Ashman and Keech (1975a) have shown that an investigation of changes in apparent  $K_m$  values and apparent  $V$  values following chemical modification can yield useful information about the reaction mechanism of the enzyme under investigation.

In particular, it is possible that an investigation of this type could provide further experimental data with which to assess the hypothetical role of the lysyl residue(s) in the biotin carboxylation site of pyruvate carboxylase described above.

Fig. IV.1. The rate of inhibition of pyruvate carboxylase with varying concentrations of cyanate.

Pyruvate carboxylase (specific activity 10 U/mg, 5.6 units per assay) was inactivated with cyanate using the procedure described in Section IV.B.2. The enzymic activity at the time intervals indicated was determined by the acetyl CoA dependent assay procedure described in Section II.B.4. The cyanate concentrations used were: 0 mM, (  $\nabla$  ); 50 mM, (  $\blacktriangledown$  ); 100 mM, (  $\square$  ); 200 mM, (  $\blacksquare$  ); and 300 mM, (  $\circ$  ).

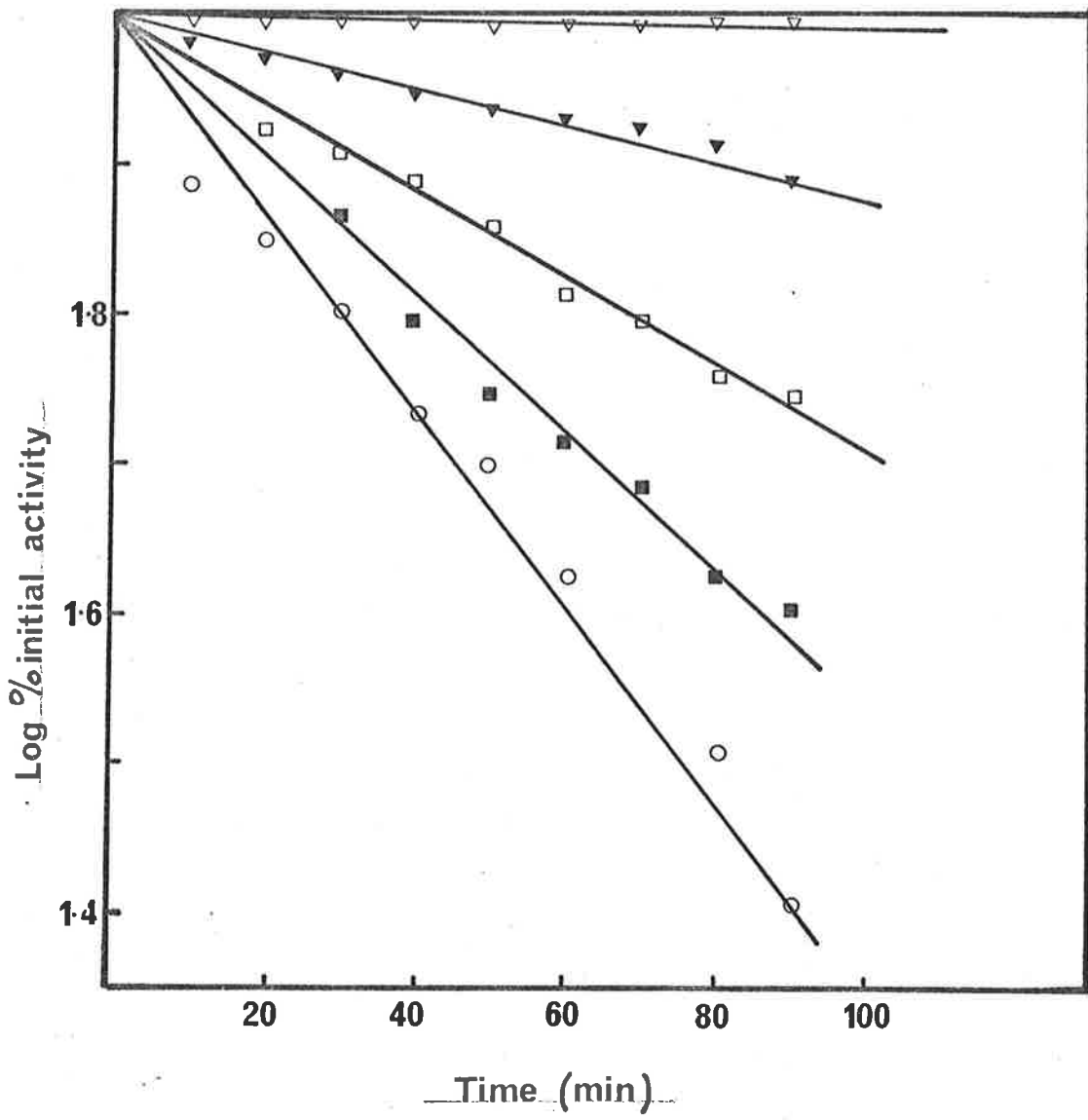


Fig. IV.2     The pseudo first-order kinetics of inactivation with respect to cyanate concentration.

The average half time ( $t_{1/2}$ ) values obtained from three experiments of the type shown in Fig. IV.1 are plotted as a function of reciprocal cyanate concentration. The error bars indicate standard errors.

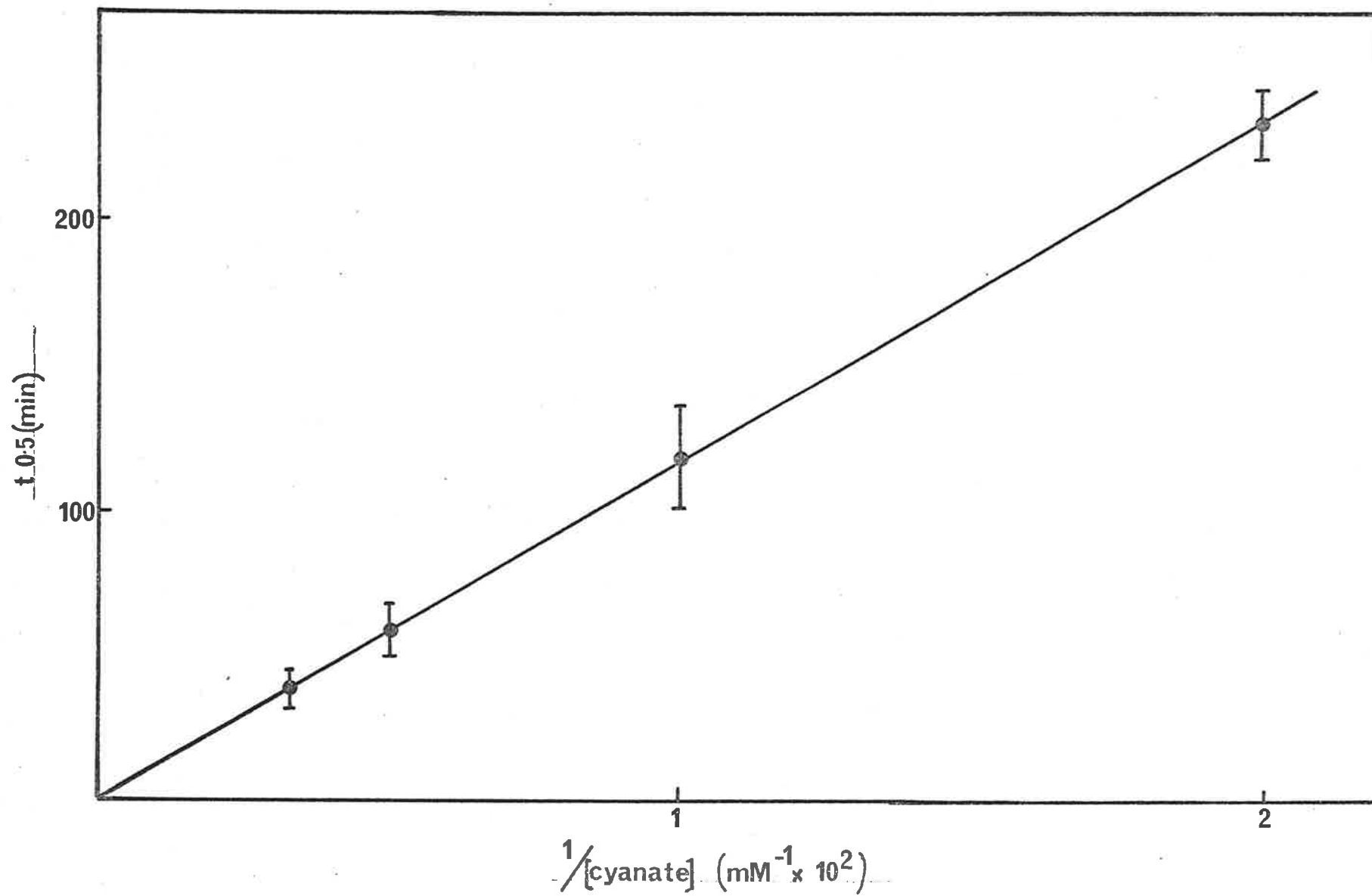




Fig. IV.3.

The data of Fig. IV.2 were replotted as  $\log_{10}$  of the reciprocal of the half time of the inactivation as a function of  $\log_{10}$  concentration of cyanate.

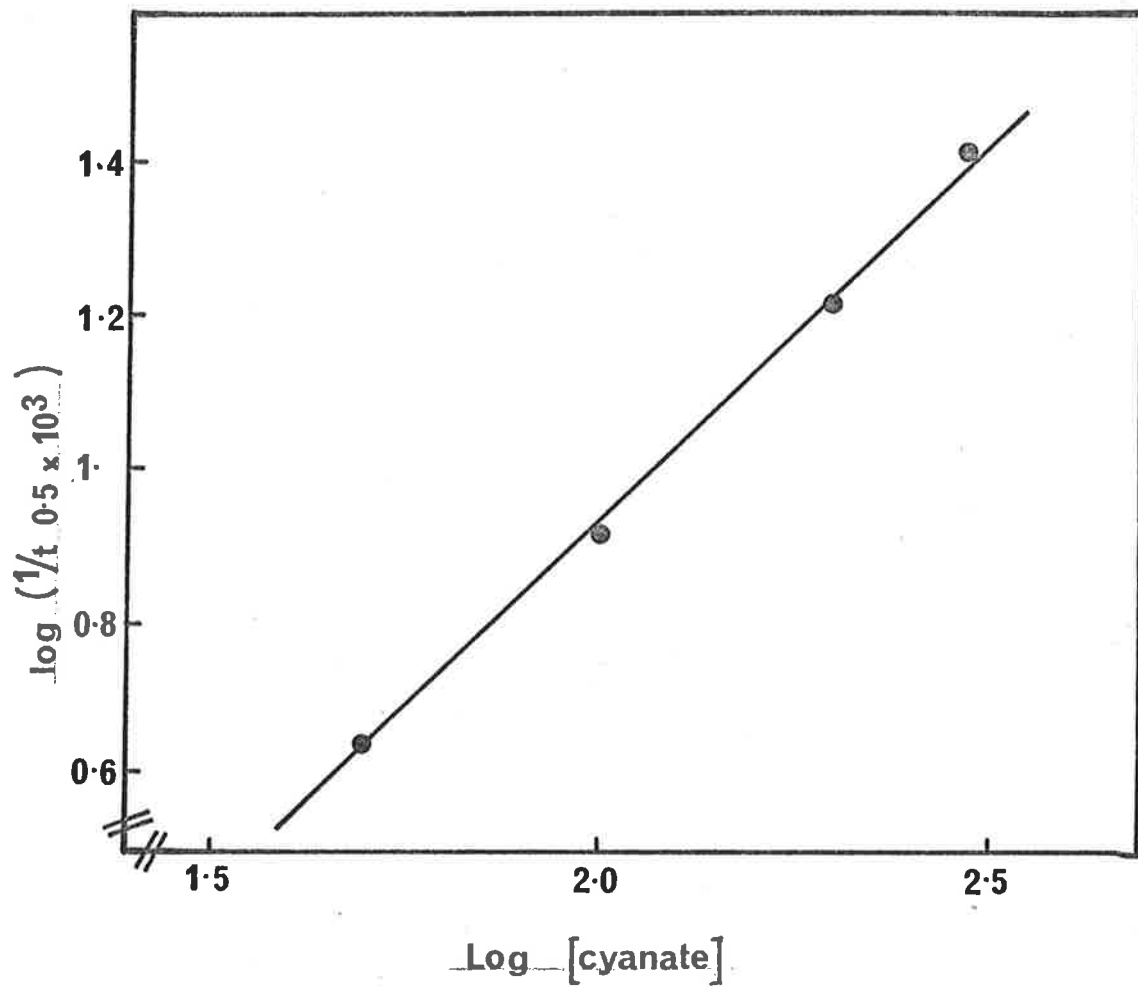


Fig. IV.4. The rate of inhibition of pyruvate carboxylase with varying concentrations of cyanate.

Pyruvate carboxylase (specific activity 10 U/mg, 5.1 units per assay) was inactivated by cyanate using the procedure described in Section IV.B.2. The enzymic activity at the time intervals indicated was determined using the acetyl CoA independent assay procedure described in Section II.B.4. The cyanate concentrations used were: 0 mM, (  $\Delta$  ); 50 mM, (  $\blacktriangle$  ); 100 mM, (  $\blacksquare$  ); 200 mM, (  $\square$  ); and 300 mM, (  $\bullet$  ).

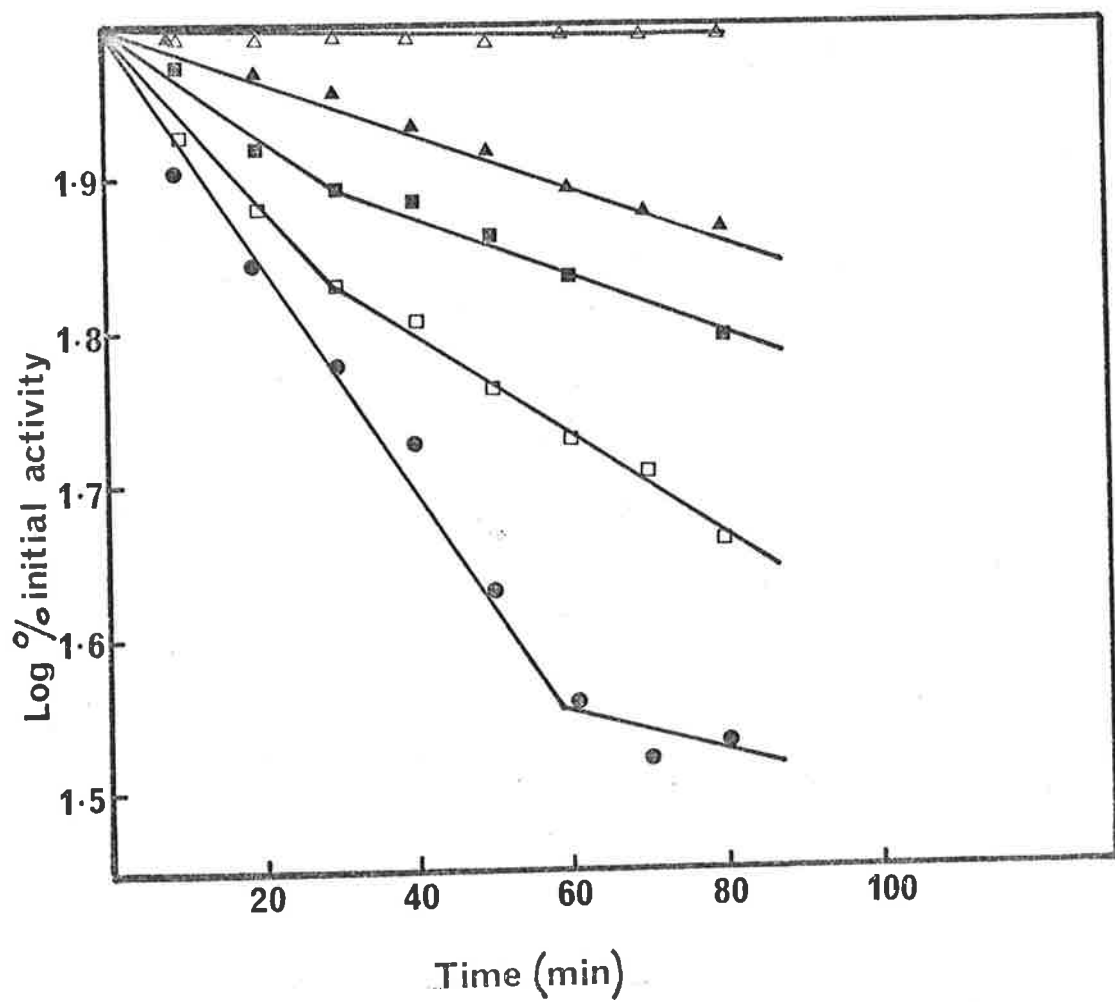


Fig. IV.5.

The data shown in Fig. IV.4 were analysed using equation IV-6, in which F was set at 0.3. The cyanate concentrations used were: 50 mM, ( ● ); 100 mM, ( ○ ); 200 mM, ( ■ ); and 300 mM, ( □ ).

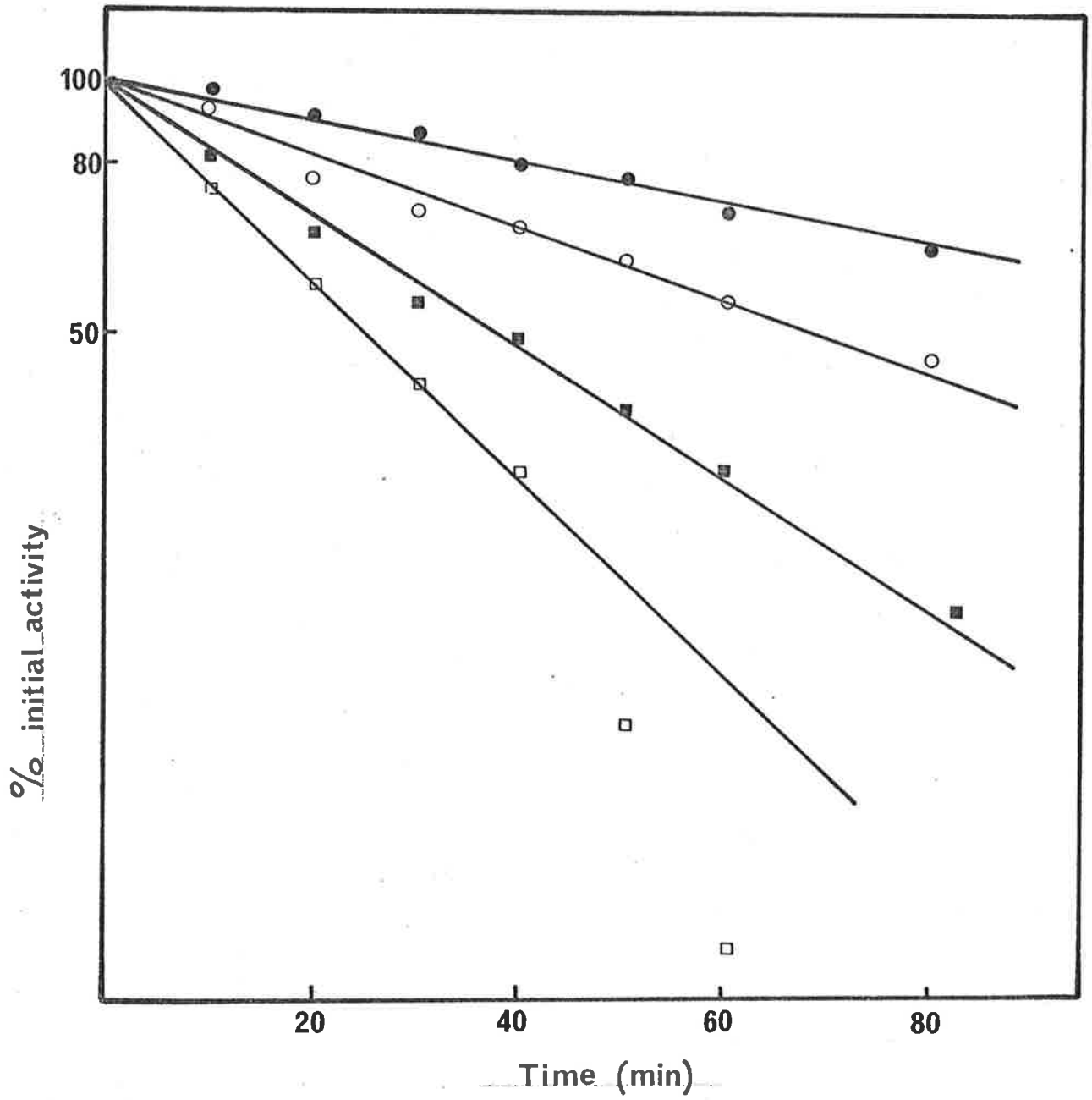


Fig. IV.6.

The data shown in Fig. IV.5 were analysed using equation IV-5. The  $\log_{10}$  of the reciprocal half time of the inactivation was plotted as a function of  $\log_{10}$  concentration of cyanate.

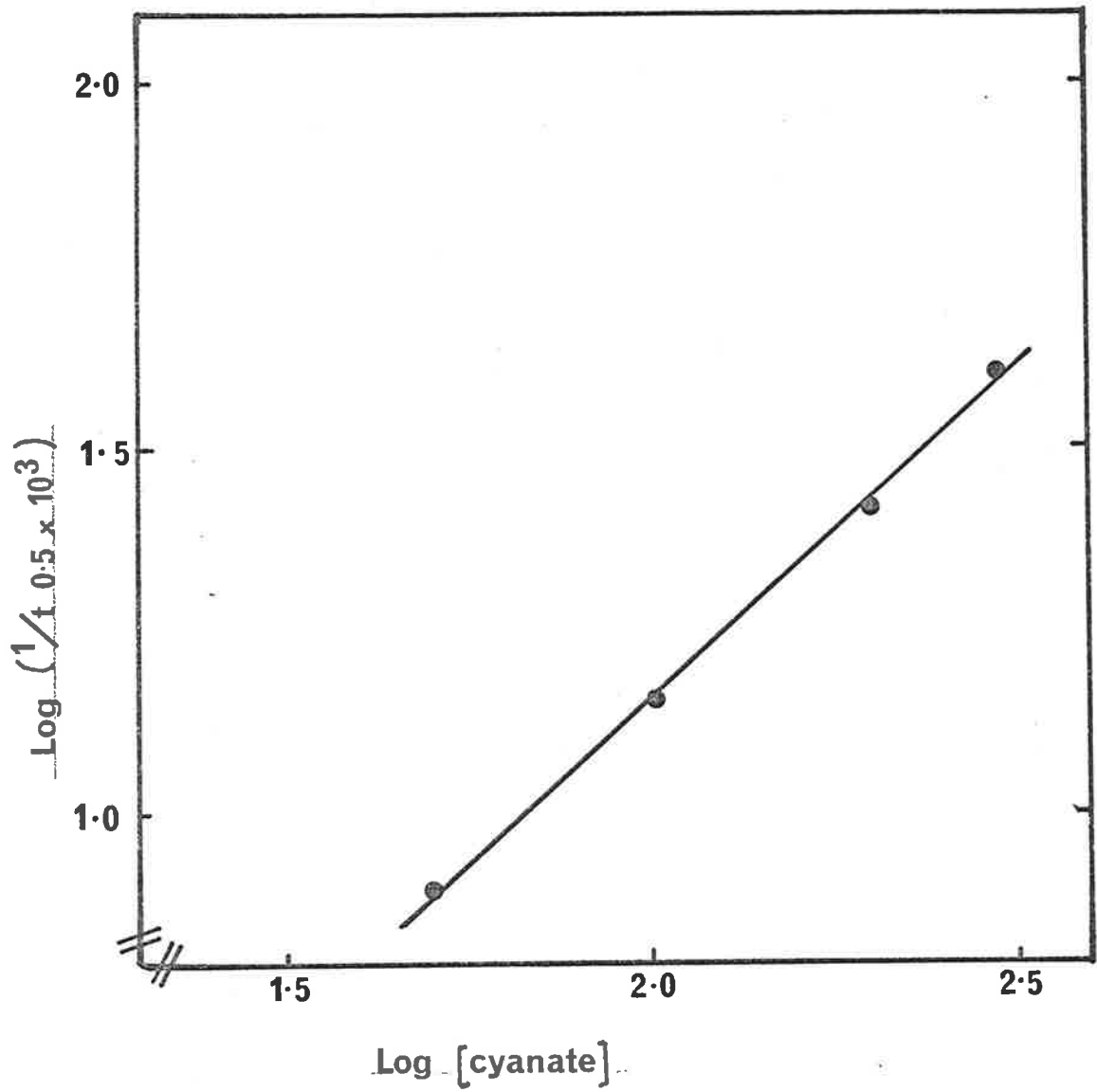




Fig. IV.7. The pseudo first-order kinetics of inactivation with respect to cyanate concentration.

The half times of inactivation obtained from the data of Fig. IV.5 were plotted as a function of reciprocal concentration of cyanate.

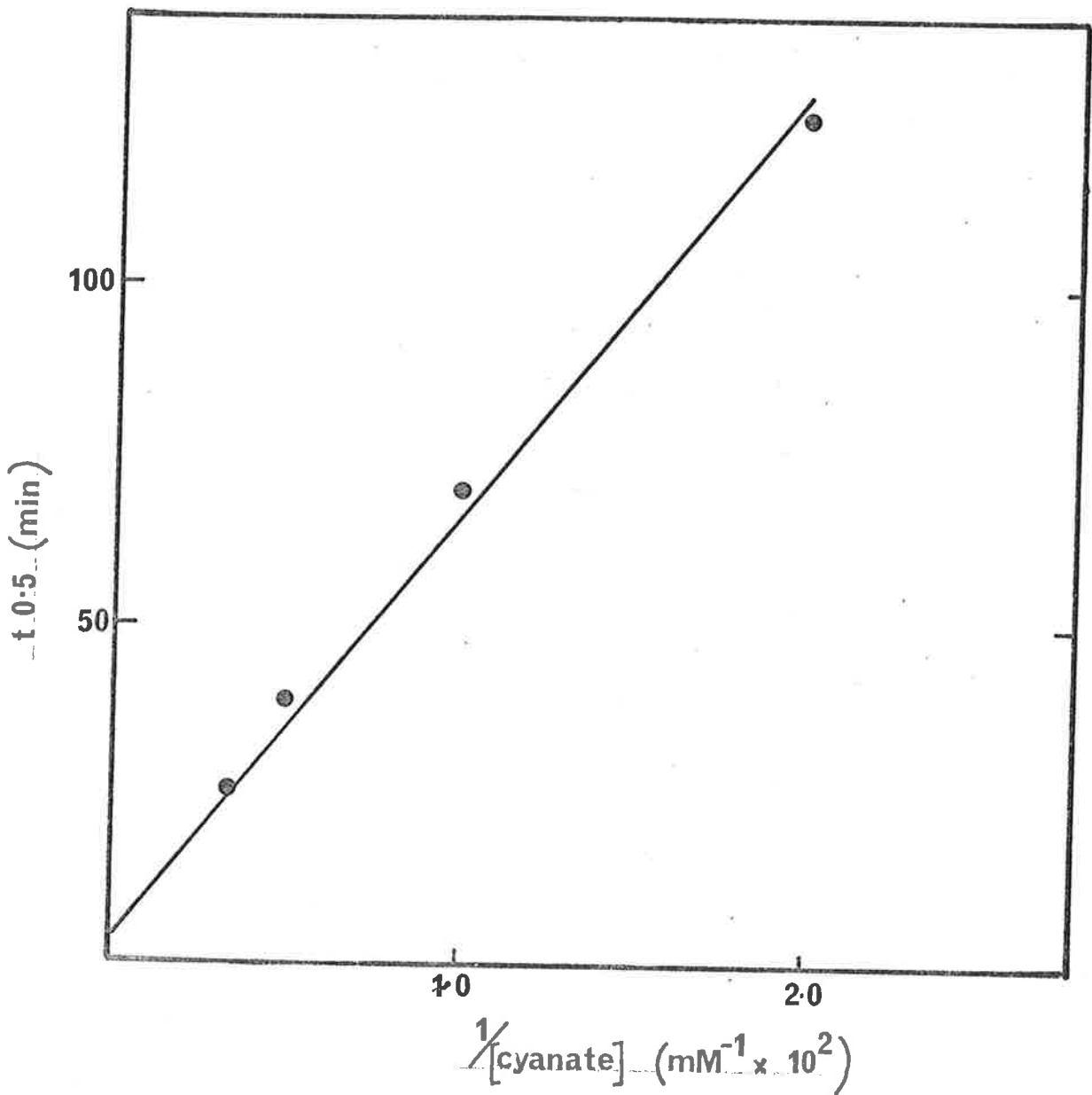


Table IV.1 The effect of acetyl CoA concentration on the rate of inhibition of pyruvate carboxylase by cyanate.

Pyruvate carboxylase (specific activity 12 U/mg, 1.8 units per inactivation tube) was inactivated with 200 mM cyanate, in the presence of varying concentrations of acetyl CoA, using the procedure described in Section IV.B.2. The enzymic activity was measured using the acetyl CoA dependent assay procedure. The rates of inactivation ( $K_{inact}$ ) were determined using the expression:  $K_{inact} = \log_e 2/t_{1/2}$ .

Acetyl CoA ( $\mu\text{M}$ )	$K_{\text{inact}} \times 10^2$ ( $\text{min}^{-1}$ )
0	$1.03 \pm 0.07$
15.3	$1.08 \pm 0.10$
22.9	$1.24 \pm 0.18$
30.6	$1.11 \pm 0.10$
45.8	$1.24 \pm 0.12$
61.1	$0.94 \pm 0.13$
76.4	$0.96 \pm 0.10$
91.7	$0.97 \pm 0.15$

Fig. IV.8 The effect of  $\text{MgATP}^{2-}$  on the rate of inhibition of pyruvate carboxylase by cyanate.

Pyruvate carboxylase (specific activity 14 U/mg, 5 units per inactivation tube) was inactivated with 200 mM cyanate in either the presence ( ■ ) or absence ( ● ) of 5 mM MgATP. In the control ( □ ) cyanate was omitted. In A the acetyl CoA dependent assay procedure was used, while in B enzymic activity was measured using the acetyl CoA independent procedure.

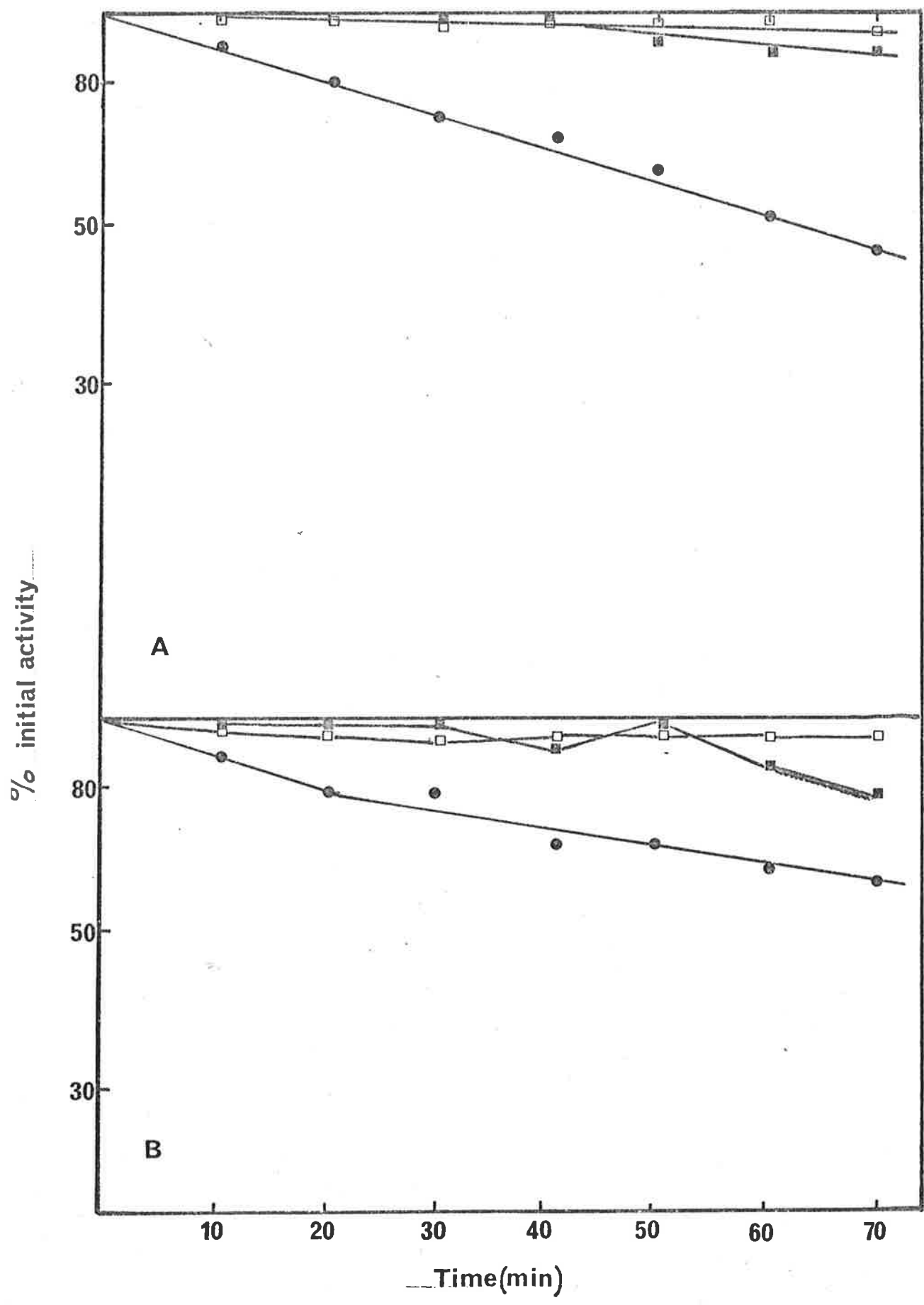


Fig. IV.9. The effect of bicarbonate concentration on the rate of inhibition pyruvate carboxylase by cyanate.

