MECHANISM AND CONTROL OF SHEEP KIDNEY MITOCHONDRIAL PEP CARBOXYKINASE

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SUMMARY

PEP carboxykinase which catalyses the reaction

$$PEP + IDP + CO_2 \overset{Mn^{2+}}{=\!=} OAA + ITP$$

was purified from sheep kidney mitochondria and some of its physical, chemical and kinetic properties have been investigated. This enzyme was shown to be similar in most respects to the equivalent enzyme from pig liver.

Amino acid analysis revealed no particular atypical features of the protein while spectral analysis indicated the absence of any cofactors. However, the molecular weight of the protein as determined by the two standard techniques, Sephadex gel filtration and sedimentation-diffusion analysis, were 48,000 and 71,100 respectively. An estimate of 72,000 was obtained from electrophoresis in the presence of sodium dodecyl sulphate, thus confirming the estimate obtained using the ultracentrifuge.

Initial velocity studies of the enzyme using an uncoupled assay system revealed non-hyperbolic saturation kinetics when particular pairs of substrates were varied, e.g., PEP and IDP or IDP and HCO_3^- or PEP and Mn^{2+}. The kinetic patterns were typical of those obtained in a system showing negative cooperativity with respect to substrate binding. However, further analysis of the system indicated that negative cooperativity as defined by Levitzki and Koshland (1969) did not fully

describe the observed kinetics, *e.g.*, (a) the manifestation of the interactions was dependent on the particular pairs of substrates varied, (b) the degree of interaction as measured by the R_s value varied with the substrate concentration (c) only one mole of substrate per mole of enzyme was bound, (d) the biphasic initial velocity patterns were eliminated if a coupled assay system was used. The similarity between the *in vivo* conditions and the uncoupled assay with respect to the concentrations of PEP and OAA suggested that the OAA would be an important effector controlling the balance between the carboxylation of PEP and the decarboxylation of OAA.

Extensive studies with the common amino acid modifying reagents have been carried out in an attempt to elucidate the amino acid residues in the active site of PEP carboxykinase. The presence of two reactive sulphydryl residues was positively identified using N-ethylmaleimide (NEM), 1-fluoro-2,4-dinitrobenzene (DNFB) and 5,5'-dithiobis(2-nitrobenzoic acid). The residue reactive towards NEM was protected by IDP (and ITP) although it was shown that while IDP binding was abolished by reaction with NEM this residue was not strictly a nucleotide binding group since it had some apparent catalytic function. Reaction with DNFB (or 2,4,6-trinitrobenzene sulphonic acid (TNBS)) was not specific as two sulphydryl groups were modified. There was also a slight reaction towards a histidine residue. Both Mn^{2+} and IDP protected against DNFB (and TNBS)

modification, so it was concluded that DNFB was reacting with that residue modified by NEM. The presence of a second reactive sulphydryl residue which was protected by Mn^{2+} was confirmed using 5,5'-dithiobis(2-nitrobenzoic acid). Because of the protection afforded by Mn^{2+} against DNFB (and TNBS) modification, it is suggested that this sulphydryl residue could be a ligand for the enzyme-bound Mn^{2+}. Studies on the modification of PEP carboxykinase with NEM, DNFB and diazobenzene sulphonic acid (DBS) at varying conditions of pH indicated a group of pK_a about 6.5. All available evidence is consistent with this group being a histidine residue. It is suggested that this group could also be a ligand for Mn^{2+} as this cation affords protection to this group against DBS modification.

Evidence pertaining to the mechanism of action of PEP carboxykinase has been obtained from N.M.R. studies on Mn^{2+} and substrate binding, initial velocity and product inhibition studies on the carboxylation reaction and an analysis of the properties and requirements of the CO_2:OAA exchange reaction. Mn^{2+} binds directly to the enzyme while PEP, IDP, ITP and OAA were shown to bind independently to the binary enzyme.Mn^{2+} complex. The nucleotide binds tightly to the enzyme even in the absence of metal ion. Kinetic analysis indicated a sequential mechanism for PEP carboxykinase. The central complex consisting of enzyme, Mn^{2+}, IDP, PEP and CO_2 is formed

by a preferred pathway where IDP binds first followed by a random addition of PEP and CO_2. However, it would appear that CO_2 is normally bound last (or released first) as the CO_2:OAA exchange reaction is more rapid than theoverall carboxylation or decarboxylation reactions. There is strong evidence for the participation of two metal ions in the catalysis. Studies on the CO_2:OAA exchange reaction have provided considerable evidence that the reaction catalysed by this enzyme occurs via a two-step mechanism with the phosphoryl transfer from PEP to IDP preceding the carboxylation of an enzyme-bound enolpyruvate intermediate to OAA. A mechanism is proposed which can account for all the available evidence and invokes the participation of metal-bound hydroxyl ions.

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 which has been previously published or written by any other person, except where due reference is made in the text.

R.J. BANNS

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ABBREVIATIONS

The following abbreviations have been used in this thesis:

ATP	adenosine-5'-triphosphate
ADP	adenosine-5'-diphosphate
B.S.A.	bovine serum albumin
CoA	coenzyme A
DBS	diazobenzene sulphonic acid
DNFB	1-fluoro-2,4-dinitrobenzoic acid
E	enzyme
E.P.R.	electron paramagnetic resonance
GTP	guanosine-5'-triphosphate
GDP	guanosine-5'-diphosphate
GSH	reduced glutathione
ITP	inosine-5'-triphosphate
IDP	inosine-5'-diphosphate
IMP	inosine-5'-monophosphate
NEM	N-ethylmaleimide
N.M.R.	nuclear magnetic resonance
OAA	oxaloacetic acid
PEP	phosphoenolpyruvate
P_i, PP_i	inorganic phosphate, inorganic pyrophosphate
PRR	proton relaxation rate
TNBS	2,4,6-trinitrobenzene sulphonic acid

Definition

The term conformational change is used in this thesis in its fullest sense to denote any spatial, electronic, hydration *etc.* changes in the protein components of the active centre.

CHAPTER 1. GENERAL INTRODUCTION

1.1. INTRODUCTION

The importance of CO_2 fixation in photosynthetic and autotrophic organisms is readily appreciated as all carbon atoms in these organisms are derived from this source. However, in the case of heterotrophic organisms, the importance of CO_2 fixation is less readily appreciated because the evolution of CO_2 from decarboxylation reactions masks the CO_2 fixation process. Wood and Werkman (1938) first demonstrated a net CO_2 uptake in heterotrophic organisms following their observation that propionic bacteria used substrate amounts of CO_2 when grown on glycerol and proposed the condensation of CO_2 and a three-carbon compound. Over the last thirty years, the phenomenon of CO_2 fixation in heterotrophic organisms has been fully recognised and extensively investigated and it is interesting to reflect that the enzyme which was largely responsible for the original CO_2 fixation observation was not characterised until 1962 (Siu and Wood, 1962). The importance of this class of reaction in heterotrophic organisms has since been demonstrated by the number of enzymes capable of net CO_2 fixation and their strategic position in the metabolic scheme.

Wood and Stjernholm (1962) defined primary CO_2 fixation as a reaction by which CO_2 is combined with some acceptor molecule to form a new carbon-carbon bond which results in a net fixation of CO_2. Thus, excluding those enzymes where CO_2 is affixed to a non-carbon atom, e.g., carbamyl

phosphate formation, these authors divided those enzymes capable of net CO_2 fixation into three classes depending on their mode of action:

(a) fixation of CO_2 by reduction,

(b) fixation of CO_2 by biotin-containing enzymes,

(c) fixation of CO_2 by enoyl compounds.

(a) Fixation of CO_2 by reduction

(i) malate dehydrogenase (decarboxylating) E.C. 1.1.1.38-40,

(ii) isocitrate dehydrogenase (decarboxylating) E.C. 1.1.1.41-42

(iii) 6-phosphogluconate dehydrogenase (decarboxylating) E.C. 1.1.1.44.

When Ochoa (1945) demonstrated the reversibility of the reaction catalysed by isocitrate dehydrogenase (E.C. 1.1.1.42; Reaction 1.1), he provided the first demonstration of a net CO_2 fixation using a purified enzyme from a heterotrophic tissue. Similarly, the malate enzyme (E.C. 1.1.1.40; Reaction 1.2) was shown to be reversible (Ochoa et al., 1948).

$$L_s\text{-isocitrate} + NADP \xrightleftharpoons{Mn^{2+}} \alpha\text{-oxoglutarate} + CO_2 + NADPH_2 \quad \ldots (1.1)$$

$$L\text{-malate} + NADP \xrightleftharpoons{Mn^{2+}} \text{pyruvate} + CO_2 + NADPH_2 \quad \ldots (1.2)$$

Analogous enzymes for (i) and (ii) will utilise NAD. Energy for carboxylation would be provided by the splitting of C-H bonds of $NADPH_2$ and $NADH_2$ although the preferred direction of reaction is decarboxylation.

(b) Fixation of CO_2 by biotin-containing enzymes

(i) pyruvate carboxylase E.C. 6.4.1.1.

(ii) acetyl-CoA carboxylase E.C. 6.4.1.2.

(iii) propionyl CoA carboxylase E.C. 6.4.1.3.

(iv) β-methylcrotonyl CoA carboxylase E.C. 6.4.1.4.

All the enzymes of this group are characterised by a two-step mechanism involving the obligatory intermediate, enzyme-CO_2 (Kaziro and Ochoa, 1961). With one exception, the free energy of hydrolysis of the terminal phosphate of ATP is used to activate the CO_2 molecule (Reactions 1.3-5).

$$\text{E.biotin} + HCO_3^- + \text{ATP} \xrightleftharpoons{Mg^{2+}} \text{E.biotin-}CO_2 + \text{ADP} + P_i \quad \ldots (1.3)$$

$$\text{E. biotin-}CO_2 + RCH_2COSCoA \rightleftharpoons \text{E. biotin} + \overset{COO^-}{\underset{|}{R}}CHCOSCoA \quad \ldots (1.4)$$

$$\underline{\text{SUM}}: \quad HCO_3^- + \text{ATP} + RCH_2COSCoA \xrightleftharpoons{Mg^{2+}} \overset{COO^-}{\underset{|}{R}}CHCOSCoA + \text{ADP} + P_i \quad \ldots (1.5)$$

The acceptor molecule is a Coenzyme A ester, e.g., acetyl CoA for acetyl CoA carboxylase, except for pyruvate carboxylase where pyruvate is the acceptor. However, pyruvate carboxylase from liver (Keech and Utter, 1963) and kidney (Ling and Keech, 1966) shows an absolute requirement for catalytic amounts of the acyl CoA ester, acetyl CoA, while microbial pyruvate carboxylases either are stimulated by this ester as in the case of yeast pyruvate carboxylase (Ruiz-Amil, et al., 1965) or are unaffected as with the enzymes from Aspergillus niger (Bloom and Johnson, 1962) and Pseudomonas citronellolis (Seubert and Remberg, 1961).

The exception to the reaction scheme detailed above (Reactions 1.3-5) is methylmalonyl CoA carboxytransferase occurring in propionic acid bacteria (Wood et al., 1963). This enzyme does not fix CO_2, but rather transfers the carboxyl group of methylmalonyl CoA to pyruvate to give OAA (Reactions 1.6-8).

methylmalonyl CoA + E.biotin $\rightleftharpoons$ E.biotin-CO_2 + propionyl CoA ... (1.6)

pyruvate + E.biotin-CO_2 $\rightleftharpoons$ E.biotin + OAA ... (1.7)

SUM: methylmalonyl CoA + pyruvate $\rightleftharpoons$ propionyl CoA + OAA ... (1.8)

This series of reactions emphasises the role of biotin as a transcarboxylating agent. The biotin is attached to the protein through the ε-NH_2 of a lysine residue and the biotin-CO_2 intermediate has been shown to be 1'-N-carboxybiotin (Knappe, Ringelmann and Lynen, 1961; Numa, Ringelmann and Lynen, 1964).

(c) Fixation of CO_2 by enol compounds

(i) phosphoenolpyruvate carboxylases

(a) phosphopyruvate carboxylase (phosphorylating)
E.C. 4.1.1.31.

(b) phosphopyruvate carboxylase (transphosphorylating)
E.C. 4.1.1.32.

(c) phosphopyruvate carboxylase (transphosphorylating)
E.C. 4.1.1.--.

(d) phosphopyruvate carboxylase (transphosphorylating)
E.C. 4.1.1.38.

(ii) ribulose diphosphate carboxylase E.C. 4.1.1.39.

(iii) phosphoribosyl-aminoimidazole carboxylase
E.C. 4.1.1.21.

(i) The subject matter of this thesis is concerned with this group of enzymes and discussion of their properties will be left to subsequent sections.

(ii) Ribulose diphosphate carboxylase catalyses a primary CO_2 fixation reaction in all photosynthetic tissues (Calvin and Basshan, 1962). Ribulose-1,5-diphosphate is carboxylated to yield two equivalents of 3-phosphoglyceric acid.

(iii) This enzyme catalyses the condensation of a carboxyl group to position 4 of the imidazole ring of 5'-phosphoribosyl-5-aminoimidazole to give 5'-phosphoribosyl-5-amino-4-imidazole carboxylate during the biosynthesis of purine nucleotides (Lukens and Buchanan, 1959).

The energy of carboxylation by this group of enzymes is provided by cleavage of the enolphosphate C-P bond in (i), the C_3-C_4 bond of the ene-diol in (ii), and the C_4-H bond of the ene-amine in (iii) (Wood and Stjernholm, 1962). In the case of PEP carboxykinase (b,c) and PEP carboxytransphosphorylase (d), the free energy of hydrolysis of the enolphosphate bond is effectively trapped by the formation of GTP (or ATP) and PP_i respectively so that these reactions are freely reversible.

1.2. FIXATION OF CO_2 BY PHOSPHOENOLPYRUVATE

This group of enzymes catalyses the formation of OAA with PEP as the CO_2 acceptor. They differ in phosphate acceptor, as shown:

$$CO_2 + PEP \xrightarrow{\begin{matrix}(a)\ H_2O \\ (b)\ GDP \\ (c)\ ADP \\ (d)\ P_i\end{matrix}\ \ Mn^{2+}} OAA + \begin{matrix}(a)\ P_i \\ (b)\ GTP \\ (c)\ ATP \\ (d)\ PP_i\end{matrix} \qquad \ldots (1.9)$$

The first two members of this group, PEP carboxylase (orthophosphate : oxaloacetate carboxy-lyase (phosphorylating), E.C. 4.1.1.31) which uses water as the phosphate acceptor

$$PEP + CO_2 + H_2O \xrightarrow{Mg^{2+}} OAA + P_i \qquad \ldots (1.10)$$

and PEP carboxykinase (GTP : oxaloacetate carboxy-lyase (transphosphorylating), E.C. 4.1.1.32) which uses GDP (or IDP) as phosphate acceptor

$$PEP + CO_2 + GDP \xrightarrow{Mn^{2+}} OAA + GTP \qquad \ldots (1.11)$$

were discovered almost simultaneously by Bandurski and Greiner (1953) in plants and Utter and Kurahashi (1954) in chicken liver respectively. The third member of this group is found in some micro-organisms, e.g., yeast (Cannata and Stoppani, 1963a,b) and is similar to the mammalian enzyme except that it uses adenine nucleotides rather than guanosine (or inosine) nucleotides. It was not until 1961 that the fourth member, PEP carboxytransphosphorylase (pyrophosphate : oxaloacetate

carboxy-lyase (transphosphorylating), E.C. 4.1.1.38) which uses inorganic phosphate as phosphate acceptor

$$PEP + CO_2 + P_i \xrightleftharpoons{Mg^{2+}} OAA + PP_i \quad \ldots (1.12)$$

was discovered by Wood and his co-workers in propionic acid bacteria (Siu, Wood and Stjernholm, 1961; Siu and Wood, 1962).

Compared with the other members of this group, PEP carboxylase shows one basic difference in that the reaction is essentially irreversible (Bandurski, 1955; Tchen, Loewus and Vennesland, 1955). The other members catalyse the reversible carboxylation of PEP. With PEP carboxykinase, the rate of decarboxylation of OAA is faster than the rate of carboxylation of PEP (Chang, et al., 1966; this thesis, Chapter 3) while with PEP carboxytransphosphorylase, carboxylation is the preferred direction (Lochmuller, Wood and Davis, 1966). These observations would therefore suggest a role for PEP carboxykinase in gluconeogenesis by forming PEP from OAA. The other members of the group would fulfil an "anaplerotic" role (Kornberg, 1965), i.e., maintaining, via OAA formation, the level of citric acid cycle intermediates which have been depleted by various synthetic reactions.

1.3. INTERCONVERSION OF PEP, OAA AND PYRUVATE AND THE METABOLIC ROLE OF PEP CARBOXYLATION REACTIONS

The first crucial step in the formation of glycogen or glucose from lactose, alanine or other pyruvate precursors in liver and kidney is the formation of PEP. This could occur through the reversal of pyruvate kinase whose reversibility has been demonstrated (Lardy and Ziegler, 1945). However, the physiological significance of this observation was questioned on energetic grounds (Krebs, 1954) and its maximal contribution in liver to PEP synthesis is only a small fraction of that needed for gluconeogenesis (Utter, 1963). The discovery of PEP carboxykinase in chicken liver (Utter and Kurahashi, 1954) provided the first link in the by-pass of pyruvate kinase by conversion of OAA to PEP. This "by-pass" would require a GTP-generating system which may be supplied by succinyl CoA synthetase (Mudge, et al., 1954; Nordlie and Lardy, 1963a) or nucleoside diphosphate kinase (Gevers, 1967).

Utter and Kurahashi (1954a) and Krebs (1954) postulated that PEP was synthesised from pyruvate as follows:

$$\text{pyruvate} + CO_2 + NADPH_2 \xrightarrow{\text{malate enzyme}} \text{malate} + NADP \quad \ldots (1.2)$$

$$\text{malate} + NAD \xrightarrow[\text{dehydrogenase}]{\text{malate}} OAA + NADH_2 \quad \ldots (1.13)$$

$$OAA + GTP \xrightarrow[\text{carboxykinase}]{\text{PEP}} PEP + GDP + CO_2 \quad \ldots (1.11)$$

<u>SUM</u>: pyruvate + GTP + $NADPH_2$ + NAD ⟶ PEP + GDP + NADP + $NADH_2$ ... (1.14)

However, several factors argue against the involvement of the malate enzyme in this scheme, <u>viz</u>., (a) washed chicken liver mitochondria were essentially devoid of malate enzyme and yet they were capable of synthesising significant amounts of PEP from pyruvate (Mendicino and Utter, 1962), (b) the $K_{m(app)}$ values for both pyruvate and HCO_3^- are high (Utter, 1963), (c) hormone administration or metabolic conditions which favour gluconeogenesis produce either no effect on this enzyme or depress it (Hardy, <u>et al</u>., 1964) and this activity is virtually absent from ruminant liver (Hanson and Ballard, 1968; Young, Thorp and de Lumen, 1969).

A re-examination of liver mitochondria resulted in the demonstration of pyruvate carboxylase (Utter and Keech, 1960; Keech and Utter, 1963) which carboxylates pyruvate to form OAA thus completing the dicarboxylic acid "by-pass" (Utter, 1963):

$$\text{pyruvate} + \text{ATP} + CO_2 \xrightarrow[\text{carboxylase}]{\text{pyruvate}} \text{OAA} + \text{ADP} + P_i \quad \ldots (1.15)$$

$$\text{OAA} + \text{GTP} \xrightarrow[\text{carboxykinase}]{\text{PEP}} \text{PEP} + \text{GDP} + CO_2 \quad \ldots (1.11)$$

<u>SUM</u>: pyruvate + ATP + GTP ⟶ PEP + GDP + ADP P_i ... (1.16)

Since two equivalents of nucleoside triphosphate are required, the energetics of the pathway are favourable for PEP synthesis and an efficient coupling of purified pyruvate carboxylase and PEP carboxykinase has been demonstrated (Utter and Keech, 1963). In contrast to the malate enzyme, the capacity of pyruvate carboxylase always appears to be sufficient to account for the observed rate of gluconeogenesis in rat liver (Henning, Seiffert and Seubert, 1963) and sheep liver (Filsell, Jarrett, Taylor and Keech, 1969).

This dicarboxylic acid pathway for PEP formation from pyruvate appears to be common to all vertebrates and yeast (Utter, 1969). However, certain bacteria possess another mechanism of PEP formation from pyruvate. Discovery of PEP carboxytransphosphorylase in propionic acid bacteria (Reaction 1.12; Siu and Wood, 1962) suggested a coupling of this enzyme and pyruvate carboxylase (Reaction 1.15) for PEP formation from pyruvate. Such a coupling was found to be energetically feasible using chicken liver pyruvate carboxylase (Wood, et al., 1966) although less efficient than the pyruvate carboxylase and PEP carboxykinase couple (Reactions 1.15; 11; 16). Cell-free extracts of these bacteria do catalyse OAA formation from pyruvate, CO_2 and ATP as expected for pyruvate carboxylase but there was a requirement for P_i (Wood, 1968). Cooper and Kornberg (1965) described the properties of a new enzyme from E. coli, PEP synthetase (Reaction 1.17), which in combination with PEP carboxytransphosphorylase would be expected to

$$\text{pyruvate} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{PEP}} \text{PEP} + \text{AMP} + P_i \quad \ldots (1.17)$$

carry out OAA synthesis. However, this would not explain the P_i requirement. Further studies by Evans and Wood (1968) demonstrated the presence of pyruvate:phosphate dikinase which had been reported in tropical grasses by Hatch and Slack (1968). This effects the reversal of the pyruvate kinase reaction by utilising the free energy of hydrolysis of the two terminal phosphate bonds of ATP (Reaction 1.18) and in combination with PEP carboxytransphosphorylase would bring about the observed OAA synthesis (Reactions 1.18; 12; 19).

$$\text{pyruvate} + \text{ATP} + P_i \xrightarrow[\text{dikinase}]{\text{pyruvate:phosphate}} \text{PEP} + \text{AMP} + \text{PPi} \quad \ldots (1.18)$$

$$\text{PEP} + CO_2 + P_i \xrightarrow[\text{transphosphorylase}]{\text{PEP carboxy-}} \text{OAA} + PP_i \quad \ldots (1.12)$$

SUM: $\text{pyruvate} + CO_2 + \text{ATP} + 2P_i \longrightarrow \text{OAA} + \text{AMP} + 2PP_i \quad \ldots (1.19)$

E. coli do not possess pyruvate carboxylase or PEP carboxytransphosphorylase although they can effect an OAA synthesis as do the propionic acid bacteria. This is achieved by PEP carboxylase (Canovas and Kornberg, 1965) coupled with

PEP synthetase (Cooper and Kornberg, 1965) (Reactions 1.17; 10; 20).

$$\text{pyruvate} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{PEP}} \text{PEP} + \text{AMP} + P_i \qquad \ldots (1.17)$$

$$\text{PEP} + CO_2 + H_2O \xrightarrow[\text{carboxylase}]{\text{PEP}} \text{OAA} + P_i \qquad \ldots (1.10)$$

SUM: $\text{pyruvate} + \text{ATP} + CO_2 + H_2O \rightleftharpoons \text{OAA} + \text{AMP} + 2P_i$ ... (1.20)

This OAA synthesis by PEP carboxylase is essential for maintaining the levels of citric acid cycle intermediates, i.e., it serves an "anaplerotic" function (Kornberg, 1965). This is demonstrated clearly by growth responses of mutants of E. coli (Amarsingham, 1959; Ashworth, Kornberg and Ward, 1965) and Salmonella typhimurium (Theodore and Englesberg, 1964) which lack PEP carboxylase. These mutants, although they possess malate enzyme and PEP carboxykinase, will only grow on pyruvate or its precursors if citric acid cycle intermediates are added to the growth medium.

The majority of plants effect the primary CO_2 fixation reaction by ribulose diphosphate carboxylase (Calvin and Bassham, 1962). However, recent evidence has shown that in

some species of grasses the first carboxylation products are OAA, aspartate and malate and not 3-phosphoglycerate (Hatch and Slack, 1966; Hatch, Slack and Johnson, 1967) consistent with PEP carboxylase catalysing the primary carboxylation reaction. It was subsequently shown that in those species where this labelling pattern occurred, ribulose diphosphate carboxylase was much less active than in other photosynthetic tissues and that the PEP carboxylase activity, present in the chloroplast, was much higher than the photosynthetic capacity of these tissues (Slack and Hatch, 1967). The requirement of PEP synthesis demanded by this new carboxylation pathway is achieved by pyruvate: phosphate dikinase (Reaction 1.18) which was demonstrated in those tissues showing this new carboxylation reaction (Hatch and Slack, 1968; Johnson and Hatch, 1968). Thus, the couple producing OAA would be:

$$\text{pyruvate} + \text{ATP} + P_i \xrightarrow[\text{dikinase}]{\text{pyruvate:phosphate}} \text{AMP} + \text{PEP} + PP_i \qquad \ldots (1.18)$$

$$\text{PEP} + CO_2 + H_2O \xrightarrow[\text{carboxylase}]{\text{PEP}} \text{OAA} + P_i \qquad \ldots (1.10)$$

SUM: $\text{pyruvate} + \text{ATP} + CO_2 + H_2O \rightleftharpoons \text{OAA} + \text{AMP} + PP_i \qquad \ldots (1.21)$

It is noteworthy that PEP carboxykinase in animals, yeast and certain bacteria and PEP carboxytransphosphorylase in the propionic acid bacteria catalyse similar reversible reactions but appear to function quite differently; the former acting as a decarboxylase and the latter as a carboxylase. Also, E. coli, propionic acid bacteria, certain other micro-organisms and plant tissues phosphorylate pyruvate directly with ATP whereas in animals, yeast and certain bacteria this is effected via OAA. On the other hand, animals, yeast and some bacteria form OAA directly from pyruvate whereas propionic acid bacteria, E. coli and certain bacteria and plant tissues achieve this via PEP.

1.4. (a) PEP CARBOXYLASE

This enzyme was first demonstrated in spinach leaves by Bandurski and Greiner (1953) and was shown to catalyse the irreversible carboxylation of PEP to OAA and P_i (Reaction 1.10). It has a wide distribution in plant tissues and micro-organisms including wheat germ (Tchen and Vennesland, 1955), peanut cotyledons (Maruyama and Lane, 1962), tropical grasses (Hatch, Slack and Johnson, 1967), E. coli (Canovas and Kornberg, 1965), Thiobacillus thiooxidans (Suzuki and Werkman, 1958) and Salmonella typhimurium (Maeba and Cook, 1965). In the topical grasses where this enzyme catalyses the primary CO_2 fixation reaction, it is located largely in the

chloroplast (Slack and Hatch, 1967). It has not been detected in animal tissues.

PEP carboxylase from S. typhimurium exists in dilute solutions as a tetramer of molecular weight 200,000 and undergoes a concentration-dependent aggregation over the range 2 mg per ml to 5 mg per ml (Maeba and Sanwal, 1965). Sodium dodecyl sulphate caused a disaggregation to a monomer unit of molecular weight 49,000 with a concomitant exposure of four sulphydryl groups. Amino acid analysis gives also a minimum molecular weight of 49,000. Sedimentation analysis of the E. coli enzyme (Smith, 1968) indicated that this enzyme existed as a dimer of molecular weight 188,000. 1 M urea caused dissociation to a monomeric unit while Mg^{2+} and aspartate, an allosteric inhibitor (Maeba and Sanwal, 1965), resulted in the formation of a tetramer. Both dimer and tetramer appear to be catalytically active. Maruyama et al. (1966) obtained a molecular weight of 350,000 from gel filtration analysis of the peanut cotyledon enzyme. Although no analysis was made of subunit structure, N.M.R. studies of Mn^{2+} binding indicated six moles of Mn^{2+} bound per 300,000 g of enzyme protein (Miller et al., 1968). This would also be consistent with a polymeric structure with a monomeric unit of about 50,000.

In all cases, PEP carboxylase is inhibited by sulphydryl reagents such as p-hydroxymercuribenzoate and stimulated by reducing agents such as GSH. This would suggest a sulphydryl

residue in the active centre although no analysis of this has been reported. Results of Maruyama et al. (1966) suggest the involvement of an imidazole residue in Mg^{2+} binding. This conclusion was derived from the susceptibility of the peanut cotyledon enzyme to diazo-benzene sulphonic acid and the presence of a residue whose pK_a = 7.3 associated with Mg^{2+} and PEP binding. Obviously, this would not exclude a sulphydryl residue being responsible for the observations as diazonium salts will react with both sulphydryl and imidazole residues (Landsteiner, 1945). Further analysis of this inhibition is required before an imidazole group is positively implicated.

Like other members of this group of enzymes, several divalent cations are active including Mg^{2+}, Mn^{2+} and Cd^{2+}. Using the paramagnetic ion, Mn^{2+}, N.M.R. studies indicated that Mn^{2+} was bound directly by the enzyme (Miller et al., 1968). Therefore, this enzyme may be classified as a Type II enzyme (Mildvan and Cohn, 1970). Addition of PEP to the enzyme.Mn^{2+} binary complex decreased the enhancement of the proton relaxation rate of water by the binary complex. This is consistent with PEP binding through the metal ion in a "metal bridge" structure (Mildvan and Cohn, 1970). Also consistent with this structure is the data of Maruyama et al. (1966) showing a group whose pK_a = 7.3 common to the binding of PEP and Mg^{2+}.

The reaction catalysed by PEP carboxylase has a sequential mechanism as no partial reactions have been demonstrated (Bandurski, 1955; Tchen and Vennesland, 1955; Maruyama and Lane, 1962) in contrast to the CO_2:OAA exchange reaction catalysed by PEP carboxykinase (Utter and Kurahashi, 1954) and PEP carboxytransphosphorylase (Wood, Davis and Willard, 1969). The keto and not the enol form of OAA is the primary carboxylation product of PEP carboxylase (Tchen, Loewus and Vennesland, 1955), while HCO_3^- appears to be the active species of CO_2 in the reaction (Maruyama *et al.*, 1966). On the other hand, the other two members of this group of enzymes both appear to use CO_2 and not HCO_3^- or CO_3^{2-} (Cooper *et al.*, 1968). Although there are inherent assumptions in the interpretation of the data of Maruyama *et al.* (1966) there are other points which favour this conclusion, *viz.*, the high pH optimum of pH 8.0 and the low Km_{app} for HCO_3^- of 3.1×10^{-4} M at pH 7.9. Those enzymes which use CO_2 all exhibit a very high Km_{app} for HCO_3^-.

A mechanism was proposed consisting of nucleophilic attack by a bicarbonate oxygen on the enol-phosphoryl phosphorus atom leading to a transition state (Fig. 1.1). The simultaneous displacement at the bicarbonate carbon and phosphoryl phosphate atoms leads to the keto form of OAA. Binding of PEP through Mn^{2+} in a manner similar to that proposed for pyruvate kinase (Mildvan and Cohn, 1966) would

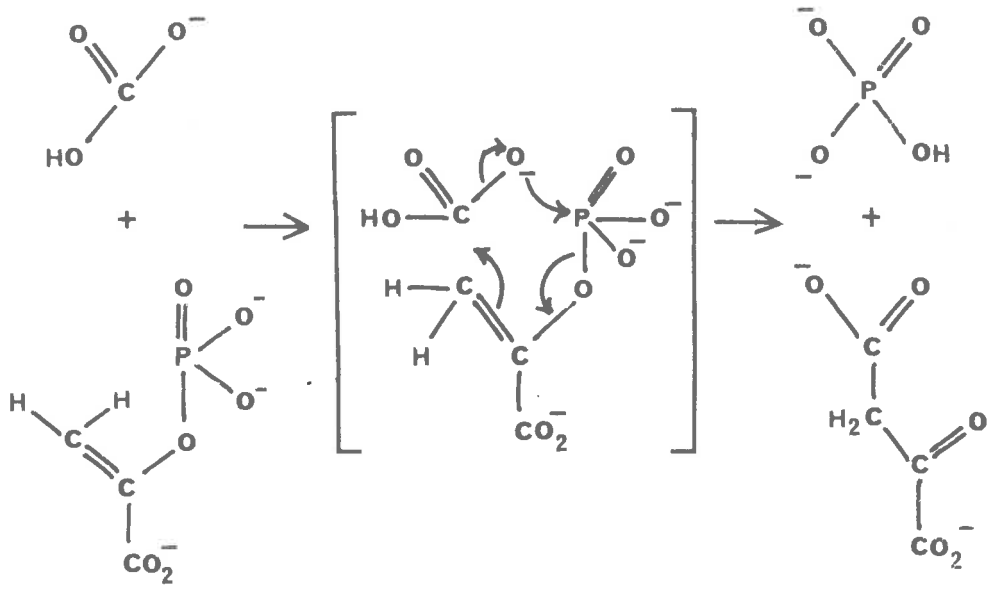


FIG. 1.1. The mechanism proposed by Maruyama et al. (1966) for peanut cotyledon PEP carboxylase.

enhance the positive character of the phosphoryl phosphorus thus facilitating nucleophilic attack by the bicarbonate oxygen.

In keeping with its important metabolic role of replenishing citric acid cycle intermediates, bacterial PEP carboxylase is subject to a multiplicity of controls. These controls are qualitatively the same for the enzymes from E. coli and S. typhimurium although no such controls have yet been reported for the enzyme from plants.

The enzyme exhibits positive cooperativity with respect to PEP (Maeba and Sanwal, 1969; Corwin and Fanning, 1968), is activated by acetyl CoA (Maeba and Sanwal, 1965; Canovas and Kornberg, 1965) and is subject to precursor activation by fructose-1,6-diphosphate (Sanwal and Maeba, 1966). Transamination of the enzymic product gives aspartate which is a powerful feed-back inhibitor of the enzyme (Maeba and Sanwal, 1965; Nishikida et al., 1965; Izui et al., 1967) while the E. coli enzyme also appears to be susceptible to other related metabolites such as malate and succinate (Corwin and Fanning, 1968). A partially specific activation by nucleotides was shown by Sanwal and Maeba (1966a) with CDP, GTP and CMP being the most active. However, the partial specificity and the high nucleoside diphosphate kinase activity would suggest that the size of the nucleotide pool is the

is the controlling factor as appears to be the case for aspartate transcarbamylase (Gerhart and Pardee, 1962) where a variety of nucleotides exert a feed-back effect on the enzyme. This nucleotide activation which is potentiated by acetyl CoAwould allow OAA formation for energy requirements under conditions where aspartate levels are high.

Some insight into the mechanism of control of PEP carboxylase by the above allosteric effectors was provided by Sanwal, Maeba and Cook (1966). The enzyme was activated by polycations such as polylysine and yet was still susceptible to modulations by aspartate and fructose-1,6-phosphate. In addition, non-polar solvents such as dioxane, ethanol, 2-propanol, dimethylsulphoxide and propylene glycol activate the enzyme reversibly and, apart from 10% (v/v) dioxane with aspartate and fructose-1,6-diphosphate, the enzyme is not desensitised by this treatment. At the low concentrations of solvents used, the prime effect would be expected to be the weakening of hydrophobic bonds (Kauzmann, 1959) while the increase in pK_a of ionising groups and the resultant alterations in ionic interactions in the protein (Iizuka and Yang, 1965) would be secondary. However, in view of the activation by polycations, this secondary effect may well be quite significant causing an effect analogous to that of the polycations on the ionic interactions in the protein. The desensitisation could result from the effect of dioxane on the hydro-

phobic interactions in the protein at the concentration of dioxane used. None of the activators or inhibitors of S. typhimurium PEP carboxylase caused any apparent change in the polymeric state of the enzyme. Thus, changes in the state of aggregation of the enzyme are not necessarily a prerequisite for, or a consequence of, activation or inhibition.

(b) PEP CARBOXYTRANSPHOSPHORYLASE

Siu and Wood (1962) first demonstrated the presence of PEP carboxytransphosphorylase in propionic acid bacteria and this remains the only reported source of the enzyme. It has been purified to near homogeneity and readily crystallises as small needles (Lochmuller, Wood and Davis, 1966). It catalyses the reversible carboxylation of PEP in the presence of P_i to give OAA and PP_i (Reaction 1.12). Carboxylation occurs seven-fold faster than decarboxylation while in the absence of HCO_3^-, the enzyme will catalyse the irreversible

$$PEP + P_i \xrightarrow{Mg^{2+}} \text{pyruvate} + PP_i \qquad \ldots (1.22)$$

dephosphorylation of PEP to pyruvate (the pyruvate reaction; Reaction 1.22). The pyruvate reaction is strongly inhibited by HCO_3^- and reducing compounds such as GSH and occurs at about one-tenth the rate of carboxylation (Lochmuller, Wood and Davis, 1966).

The native protein has an apparent molecular weight of 430,000 although an active form of the enzyme with about one-third the specific activity of the larger molecule has been observed (Wood, Davis and Willard, 1969). The smaller unit has a sedimentation coefficient of 7.4S compared with 15.2S for the native enzyme. Treatment of the enzyme with 6 M urea resulted in an inactive 7.0S protein unit (Lochmuller, Wood and Davis, 1966). These results would suggest that the native enzyme is probably tetrameric.

The requirement for reducing compounds such as GSH for maximum activity is characteristic of this enzyme group. It would suggest the presence in the active site of a sulphydryl group whose integrity is essential for activity.

The metal ion requirement is somewhat more complex than that for the other enzymes of this group although the cations which are active, Mg^{2+}, Mn^{2+} and Co^{2+}, are characteristic. These metal ions are active with both the CO_2 fixation and pyruvate reactions while both reactions are inhibited by 10^{-5} M EDTA even in the presence of 12 mM Mg^{2+} (Davis, Willard and Wood, 1969). However, 10^{-4} M EDTA causes only 10% inhibition of the CO_2:OAA exchange reaction in the presence of PP_i (Wood, Davis and Willard, 1969) suggesting that the enzyme still retains its active conformation. Thus, it was postulated that two metal ions are required: Type I which is dissociable (Mg^{2+}, Co^{2+} and Mn^{2+}) and a firmly bound metal, Type II, as

yet unidentified, whose function is blocked by chelators (Davis, Willard and Wood, 1969). The inhibition by EDTA is not relieved by dialysis. Although Co^{2+} will relieve the EDTA inhibition, this relief was not the result of Co^{2+} binding to an apoenzyme (Willard, Davis and Wood, 1969).

The requirement for Cu^{2+} to relieve the EDTA inhibition of the pyruvate reaction led Davis, Willard and Wood (1969) to propose a heavy metal ion requirement for the pyruvate reaction. However, such a metal ion would need to be firmly bound to the enzyme as the deactivation of the pyruvate reaction by thiol compounds was reversible by dialysis with no Cu^{2+} requirement. Co^{2+} was capable of removing EDTA, thus the Cu^{2+} requirement was additional to EDTA removal. Perhaps a more attractive explanation would be that Cu^{2+} reversibly oxidises a susceptible group on the enzyme in the presence of molecular oxygen. EDTA treatment removes the trace amounts of Cu^{2+}; thus, the pyruvate reaction is inactive until oxidation is promoted by added Cu^{2+}. Such an oxidation would need to be reversible by thiol compounds. Therefore, oxidation of a sulphydryl group to a disulphide or even a sulphone would explain the data. Kobashi and Horecker (1967) have reported the reversible oxidation of sulphydryl residues of rabbit muscle aldolase by Cu^{2+} complexes and molecular oxygen. Also, Morgan *et al.* (1969) reported an irreversible

inactivation of pig and ox liver carboxylesterases in the presence of hydroxylamine, molecule O_2 and trace amounts of Cu^{2+} although, in this case, studies with model systems indicated oxidation of tyrosine residues (Fernley and Zerner, 1968).

As is the case for PEP carboxykinase, CO_2 was the active species of CO_2 involved in the enzymic carboxylation of PEP by PEP carboxytransphosphorylase (Cooper et al., 1968).

Wood, Davis and Willard (1969) proposed the reaction mechanism detailed in Fig. 1.2. The CO_2:OAA exchange reaction which requires PP_i and Type I metal for maximum activity would occur by Reaction 1.23 where Mg^{2+} forms a bridge between enzyme and pyrophosphate. OAA combines with this complex to form pyrophosphoenol-OAA which is then reversibly decarboxylated to enzyme-bound pyrophosphoenolpyruvate (I). It is this intermediate which is common to both the carboxylation and pyruvate reactions. Type I metal ion facilitates hydrolysis of the pyrophosphate bond so that PEP becomes bound through the Type II metal ion and the remaining phosphate bound through Mg^{2+} (Reaction 1.24), i.e., carboxylation and decarboxylation occur via Reactions 1.23-24. In the pyruvate reaction, H_2O replaces CO_2 and hydrolysis of the enzyme.pyrophosphoenolpyruvate intermediate (I) occurs yielding pyruvate and PP_i (Reactions 1.24-25).

The P_i-PEP exchange reaction, which requires both Type I and II metal ions and CO_2, occurs via the reversal of the whole

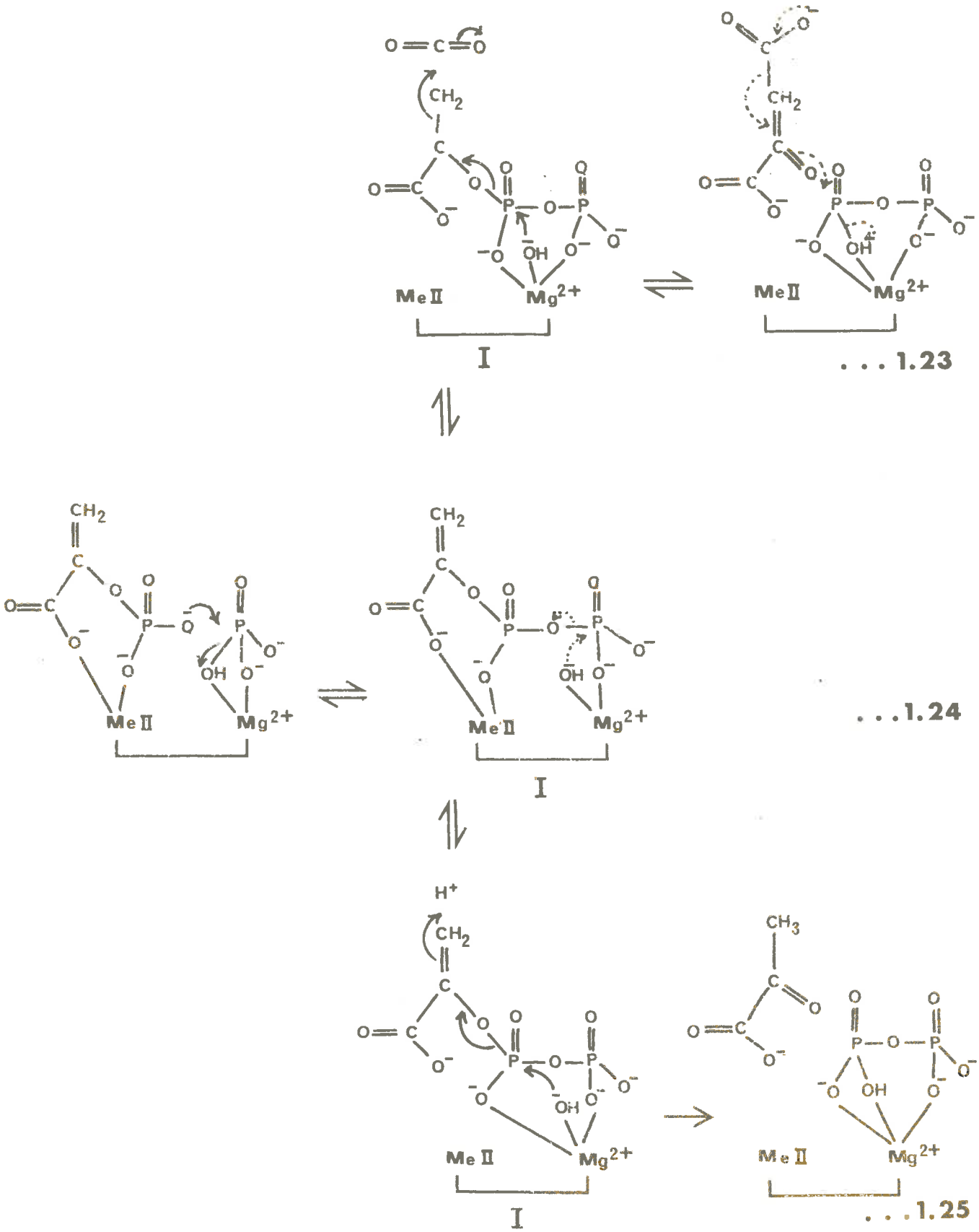


FIG. 1.2. The mechanism proposed by Wood, Davis and Willard (1969) for PEP carboxytransphosphorylase. Dotted arrows show the electron shifts for the decarboxylation of OAA.

reaction and is not strictly a partial reaction. This is also the case for the PP_i:PEP and P_i:PP_i exchange reactions. In fact, the only partial reaction would appear to be a CO_2:OAA exchange reaction in the presence of Mg^{2+}, although this was not recognised by these workers. Addition of PP_i stimulates this exchange at least 30-fold. The fact that 10^{-4} M EDTA only inhibits the exchange reaction about 10% while the full reaction is almost completely inhibited would indicate that the exchange reaction does not rely on the reversal of the complete reaction. Thus, despite the fact that PP_i would turnover in the absence of EDTA, since the decarboxylation system would be complete, this substrate activates the exchange reaction per se, i.e., PP_i must produce a more favourable protein conformation. This would be analogous to the synergistic effect proposed by Bridger, Millen and Boyer (1968) to explain why exchange reactions could be slower than the overall reaction. These workers proposed that in the absence of a particular component of the overall reaction which is not involved in the partial reaction, the enzyme conformation may not be ideal for the full expression of activity.

It is noteworthy that the results of the partial reactions of sheep kidney mitochondrial PEP carboxykinase are very similar to those discussed above for PEP carboxytransphorylase. PEP carboxykinase catalyses a Mn^{2+}-dependent CO_2:OAA exchange

reaction and initial velocity studies of the concentration dependence for the three reactants, CO_2, OAA, and Mn^{2+}, confirmed that this reaction was enzymically catalysed. ITP stimulated the exchange rate 30-fold and this rate was greater than the rate of ITP turnover, while IDP also stimulated the exchange reaction but without undergoing turnover itself. Furthermore, the exchange reaction did not rely on the reversal of the complete reaction (see Chapter 6). Finally, there is evidence that catalysis also requires two metal ions. Thus, it is tempting to conclude that these two enzymes which catalyse such similar reversible reactions have very similar mechanisms. This similarity will be discussed further in the thesis.

The physiological importance of the pyruvate reaction is seriously questionable as these bacteria show high pyruvate kinase activity (Davis, Willard and Wood, 1969). No mechanism for control of PEP carboxytransphosphorylase has been described apart from a suggestion that PP_i may fulfil this role (Davis, Willard and Wood, 1969) as PP_i is a strong inhibitor of both the carboxylation and decarboxylation reactions (Lochmuller, Wood and Davis, 1966). However, this inhibition could be largely due to an ionic strength effect.

(c) PEP CARBOXYKINASE

PEP carboxykinase is the enzyme of particular interest in this thesis and was first detected by Utter and Kurahashi (1954) in chicken liver. It has since been shown to occur in several other species. This activity was originally thought to be confined to liver and kidney (Utter, 1959) but it has subsequently been shown to have significant activity in white muscle (Opie and Newsholme, 1967) and adipose tissue (Ballard and Hanson, 1967). In addition to animal sources, it has been detected in wheat germ (Tchon and Vennesland, 1955), yeast (Cannata and Stoppani, 1963a,b,c) and a variety of micro-organisms (Bates and Werkman, 1960; Uchida and Kikuchi, 1966; Shrago and Shug, 1966; Wright and Sanwal, 1969).

In mammalian tissues, the intracellular distribution of the enzyme varies greatly from species to species with more than 90% of the enzyme localised in the cytosol of rat, mouse and hamster liver (Nordlie and Lardy, 1963) while in the livers of pigeons (Gevers, 1967) and chickens (Utter, 1959) the activity is almost exclusively mitochondrial. Most other species have significant activities in both cellular fractions of liver (Nordlie and Lardy, 1963; Ballard, Hanson and Kronfeld, 1968; Chang and Lane, 1966; Taylor, Wallace and Keech, 1970). The mitochondrial activity is located in the matrix (Landriscina et al., 1970; Taylor, Wallace

and Keech, 1970).

The relationship between cytosol and mitochondrial activities of the enzyme also seems to vary from species to species. In most species where the effects of diet and hormonal changes have been tested on hepatic PEP carboxykinase, only the cytosol activity is adaptive and this activity changes in the same direction as the overall gluconeogenic flux (Gevers, 1967; Lardy et al., 1964; Nordlie, Varricchio and Holten, 1965). Also, only the cytosol activity of T. pyriformis is repressible by glucose (Shrago and Shug, 1966). This differential response would suggest that the two enzymes were different proteins and this has been shown for guinea pig (Holten and Nordlie, 1965) and rat (Ballard and Hanson, 1969). However, both cytosol and mitochondrial activities of sheep liver (Taylor, Wallace and Keech, 1970) and pig liver (Swiatek et al., 1970) vary together, possibly indicating that one protein species exists in these animals.

The mammalian enzyme catalyses the reversible GTP- (or ITP-) dependent decarboxylation of OAA to PEP and GDP (or IDP) (Reaction 1.11). However, the bacterial enzyme shows a different nucleotide specificity from that of the mammalian enzyme. The enzyme from yeast (Cannata and Stoppani, 1963a) and Rhodopseudomonas spheroides (Uchida and Kikuchi, 1966) has an absolute requirement for adenine nucleotides

while that from R. rubrum (Bates and Werkman, 1960) and T. pyriformis (Shrago and Shug, 1966) is active with either adenine or guanine nucleotides but the guanine nucleotides are preferred in the former case, the adenine nucleotides in the latter.

PEP carboxykinase has been prepared from pig liver mitochondria (Chang and Lane, 1966) and rat liver cytosol (Ballard and Hanson, 1969) in a homogeneous state and has a molecular weight of 73,300 and 74,500 respectively. The yeast enzyme has been obtained crystalline and has a molecular weight of 252,000 (Cannata, 1970). The pig liver and yeast enzymes show considerable differences in amino acid composition, despite marked similarities in catalytic properties. No evidence of subunit structure for the enzymes has been reported.

PEP carboxykinase from all sources shows a susceptibility to thiol reagents such as p-hydroxymercuribenzoate and a requirement for thiol compounds such as GSH for maximum activity. This would suggest that the integrity of a sulphydryl residue is essential for activity. An analysis of this requirement has been reported by Barns and Keech (1968) who showed that one sulphydryl residue was modified by N-ethylmaleimide (NEM) and this residue had an apparent catalytic function.

Using Mn^{2+} at pH 7.6, Cooper *et al.* (1968) demonstrated that the active species of CO_2 used by PEP carboxykinase was CO_2 in common with PEP carboxytransphosphorylase. This would be in accord with its optimum pH of 6.5 for carboxylation with Mn^{2+} as the activating cation. However, in Chapter 3 it is shown that, with Mg^{2+} as the cation, the pH optimum is shifted to 7.5. This could suggest that a different CO_2 species was utilised by the different metal ions as the CO_2 concentration would be decreased about 7-fold at pH 7.5 relative to pH 6.5. Yeast PEP carboxykinase would also appear to use CO_2 in accord with the low pH optimum of 5.6 - 6.0 (Cannata and Stoppani, 1963a) whereas it is doubtful that CO_2 would be the active species for the enzyme from *T. pyriformis* as it shows maximum activity at pH 9.5 in the assay of Shrago and Shug (1966).

The metal requirement is again characteristic of this group of enzymes with Mn^{2+}, Mg^{2+} and Co^{2+} all active. The specificity for the metal ion varies with the source of the enzyme, *e.g.*, the yeast enzyme is activated by Zn^{2+} (60% as effective as Mn^{2+}) in contrast to that from other sources (Cannata and Stoppani, 1963b). Within the one tissue, the specificity varies from cytosol to mitochondria, from carboxylation to decarboxylation and with pH. Holten and Nordlie (1965) showed that both cytosol and mitochondrial guinea pig liver activities were specific for Mn^{2+} in the carboxylation direction. However, for the decarboxylation reaction, the cytosol activity

was more specific for Mn^{2+} at all concentrations and pH values tested while the mitochondrial enzyme was more active with Mg^{2+} above 4 mM at pH 8.0 and above. Also, significant was the synergistic effect of Mn^{2+} and Mg^{2+} shown for the decarboxylation reaction at pH 8.0 by both guinea pig liver activities (Holten and Nordlie, 1965) and by the rat cytosol activity (Foster et al., 1967) and, especially in the latter case, the effects of the two metal ions were essentially additive. Such data would be consistent with the involvement of two metal ions in catalysis (cf. PEP carboxytransphosphorylase).

Miller et al. (1968) used equilibrium dialysis, gel filtration and proton relaxation rates of water (PRR) to study the binding of Mn^{2+} and substrates to pig liver mitochondrial PEP carboxykinase. A binary enzyme.Mn^{2+} complex was shown by PRR studies thus classifying the enzyme as Type II (Mildvan and Cohn, 1970). Ternary enzyme.Mn^{2+}.substrate complexes were detected for IDP, ITP, PEP and OAA. Mn^{2+} was shown to enhance markedly PEP binding but to inhibit IDP binding. Analysis of the PRR data on the formation of the PEP and IDP ternary complexes led these workers to conclude that the protein displaces two of the six water molecules co-ordinated to Mn^{2+}, PEP two and IDP one, i.e., a metal-bridge structure (Mildvan and Cohn, 1970) is proposed for the binding of PEP and IDP. Although the data is consistent with this proposal,

especially with respect to PEP binding which is markedly enhanced by Mn^{2+}, it does not eliminate the concept of two metal ions participating in catalysis (Foster et al., 1967). In fact, Miller et al. (1968) report the formation of higher order complexes of the form enzyme.Mn^{2+}.substrate.Mn^{2+} for GDP and GTP consistent with the substrate for the enzyme being $Mn.IDP^{-}$ or $Mn.ITP^{2-}$. Additional evidence presented in this thesis will add weight to the concept that PEP is bound through Mn^{2+} but that $Mn.IDP^{-}$ is the substrate for carboxylation and not IDP^{3-} as proposed by Miller et al. (1968).

Chang et al. (1966) demonstrated that the only exchange catalysed by pig liver mitochondrial PEP carboxykinase was a Mn^{2+} - and ITP-dependent CO_2-OAA exchange reaction. This particular reaction appears to be catalysed by this enzyme from all sources when the appropriate nucleoside triphosphate is present. Such a finding would suggest a sequential mechanism with the dissociation of IDP or PEP the rate limiting step. In addition, Miller and Lane (1968) demonstrated that ^{18}O of $H_2{}^{18}O$ was not incorporated into the phosphoryl group of PEP or IDP during decarboxylation. This further suggests a concerted mechanism for the phosphoryl transfer and eliminates any mechanism involving hydrolysis of a phosphate bond of an intermediate. The inherent assumption in such an experiment is that the enzyme-bound water is in equilibrium with the water of the solvent and that hydrolysis is not

effected by water generated during the reaction and immediately reused without equilibrium. The concerted mechanism for the conversion of substrates to products proposed by these workers is shown in Fig. 1.3. PEP undergoes nucleophilic attack on its phosphoryl group, tautomerises and accepts the carbonium ion form of CO_2 analogous to the pyruvate kinase reaction where PEP accepts a proton instead of the carbonium ion (Mildvan and Cohn, 1966).