Pyruvate carboxylase (specific activity 12 U/mg, 2.7 units per inactivation tube) was inactivated with 200 mM cyanate, in the presence of the concentrations of sodium bicarbonate indicated, using the procedure described in Section IV.B.2. Enzymic activity was measured by the acetyl CoA dependent assay procedure.

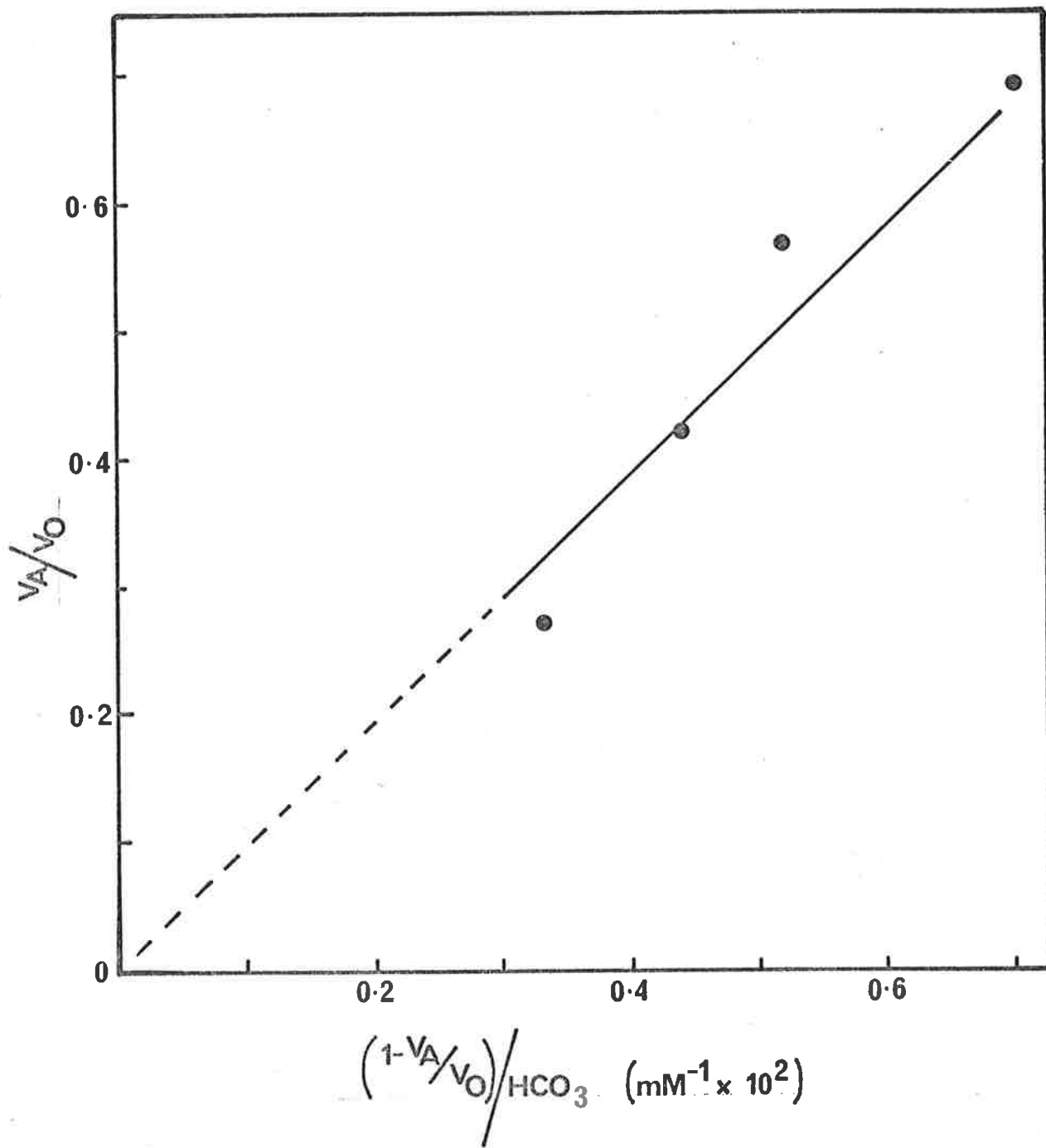




Fig. IV.10. The effects of MgATP<sup>2-</sup> and MgADP concentrations on the rate of inhibition of pyruvate carboxylase by cyanate.

Pyruvate carboxylase was inactivated with 200 mM cyanate, in the presence of the indicated concentrations of MgATP<sup>2-</sup> and MgADP, by the procedure described in Section IV.B.2. Enzymic activity was measured using the acetyl CoA dependent assay procedure. When MgATP<sup>2-</sup> was used ( ● ), 2-5 units pyruvate carboxylase (specific activity 22 U/mg) were present in each tube, while 2.0 units pyruvate carboxylase (specific activity 10 U/mg) per tube were used when the effect of MgADP was investigated ( ○ ).

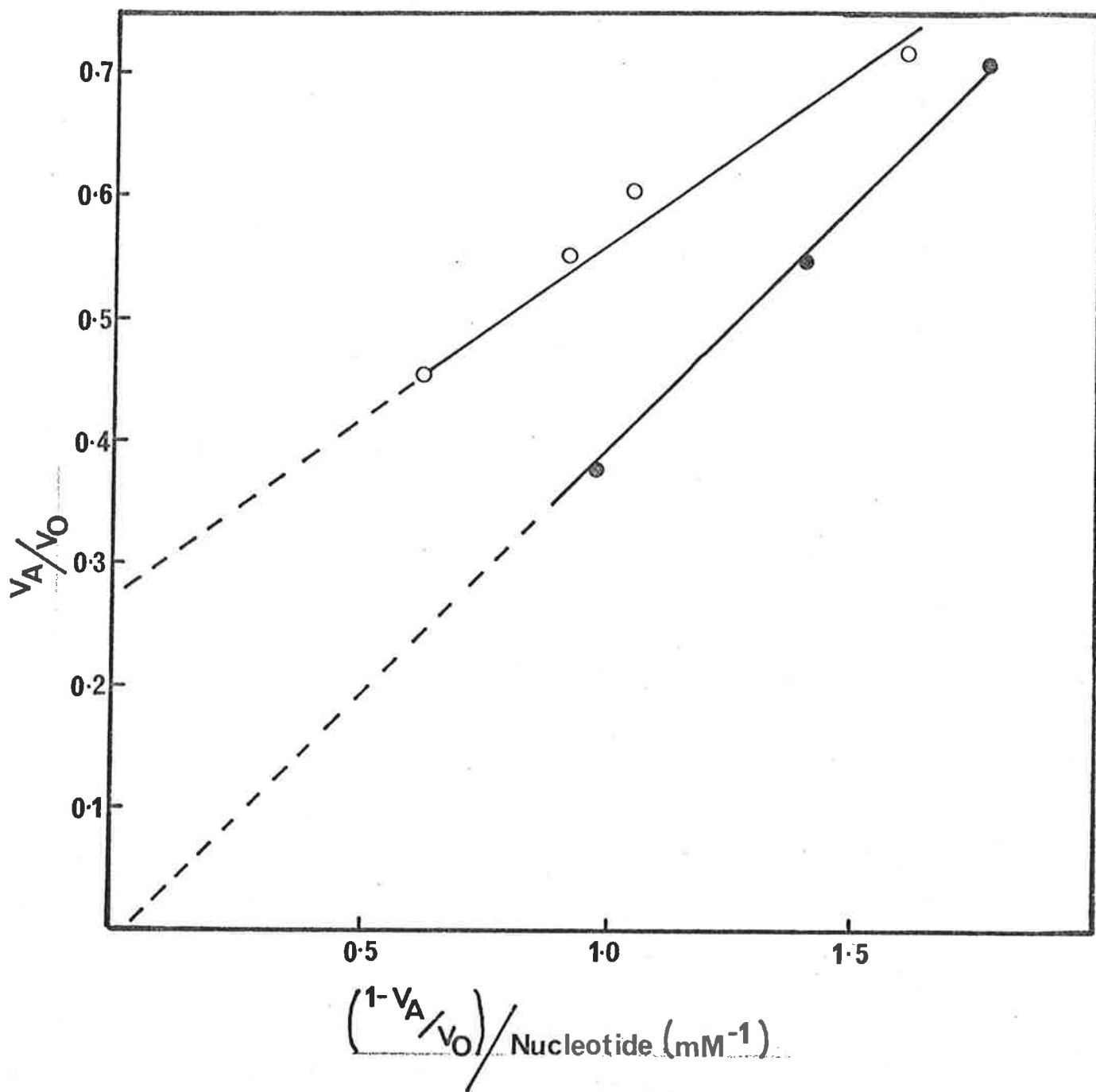


Fig. IV.11. The effect of  $Mg^{2+}$  concentration on the rate of inactivation of pyruvate carboxylase by cyanate.

Pyruvate carboxylase (specific activity 12 U/mg, 1.5 units per inactivation tube) was inactivated with 200 mM cyanate, in the presence of varying concentrations of  $MgCl_2$ , using the procedure described in Section IU.B.2. The enzyme activity was measured using the acetyl CoA dependent assay procedure.  $V_A$  and  $V_0$  represent the rates of inactivation in the presence and absence of  $MgCl_2$  respectively.

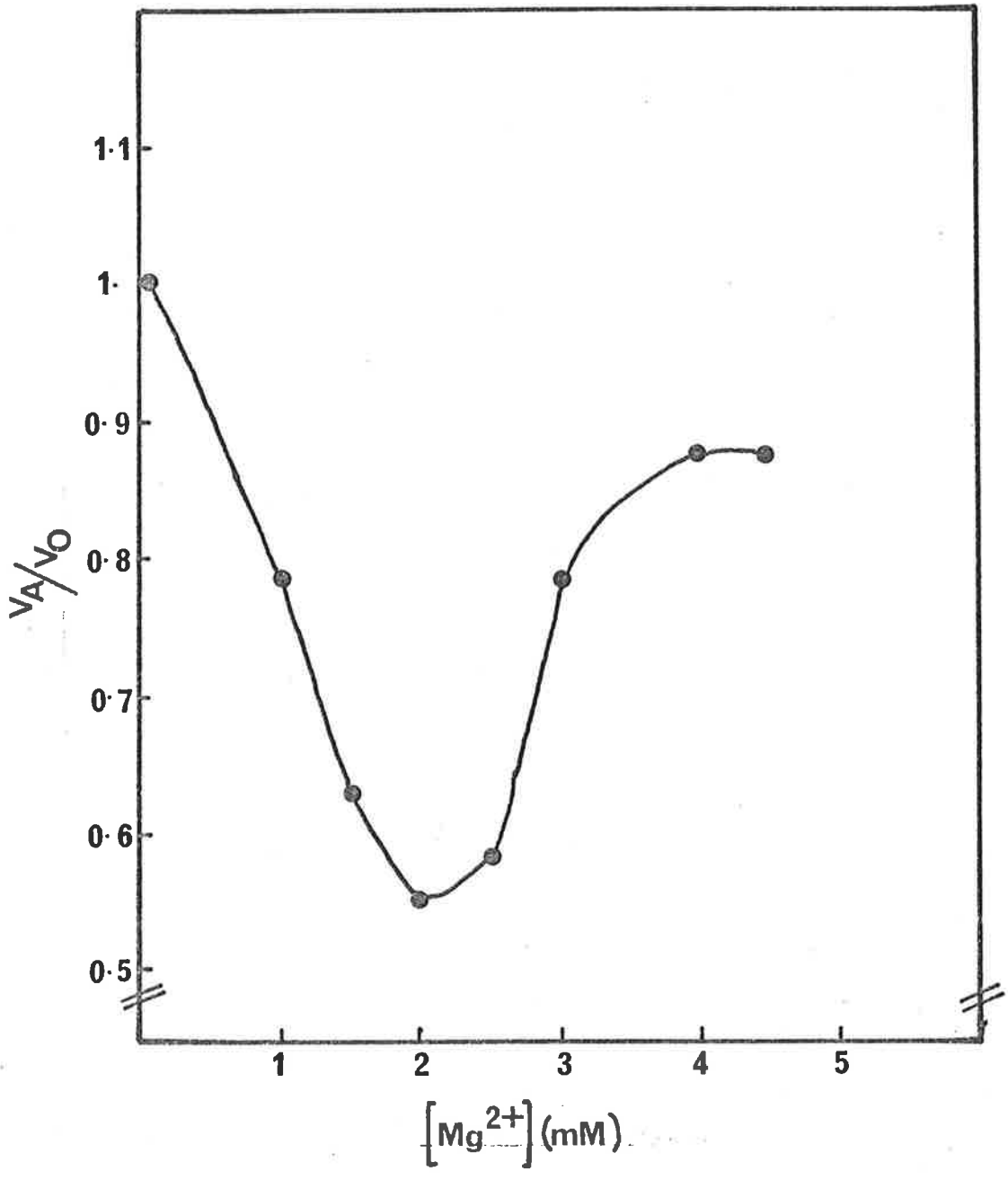


Table IV.2. The effect of cyanate modification on the isotopic exchange reactions catalysed by pyruvate carboxylase.

Pyruvate carboxylase (specific activity 22 U/mg, 54 units) was inactivated with 200 mM cyanate using the procedure described in Section IV.B.2. The inactivation was allowed to proceed until 38% of initial activity had been lost. The enzyme was purified from contaminating cyanate by Sephadex G-25 gel filtration. Aliquots of the purified enzyme were used to initiate the ATP/P<sub>i</sub> and pyruvate/oxaloacetate isotopic exchange reactions which were measured by the procedures described in Sections II.B.5 and II.B.6. The rate of enzyme catalysed oxaloacetate decarboxylation in the absence of pyruvate was determined by the procedure described in Section V.B.

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	Unmodified enzyme	Modified enzyme
Overall reaction (%)	100	60
ATP/P <sub>i</sub> exchange reaction (nmole/min/mg)	2.1 ± 0.36	0.56 ± 0.1
pyruvate/oxaloacetate exchange reaction (μmole/min/mg)	11.2 ± 0.8	16.4 ± 0.4
oxaloacetate decarboxylation (nmole/min/mg)	394 ± 28	326 ± 8

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CHAPTER V

THE MECHANISM OF THE OVERALL REACTION

V.A.        INTRODUCTION

Although the reaction pathway of the pyruvate carboxylase catalysed reaction has been under investigation for nearly two decades, there are a number of features of the reaction which remain to be elucidated. For example, from kinetic studies aimed at elucidating the overall reaction pathway, a consistent feature of the data obtained has been that when pyruvate was the varied substrate, double reciprocal plots of the initial rate of oxaloacetate synthesis plotted as a function of pyruvate concentration were concave downwards. (Taylor et al., 1969; Cazzulo et al., 1970; McLure et al., 1971a; Seufert et al., 1971; Barden et al., 1972; Warren and Tipton, 1974b).

The original postulates advanced to explain this apparent substrate activation have not been adequately supported by experimental data. Taylor et al., (1969) suggested negative co-operativity of binding of pyruvate as a possible explanation, but the demonstration by Nakashima et al., (1975) that the active monomeric form of rat liver pyruvate carboxylase exhibited non-linear double reciprocal plots negated any explanation based on subunit interactions. In addition, the suggestion that the non-classical kinetic profile was due to a pyruvate induced interaction between the biotin carboxylation and pyruvate carboxylation sites (McClure et al., 1971c) was inconsistent with the observation of a non-classical pyruvate saturation curve in the pyruvate/oxaloacetate isotopic exchange reaction, where apparently only the pyruvate carboxylation site is involved (Scrutton et al., 1965).

Another aspect of the reaction which has not been unambiguously described is the order of binding of substrates and release of products.



As was discussed in Chapter I, the experimental data obtained was not entirely consistent with either the proposed ping-pong or sequential reaction pathways.

In order to resolve these problems, new experimental approaches have been used in an attempt to elucidate the reaction sequence of the pyruvate carboxylase reaction. The results of this investigation are presented in this Chapter.

## V.B. MATERIALS AND METHODS

### V.B.1. General Methods

Purification of nucleotides and pyruvate carboxylase was performed as described in Chapter II. The procedure for determination of radioactivity was described in Section II.B.3.

### V.B.2. Assay Methods

#### V.B.2.a. Overall reaction assay procedures.

When the rate of ketoacid product synthesis only, was measured, the assay procedure described in Section II.B.4 was followed. When both oxaloacetate and orthophosphate synthesised were measured, the assay system contained, (in  $\mu\text{mole}$ ), in a final volume of 1.5 ml; tris Cl, pH 8.4, (150);  $\text{MgCl}_2$ , (12); acetyl CoA, (0.5); [ $^{14}\text{C}$ ]bicarbonate, sodium salt, (15),  $1.5 \times 10^5$  cpm/ $\mu\text{mole}$ ; [ $\gamma$ - $^{32}\text{P}$ ]ATP, (3.3),  $4.9 \times 10^3$  cpm/ $\mu\text{mole}$ ; pyruvate as indicated, and 0.12 units of pyruvate carboxylase (specific activity 12-20 U/mg). After two minutes reaction time at  $30^\circ\text{C}$ , 0.3 ml was removed into formic acid at  $0^\circ\text{C}$  for orthophosphate determination, and 0.15 ml saturated dinitrophenylhydrazine in 6M HCl was added to the remainder of the mixture for oxaloacetate determination. The amount of oxaloacetate formed was

determined by the procedure outlined in Section II.B.3 while the amount of orthophosphate released was determined either by the method of Bais (1975), or by chromatographic separation of radioactive orthophosphate from [ $\gamma$ - $^{32}$ P]-ATP using polyethyleneimine paper, with 0.2M  $\text{NH}_4\text{HCO}_3$  as developing solvent. In the controls, pyruvate carboxylase was incubated with excess avidin prior to its addition to the reaction mixtures.

When only the rate of orthophosphate release from [ $\gamma$ - $^{32}$ P]ATP was measured, the assay system described in Section II.B.3 was used. After five minutes reaction at 30°C, the reaction was quenched by addition of 0.05 ml 6M HCl. Carrier orthophosphate (20  $\mu$ moles) was added, and the orthophosphate in a 0.25 ml aliquot was extracted into the organic phase of a water-saturated iso-butanol (4 ml), ammonium molybdate (1 ml), (40 mM in 1.25M  $\text{H}_2\text{SO}_4$ ) separation system. Duplicate samples were taken and their radioactivity determined. Non-specific  $\text{MgATP}^{2-}$  hydrolysis was corrected for using the avidin control described above.

#### V.B.2.b. Partial reaction assays.

The conditions used for measuring the pyruvate/oxaloacetate isotopic exchange reaction have been described in Section II.B.6. In addition an aliquot of the exchange reaction mixture was used to determine the pyruvate concentration by measuring the change of absorbance at 340 nm in the presence of NADH and lactate dehydrogenase. In the control, the enzyme was incubated with avidin prior to the addition to the reaction mixture.

The assay system for measuring the rate of  $\text{MgATP}^{2-}$  hydrolysis in the absence of pyruvate contained, (in  $\mu$ moles), in a final volume of 0.5 ml: tris Cl, pH 8.4, (50);  $\text{MgCl}_2$ , (2.5); bicarbonate, sodium salt, (5);

acetyl CoA, (0.125); [ $\gamma$ - $^{32}$ P]-ATP, (1.0) ( $2 \times 10^6$  cpm/ $\mu$ mole), and two units of pyruvate carboxylase. After ten minutes at 30°C, the reaction was stopped by addition of 0.05 ml 5M formic acid, and orthophosphate separated from the [ $\gamma$ - $^{32}$ P]-ATP by the chromatographic procedure described above. The rate of hydrolysis was proportional to enzyme concentration, and linear with time under the conditions described.

#### V.B.3. Preparation of carboxybiotin enzyme

The reaction mix for preparation of carboxylation enzyme contained, (in  $\mu$ moles), in a final volume of 0.5 ml: tris Cl, pH 8.4, (50);  $MgCl_2$ , (4); ATP, (1.25); bicarbonate, sodium salt, (20),  $5.5 \times 10^7$  cpm/ $\mu$ mole; acetyl CoA, (0.125), and pyruvate carboxylase, specific activity 12.5 U/mg, 40 units. After twenty minutes incubation at 0°C, the carboxybiotin enzyme was purified by gel filtration on a Sephadex G25 column (20x1cm), previously equilibrated with 0.1M tris Cl, pH 8.4, at 4°C.

#### V.B.4. Derivation of rate equations

Rate equations describing dependence of the initial velocity release of products on substrate concentrations were derived by the Wang algebra technique of Indge and Childs (1975). The equations were checked by deriving them twice using two different sets of enzyme form nodes.

#### V.B.5. Data Processing

When appropriate, the slopes and intercepts of lines were obtained from a least mean squares analysis, using a Fortran computer program. Simulation of the enzyme kinetics as a function of substrate concentration, in terms of the derived rate equations, was performed using a Fortran computer program which calculated values of  $1/v$  for any

given set of values of the rate constants, using an appropriate range of substrate concentration. When appropriate, initial velocity data were analysed by computing the hyperbolae of best fit, using the Fortran program HYPER (Cleland, 1963a).

V.C.        RESULTS

V.C.1.      Stoichiometry of the overall reaction.

Hitherto, all investigators of the pyruvate carboxylase reaction have determined enzymic activity by measuring the rate of production of oxaloacetate. The validity of this assay stems from the stoichiometric relationship between synthesis of oxaloacetate and orthophosphate release established by Utter and Keech (1960), and it has been assumed that this stoichiometry remains constant under all experimental conditions. In order to test this assumption, the dependence of both oxaloacetate synthesis and orthophosphate release on pyruvate concentration was determined. The data obtained are presented in Fig. V.1 in the form of double reciprocal plots. It can be seen that, as reported previously, the plot obtained using the rate of oxaloacetate synthesis is non-linear with downward curvature, whereas that for the amount of orthophosphate release is apparently a straight line. Furthermore, when the ratio of the amount of orthophosphate released to the amount of oxaloacetate produced is plotted as a function of pyruvate concentration (Fig. V.2), it can be seen that at saturating levels of pyruvate the ratio approached unity, but as the pyruvate concentration was decreased, the ratio increased. This lack of a one-to-one stoichiometric product release cannot be explained by an ATPase contaminant in the enzyme preparations as the avidin-treated control did not liberate any orthophosphate.

One way of explaining the above data is to postulate that the

carboxybiotin enzyme complex is unstable, and undergoes hydrolysis. A possible reaction mechanism, incorporating this hydrolysis side reaction, is shown diagrammatically in Fig. V.3. At low levels of pyruvate, hydrolysis of a significant proportion of the E-biotinCO<sub>2</sub> complex would occur. However, as the level of pyruvate is increased, a greater proportion of the E-biotinCO<sub>2</sub> complex is involved in pyruvate carboxylation, and the proportion undergoing hydrolysis is decreased, thus permitting the ratio of the reaction products to approach one.

V.C.2. Localisation of the site of enzyme-biotinCO<sub>2</sub> hydrolysis.

Although the data presented above provided compelling evidence that a hydrolytic breakdown of the enzyme-biotinCO<sub>2</sub> complex occurs, it did not indicate whether the hydrolysis occurs at the biotin carboxylation site, at the transcarboxylation site, or during movement of carboxybiotin between these two sites. To resolve this question, the following experiments were conducted. First of all, the rate of MgATP<sup>2-</sup> hydrolysis in the absence of pyruvate was determined by measuring the rate of release of [<sup>32</sup>P]orthophosphate from [ $\gamma$ -<sup>32</sup>P]ATP. Extrapolation of the data presented in Fig. V.2 to zero pyruvate concentration suggests that the rate of ATP hydrolysis would be relatively high in the absence of pyruvate. However, the results presented in Table V.1 show that the low rate of ATP hydrolysis (3.6nmole/min/unit) could not account for the orthophosphate produced in excess of the oxaloacetate synthesised in the overall reaction (100 nmole/min/unit at 0.42 mM pyruvate, Fig. V.1).

Similarly, the slow rate of decay of the enzyme-biotinCO<sub>2</sub> complex, prepared by incubating enzyme as described in Section V.B.3, 0.5 nmole/min/unit at 16.5°C (Fig. V.4), could not account for the high rate of MgATP<sup>2-</sup>

hydrolysis in the overall reaction at low levels of pyruvate. Together, these data exclude the biotin carboxylation site as the locus of hydrolysis.

To ascertain whether the hydrolytic breakdown occurred at the transcarboxylation site, both the rate and stoichiometry of the pyruvate/oxaloacetate isotopic exchange reaction were determined. If hydrolysis of the enzyme-biotin  $\text{CO}_2$  complex occurs preferentially at low levels of pyruvate, then a marked difference in the change of concentration of oxaloacetate should occur at low concentrations compared to high levels of pyruvate during the course of the reaction. Data presented in V.5 confirm that this is the case. It can be seen that the plot of  $1/v$  against  $1/\text{pyr}$  is concave downward, as has been observed previously (Scrutton et al., 1965). Moreover, when the change in pyruvate concentration occurring during the exchange was plotted as a function of the initial pyruvate concentration (Fig. V.5 inset) it can be seen that at low initial pyruvate concentrations, there was a pronounced increase in pyruvate concentration, indicating a relatively rapid rate of oxaloacetate decarboxylation. However, as the initial pyruvate concentration increased, the rate of oxaloacetate decarboxylation decreased.

These data established the site of hydrolysis as the transcarboxylation site, but raise the additional question of whether the lack of stoichiometry is due to hydrolysis of a carboxybiotinenzyme species, or to decarboxylation of oxaloacetate. To decide between these two possibilities, the rate of decarboxylation of oxoacetate was determined by incubating the enzyme with oxaloacetate, and following the rate of pyruvate formation by coupling with NADH and lactate dehydrogenase. Once again, the rate of oxaloacetate decarboxylation (8 nmole/min/unit) was too slow to account for the lack of stoichiometry in either the overall reaction or the exchange reaction. The conclusions to be drawn from these

data is that the enzyme-biotin  $\text{CO}_2$  complex is stable in the absence of pyruvate, which implies that the presence of pyruvate is necessary for hydrolysis to occur. Obviously then, the mechanism described in Fig. V.3 required modification in order to be consistent with the additional evidence.

### V.C.3. The transcarboxylation reaction sequence.

Qualitatively, the sequence of events presented in Fig. V.6 satisfies the experimental evidence presented above. In this model, it is implied that the binding of pyruvate shifts the carboxy-biotin moiety to the transcarboxylation site in readiness for pyruvate carboxylation. However, if pyruvate dissociates before carboxylation occurs, then the resulting E-biotin- $\text{CO}_2$  complex hydrolyses to E-biotin.

Assuming that  $\text{MgATP}^{2-}$  and  $\text{HCO}_3^-$  are present at saturating levels, equation (V-1) describes the dependence of oxaloacetate production on pyruvate concentration.

$$v = \frac{V}{1 + \frac{k_3}{k_4\text{py}+k_5} + \frac{(k_2+k_6)k_4\text{py}+(k_2+k_3+k_6)k_5}{k_1\text{py}(k_4\text{py}+k_5)}} \quad (\text{V-1})$$

This equation may be simplified by considering that the binding of pyruvate is a rapid equilibrium process. This is consistent with the nuclear magnetic resonance studies of Mildvan and Scrutton (1966), who showed that in the case of the chicken liver enzyme, pyruvate moves in and out of the active site at a rate which is two orders of magnitude faster than the overall reaction. Furthermore, the observation of a low rate of oxaloacetate decarboxylation in the absence of pyruvate is consistent with rapid equilibrium binding. The point here is that as rate constant  $k_2$  has a large numerical value, the rate of formation of E-biotin- $\text{CO}_2$  greatly exceeds

that of the unstable E-biotin  $\text{CO}_2$  complex under conditions of essentially zero pyruvate concentration.

Hence, assuming rapid equilibrium binding of pyruvate, it follows that

$$k_2 \gg k_3, k_6 .$$

Therefore, equation V-1 may be simplified to:

$$v = \frac{V}{1 + \frac{k_2}{k_1 \text{pyr}} + \frac{k_3}{k_4 \text{pyr} + k_5}} \dots \quad (\text{V-2})$$

Assuming that equation V-2 correctly describes the dependence of the pyruvate carboxylase reaction on pyruvate concentration, it is possible to deduce values for the ratios of various rate constants by examining experimental data. For example at low concentrations of pyruvate,

$$k_4 \text{pyr} + k_5 \approx k_5$$

and equation V-2 becomes

$$v = \frac{V}{1 + \frac{k_3}{k_5} + \frac{k_2}{k_1 \text{pyr}}} \quad (\text{V-3})$$

This equation describes a straight line when  $1/v_{\text{OAA}}$  is plotted as a function of  $1/\text{pyr}$ . This line corresponds to region "A" of the double reciprocal plot shown in Fig. IV.1, and the slope of the line is  $\frac{1}{V} \cdot \frac{k_2}{k_1}$ . Similarly, at high pyruvate concentration,

$$k_4 \text{pyr} + k_5 \approx k_4 \text{pyr}$$

and

$$v_{\text{OAA}} = \frac{V}{1 + \frac{k_3}{k_4 \text{pyr}} + \frac{k_2}{k_1 \text{pyr}}} \dots \quad (\text{V-4})$$

This equation describes region "B" of the double reciprocal plot, and the slope of this line is  $\frac{1}{V} \cdot \left( \frac{k_3}{k_4} + \frac{k_2}{k_1} \right)$ . It is possible to obtain further estimates of the ratios of rate constants by considering the ratios of the intercepts of the lines of regions "A" and "B". From equation V-3 the



intercept of the region "A" line is

$$\frac{1}{V} \left( 1 + \frac{k_3}{k_5} \right)$$

and from equation V-4 the intercept of the region "B" line is  $1/V$ . Therefore the ratio of intercept "A" to intercept "B" is given by the expression  $1 + \frac{k_3}{k_5}$ . From the data of a number of experiments, values for  $\frac{1}{V} \cdot \frac{k_2}{k_1}$ ,  $\frac{1}{V} \left( \frac{k_2}{k_1} + \frac{k_3}{k_4} \right)$ , and  $k_3/k_5$  of  $0.47 \pm 0.04$ ,  $1.16 \pm 0.04$  and  $1.7 \pm 0.18$  respectively were obtained. These values were used to set appropriate boundary values on the rate constants during an attempt to obtain suitable values of the rate constants in equation V-2 for a simulation of the data shown in Fig. IV.1. Evaluating the rate constants was done by calculating theoretical initial velocities using equation V-4 for a range of values of the rate constants, and comparing these theoretical velocities with the experimental values. This process was repeated until a set of values were obtained giving a least mean squares best fit of experimental and theoretical data (Fig. V.7). The dependence of the pyruvate/oxaloacetate isotopic exchange reaction on pyruvate concentration may also be described by equation V-2. This is because, under the initial velocity conditions used, the amount of [ $^{14}\text{C}$ ] oxaloacetate would be negligible and hence  $k_6 \cdot [^{14}\text{C}] \text{oxaloacetate} \approx 0$ . This is the same initial velocity assumption that was made in deriving equation V-2 and therefore the same equation applies to this exchange reaction.

Using the values of the rate constants obtained from simulation of the overall reaction, a similar curve was generated for the exchange reaction data (Fig. V.7). However, the fit in this case was not as good, as the pyruvate concentrations used varied during the course of the experiment, because of the decarboxylation of oxaloacetate (Fig. V.4). The changing pyruvate concentration meant that the specific activity of the [ $^{14}\text{C}$ ]pyruvate was not constant throughout the experiment. Therefore,

further attempts to optimise fit of the experimental data to simulated curves were not carried out.

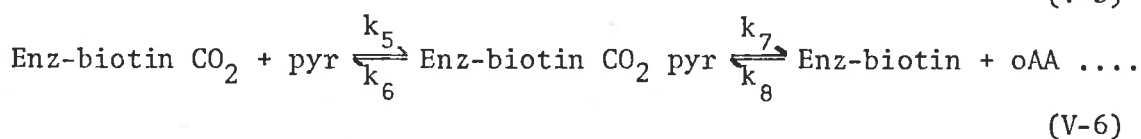
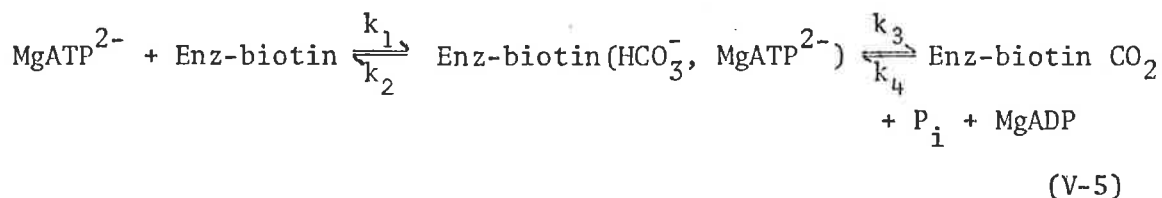
One interesting facet of the reaction sequence presented here concerns the report of Brunette et al., (1972), who have shown that pyruvate carboxylase from the liver of a patient suffering from pyruvate accumulation in the blood, with concurrent lactic acidosis, did not exhibit the expected non-linear pyruvate saturation kinetics. From the model presented in Fig. V.6, one would predict that the pathway for hydrolytic breakdown of the enzyme-substrate complex was blocked; more precisely  $k_5 = 0$ . This can be seen from the computer generated curves of Fig. V.8, where the value of  $k_5$  is reduced from 800 to 0  $\text{min}^{-1}$ , and as the value of  $k_5$  decreases, the plot tends to a straight line. The reason for the decreased velocity at low levels of pyruvate is that under these conditions, the enzyme species  $E \cdot \text{CO}_2$  is a dead end complex, removing more and more enzyme from the carboxylating pathway as the pyruvate concentration is reduced. This explanation is consistent with pyruvate accumulation in the blood, as the intramitochondrial pyruvate concentration of 0.1 mM (Williamson et al., 1967), is low compared to the Michaelis constant of 0.24 mM (Scrutton and White, 1974), reported for this substrate in human pyruvate carboxylase.

#### V.C.4. Alternative substrate kinetics of pyruvate carboxylase.

##### V.C.4.a. Theory

The use of alternative substrates has proved a powerful tool in investigations of the reaction pathways of enzyme catalysed reactions (Webb et al., 1976). In this section the theory of the use of alternative substrate kinetics is developed with respect to the pyruvate carboxylase reaction.

Under conditions where  $\text{HCO}_3^-$  is present at saturating concentrations the ping-pong reaction pathway may be represented by equations V-5 and V-6.



Assuming initial velocity conditions, equation V-7

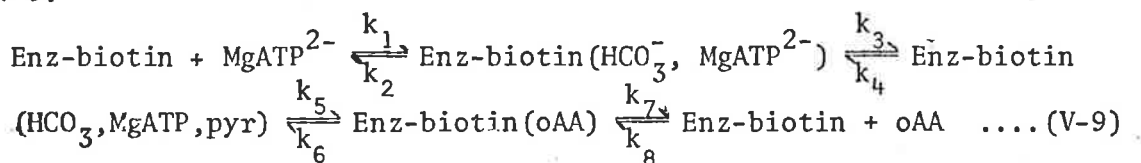
$$\frac{E_t}{v} = K_\alpha \cdot \frac{1}{\text{MgATP}^{2-}} + K_\beta \cdot \frac{1}{\text{pyr}} + \left( \frac{1}{k_3} + \frac{1}{k_7} \right) \dots \quad (\text{V-7})$$

describes the dependence of rate ( $v$ ) of carboxylated product synthesis on the concentrations of the substrates, where  $E_t$ ,  $K_\alpha$  and  $K_\beta$  represent total enzyme concentration, the slope term when  $\text{MgATP}^{2-}$  is the varied substrate (i.e.  $(k_2+k_3)/k_1k_3$ ), and the slope term when pyruvate is the varied substrate (i.e.  $(k_6+k_7)/k_5k_7$ ) respectively. If an alternative keto-acid substrate A is used, the rate constants  $k_5$ ,  $k_6$ , and  $k_7$  will assume new values, i.e.  $k'_5$ ,  $k'_6$  and  $k'_7$ , and equation V-7 will become, -

$$\frac{E_t}{v'} = K_\alpha \cdot \frac{1}{\text{MgATP}} + K'_\beta \cdot \frac{1}{A} + \left( \frac{1}{k_3} + \frac{1}{k'_7} \right) \dots \quad (\text{V-8})$$

where  $v'$  and  $K'_\beta$  represent the rate of synthesis of the alternative carboxylated product and  $(k'_6 + k'_7)/k'_5k'_7$  respectively.

Similarly the sequential reaction pathway may be represented by equation V-9.



Assuming initial velocity conditions, equation V-10 describes the dependence of the rate ( $v$ ) of carboxylated product synthesis, where

$K'_\alpha$  and  $K'_\beta$  represent  $(1 + \frac{k_2}{k_3} \cdot \frac{1}{\text{pyr}} + \frac{k_2 k_4}{k_3 k_5} \frac{1}{\text{pyr}})$  and  $(1 + \frac{k_4}{k_5})$  respectively;

$$\frac{E_t}{v} = K'_\alpha \cdot \frac{1}{k_1 \text{MgATP}^{2-}} + K'_\beta \cdot \frac{1}{\text{pyr}} + \left(\frac{1}{k_5} + \frac{1}{k_7}\right) \dots \quad (\text{V-10})$$

In this case, if an alternative keto-acid substrate is used, rate constants  $k_3$ ,  $k_4$ , and  $k_5$  will assume different values, and therefore the numerical values of both  $K'_\alpha$  and  $K'_\beta$  depend upon the substrate used.

#### V.C.4.b. Results

Keech and Utter (1963) have reported that 2-ketobutyrate could substitute for pyruvate as the keto acid acceptor substrate of the pyruvate carboxylase reaction. More recently, Cheung and Walsh (1975) showed that 3-fluoropyruvate could be carboxylated by chicken liver pyruvate carboxylase. Therefore, equations V-8 and V-9 predict that, if the postulated ping-pong reaction pathway is correct, plots of  $\frac{1}{v}$  versus  $\frac{1}{\text{MgATP}}$ , for fixed concentrations of pyruvate or one of the above alternative substrates should have identical slopes of numerical value  $K'_\alpha$ . Conversely, the double reciprocal plots for a sequential reaction pathway should have different slopes, since the numerical value of the slope term in equation V-10, i.e.  $K'_\alpha$ , depends on the keto-acid substrate used. The results presented in Table V.2 show that the slopes of the lines for the alternative substrates are significantly different. Similar results were obtained using chicken pyruvate carboxylase (assayed under identical conditions, except tris Cl buffer pH 7.8 was used); the slope ratios were 0.52 and 2.42 for 2-ketobutyrate and 3-fluoropyruvate respectively.

Such data clearly eliminates the proposed ping-pong reaction sequence as a valid mechanism of pyruvate carboxylase.

V.C.5. Orthophosphate release kinetics of pyruvate carboxylase

To confirm the conclusion drawn from the alternate substrate approach, additional evidence for a sequential pathway was sought by re-examining the initial velocity studies carried out previously, (McLure *et al.*, 1971b; Barden *et al.*, 1972; Ashman and Keech, 1975). As was described in Section V.C.1, kinetic data obtained at non-saturating levels of pyruvate using the rate of oxaloacetate synthesis only as a measure of reaction velocity are difficult to interpret because of the lack of stoichiometry between orthophosphate and oxaloacetate released. To avoid these complications, the dependence of initial velocity on  $\text{MgATP}^{2-}$  (or bicarbonate) concentration, at various fixed pyruvate concentrations was determined, monitoring the rate of orthophosphate release instead of oxaloacetate synthesis.

The results of these experiments are shown in Fig. V.9, and it can be seen that the data, in double reciprocal form, yielded families of intersecting lines. This pattern is inconsistent with the proposed ping-pong reaction sequence, but is consistent with a sequential pathway.

The dependence of the rate of orthophosphate release on pyruvate concentration, determined at various fixed concentrations of  $\text{MgATP}^{2-}$  is shown in Fig. V-10. Clearly, from Fig. V-10, at low levels of  $\text{MgATP}^{2-}$ , the double reciprocal plots are concave downwards, whereas, at higher  $\text{MgATP}^{2-}$  concentrations, the data may be described by a straight line in double reciprocal form. This was especially evident when a relatively small range of pyruvate concentrations was used, as shown in Fig. V.1. However, when a large pyruvate concentration range was used, significant downward curvature of the double reciprocal plots was observed, even at high  $\text{MgATP}^{2-}$  concentrations, as is shown in Fig. V-11.

V.C. . The pyruvate carboxylase reaction pathway.

In view of the above data, any proposed pyruvate carboxylase reaction pathway must yield steady-state rate equations which describe the following features of the dependence of initial velocity product release on substrate concentrations:

a) intersecting families of straight lines, in double reciprocal form, when the dependence of either orthophosphate or oxaloacetate synthesis on either varying  $\text{MgATP}^{2-}$  or bicarbonate concentration is determined, at various fixed pyruvate concentrations.

b) concave downwards families of lines, in double reciprocal form, when the dependence of orthophosphate or oxaloacetate synthesis on varying pyruvate concentration is determined, at fixed concentrations of  $\text{MgATP}^{2-}$  or bicarbonate.

c) the ratio of orthophosphate released to oxaloacetate synthesised should exceed one at low pyruvate concentrations and should approach one as the pyruvate concentration increases.

A reaction pathway which may satisfy these criteria is shown as Fig. V.12. Because significant hydrolysis of the enzyme species  $\text{E} \cdot \text{CO}_2$  occurs, by the pathway described by rate constant  $K_{11}$ , the ratio of orthophosphate to oxaloacetate release will exceed one. However, as the pyruvate concentration increases the term  $K_{9\text{pyr}}$  becomes numerically greater than  $K_{10}$ , and therefore the steady-state concentration of enzyme species  $\text{E} \cdot \text{CO}_2$  decreases, leading to a decreased rate of hydrolysis. Therefore, this pathway satisfies requirement (c) above.

Equations (V-11) and (V-12) describe the dependence of initial velocity synthesis of orthophosphate and oxaloacetate on the concentrations of varying  $\text{MgATP}^{2-}$  pyruvate, and bicarbonate, assuming  $\text{Mg}^{2+}$  and acetyl CoA are present at saturating levels, and that the initial binding of pyruvate

is a rapid equilibrium process.

$$\frac{V}{v_{P_i}} = 1 + \frac{(k_{10} + k_{9\text{pyr}} + k_{11})k_7}{k_{10}k_{11} + k_8k_{11} + k_8k_9\text{pyr}} + \frac{k_6}{k_5\text{pyr}} \left[ 1 + \frac{k_4}{k_3\text{HCO}_3} + \frac{k_2k_4}{k_1k_3\text{ATP}\cdot\text{HCO}_3} \right] \\ + \frac{k_7}{k_3\text{HCO}_3} + \frac{k_2k_7}{k_1k_3\text{ATP}\cdot\text{HCO}_3} + \frac{k_7}{k_1\text{ATP}} \dots \quad (\text{V-11})$$

$$\frac{V}{v_{\text{OAA}}} = 1 + \frac{k_8}{k_1\text{ATP}} + \frac{k_2k_8}{k_1k_3\text{ATP}\cdot\text{HCO}_3} + \frac{k_8}{k_3\text{HCO}_3} + \frac{k_{10}}{k_9\text{pyr} + k_{11}} \\ + \frac{k}{k_7} \left[ 1 + \frac{k_2k_4k_6}{k_1k_3k_5\text{ATP}\cdot\text{HCO}_3\cdot\text{pyr}} + \frac{k_4k_6}{k_3k_5\text{HCO}_3\cdot\text{pyr}} + \frac{k_6}{k_5\text{pyr}} \right] \\ + \frac{k_{10}k_{11}}{k_9\text{P} + k_{11}} \left[ 1/k_7 + \frac{k_6}{k_5k_7\text{pyr}} + \frac{k_4k_6}{k_3k_5k_7\text{pyr}\cdot\text{HCO}_3} + \frac{k_2k_4k_6}{k_1k_3k_5k_7\text{ATP}\cdot\text{HCO}_3\cdot\text{pyr}} \right] \\ + \frac{1}{k_3\text{HCO}_3} + \frac{k_2}{k_1k_3\text{ATP}\cdot\text{HCO}_3} + \frac{1}{k_1\text{ATP}} \dots \quad (\text{V-12})$$

where  $V$ ,  $v_{P_i}$ , and  $v_{\text{OAA}}$  represent the maximum velocity, the rate of orthophosphate release, and the rate of oxaloacetate release respectively.

The symbols  $\text{pyr}$ ,  $\text{HCO}_3$  and  $\text{ATP}$  represent the concentrations of pyruvate, bicarbonate, and  $\text{MgATP}^{2-}$  respectively.

Inspection of the equations reveals that double reciprocal plots of  $\frac{1}{v_{P_i}}$  and  $\frac{1}{v_{\text{OAA}}}$  against either  $\frac{1}{\text{MgATP}^{2-}}$  or  $\frac{1}{\text{HCO}_3}$  will give families of intersecting straight lines. Thus the proposed reaction pathway satisfies requirement (a) above.

Equation (V-12) can be transformed into equation (V-13) which describes the dependence of  $1/v_{P_i}$  on varying  $1/\text{pyr}$ , at fixed concentrations of  $\text{MgATP}^{2-}$  and bicarbonate.

$$\frac{V}{v_{P_i}} = \frac{a_0 + a_1 \frac{1}{\text{pyr}}}{b_0 + b_1 \frac{1}{\text{pyr}}} + \frac{c}{\text{pyr}} + d \quad (\text{V-13})$$

where  $a_0, a_1, b_0, b_1, c$  and  $d$  represent  $k_7 k_9, k_7 (k_{10} + k_{11}), k_8 k_9, k_{11} (k_8 + k_{10}), k_6/k_5 (1 + \frac{k_4}{k_3 \text{HCO}_3} + \frac{k_2 k_4}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3})$  and  $k_7 (\frac{1}{k_3 \text{HCO}_3} + \frac{1}{k_1 \text{ATP}} + \frac{k_2}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3})$  respectively.

Now, a concave downward plot of  $\frac{1}{v_{P_i}}$  against  $\frac{1}{\text{pyr}}$  will be obtained if  $\frac{d^2 \frac{V}{v_{P_i}}}{d(\frac{1}{\text{pyr}})^2}$  is negative. From equation (V-13):

$$\frac{d^2 \frac{V}{v_{P_i}}}{d(\frac{1}{\text{pyr}})^2} = \frac{(a_0 b_1 - a_1 b_0) (2 b_1 \frac{1}{\text{pyr}} + 2 b_0 b_1)}{(\frac{b_1}{\text{pyr}} + b_0)^4}$$

Hence a concave downward plot will be obtained if  $a_0 b_1 - a_1 b_0$  is negative. This condition holds if  $k_8$  is greater than  $k_{11}$ . Therefore, the proposed pathway is potentially capable of satisfying the first part of requirement (b) above.

Similarly, equation (V-12) can be transformed into equation (V-14) which describes the dependence of  $\frac{1}{v_{\text{OAA}}}$  on  $\frac{1}{\text{pyr}}$  at fixed concentrations of  $\text{MgATP}^{2-}$  and bicarbonate:

$$\frac{V}{v_{\text{OAA}}} = 1 + \frac{a_1 \cdot \frac{1}{\text{pyr}} + a_2 \cdot (\frac{1}{\text{pyr}})^2}{k_9 + k_{11} \cdot \frac{1}{\text{pyr}}} + \frac{b}{\text{pyr}} + c \quad (\text{V-14})$$

where  $a_1, a_2, b,$  and  $c$  represent  $k_{10} \left( 1 + \frac{k_{11}}{k_7} + \frac{k_{11}}{k_3 \text{HCO}_3} + \frac{k_{11}}{k_1 \text{ATP}} + \frac{k_2 k_{11}}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3} \right),$

$$\frac{k_6 k_{10} k_{11}}{k_5 k_7} \left( 1 + \frac{k_4}{k_3 \text{HCO}_3} + \frac{k_2 k_4}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3} \right), \frac{k_6 k_8}{k_5 k_7} \left( 1 + \frac{k_2 k_4}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3} + \frac{k_4}{k_3 \text{HCO}_3} \right) \text{ and}$$

$$\frac{k_8}{k_1 \text{ATP}} + \frac{k_2 k_8}{k_1 k_3 \text{ATP} \cdot \text{HCO}_3} + \frac{k_8}{k_3 \text{ATP}} + \frac{k_8}{k_7} \text{ respectively.}$$

In this case the second derivative is given by the expression:

$$0 \quad \frac{d^2 \frac{V}{v_{\text{AA}}}}{d(\frac{1}{\text{pyr}})^2} = \frac{2a_2 k_9^3 + 2a_2 k_9^2 k_{11} \cdot \frac{1}{\text{pyr}} - 2k_9^2 k_{11} a_1 - 2a_1 k_9 k_{11}^2 \cdot \frac{1}{\text{pyr}}}{(k_9 + k_{11} \cdot \frac{1}{\text{pyr}})^4}$$



This expression will always be negative, provided  $k_{11}a_1$  is greater than  $k_9a_2$ . Therefore, the proposed reaction pathway is also potentially consistent with the second part of requirement (b) above.

Although the mechanism depicted in Fig. V.12 appears to be consistent with the primary plot initial velocity data, as described above, a closer inspection reveals that it is inconsistent with the slope replots in Figs. V.13 and V.14. Inspection of equation (V-11) shows that there should be a linear relationship between the slopes of the  $\frac{1}{v_{p_i}}$  lines against both  $\frac{1}{MgATP}$  and  $\frac{1}{HCO_3}$ , plotted as a function of various levels of pyruvate, whereas a concave downwards line was obtained for the variable  $MgATP^{2-}$  case.

Thus, it is clear that the mechanism shown in Fig. V.12 must be modified in order to be consistent with the slope replot data. A possible modified mechanism is shown in Fig. V.15. This mechanism incorporates a suggestion made in Section I.A.3.b; that, at low concentrations of pyruvate, orthophosphate and MgADP may be released from the enzyme prior to pyruvate binding, whereas at high pyruvate concentrations these products are not released until pyruvate has bound.

Equation (V-15) describes the dependence of orthophosphate release on the  $MgATP^{2-}$ , bicarbonate, and pyruvate concentrations, assuming  $Mg^{2+}$  and acetyl CoA are present at saturating levels, and that the initial binding of pyruvate is a rapid equilibrium process.

$$\frac{v}{v_{p_i}} = 1 + \frac{k_5k_7}{k_7k_8pyr + k_6k_8pyr^2} + \frac{k_4k_7 + k_6k_{10}pyr}{k_3k_7HCO_3 + k_3k_6HCO_3 \cdot pyr} + \frac{k_2k_6k_{10}pyr + k_2k_4k_7 + k_2k_6k_5pyr + k_2k_7k_5}{k_1k_3k_7ATP \cdot HCO_3 + k_1k_3k_6ATP \cdot HCO_3 \cdot pyr} + \frac{k_6k_{10}pyr + k_5k_7}{k_1k_7ATP + k_1k_6ATP \cdot pyr} + \frac{k_6k_8pyr^2(k_{10}k_{12}pyr + k_{11}k_{14} + k_{10}k_{11})}{(k_7k_8pyr + k_6k_8pyr^2)(k_{12}k_{13}pyr + k_{11}k_{14} + k_{13}k_{14})}$$

$$\begin{aligned}
& + \frac{(k_{10}k_{12}\text{pyr}+k_{10}k_{14})(k_6k_9\text{pyr}+k_5k_9)}{(k_7k_8\text{pyr}+k_6k_8\text{pyr}^2)(k_{12}k_{13}\text{pyr}+k_{11}k_{14}+k_{13}k_{14})} \\
& + \frac{k_5k_7(k_8k_{12}\text{pyr}^2+k_8k_{14}\text{pyr}+k_8k_{11}\text{pyr}+k_9k_{12}\text{pyr}+k_9k_{14})}{(k_7k_8\text{pyr}+k_6k_8\text{pyr}^2)(k_{12}k_{13}\text{pyr}+k_{11}k_{14}+k_{13}k_{14})} \quad \dots (V-15)
\end{aligned}$$

The dependence of the slope of the line obtained by plotting  $\frac{1}{v_{p_i}}$  against  $\frac{1}{\text{HCO}_3}$  on various fixed pyruvate concentrations is given by the expression:

$$S1(\text{HCO}_3) = \frac{a_1 \cdot \frac{1}{\text{pyr}} + a_0}{b_1 \cdot \frac{1}{\text{pyr}} + b_0} + \frac{c_1 \cdot \frac{1}{\text{pyr}} + c_0}{d_1 \cdot \frac{1}{\text{pyr}} + d_0} \quad \dots (V-16)$$

where  $a_0$ ,  $a_1$ ,  $b_0$ ,  $b_1$ ,  $c_0$ ,  $c_1$ ,  $d_0$ , and  $d_1$  represent  $k_2k_6k_{10} + k_2k_6k_5$ ,  $k_2k_4k_7 + k_2k_4k_5$ ,  $k_1k_3k_6$ ,  $k_1k_3k_7$ ,  $k_6k_{10}$ ,  $k_4k_7$ ,  $k_3k_6$ , and  $k_3k_7$  respectively.

Now, the data shown in Fig. V.13 suggest that there is probably a linear relationship between  $S1(\text{HCO}_3)$  and  $1/\text{pyr}$ . This will be the case if  $\frac{d^2S1(\text{HCO}_3)}{d(1/\text{pyr})^2}$  is equal to zero. Analysis of equation (V-16) reveals that this will be the case if  $c_1d_0$  is equal to  $c_0d_1$ , which implies that  $k_4$  is equal to  $k_{10}$ . Hence, the modified mechanism is potentially consistent with the first of the slope replot requirements.

The dependence of the slope of the line obtained by plotting  $\frac{1}{v_{p_i}}$  against  $\frac{1}{\text{MgATP}}$  as a function of various fixed pyruvate concentrations is given by the expression:

$$S1(\text{MgATP}) = \frac{a_1 \cdot \frac{1}{\text{pyr}} + a_0}{b_1 \cdot \frac{1}{\text{pyr}} + b_0} + \frac{c_1 \cdot \frac{1}{\text{pyr}} + c_0}{d_1 \cdot \frac{1}{\text{pyr}} + d_0} \quad (V-17)$$

where  $a_1$ ,  $a_0$ ,  $b_1$ ,  $b_0$ , have the values defined above, and  $\bar{c}_0$ ,  $c_1$ ,  $d_0$ , and  $d_1$  represent  $k_6k_{10}$ ,  $k_5k_7$ ,  $k_1k_6$ , and  $k_1k_7$  respectively. A similar analysis of this equation reveals that the concave downward relationship between the slope and  $1/\text{pyr}$  shown in Fig. V.14, will occur if  $k_4$  is equal to  $k_{10}$  and  $k_5$  is greater than  $k_{10}$ . Hence, the modified mechanism is also potentially

consistent with the second of the slope replot requirements.

It is appreciated that this mathematical analysis does not prove that the mechanism shown in Fig. V.15 is correct. However, the mechanism does suggest a number of new experimental approaches which may be able to provide suitably rigorous tests of the proposed mechanism. In particular, a careful study of the patterns of MgADP and orthophosphate product inhibition should shed some light on the pyruvate carboxylase reaction pathway. The mechanism suggests that, at low pyruvate concentrations, the reaction proceeds via a non-sequential pathway, whereas at high pyruvate concentrations it proceeds via a sequential pathway. Hence, use of MgADP and orthophosphate as product inhibitors, at low pyruvate concentrations, should give rise to the product inhibition pattern predicted by Barden et al., (1972) for the non-sequential reaction pathway. However, similar product inhibition experiments, conducted at a high pyruvate concentration, should give a pattern diagnostic of a sequential reaction pathway.

In addition, use of alternate substrates may provide a useful tool, in defining the reaction pathway. Using the theory described in Section V.C.4.a., one might expect that double reciprocal plots indicative of a non-sequential pathway might be obtained at low keto-acid concentrations, while the data presented in Section V.C.4.b show that the pyruvate carboxylase reaction does proceed via a sequential pathway at high keto-acid concentrations.

## V.D. DISCUSSION

### V.D.1. Extension of the pyruvate carboxylase mechanism to other enzymes.

Steady state kinetic data giving concave downwards double

reciprocal plots has been obtained with numerous enzyme systems (for review see Levitski and Koshland, 1969). Two particularly interesting enzymes in this regard are bacterial deoxythymidine kinase (Okazaki and Kornberg, 1964), and human erythrocyte hexokinase (Rijkson and Staal, 1976). In both cases, concave downward plots were obtained when the reciprocal rate of phosphorylated product formation was plotted as a function of the reciprocal acceptor substrate concentration. There is thus an analogy with the double reciprocal plots obtained for pyruvate carboxylase, with the acceptor substrate (pyruvate), variable. It is tempting to speculate that, in the case of the two kinases, hydrolytic breakdown of an intermediate phosphoryl-enzyme species may account for the observed kinetic behaviour of these enzymes. A possible mechanism for these enzymes is shown in Fig. V.16, in which D, D', A, and A' represent the donor nucleotide triphosphate, product nucleotide diphosphate, acceptor substrate, and phosphorylated product respectively. Equation V-15, describes the dependence of initial velocity phosphorylated product synthesis ( $v$ ) on the concentrations of the substrates:

$$\frac{v}{E_t} = \frac{\alpha_1 A + \alpha_2 A^2}{\beta_0 + \beta_1 A + \beta_2 A^2}$$

where  $E_t$ ,  $\alpha_1$ ,  $\alpha_2$ ,  $\beta_0$ ,  $\beta_1$ , and  $\beta_2$  represent the total enzyme concentration,  $k_1 k_3 k_5 k_9$ ,  $k_1 k_3 k_5 k_7$ ,  $(k_6 k_9 + k_8 k_9)(k_2 k_5 + k_1 k_5 D) + (k_2 k_4 + k_1 k_4 D) k_8 k_9$ ,  $k_6 k_7 (k_2 k_5 + k_1 k_5 D) + (k_6 k_9 + k_8 k_9) \cdot (k_3 k_5 + k_1 k_3 D) + k_1 k_3 k_5 k_9 D + k_1 k_3 k_5 k_8 D$ , and  $k_3 k_5 k_6 k_7 + k_1 k_3 k_5 k_7 D + k_1 k_3 k_6 k_7 D$  respectively.

Using this equation, the double reciprocal plot shown as Fig. V.17 was constructed. Thus, a mechanism of this type is consistent with the experimental initial velocity data.

The postulated hydrolysis of a phosphoryl-enzyme intermediate (in which phosphate may be attached either covalently or non-covalently to

the enzyme), would be simple to test experimentally. The demonstration of acceptor substrate-dependent lack of stoichiometry between ADP and phosphorylated product released, and/or demonstration of formation of [ $^{32}\text{P}$ ]orthophosphate from [ $\gamma\text{-}^{32}\text{P}$ ]ATP would be strong evidence in favour of the proposed mechanism. In the latter case, it would be necessary to show that the release of labelled orthophosphate varied inversely with the acceptor substrate concentration.

V.D.2. Possible physiological significance of the pyruvate carboxylase mechanism.

Although the significance of the atypical kinetic behaviour of pyruvate carboxylase for the proposed reaction pathways has been stressed in this thesis, it is reasonable to speculate on the kinetic behaviour as a possible form of metabolic control. Pyruvate carboxylase is known to be involved in (a) gluconeogenesis from pyruvate and its precursors (Keech and Utter, 1963), (b) lipogenesis from pyruvate via the citrate-carrier system (Hanson and Ballard, 1967), and (c) an anapleurotic role in maintaining the tricarboxylic acid cycle intermediates at function levels (Kornberg, 1966). One might expect that to span these diverse roles across a range of pyruvate concentrations and physiological conditions, isoenzymes might have evolved, but no evidence for this has been obtained (Scrutton and Fatabene, 1975).

On the other hand, there is a considerable body of evidence to show that the activity of the enzyme is modulated by varying concentrations of acetyl CoA. Superimposed on this, there appears to be, from the results presented above, an additional form of regulation: a control mechanism which can be considered analogous to a "slipping clutch".

The weaknesses in the argument are first of all, the uncertainty of the intramitochondrial concentrations of both pyruvate and acetyl CoA, and second, the effect of different levels of acetyl CoA on the concentrations of pyruvate which will yield half-maximal velocities. The work of Ashman and Keech (1975), and results presented in this thesis (Chapter VI) has shown that a major effect of acetyl CoA is facilitation of pyruvate binding to the enzyme. Thus, it is possible that the in vivo effect of acetyl CoA is mediated via pyruvate binding.

Although pyruvate carboxylation is energetically inefficient at low levels of pyruvate, because of the relatively high rate of  $\text{MgATP}^{2-}$  hydrolysis, relative to oxaloacetate synthesis, this form of control permits a single protein to serve a multi-purpose function. Presumably, even at the low levels of pyruvate normally found in the cell (Williamson et al., 1967) sufficient oxaloacetate can be synthesised to maintain the essential anapleurotic role. However, in response to elevated pyruvate levels, e.g., following muscular activity, or under gluconeogenic conditions, there is a hypercompensatory increase in enzymic efficiency, to remove the excess substrate. The energy expenditure of the substrate induced variation in stoichiometry described herein is a somewhat analagous situation to the futile cycles (Friedman et al., 1971; Newsholme et al., 1972, and Clark et al., 1973), for which a regulatory function has been proposed (Newsholme and Gevers, 1967; Scrutton and Utter, 1968). Furthermore, it is probably a most effective biological solution to the problem of regulation of this enzyme to changing substrate concentrations.

Finally, although glucagon is known to potentiate gluconeogenesis at the level of pyruvate carboxylase, no direct effect on the enzyme has been observed. Recently however, Yamazaki and Haynes (1975) have shown that this hormone facilitates the transport of pyruvate across the mito-

chondrial membranes. Taken in conjunction with the substrate activation mechanism proposed here, their report provides a plausible explanation for the hormonal-induced stimulation of gluconeogenesis.

V.D.3. The significance of the exchange reactions.

It is appreciated that any proposition supporting a sequential pathway for this enzyme must also provide some explanation for the fact that the enzyme catalyses isotopic exchange reactions in the absence of components of the other partial reaction. This contingency can be met if it is assumed that  $\text{MgADP}^-$  and orthophosphate are released at a significant rate only after the binding of pyruvate. This requirement provides a plausible explanation for the low rate of the  $\text{ATP}/\text{P}_i$  isotopic exchange reaction, (Scrutton et al., 1965; McLure et al., 1971a; Ashman and Keech, 1975) with regard to the very rapid pyruvate/oxaloacetate isotopic exchange reaction (McLure et al., 1971a; Ashman and Keech, 1975), this is not inconsistent with a sequential pathway if the active site is considered to consist of two spatially distinct subsites, as is suggested from product inhibition studies (McLure et al., 1971a; Barden et al., 1972; Ashman and Keech, 1975), and as may be inferred from magnetic resonance studies (Mildvan et al., 1966; Reed and Scrutton, 1974).

Furthermore, the affinity labelling and chemical modification studies presented in this thesis (Chapters III and IV) show that it is possible to irreversibly inhibit the  $\text{ATP}/\text{P}_i$  isotopic exchange reaction without causing concomitant inhibition of the pyruvate/oxaloacetate isotopic exchange reaction. The simplest interpretation of these results is to postulate the existence of spatially distinct sites for  $\text{MgATP}^{2-}$ -dependent biotin carboxylation and pyruvate transcarboxylation.

The point here is that, although in the overall reaction,

release of MgADP and orthophosphate occurs after pyruvate binding at least at high pyruvate concentrations, the separation of the sites implies there is no mechanistic (in the sense of chemical interactions between the substrates and active site amino acid residues), requirement for this order of release to be maintained under all experimental conditions.

V.D.4. The mechanism of carboxyl transfer to pyruvate.

As was discussed in Section I.B.2.c, the nature of the group(s) responsible for activation of pyruvate prior to transfer of the carboxyl group from 1'-N-carboxyl-biotin has not been clearly established. Some data is consistent with the enzyme bound metal ion (usually  $Mn^{2+}$ ) facilitating pyruvate carboxylation because of its electron withdrawing properties (Mildvan et al., 1966; Mildvan and Scrutton, 1967). On the other hand, Retey and Lynen (1965) have suggested that carboxy-biotin is the species that activates pyruvate. This suggestion is consistent with the retention of configuration around the substituted carbon atom observed for a number of biotin carboxylases (Arigioni et al., 1966; Prescott and Rabinowitz, 1968; Rose, 1970; and Cheung et al., 1975). As was noted by Rose (1970) this retention of configuration implies that pyruvate carboxylation is a concerted process; i.e., once pyruvate is committed to carboxylation, no kinetically significant intermediates occur until oxaloacetate is synthesised.