Furthermore, Miller and Lane (1968) attempted a kinetic analysis of the order of addition of substrates and release of products and produced evidence to suggest a mixed ordered-random addition of substrates, i.e., when Mn^{2+} was bound, either PEP, IDP or CO_2 might bind next but if IDP or CO_2 bound prior to PEP, the PEP site was unavailable and a dead-end complex (Cleland, 1963b) was formed. However, the conditions used for this analysis were not ideal as often three substrates were varying at non-saturating levels making interpretation of the results difficult and subject to error. This criticism does not invalidate their concerted reaction but only perhaps the order of substrate addition. Felicioli, Barsacchi and Ipata (1970) proposed an ordered Bi Ter (Cleland, 1963a) mechanism for chicken liver mitochondrial PEP carboxykinase. However, the binding characteristics of the pig liver mitochondrial enzyme (Miller et al., 1968) and sheep kidney mitochondrial enzyme (this thesis) show random binding of substrates. This would preclude such an ordered Bi Ter

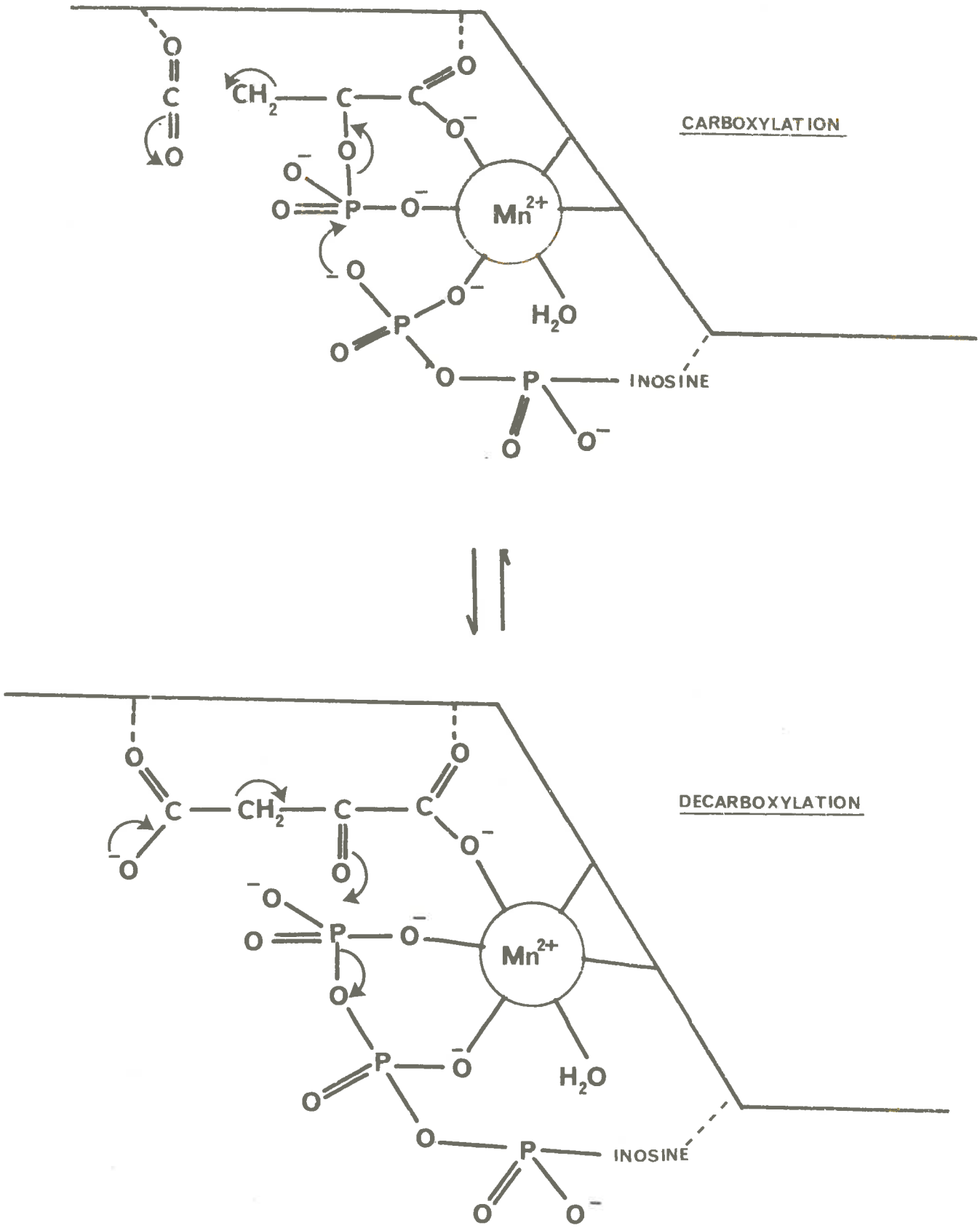


FIG. 1.3. The mechanism proposed by Miller and Lane (1968) for pig liver mitochondrial PEP carboxykinase.

mechanism. Therefore, although PEP carboxykinases from various sources catalyse similar over-all reactions and partial reactions, they may show differences in the order of addition of substrates and release of products.

Perhaps the most urgent need at the moment is an evaluation of the CO_2.OAA exchange reaction in the absence of nucleoside triphosphate. To date, this has been dismissed as not being significant perhaps largely because of the much higher activity of this reaction in the presence of nucleoside triphosphate. It has been shown in this thesis that sheep kidney mitochondrial PEP carboxykinase will catalyse the exchange reaction in the absence of ITP, but the presence of a nucleotide greatly stimulates this activity. The related enzyme, PEP carboxytransphosphorylase, also appears to catalyse this reaction in the absence of PP_i although this was not recognised probably because of the 30-fold stimulation with PP_i (Wood, Davis and Willard, 1969). If this exchange reaction in the absence of nucleotide is real, then it implies that nucleotide is not essential for the exchange reaction. Rather, the nucleotide becomes an activator and its turnover becomes secondary to its primary activating effect (Bridger, Millen and Boyer, 1968). Furthermore, this partial reaction would also have to be taken into account in any proposed mechanism. It is to be remembered that nucleoside diphosphate will stimulate decarboxylation of OAA (Chang and Lane, 1966;

Cannata and Stopanni, 1963a) and, with the sheep kidney mitochondrial enzyme at least, will stimulate the CO_2:OAA exchange reaction without turnover of itself. Therefore, phosphoryl transfer or splitting of a high energy phosphate bond would not appear to be essential for decarboxylation or carboxylation *per se* (see Chapter 6).

It would be expected that PEP carboxykinase would be subject to several control mechanisms considering its key position in gluconeogenesis. However, attempts to detect any metabolic effectors have so far failed in mammalian systems while in micro-organisms only one example of an acute control has been reported, *viz.*, an allosteric inhibition by $NADH_2$ in *E. coli* (Wright and Sanwal, 1969). In this case, sigmoidal saturation kinetics, shown with PEP, HCO_3^- and OAA, were detected only in the presence of the effector and this was not the result of any change in the polymeric state of the enzyme. *E. coli* cells undergoing rapid glycolysis showed elevated levels of $NADH_2$ (2.05 mM) compared with cells grown on succinate (1.2 mM). Therefore, this inhibition could be physiologically significant although the concentration dependence of the $NADH_2$ inhibition was not reported. However, these workers did not indicate whether their enzyme preparation was contaminated with malic dehydrogenase as low levels of this activity could produce the observed effects.

Although this remains the only reported case of acute control of PEP carboxykinase, there are numerous reports of adaptive control via changes in enzyme levels. In general, any changes in enzyme level which occur are in the same direction as overall gluconeogenesis, e.g., bacteria and mammals both show a depression of PEP carboxykinase when the organism is fed a high glucose diet (Shrago and Shug, 1966; Shrago, Young and Lardy, 1967; Shrago and Shug, 1969; de Torrontegui, Palacian and Losada, 1966). On the other hand, fasting (Shrago et al., 1963) or feeding on a diet low in carbohydrate increases PEP carboxykinase in rats while refeeding fasted rats with a diet containing sufficient carbohydrate decreases the elevated enzyme levels (Young et al., 1964). While the rat remains the most extensively studied animal in this field, similar increases with fasting are seen in the cytosol activity of guinea pig liver (Lardy et al., 1964). Thus, the activity of hepatic PEP carboxykinase in most species is related to the metabolic state of the organism.

Obviously, changes in the metabolic state of an animal would alter the hormonal balance. Therefore, administration of hormones related to carbohydrate metabolism would be expected to alter the PEP carboxykinase levels. Fasting an adrenalectomised rat still shows the elevated hepatic enzymic levels (Foster et al., 1966) suggesting that the adrenocortical hormones are not solely responsible for the enhanced enzyme levels.

Administration of insulin depresses the elevated levels of a diabetic rat (Shrago et al., 1963) while blocking insulin secretion with mannoheptulose (Simon et al., 1962; Coore et al., 1963) causes a rapid increase in hepatic PEP carboxykinase levels in the rat (Shrago et al., 1963) and the guinea pig (Lardy et al., 1964). All increases in enzyme levels are blocked by puromycin and actinomycin D indicating that the adaptive changes involve increased enzyme synthesis although the relative contribution of enzyme synthesis and degradation most likely vary from one condition to another.

Foetal livers from both rats and guinea pigs contain only the mitochondrial PEP carboxykinase activity but do not carry out gluconeogenesis. They only develop the cytosol activity at birth simultaneously with the development of gluconeogenesis (Ballard and Hanson, 1967a). This is in accord with the observation that the cytosol activity is usually the only target for adaptive control (Lardy et al., 1964; Nordlie, Varricchio and Nolten, 1965; Gevers, 1967; Johnson, Ebert and Ray, 1970) although with pig and sheep liver, both mitochondrial and cytosol activities vary together (Swiatek et al., 1970; Taylor, Wallace and Keech, 1970).

What then is the role of the mitochondrial PEP carboxykinase in those tissues such as rabbit and guinea pig liver where there is high PEP carboxykinase activity in the cytosol? From studies on avian and lamb liver, Utter, Keech and Scrutton (1964) proposed that both malate and PEP were transported to the cytosol for conversion to carbohydrate. This would

provide a role for both mitochondrial (synthesis of PEP) and the cytosol activities (utilisation of OAA from malate). Lardy, Paetkau and Walter (1965) and Haynes (1965) proposed an alternative scheme where malate and aspartate were transported to the cytosol in accord with the almost complete localisation of PEP carboxykinase in the cytosol of rat liver (Nordlie and Lardy, 1963). Garber and Ballard (1969) reported that isolated guinea pig liver mitochondria accumulated PEP to a concentration of 4 mM before additional PEP was transported out. This concentration is much higher than the 0.1 - 0.2 mM range reported for whole liver (Ray, Foster and Lardy, 1966; Rolleston and Newsholme, 1967; Weidemann, Hems and Krebs, 1969; Baird and Heitzman, 1970) while the mitochondrial levels of OAA are considerably below the 1 - 10 μM range reported for whole tissues (Williamson, Lund and Krebs, 1967; Williamson, 1969). Therefore, it would appear that the carboxylation reaction of mitochondrial PEP carboxykinase could be important <u>in vivo</u> judging by the relative levels of PEP and OAA. In support of this, Johnson, Ebert and Ray (1970) reported that isolated rabbit liver mitochondria incubated with pyruvate and HCO_3^- liberated large amounts of malate and aspartate (if given NH_4^+) to the external medium but only small amounts of PEP. It would appear that gluconeogenesis proceeds, in general, in a similar manner in both rats and rabbits despite the presence of a mitochondrial PEP carboxykinase activity in

rabbits. An important "anaplerotic" role is therefore indicated for the mitochondrial activity, a role for which dramatic changes are not necessary.

1.5. AIMS OF THIS PROJECT

Extensive studies have established a significant role for PEP carboxykinase in both renal and hepatic gluconeogenesis especially in the case of the cytosol activity. Renal gluconeogenesis appears to be as active as the hepatic pathway although the quantitative contribution of the kidney cortex to glucose production is only about 5 - 10% of the total glucose production because of the differences in tissue weight (Scrutton and Utter, 1968).

Filsell *et al.* (1969) reported that sheep, made diabetic by administration of alloxan or by pancreatectomy, showed the expected increases in PEP carboxykinase levels in liver although no change occurred in the kidney. This observation indicates that the control of the adaptive response which operates in the diabetic liver does not appear to be present in the kidney, possibly indicative of different enzyme species being present in the twotissues.

Hepatic PEP carboxykinases from a variety of species are remarkably similar with respect to kinetic and physical properties, while only subtle differences exist between cytosol and mitochondrial activities from the same cell

(Holten and Nordlie, 1965; Ballard and Hanson, 1969). Therefore, an investigation of the sheep kidney mitochondrial PEP carboxykinase was initiated so that a detailed comparison of the hepatic and renal activities could be achieved. This would allow an evaluation of the two protein species and the need for different control mechanisms in liver and kidney. This investigation will cover the physical properties of this enzyme and the methods of control of its activity as well as attempts to elucidate the mechanism of the catalytic reaction both with respect to the pathway in converting substrate to product and with respect to the mode of interaction and role of the functional amino acid residues within the active centre of the enzyme.

Apart from the comparison with the equivalent hepatic activities, such a study allows a comparison of the methods of carboxylation of two related classes of enzymes, viz., the biotin-containing enzymes such as pyruvate carboxylase, and the non-biotin PEP-carboxylating enzymes such as PEP carboxykinase. Both of these groups of enzymes utilise the free energy of an O-P bond cleavage to achieve the formation of a new C-C bond between "CO_2" and the carbon adjacent to a carbonyl group. (In the case of the biotin enzyme, methylmalonyl CoA carboxytransphosphorylase, a C-C bond cleavage replaces the O-P bond cleavage.)

Studies on non-enzymic carboxylation reactions suggest two possible modes of activation of the reactants, viz., (i) electrophilic activation of CO_2 by a Lewis acid, and (ii) nucleophilic activation of the CO_2 acceptor (Kosower, 1962). Carboxylation by biotin enzymes is a two step mechanism including an activation of the "CO_2" to give the enzyme.biotin-CO_2 intermediate followed by transfer of the CO_2 to the acceptor molecule, i.e., biotin enzymes rely primarily on mechanism (i), the activation of "CO_2". On the other hand, the PEP carboxylating enzymes do not form an enzyme-bound CO_2 intermediate. Furthermore, PEP is an activated substrate with the pyruvate moiety held in the enol configuration so that it would be necessary for the enzyme to maintain the enolpyruvate configuration, i.e., mechanism (ii) would predominate, the activition of the CO_2 acceptor. Therefore, a detailed study of the mechanism of PEP carboxykinase would allow more insight into the process of CO_2 fixation.

CHAPTER 2. MATERIALS AND METHODS

MATERIALS

2.1. Enzyme Purification

(a) Sheep kidneys were obtained from the Metropolitan Export and Abattoirs Board and stored in ice as soon as possible after slaughter of the animal.

(b) Phosphate buffers were prepared from the potassium salts.

(c) Reduced glutathione (GSH) was obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

(d) Ethylene diamine tetra-acetic acid (EDTA, disodium salt) was obtained from British Drug Houses.

(e) Ammonium Sulphate (Enzyme grade) was purchased from Mann Research Laboratories.

(f) All grades of Sephadex were products of Pharmacia, Sweden. The Sephadex G25 column was calibrated with Blue Dextran and potassium ferricyanide in 5 ml of equilibrating buffer. SE-Sephadex (C50) was regenerated by successively washing with 10^{-1} M NaOH, water (until neutral), 10^{-1} M HCl, water (until neutral), 10^{-1} M KOH, 6×10^{-2} M KOH and adjusted to pH 6.2 with H_3PO_4, and finally 6×10^{-2} M phosphate (K^+), pH 6.2, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH. DEAE-Sephadex (A50) was similarly recycled except that 5×10^{-2} M H_3PO_4 replaced 6×10^{-2} M KOH and was adjusted to pH 6.8 with KOH. The final wash was 5×10^{-2} M phosphate (K^+), pH 6.8, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH.

2.2. Assays of PEP carboxykinase

(a) P-enolpyruvate (PEP, monocyclohexylammonium salt) was synthesised by the method of Clark and Kirby (1963).

(b) Inosine diphosphate (IDP) was a product of Sigma Chemical Co., U.S.A.

(c) Inosine triphosphate (ITP) was a product of Sigma Chemical Co., U.S.A.

(d) Guanosine triphosphate (GTP) was a product of P.L. Biochemicals, U.S.A.

(e) $NaH^{14}CO_3$. $Ba^{14}CO_3$ was obtained from the Radiochemical Centre, Amersham, Great Britain and $^{14}CO_2$ distilled under vacuo from perchloric acid into an equivalent amount of NaOH. To ensure complete liberation of $^{14}CO_2$, the distillation flask was warmed. The solution of $NaH^{14}CO_3$ was diluted to 50 μC per ml with 0.2 M $NaHCO_3$ freshly prepared with CO_2-free glass-distilled water. The specific activity of the $NaH^{14}CO_3$ solution was determined by dilution in glass-distilled water which had been neutralised by 1 M tris and which contained carrier $NaHCO_3$. Aliquots of this solution were dried on 1 inch squares of Whatman 3 MM paper previously impregnated with $BaCl_2$ and counted in a Packard-Tricarb scintillation spectrometer.

(f) Oxaloacetic acid (OAA) was purchased from the California Corporation for Biochemical Research. Solutions,

prepared immediately before use, were adjusted to pH 6.5 with 1 M tris.

(g) Manganese chloride ($MnCl_2$, A.R.) was purchased from British Drug Houses.

(h) Imidazole (iminazole) was a product of British Drug Houses.

(i) Coupling components. Sodium glutamate, pyridoxal phosphate, NADH, and ADP were all products of Sigma Chemical Co., U.S.A., while aspartate transaminase, malic dehydrogenase, lactic dehydrogenase and pyruvic kinase were purchased from the California Corporation for Biochemical Research, U.S.A.

2.3. N.M.R. and Binding Studies

(a) Imidazole (iminazole), purchased from Sigma Chemical Co., U.S.A., was used for the N.M.R. studies, otherwise as in Section 2.2 (h).

(b) N-ethyl morpholine, purchased from Eastman Organic Chemicals, U.S.A., was purified by distillation under reduced pressure. The pH of solutions was adjusted with constant boiling HCl.

(c) Guanosine diphosphate (GDP) was a product of Sigma Chemical Co., U.S.A.

(d) ^{32}P-Labelled compounds were all generous gifts of Dr. R.H. Symons of this Department.

2.4. Chemical Inhibitor Studies

(a) Inhibitors. N-ethylmaleimide, p-hydroxymercuribenzoic acid, p-hydroxymercuriphenylsulphonic acid, 5,5'-dithiobis (2-nitrobenzoic acid), 1-fluoro-2,4-dinitrobenzoic acid were all products of Sigma Chemical Co., U.S.A. Iodoacetamide, and methyl iodide, distilled prior to use to remove traces of mercury, were both purchased from British Drug Houses, Ltd. Iodosobenzoic acid, diazobenzene sulphonic acid were purchased from K. and K. Labs., Inc., U.S.A. Diethylpyrocarbonic acid was obtained from Flucka, AG., Switzerland.

(b) N-ethyl-1-^{14}C-maleimide was purchased from Schwarz Bioresearch, Inc., U.S.A.

(c) 1-fluoro-2,4-dinitro-^{14}C-benzoic acid was a product of Schwarz Bioresearch, Inc., U.S.A.

(d) 3,3'-dimethylglutaric acid was a product of Sigma Chemical Co., U.S.A.

(e) Tris(hydroxymethyl)-aminomethane (tris, Trizma Grade) was purchased from Sigma Chemical Co., U.S.A.

(f) S-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine was prepared by reacting N-ethylmaleimide and L-cysteine. (Smyth, Blumenfeld and Konigsberg (1964)).

(g) S-(1,2-dicarboxyethyl)-L-cysteine was prepared from maleic anhydride and L-cysteine according to the method of Smyth, Blumenfeld and Konigsberg (1964).

(h) DNP-amino acids were purchased from Mann Research

Labs., U.S.A., except im-DNP-L-histidine which was prepared from N-acetylhistidine and DNFB by the method of Margoliash (1955).

(i) 2,4-dinitrothiophenol was prepared by mixing equimolar amounts of 1-chloro-2,4-dinitrobenzene and sodium sulphide in acetone with stirring at room temperature. The acetone was removed on a rotary evaporater and the residue dissolved in 1 M NH_4OH containing 10 ml 2-mercapto-ethanol. 2,4-dinitrothiophenol was precipitated by acidification with formic acid and the precipitate recovered by filtration. 2,4-dinitrothiophenol was further purified by dissolving the precipitate in 0.1 M NH_4OH containing 10 ml 2-mercaptoethanol and acidifying with formic acid. The precipitate was recovered by filtration, washed with cold 0.1 M formic acid, and dried under vacuo for 72 hr.

2.5. Scintillation Counting

(a) Scintillation fluid. The Packard Instrument Co., U.S.A. supplied PPO (2,5-diphenyl oxazole) and dimethylPOPOP (1,4-bis-2(4-methyl-5-phenyl-oxazoylyl)-benzene). These compounds were dissolved in sulphur-free toluene according to the method of Bousquet and Christian (1960).

METHODS

2.6. Protein Determination

Protein was measured spectrophotometrically by the method of Warburg and Christian as described by Layne (1957) except during the purification where elution of protein from a chromatographic support was followed as the absorbance at 280 mμ in a 1 cm light path.

2.7. PEP Carboxykinase Assays

Three assay methods can be used to determine PEP carboxykinase activity: (i) the IDP- and Mn^{2+}-dependent carboxylation of PEP (ii) the Mn^{2+}-dependent $H^{14}CO_3^-$: OAA exchange reaction (iii) the ITP- and Mn^{2+}-dependent decarboxylation of OAA.

(i) The carboxylation of PEP was followed by the incorporation of $H^{14}CO_3^-$ into OAA which was converted to aspartate by aspartate transaminase. Aliquots of the enzyme (up to 0.04 units for a 5 min. assay) were incubated in reaction mixtures (total volume 0.5 ml) containing (in μmoles); imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $NaH^{14}CO_3$ (1.25 μC), 5; PEP, 0.4; IDP, 0.5; $MnCl_2$, 1.5; GSH, 0.8; sodium glutamate, 5; pyridoxal phosphate, 0.02; aspartate transaminase, 17 μg (approx. 4.5 units). After incubation for 5-10 min. (as indicated in the text) at 30°, the reaction was stopped with 0.25 ml of a 10% (w/v) trichloroacetic acid solution. After centrifuging to remove denatured protein,

0.05 ml aliquots of the protein-free supernatant were dried for 5 min. at 100° on 1 inch squares of Whatman 3 MM filter paper and counted in a Packard-Tricarb scintillation spectrometer. Each assay was counted in triplicate using the counting medium described by Bousquet and Christian (1960).

This assay was used routinely in preference to the decarboxylation assay because of its greater sensitivity and convenience. Furthermore, use of this assay obviates the problems associated with the instability of OAA.

(ii) PEP carboxykinase catalyses a Mn^{2+}-dependent exchange between $H^{14}CO_3^-$ and OAA which is stimulated by inosine (or guanosine) di- and tri-phosphates. Aliquots of the enzyme (up to 0.06 units for a 2 min. incubation) were incubated in reaction mixtures (total volume 0.5 ml) containing (in μmoles); imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $MnCl_2$, 2; ITP, 1; OAA, 1; $NaH^{14}CO_3$ (2.5 μC), 10; GSH, 0.8. All solutions were previously adjusted to pH 6.5. The reaction was initiated by the addition of OAA, incubated at 30° for 2 - 4 min. (as detailed in the text) and stopped by the addition of 0.05 ml of 6 N HCl saturated with 2,4-dinitrophenylhydrazine. The ^{14}C-OAA is stabilised by conversion to the 2,4-dinitrophenylhydrazone. The assay solutions were processed in triplicate as for the carboxylation assay, and all radioactive counting was corrected for quenching by the channels

ratio method.

(iii) The ITP- and Mn^{2+}-dependent decarboxylation of OAA leads to the formation of IDP and PEP. The spectrophotometric assay of Chang and Lane (1966) was tried and found to be unsatisfactory. The non-enzymic rate of decarboxylation of OAA is dependent on the concentration of free Mn^{2+} or Mg^{2+} and, to minimise this, excess ITP must be present which is inhibitory. The decarboxylation assay used was modified from that ofSeubert and Muth (1965). Aliquots of the enzyme (up to 0.03 units) were incubated in reaction mixtures (total volume 0.5 ml) containing (in μmoles); imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; ITP, 0.5; OAA, 1; $MnCl_2$, 1; GSH, 0.8. All solutions were previously adjusted to pH 6.5. The reaction was initiated by the addition of OAA, incubated at 30° for 5 min. and stopped by the addition of 15 mg sodium borohydride. The solutions were immediately placed in an ice bath for 2 min. and 0.2 ml 15% (w/v) perchloric acid were then added. These were neutralised with $KHCO_3$, centrifuged to remove precipitated material and the supernatant retained for PEP estimations.

PEP was estimated spectrophotometrically by NADH oxidation in a system containing pyruvate kinase and lactate dehydrogenase. Aliquots of the supernatant were added to a solution (total volume 1 ml) containing (in μmoles); tris (Cl^-),

pH 7.4; 50; KCl, 10; ADP, 0.5; $MgCl_2$, 1; NADH, 0.25; pyruvate kinase, 1 unit; lactate dehydrogenase, 1 unit; and the oxidation of NADH was followed at 340 mμ.

2.8. Expression of Enzymic Activity

Enzymic activity is expressed in units. One unit of enzymic activity denotes the carboxylation of 1 μmole of PEP per min. at 30° under the standard assay conditions described in Section 2.7 (i) above.

Specific activity is expressed as units per mg of protein.

2.9. Preparation of Freeze-dried Mitochondria

Freeze-dried mitochondria were prepared from the cortex of fresh sheep kidneys which had been stored in ice as soon as possible after slaughter of the animals. The temperature at all stages of the preparation was maintained at 0°. The cortex was separated from the medulla, connective and adipose tissue and homogenised in a Waring Blendor with 3.5 vol. (w/v) of 0.25 M sucrose containing 10^{-4} M EDTA for 1 min. The homogenate was centrifuged at 600 x g for 20 min. The supernatant solutions were combined and centrifuged at 23,000 x g for 15 min. The mitochondrial precipitate was suspended in 10^{-4} M EDTA to a final volume of half that of the original homogenate and then recentrifuged at 23,000 x g for 20 min.

The final residue was suspended in a minimum volume of 10^{-4} M EDTA, frozen quickly in a dry ice/ethanol mixture and placed on a vacuum line overnight. The freeze dried mitochondria were powdered in a Waring Blendor and stored at room temperature in an evacuated dessicator.

CHAPTER 3. PURIFICATION AND SPECIFICITY PROPERTIES

3.1. INTRODUCTION

PEP carboxykinase has been prepared from pig liver mitochondria in a homogeneous state by Chang and Lane (1966). The method used by these workers included an ammonium sulphate fractionation and chromatography on DEAE-cellulose, pH 7.0, cellulose phosphate, pH 7.0, and hydroxylapatite, pH 7.0. Attempts to purify sheep kidney mitochondrial PEP carboxykinase by this method were unsuccessful as the enzyme did not bind to cellulose phosphate at pH 7.0 while chromatography on hydroxylapatite at pH 7.0 resulted in only 10 - 20% recovery of enzymic activity. In fact, recoveries of activity were always low if the enzyme was bound to a chromatographic support, irrespective of its nature, and subsequently eluted. Recently, Ballard and Hanson (1969) have purified the rat liver cytosol PEP carboxykinase. Again, their method could not be adapted to the sheep kidney mitochondrial enzyme as this enzyme was not bound by DEAE-cellulose at pH 8.0 nor does calcium phosphate gel adsorb the enzyme. In addition, it was found that iso-electric focusing, an essential part of the purification of the rat cytosol enzyme, usually resulted in a decrease in specific activity of the sheep kidney mitochondrial enzyme.

Thus, a new purification method had to be developed which would by-pass the problem of the instability of the enzyme when bound to a chromatographic support. This was

achieved using the ion-exchange Sephadex gels under conditions of pH and ionic strength where the enzyme was retarded but not bound. This method resulted in enzyme preparations of high specific activity with good recoveries.

3.2.A. ISOLATION OF PEP CARBOXYKINASE

All operations were conducted at 0 - 4°. The results obtained in a typical purification run are summarised in Table 3.1.

(a) Extraction and ammonium sulphate fractionation

Mitochondrial powder (50.0 gm) was extracted with 1 litre of 5×10^{-2} M phosphate (K^+), pH 6.0, containing 5×10^{-4} M EDTA and 5×10^{-4} M, GSH, with stirring for 15 min. and the insoluble material was removed by centrifuging at 23,000 x g for 15 min. Solid ammonium sulphate (24.3 g per 100 ml of extract) was added to the extract slowly with stirring, the pH being maintained at pH 6.5 with KOH. After standing for 10 min., the precipitated protein was removed by centrifuging at 23,000 x g for 15 min. and the supernatant recovered. Further solid ammonium sulphate was added (13.2 g per 100 ml of supernatant) to the supernatant with stirring, maintaining the pH at 6.5. The precipitated protein was recovered by centrifuging at 23,000 x g for 15 min. The pellet was taken up in a minimum volume of 2×10^{-2} M phosphate (K^+), pH 6.8, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH, and the dissolution of the

TABLE 3.1: PURIFICATION OF SHEEP KIDNEY MITOCHONDRIAL PEP CARBOXYLASE

Treatment	Protein (mg)	$\frac{A_{280m\mu}}{A_{260m\mu}}$	Activity (units)	Specific Activity (units/mg)	Yield %
Extract, pH 6.0	13565	0.88	826	0.06	100
Ammonium Sulphate Fractionation ..	4443	1.19	728	0.16	88.6
Sephadex G100, pH 6.8 ..	450	1.58	458	1.02	56.8
SE-Sephadex, pH 6.2 ..	58.6	1.76	186	3.1	23.5
DEAE-Sephadex, pH 6.8 ..	21.2	1.77	101	4.75	12.2

pellet was facilitated by dialysis against the above buffer for 1 h. At this stage, the volume of the enzyme solution was 30 - 35 ml.

(b) Sephadex G100 gel filtration

The enzyme solution was applied to a Sephadex G100 column (6 x 85 cm) equilibrated with 2×10^{-2} M phosphate (K^+), pH 6.8, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH, and eluted with the same buffer. The void volume of this column was 670 ml and the peak of enzymic activity appeared after a further 470 ml. The enzymic activity was detected using the $^{14}CO_2$-fixation assay (Section 2.7 (i)) and the protein concentration was monitored at 280 mμ. The most active fractions were pooled and the protein precipitated with solid ammonium sulphate (39.0 g per 100 ml of solution) at pH 6.5. The pellet, recovered after centrifuging at 23,000 x g for 15 min., was dissolved in 6×10^{-2} M phosphate (K^+), pH 6.2, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH (total volume, 5.0 ml). This solution was desalted on a Sephadex G25 column (2.6 x 26 cm) equilibrated with the above buffer. The first 55 ml of eluate were discarded and the enzyme was recovered in the following 15 ml. This enzyme solution was then concentrated to about 3 ml on a Sartorius vacuum-filtration membrane. A typical elution profile from Sephadex G100 is shown in Fig. 3.1.

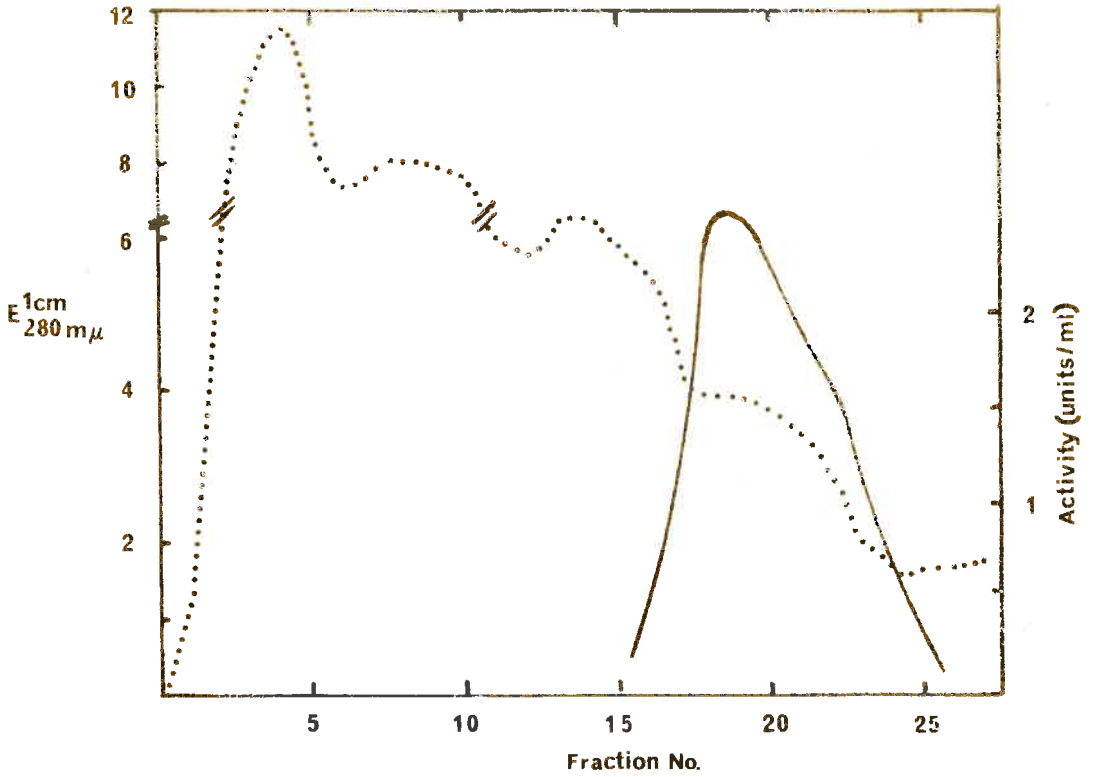


FIG. 3.1. The protein and activity profiles resulting from PEP carboxykinase gel filtration on Sephadex G100, pH 6.8. Aliquots (22 ml) were collected. Flow rate was about 60 ml per hr. (———), enzymic activity; (.....), protein, determined as absorbance at 280 mμ.

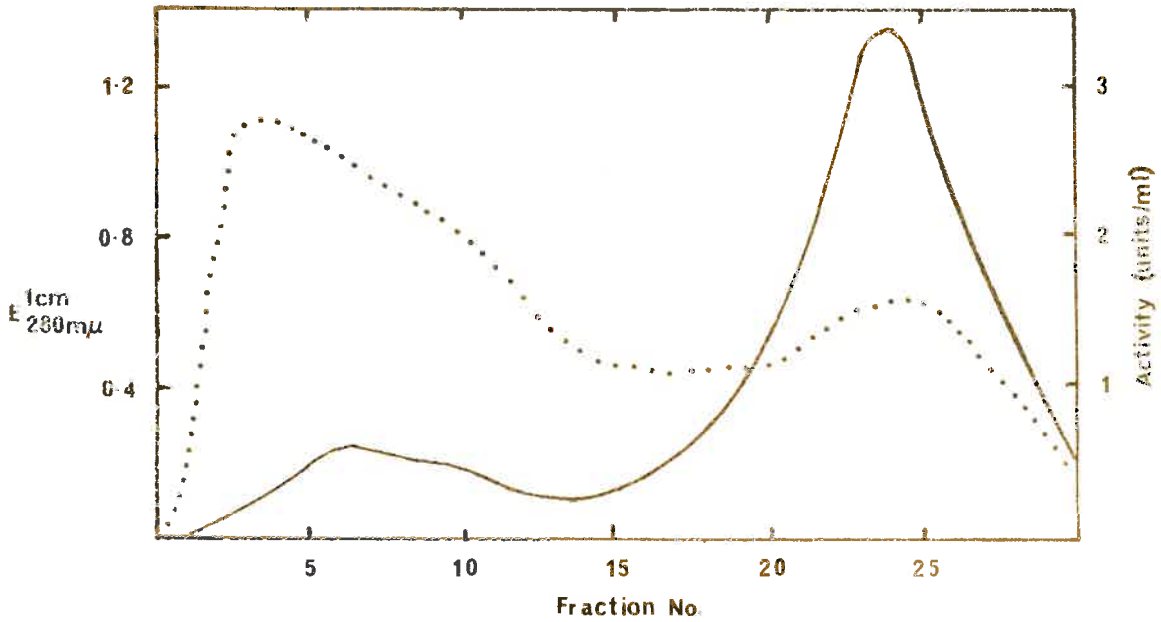


FIG. 3.2. The protein and activity profiles resulting from PEP carboxykinase chromatography on SE-Sephadex, pH 6.2. Flow rate was about 30 ml per hr and 10 ml aliquots were collected. (———), enzymic activity; (.....), protein, determined as absorbance at 280 mμ.

(c) SE-Sephadex Chromatography

The enzyme solution was applied to an SE-Sephadex column (C50, 4 x 56 cm) previously equilibrated with 6 x 10^{-2} M phosphate (K^+), pH 6.2, containing 5 x 10^{-4} M EDTA and 5 x 10^{-4} M GSH, and eluted with the same buffer. The protein and enzymic activity were monitored as described for the Sephadex G100 column. The tubes with the highest specific activity were pooled and the protein precipitated with solid ammonium sulphate (39.0 g per 100 ml of solution) at pH 6.5. The protein was recovered, freed from ammonium sulphate and concentrated to about 3 ml as described above except that the buffer was 5 x 10^{-2} M phosphate (K^+), pH 6.8, containing 5 x 10^{-4} M EDTA and 5 x 10^{-4} M GSH. A typical elution profile from SE-Sephadex is shown in Fig. 3.2.

(d) DEAE-Sephadex Chromatography

The enzyme solution was applied to a DEAE-Sephadex column (A50, 30 x 2.7 cm) previously equilibrated with 5 x 10^{-4} M phosphate (K^+), pH 6.8, containing 5 x 10^{-4} M GSH, and eluted with the same buffer. The protein and enzymic activity were monitored as described in (b) above. The tubes with the highest specific activity were pooled and the enzyme was stored as a suspension in ammonium sulphate (40 g per 100 ml of solution, pH 6.5). The enzyme was stable for several months in this form. A typical elution profile from DEAE-Sephadex is shown in Fig. 3.3.

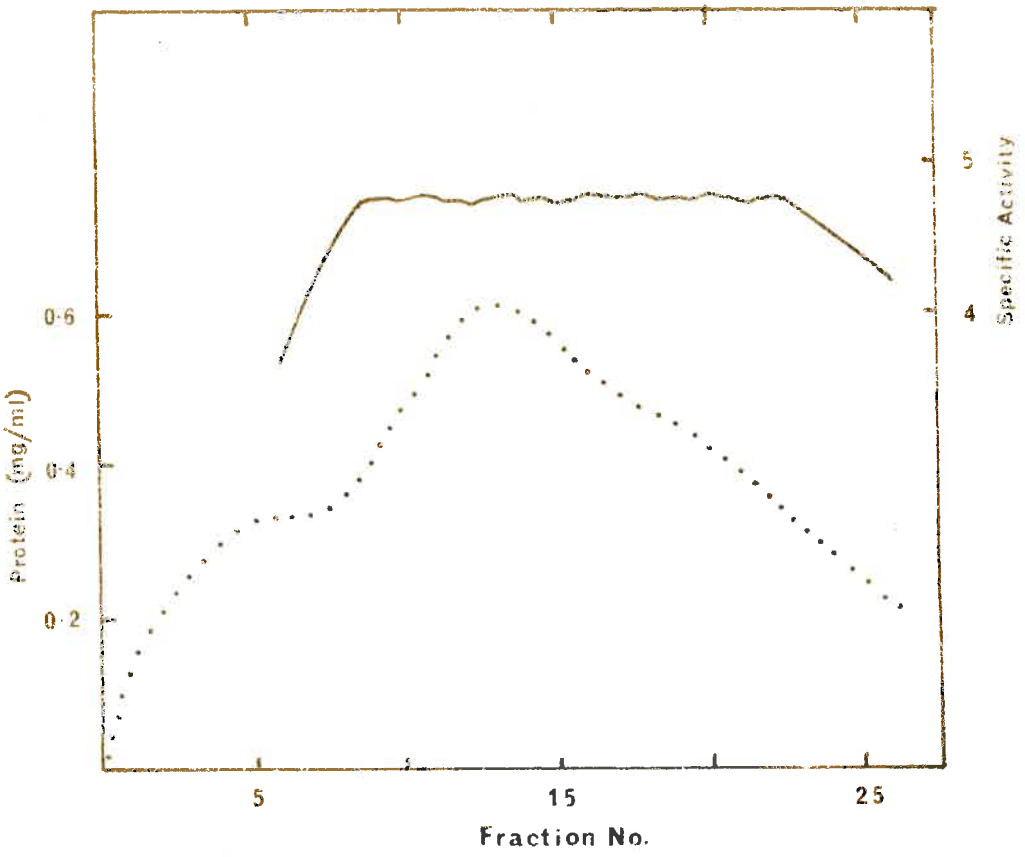


FIG. 3.3. Chromatography of PEP carboxykinase on DEAE-Sephadex, pH 6.8. Flow rate was about 15 ml per hr and 4 ml aliquots were collected. (———), specific activity; (.....), protein, determined as absorbance at 280 mμ.

3.2.B. PHYSICAL PROPERTIES

(a) Molecular weight of PEP carboxykinase

An initial molecular weight study was carried out using Sephadex gel filtration. A Sephadex G150 column (185 x 1.5 cm) was equilibrated with a 5×10^{-2} M phosphate solution, pH 6.8, containing 5×10^{-4} M EDTA and 5×10^{-4} M GSH, and calibrated with cytochrome C, pancreatic ribonuclease, chymotrypsinogen, ovalbumin, bovine serum albumin monomer and dimer and cytochrome b_2. Blue dextran gave the void volume (V_o). PEP carboxykinase was located by the CO_2-fixation assay (Section 2.7 (i)). The results of this analysis are shown in Fig. 3.4 plotted according to the method of Andrews (1964). The molecular weight estimate obtained was 47,800.

Molecular weights (M) of proteins may also be determined using the Svedberg Equation (Svedberg, 1940)

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

where R, T, s, D, $\bar{\nu}$ and ρ are the gas constant, the absolute temperature, the sedimentation coefficient, the diffusion coefficient, the partial specific volume and the density of the solution, respectively. Therefore, a knowledge of s, D and $\bar{\nu}$ are required for a determination of the molecular weight.

PEP carboxykinase (1.0 - 7.5 mg per ml in a solution

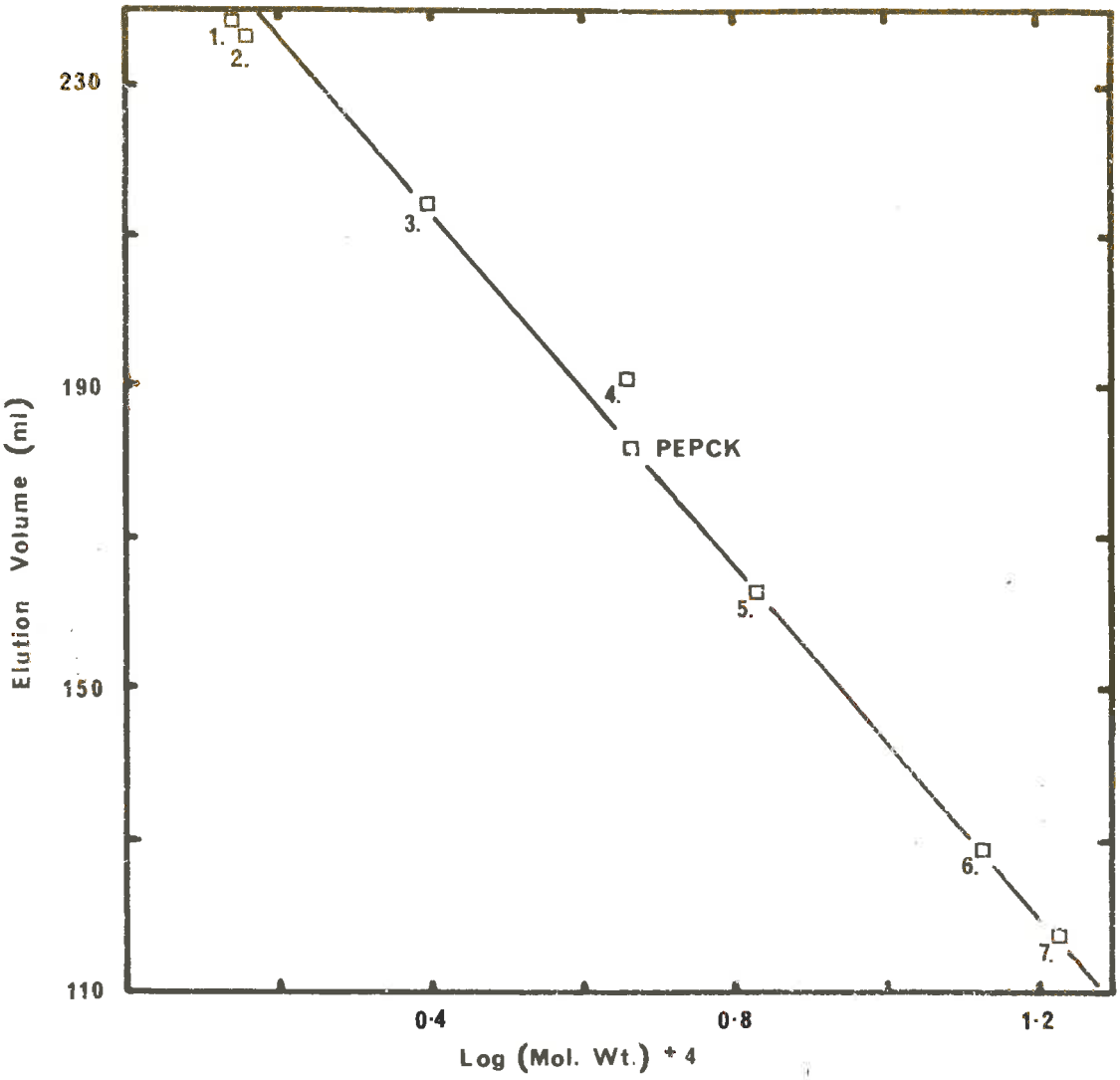


FIG. 3.4. Determination of the molecular weight of PEP carboxykinase by gel filtration. A Sephadex G150 column (185 x 1.5 cm), previously equilibrated with 5 x 10^{-2}M phosphate (K^+), pH 6.8, containing 5 x 10^{-4}M EDTA and 5 x 10^{-4}M GSH, was calibrated with the following proteins: 1, cytochrome c; 2, pancreatic ribonuclease; 3, chymotrypsinogen; 4, ovalbumin; 5, B.S.A. monomer; 6, B.S.A. dimer; 7, cytochrome b_2. Elution volume was plotted against log molecular weight (Andrews, 1964). PEP carboxykinase was detected with the CO_2-fixation assay.

containing 0.05 M phosphate (K^+), 10^{-4} M EDTA and 10^{-4} M GSH, pH 6.8) was centrifuged at 59,780 rpm in the Beckman Model E ultracentrifuge using the AN-F rotor. Photographs of the schlieren patterns were taken every 8 - 16 min. after the rotor attained maximum velocity and these were analysed to give the distance (r) moved by the peaks from the centre of the rotor.

Log r was plotted as a function of time. The slope of these straight line plots is related to the sedimentation coefficient and the velocity of the rotor by

$$s_{obs} = (\text{Slope})\left(\frac{2.303/60}{(2\pi(\text{rpm}/60))^2}\right) \qquad \ldots (3.2)$$

$s_{20,w}$, the sedimentation coefficient the protein would have in a solvent with the density and viscosity of water at 20°, is related to s_{obs} by

$$s_{20,w} = s_{obs}\left(\frac{\eta_t}{\eta_{20}}\right)\left(\frac{\eta}{\eta_w}\right)\left(\frac{1 - \bar{\nu}\rho_{20,w}}{1 - \bar{\nu}\rho_t}\right) \qquad \ldots (3.3)$$

where η_t/η_{20} is the viscosity of water at t° relative to that at 20°, η/η_w is the viscosity of the solution relative to that of water and $\rho_{20,w}$ and ρ_t are the densities of water at 20° and solvent at t°, respectively. The value $s_{20,w}$ is dependent on the protein concentration. Therefore, the value $s^{o}_{20,w}$, the sedimentation coefficient at zero protein concentration, is obtained by plotting $s_{20,w}$ as a function of the protein concentration and extrapolating to zero concentra-

tion. The value for $s^{o}_{20,w}$ obtained for sheep kidney mitochondrial PEP carboxykinase was 4.58S compared with 5.21S for the pig liver mitochondrial enzyme (Chang and Lane, 1966). The equation

$$s_{20,w} = s^{o}_{20,w} - k.c. \qquad \ldots (3.4)$$

where c is the protein concentration in mg per ml and k = 0.03135 mg^{-1} ml describes the concentration dependence of the sedimentation coefficient.

The diffusion coefficient (D) of PEP carboxykinase was obtained by the method of Kawahara (1969) using a double sector synthetic boundary cell in the AN-D rotor. Enzyme (0.15 ml, 3 - 5 mg per ml in a solution containing 0.05 M phosphate (K^+), 10^{-4} M EDTA and 10^{-4} M GSH, pH 6.8) was loaded into one sector of the cell and 0.5 ml of the solvent into the other. The cell was rotated at 12,590 rpm and photographs of the schlieren patterns were taken every 4 - 8 min. after the rotor reached the preset velocity. The ratio $\left(\frac{\text{area}(A)}{\text{height}(H)}\right)^2$ was obtained from the schlieren patterns and plotted against time. The slope of this line is $4\pi D_{obs}$ from which $D_{20,w}$ was found to be 5.848×10^{-7}.

Applying the Svedberg Equation (Eqn. 3.1) with $s^{o}_{20,w}$ equal to 4.58S, $D_{20,w}$ equal to 5.848×10^{-7} and $\bar{\nu}$ equal to 0.730 (obtained from the amino acid composition (Section 3.2B (b)) by the method of Cohn and Edsall, 1943), the molecular

weight of sheep kidney mitochondrial PEP carboxykinase was calculated to be 71,100.

Since these two methods gave such different estimates, the molecular weight of PEP carboxykinase was determined by the method of Shapiro *et al.* (1967) where the protein was subjected to polyacrylamide electrophoresis in the presence of the anionic detergent, sodium dodecyl sulphate. The system was calibrated with the following proteins: catalase, ribonuclease, ovalbumin, myoglobin, glyceraldehyde-3-phosphate dehydrogenase, B.S.A., glutamate dehydrogenase, liver and yeast alcohol dehydrogenase, lactate dehydrogenase, carbonic anhydrase, L-amino acid oxidase and pyruvate kinase. PEP carboxykinase (200 μg) was dissolved in 0.4 ml of 0.01 M phosphate (Na^+), pH 7.0, containing 0.1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. The proteins were electrophoresed on 10% acrylamide gels contained normal amounts of cross-linker at 8 ma per tube (Weber and Osborn, 1969) until the marker dye had moved about three-quarters of the way through the gels. After electrophoresis, the length of the gel and the distance moved by the marker dye were measured. The gels were stained with Coomassie brilliant blue and then destained in acetic acid/methanol/water (3/2/35: v/v/v). The length of the gels after destaining and the positions of the blue protein zones were recorded. Using the expression

$$\text{Mobility} = \left(\frac{\text{distance of protein migration}}{\text{length after destaining}}\right) \times \left(\frac{\text{length before staining}}{\text{distance of dye migration}}\right) \quad \ldots (3.5)$$

the mobilities were calculated and plotted against log molecular weight. From this method, the molecular weight of PEP carboxykinase was found to be 72,000 in agreement with the estimate obtained with the ultracentrifuge.

(b) Amino acid analysis of PEP carboxykinase

Prior to acid hydrolysis, the protein was carboxymethylated by the method of Milne and Wells (1970) modified from that of Crestfield, Moore and Stein (1963). Protein, about 5 mg, was dissolved in 1 ml of a solution containing 8.0 M urea, 0.6 M tris (Cl^-) and 0.01 M EDTA, pH 8.6. The urea solution was previously deionised by chromatography on a mixed bed resin immediately before use. 2-Mercaptoethanol, 10 µl, was then added and the protein was reduced at 37° for 4 hr. Iodoacetic acid, 26.8 mg in 0.1 ml of 1 M NaOH, was added. After 15 min., the solution was transferred to dialysis tubing and dialysed exhaustively against 0.01 M NH_4HCO_3. The solution was protected from light during carboxymethylation and dialysis.

The protein was hydrolysed under vacuum in glass-distilled

constant boiling point HCl at 105 - 110°. Hydrolysates were dried by rotary evaporation. The hydrolysates were analysed with a Beckman 120C Amino Acid Analyser using a single column with gradient elution according to the method of Piez and Morris (1960). The amino acid composition of sheep kidney mitochondrial PEP carboxykinase is shown in Table 3.2.

3.2.C. SPECIFICITY PROPERTIES OF PEP CARBOXYKINASE

(a) Identification of the products of the reactions catalysed by PEP carboxykinase

The Mn^{2+}- and IDP-dependent carboxylation of PEP yields OAA as the carboxylation product. If no coupling system were used, the product may be stabilised by the addition of 2,4-dinitrophenylhydrazine in 6 M HCl giving the 2,4-dinitrophenylhydrazone. However, if the aspartate transaminase or the malate dehydrogenase coupling systems were used, the OAA would be converted to aspartate or malate respectively. (Formic acid, instead of trichloroacetic acid, was used to stop the reactions here to overcome salt problems in the chromatography.)

Samples of these three reaction mixtures obtained using $H^{14}CO_3^-$ were chromatographed on Whatman 3 MM paper in the solvent, 1-butanol/propionic acid/water (10/5/7, v/v/v) for 15 hr. One radioactive band was found in each case. The radioactivity was eluted with a 10^{-5} M EDTA solution.

TABLE 3.2: AMINO ACID COMPOSITION OF THE CARBOXYKINASE

Amino acid	Residues per mole (sheep kidney)[a]	Residues per mole (pig liver)[b]
Cysteine[c]	13	15
Aspartic acid	57	53
Threonine[d]	38	31
Serine[d]	39	31
Glutamic acid	79	70
Proline[d]	54	56
Glycine	63	64
Alanine	55	55
Valine	42	44
Methionine	14	20
Isoleucine	29	28
Leucine	52	58
Tyrosine	12	12
Phenylalanine	29	27
Lysine	34	26
Histidine	13	12
Tryptophan[e]	13	13
Arginine	38	48

a. Molecular weight, 71,100; b. Molecular weight, 73,300 (Chang and Lane, 1966); c. Determined as carboxymethylcysteine;
d. Corrected for destruction during hydrolysis;
e. Determined spectrophotometrically by the method of

concentrated and rechromatographed with the appropriate internal standards, in the solvent systems, 1-butanol/propionic acid/water (10/5/7, v/v/v) or ethanol/conc. ammonia/water (80/4/16, v/v/v) on Whatman No.2 paper. In each case, the radioactivity corresponded exactly with the internal standard. Thus, the product of sheep kidney mitochondrial PEP carboxykinase was OAA as expected. The standard sample of OAA-2,4-dinitrophenylhydrazone was prepared by the method of Clift and Cook (1932). (M.Pt. = 211° (dec.): Lit. value = 211° (dec.)).

The product of the ITP- and Mn^{2+}-dependent decarboxylation of OAA was shown to be PEP by being utilised by pyruvate kinase. In addition, IDP (or GDP) can partially replace ITP (or GTP) in the decarboxylation reaction. However, in this case, pyruvate was the product and not PEP as shown by being utilised by lactate dehydrogenase. This is in agreement with reported observations with the equivalent enzyme from pig liver mitochondria (Chang and Lane, 1966) and yeast (Cannata and Stoppani, 1963c), although in the latter case adenine nucleotides are favoured such that ADP replaces ATP.

(b) Relationship between pH and activity

The effect of pH on the activity of PEP carboxykinase was examined over the range, pH = 5.7 - 8.8, for both the CO_2-fixation reaction (Fig. 3.5) and the CO_2:OAA exchange activity (Fig. 3.6) in the presence of Mn^{2+} or Mg^{2+} as the

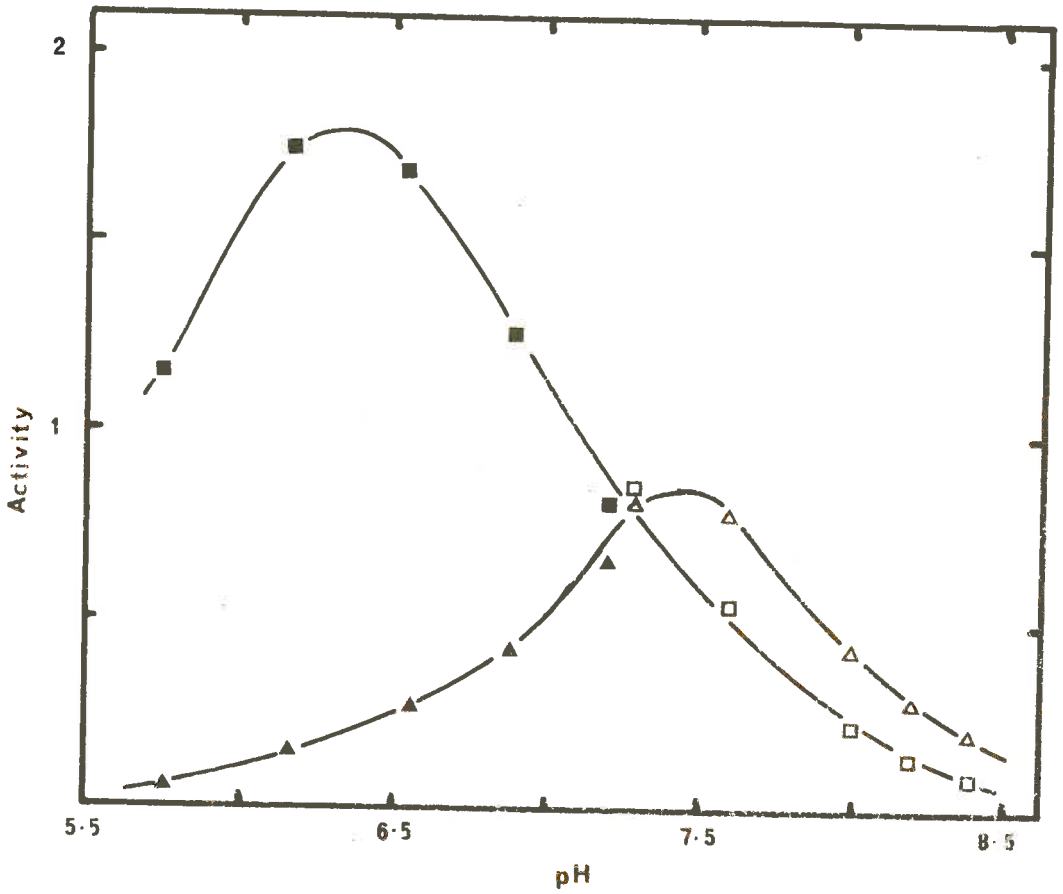


FIG. 3.5. Effect of pH on the PEP carboxylation activity with Mn^{2+} or Mg^{2+} as the activating divalent cation. Assay conditions were as described in Section 2.7.(i) except that the buffers used were 3,3'-dimethylglutarate (K^+) (closed symbols) and tris (Cl^-) (open symbols), both at 0.1M. (■——■), Mn^{2+}; (▲——▲), Mg^{2+}.