However, the nuclear magnetic resonance studies of Mildvan and Scrutton (1967) show that pyruvate moves in and out of its binding site at a rate several orders of magnitude greater than the rate of carboxylation. This result implies that pyruvate binding is not synonymous with pyruvate carboxylation. Also, recent studies in this laboratory have shown that pyruvate induces a large change in the circular dichroism spectrum of



of pyruvate carboxylase (Duc, unpublished results), suggesting that the enzyme undergoes a significant conformational change on pyruvate binding.

These results may be used to provide a possible mechanistic interpretation of the proposed reaction pathway described above. The low rate of  $\text{MgATP}^{2-}$  hydrolysis in the absence of pyruvate (Table V.1) suggests that carboxybiotin in the biotin carboxylation site is resistant to hydrolysis. On the other hand, it has been shown that carboxybiotin in the pyruvate carboxylation site is labile to hydrolysis.

It is possible that the observed change in the enzyme's three dimensional structure on pyruvate binding reflects the movement of carboxybiotin from the biotin carboxylation site to the pyruvate carboxylation site.

The observed lability of carboxybiotin in the pyruvate carboxylation site suggests that carboxybiotin in this site is activated, (possibly by the sulphhydryl) residue identified by Hudson et al., 1977) so that carboxyl transfer to pyruvate can occur. However, if pyruvate dissociates from the enzyme, then the carboxybiotin may remain in the activated state, and thus be labile to hydrolysis.

Fig. V.1.

Reciprocal plots of the velocity of the pyruvate carboxylase reaction plotted as a function of pyruvate concentration; the reaction velocity, determined by the procedure described in Section V.B.2.a, was measured as either orthophosphate released ( ○ ) or as oxaloacetate produced ( ● ).

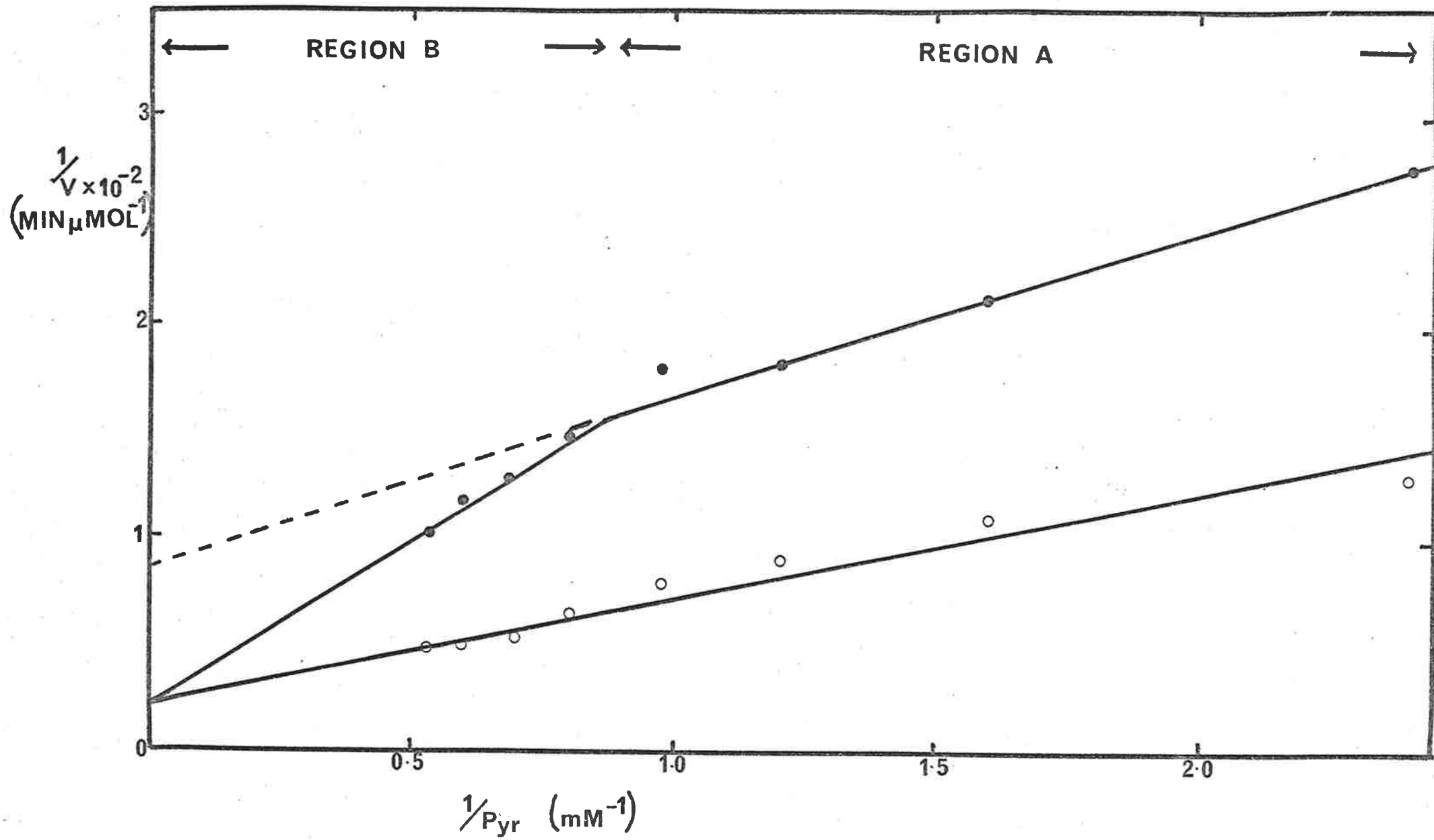


Fig. V.2. Stoichiometry of the products of the overall reaction.

From the data of a number of experiments of the type shown in Fig. V.1 the mean ratio of orthophosphate released to oxaloacetate produced is plotted as a function of pyruvate concentration. The error bars indicate standard errors.

$\frac{P_i}{OAA}$

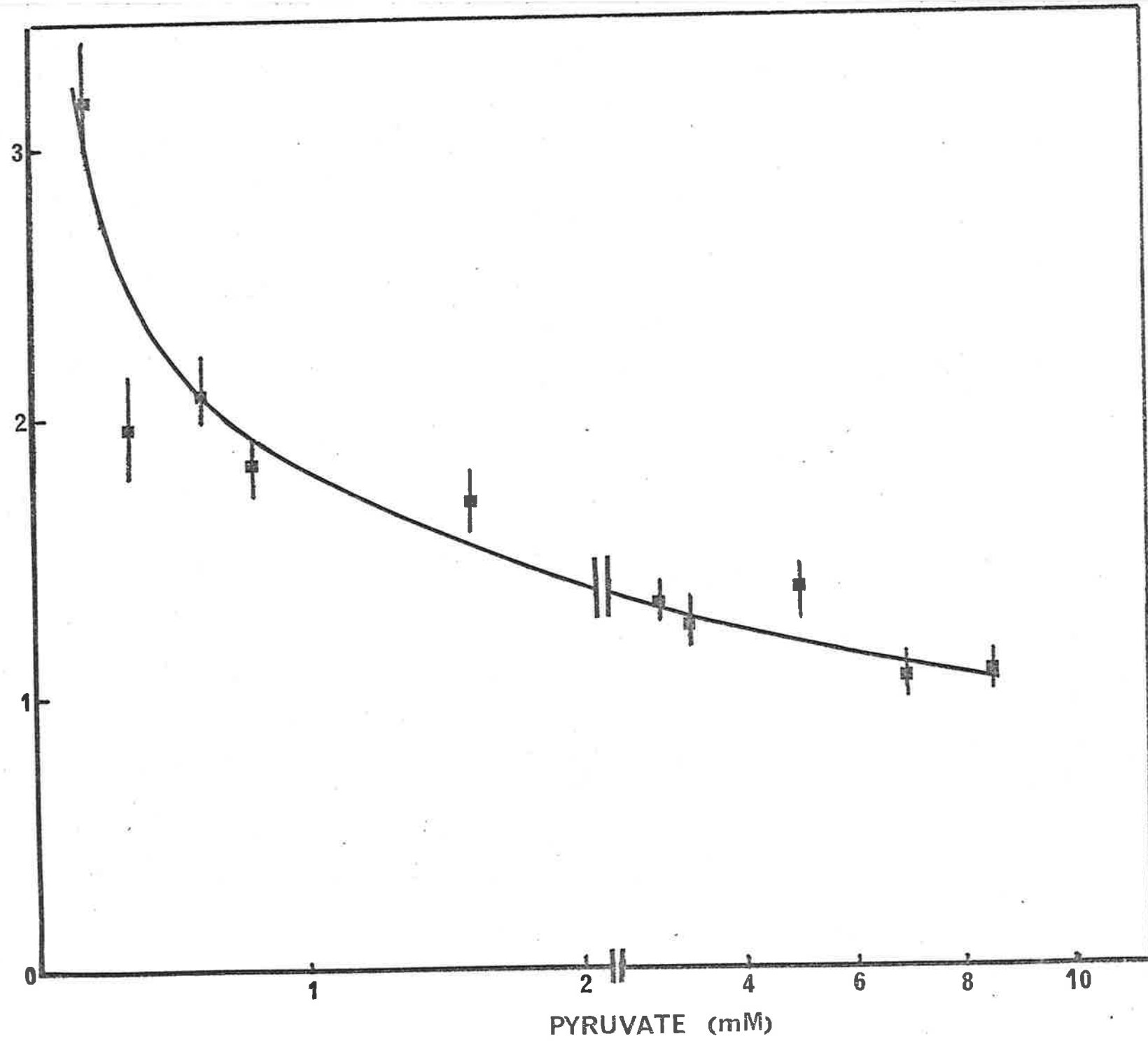


Fig. V.3. General scheme for the pyruvate carboxylase reaction.

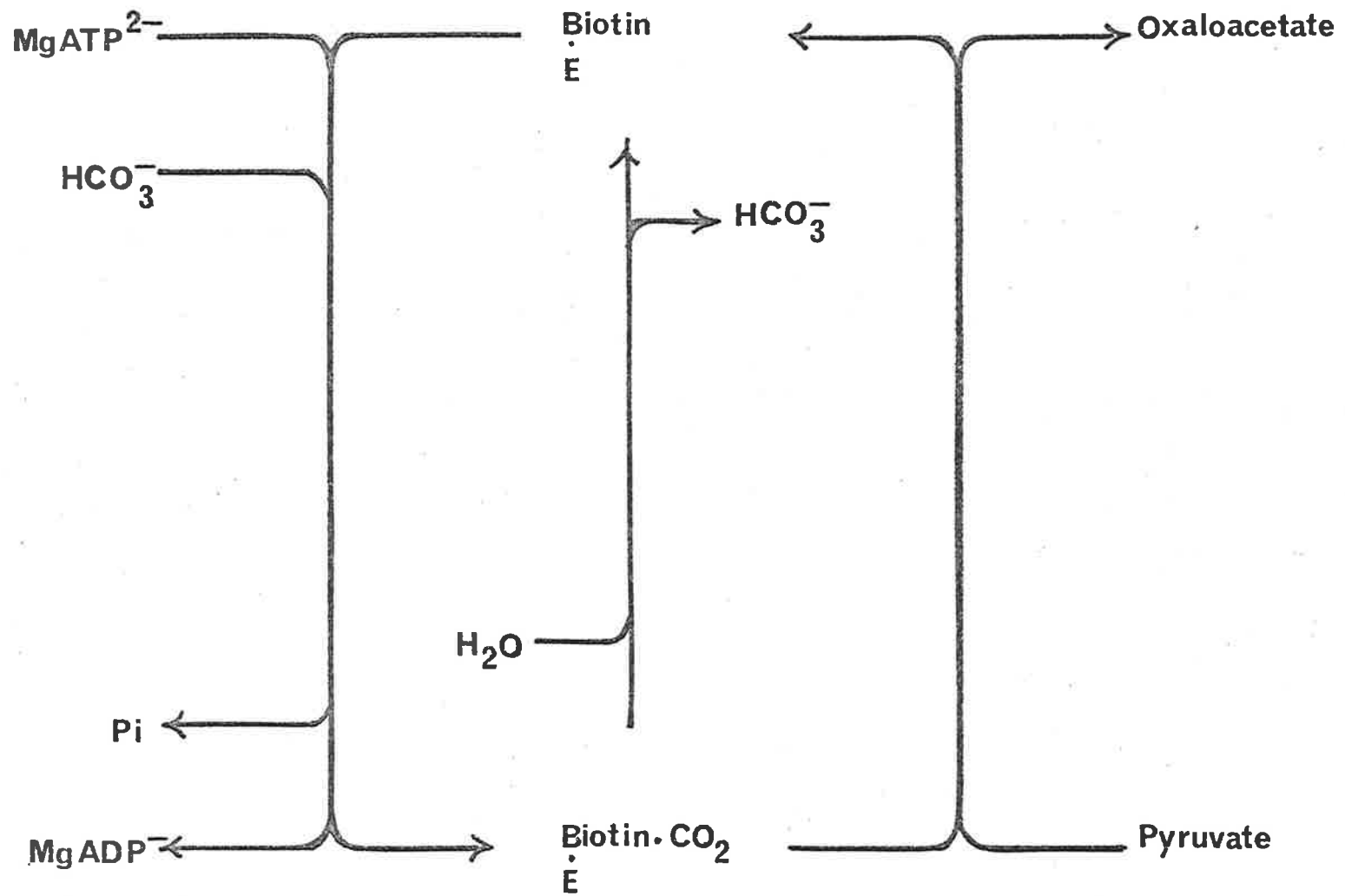


Table V.1. Hydrolysis of  $[\gamma\text{-}^{32}\text{P}]\text{MgATP}^{2-}$  by pyruvate carboxylase.

The rate of hydrolysis was determined by the procedure described in Section V.B.2.b.



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	nmole/min/unit
Complete system	3.66
-Mg <sup>2+</sup>	0
-acetyl CoA	1.24
Complete + avidin-treated enzyme	0

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Fig. V.4. Decay of the E-biotin CO<sub>2</sub> complex.

The E-biotin CO<sub>2</sub> complex was prepared as described in Section V.B.3, and held at 16.5°C. At the specified time intervals 0.25 ml samples were removed into 0.25 ml of 0.1 M tris Cl, pH 8.4, containing 5 μmoles of pyruvate. The radioactivity incorporated into oxaloacetate was determined by the procedure described in Section II. The rate of decay of the E-biotin CO<sub>2</sub> complex was calculated from the expression:  $k_1 = 0.693/t_{0.5}$ .

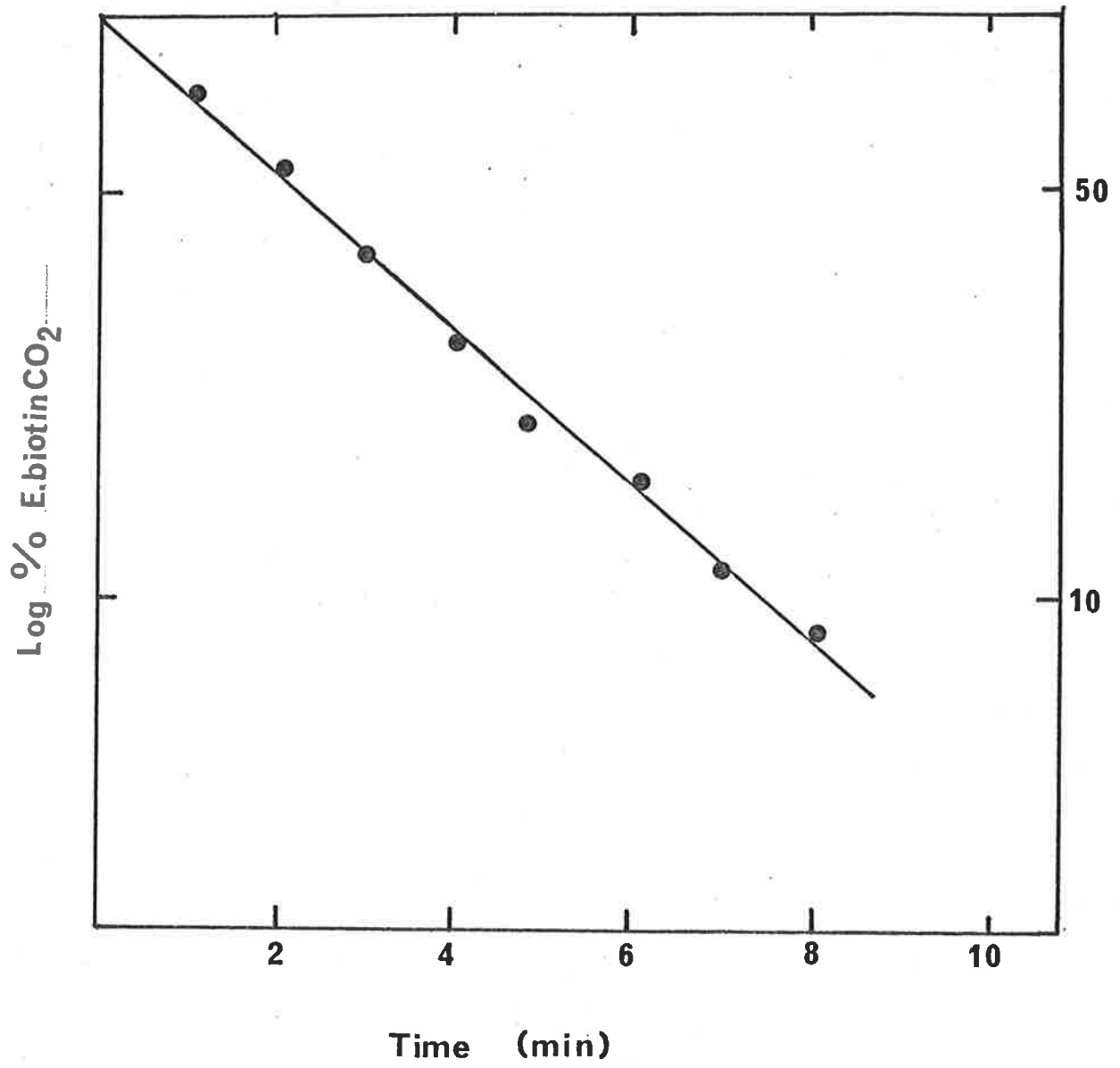


Fig. V.5. The kinetic behaviour and stoichiometry of the pyruvate/oxaloacetate isotopic exchange reaction.

The rate of the pyruvate/oxaloacetate concentration, at the indicated initial pyruvate concentrations, was determined by the procedure described in Section II. B.6.

Inset: The change in pyruvate concentration was obtained by deducting the concentration in the avidin controls from the concentration in the assay solutions at the end of the experiment.

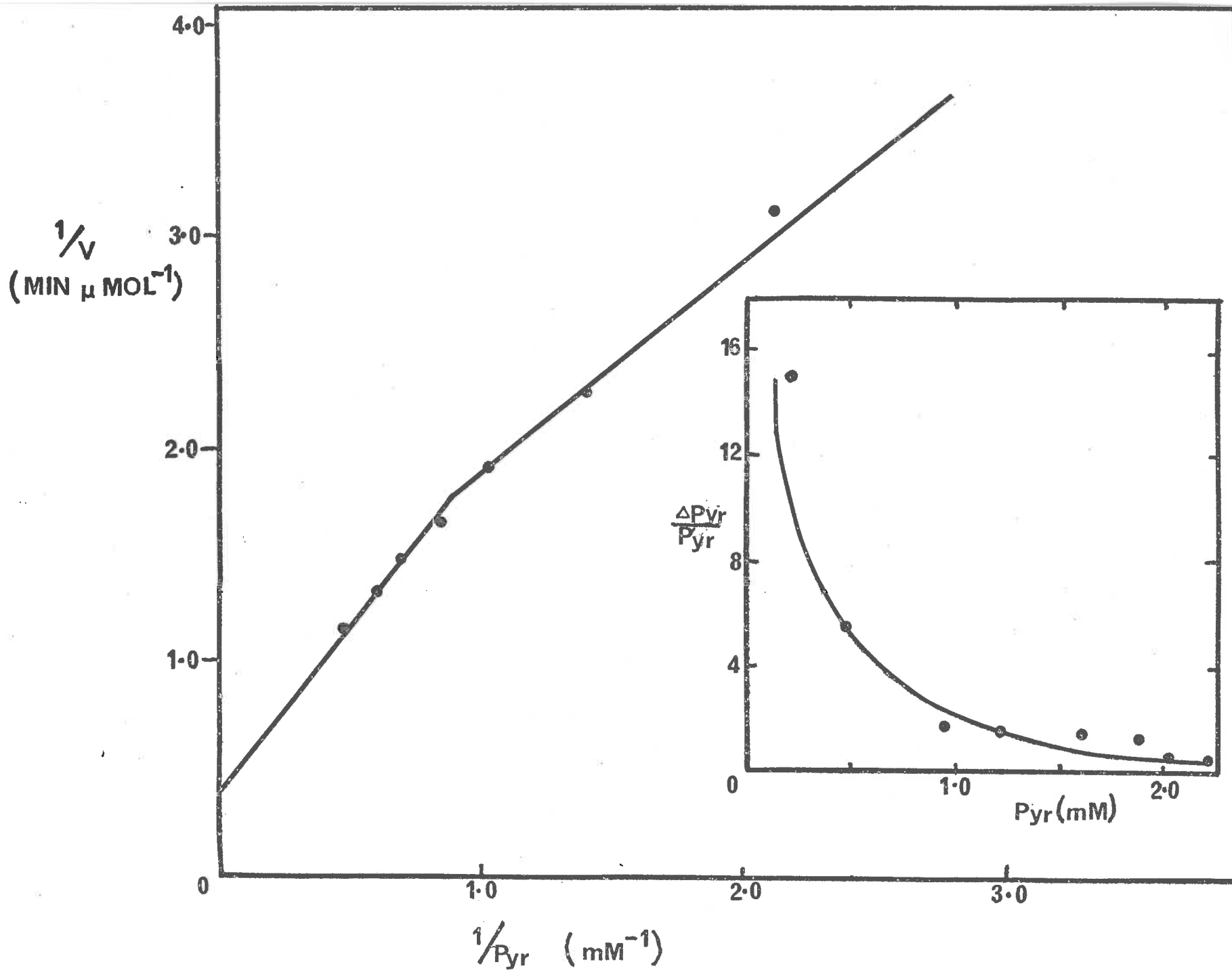


Fig. V.6. Postulated reaction pathway for the pyruvate trans-carboxylation reaction of pyruvate carboxylase.

It should be noted that E-biotin CO<sub>2</sub> and E-biotin...CO<sub>2</sub> represent different forms of the carboxylated enzyme.

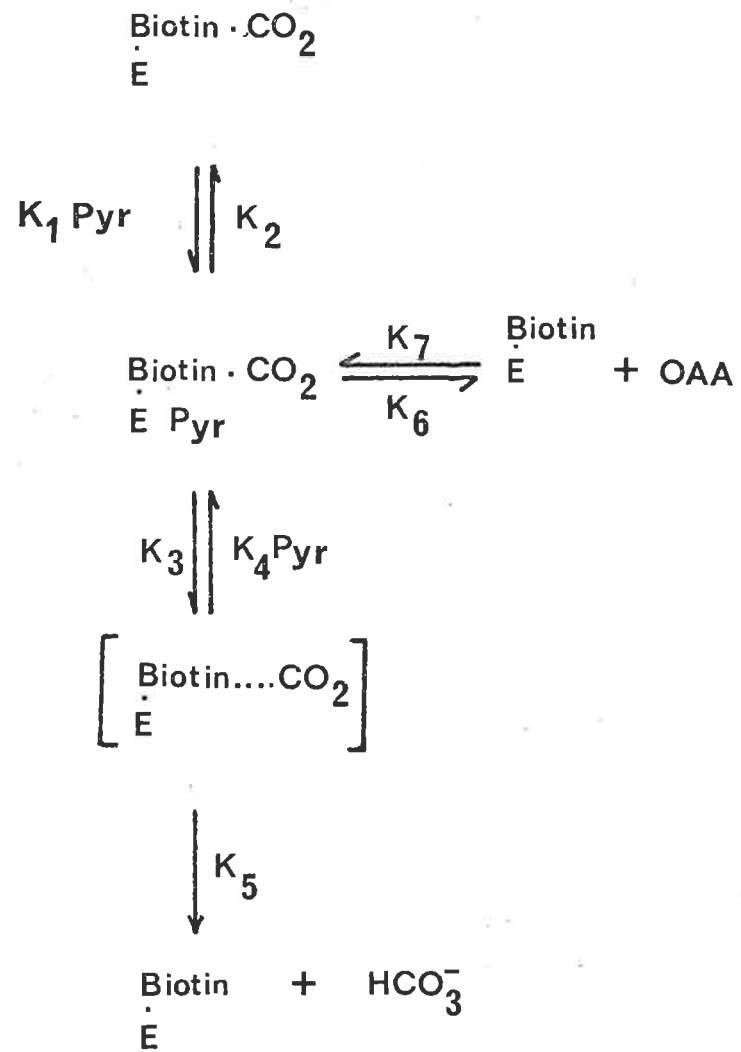


Fig. V.7 Comparison of the derived rate equation and kinetics data obtained with pyruvate as the variable substrate.

The experimental data: ( ● ) were those shown in Fig. V.1 and ( ■ ) were those shown in Fig. V.5. The rate constants used to generate the theoretical curves for the overall reaction ( ● ) and the pyruvate/oxaloacetate isotopic exchange reaction were:  $k_1 = 5.5 \times 10^5 \mu\text{mole}^{-1} \text{min}^{-1}$ ,  $k_2 = 2.5 \times 10^5 \mu\text{mole}^{-1} \text{min}^{-1}$ ,  $k_3 = 7.1 \times 10^3 \text{min}^{-1}$ ,  $k_4 = 2.1 \times 10^3 \mu\text{mole}^{-1} \text{min}^{-1}$ ,  $k_5 = 8 \times 10^2 \text{min}^{-1}$ , and  $k_6 = 4 \times 10^3 \text{min}^{-1}$ .



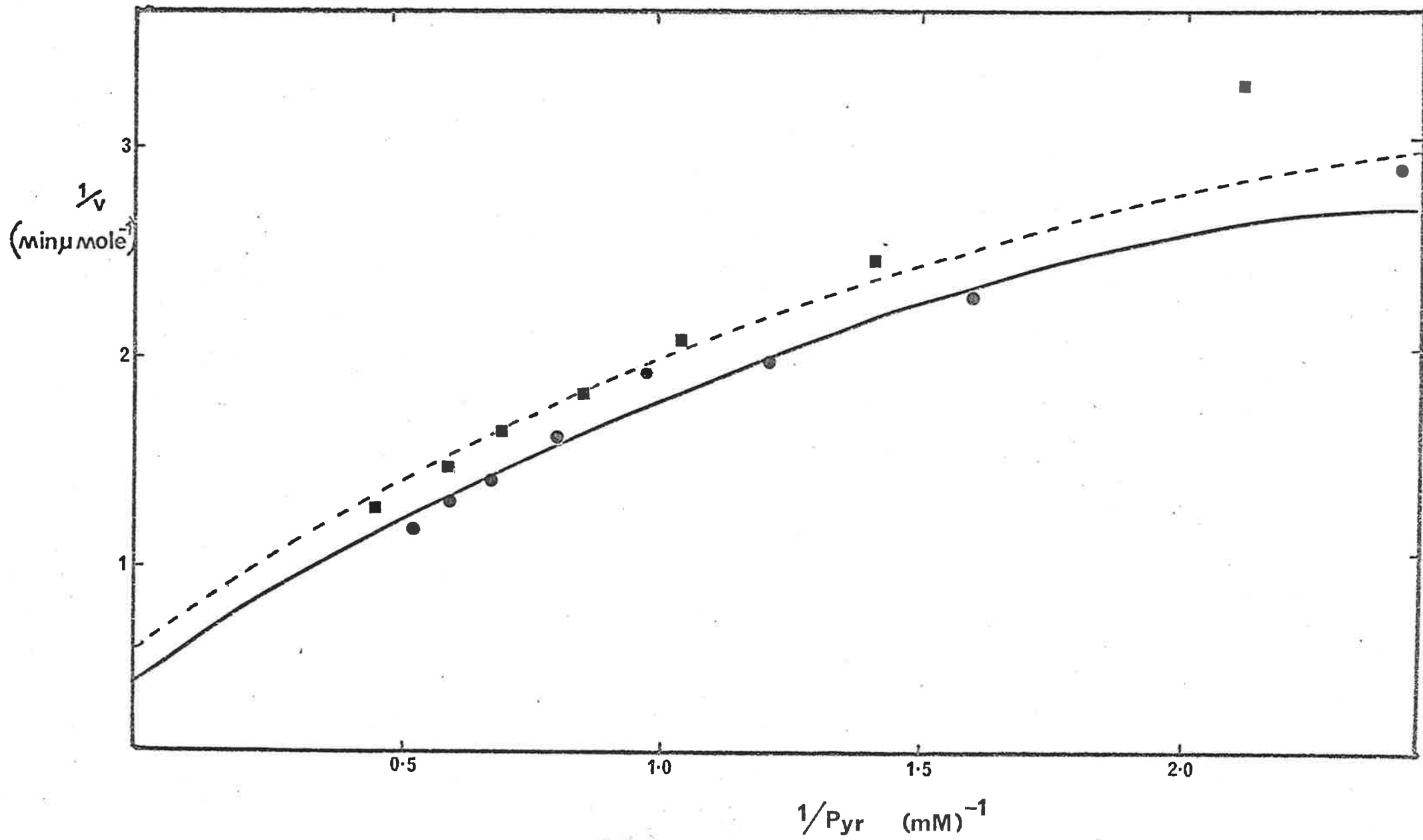


Fig. V.8

Theoretical curves generated from equation V-4 showing the effect of decreasing the value of  $k_5$ , while  $k_1$ ,  $k_2$ ,  $k_3$ ,  $k_4$ , and  $k_6$  were held constant at the values used in Fig. V.7. The values of  $k_5$  used were:  $0.0 \text{ min}^{-1}$ , (  $\blacktriangle$  );  $2 \times 10^2 \text{ min}^{-1}$ , (  $\square$  );  $5 \times 10^2 \text{ min}^{-1}$ , (  $\bullet$  ); and  $8 \times 10^2 \text{ min}^{-1}$ , (  $\nabla$  ).

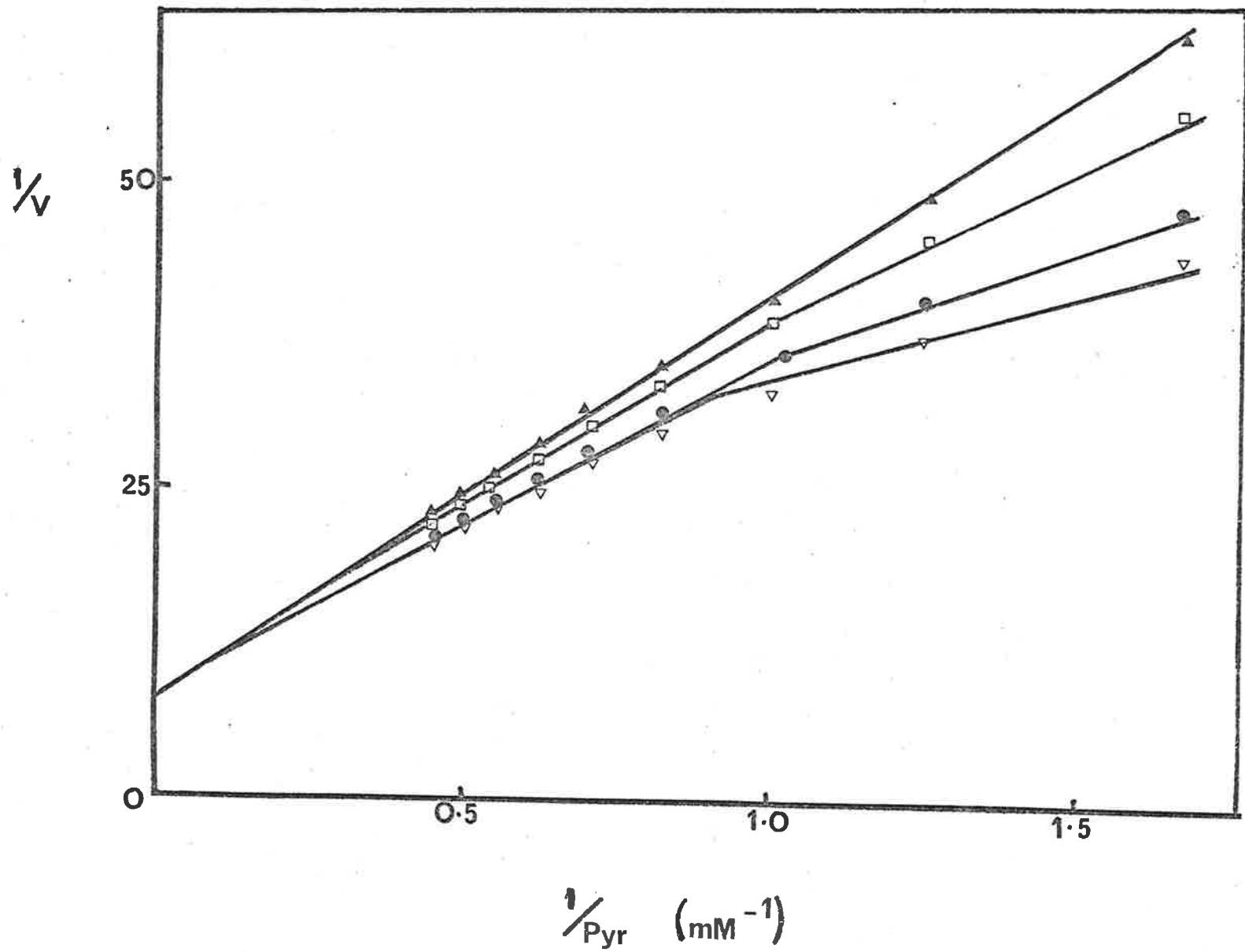


Table V.2. Alternative keto-acid kinetic constants.