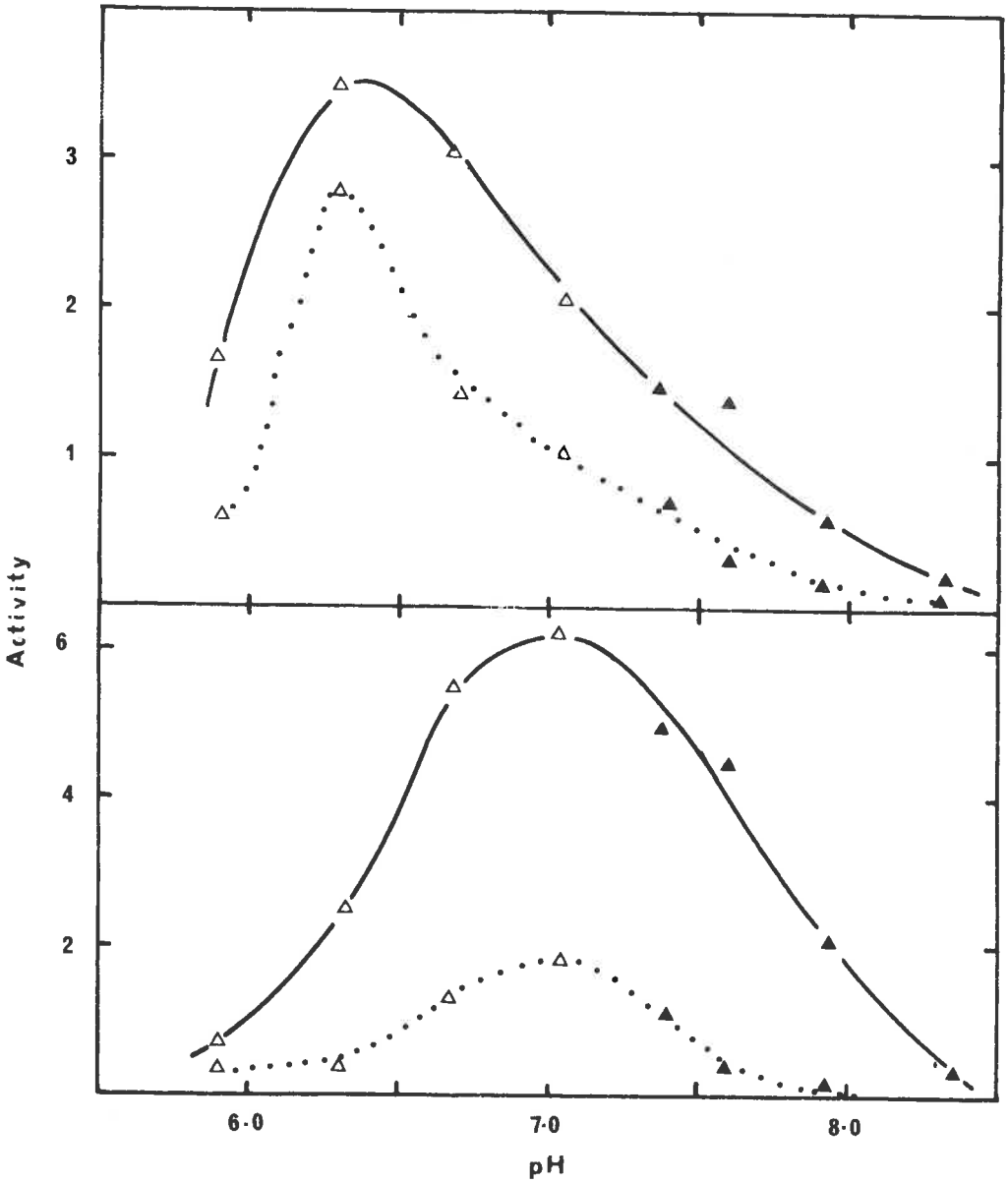


FIG. 3.6. Effect of pH on the CO_2:OAA exchange activity with Mn^{2+} or Mg^{2+} as the activating divalent cation and in the presence of ITP (top) or IDP (bottom). Assay conditions were as described in Section 2.7.(ii) except that the buffers used (0.1M) were 3,3'-dimethylglutarate (K^+) (closed symbols) and tris (Cl^-) (open symbols). (———), Mn^{2+}; (———), Mg^{2+}.

activating metal ion. With the carboxylation reaction, the pH optimum is 6.5 with Mn^{2+} but moves to pH 7.5 with Mg^{2+} with a much lower activity being observed. However, in the case of the CO_2:OAA exchange activity, the pH optimum is independent of the divalent cation but varies with the nucleotide, viz., pH 6.5 with the triphosphate but pH 7.0 with the diphosphate.

(c) Comparison of the rates of the enzyme-catalysed reactions with inosine or guanosine nucleotides and Mn^{2+} or Mg^{2+}

The relative magnitudes of the carboxylation reaction, the CO_2:OAA exchange reaction and the decarboxylation reaction were assessed using either inosine or guanosine nucleotides in the presence of Mn^{2+} or Mg^{2+} as the activating metal ion. The results of this investigation are shown in Table 3.3. The exchange reaction was faster than the decarboxylation reaction which, in turn, was more rapid than the carboxylation reaction. This is in agreement with the results of Chang et al. (1966) and Policioli et al. (1966) for the guinea pig liver and chicken liver mitochondrial activities respectively. However, there are species differences with respect to the relative rates of the reactions as Chang et al. (1966) report up to a 32-fold difference between the exchange and carboxylation activities for the pig liver enzyme compared with only

TABLE 3.3. COMPARISON OF THE RATES OF THE ENZYME-CATALYSED REACTIONS WITH INOSINE OR GUANOSINE NUCLEOTIDES AND Mn^{2+} AND Mg^{2+}

Components		% Activity[a]
Carboxylation:	IDP + Mn^{2+}	100
	GDP + Mn^{2+}	83
	IDP + Mg^{2+}	3
	GDP + Mg^{2+}	5
	IDP + Mn^{2+} + Mg^{2+}	46
	GDP + Mn^{2+} + Mg^{2+}	46
CO_2/OAA Exchange:	ITP + Mn^{2+}	260
	GTP + Mn^{2+}	213
	IDP + Mn^{2+}	132
	GDP + Mn^{2+}	140
	ITP + Mg^{2+}	185
	GTP + Mg^{2+}	161
	IDP + Mg^{2+}	8
	- + Mn^{2+}	8
	- + Mg^{2+}	Trace
Decarboxylation:	ITP + Mn^{2+}	130
	GTP + Mn^{2+}	68
	ITP + Mg^{2+}	Trace
	GTP + Mg^{2+}	Trace
	ITP + Mn^{2+} + Mg^{2+}	131
	GTP + Mn^{2+} + Mg^{2+}	68

a. Expressed as % activity relative to carboxylation with IDP and Mn^{2+}.

Standard assay conditions were as described in Section 2.7 except that nucleotide and metal ion were added as indicated above. When both cations were present, each was added to a concentration half that normally present.

a 2.6-fold difference for the sheep kidney enzyme.

3.3. DISCUSSION

Physical properties. The purification scheme reported here yields enzyme with is almost homogeneous as indicated by electrophoretic analysis in the presence of sodium dodecyl sulphate. A minor contaminant of slightly lower molecular weight amounting to only about 5% of the total protein was detected. The difficulty of purifying this enzyme was markedly increased by its instability when bound to any chromatographic support. This is in constrast to the pig liver mitochondrial (Chang and Lane, 1966) and the rat liver cytosol (Ballard and Hanson, 1969) enzymes. In both these cases, the purification involves binding the enzyme to a chromatographic support followed by subsequent elution.

Many other techniques have been tried for the sheep kidney enzyme but with limited or negligible success. These include calcium phosphate and protamine sulphate adsorption, CM-cellulose, Phospho-cellulose, DEAE-cellulose, QAE-sephadex and hydroxyl-apatite chromatography, iso-electric focusing, ammonium sulphate back-extraction and preparative gel electrophoresis. However, with the observation that the enzyme behaves as a molecule of 48,000 molecular weight on Sephadex gel filtration, substantial purification should be achieved by rechromato-

graphy on a Bio-Gel P column where the protein should now behave as a particle of molecular weight 71,000 if a specific interaction of the enzyme and dextran causes the retardation. The apparent low molecular weight on Sephadex was not detected until very late in this work so that it was not possible to take advantage of the observation.

Molecular weight estimates, obtained from Sephadex gel filtration, have been reported which were either greater or less than the true molecular weight (Andrews, 1964; Milne and Wells, 1970). This emphasises the need to confirm such an estimate obtained from Sephadex gel filtration. The possibility that the presence of carbohydrate in the enzyme was responsible for the retardation on Sephadex was rejected after Molisch and Thymol tests gave negative results for carbohydrate. Also, higher ionic strength (0.3 M) or a change of buffer (tris (Cl^-), pH 7.4) did not alter the anomolous behaviour observed. This would eliminate ionic or specific ion effects as the cause.

The molecular weight of the sheep kidney enzyme is in close agreement with that of the pig liver mitochondrial enzyme (73,300) and the rat cytosol enzyme (74,500). It is interesting to note that the latter estimate was obtained by gel filtration so that the possibility exists that the molecular weight has been underestimated.

Despite the good agreement between the molecular weights and the catalytic properties of the sheep kidney and pig liver enzymes, the two proteins show significant differences in their amino acid compositions (Table 3.2). The main differences are that the sheep kidney activity has more acidic, more lysine and fewer arginine residues (although total basic residues are essentially the same). To account for the inability of sheep kidney mitochondrial PEP carboxykinase to bind to DEAE-cellulose at pH 7.0 - 8.0 and the low rate of migration of the protein on polyacrylamide electrophoresis at pH 7.5 and 9.5, despite the large excess of acidic residues, most of these groups must exist as amides.

Specificity properties. At pH 6.5, Mg^{2+} was only effective in the CO_2:OAA exchange reaction (Table 3.3). In fact, it appeared to compete with Mn^{2+} in the carboxylation reaction but not in the decarboxylation reaction. However, Mg^{2+} does activate PEP carboxykinase although at a higher pH than does Mn^{2+}. This observation could be important mechanistically and could reflect either different CO_2 species being utilised or the different pK_a's of metal-bound water molecules. These metal-bound hydroxyl ions have been proposed as active species of the PEP carboxykinase reaction (Chapter 6). These complexes are not required for the CO_2:OAA exchange and so the pH differences with the two metal ions are not shown for this

activity.

For the three activities, the inosine nucleotides were more active than the guanosine nucleotides except perhaps for the IDP- or GDP-stimulated CO_2:OAA exchange in the presence of Mn^{2+} where GDP was slightly more active. Chang and Lane (1966) reported that the pig liver mitochondrial activity was more active with the guanosine nucleotides than with inosine nucleotides. As the carboxylation assay used by these workers was essentially the same as that used here, the differential reactivities towards inosine and guanosine nucleotides could represent real differences in specificity between the two enzyme species.

It is significant that Mg^{2+} was ineffective in activating the CO_2:OAA exchange reaction at pH 6.5. If the nucleotide-independent exchange activity is due to contamination by traces of ITP or GTP, then Mg^{2+} should activate the exchange reaction in the absence of added nucleotide. However, this is not so. Mn^{2+} activates the CO_2:OAA exchange reaction in the presence of the triphosphate or diphosphate although Mg^{2+} can only effectively activate this reaction in the presence of triphosphate. If thediphosphate was contaminated with triphosphate and the exchange activity was dependent on the triphosphate, the exchange activity with IDP and Mg^{2+} would be expected to be

$$\frac{185 \times 132}{260}\ \% = 94\%$$

relative to carboxylation with IDP and Mn^{2+} as 100%. Instead, the relative activity was only 8%. Furthermore, it is obvious from Fig. 3.6 that the ITP- and IDP-stimulated exchange activities are distinct reactions and do not occur because of contamination of one with the other. Therefore, it does appear that the Mn^{2+}-dependent CO_2:OAA exchange activity is a real activity and that this reaction is stimulated by nucleoside di- and tri-phosphates (cf. Bridger, Millen and Boyer, 1968). With regard the nucleotide-independent activity, attempts to detect trace amounts of contaminating nucleotides have been unsuccessful, i.e., this activity is a true partial reaction of PEP carboxykinase.

CHAPTER 4. N.M.R. STUDIES ON Mn^{2+} ACTIVATION OF PEP CARBOXYKINASE

4.1. INTRODUCTION

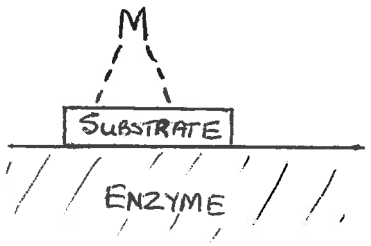
As PEP carboxykinase is activated by the paramagnetic manganous ion, it is possible to use pulsed nuclear magnetic resonance to observe the interactions of the metal ion with enzyme protein and/or substrates. Such a paramagnetic species profoundly increases both the longitudinal ($\frac{1}{T_1}$) and transverse ($\frac{1}{T_2}$) relaxation rates of magnetic nuclei, such as water protons, located in their immediate environment. However, in the results presented in this chapter, only the longitudinal relaxation time (T_1) of water protons was measured.

Pulsed nuclear magnetic resonance (N.M.R.) was initially employed by Eisinger *et al.* (1961, 1962) to investigate the interactions of various paramagnetic ions with DNA. This technique was extended to include proteins, such as creatine kinase, enolase and bovine serum albumin by Cohn and Leigh (1962), Cohn (1963) and Mildvan and Cohn (1963). Their aim was to establish the interaction of the metal ion, manganese, with the protein and/or substrates. In the case of enolase, kinetic investigations had indicated a direct interaction of the activating cation with the enzyme (Malstrom, 1961; Wold and Ballou, 1957) and this was confirmed by Cohn (1963) using this N.M.R. technique. A large enhancement of the proton relaxation rate (P.R.R.) of water protons was observed on addition of enolase to a manganous chloride solution. On the other hand, the kinetics of creatine kinase suggested that the metal ion interacted with the nucleotide but not the enzyme

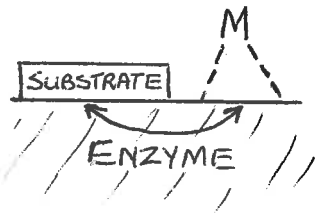
(Noda et al., 1960; Mihei et al., 1961; Morrison and O'Sullivan, 1965). Confirmation was obtained from the observation that a significantly increased enhancement of the P.R.R. of water protons was only noted when creatine kinase, metal ion and nucleotide were present (Cohn and Leigh, 1962). These observations then led Cohn (1963) to propose two types of manganous-activated enzymes with respect to the nature of the metal ion complexes: Type I (e.g., creatine kinase) where the enhancement of the P.R.R. (ϵ_b) of the binary metal-enzyme complex (if any) is much less than the enhancement (ϵ_t) of the ternary enzyme-metal-substrate complex; Type II (e.g., enolase) where ϵ_b is much greater than ϵ_t. Type I enzymes appear to bind the metal ion through the substrates while Type II enzymes bind the metal directly. Table 4.1 lists some examples of these two types of enzyme. Type I has been designated a "substrate-bridge" structure and Type II as a "metal-bridge" structure while a third type, Type III, has been considered as an "enzyme-bridge" structure (Mildvan and Cohn (1970)). In this case, a substrate alters the enhancement of the P.R.R. of the binary or ternary complex without binding to the metal directly. This is presumably effected via a conformational change of some nature such that the environment of the metal ion is now altered. An example of the Type III structure is the addition of creatine to the ternary Mn.ADP.creatine kinase complex. A schematic representation of the three structures is shown below:

TABLE 4.1: CLASSIFICATION OF ENZYMES ACCORDING TO STRUCTURE OF COMPLEXES CONTAINING ENZYME, METAL AND SUBSTRATE

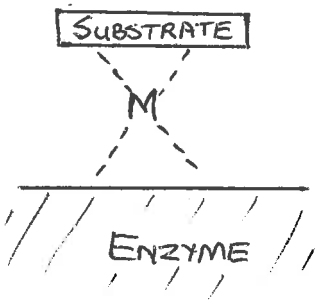
Type I	Type II
Creatine kinase (muscle and brain)	Pyruvate kinase (muscle and yeast)
Adenylate kinase	P-enolpyruvate carboxykinase
Arginine kinase	P-enolpyruvate carboxylase
Hexokinase	Pyruvate carboxylase
Phosphoglycerate kinase	Enolase



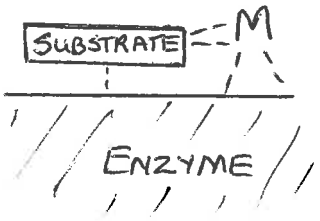
TYPE I



TYPE III



OR



TYPE II

To distinguish between Type II and Type III structures is difficult using only P.R.R. studies and requires the use of continuous wave N.M.R. spectroscopy (Mildvan and Cohn, 1970).

The continuous wave N.M.R. technique allows the detection of a spectrum of a particular nuclei being observed. Proton, ^{31}P, ^{15}N, ^{19}F, and ^{205}Tl spectra have all proved useful. The P.R.R. of water due to such a group, e.g., on the substrate, can be used to establish an interaction between this group and another compound by the perturbation of the P.R.R. ($^{1}/T_1$ and $^{1}/T_2$). A broadening of the spectrum of this group is also indicative of an interaction with this group and

the added compound. Under favourable circumstances, this broadening can be used to calculate the distance between the interacting groups. This approach has provided direct evidence for the "metal-bridge" structure with pyruvate kinase (Mildvan, Leigh and Cohn, 1967) and pyruvate carboxylase (Mildvan and Scrutton, 1967). A further development of this field has been the use of free radical "spin labels" introduced by Hamilton and McConnell (1968). These were originally developed for detecting small changes in the protein electron paramagnetic resonance (E.P.R.) spectra following a perturbation. However, these labels also influence the P.R.R. of protons in their environment and can be monitored either way. Again, the E.P.R. spectra allow the calculation of distances between interacting groups thus providing direct insight into the topographyof the active sites of enzymes. Both "spin-labelled" substrate analogues, e.g., the NAD analogue in the investigation of alcohol dehydrogenase (Weiner and Mildvan, 1969), and "spin-labelled" sulphydryl reagents, e.g., creatine kinase labelled with an iodoacetamide analogue (Taylor, Leigh and Cohn, 1969) have been used successfully.

Miller et al. (1968) have carried out P.R.R. studies on the interaction of Mn^{2+} with the pig liver mitochondrial PEP carboxykinase. They established that it was a Type II enzyme ($\in_b$ = 14.2 at pH 7.5) and the observed enhancement was reduced in the ternary complexes with PEP ($\in_t$ = 6.7) and IDP

(ϵ_t = 10.4). One mole of Mn^{2+} was bound per mole of enzyme. Ternary complexes of enzyme and Mn^{2+} with OAA or ITP were also demonstrated.

A temperature study of the mechanism of relaxation of the three complexes, enzyme.Mn^{2+}, enzyme.Mn^{2+}.PEP and enzyme.Mn^{2+}.IDP, showed that the P.R.R. of these complexes all showed a positive temperature coefficient. The simplest explanation of this behaviour, based on the studies of Lux and Neiboom (1964), was considered to be that the P.R.R. of all three complexes was determined by the rate of exchange of water protons between the bulk solvent and the coordination sphere of the enzyme-bound Mn^{2+}. Then the relative rates of water proton exchange for the respective complexes could reflect the relative coordination numbers for Mn^{2+} in these complexes, i.e., enzyme-bound Mn^{2+} retained half of its water of hydration in the PEP ternary complex and three-quarters in the IDP complex. On the basis of these arguments, Miller et al. (1968) proposed a model in which Mn^{2+} was chelated to the enzyme by two ligands, PEP displaced two of the four remaining water molecules and IDP one further molecule. It should be noted, however, that the interpretation of the P.R.R. data is not unique. If the P.R.R. is dominated by τ_s, the electron spin correlation time, a positive temperature coefficient will also be observed (Mildvan and Cohn, 1970) and this possibility was not explored for any of the complexes.

A "metal-bridge" complex was certainly suggested by their observation that Mn^{2+} enhanced the affinity of PEP for the enzyme. However, Mn^{2+} decreased the affinity of IDP for the enzyme. This observation was a little more difficult to rationalise in terms of a "metal-bridge" structure. Their results, although consistent with their proposed scheme, are presumably also consistent with a scheme where two moles of Mn^{2+} are involved, one forming a "metal-bridge" with PEP, i.e., the Type II structure, and the other involved in a "substrate-bridge" structure with IDP. Thus, with this scheme, more water molecules in the hydration shell of Mn^{2+} would be lost in the PEP ternary complex (where Mn^{2+} binds to both enzyme and PEP) than with IDP (where Mn^{2+} binds only to nucleotide). In support of this scheme, both kinetic and chemical modification studies suggest the involvement of two moles of Mn^{2+} (see Chapter 6).

4.2. THEORY

A 1 M solution of Mn^{2+} has a P.R.R. approx. 10,000 times greater than that of pure water. In pure water, the principal mechanism of proton relaxation is magnetic dipolar interaction between protons. In the presence of the paramagnetic manganous ion, the P.R.R. is increased because the relaxation time (T_1) is dominated by the larger proton-electron interaction between the water protons and the unpaired electrons of the Mn^{2+}

ion, i.e., the major contribution to the relaxation occurs in the hydration sphere of ions such as Mn^{2+}. Because of the rapid exchange of protons in the hydration sphere with those in the bulk of the solution, the observed relaxation rate ($^1/T_1$) is due to the average of the different local environments of the protons. Correction for the effect due to the bulk of the solution gives the paramagnetic contribution to the observed relaxation rate ($^1/T_{1p}$). The number of water molecules coordinated to the paramagnetic ion is an essential factor in the effect of the ions on the P.R.R. Thus, if a protein were to contain a metal ion which was not in contact with solution, it would have no hydration sphere and thus would not influence the P.R.R. of the bulk solution.

If ligands such as EDTA were to replace the water in the Mn^{2+} hydration sphere, a decrease in the observed P.R.R. would be expected because fewer water molecules are now in close proximity to the metal ion and T_1 tends towards that for pure water. However, in many manganese complexes, the reverse occurs, e.g., for the complexes Mn.ADP$^-$ and Mn.ATP^{2-}, the P.R.R. is slightly increased. When Mn^{2+} is bound to a macromolecule, enhancements of the P.R.R. of up to twenty-fold have been recorded.

The term enhancement factor, ϵ^*, has been introduced (Eisinger et al., 1962) to quantitate the changes in P.R.R. under different conditions and is defined as the ratio of the

relaxation rates of the water protons in the presence and absence of complexing agent.

$$\epsilon^* = \frac{R_1^*}{R_1} = \frac{{}^1/T_1^* - {}^1/T_1^*(0)}{{}^1/T_1 - {}^1/T_1(0)} = \frac{{}^1/T_{1P}^*}{{}^1/T_{1P}} \quad \ldots (4.1)$$

where R_1 is the P.R.R. due to Mn^{2+}, T_1 and $T_1(0)$ are the observed longitudinal relaxation times in the presence and absence of Mn^{2+} respectively, ${}^1/T_{1P}$ is the paramagnetic contribution to the P.R.R. and * denotes the presence of complexing agent.

According to Luz and Meiboom (1964), the paragmagnetic contribution (${}^1/T_{1P}$) to the observed P.R.R. is given by

$$\frac{1}{T_{1P}} = \frac{P}{T_{1M} + \tau_M} \quad \ldots (4.2)$$

where is the mole fraction of water protons in the first coordination sphere, T_{1M} is the relaxation time of a water proton in the first coordination sphere and τ_M is the resident time of a water proton in the first coordination sphere.

T_{1M} is inversely proportional to the correlation time τ_c defined as (Bloemberger and Morgan, 1961):

$$\frac{1}{\tau_c} = \frac{1}{\tau_{r'}} + \frac{1}{\tau_s} \quad \ldots (4.3)$$

where τ_r, is the rotational correlation time of the hydrated ion or complex and τ_s is the electron spin relaxation time of the paramagnetic ion. For the manganous ion, the correlation time (τ_c) is dominated by τ_r, as τ_s is of the order of 10^{-8} sec. and τ_r, 10^{-11} sec.

On binding of Mn^{2+} to a macromolecule, p will always decrease tending to give a decreased P.R.R., τ_M may either increase or decrease but usually within one order of magnitude of the value of free Mn^{2+}, but τ_c may vary by as much as three orders of magnitude. Thus, it is this term in T_{1M} which changes drastically when one observes an increased enhancement for a Mn-protein complex. Further information on the relative contribution of these terms may come from studies at variable temperature and/or frequency. Thus, τ_r, has a positive temperature coefficient, τ_s and $^1/T_{1M}$ have negative temperature coefficients while $^1/T_{1M}$ usually has a much larger energy of activation than τ_s (Maldvan and Cohn, 1970).

4.3. METHODS

Analar $MnCl_2$ was obtained from B.D.H. Ltd., treated with dithizone and standardised (Morrison et al., 1961). All other chemicals used were as described in Chapter II. PEP carboxykinase, specific activity 3-5 units per mg of protein, prepared by the method described in Chapter 3, was centrifuged

and the pellet dissolved in 0.15 ml of either 0.1 M imidazole Cl^-, pH 6.5, or 0.05 M N-ethyl-morpholine Cl^-, pH 7.5. The enzyme solution was passed through a Sephadex G25 column (85 x 5 mm) equilibrated with the appropriate buffer and 0.3 ml of eluant was collected after the void volume. Protein concentration was determined as described in Chapter 2.

(a) Determination of the P.R.R.

Proton relaxation times of solutions (volume 0.1 to 0.2 ml) were measured with a pulsed apparatus of the type described by Carr and Purcell (1954) operating at 30 megacycles per sec. and assembled by Mr. K. Marsden of the Physics Department, University of New South Wales. The position of the null (τ_{null}) was determined visually on a Tetronix Inc. Type 535 Oscilloscope and readings were taken from an Elron Minicounter. The null reading is related to the relaxation time (T_1) by $\tau_{null} \times 1.41 = T_1$ sec. Temperature was 20°.

(b) Definitions

ϵ_f = enhancement of free Mn^{2+} = 1

ϵ_a = enhancement of binary Mn.S complex

ϵ_b = enhancement of binary E.Mn complex

ϵ_t = enhancement of ternary E.Mn.S complex

ϵ^* = observed enhancement at finite [E] and [S]

ϵ^*_c = extrapolated enhancement at infinite [S] and finite [E]

Subscripts f, b and t refer to free, complexed and total species.

Equation 4.1 gave the expression for the observed enhancement of the P.R.R. This value is a weighted average of each paramagnetic species present. Thus,

$$\epsilon^{*} = \frac{Mn_f}{Mn_t} \cdot \epsilon_f + \frac{Mn_b}{Mn_t} \cdot \epsilon_b \qquad \ldots (4.4)$$

By definition, $\epsilon_f = 1$, thus Equation 4.4 becomes

$$\epsilon^{*} = \frac{Mn_f}{Mn_t} + \frac{Mn_b}{Mn_t} \cdot \epsilon_b \qquad \ldots (4.5)$$

For a system containing substrate, the expression for the enhancement is the sum of four terms.

$$\epsilon^{*} = \frac{Mn_f}{Mn_t} + \frac{Mn.S}{Mn_t} \cdot \epsilon_a + \frac{E.Mn}{Mn_t} \cdot \epsilon_b \; \frac{E.Mn.S}{Mn_t} \cdot \epsilon_t \qquad \ldots (4.6)$$

where Mn.S, E.Mn and E.Mn.S are the manganese-substrate, enzyme-manganese and the enzyme-manganese-substrate complexes, respectively.

In addition, the following dissociation constants are defined:

$$K_D = \frac{[E]_f\,[Mn]_f}{[E.Mn]} \qquad \ldots (4.7)$$

$$K_1 = \frac{[Mn]_f\,[S]_f}{[Mn.S]} \qquad \ldots (4.8)$$

$$K_2 = \frac{[E]_f\,[Mn.S]_f}{[E.Mn.S]} \qquad \ldots (4.9)$$

$$K_S = \frac{[E]_f\,[S]_f}{[E.S]} \qquad \ldots (4.10)$$

$$K_3 = \frac{[E.Mn]_f\,[S]_f}{[E.Mn.S]} \qquad \ldots (4.11)$$

4.4. RESULTS

(a) Binary Enzyme-Mn^{2+} Complex

Figures 4.1 and 4.2 show the results of a titration of constant PEP carboxykinase with Mn^{2+} at pH 7.5. In Figure 4.1, the reciprocal of the observed enhancement (ϵ^*) is plotted against the total Mn^{2+} concentration (Mildvan and Cohn, 1963). A straight line is obtained which, when extrapolated to zero Mn^{2+} concentration, yields a value for $_b$ of 8.3. Applying Equation 4.5 allows the replot of the data of Figure 4.1 according to the method of Hughes and Klotz (1956) (Fig. 4.2). This gives a straight line, whose ordinate intercept gives $\frac{1}{n}$ where n is the number of binding sites,

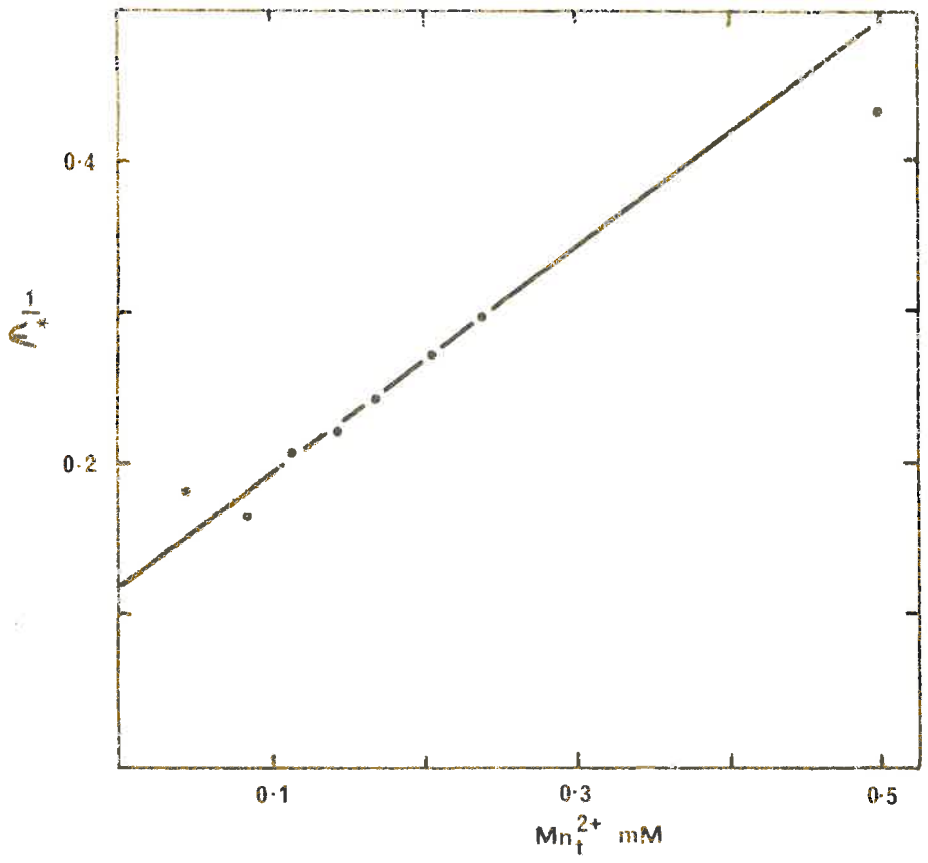


FIG. 4.1. Titration of PEP carboxykinase with Mn^{2+} at pH 7.5. Each solution contained 0.08M N-ethylmorpholine (Cl^-), pH 7.5, 0.115mM enzyme and Mn^{2+} as indicated. Temperature was 20°.

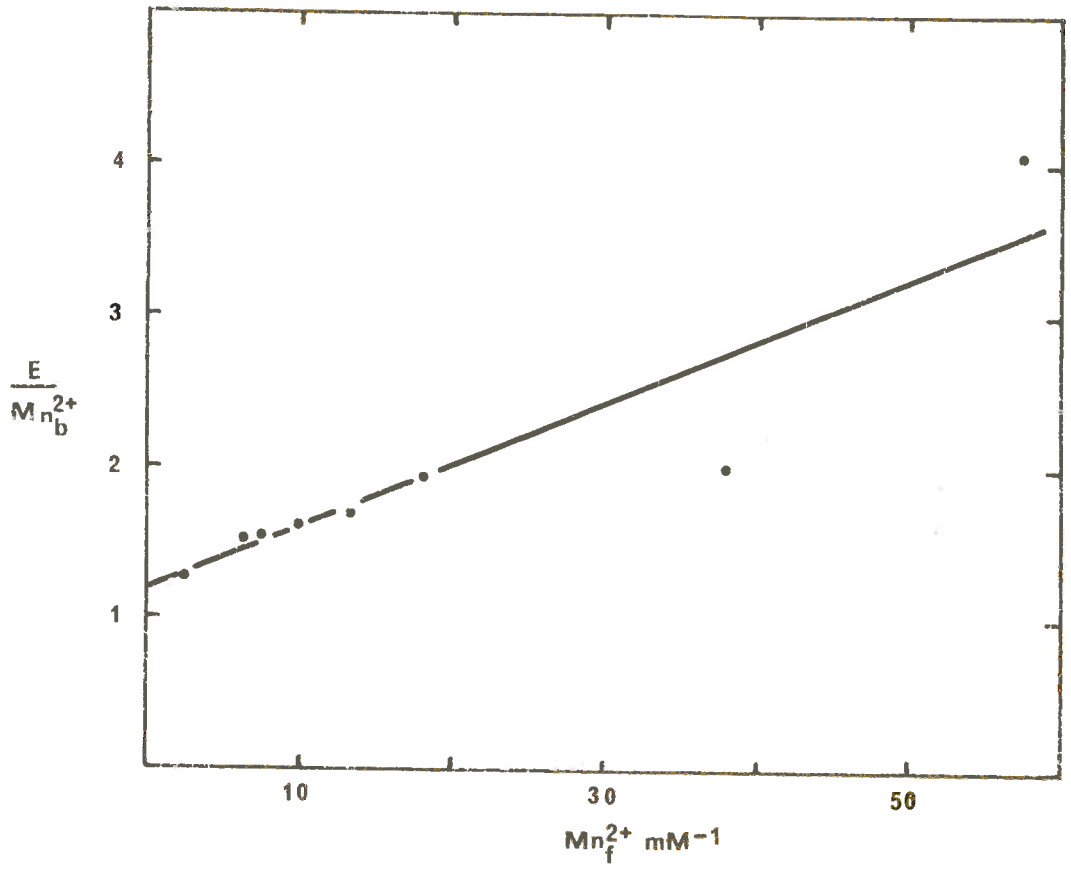


FIG. 4.2. Hughes-Klotz (1956) plot of the results of Fig. 4.2. The intercept on the ordinate gives $^1/n$, where n is the number of Mn^{2+} binding sites, and the intercept on the abscissa gives $-^1/K_D$, where K_D is the dissociation constant for the enzyme-Mn^{2+} complex.

while the abscissa intercept gives $-1/K_D$. From this graph, n = 0.83 which is effectively one. Thus, one mole of Mn^{2+} binds per mole of enzyme with a K_D of 3.45×10^{-5}M.

Fig. 4.3 shows the results of a titration of constant Mn^{2+} with PEP carboxykinase at pH 7.5. A double reciprocal plot of enhancement against enzyme gives a straight line yielding a value for ϵ_b of 8.3. Applying Equation 4.5 and substituting directly into Equation 4.7 gives an average value for K_D of 5.52×10^{-5} M which is in good agreement with that obtained in the first titration.

The two previous titrations were repeated at pH 6.5 and the results are presented in Figures 4.4 - 6. The enhancements at this pH were much smaller and thus the measurements were less precise. Also, at this pH there was a tendency for the protein to precipitate. However, values for ϵ_b of 3.2 and 2.9 were obtained from Figures 4.4 and 4.6 respectively. Titration with Mn^{2+} gave a value for K_D of 5.9×10^{-5} M with one mole of Mn^{2+} bound per mole of enzyme (n = 0.88, Fig. 4.5). Titration with enzyme gave an average value for K_D of 8.58×10^{-5} M which is in reasonable agreement with the value obtained from the other titration. Thus, there is little change in the affinity of the enzyme for Mn^{2+} over the pH range 6.5 to 7.5.

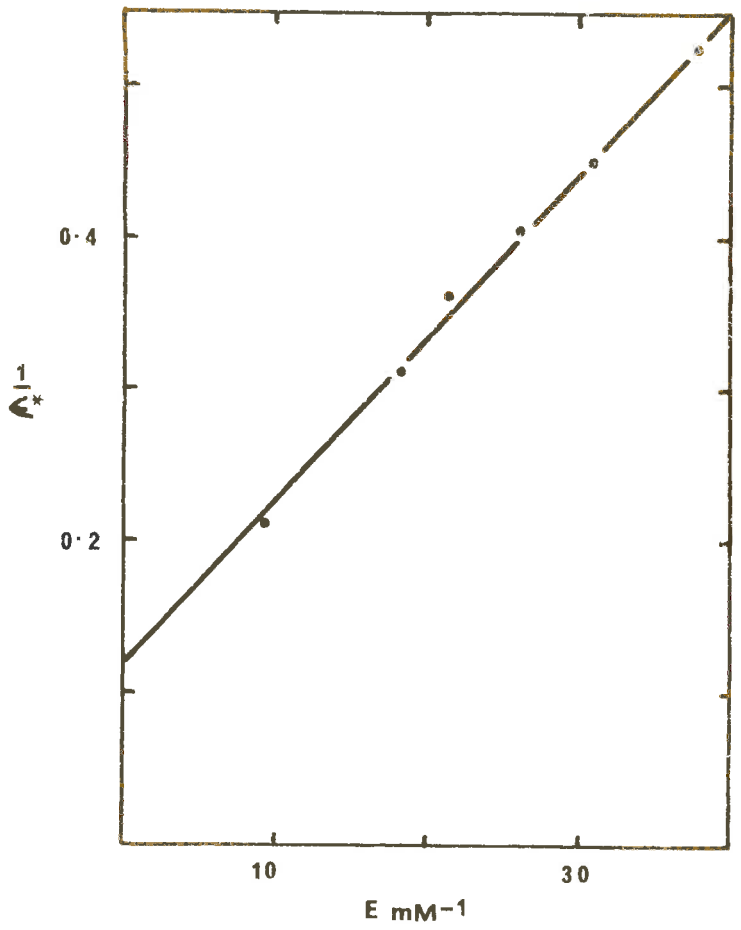
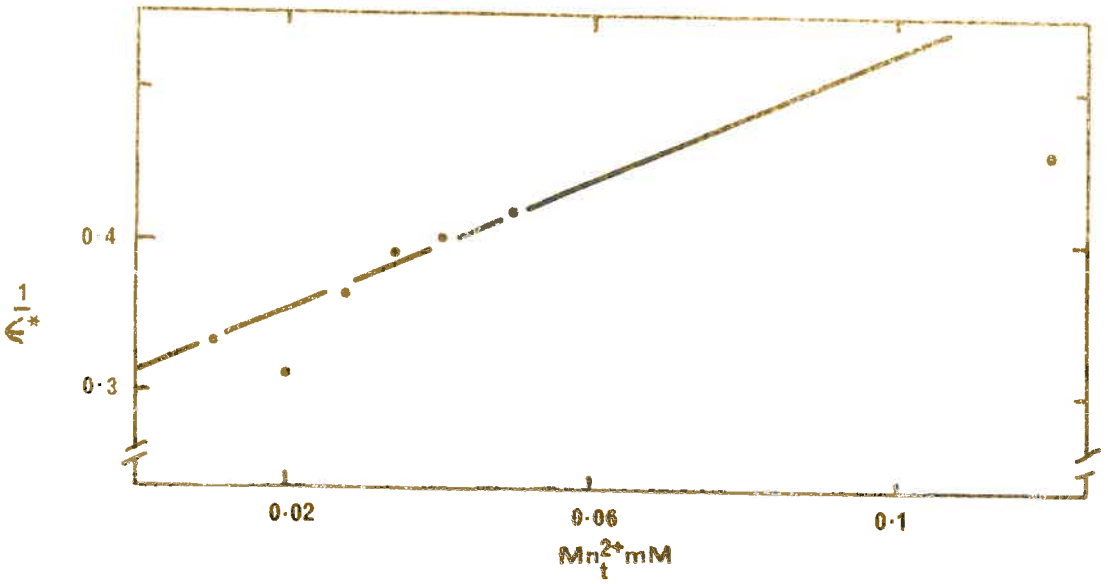


FIG. 4.3. Titration of Mn^{2+} with PEP carboxykinase at pH 7.5. Each solution contained 0.08M N-ethylmorpholine (Cl^-), pH 7.5, 0.12mM $MnCl_2$ and enzyme as indicated. Temperature was 20°.



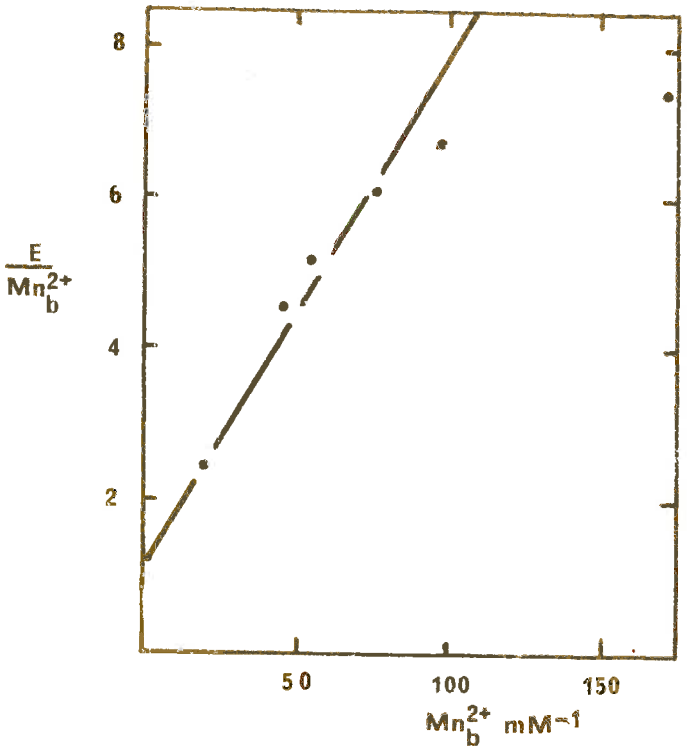


FIG. 4.4. (top) Titration of PEP carboxykinase with Mn^{2+} at pH 6.5. Each solution contained 0.15M imidazole (Cl^-), pH 6.5, 0.16mM enzyme and Mn^{2+} as indicated. Temperature was 20°.

FIG. 4.5. (bottom) Hughes-Klotz (1956) plot of the results of Fig. 4.4. This plot was analysed as for Fig. 4.2.

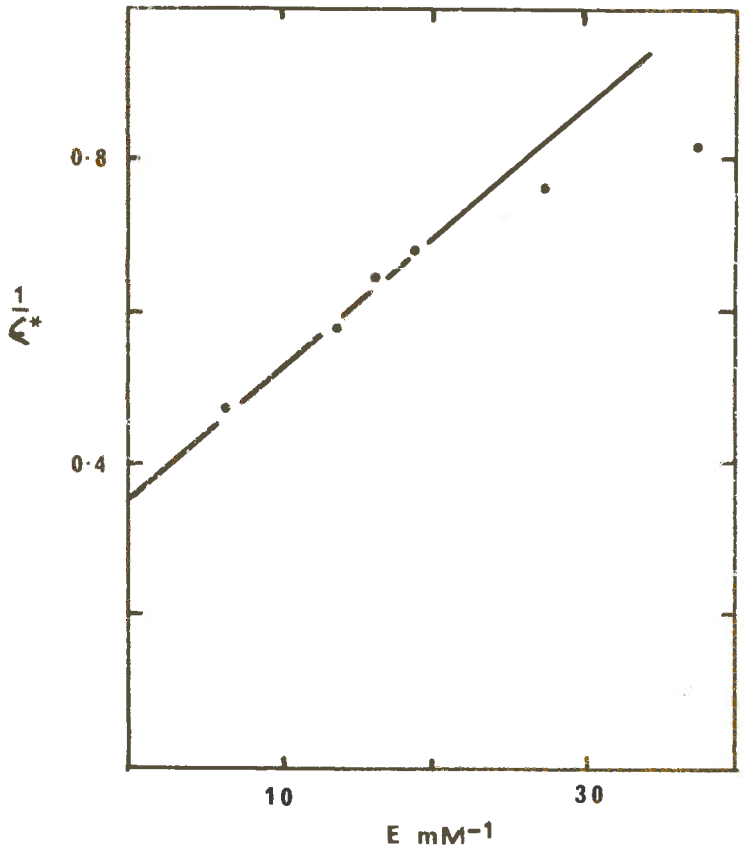


FIG. 4.6. Titration of Mn^{2+} with PEP carboxykinase at pH 6.5. Each solution contained 0.15M imidazole (Cl^-), pH 6.5, 0.12mM $MnCl_2$ and enzyme as indicated. Temperature was 20°.

(b) Ternary Enzyme-Mn-PEP Complex

At pH 7.5, addition of PEP to the binary enzyme. Mn^{2+} complex caused a decrease in the observed enhancement. The effect was observed in the presence and absence of IDP and $NaHCO_3$. Titration of the binary complex with PEP was carried out at three enzyme concentrations. A plot of enhancement (ϵ^*) against the reciprocal of the PEP concentration allows the calculation of values for ϵ_c^* by extrapolation, to infinite PEP (Mildvan and Cohn, 1966) (Fig. 4.7). A replot of the reciprocal of ϵ_c^* against the reciprocal enzyme concentration gives, on extrapolation to infinite enzyme concentrations, a value for ϵ_t of 6.4 (Mildvan and Cohn, 1966) (Fig. 4.8). Using Procedure III (Mildvan and Cohn, 1966) the value for K_3 obtained from this data was 1.13×10^{-5} M and Procedure I gives $K_2 = 1.26 \times 10^{-4}$ M while substitution in Equation 8 (Mildvan and Cohn, 1966) gives 2.27×10^{-4} M for K_2.

At pH 6.5, there was a small increase (approx. 5%) in the P.R.R. on addition of 0.1 - 0.2 mM PEP to the binary complex but the difference was too small to titrate.

(c) Ternary Enzyme-Mn-IDP Complex

In this case, there was a small decrease (approx. 5% in the enhancement on addition of 0.1 mM IDP to the binary complex at pH 7.5. At pH 6.5, there was an increase in P.R.R. and the results of titration of two concentrations of enzyme

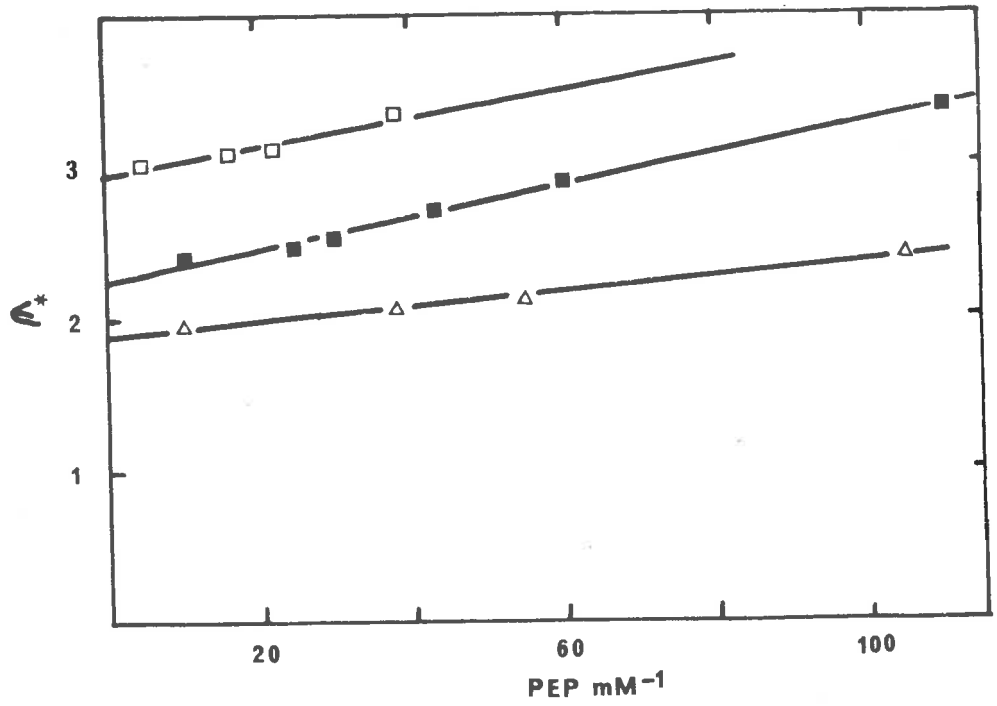


FIG. 4.7. Titration of 0.12mM Mn^{2+} with PEP at three enzyme concentrations. The observed enhancement (ϵ^*) is plotted against the reciprocal PEP concentration. Linear extrapolation to infinite PEP gives ϵ_c^*. (Mildvan and Cohn, 1966). Each solution contained 0.07M N-ethylmorpholine (Cl^-), pH 7.5. Temperature was 20°. PEP carboxykinase concentrations were: (□——□), 0.115mM; (■——■), 0.083mM; (△——△), 0.057mM.

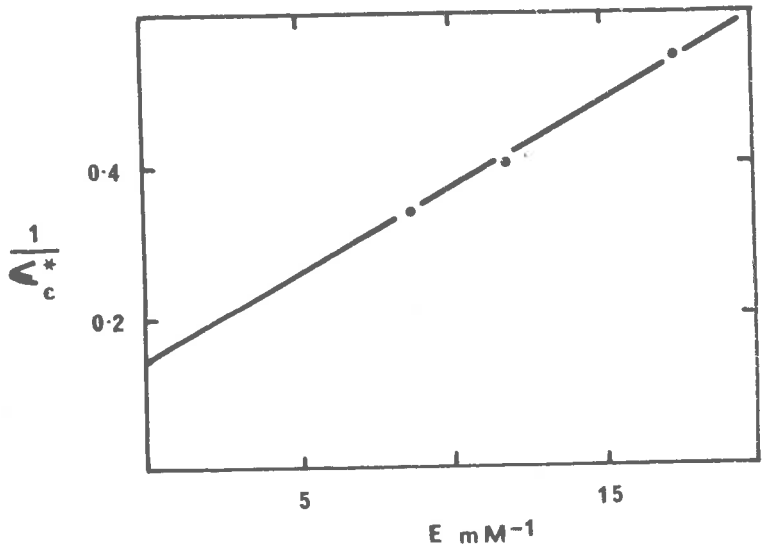


FIG. 4.8. Double reciprocal plot of ϵ_c^* obtained from Fig. 4.7 against enzyme concentration. Linear extrapolation to infinite enzyme concentration gives ϵ_t (Mildvan and Cohn, 1966).

with IDP at constant Mn^{2+} are shown in Figure 4.9. This gives an estimate for K_3 of 1.94×10^{-5} M (Procedure III of Mildvan and Cohn, 1966) and ϵ_t would approximate 4.0.

4.5. DISCUSSION

Table 4.2 presents a summary of the results obtained in this study. These show that the sheep kidney mitochondrial PEP carboxykinase is similar to pig liver mitochondrial PEP carboxykinase (Miller et al., 1968) with respect to Mn^{2+} and substrate binding.

At pH 6.5 and 7.5, the sheep kidney mitochondrial enzyme binds one mole of Mn^{2+} per mole although ϵ_b at pH 7.5 (8.3) is much higher than at pH 6.5 (3.0). Thus, this enzyme belongs to the Type II group of enzymes (Mildvan and Cohn, 1970) where a "metal-bridge" structure presumably exists. Such a structure has been clearly demonstrated now for two Type II enzymes, pyruvate kinase (Mildvan, Leigh and Cohn, 1967; Mildvan and Scrutton, 1967) and pyruvate carboxylase (Mildvan and Scrutton, 1967). The fact that Ca^{2+} can not activate PEP carboxykinase (Chapter 6) is consistent with its classification as a Type II enzyme (Cohn, 1963).

The observed ϵ_b of 8.3 is lower than that reported for other Type II enzymes, namely, peanut cotyledon PEP carboxylase (13.8) and PEP carboxykinase from pig liver mitochondria (14.2)

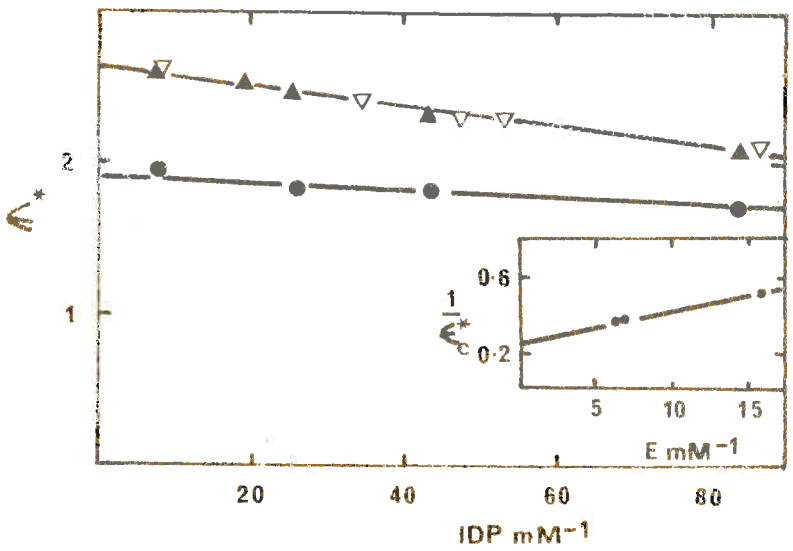


FIG. 4.9. Titration of 0.12mM Mn^{2+} with IDP at three enzyme concentrations. ϵ^* is plotted against the reciprocal IDP concentration. Linear extrapolation to infinite IDP concentration gives ϵ_c^* (Mildvan and Cohn, 1966). Temperature was 20°. Each solution contained: 0.08M imidazole (Cl^-), pH 6.5, and 0.156mM enzyme (▽——▽); 0.15M imidazole (Cl^-), pH 6.5, and 0.16mM enzyme (▲——▲); 0.13M imidazole (Cl^-), pH 6.5, and 0.063mM enzyme (●——●). Inset. Double reciprocal plot of ϵ_c^* against enzyme concentration. Linear extrapolation to infinite enzyme concentration gives ϵ_t (Mildvan and Cohn, 1966).

TABLE 4.2: SUMMARY OF N.M.R. RESULTS

Substrate	pH	ϵ_b	ϵ_t	K_D(M)	K_2(M)	K_3(M)
Mn^{2+}	6.5	3.0		5.9×10^{-5}		
Mn^{2+}	7.5	8.3		3.45×10^{-5}		
PEP	7.5		6.4		1.26×10^{-4}[a]	1.13×10^{-5}[c]
					2.27×10^{-4}[b]	
IDP	6.5		4.0			1.94×10^{-5}[c]

a, Procedure I of Mildvan and Cohn (1966);

b, Substitution in Equation 8 of Mildvan and Cohn (1966);

c, Procedure III of Mildvan and Cohn (1966).

(Miller et al., 1968), yeast enolase (13.8) (Cohn, 1963) and rabbit muscle pyruvate kinase (32.7) (Mildvan and Cohn, 1965). However, it is to be expected that variations such as these will occur from one protein to another reflecting subtle differences in the microenvironment of the Mn^{2+} ion.

The difference in ϵ_b at the two pH values is significant and would suggest that at pH 6.5 the environment of the Mn^{2+} was more "open" or more accessible to solvent than at pH 7.5. An alternative explanation is that a group responsible for Mn^{2+} binding ionises over this pH range such that Mn^{2+} is now bound less firmly. This does not appear to be the case as the difference in K_D values at the two conditions of pH is small and is within experimental error. Cohn (1963) found a similar change in ϵ^* with pH for Mn^{2+} binding to bovine serum albumin. In this case, ϵ^* was chiefly affected by a group of $pK_a \approx 7$ while K_D was not affected by this group but was strongly affected by a group of $pK_a \approx 8.5$.