The dependence of carboxylated keto-acid product synthesis was determined as described in Section V.B.2.a, using 0.05 units of pyruvate carboxylase, specific activity 12 U/mg per assay. The  $\text{MgATP}^{2-}$  concentration range was 0.02 mM to 1.8 mM, while the keto-acid and bicarbonate concentrations were 10 mM. The data was plotted in double reciprocal form, and the slopes and intercepts of the lines obtained by a least mean squares analysis. The data shown represents the mean values obtained from duplicate experiments. The slope ratio represents the slope of the line relative to that using pyruvate as the keto-acid substrate.

Keto-acid substrate	Slope	Slope ratio	V(%)
pyruvate	0.035 ± 0.0004	1.00	100
2-ketobutyrate	0.076 ± 0.004*	2.17 ± 0.14	4
3-fluoropyruvate	0.350 ± 0.014**	10.3 ± 0.5	5

By analysis of covariance (Snedcor and Cochran, 1967) the slopes for the alternate substrates are significantly different from that for pyruvate.

\*  $F_{1,31} = 6.4$ ,  $P < 0.05$

\*\*  $F_{1,32} = 383.9$ ,  $P < 0.001$

Fig. V.9.A Reciprocal plots of orthophosphate release as a function of varying  $\text{MgATP}^{2-}$  concentrations at several fixed levels of pyruvate.

Pyruvate carboxylase (specific activity 12 U/mg, 0.06 units) was assayed as described in Section V.B.2.a. The specific activity of the  $[\gamma\text{-}^{32}\text{P}]\text{ATP}$  was  $3.5 \times 10^3$  cpm/nmole. The pyruvate concentrations used were: 0.5 mM, ( ● ); 0.8 mM, ( ○ ); 1.0 mM, ( □ ); 5.0 mM, ( ■ ); 10.0 mM, ( ▲ ); and 20.0 mM, ( △ ).

Fig. V.9.B. Reciprocal plots of orthophosphate release as a function of varying  $\text{HCO}_3^-$  concentrations at several fixed levels of pyruvate.

Pyruvate carboxylase, (specific activity 12 U/mg, 0.05 units) was assayed as described in Section V.B.2.a. The  $[\gamma\text{-}^{32}\text{P}]\text{MgATP}^{2-}$  concentration was 1 mM ( $1.1 \times 10^3$  cpm/nmole). The pyruvate concentrations used were: 0.5 mM, ( ● ); 0.8 mM, ( ○ ); 1.0 mM, ( □ ); 5.0 mM, ( ■ ); and 20 mM, ( ▼ ).

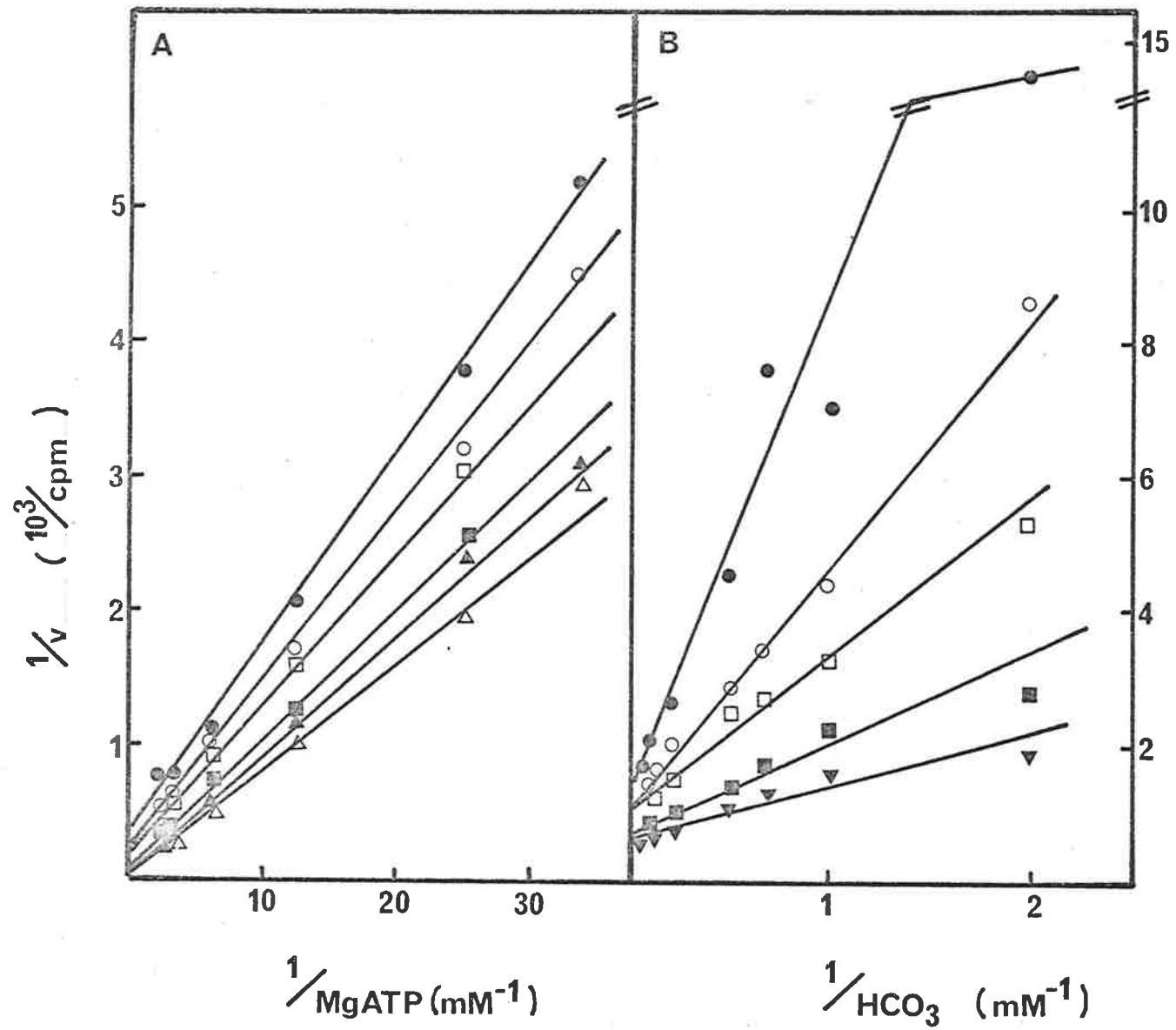




Fig. V.10 Reciprocal plots of orthophosphate release as a function of varying pyruvate concentrations, at various fixed levels of  $\text{MgATP}^{2-}$ .

The data shown are some of those of Fig. V.9.A, plotted as a function of pyruvate instead of  $\text{MgATP}^{2-}$ . The  $\text{MgATP}$  concentrations shown were: 0.03 mM, ( ■ ); 0.04 mM, ( □ ); 0.08 mM, ( ● ), and 0.40 mM, ( ○ ).

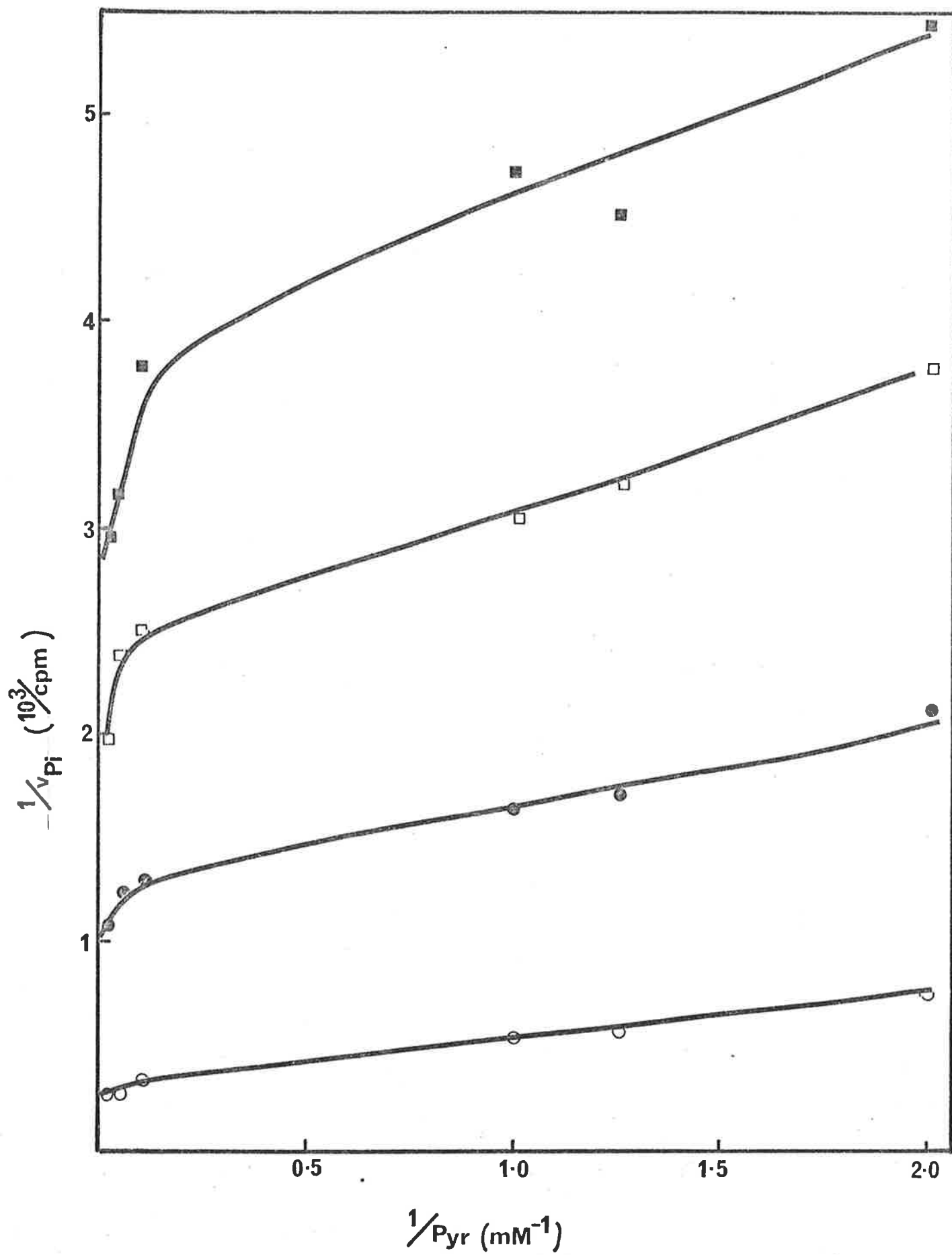


Fig. V.11. Reciprocal plots of orthophosphate release as a function of pyruvate concentration.

Pyruvate carboxylase (specific activity 12 U/mg, 0.065 units), was assayed as described in Section V.B.2.a. The  $[\gamma\text{-}^{32}\text{P}]\text{MgATP}$  concentration was 1 mM ( $1.2 \times 10^3$  cpm/nmole).

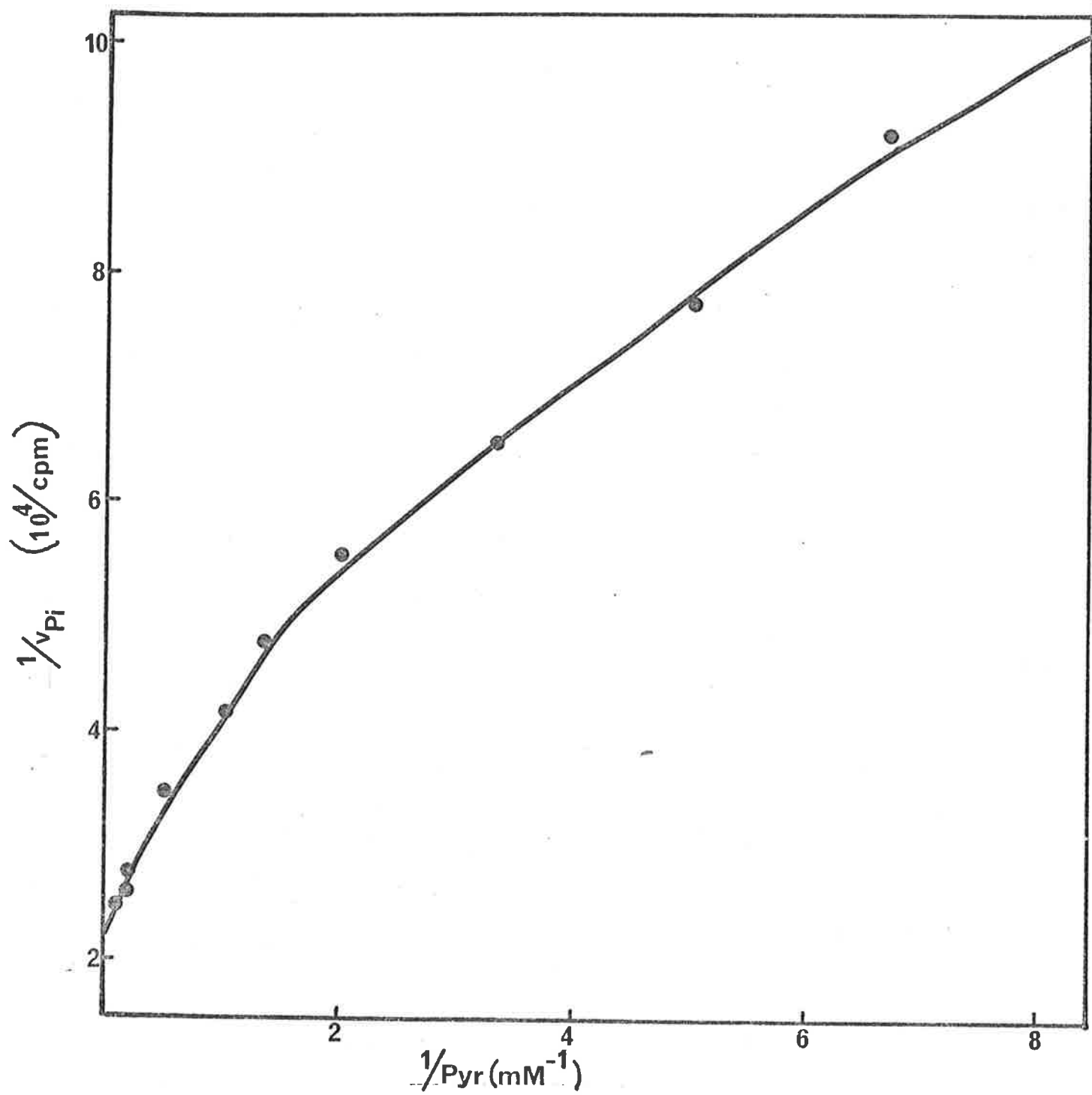


Fig. V.12. Postulated reaction pathway for the pyruvate carboxylase catalysed reaction. I.

$ECO_2$  and  $E..CO_2$  represent different forms of the carboxybiotin enzyme complex.

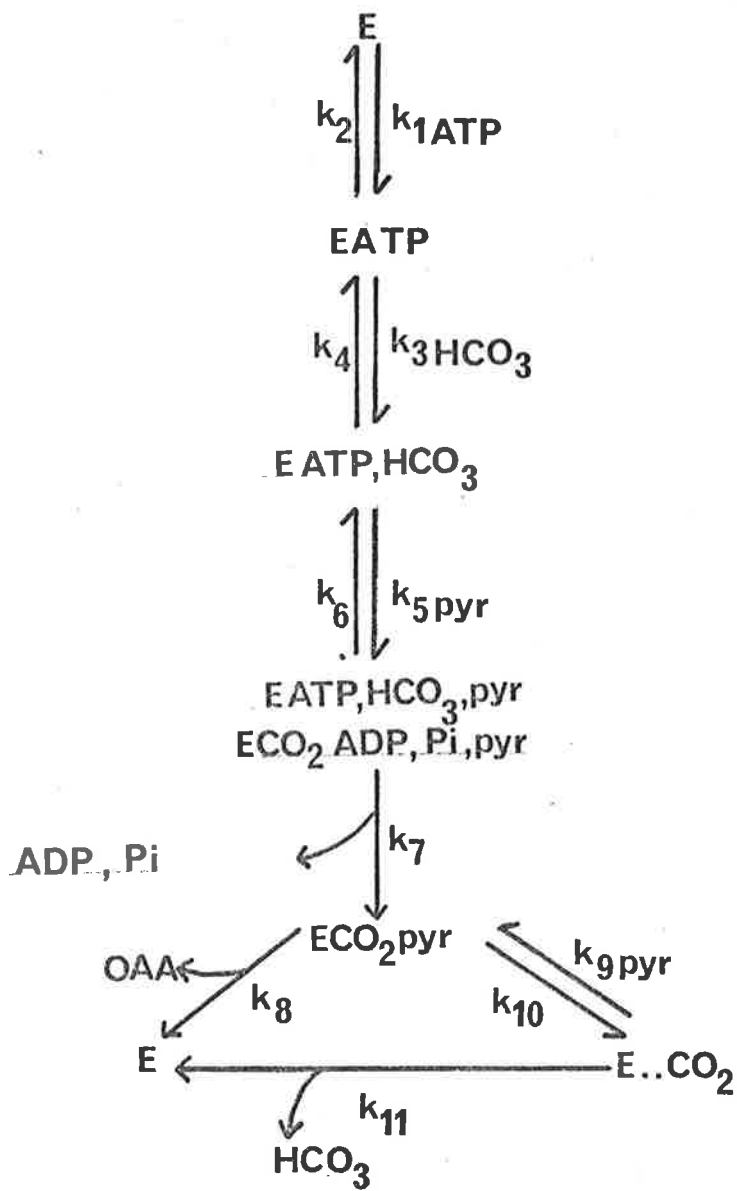


Fig. V.13. Secondary plots of the data shown in Fig. V.9.A.

The data were analysed using the Fortran program HYPER  
(Cleland, 1963a).

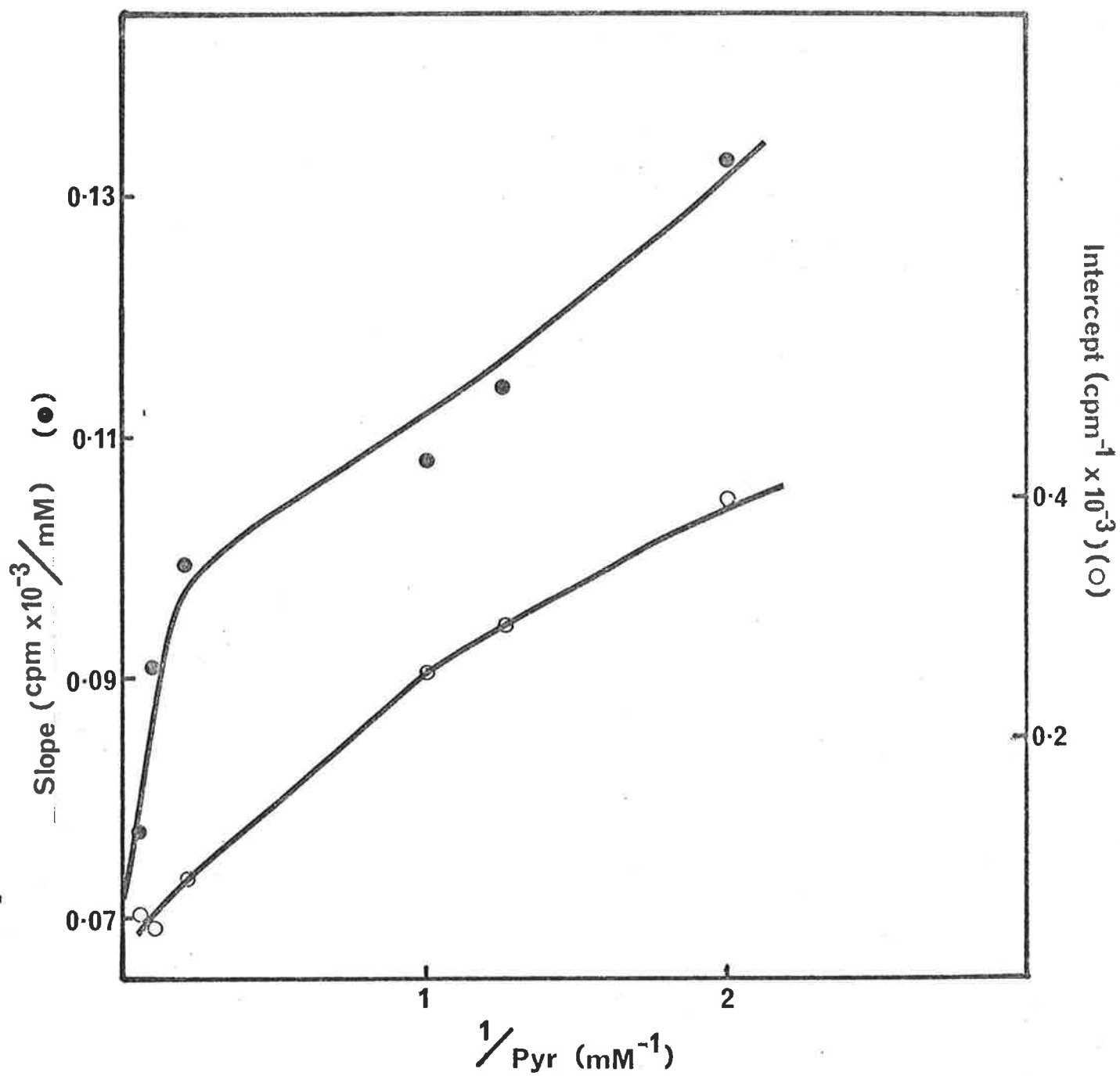




Fig. V.14. Secondary plots of the data shown in Fig. V.9.B.

The data shown were analysed using the Fortran program HYPER  
(Cleland, 1963a).

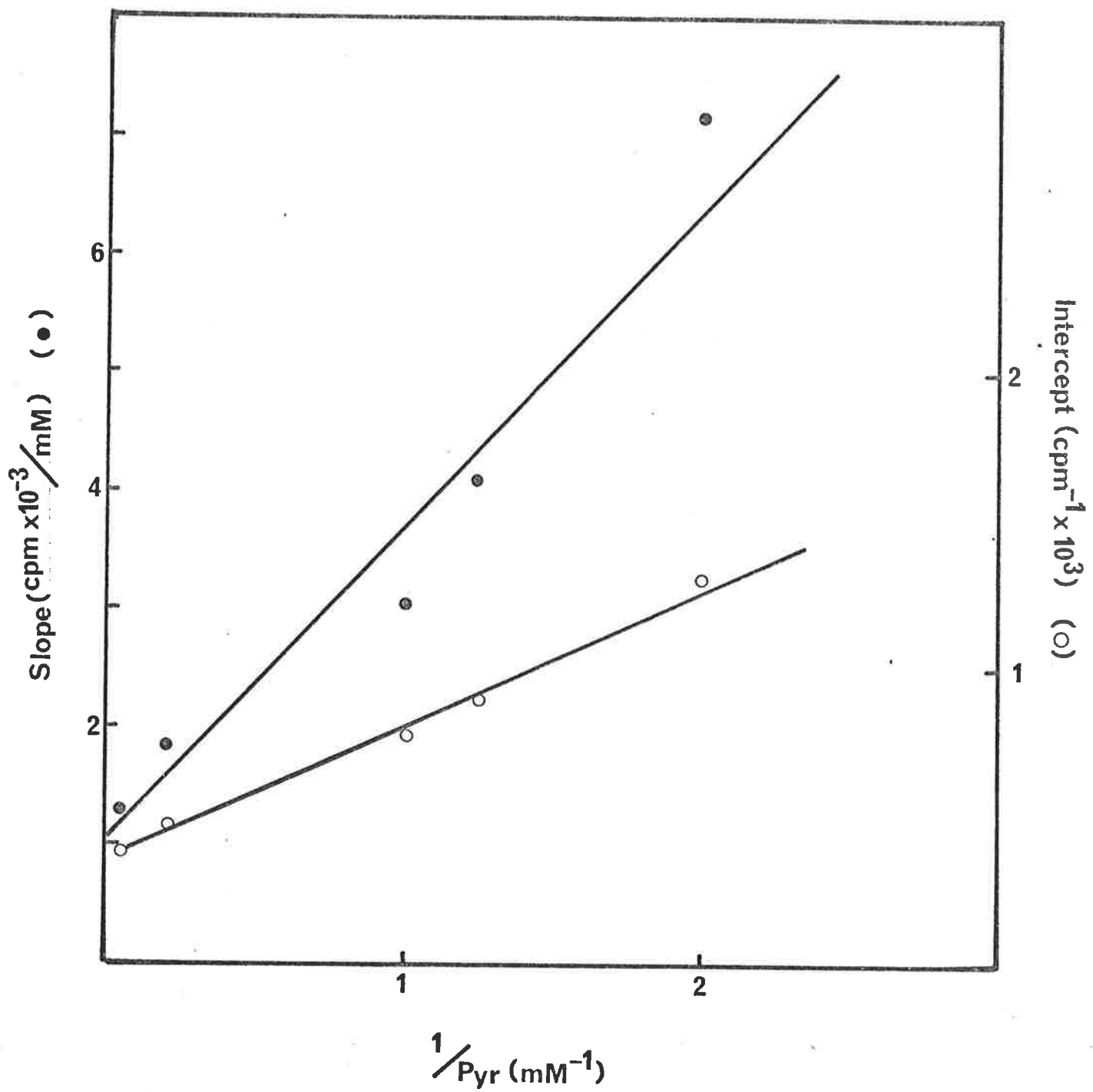


Fig. V.15. Postulated reaction pathway for the pyruvate carboxylase reaction. II.

$ECO_2$  and  $E..CO_2$  represent different forms of the carboxybiotin enzyme complex.

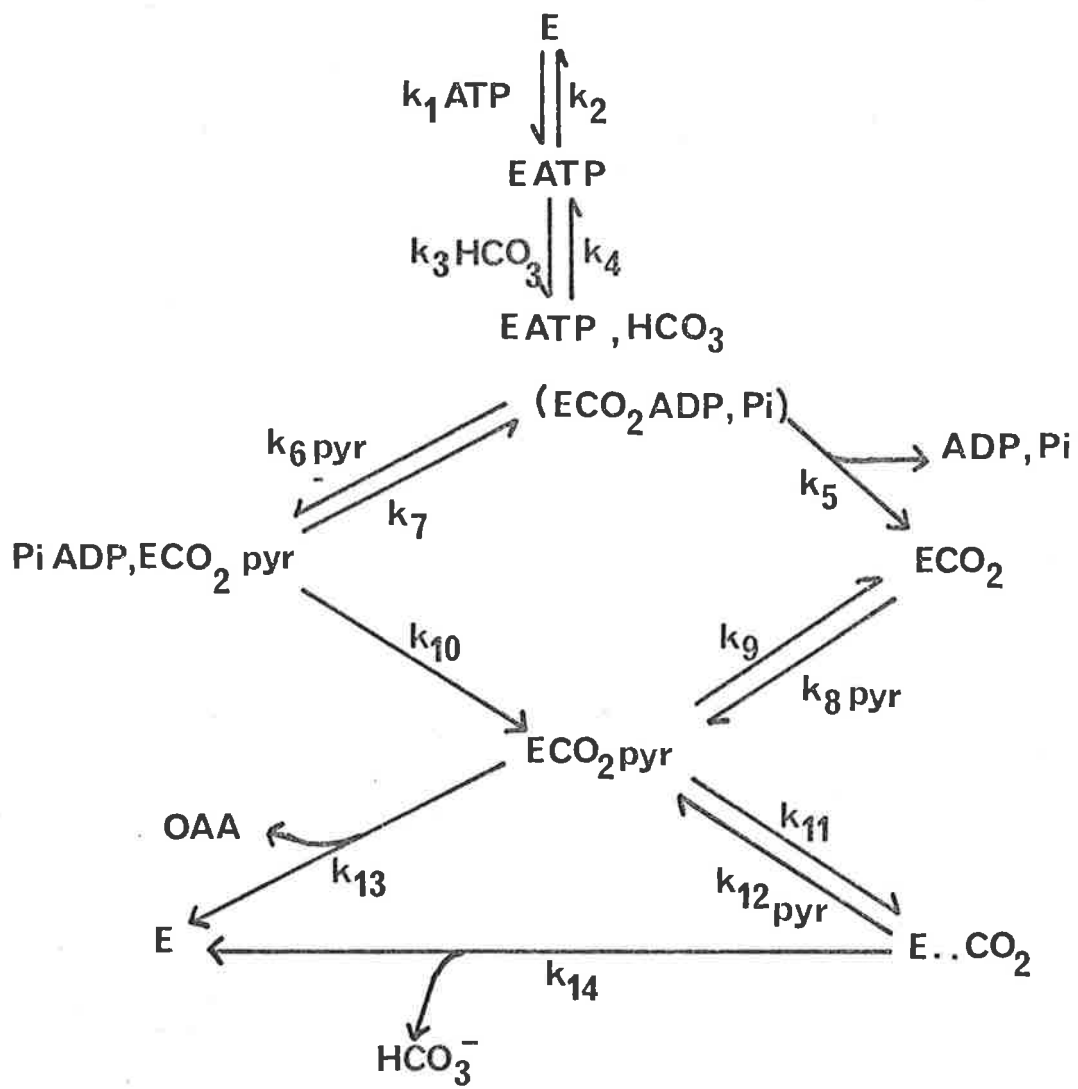


Fig. V.16. Postulated reaction pathway for deoxythymidine kinase  
and hexokinase. I.