At pH 7.5, addition of IDP and ITP to the binary enzyme. Mn^{2+} complex causes a small decrease in ϵ^* although the decrease was too small to warrant a detailed titration. Miller et al. (1968) reported much larger decreases with the pig liver mitochondrial enzyme under similar conditions. At pH 6.5, addition of IDP to the binary enzyme.Mn^{2+} complex resulted in an increase in ϵ^*. This may indicate an altered mode of binding of Mn^{2+} or an altered environment because of a conformational change in the protein. If an altered mode

of binding occurred, this could be achieved by a second mole of Mn^{2+} binding through the nucleotide to give an enzyme.Mn^{2+}. substrate.Mn^{2+} complex or a relocation of the bound Mn^{2+} to give the ternary enzyme.IDP.Mn^{2+} complex. In support of such structures, Miller *et al.* (1968) have reported the formation of higher order complexes of the type enzyme.Mn^{2+}. substrate.Mn^{2+} for both GDP and GTP with pig liver mitochondrial PEP carboxykinase consistent with the metal complex of the nucleotide being the true substrate for the enzyme.

While the data is consistent with an altered mode of Mn^{2+} binding, it does not exclude an altered environment being responsible for the observed increase in $\in^{*}$ as there is evidence for an IDP-induced conformational change. It has been shown that IDP (and ITP) induces a significant increase in the rate of the CO_2:OAA exchange reaction (and OAA decarboxylation) without undergoing reaction itself. This stimulation of the exchange reaction is completely independent of the decarboxylation reaction (see Chapter 6), *i.e.*, IDP (and ITP) stimulates the CO_2:OAA exchange reaction *per se* by inducing a more favourable environment or conformation.

Miller *et al.* (1968) analysed the observed decrease in enhancement at pH 7.5 with IDP in terms of a displacement of one water molecule from the hydration shell of Mn^{2+} similar to the proposed scheme for pyruvate kinase (Mildvan and

and Cohn, 1966). However, this explanation could not account for an increase in $\in^{*}$ at pH 6.5 and it is perhaps fortuitous that such a "simplified" interpretation is feasible.

Similar considerations of the data obtained with PEP are possible. At pH 6.5, an increase in $\in^{*}$ too small for a full titration is observed on addition of PEP to the binary enzyme.Mn^{2+} complex. At pH 7.5, a decrease in the P.R.R. enhancement is observed.

The magnitude of the decrease observed by Miller *et al.* (1968) under similar circumstances was greater ($\in_b : \in_t$ = 14.7 : 6.7) than that reported here ($\in_b : \in_t$ = 8.3 : 6.4). These workers interpreted the decrease in terms of the loss of two water molecules from the hydration sphere of Mn^{2+}. Applying this approach to the sheep kidney enzyme we find a decrease in enhancement from 8.3 to 6.4, *i.e.*, a 25% decrease, consistent with the loss of one water ligand if we assume that the protein provides two ligands for Mn^{2+}. Such a figure would surely be a minimum and has been proposed as the figure for pyruvate kinase (Mildvan and Cohn, 1965). Furthermore, Mildvan and Cohn (1966) have proposed that PEP only provides the one ligand for Mn^{2+} in pyruvate kinase in contrast to the two proposed for pig liver mitochondrial PEP carboxykinase. Thus, the data for the sheep kidney enzyme is consistent with a structure for the ternary enzyme.Mn^{2+}.PEP complex similar to that proposed for pyruvate kinase. At pH 6.5, the predominant

influence on the enhancement of the P.R.R. would again be conformational considerations as was the case with IDP.

Comparison of the behaviour at pH 6.5 and 7.5 on addition of PEP or IDP to the binary enzyme.Mn^{2+} complex show that the changes in ϵ^* occur in opposite directions, i.e., ϵ^* decreases in both cases at pH 7.5 but increases in both cases at pH 6.5. This provides strong evidence for conformational changes in the enzyme induced by substrate binding especially at pH 6.5. Such induced conformational changes would be in accord with the "induced-fit" theory proposed by Koshland (1958).

No effect on ϵ^* could be detected with $NaHCO_3$ although ternary complexes were detected with ITP and OAA in addition to IDP and PEP. From these studies, it was concluded that PEP, IDP, OAA and ITP can all bind independently although the requirement for Mn^{2+} for the binding of these substrates cannot be established from this data.

CHAPTER 5. CONTROL OF PEP CARBOXYKINASE.

5.1. INTRODUCTION

It would be expected that PEP carboxykinase would be subject to several control mechanisms considering its key position in gluconeogenesis. However, attempts to detect any metabolic effectors have so far failed in mammalian systems while in microorganisms only one example of an acute control has been reported, viz., an allosteric inhibition by $NADH_2$ in E. coli (Wright and Sanwal, 1969). In this case, sigmoidal saturation kinetics, shown with PEP, HCO_3^- and OAA, are detected only in the presence of the effector and this is not the result of any change in the polymeric state of the enzyme. However, these authors gave no indication of whether their enzyme preparation was contaminated with malate dehydrogenase as low levels of this activity with the added $NADH_2$ could produce the observed effects. On the other hand, there are numerous examples of adaptive control via changes in enzyme levels in response to perturbations in the metabolic and/or hormonal states of an organism and, in general, any changes which do occur are in the same direction as overall gluconeogenesis (Scrutton and Utter, 1968; Shrago and Shug, 1966; Shrago and Shug, 1969; de Torrontegui, Palacian and Losada, 1966).

When initial velocities of sheep kidney mitochondrial

PEP carboxykinase were measured with an uncoupled carboxylation assay system and plotted as a function of PEP concentration, a normal rectangular hyperbola was obtained when all other substrates were at saturating levels. However, if IDP was present at fixed non-saturating concentrations, deviations from a rectangular hyperbola were consistently observed. Double reciprocal plots of this data were biphasic with apparent substrate activation. Furthermore, other combinations of substrates also showed similar results. These data would suggest that PEP carboxykinase exhibited negative cooperativity with respect to IDP (or PEP) binding (Levitzki and Koshland, 1969), and that this property might be important in the control of the enzyme *in vivo*.

However, subsequent analysis showed that these kinetic effects could be eliminated if a coupled assay system were used to convert OAA to aspartate or malate. It therefore appears that the non-Michaelis-Menten kinetics result from an accumulation of OAA, *i.e.*, OAA is a powerful product inhibitor. It is proposed that OAA acts as an effective inhibitor of its own synthesis.

5.2. MATERIALS AND METHODS

PEP carboxykinase used in this section was prepared as detailed in Chapter 3 and had a specific activity of 2 - 2.5 units per mg protein (Section 2.8).

(a) Initial velocity studies on the uncoupled carboxylation reaction

The carboxylation of PEP was followed by the incorporation of $^{14}CO_2$ into OAA which was stabilised as the 2,4-dinitrophenylhydrazone. The 0.5 ml reaction mixtures contained (in μmoles) : imidazole (Cl^-), pH 6.5 (adjusted at 30°), or N-ethylmorpholine (Cl^-), pH 7.5, 50; GSH, 0.8; PEP carboxykinase, up to 0.01 units; $MnCl_2$ or $MgCl_2$, PEP, IDP and $NaH^{14}CO_3$ (6×10^5 cpm per μmole) as indicated in the legends to the figures. The reaction was initiated by the addition of PEP carboxykinase (up to 0.015 units), incubated for 5 min at 30° and stopped by the addition of 0.05 ml of 6 N HCl saturated with 2,4-dinitrophenylhydrazine. Each assay solution was processed in triplicate as described in Section 2.7.ii.

Reaction velocity data were processed on a CDC 6400 digital computer using the HYPERB program of Cleland (1963d) where the computer analyses were performed on a best fit to the hyperbolic form of rate equations (Equation 5.1)

$$v = \frac{V\,S}{K + S} \qquad \ldots (5.1)$$

assuming equal variance for the velocities. In those cases where biphasic double reciprocal plots of velocity against substrate concentration were obtained, the data corresponding to the two linear regions were analysed separately.

(b) GDP binding to PEP carboxykinase

The apparatus and method used was that described by Colowick and Womack (1969). PEP carboxykinase dissolved in 0.025 M imidazole (Cl^-), pH 6.5, and β-^{32}P-GDP were added to the upper chamber, while 0.025 M imidazole (Cl^-), pH 6.5, was pumped through the lower chamber at the rate of 8 ml per min. The effluent was collected (2 ml fractions) for measurement of radioactivity. The concentration of GDP in the upper chamber was progressively increased with 5 - 10 µl aliquots of unlabelled GDP every two min. Aliquots (1 ml) of the effluent were added to 9 ml of Triton X100 counting medium and the radioactivity was determined in a Packard-Tricarb scintillation spectrometer.

The steady-state concentration of radioactivity in the effluent at any given substrate concentration is taken as a measure of the fraction of the total substrate in the upper chamber in the freely diffusible state. When excess substrate (about 100 fold relative to the enzyme concentration) is added, the radioactivity in the effluent is taken to be that corresponding to 100% of the substrate in the free state. This allows the calculation of the concentration of the free and

bound substrate at each GDP concentration.

(c) Triton X100 counting medium

The counting medium used consisted of 2 volumes of scintillation fluid (Section 2.5) and 1 volume of Triton X100.

5.3. RESULTS

The initial velocity patterns shown in Fig. 5.1 were obtained when PEP and IDP were the variable and fixed variable substrates respectively. A deviation from a rectangular hyperbola was consistently observed and this effect became more pronounced as the concentration of IDP was decreased. The same data presented as a double reciprocal plot are shown in Fig. 5.2 where a biphasic plot is obtained with an inflection point at 0.2 mM PEP. From this plot, R_s values (the ratio of PEP concentrations at 90% and 10% of the maximum velocity) of 93.5, 155 and 174 were obtained at the IDP concentrations of 0.8 mM, 0.2 mM and 0.1 mM respectively as opposed to a value of 81 for Michaelis-Menten kinetics. The progressive increase in the R_s value confirms that deviations from hyperbolic saturation by PEP became more pronounced as the IDP concentration decreased. Hill plots of the initial velocity data obtained with 0.8 mM and 0.2 mM are presented in Fig. 5.3. With 0.8 mM IDP, the plot was essentially linear with unit slope in accord with an R_s value of 93.5 whereas with 0.2 mM IDP, a triphasic curve was obtained with the slope decreasing to 0.37 in the region

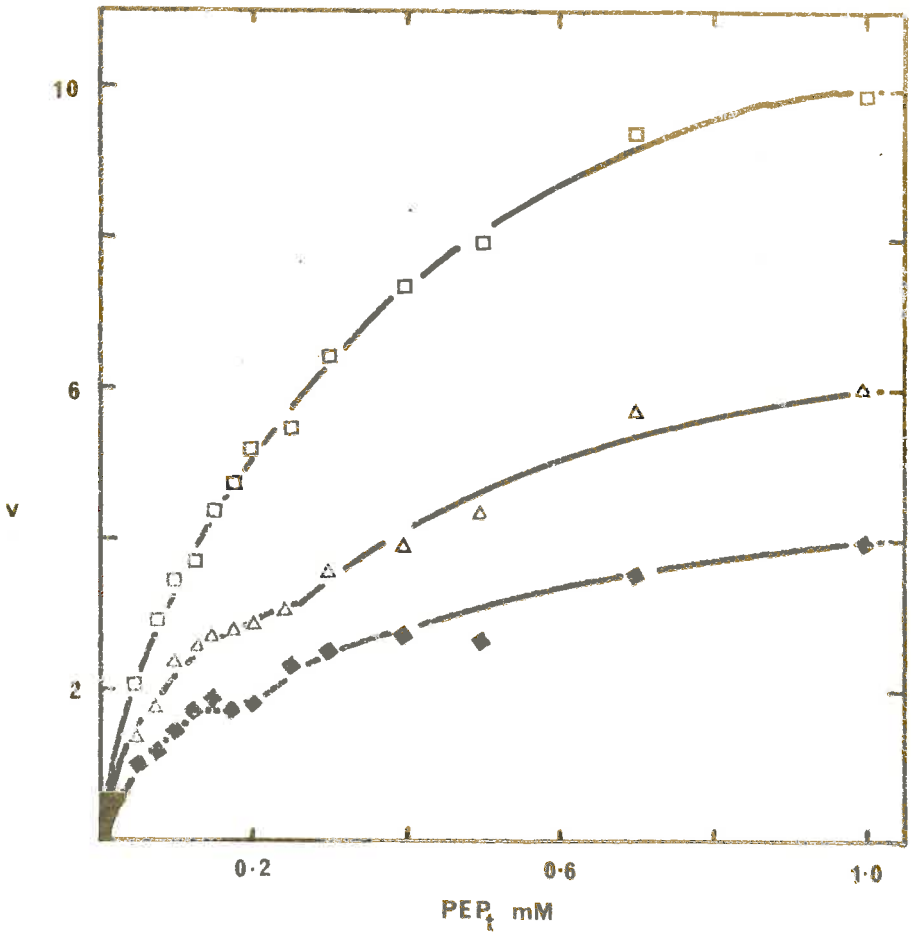


FIG. 5.1. Initial velocity as a function of PEP concentration with Mn^{2+} at pH 6.5. PEP concentration was varied at three IDP concentrations: (□——□), 0.8mM; (△——△), 0.2mM; (◆——◆), 0.1mM. The points are the experimental values, the lines are the computer-determined lines of best fit using the HYPERB program of Cleland (1963d) (see Section 5.2.(a)). Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $MnCl_2$, 1; $NaH^{14}CO_3$, 5; GSH, 0.8; PEP and IDP as indicated; PEP carboxykinase, 0.005 units. Incubation was for 5 min at 30° and the reaction stopped with 0.05 ml of 6M HCl saturated with 2,4-dinitrophenylhydrazine. The radioactivity determinations were corrected for quenching by the channels ratio method.

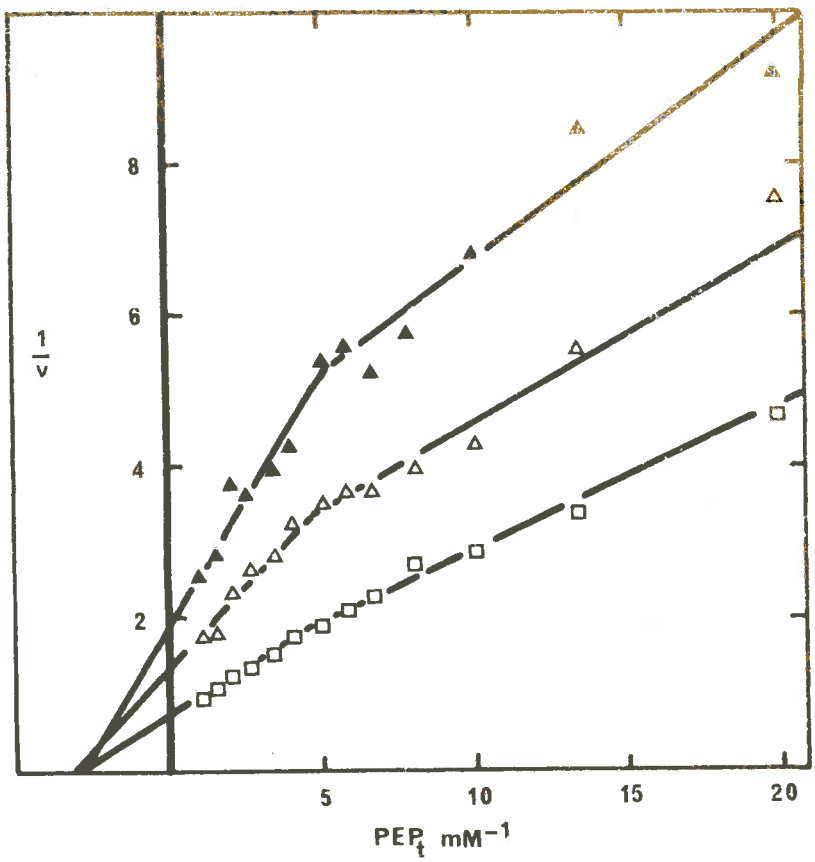


FIG. 5.2. Double reciprocal plot of initial velocity against PEP concentration at pH 6.5. Data is from Fig. 5.1. (□——□), 0.8mM IDP; (△——△), 0.2mM IDP; (▲——▲), 0.1mM IDP.

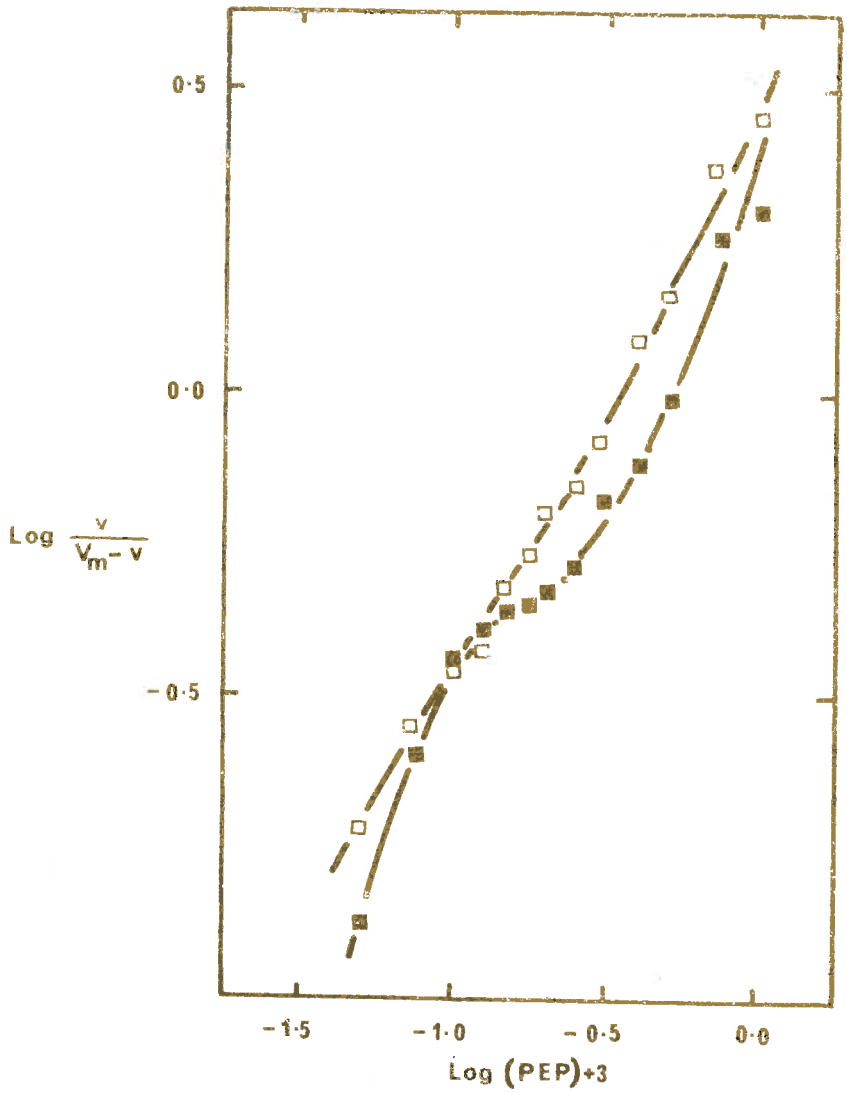


FIG. 5.3. Hill plot for the data presented in Fig. 5.1. (□——□), 0.8mM IDP; (■——■), 0.2mM IDP.

of 0.2 mM PEP.

The pH optimum of PEP carboxykinase was dependent on the activating metal ion, pH 6.5 with Mn^{2+} and pH 7.5 with Mg^{2+} (Chapter 3), whereas the pH of the kidney mitochondrion would be closer to pH 7.5 with Mg^{2+} in excess of Mn^{2+}. It is therefore possible that the observed initial velocity patterns result from the choice of pH and divalent cation for the kinetic studies. To eliminate this possibility, the initial velocity patterns were investigated at pH 7.5 with Mn^{2+} and Mg^{2+} (Figs. 5.4, 5.5). Essentially the same behaviour was obtained in both cases with the inflection point at 0.2 mM PEP as before. It would therefore appear that the biphasic kinetic patterns are a property of the enzyme and not of the choice of divalent cation or pH.

This biphasic initial velocity pattern was also obtained when IDP and HCO_3^- were the variable and fixed variable substrates respectively, but not when PEP and HCO_3^- were varied (Figs. 5.6 - 7). In the former case, the inflection in the double reciprocal plot occurred at 0.2 mM IDP. In contrast to the results when PEP and IDP were varied, the magnitude of the deviation from Michaelis-Menten kinetics was apparently independent of the HCO_3^- concentration as indicated by a constant R_s value of 125. It would appear that non-linear double reciprocal plots are only obtained when IDP is non-saturating while the R_s value is constant if PEP is held constant (Fig. 5.6) but varies if PEP varies (Fig. 5.2; 5.4 - 5).

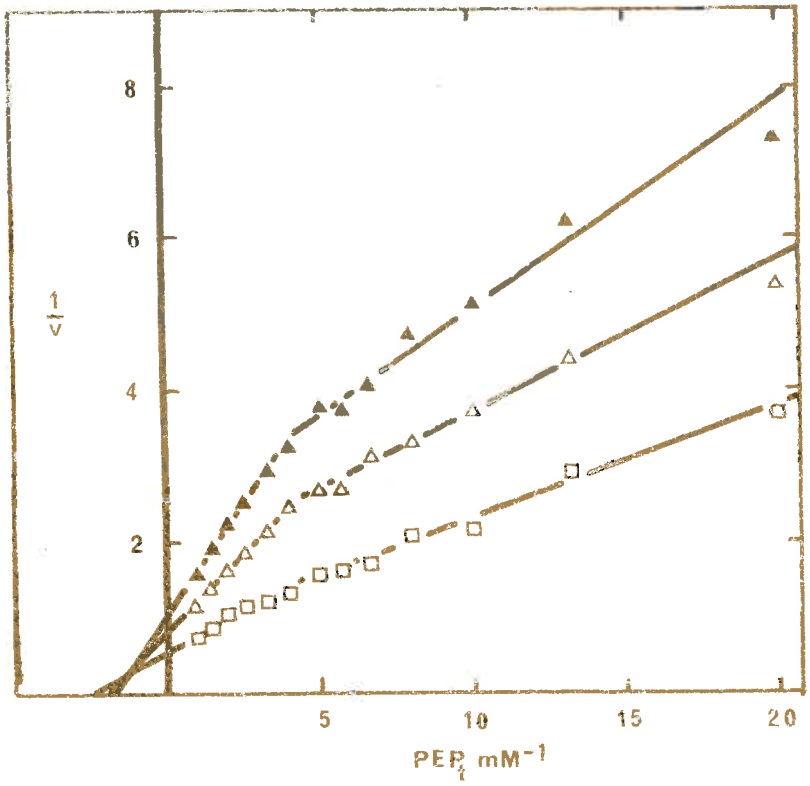


FIG. 5.4. Double reciprocal plot of initial velocity against PEP concentration with Mn^{2+} at pH 7.5. PEP concentration was varied at the three IDP concentrations; (□———□), 0.8mM; (△———△), 0.2mM; (▲———▲), 0.1mM. Conditions were as for Fig. 5.1. except that the buffer was N-ethylmorpholine (Cl^-), pH 7.5, and 0.01 units of enzyme were added.

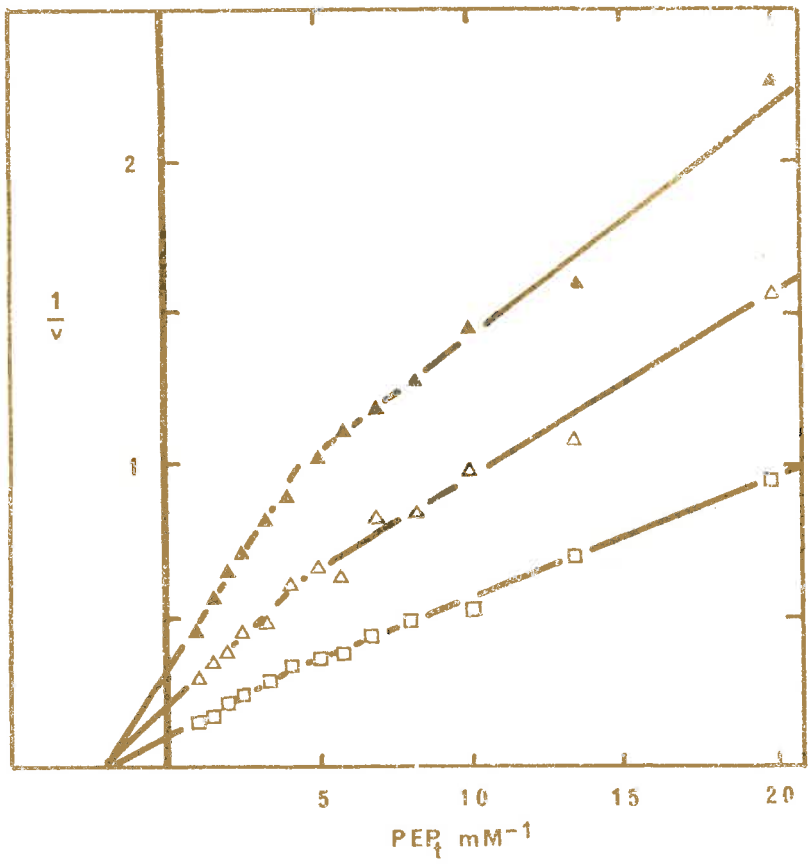


FIG. 5.5. Double reciprocal plot of initial velocity against PEP concentration with Mg^{2+} at pH 7.5. PEP concentration was varied at the three IDP concentrations: (□——□), 0.8mM; (△——△), 0.2mM; (▲——▲), 0.1mM. Conditions were as for Fig. 5.4. except 2mM $MgCl_2$ and 0.005 units of enzyme were used.

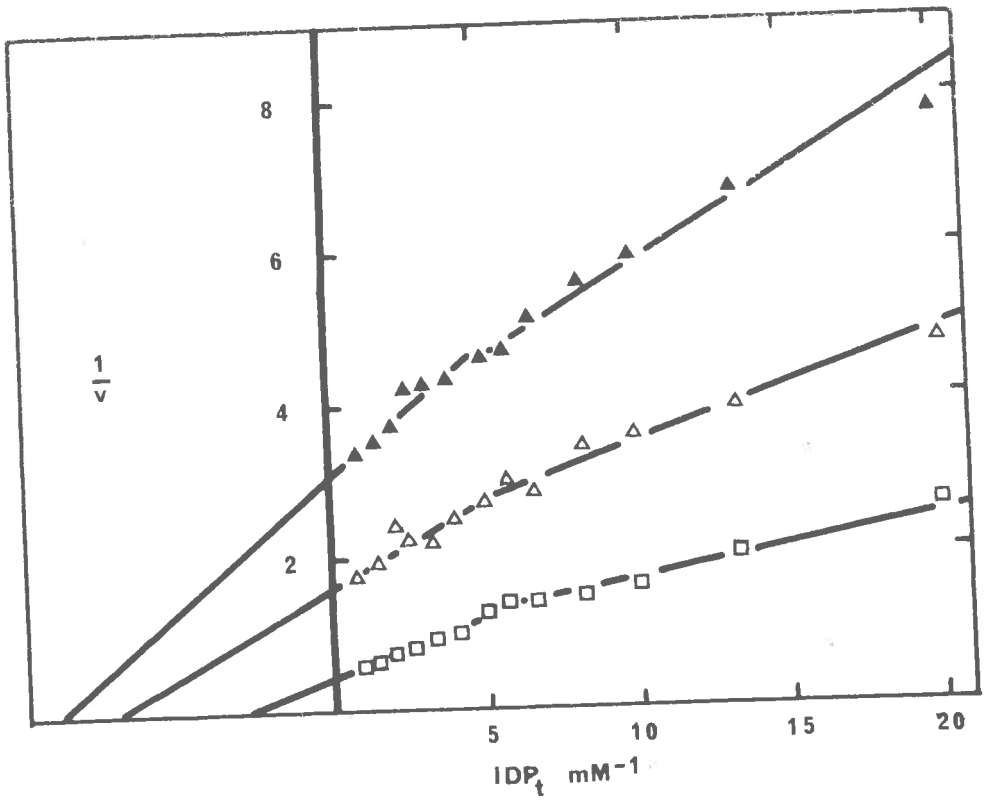


FIG. 5.6. Double reciprocal plots of initial velocity against IDP concentration. IDP concentration was varied at three fixed levels of HCO_3^-: (□——□), 8.0mM; (△——△), 2.0mM; (▲——▲), 1.0mM. Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $MnCl_2$, 1; PEP, 0.5; GSH, 0.8; IDP and $H^{14}CO_3^-$ as indicated; 0.005 units of enzyme. Assay conditions were as for Fig. 5.1.