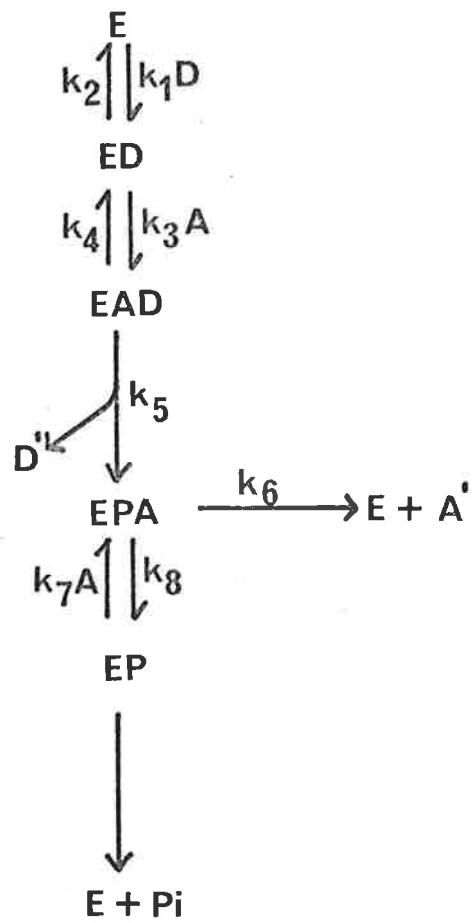
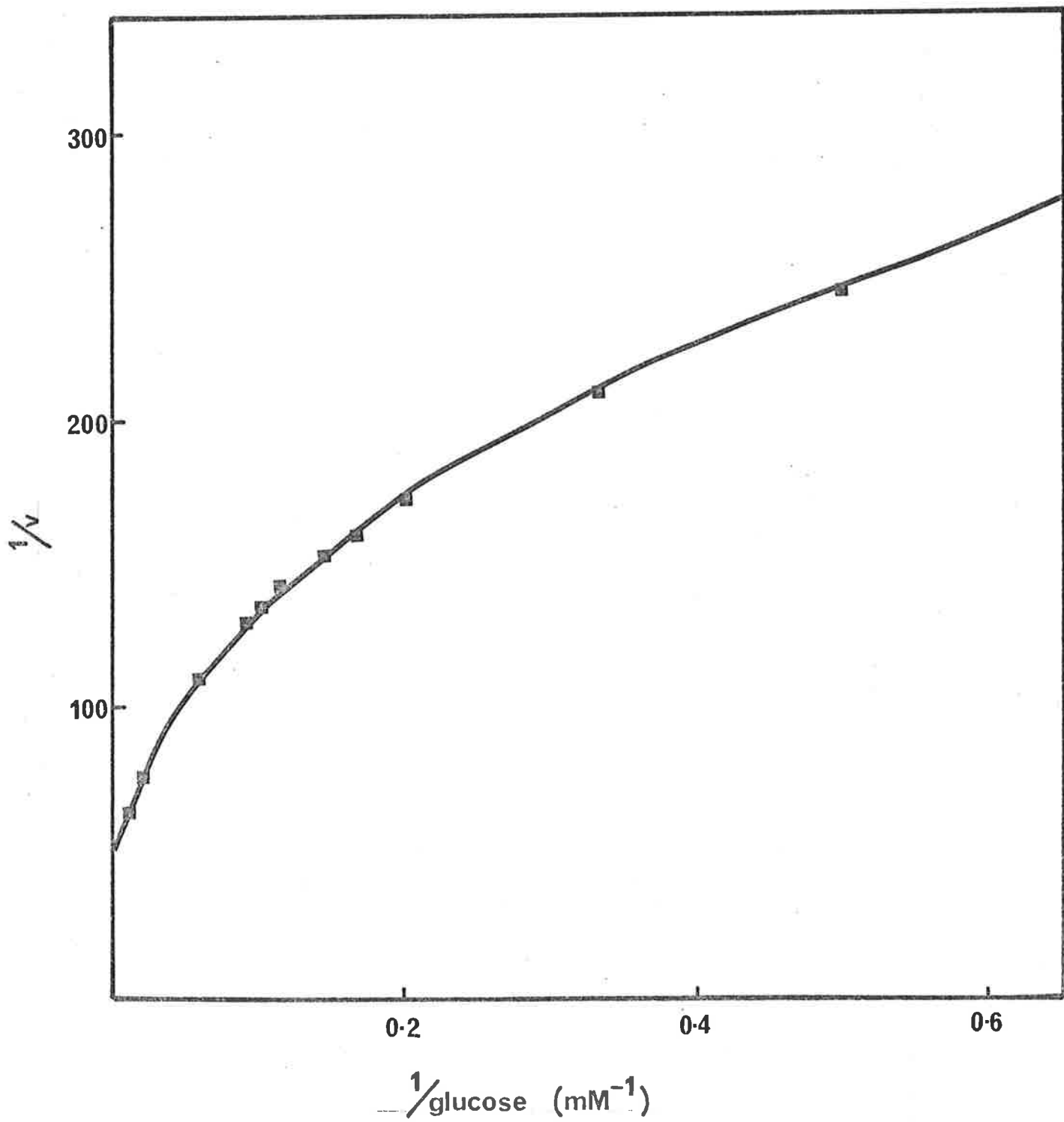


Fig. V.17. Postulated reaction pathway for deoxythymidine kinase and hexokinase. II.

The values of the rate constants used were:  $k_1 = 1 \times 10^3 \text{ mM}^{-1} \text{ min}^{-1}$ ,  $k_2 = 0.5 \times 10^3 \text{ min}^{-1}$ ,  $k_3 = 1 \times 10^3 \text{ mM}^{-1} \text{ min}^{-1}$ ,  $k_4 = 0.5 \times 10^3 \text{ mM}^{-1} \text{ min}^{-1}$ ,  $k_5 = 1 \times 10^3 \text{ min}^{-1}$ ,  $k_6 = 0.2 \times 10^3 \text{ min}^{-1}$ ,  $k_7 = 1 \times 10^2 \text{ mM}^{-1} \text{ min}^{-1}$ ,  $k_8 = 3 \times 10^3 \text{ min}^{-1}$ ,  $k_9 = 1 \times 10^3 \text{ min}^{-1}$ , while the concentration of the donor substrate was set at 10 mM.





CHAPTER VI

THE INTERACTION OF ACETYL CoA  
WITH PYRUVATE CARBOXYLASE

VI.A INTRODUCTION

Since the discovery of pyruvate carboxylase in 1960 (Utter and Keech, 1960) the locus of action, and the manner by which acetyl CoA exerts its activating effect on the enzymic reaction have been the subject of much speculation. However, very little unequivocal evidence has been obtained to provide a clear understanding of its role in the reaction mechanism.

Since the first reports of a sigmoidal reaction velocity response to increasing concentrations of acetyl CoA (Barritt et al., 1966; Scrutton and Utter, 1967) it has been assumed that the binding of the ligand is a homotropic co-operative process. The lack of rigour implicit in extracting information on the binding of acetyl CoA from its effects on the reaction velocity has already been discussed (Section I.C). Indeed, some experimental evidence suggests that there is no co-operativity of binding of acetyl CoA. The observation by Nakashima et al., (1975) that active monomers of rat liver pyruvate carboxylase exhibit a sigmoidal shaped velocity profile, together with the report of Frey and Utter (1977) that only 3 to 4 molecules of acetyl CoA bind to the tetrameric form of chicken liver pyruvate carboxylase suggest that homotropic co-operativity is a most unlikely explanation for the non-classical acetyl CoA kinetic behaviour.

A similar conclusion may be drawn from the report of Griffiths (1977) on the fit of calculated curves of pyruvate carboxylase activity to the data of Barritt et al., (1976) showing the in vivo dependence of reaction velocity on intra-mitochondrial acetyl CoA concentration. The best fit was obtained when a Hill coefficient of unity (indicating lack of co-operativity) was used in the expression for acetyl CoA saturation.

In addition, it has recently been shown in this laboratory, that there is a hyperbolic relationship between the rate of the ATP/P<sub>i</sub> isotopic

exchange reaction and acetyl CoA concentration, for sheep liver pyruvate carboxylase (P.R. Clements, unpublished results).

The only report of direct evidence for co-operativity of binding of acetyl CoA to pyruvate carboxylase is that of Frey and Utter (1977). Using the rapid flow dialysis method of Colowick and Womack (1969), these workers obtained data they interpreted as showing that the binding of acetyl CoA to the chicken enzyme was a co-operative process characterised by a dissociation constant of 13.9  $\mu\text{M}$  and a Hill coefficient of 1.9. A number of features of this interpretation are worthy of comment.

Firstly, the binding dissociation constant of 13.9  $\mu\text{M}$  was not significantly different from the activation constant of 13.3  $\mu\text{M}$  for the catalytic reaction measured under the same buffer conditions. In view of the effect of  $\text{Mg}^{2+}$  in lowering the activation constant for acetyl CoA in the catalytic reaction reported by Warren and Tipton (1974d), and Barden and Scrutton (1974), it is surprising that the binding dissociation constant, determined in the absence of  $\text{Mg}^{2+}$ , is the same as the activation constant obtained for the catalytic reaction, where the  $\text{Mg}^{2+}$  concentration was 4 mM (Scrutton et al., 1969).

A more serious problem is the extent of deacylation of acetyl CoA occurring during a flow dialysis experiment. If significant deacylation of acetyl CoA occurred during an experiment, then not only would the [ $^{14}\text{C}$ ] acetyl CoA concentration change, but the [ $^{14}\text{C}$ ]acetate formed would be expected to diffuse out of the flow cell at a rate nearly three times that of the remaining unbound [ $^{14}\text{C}$ ]acetyl CoA. Although Frey and Utter (1977) determined a rate of acetyl CoA deacylation, they did not report a  $K_m$  value for acetyl CoA in this reaction. The point here is that, if the  $K_m$  value is within the acetyl CoA concentration range used in the binding studies,

then the amount of [ $^{14}\text{C}$ ]acetate produced would be large enough to make an unequivocal interpretation of their data, in terms of the binding parameters of acetyl CoA, difficult to obtain. In order to resolve this uncertainty, the dependence of chicken pyruvate carboxylase deacylase activity on acetyl CoA concentration, using the assay conditions of Frey and Utter (1977), has been investigated, and the results of this investigation are presented below.

Another feature of the study of Frey and Utter worthy of comment is their report of only two  $\text{MgATP}^{2-}$  binding sites per pyruvate carboxylase tetramer. In view of the endogenous bicarbonate present in their buffer solutions, it is likely that at the high enzyme concentrations used, significant hydrolysis of the  $\text{MgATP}^{2-}$  could occur during a binding study experiment. Until a more detailed investigation of  $\text{MgATP}^{2-}$  binding is reported, it would seem reasonable to view the preliminary results obtained by Frey and Utter (1977) with some caution.

Because of the uncertainty as to the mode of interaction of acetyl CoA with pyruvate carboxylase, new experimental approaches to the problem were investigated. The results of such investigations are presented in this chapter, and show that the binding of acetyl CoA to pyruvate carboxylase is a non-cooperative process.

## VI.B MATERIALS AND METHODS

### VI.B.1. Assay Methods

#### VI.B.1.a. Overall reaction

When the rate of oxaloacetate synthesis only was measured, the assay procedure described in Section II.B.4 was used; with the altered substrate concentrations indicated in legends to figures and tables. When both oxaloacetate and ortho-phosphate were determined the procedure described in Section V.B.2.a. was used, except the assay mix contained, (in  $\mu\text{moles}$ )

in a final volume of 0.5 ml; tris Cl, pH 8.4, (45);  $MgCl_2$ , (4.7);  $NH_4Cl$ , (88); [ $^{14}C$ ]bicarbonate, sodium salt, (35), ( $7.8-9.3 \times 10^5$  cpm/ $\mu$ mole); [ $\gamma$ - $^{32}P$ ]ATP, (1.23) ( $0.5-1.0 \times 10^5$  cpm/ $\mu$ mole), pyruvate as indicated, and 2 to 2.5 units of pyruvate carboxylase.

#### VI.B.1.b. Acetyl CoA deacylase assay procedure

This assay procedure entails the determination of the [ $1-^{14}C$ ]acetate produced on deacylation of [ $1-^{14}C$ ]acetyl CoA. The unreacted acetyl CoA is removed from solution by adsorption to activated charcoal. The assay solution contained, (in  $\mu$ mole), in a final volume of 0.4 ml: tris Cl, pH 8.4, (40); ATP, (1);  $MgCl_2$ , (2.5); pyruvate, sodium salt, (4); [ $1-^{14}C$ ]acetyl CoA,  $5.6 \times 10^5$  cpm/ $\mu$ mole, as indicated, and 2-3 units of pyruvate carboxylase. Assay tubes were equilibrated at 30°C and the reaction was initiated by addition of enzyme. After a twenty minute incubation the reaction was stopped by addition of 0.05 ml 6M HCl. Carrier sodium acetate (15  $\mu$ mole) was added, followed by the addition of 0.3 ml of an activated charcoal suspension (100 mg/ml in 1M HCl). The resulting suspension was allowed to stand for five minutes and the charcoal was then removed by centrifuging. Samples of the supernatant were taken and their radioactivity determined using the Triton scintillant described in Section II.B.4. The extent of non-specific acetyl CoA deacylation was determined using control assays in which pyruvate carboxylase was omitted. Under these conditions the reaction was linear with time for at least twenty minutes. The agreement of duplicate assays using this procedure was usually better than 5%. The pyruvate carboxylase deacylase activity of chicken enzyme was determined in the same way, except Buffer I of Frey and Utter (1977) was used.

### VI.B.2. Analysis of data

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

### VI.B.3. Reacting enzyme sedimentation

Reacting enzyme sedimentation was carried out at 20°C using a Beckman Model E centrifuge. The experiments were carried out at either  $6.0 \times 10^4$  or  $5.2 \times 10^4$  rpm, using an Ft-1 rotor equipped with Vinograd single sector cells with sapphire windows. The assay mix in the cell, final volume approximately 3.4 ml, contained 150 mM tris Cl, pH 8.4, 2.5 mM ATP, 8.0 mM  $MgCl_2$ , 10 mM Na pyruvate, 10 mM  $K^+$  bicarbonate, 0.2 mM NADH, 200  $\mu$ M acetyl CoA, and 0.5 units/ml malate dehydrogenase. A capillary tube containing 0.001-0.002 units of pyruvate carboxylase in the indicated buffer was inserted into the cell. At the start of the run the enzyme layered on top of the assay solution. The position of the enzyme band during sedimentation was detected by scanning the cell at 359 nm at the time intervals indicated in legends to the figures. The  $S_{20}$  value was determined from the slopes of plots of  $\log_e$  (radial distance) against time. The value of  $S_{20,w}$  was obtained using a value of 0.733 for the partial specific volume of pyruvate carboxylase (Bais, 1974).

### VI.B.4. Preparation of [ $^{14}C$ ]acetylated pyruvate carboxylase

The assay mix contained, in  $\mu$ moles, in a final volume of 0.5 mls;

tris Cl, pH 8.4, 50; [ $^{14}\text{C}$ ]acetyl CoA 0.1 ( $9.75 \times 10^5$  cpm/ $\mu\text{mole}$ ), and pyruvate carboxylase, specific activity 20 U/mg, 8 units. Assay tubes were incubated at  $30^\circ\text{C}$  and the reaction initiated by addition of enzyme. After a 10 minute incubation the reaction mixture was either transferred to a dialysis bag and dialysed for 24 hours at  $4^\circ\text{C}$  against 0.1M tris Cl, pH 8.4, or the reaction was stopped by addition of 0.25 ml 20% ( $^{\text{W}}/\text{v}$ ) TCA. When the latter procedure was followed, 0.1 ml bovine serum albumin (10 mg/ml in 0.1M tris Cl, pH 8.4) was then added, and the reaction mix transferred to a Whatman GF/A glass fibre disk. The disk was then washed with 10% ( $^{\text{W}}/\text{v}$ ) TCA (50 ml), and ether (50 ml), after which its radioactivity was determined using the toluene scintillant. When the dialysis procedure was followed, samples of the dialysate were taken and their protein concentration and radioactivity determined. Control assays, in which pyruvate carboxylase was omitted, were used to measure non-specific [ $^{14}\text{C}$ ]acetate levels in both the dialysis and glass fibre disc procedures.

#### VI.B.5. Preparation of [ $^{14}\text{C}$ ]-thiocyanato pyruvate carboxylase.

The assay procedure described in Section VI.B.4 was followed, except unlabelled acetyl CoA was used. After the 10 minute incubation 2  $\mu\text{moles}$  of [ $^{14}\text{C}$ ]cyanide ( $\text{K}^+$  salt,  $1.85 \times 10^6$  cpm/ $\mu\text{mole}$ ) were added, and the mixture allowed to stand for 3 hours at  $20^\circ\text{C}$ . The reaction mixture was then dialysed against 0.1 M tris Cl, pH 8.4, for 24 hours at  $4^\circ\text{C}$ . Samples of the dialysate were then taken and their protein concentration and radioactivity determined. Control assays, in which acetyl CoA was omitted, were used to measure any non-specific reaction of cyanide with pyruvate carboxylase. This method is similar to that described by Wagner and Yount, (1975a,b).

VI.C. RESULTSVI.C.1. The binding of acetyl CoA to pyruvate carboxylase

In view of the difficulties and uncertainties associated with the interpretation of the direct binding study data discussed in Section VI.A, the binding of acetyl CoA to pyruvate carboxylase was measured using two indirect techniques: the protection afforded by acetyl CoA against covalent modification of the enzyme by TNBS, and the initial velocity kinetics of the pyruvate carboxylase catalysed acetyl CoA deacylase reaction.

VI.C.1.a. Protection against TNBS modification

In using this approach, use was made of the fact that the stimulatory response of acetyl CoA can be removed in several species of pyruvate carboxylase by prior treatment of the enzyme with TNBS (Ashman *et al.*, 1973; Scrutton and White, 1973). Furthermore, enzyme completely saturated with acetyl CoA is not inactivated by TNBS. These observations imply that the modification occurs at the acetyl CoA binding site, and that if the enzyme is treated with TNBS in the presence of acetyl CoA, then only that fraction of the enzyme without bound acetyl CoA is susceptible to inactivation.

Now, if the saturation of pyruvate carboxylase with acetyl CoA can be described by the empirical Hill equation, then equation VI-1 describes the dependence of the saturation ( $\bar{Y}$ ) on the acetyl CoA concentration.

$$\bar{Y} = \frac{1}{1 + \left(\frac{K_a}{A}\right)^{n_H}} \quad \text{VI-1}$$

where  $K_a$ ,  $A$  and  $n_H$  represent the dissociation constant, the acetyl CoA concentration, and the Hill  $n$  coefficient, respectively.



Given that

$$\bar{Y} = \frac{EA}{E_T} \dots\dots \quad \text{VI-2}$$

where EA and  $E_T$  represent the concentration of enzyme with bound acetyl CoA, and the total enzyme concentration respectively, insertion of equation VI-2 into VI-1, and re-arranging, gives:

$$E_F = \frac{E_T}{1 + \left(\frac{A}{K_a}\right)^{n_H}} \dots\dots \quad \text{VI-3}$$

where  $E_F$  represents the concentration of enzyme without bound acetyl CoA.

Now, for the TNBS inactivation,

$$K_{\text{inact}} = K_1 E_F \dots\dots \quad \text{VI-4}$$

where  $K_{\text{inact}}$  is the pseudo first order rate constant which may be derived from the expression  $\ln 2 / t_{0.5}$  where  $t_{0.5}$  is the time for inactivating 50% of the enzyme;  $K_1$  is a true first order rate constant, and  $E_F$  is the concentration of enzyme free of acetyl CoA. Inserting equation VI-4 into equation VI-3, and rearranging, we have;

$$\frac{K_{\text{inact}}^0}{K_{\text{inact}}^a} - 1 = A^{n_H} / K_a^{n_H} \dots\dots \quad \text{VI-5}$$

where  $K_{\text{inact}}^a$  and  $K_{\text{inact}}^0$  are the rate constants for the inactivation process in the presence and absence of acetyl CoA.

It follows that

$$\log \left( \frac{K_{\text{inact}}^0}{K_{\text{inact}}^a} - 1 \right) = n_H \log A - n_H \log K_a \quad \text{VI-6}$$

Therefore, the slope of the line obtained by plotting  $\log \left( \frac{K_{\text{inact}}^0}{K_{\text{inact}}^a} - 1 \right)$  as a function of  $\log A$  will be  $n_H$ , and the intercept on the ordinate will be  $-n_H \log K_a$ . Fig. VI.1 presents data obtained from experiments designed to measure the rate of inactivation of the enzyme by TNBS in the

presence of varying concentrations of acetyl CoA. Pyruvate and  $MgCl_2$  were present because it was hoped to obtain data for acetyl CoA binding under conditions similar to those of a normal pyruvate carboxylase assay.  $MgATP^{2-}$  and bicarbonate were omitted because of their stimulation of the acetyl CoA deacylase reaction of pyruvate carboxylase (Ashman, 1973). The rate constants obtained from Fig. VI-1 were used to construct the linear regression in the log-log plot (Fig. VI.2) according to equation VI-6. The slope of this line was  $1.07 \pm 0.11$  and the  $K_a$  value was  $13.8 \pm 1.4 \mu M$ . These results suggest that, under these conditions, acetyl CoA binds to pyruvate carboxylase in a non-cooperative manner, because the  $n_H$  value was not significantly different from one. The results obtained from similar experiments using rat liver pyruvate carboxylase showed a different mode of acetyl CoA binding; an  $n_H$  value of 2.4 and a  $K_a$  value of  $310 \mu M$  were obtained from TNBS modification data plotted in the form of Fig. VI.2 (Scrutton and White, 1973). However, in this case the modification was conducted in the absence of  $Mg^{2+}$ , which suggests the hypothesis that this cation alters the nature of acetyl CoA binding to the enzyme. This hypothesis has been tested for the sheep liver enzyme (Duc, 1976). Analysis of TNBS protection data, obtained in the absence of  $Mg^{2+}$ , using equation VI-6, yielded an  $n_H$  value of 2.0 and a  $K_a$  value of  $50 \mu M$ ;  $Mg^{2+}$  alone had no effect on the rate of inactivation by TNBS. Furthermore, when the effect of  $Mg^{2+}$  on the acetyl CoA activation of the overall reaction was investigated, it was found that the  $n_H$  value for acetyl CoA decreased from 2.28 to 1.57, as the free  $Mg^{2+}$  concentration increased from 0.01 mM to 1.0 mM. These observations are consistent with the hypothesis that  $Mg^{2+}$  alters the mode of binding of acetyl CoA to the enzyme. Although the interpretation of the direct binding data of Frey and Utter (1977) is difficult, the  $n_H$  value greater than one obtained from TNBS protection data in the absence of  $Mg^{2+}$

suggests that positive co-operativity of binding of acetyl CoA may have contributed to the shape of the curve obtained for the chicken enzyme, although the problem of acetyl CoA deacylation precludes any definite conclusion in this regard. In any case, it would appear that binding data, obtained directly or indirectly, in the absence of  $Mg^{2+}$ , is not necessarily relevant to understanding how acetyl CoA binds to pyruvate carboxylase under overall reaction assay conditions, where the level of free  $Mg^{2+}$  is 4-5 mM.

#### VI.C.1.b. Acetyl CoA deacylase kinetics

High specific activity preparations of pyruvate carboxylase isolated from either sheep (Ashman, 1973), or chicken mitochondria (Scrutton and Utter, 1967) catalyse the slow deacylation of acetyl CoA to CoASH and acetate. The rate of deacylation was found to be enhanced by substrates of the carboxylation reaction, which implies the deacylase activity is associated with pyruvate carboxylase, rather than a contaminating enzyme in the preparations used. Also, the observation by Frey and Utter (1977) of 3-4 acetyl CoA binding sites per tetrameric pyruvate carboxylase molecule suggests that deacylation occurs at the acetyl CoA activator site, rather than at an additional catalytic site. (Although the deacylation of acetyl CoA does not appear to allow accurate interpretation of the binding data at low concentrations of acetyl CoA, the value for the number of acetyl CoA binding sites was obtained from high acetyl CoA concentration data, where the deacylation would be proportionately less significant.)

Therefore, if acetyl CoA binding to pyruvate carboxylase under carboxylation assay conditions is a classical Michaelis-Menten process, a hyperbolic response of the rate of deacylation to acetyl CoA concentration would be expected. This is the case even though pyruvate carboxylase is

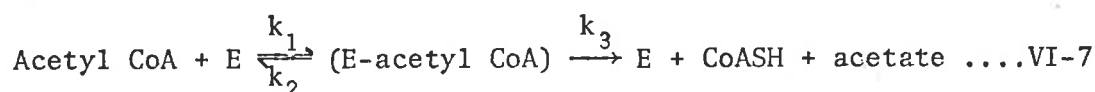
a tetrameric enzyme, with 4 acetyl CoA binding sites per tetramer, because, as has been shown by Childs and Bardsley (1975) a sigmoid curve can not result from one single substrate enzyme with multiple independent active sites.

Fig. VI.3 presents data from experiments designed to measure the rate of deacylation as a function of acetyl CoA concentration.  $\text{MgATP}^{2-}$ ,  $\text{Mg}^{2+}$ , and pyruvate were included in order to obtain a measure of the binding of acetyl CoA under conditions approaching those of the carboxylation assay. Bicarbonate was omitted, because at the high enzyme concentration used, rapid depletion of the MgATP, pyruvate and bicarbonate would have occurred, with rapid formation of the products of the carboxylation reaction. The slope of the line shown in Fig. VI.3 was  $0.147 \pm 0.004$  and the vertical intercept  $0.36 \pm 0.03$ , yielding a  $K_m$  value of  $40.9 \pm 4.7 \mu\text{M}$  for acetyl CoA deacylation under these conditions. The relatively low standard errors of the parameters of the straight line suggest that there is a hyperbolic response of the rate of deacylation to acetyl CoA concentration, which in turn implies the binding of acetyl CoA to the enzyme under these conditions is a Michaelis-Menten process.

In order to assess the effect of acetyl CoA deacylation on the interpretation of the binding data of Frey and Utter (1977), the dependence of chicken pyruvate carboxylase catalysed deacylation on acetyl CoA concentration was investigated. The results presented in Fig. VI.4 suggest that, under the conditions used by Frey and Utter (1977), there is a hyperbolic response of the rate of deacylation to acetyl CoA concentration, which is consistent with the non-cooperative binding of acetyl CoA to the chicken enzyme. Moreover, the  $K_m$  value of  $54 \mu\text{M}$  obtained from a linear regression analysis of the data shown in Fig. VI.4 may be used, in conjunction with the maximum rate of deacylation of  $4 \text{ nmole/min/mg}$  reported

by Frey and Utter (1977), to estimate the extent of deacylation occurring during a binding study experiment. At an acetyl CoA concentration of 20  $\mu\text{M}$ , a protein concentration of 2.5 mg/ml, and a 2.5 minute time period, it is possible to calculate, using the  $K_m$  and  $V$  values given above, that approximately 3.4 nmoles of [ $^{14}\text{C}$ ]acetate will have been produced by the time the unlabelled acetyl CoA was added. However, given that the rate of diffusion of a molecule is proportional to the cube root of its molecular weight, the [ $^{14}\text{C}$ ]acetate would be expected to diffuse across the binding cell membrane at 2.4 times the rate of the [ $^{14}\text{C}$ ]acetyl CoA. Hence, given that 10 nmoles of [ $^{14}\text{C}$ ]acetyl CoA would be initially present in the flow cell (volume 0.5 ml), over 80% of the radioactive material in the effluent from the flow cell would be expected to represent [ $^{14}\text{C}$ ]acetate, rather than [ $^{14}\text{C}$ ]acetate CoA. Thus, it may be concluded that a valid interpretation of the low acetyl CoA concentration data of Frey and Utter (1977) does not appear to be possible. It is therefore difficult to assess the significance of the  $n_H$  values of 1.9 reported by these workers.

A feature of the deacylase data reported above worthy of comment is the observation that the  $K_m$  values of acetyl CoA in the deacylase reaction are higher than the  $K_a$  values reported for activation of the catalytic reaction. This difference may be explained if it is assumed that acetyl CoA deacylation proceeds by a Briggs-Haldane reaction pathway, as is shown in equation VI-7



Equation VI-8 describes the dependence of the rate of deacylation on acetyl CoA concentration, in terms of the pathway shown in Equation VI-7:

$$v = \frac{k_3 E_T}{1 + \frac{k_2 + k_3}{k_1} [\text{acetyl CoA}]} \dots \text{VI-8}$$

where  $v$  and  $E_T$  represent the rate of deacylation and the total enzyme concentration respectively. From equations VI-7 and VI-8 the Michaelis constant ( $K_m$ ) and dissociation constant ( $K_a$ ) for acetyl CoA are given by the expressions

$$K_m = \frac{k_2 + k_3}{k_1}$$

$$K_a = \frac{k_2}{k_1}$$

Thus, the  $K_m$  value for acetyl CoA in the deacylation reaction would be expected to be greater than the  $K_a$  value for acetyl CoA in the catalytic reaction.

The results obtained using two independent experimental approaches, TNBS modification, and the deacylase reaction, suggest that acetyl CoA binds to pyruvate carboxylase in a non-cooperative, Michaelis-Menten manner, under conditions similar to the carboxylation reaction assay. The question now arises: how can the non-classical acetyl CoA-velocity saturation profile arise in the carboxylation reaction if acetyl CoA binds to the enzyme in a non-cooperative manner. It is possible to provide a plausible answer by extending the studies of Ashman et al., (1972) on the effects of acetyl CoA on sheep pyruvate carboxylase in the manner described in Sections VI.C.2 onwards below.

#### VI.C.1.c. The mechanism of deacylation

The mechanism of the pyruvate carboxylase catalysed acetyl CoA deacylation has not been extensively investigated. One hypothetical mechanism involves transfer of an acetyl group from acetyl CoA to an appropriate residue in the acetyl CoA binding site, followed by hydrolytic cleavage of the group from the acetylated amino acid.

In order to test this possibility, experiments were carried out

in which pyruvate carboxylase was incubated with [ $^{14}\text{C}$ ]acetyl CoA labelled in the acetyl moiety. The protein was removed from unreacted acetyl CoA by either dialysis against 0.1 M tris Cl, pH 8.4, gel filtration on Sephadex G50, or trichloroacetic acid precipitation after which the protein was trapped on a glass fibre disc. When the radioactivity associated with the protein isolated by these three procedures was determined, only background levels were observed. Thus, if an acetyl-enzyme intermediate forms during the acetyl CoA deacylase reaction, it must be too labile to be easily detected.

Another possible mechanism of acetyl CoA deacylation involves thiolysis of acetyl CoA by an enzyme sulphhydryl residue, leading to formation of a disulphide bond between the CoASH moiety and the sulphhydryl group. In order to determine if any such disulphide bond formation occurs, pyruvate carboxylase, specific activity 20 U/mg, 8 units, was treated with acetyl CoA, and then [ $^{14}\text{C}$ ]cyanide as described in Section VI.B.5. The use of cyanide to displace thionucleotides from disulphide bond linkages to proteins has been investigated by Wagner and Yount (1975a,b) who were able to detect disulphide bond formation in an affinity labelled myosin ATPase preparation.

However, in this case the radioactivity of control and experimental samples was not significantly different. Thus it may be concluded that acetyl CoA deacylation probably does not involve disulphide bond formation.

#### VI.C.2      Effect of pyruvate concentration on the acetyl CoA activation.

Previous investigations of the acetyl CoA activation of sheep pyruvate carboxylase have shown that the presence of acetyl CoA decreases the  $K_m$  value for pyruvate eight-fold (Ashman et al., 1972). This finding

suggested a contributing factor to the sigmoid profile obtained when  $v$  is plotted as a function of varying acetyl CoA concentration; namely, the fixed pyruvate concentration used in the assay solution, although saturating at high levels of acetyl CoA, becomes non-saturating as the acetyl CoA concentration is decreased. Thus the observed rate of pyruvate carboxylation would be expected to be lower, at low levels of acetyl CoA, than the classical acetyl CoA binding data would predict. This decreased rate would therefore contribute to the sigmoid nature of the velocity response curve. In order to test this hypothesis, experiments were carried out to determine the effect of various pyruvate concentrations on the sigmoidicity of the acetyl CoA-velocity profile. The prediction from the above explanation would be that higher levels of pyruvate would decrease the degree of sigmoidicity, while low levels would increase sigmoidicity.

The experimental evidence confirmed this prediction. The data obtained are presented in double reciprocal form in Fig. VI.5. It can be seen that at high fixed levels of pyruvate the deviation from linearity was least, but as the pyruvate concentration was decreased, the deviation from linearity increased. The effect of different levels of pyruvate on the  $n_H$  value for acetyl CoA obtained from these data with the sheep enzyme are summarized in Table VI.1 where it can be seen that at low levels of pyruvate the  $n_H$  value is 3.16, but decreases to 2.41 at the highest pyruvate concentration. Table VI.1 also shows that the effect of variable pyruvate concentration on the acetyl CoA kinetics is not peculiar to the sheep enzyme, since the same trend was observed with the enzyme isolated from chicken liver mitochondria.

The conclusion to be drawn from these data is that, within limits, the  $n_H$  value for acetyl CoA is a function of the fixed pyruvate concentration used in measuring the reaction velocity. The reason that the  $n_H$  value does not decrease to a limiting value of 1.0 is because, in



addition to the effect of acetyl CoA on pyruvate binding, other factors are operating; these will be discussed in subsequent sections.

In order to test the hypothesis that acetyl CoA stimulates the binding of pyruvate, the rates of synthesis of oxaloacetate and orthophosphate as a function of pyruvate concentration, in the absence of acetyl CoA, were determined. The results of a typical experiment are shown in Fig. VI.6. The profiles shown are qualitatively similar to those obtained using saturating levels of acetyl CoA (Fig. V.1). This similarity implies that the fundamental mode of action of pyruvate, as discussed in the previous chapter, is independent of the presence of acetyl CoA. However, there are quantitative differences between the pyruvate interactions with pyruvate carboxylase in the presence or absence of acetyl CoA. This is illustrated in Fig. VI.7, where the orthophosphate/oxaloacetate ratio is plotted as a function of pyruvate concentration, using data obtained in the presence and absence of acetyl CoA. In the absence of acetyl CoA the product ratio approaches one at a much higher pyruvate concentration than in the presence of saturating levels of acetyl CoA. The conclusion to be drawn from this comparison is that acetyl CoA facilitates the binding of pyruvate to the enzyme, such that the product ratio approaches one at lower levels of pyruvate than in the absence of acetyl CoA.

### VI.C.3. Effect of bicarbonate concentration on the acetyl CoA activation.

As well as the effect of the presence or absence of acetyl CoA on pyruvate binding, Ashman et al., (1972) also found that the presence of acetyl CoA decreases the  $K_m$  value for bicarbonate by about an order of magnitude. Using reasoning analogous to that above, this finding suggested another contributing factor to the sigmoid acetyl CoA-velocity profile; the

fixed bicarbonate concentration used in the assay solution becomes non-saturating as the acetyl CoA concentration is decreased. Thus, low levels of bicarbonate would be expected to act in a similar manner to low pyruvate levels in increasing the degree of sigmoidicity, whereas high bicarbonate levels would decrease the sigmoidicity. Accordingly, experiments were carried out to determine the effect of various bicarbonate concentrations on the sigmoidicity of the acetyl CoA-velocity profile. The results of a typical experiment of this type are summarised in Table VI.2, where it can be seen that at low levels of bicarbonate the  $n_H$  value is 2.00, but decreases to 1.37 at the highest bicarbonate concentration. This data is consistent with the effect of non-saturating bicarbonate described above, showing that non-saturating bicarbonate is another contributing factor to the sigmoid acetyl CoA velocity profile.

VI.C.4. Effect of  $MgATP^{2-}$  concentration on the acetyl CoA activation.

The postulated effect of non-saturating substrate concentrations on the acetyl CoA velocity profile may be tested by investigation of the effect of various fixed  $MgATP^{2-}$  concentrations on the sigmoidicity of the acetyl CoA curve. The point here is that Ashman et al., (1972) found that the  $K_m$  for  $MgATP^{2-}$  was not significantly different in the presence or absence of acetyl CoA, implying that acetyl CoA has no effect on the binding of  $MgATP^{2-}$  to pyruvate carboxylase. Therefore, the  $MgATP^{2-}$  concentration used would remain saturating regardless of the acetyl CoA concentration, and so varying the fixed  $MgATP^{2-}$  concentration would be expected to have no effect on the sigmoidicity of the acetyl CoA velocity profile. Experiments to test this prediction were carried out, and the results summarised in Table VI.3. show that the  $MgATP^{2-}$  concentration used

in the assay has no significant effect on the  $n_H$  value for acetyl CoA activation. This negative result suggests that the interpretation of the results of Ashman et al., in terms of the shape of the acetyl CoA velocity profile is substantially correct. The increase in the  $K_a$  for acetyl CoA, without a significant decrease in the  $V_m$  implies that  $MgATP^{2-}$  is acting as a competitive inhibitor with respect to acetyl CoA. As the nucleotide end of the acetyl CoA molecule is not completely dissimilar to ATP, it is not unreasonable to suppose that  $MgATP$  is able to bind at the acetyl CoA binding site to some extent. This interpretation is consistent with the observation of Barritt (1967) that adenosine is a partially competitive inhibitor of acetyl CoA; presumably adenosine inhibits pyruvate carboxylation by binding at both the ATP and acetyl CoA sites.

#### VI.C.5. Effect of KCl on the acetyl CoA activation

The effect of monovalent cation concentration on the shape of the acetyl CoA profile was also investigated. It was found that KCl, in the range 0-15 mM had no significant effect on the  $n_H$  value for acetyl CoA. This finding was consistent with the observation of Barden and Scrutton (1973), that the apparent  $K_a$  for  $K^+$  was essentially independent of the acetyl CoA concentration.

#### VI.C.6. Effect of dilution inactivation on the acetyl CoA activation.

In previous sections evidence has been presented to account for the sigmoid acetyl CoA kinetics exhibited by pyruvate carboxylase. However, another factor not considered previously is that pyruvate carboxylase is subject to dilution inactivation (Ashman et al., 1972).

When an aliquot of enzyme is removed from a concentrated solution, and diluted below 4 enzyme units per ml, a lower than expected level of enzyme activity is observed. Acetyl CoA has been shown to protect against this inactivation process. Therefore, the possibility existed that at low levels of acetyl CoA some inactivation of the enzyme might occur during the course of the experiment. As was discussed in Section I.C.3, if a classically binding activator or substrate prevents such an inactivation process, then a sigmoid velocity profile would be observed (Harding, 1969; Hemphill et al., 1971; Fischer and Keleti, 1975). The possibility that dilution inactivation contributes to the shape of the acetyl CoA velocity profile was tested in two ways. Firstly, the linearity of oxaloacetate synthesis with time was examined, at various acetyl CoA concentrations. The results presented in Fig. VI.8 show that at low acetyl CoA concentrations there is some deviation from linearity, implying that at these low acetyl CoA levels the conventional five minute assay results in an underestimate of the initial velocity of oxaloacetate synthesis, thus contributing to the observed sigmoid curve.

The effect of dilution inactivation on the sigmoidicity of the acetyl CoA velocity profile was also investigated by determining the effect of the time of the assay on the  $n_H$  value for acetyl CoA. The results summarised in Table VI.4 show that decreasing the period of the assay results in a decreased  $n_H$  value for acetyl CoA. This finding was consistent with the non-linearity of the reaction with time described above, because this non-linearity becomes less significant as the duration of the assay is reduced.

The concentration dependence of the dilution inactivation process suggested the possibility that lower order polymers (dimers or monomers) of

pyruvate carboxylase, with lower catalytic activity, might be formed during the dilution inactivation process. The low concentrations of enzyme necessary for dilution inactivation meant that the orthodox techniques of gel filtration or analytic ultracentrifugation with a schlieren optic system would not be sensitive enough to detect presumptive lower molecular weight forms of pyruvate carboxylase. However, the reacting enzyme sedimentation technique (Cohen et al., 1967), in which only catalytically active enzyme is detected, may be used in this case. The reacting forms of pyruvate carboxylase isolated from Pseudomonas citronellolis, yeast, and chicken liver have been studied using this technique (Taylor et al., 1972; 1974).

Experiments designed to detect any lower molecular weight catalytically active forms of pyruvate carboxylase generated during dilution inactivation have been carried out; the results are summarised in Table VI.5. These results show that enzyme diluted in the presence of saturating acetyl CoA, without detectable dilution inactivation had a unique sedimentation coefficient of  $15.27 \pm 0.77$  S. This value is not significantly different from that of  $15.06 \pm 0.235$  obtained by Bais (1974) from analytical ultracentrifugation studies of sheep pyruvate carboxylase at high protein concentration, where pyruvate carboxylase is a tetramer of molecular weight  $4.8 \times 10^5$ . When pyruvate carboxylase was diluted in the absence of acetyl CoA, with 63% loss of catalytic activity, a sedimentation value of  $14.55 \pm 0.68$  was obtained. No smaller catalytically active forms of the enzyme were detectable. Thus it may be concluded that dilution inactivation does not involve formation of lower molecular weight, catalytically active forms of pyruvate carboxylase. The observation that acetyl CoA cannot reverse dilution inactivation (Ashman et al., 1972) suggests that lower molecular weight forms, if present, would have been detectable, even though the ultracentrifuge assay mixture contained

saturating levels of acetyl CoA.

VI.C.7. Conditions under which sigmoid velocity profiles are eliminated.

The previous experiments have provided evidence to account for the sigmoid velocity curves observed with varying acetyl CoA concentrations. Therefore, confirmation of the conclusions drawn from the data was sought by designing an experiment in which all substrates and activators were present at saturating levels regardless of the acetyl CoA concentration. Furthermore, the enzyme concentration was raised to a level where dilution inactivation did not occur. Under these conditions the only effect acetyl CoA could have was on the amount of enzyme catalysing the reaction at the acetyl CoA dependent rate compared to the acetyl CoA independent rate.

It was found (Fig. VI.9) that the reciprocal of velocity plotted as a function of the reciprocal of acetyl CoA concentration gave a concave downward curve. This result demonstrated, first of all, that provided the factors discussed above no longer operate a sigmoid velocity profile (or a concave upward double reciprocal plot) is not observed. Secondly, it eliminated the possibility that acetyl CoA bound to the enzyme in a co-operative manner, since that would have resulted in a concave upward double reciprocal plot. Under these conditions, the fractions of the total enzyme present with and without bound acetyl CoA present are given by the expressions

$$E_A = \frac{E_T}{1 + \frac{K_a}{A}}$$

$$E_O = \frac{E_T}{1 + \frac{A}{K_a}}$$

If the velocity constants of the acetyl CoA dependent and independent activities are  $K_\alpha$  and  $K_\beta$  respectively, then the observed velocity ( $v$ ) will be given by the expression:

$$v = \frac{K_\alpha E_T}{1 + \frac{K_a}{A}} + \frac{K_\beta E_T}{1 + A/K_a}$$

Now  $K_\alpha E_T$  and  $K_\beta E_T$  are the maximum velocities of the acetyl CoA dependent and independent reactions respectively. Therefore

$$v = \frac{V_D}{1 + \frac{K_a}{A}} + \frac{V_I}{1 + A/K_a}$$

where  $V_D$  and  $V_I$  are the maximum velocities of the dependent and independent activities. This equation may be re-arranged to give:

$$\frac{1}{v} = \frac{1 + \frac{K_a}{A}}{V_D + K_a \frac{V_I}{A}}$$

Now, the shape of the double reciprocal plot is defined by the sign of the second derivative of  $\frac{1}{v}$  with respect to  $1/A$ ; a negative value implies a concave downward curve, whereas the derivative is positive for a concave upward curve. The second derivative is given by the expression:

$$\frac{d^2 \frac{1}{v}}{d(\frac{1}{A})^2} = \frac{K_a (V_I - V_D) (2K_a V_D V_I + 2V_I^2 K_a^2 \cdot \frac{1}{A})}{(V_D + \frac{K_a V_I}{A})^4}$$

Now  $V_D > V_I$ , and therefore the second derivative is always negative, implying a concave downwards response of the reciprocal velocity to the reciprocal acetyl CoA concentration, which is what was found experimentally.

Thus it would appear that attempts to demonstrate a linear response of  $1/v$  to the reciprocal acetyl CoA concentration will be unsuccessful, because when the factors contributing to the sigmoid curve are corrected for, the acetyl CoA independent activity becomes significant

compared to the dependent activity, giving rise to the concave downwards response discussed theoretically and experimentally above.

#### VI.D DISCUSSION

The evidence that acetyl CoA is indeed an allosteric activator of the vertebrate enzymes is now quite convincing. The demonstration of substantial acetyl CoA independent catalytic activity of pyruvate carboxylase from sheep kidney (Ashman et al., 1972), and rat liver (Scrutton and White, 1972) eliminated the possibility that this compound participated directly in the reaction sequence. In addition, Ashman (1973) has shown that the apparently "absolute" requirement of the sheep kidney enzyme for acetyl CoA (Ling and Keech, 1966, Barritt et al., 1966) arose because of differences in the optimal conditions for activity in the presence and absence of the activator. It is possible that further investigation will reveal a similar explanation for the "absolute" requirement of chicken liver enzyme for acetyl CoA.

Since the development of the concept of co-operativity of binding by Monod et al., (1967), the observation of non-classical saturation kinetics for a given enzyme has usually been explained by asserting that the ligand binding is a co-operative process. However, as has been discussed in Section I.C.3, it has often been overlooked that the relationship between the equations describing non-classical binding and non-classical saturation kinetics is not necessarily simple. Thus it is in general not valid to infer that ligand binding is a co-operative process, from the observation of non-classical saturation kinetics.

The data presented in this Chapter appear to show that the binding of acetyl CoA to sheep liver pyruvate carboxylase, under conditions approaching those of an initial velocity assay, is a non-cooperative process.



The observed non-classical acetyl CoA kinetics can be explained adequately by the effect of acetyl CoA on pyruvate and bicarbonate binding, and, to a lesser extent, its ability to protect the enzyme against dilution inactivation.

In view of the lack of experimental evidence to support the concept of homotropic co-operativity of binding of acetyl CoA, the demonstration that acetyl CoA facilitates the binding of pyruvate provides a plausible alternate explanation for the regulation of pyruvate carboxylase activity. If the activity of an enzyme is to be regulated by variations in the concentrations of a metabolite, then that metabolite must satisfy two criteria. Firstly, it must normally be present in the cell at non-saturating levels, and secondly its in vivo concentration must fluctuate with the metabolic state of the cell. Pyruvate and acetyl CoA satisfy these criteria with respect to the regulation of pyruvate carboxylase activity. When gluconeogenesis is stimulated, as for example, by glucagon, pyruvate uptake by mitochondria will increase (Titheradge and Coore, 1976) and effect an increase in the activity and efficiency of pyruvate carboxylase, possibly by the mechanism discussed in Section V.D.2. This increase will be further enhanced by the increased level of acetyl CoA generated by the action of pyruvate dehydrogenase. This "double effect" of increasing pyruvate levels thus leads to a very efficient method of regulating pyruvate carboxylase activity in the cell.

Fig. VI.1 TNBS modification in the presence of varying concentrations of acetyl CoA.

Inactivation was carried out at room temperature in a solution containing 100 mM tris Cl, pH 8.4, 4 mM MgCl<sub>2</sub>, 10 mM sodium pyruvate, and 0.15 mM TNBS. Pyruvate carboxylase (specific activity 29 U/mg, 2.35 units) was added and at the time intervals indicated 0.01 ml samples were removed and assayed immediately using the procedure described in Section II.B.4. The acetyl CoA concentrations used were: 0 μM, ( ● ); 3.6 μM, ( △ ); 7.2 μM, ( ▼ ); 14.3 μM, ( ▲ ); 18.6 μM, ( ○ ); 21.5 μM, ( ◆ ); 28.7 μM, ( □ ); and 35.9 μM, ( ▲ ). In the control ( ■ ), TNBS was omitted.

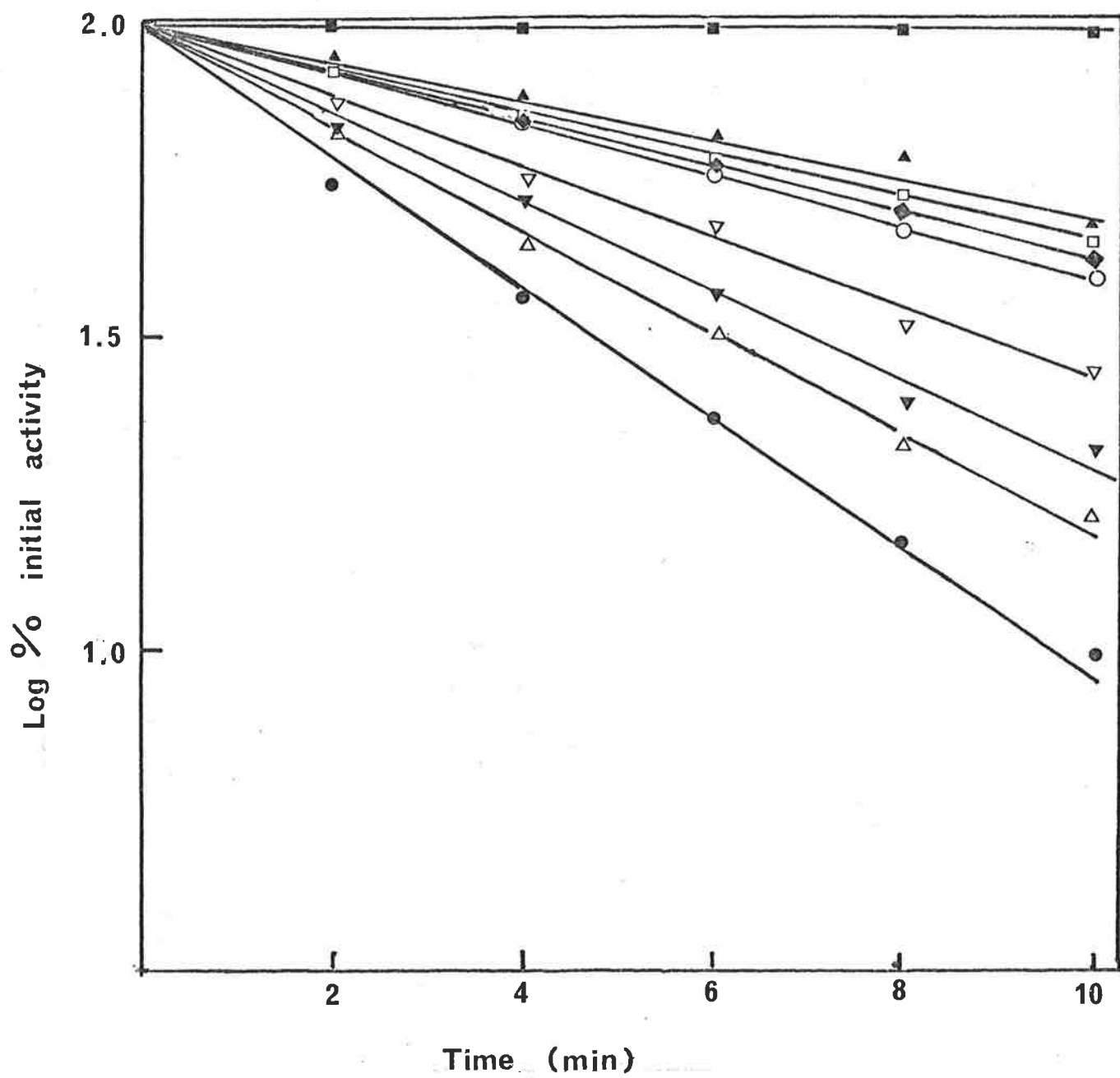


Fig. VI.2 Replot of the data shown in Fig. VI.1, according to Equation VI.6.

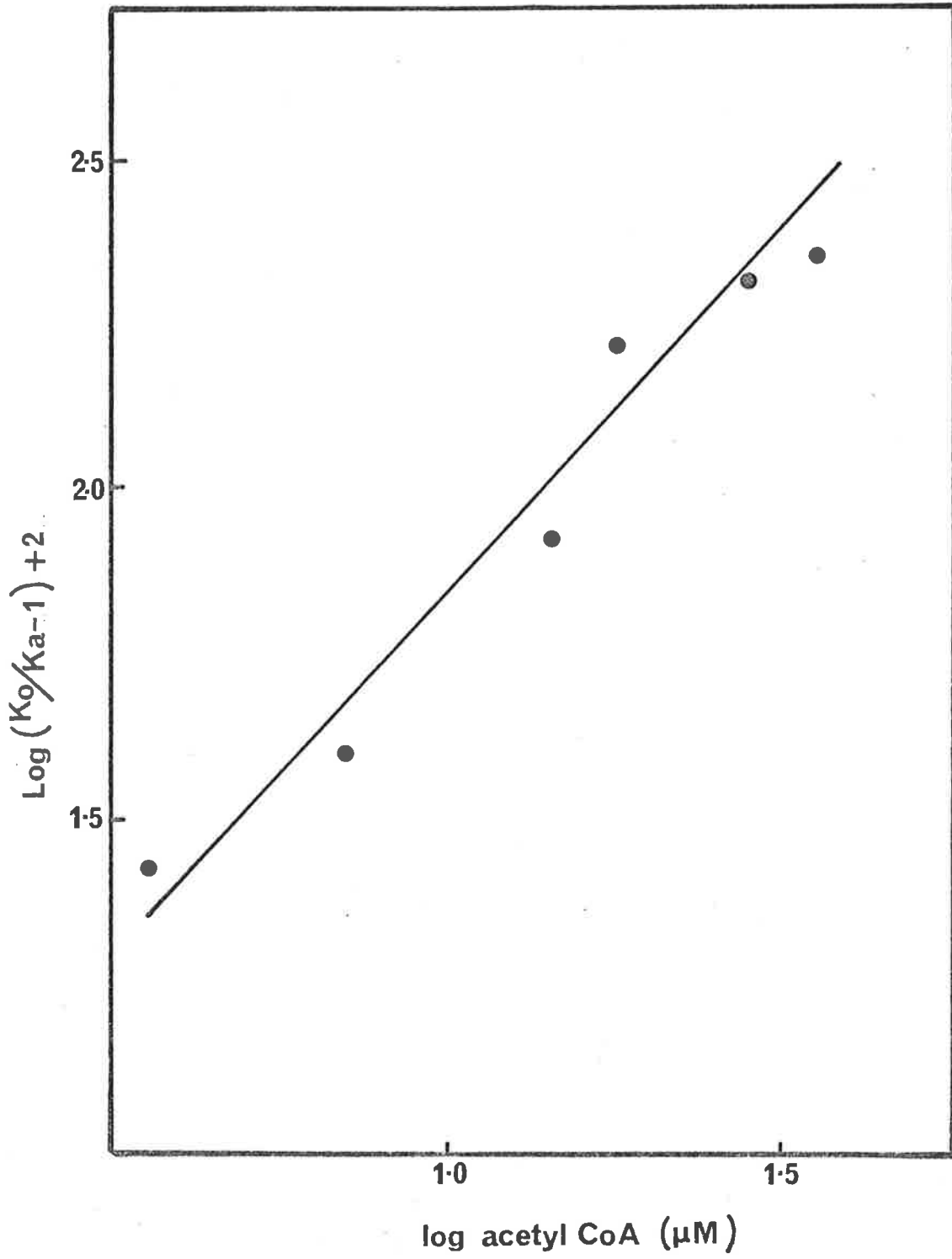


Fig. VI.3 The dependence of the rate of acetyl CoA deacylation  
on acetyl CoA concentration.

The rate of acetyl CoA deacylation at the acetyl CoA concentrations indicated was determined using the procedure described in Section VI.B.1.b, using 2.5 units of pyruvate carboxylase (specific activity 19 U/mg).

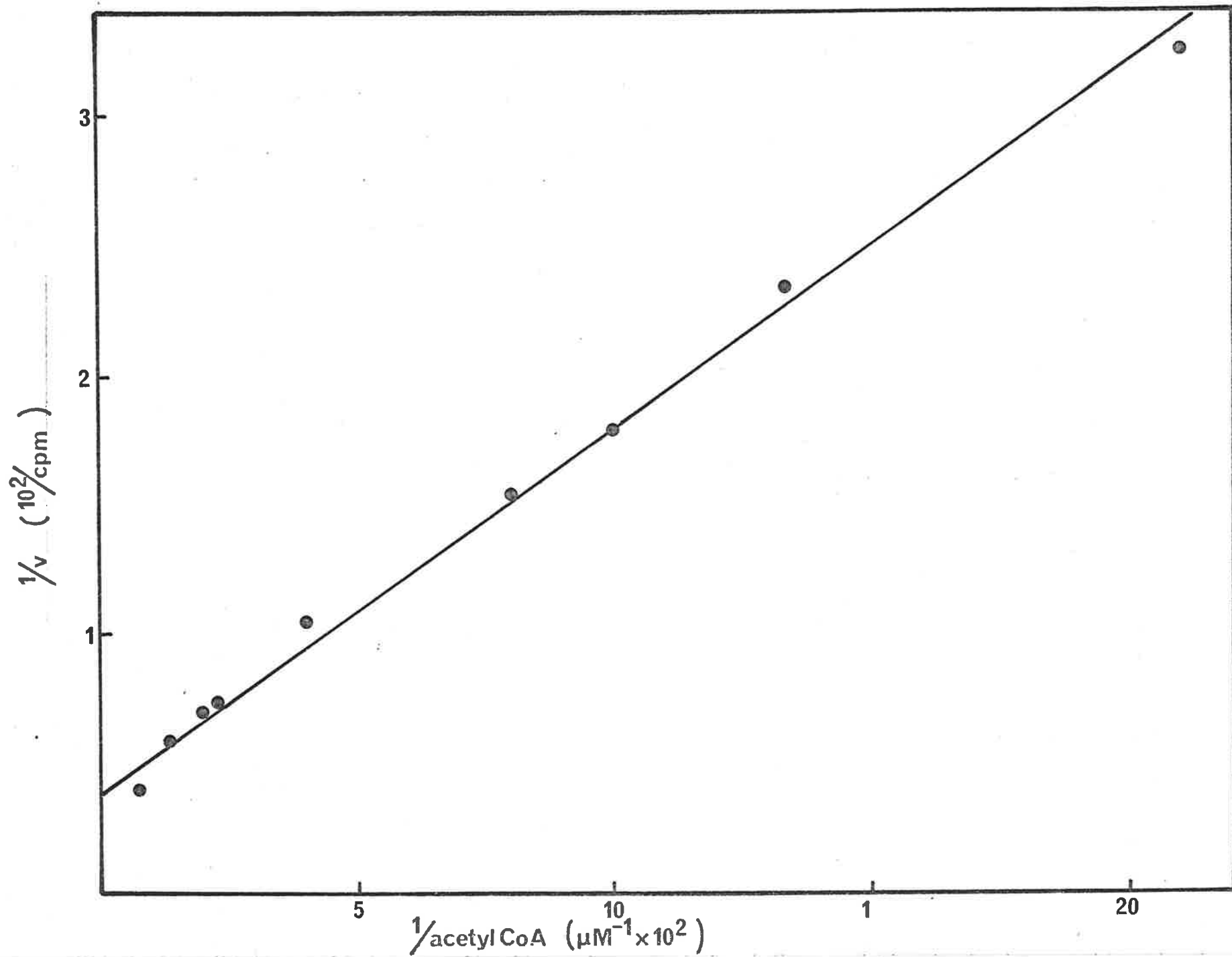


Fig. VI.4 The dependence of the rate of acetyl CoA deacylation on acetyl CoA concentration for chicken pyruvate carboxylase.

The rate of acetyl CoA deacylation at the acetyl CoA concentrations indicated was determined using the procedure described in Section VI.B.1.b, except the assay mix contained (in  $\mu$ moles), in a final volume of 0.4 ml :  $K^+$  HEPES, pH 7.2, (4); KCl, (80); EDTA, (0.4); acetyl CoA ( $1.34 \times 10^3$  cpm/nmole), and chicken pyruvate carboxylase (specific activity 25 U/mg, 10 units). The reaction was allowed to proceed for eight minutes before addition of the 6M HCl.



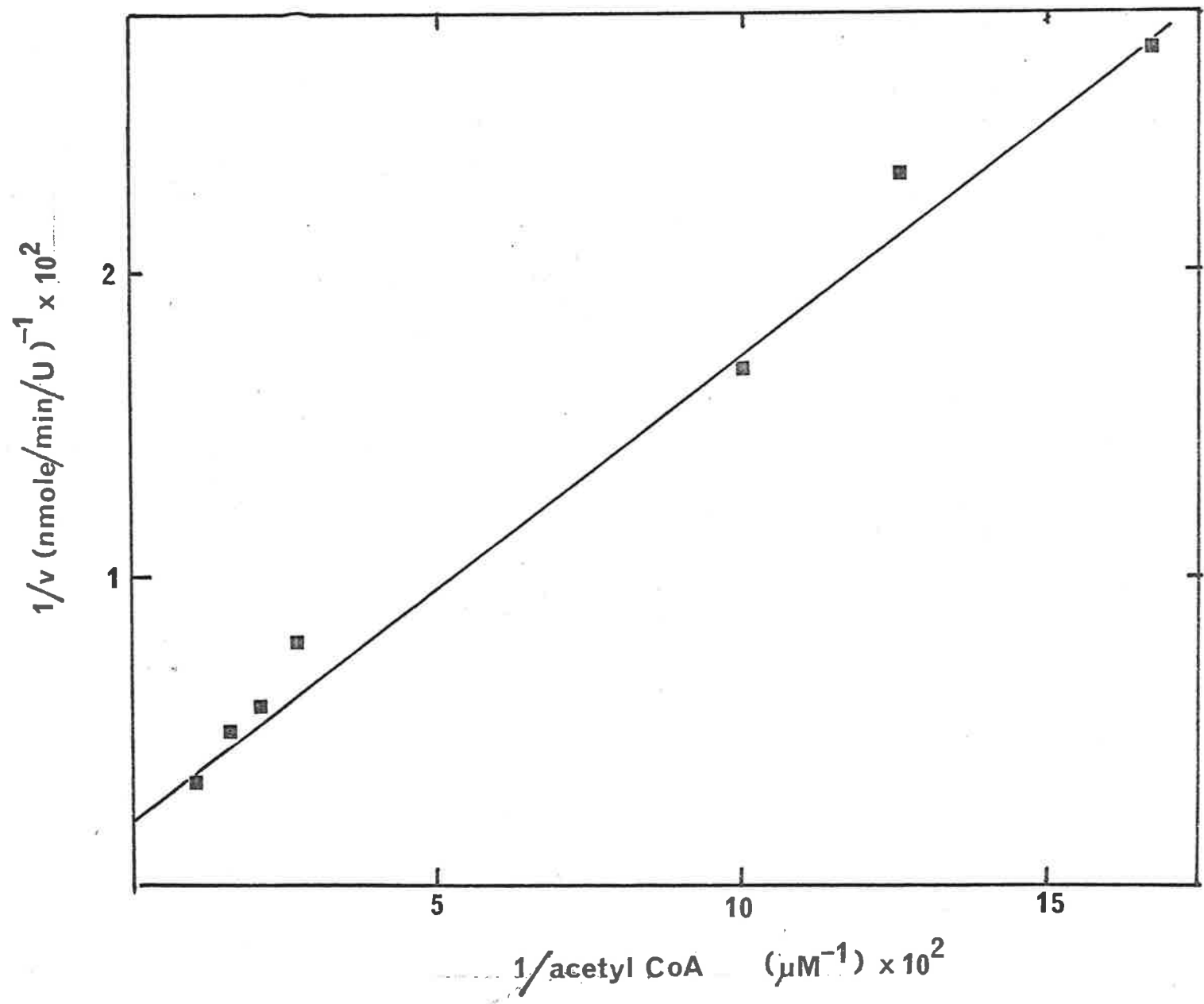


Fig. VI.5 The effect of pyruvate concentration on activation of pyruvate carboxylase by acetyl CoA.

The rate of oxaloacetate synthesis at the acetyl CoA concentrations indicated was determined by the procedure described in Section II.B.4, using 0.01 units of pyruvate carboxylase (specific activity 17 U/mg) per assay. The concentrations of pyruvate were : 0.95 mM, ( ● ); 3.18 mM, ( ○ ); 4.76  $\mu$ M, ( ▲ ); 9.52 mM, ( △ ); and 19.05 mM, ( ■ ).

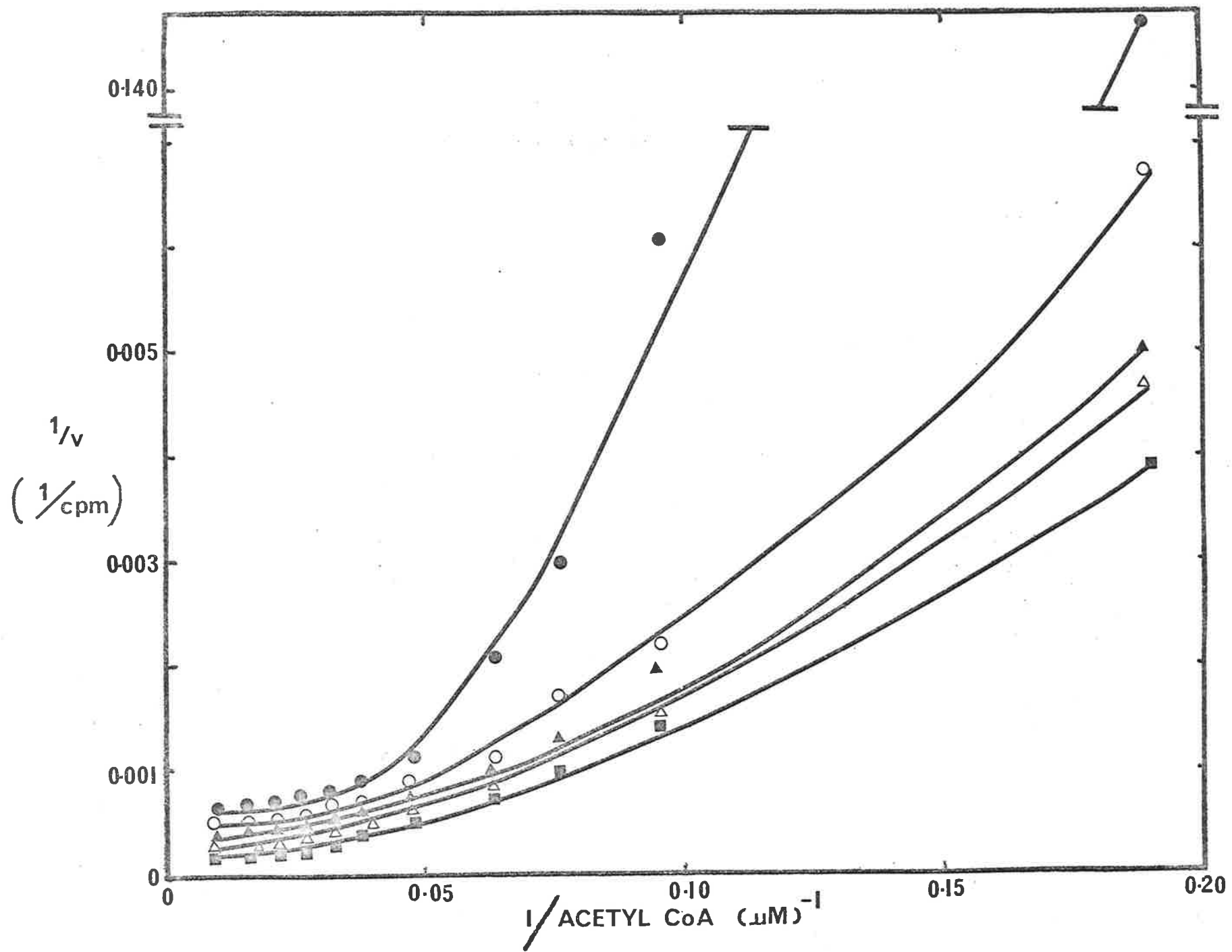


Table. VI.1 Effect of pyruvate concentration on the  $n_H$  value for acetyl CoA activation of the catalytic reaction.

The  $n_H$  values for the sheep enzyme were obtained from the data shown in Fig. VI.5, while the chicken enzyme data was obtained using the assay procedure described in the legend to Fig. VI.5, except tris Cl, pH 7.8 was used.

Pyruvate (mM)	$n_H$	
	sheep enzyme	chicken enzyme
0.95	3.16	-
1.00	-	4.20
3.00	-	3.48
3.18	2.77	-
4.76	2.15	-
9.52	2.52	-
10.00	-	2.24
19.05	2.41	-
20.00	-	2.22
30.00	-	1.84
61.60	-	1.85

Fig. VI.6 The dependence of the reaction rate on pyruvate concentration using the acetyl CoA independent conditions.

The rates of oxaloacetate synthesis ( ● ), and orthophosphate synthesis ( ○ ) at the pyruvate concentrations indicated were determined by the procedure described in Section VI.B.1.a using 2 units pyruvate carboxylase (specific activity 22 U/mg) per assay.

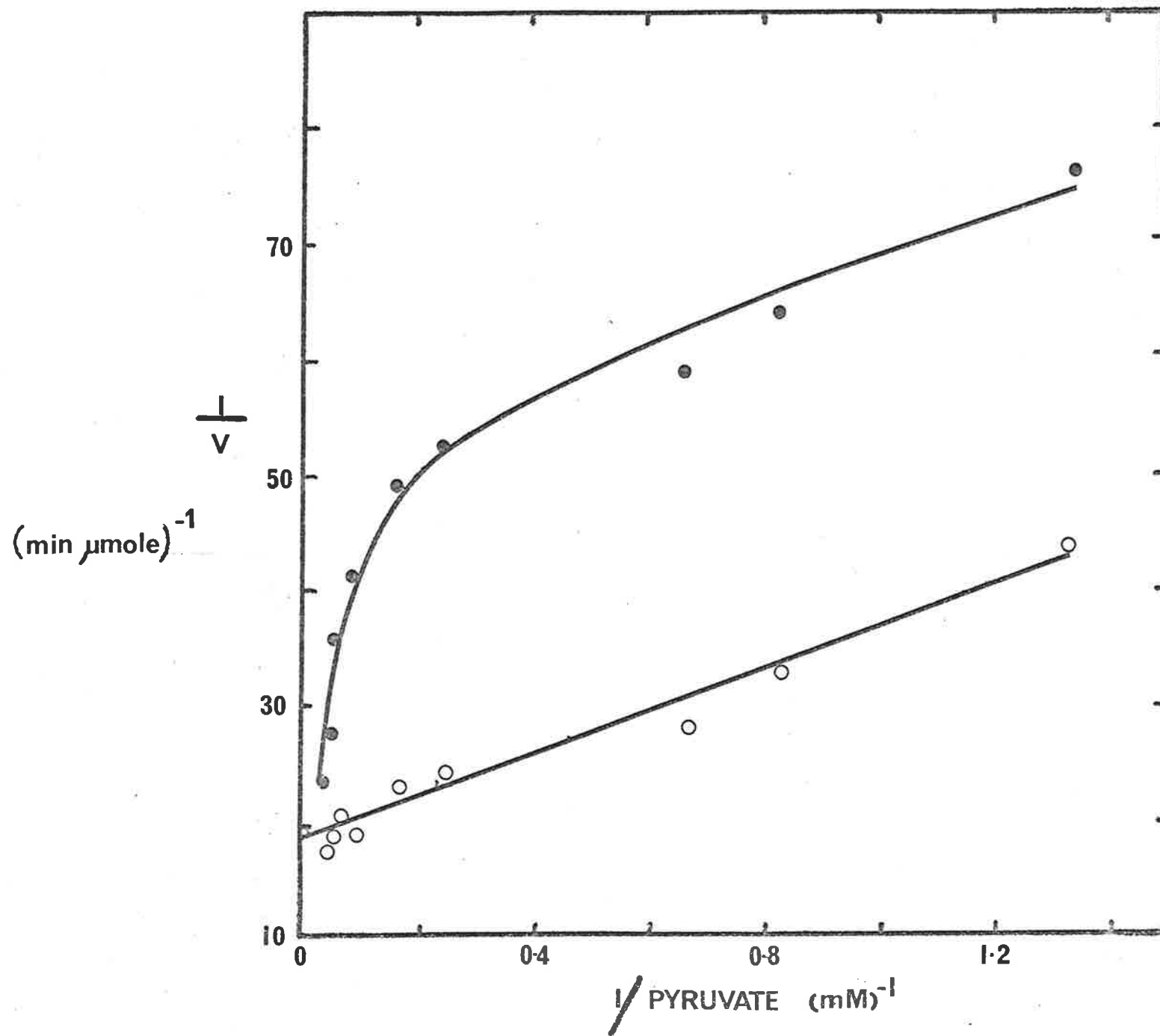


Fig. VI.7 Effect of pyruvate concentration on the orthophosphate/oxaloacetate ratio in the presence and absence of acetyl CoA.

Data for the product ratio in the absence of acetyl CoA ( ● ) were obtained from Fig. VI,6, and the data obtained in the presence of acetyl CoA ( ■ ) were obtained from Fig. V.2.



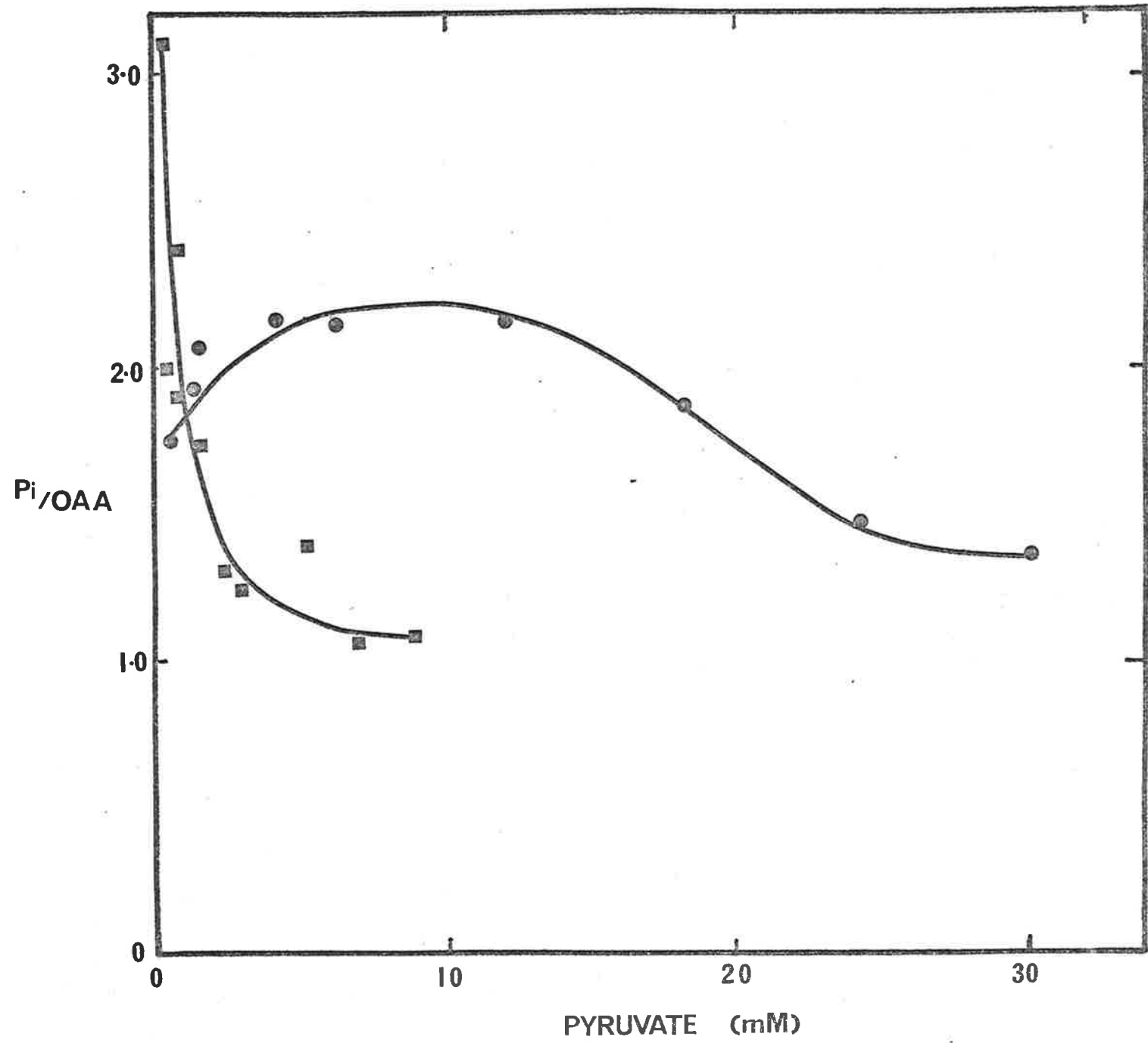


Table VI.2 Effect of bicarbonate concentration on the  $n_H$  value for acetyl CoA activation of the catalytic reaction.

The rate of oxaloacetate synthesis was determined by the procedure described in Section II.B.4, using 0.07 units pyruvate carboxylase (specific activity 10 U/mg) per assay. The acetyl CoA concentration range was 4-105  $\mu$ M, while the pyruvate concentration was 20 mM.

Bicarbonate (mM)	$n_H$
10	2,00
20	1.70
30	1.49
40	1.33
50	1.37

Table VI.3 Effect of MgATP<sup>2-</sup> concentration on the  $n_H$  and  $K_a$  values for acetyl CoA activation of the catalytic reaction.

The rate of oxaloacetate synthesis was determined by the procedure described in Section II.B.4 using 0.07 units of pyruvate carboxylase (specific activity 7 U/mg) per assay. The acetyl CoA concentration range used was 4-75  $\mu$ M and the pyruvate and bicarbonate concentrations were 20 mM.

MgATP <sup>2-</sup> (mM)	K <sub>a</sub> (μM)	n <sub>H</sub>
2.5	11.1	1.81
5.0	14.4	1.66
7.5	15.9	1.78
10.0	18.3	1.90
12.5	24.0	1.75

Fig. VI.8 Time course of the reaction determined at various concentrations of acetyl CoA.

The amount of oxaloacetate synthesised at the time periods indicated was determined by the procedure described in Section II.B.4, using 0.015 units of pyruvate carboxylase (specific activity 14 U/mg) per assay. The acetyl CoA concentrations were: 15.3  $\mu\text{M}$ , ( ● ); 40.7  $\mu\text{M}$ , ( ○ ); and 91.6  $\mu\text{M}$ , ( ■ ).

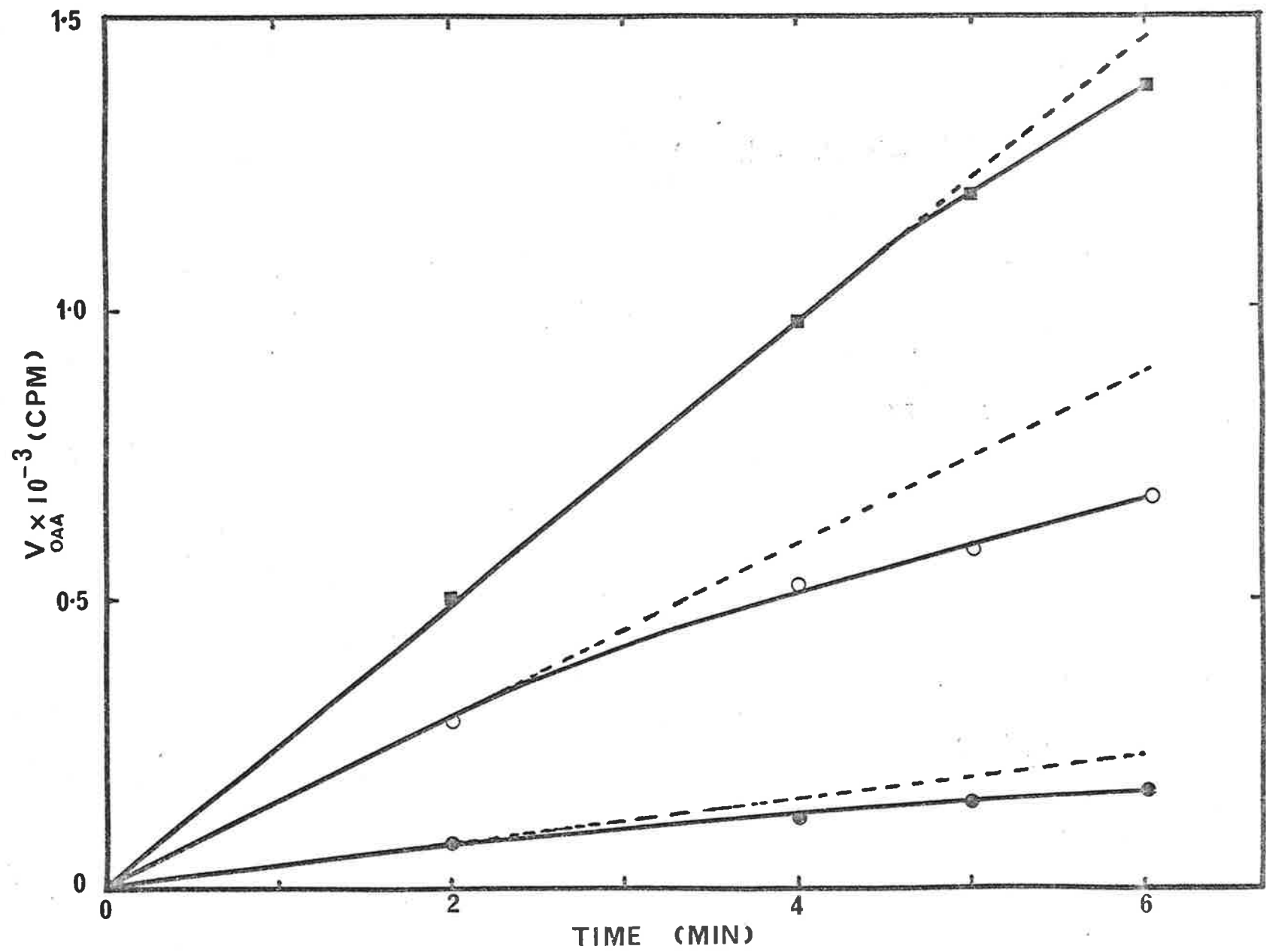


Table. VI.4 Effect of the assay time on the  $n_H$  value for acetyl CoA activation of the overall reaction.

The rate of oxaloacetate synthesis was determined by the procedure described in Section II.B.4 using 0.08 units of pyruvate carboxylase, (specific activity 10 U/mg) per assay. The acetyl CoA concentration range, pyruvate concentration, and bicarbonate concentration were the same as those described in the legend to Table VI.3.



Assay time (min)	$n_H$
0.5	1.61
1.5	1.73
2.0	1.72
4.0	1.84

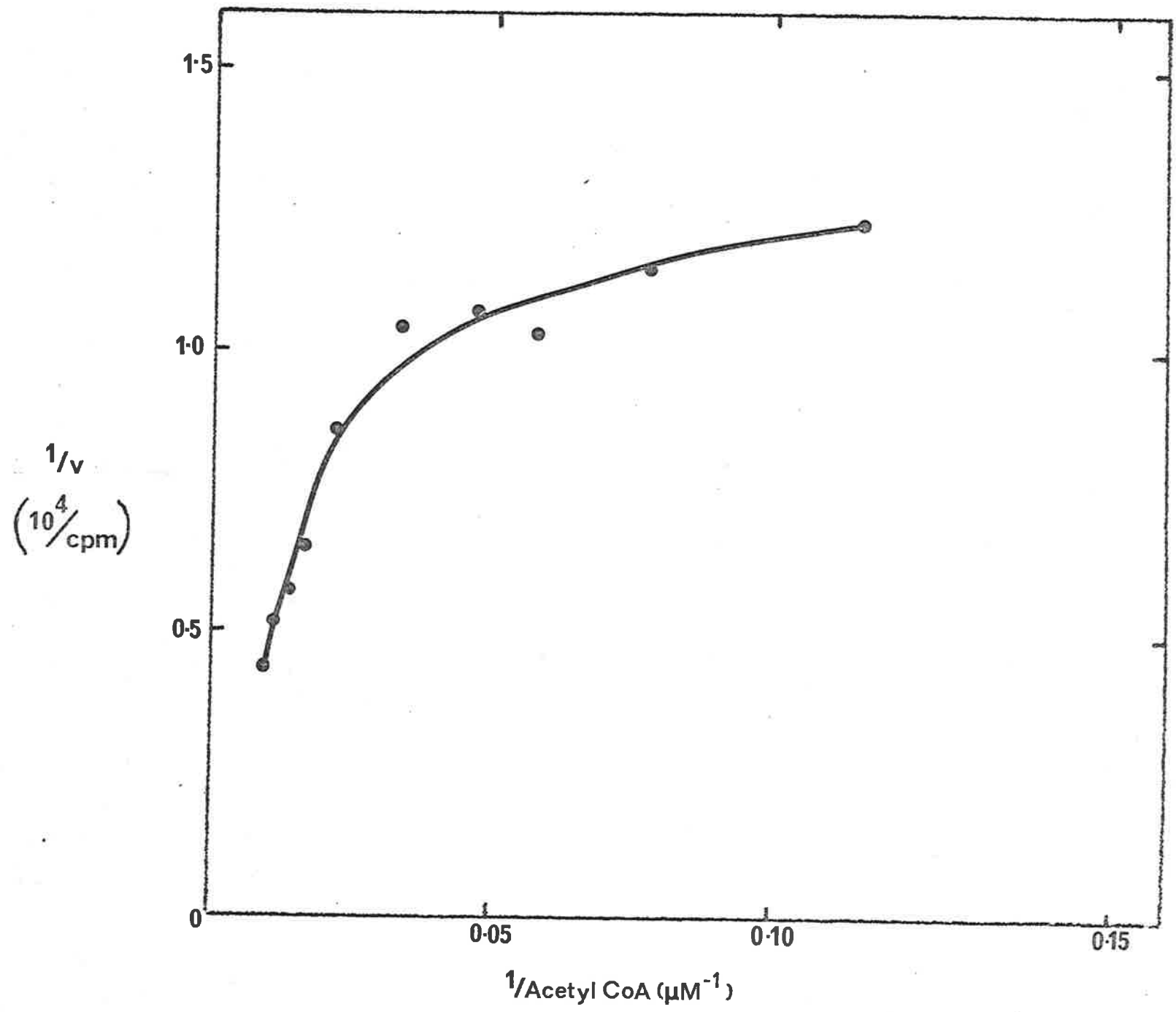
Table VI.5 Effect of dilution inactivation on the sedimentation coefficient of pyruvate carboxylase.

Pyruvate carboxylase (specific activity 8 U/mg) was diluted into 100 mM tris Cl, pH 8.4, containing 0.2 mM NADH, either in the presence or absence of acetyl CoA. The enzyme concentration was determined immediately after dilution, and just prior to commencing the ultracentrifuge run. The sedimentation coefficients were determined as described in Section VI.B.3.

Initial concentration of enzyme (U/ml)	Final concentration of enzyme (U/ml)	Acetyl CoA ( $\mu$ m)	S <sub>20,w</sub>
0.104	0.1035	200	14.8 $\pm$ 0.4
0.164	0.164	250	14.2 $\pm$ 0.3
0.257	0.255	250	16.8 $\pm$ 0.4
0.257	0.096	0	14.6 $\pm$ 0.7

Fig. VI.9 The dependence of the reaction rate on acetyl CoA concentration at high enzyme and substrate concentrations.

The rate of oxaloacetate synthesis at the acetyl CoA concentrations indicated was determined using the procedure described in Section II.B.4, except the assay mixture contained, (in  $\mu$ moles) in a final volume of 0.5 ml: tris Cl, pH 8.4 (40); ATP, (4); [ $^{14}$ C]bicarbonate, sodium salt, (37.5,  $9.86 \times 10^5$  cpm/ $\mu$ mole);  $MgCl_2$ , (17); pyruvate, sodium salt, (36.5);  $NH_4Cl$ , (83.5); and 2.6 units of pyruvate carboxylase (specific activity 27 U/mg). The reaction time was 0.5 minutes.



CHAPTER VII

GENERAL DISCUSSION

## VII GENERAL DISCUSSION

The results presented in this thesis have been discussed in detail at the end of each chapter. In this section an overview of the project is presented, and selected possible areas of investigation are presented.

### VII.A. THE REACTION PATHWAY

The results in Chapter V above show that the non-classical pyruvate saturation kinetics arise because the stoichiometry of the pyruvate carboxylase reaction depends upon the pyruvate concentration. At low pyruvate concentrations, the ratio of orthophosphate released to oxaloacetate produced exceeds unity, but it approaches one as the pyruvate concentration increases.

Also, evidence was presented which showed that, at high ketoacid substrate concentrations, the pyruvate carboxylase reaction proceeds via a sequential pathway. However, a mathematical analysis of the data suggested that the reaction may proceed via a non-sequential pathway at low concentrations of pyruvate. If the experimental tests outlined in Section V.C.7 confirm this prediction, then the conflicting evidence for sequential and non-sequential pathways may be resolved. It is possible that the data consistent with a non-sequential reaction pathway was obtained at low pyruvate concentrations, when it is possible that orthophosphate and MgADP may dissociate from the enzyme prior to pyruvate binding. On the other hand, the data presented by Warren and Tipton (1974b,c) may have been obtained at pyruvate concentrations high enough for the sequential reaction pathway to be preferred.

VII.B. THE REACTION MECHANISM

The kinetic studies described in this thesis have shown that the sequence of the reaction catalysed by sheep liver pyruvate carboxylase is similar to that described by Warren and Tipton (1974b,c) for pyruvate carboxylase isolated from pig liver. The reaction appears to be of the sequential type, in which no products are released at a significant rate until all the substrates have bound to the enzyme, at least at high levels of the keto-acid substrate.

As was discussed in Section V.D.3, the demonstration of ATP/P<sub>i</sub> and pyruvate/oxaloacetate isotopic exchange reactions catalysed by pyruvate carboxylase is consistent with the sequential reaction pathway, if the biotin carboxylation and pyruvate carboxylation sites are spatially distinct on the enzyme surface. A considerable degree of separation has been inferred from the ability of the biotin ring to "commute" between sites as far apart as 28 Å, and by analogy with the related biotin containing enzymes, transcarboxylase and acetyl CoA carboxylase, (from *Escherichia coli*) which have the analogous two binding sites on separate subunits (Moss and Lane, 1971; Chuang et al., 1975). However, in a recent magnetic resonance study of transcarboxylase, the distance from the methyl carbon of pyruvate and the methylene carbon of propionyl CoA, which is a measure of the distance between the carboxyl transfer takes place, was estimated to be about 7 Å (Fung et al., 1976). It was concluded that the major role of the 14 Å arm of carboxybiotin was not to permit a major carboxyl migration, but rather to allow carboxybiotin to transverse the interface between the subunits, and to insert itself between the keto-acid and CoA sites.

Some work currently in progress in this laboratory seems likely to shed further light on the relationship between the biotin prosthetic group and the biotin carboxylation and pyruvate carboxylation site. The



sequence of the biotin-containing tryptic peptide isolated from sheep liver pyruvate carboxylase has been determined (Rylatt et al., 1977). It should prove possible to obtain an extended sequence around the biotin moiety by reversibly blocking either the arginyl residues of the protein with phenylglyoxal (Means and Feeney, 1971), or the lysyl residues with citraconic anhydride (Gibbons and Perham, 1974). The purification procedure developed by Rylatt et al., (1977) could then be used to obtain a biotin containing tryptic peptide from enzyme so modified, extended beyond the arginyl residue at the C-terminal end of the peptide from unmodified enzyme and/or beyond the N-terminal of the peptide. After removal of the blocking group, and subsequent trypsin digestion, it should be possible to determine the sequences of amino acids between the ends of the peptide from unmodified enzyme and the next trypsin cleavage sites. Use of this procedure in an iterative manner may allow determination of the sequence of at least fifty residues each side of the biotin moiety. The configuration prediction schemes developed by Chou and Fasman (1974a,b) and Lim (1974 a,b), could then be used to construct a possible three dimensional model of the polypeptide chain surrounding the biotin group. A stereochemical analysis of this model may reveal to what extent the biotin group is able to move, and hence would give some idea of the degree of separation of the biotin carboxylation and pyruvate carboxylation sites.

The use of nuclear magnetic resonance and electron spin resonance may permit a more direct measurement of the degree of separation of the sites. The presence of a reactive sulphhydryl residue in the pyruvate carboxylation site of sheep liver pyruvate carboxylase (Hudson et al., 1975) means that it should be possible to prepare a spin-label modified enzyme, using a spin label such as 4-(iodoacetimido)-2,2,6,6-tetramethyl piperidino-oxyl. The distance between the spin label and the MgATP binding

site could then be determined by examining the electron spin resonance spectrum of spin labelled enzyme, titrated with MgATP, using an approach similar to that of Jones et al., (1973) in their study of phosphofructokinase. In this way it should be possible to obtain an estimate of the distance between the two sites.

Determination of the amino acid sequence of a tryptic peptide containing the pyruvate carboxylation site sulphhydryl residue is presently in progress in this laboratory (N.H. Goss, unpublished results). Once this information is available, and the sequence of the oATP peptide is available, it should be possible to use this data, in conjunction with that obtained for the biotin-containing peptide, and the magnetic resonance studies, to obtain a more detailed picture of the active site of pyruvate carboxylase. Once this has been done it may then be possible to correlate the kinetic data presented in this thesis, and that currently available, with the chemistry of the catalytic processes facilitated by the enzyme.

#### VII.C. THE CHEMISTRY OF BIOTIN CARBOXYLATION

The affinity labelling and chemical modification studies presented in this thesis have demonstrated the existence of at least one lysyl residue at or near the binding site for MgATP<sup>2-</sup> and bicarbonate. It appears likely that this residue(s) fulfil a catalytic role in facilitating nucleophilic attack of the bicarbonate on to the terminal phosphate of MgATP<sup>2-</sup>, allowing formation of (possibly transient) carbonyl phosphate which is the reagent which carboxylates biotin at the 1'-N position on the ureido ring (Ashman and Keech, 1975).

In order to further characterise the molecular events occurring during biotin carboxylation, it will be necessary to identify the other amino acid residues involved in the binding and catalytic processes of

biotin carboxylation. The use of other  $\text{MgATP}^{2-}$  based affinity labels seems likely to be useful in characterising these processes. The potential affinity labels, 6-thioinosine triphosphate, 8-azido ATP, and 6-azido ATP are currently under investigation in this laboratory. The use of the first mentioned analog stems from the observation that the 6-amino group of  $\text{MgATP}^{2-}$  appears to be necessary for the nucleotide to bind to the enzyme with a relatively high affinity (Scrutton and Utter, 1965). It is possible that this requirement arises because the 6-amino group of the purine moiety of  $\text{MgATP}^{2-}$  forms a hydrogen bond with the sulphhydryl group of a cysteinyl residue in the  $\text{MgATP}$  binding site. It has been suggested that this kind of interaction occurs in the ATP binding protein of myosin (Wagner and Yount, 1975a,b) and in the  $\text{Na}^+/\text{K}^+$  activated ATPase (Patzelt-Wenczler et al., 1975). Thus, if the pyruvate carboxylase biotin carboxylation site contains such a sulphhydryl group, then it may be able to react with the 6-thio group of 6-thioinosine triphosphate to form a disulphide bond.

Detection of such disulphide bond formation has been facilitated by the discovery that cyanide is able to displace the thionucleotide moiety, giving rise to a thio-cyanato enzyme derivative (Wagner and Yount, 1975a,b). Thus, if pyruvate carboxylase is treated with  $[\text{}^{14}\text{C}]$ cyanide following incubation with 6-thioinosine triphosphate, then the extent of labelling by the nucleotide should be able to be readily determined.

The azido ATP analogues may act as photoaffinity labels; the azido group can be converted to a highly reactive nitrene on photolysis. The presence of this highly reactive group may allow specific labelling of the protein even if there are no reactive amino acid residues appropriately situated in the  $\text{MgATP}^{2-}$  binding site. The use of these affinity labels should allow isolation and sequencing of more labelled peptides comprising

part of the biotin carboxylation site; this information will then be able to be combined with the other data discussed above to yield an increasingly complete picture of the active site of the enzyme.

#### VII.D. THE ACTIVATION OF PYRUVATE CARBOXYLASE BY ACETYL CoA.

The results presented in this thesis have shown that the binding of acetyl CoA to pyruvate carboxylase is a non-co-operative process, and that the observed non-classical acetyl CoA saturation profile arises because of the effects of acetyl CoA on the binding of pyruvate and bicarbonate, and on the process of dilution inactivation. However, there remains a number of aspects of the interaction of acetyl CoA with pyruvate carboxylase which warrant further investigation.

Firstly, the observation of Frey and Utter (1977) of a lag phase in initial velocity studies suggests that there may be slow isomerisation between two forms of the enzyme, possibly of the general type described by Ainslie et al., (1972). If the two such forms of the enzyme differ in their affinities for acetyl CoA, then the degree of "co-operativity" observed in kinetic studies should be higher than that observed in experiments designed to measure the binding of acetyl CoA (Frey and Utter, 1977). Thus, characterisation of the two forms of the enzyme, if in fact they exist, would shed further light on the nature of the non-classical acetyl CoA saturation profile.

Moreover, if the prediction that the pyruvate carboxylase reaction proceeds via either sequential or non-sequential pathways, depending on the pyruvate concentration, is confirmed, then an investigation of the effect of varying acetyl CoA concentrations on the relative rates of the two pathways would contribute to understanding how the non-classical acetyl CoA saturation profile arises. In view of the effect of acetyl CoA on pyruvate

binding reported in this thesis, it might be expected that an increase in the acetyl CoA concentration would facilitate the sequential pathway. In this case, it is possible that the effect of acetyl CoA on the reaction velocity may be explicable, in part, by the alternate reaction pathway allosterism model of Sweeney and Fisher (1968), as has already been suggested by Ashman et al., (1972).

Another aspect of the interaction of acetyl CoA with the enzyme, viz, investigation of changes in the enzyme's structure upon binding of acetyl CoA, is currently in progress in this laboratory. It has been shown, using the technique of circular dichroism, that dilution inactivation is associated with an increase in the  $\alpha$ -helical content of pyruvate carboxylase. Furthermore, when pyruvate carboxylase is diluted in the presence of saturating levels of acetyl CoA, the increase in  $\alpha$ -helical content is not observed. However, acetyl CoA has no effect on the  $\alpha$ -helical content of pyruvate carboxylase maintained at a concentration at which dilution inactivation does not occur (P.R. Clements, manuscript in preparation).

These results imply that acetyl CoA binding does not lead to gross changes in the structure of the polypeptide chain backbone of pyruvate carboxylase. Indeed, the effect of acetyl CoA on dilution inactivation is such that it prevents the change in the protein's structure associated with dilution inactivation.

It has also been shown that acetyl CoA induces a change in the fluorescence of the enzyme bound fluorescent probe, 1-anilinonaphthalene-8-sulphonate (J.A. Duc, unpublished results). This probe is known to bind preferentially to hydrophobic regions of proteins, and the intensity of its fluorescence emission depends on the polarity of the environment.

Thus, the effect of acetyl CoA on the 1-anilinonaphthalene-8-sulphonate fluorescence, coupled with the difference spectrum reported by

Frey and Utter (1977) suggests that changes in the environments of some hydrophobic amino acid residues, such as tyrosine, occur on acetyl CoA binding. In the light of these results, and those presented in Chapter VI above, it is possible that an investigation of the nature and orientation of aromatic amino acid residues near the bicarbonate and pyruvate binding sites could contribute to understanding the nature of acetyl CoA activation of pyruvate carboxylase.

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PUBLICATIONS

## 1. Papers published.

- a) Pyruvate carboxylase: affinity labelling of the magnesium adenosine triphosphate binding site.  
(with D.B. Keech and J.C. Wallace).  
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- b) Pyruvate carboxylase: mechanism of the second partial reaction.  
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Arch. Biochem. Biophys. 176, 709, (1976).
- c) Pyruvate carboxylase: A reappraisal of the reaction sequence.  
(with D.B. Keech and J.C. Wallace).  
Biochem. J., in the press.
- d) Pyruvate carboxylase: Explanation for non-classical acetyl CoA kinetics.  
(with A.J. Campbell, J.C. Wallace, and D.B. Keech).  
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## 2. Papers presented at meetings.

- a) Pyruvate carboxylase: affinity labelling of the MgATP binding site.  
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- b) Pyruvate carboxylase: Explanation for the non-classical pyruvate saturation kinetics.  
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- c) Pyruvate carboxylase: Explanation for the non-classical acetyl CoA saturation kinetics.  
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- d) Pyruvate carboxylase: Explanation for the non-classical acetyl CoA saturation kinetics.  
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