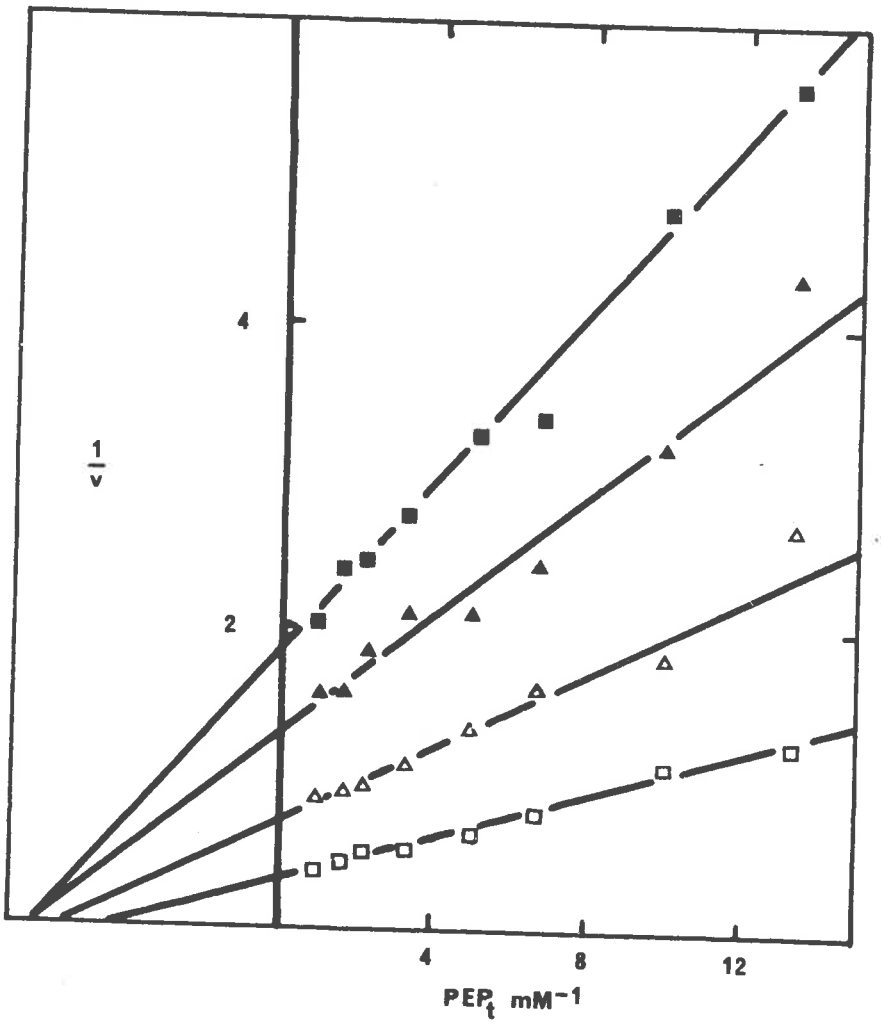


FIG. 5.7. Double reciprocal plots of initial velocity against PEP concentration. PEP concentration was varied at four fixed levels of HCO_3^-: (□——□), 8.0mM; (△——△), 3.0mM; (▲——▲), 1.5mM; (■——■), 1.0mM. Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $MnCl_2$, 1; IDP, 0.5; GSH, 0.8; PEP and $H^{14}CO_3^-$ as indicated; 0.005 units of enzyme. Assay conditions were as for Fig. 5.1.

The situation becomes more complex if the metal ion is held at certain fixed concentrations while the other three substrates are varied (Figs. 5.8 - 10). Apparent negative cooperativity was only shown when PEP was varied but not when IDP was varied. The observed interaction when PEP was the variable could be explained by Mn.IDP$^-$ being the true substrate (see Chapter 6) such that decreasing Mn^{2+} concentrations also decreased the Mn.IDP$^-$ levels (cf. Fig. 5.1). No explanation can be put forward for the lack of any interaction when IDP and Mn^{2+} were the covariables.

This system satisfies the criteria defined by Levitzki and Koshland (1969) for negative cooperativity in substrate binding: (a) the plot of initial velocity against substrate concentration shows an intermediary plateau region, (b) the double reciprocal plot of velocity against substrate concentration is biphasic with increasing slope at high substrate concentrations, (c) the R_s value is greater than 81 and (d) the Hill plot is non-linear with an intermediate region of slope much less than unity.

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968) and E. coli alkaline phosphatase (Simpson and Vallee, 1970) both possess these catalytic features. Furthermore, both enzymes have been shown to exhibit negative cooperativity with respect to substrate binding. In the former case, four moles of NAD per mole of enzyme

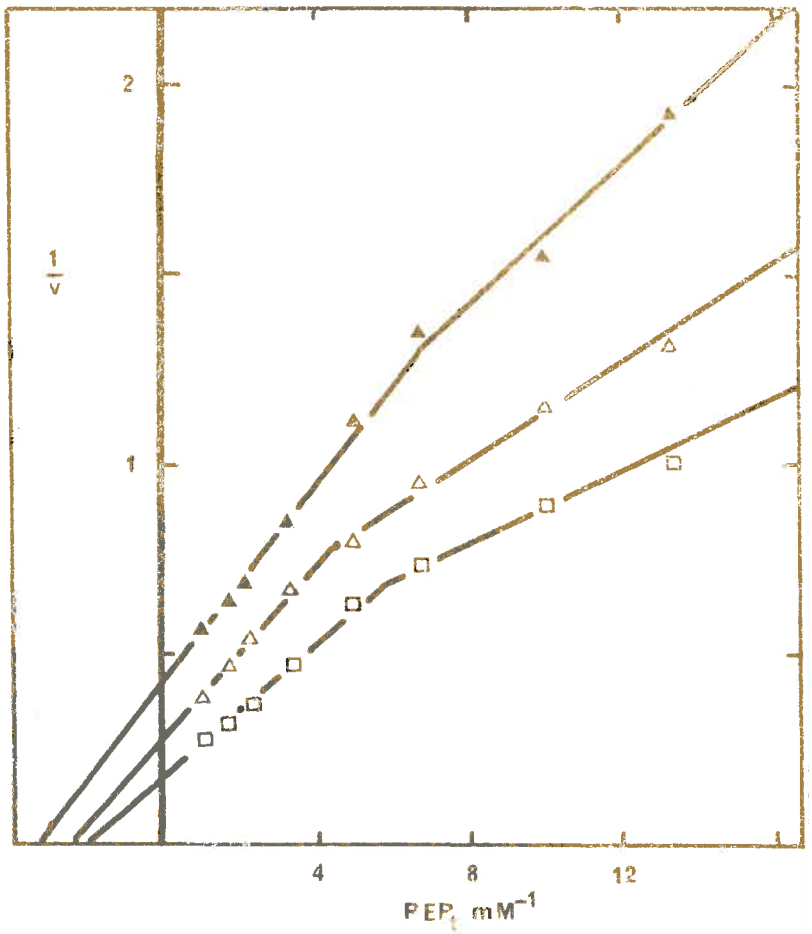


FIG. 5.8. Double reciprocal plot of initial velocity against PEP concentration. PEP concentration was varied at three fixed levels of $MnCl_2$: (□——□), 1.6mM; (△——△), 0.4mM; (▲——▲), 0.2mM. Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; IDP, 0.5; $H^{14}CO_3^-$, 5; GSH, 0.8; PEP and $MnCl_2$ as indicated; 0.003 units of enzyme. Assay conditions were as for Fig. 5.1.

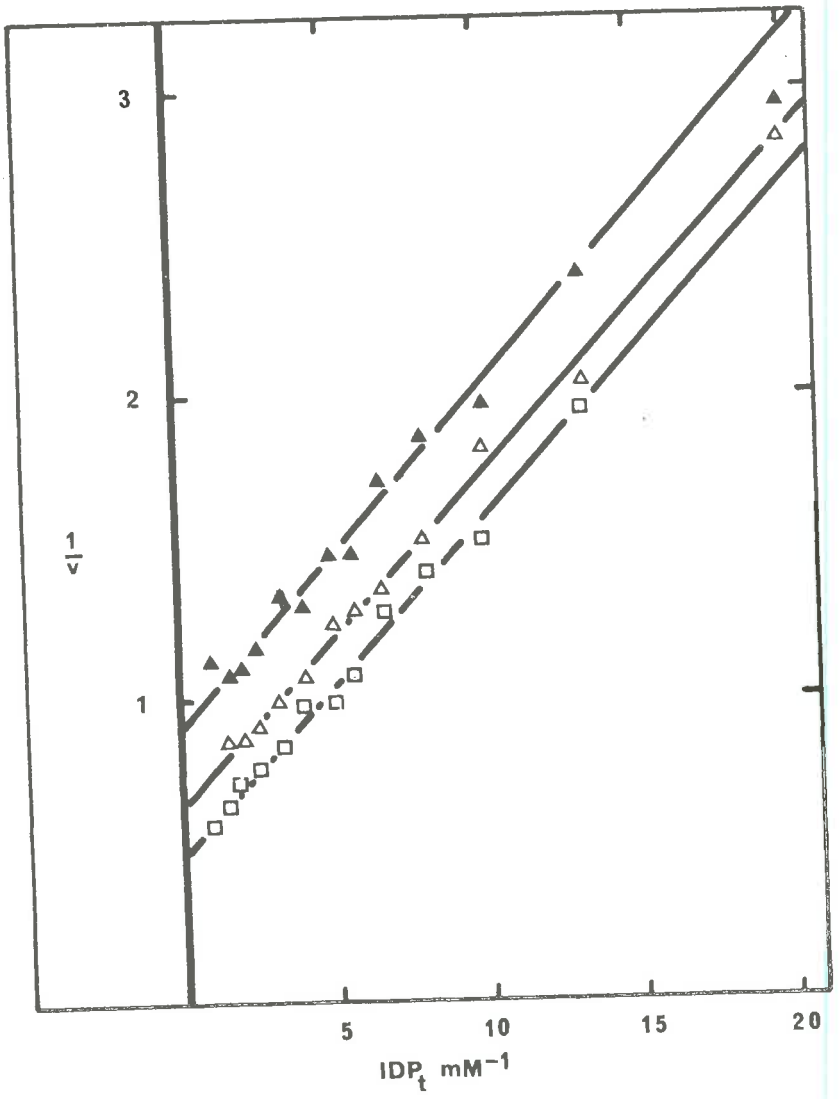


FIG. 5.9. Double reciprocal plot of initial velocity against IDP concentration. IDP concentration was varied at three fixed levels of $MnCl_2$: (□——□), 1.6mM; (△——△), 0.4mM; (▲——▲), 0.2mM. Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; PEP, 0.5; $H^{14}CO_3^-$, 5; GSH, 0.8; IDP and $MnCl_2$ as indicated; 0.005 units of enzyme. Assay conditions were as for Fig. 5.1.

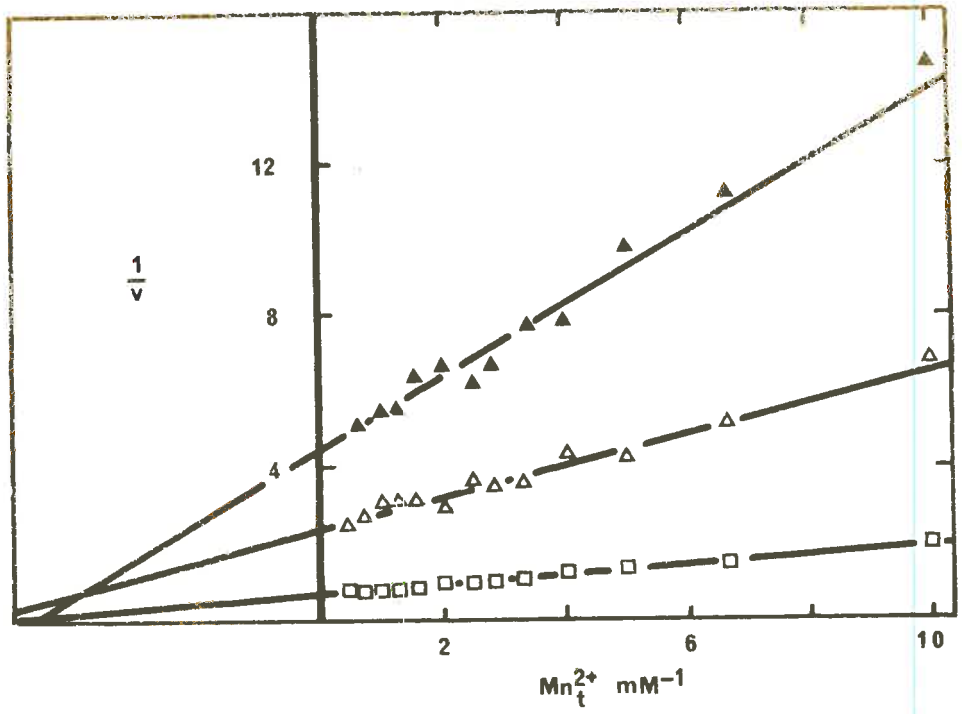


FIG. 5.10. Double reciprocal plot of initial velocity against $MnCl_2$ concentration. $MnCl_2$ concentration was varied at three fixed levels of HCO_3^-: (□——□), 8.0mM; (△——△), 2.0mM; (▲——▲), 1.0mM. Assays contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; PEP, 0.5; IDP, 0.5; GSH, 0.8; $MnCl_2$ and $H^{14}CO_3^-$ as indicated; 0.005 units of enzyme. Assay conditions were as for Fig. 5.1.

are bound with progressively decreasing affinity. In the latter case, two moles of PP_i per mole of enzyme are bound in a discontinuous manner with the second molecule bound less readily than the first. In fact, the example of alkaline phosphatase illustrates the limited application of the theoretical treatment of negative cooperativity by Teipel and Koshland (1969). These workers restricted their treatment to a rapid random situation and showed that more than two substrate sites were necessary for this interaction to occur.

The binding of GDP to PEP carboxykinase was investigated and showed only one mole of GDP bound per mole of enzyme ($n = 0.92$; Fig. 5.11). This is contrary to that expected for negative cooperativity as this concept requires a minimum of two molecules of substrate per enzyme molecule to achieve an interaction between binding sites.

If the basis of the biphasic initial velocity patterns was negative cooperativity with respect to IDP or PEP binding, these patterns would not be altered if a coupled assay were employed. With PEP carboxykinase, conversion of OAA to aspartate by the addition of aspartate transaminase, glutamate and pyridoxal phosphate resulted in linear double reciprocal plots as well as a marked increase in observed activity (Fig. 5.12). Addition of the coupling components either separately or in various combinations had no effect on the kinetics of the uncoupled assay unless the complete coupling

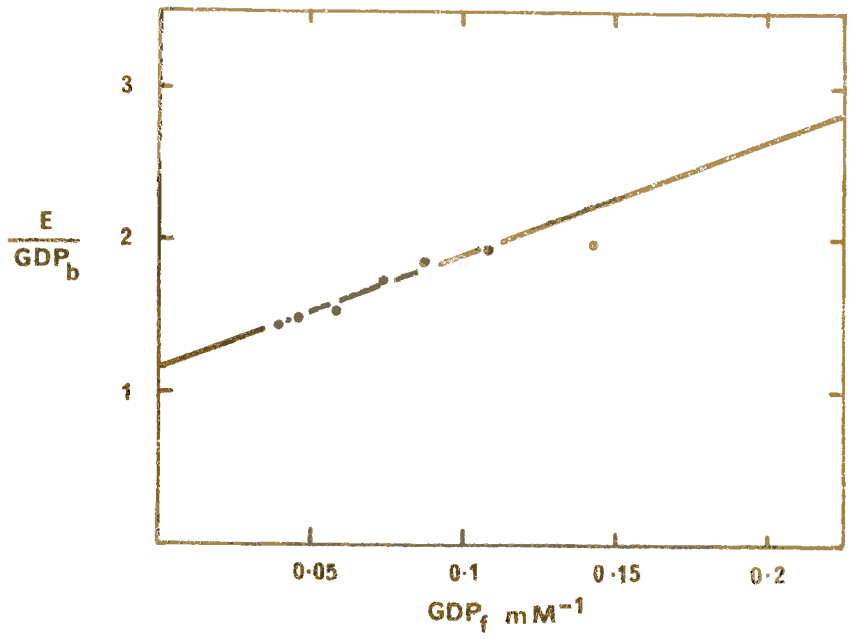


FIG. 5.11. Binding of GDP to PEP carboxykinase. A Hughes-Klotz (1956) plot of the data obtained for the binding of β-^{32}P-GDP to PEP carboxykinase using the apparatus and method described by Colowick and Womack (1969). The intercept on the ordinate gives $^{1}/n$, where n is the number of GDP binding sites, and the intercept on the abscissa gives $-^{1}/K_D$, where K_D is the dissociation constant of the enzyme.GDP complex. The buffer used was 0.025M imidazole (Cl^-), pH 6.5, and the temperature was 20^{o}.

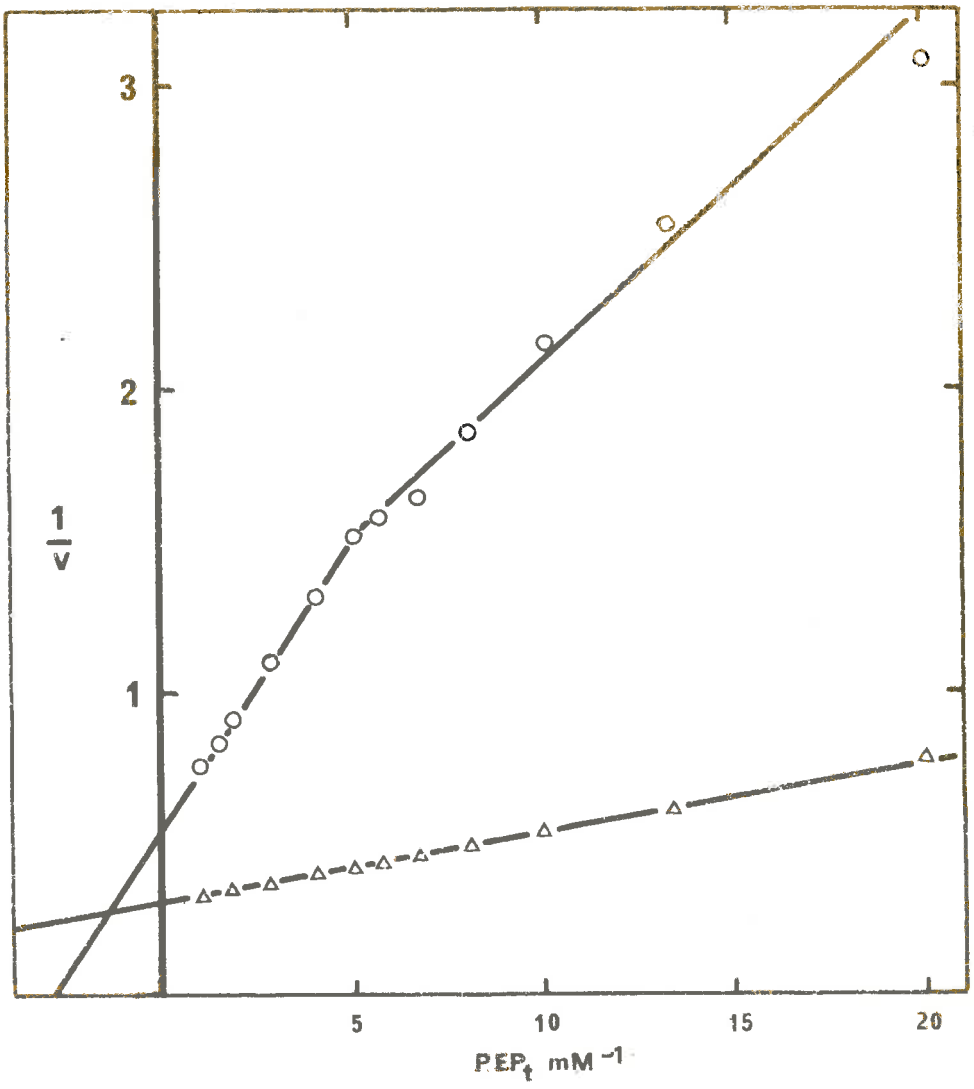


FIG. 5.12. Comparison of the coupled and uncoupled assay procedures. (o——o), coupled assay procedure; (o——o), uncoupled assay procedure. Assay mixtures contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; IDP, 0.5; PEP (various amounts); $MnCl_2$, 2; $NaH^{14}CO_3$ (100 μC per μmole), 10; GSH, 0.8; enzyme, 0.01 units; plus, in the coupled procedure, sodium glutamate, 10; pyridoxal phosphate, 0.02; aspartate transaminase, 4.5 units. Incubation was for 5 min at 30° and the reaction stopped with 0.05 ml of 6M HCl saturated with 2,4-dinitrophenylhydrazine. The radioactivity determinations were corrected for quenching by the channels ratio method.

system was present. This effect was verified with a second coupled system where OAA was converted to malate and results similar to those obtained with the aspartate transaminase couple were obtained (Fig. 5.13). These last two observations would argue against sheep kidney mitochondrial PEP carboxykinase exhibiting negative cooperativity as defined by Levitzki and Koshland (1969).

5.4. DISCUSSION

When PEP and HCO_3^- or IDP and Mn^{2+} or HCO_3^- and Mn^{2+} were covariables, the initial velocity patterns obtained with an uncoupled assay system were families of straight lines. However, when PEP and IDP or IDP and HCO_3^- or PEP and Mn^{2+} were the variable pairs, the double reciprocal plots were non-linear showing increasing slope at high substrate concentrations. In these cases, the criteria of negative cooperativity in substrate binding as defined by Levitzki and Koshland (1969) are satisfied, namely, the plot of initial velocity against substrate concentration shows an intermediary plateau, the double reciprocal plot is biphasic with increasing slope at high substrate concentration, the R_s value is greater than 81 and the Hill plot is non-linear with an intermediate region of slope less than unity.

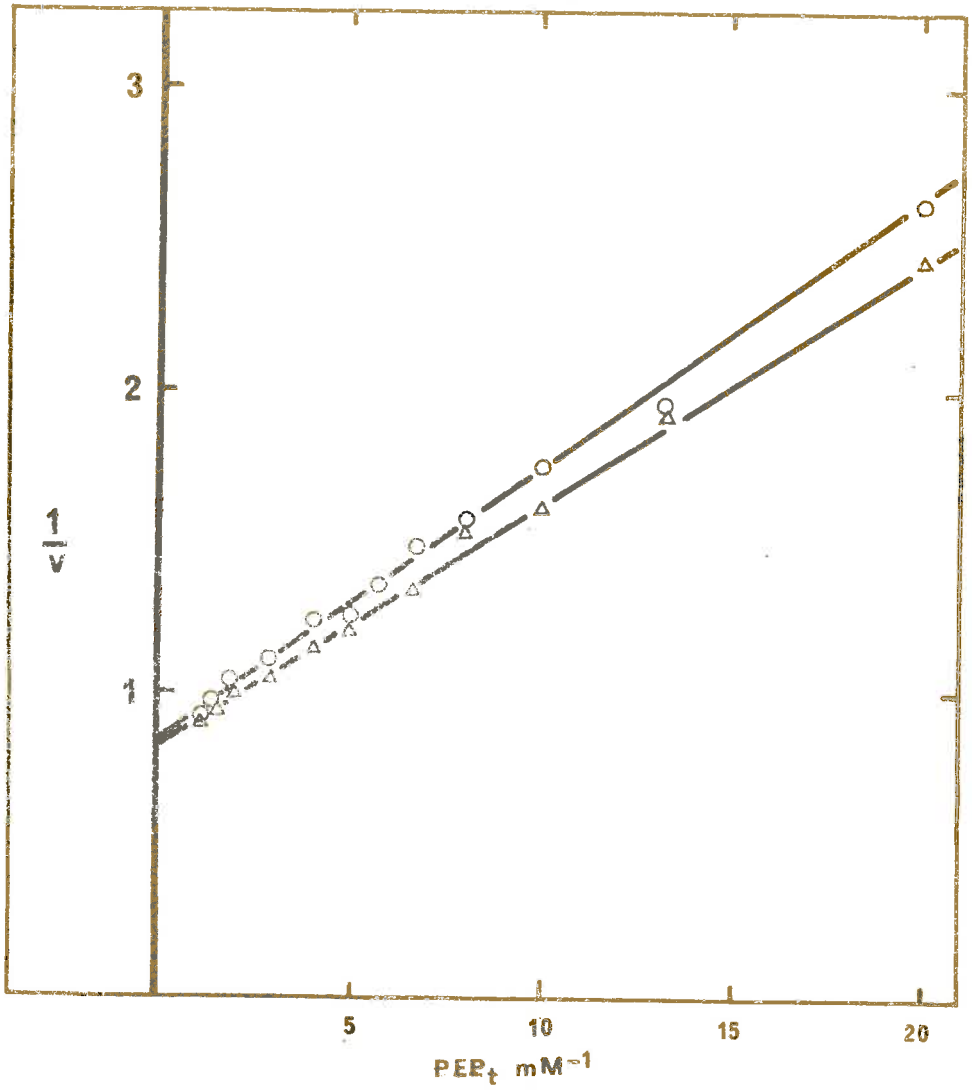


FIG. 5.13. Comparison of the aspartate transaminase and malate dehydrogenase coupled procedures. Reaction mixtures were as for the coupled procedure of Fig. 5.12 except that, for the malate dehydrogenase coupled procedure, the additional components were : malate dehydrogenase, 4 units; $NADH_2$, 1 µmole. $NaH^{14}CO_3$ was 50 µC per µmole. The assays were stopped after 5 min at 30° with 0.2 ml of a 10% (w/v) solution of trichloroacetic acid. (o——o), malate dehydrogenase; (Δ——Δ), aspartate transaminase.

However, four factors argue against the concept of negative cooperativity satisfactorily explaining the observed results. These are (a) the change in the R_s value for the data of Fig. 5.1, (b) the manifestation of the interaction appears to be dependent on the pair of substrates varied, (c) only one mole of IDP is bound per mole of PEP carboxykinase and (d) the biphasic initial velocity patterns are eliminated if a coupled assay system is used.

With a negative homotropic cooperative interaction, the R_s value would remain constant at a figure greater than 81 regardless of the concentration of the other substrates. This is the case for glutamate dehydrogenase which shows negative cooperativity with respect to NAD (I'eJohn and Jackson, 1968). If the plots of velocity against NAD concentration at various glutamate concentrations are normalised, the curves become identical. In the more complex case of pyruvate carboxylase, which exhibits positive cooperativity with respect to acetyl CoA (Barritt et al., 1966; Scrutton and Utter, 1967; Cazzulo and Stoppani, 1968) and $Mg.ATP^{2-}$ and Mg^{2+} (Keech and Barritt, 1967; Cazzulo and Stoppani, 1969), this enzyme also shows negative cooperativity with respect to pyruvate while the R_s value for pyruvate was essentially unchanged with pH and over a ten fold range of fixed levels of either acetyl CoA or $Mg.ATP^{2-}$ (Taylor, Nielsen and Keech, 1969). Similarly, with CTP synthetase, the R_s value for glutamine

appears to be independent of the concentration of GTP, an allosteric activator (Levitzki and Koshland, 1969).

In the case of a negative heterotrophic cooperative interaction, the R_s value would be expected to vary where the degree of interaction of one effector is dependent on the concentration of a second effector. However, there is no apparent interaction when IDP is varied at fixed Mn^{2+} levels while in all other cases where IDP is varied (PEP and HCO_3^- the covariables) there is clearly some interaction. PEP can not be implicated as the prime effector because when PEP is varied, the interaction is only apparent when IDP and Mn^{2+} are the covariables. Any interactions would therefore appear to depend on the pair of substrates varied rather than being attributable to IDP (or PEP) in particular. This conclusion is further borne out by the fact that only one mole of IDP is bound per mole of PEP carboxykinase.

Sheep kidney mitochondrial pyruvate carboxylase shows negative cooperativity with respect to pyruvate similar to that shown by the equivalent liver enzyme (Taylor, Nielsen and Keech, 1969). The assay employed to determine the initial velocities of pyruvate carboxylase was an uncoupled one similar to that used here. However, the interactions were not eliminated when the aspartate transaminase couple was included in the assay (Nielsen, 1970). Therefore, the results with the coupled assay would eliminate any possibility of the

concept of negative cooperativity as defined by Levitzki and Koshland (1969) being operative in sheep kidney mitochondrial PEP carboxykinase. What this finding does suggest is a powerful product inhibition by OAA. This inhibition could readily be effected by altering the pathway for the formation of the central complex consisting of enzyme, Mn^{2+}, PEP, IDP and CO_2 (see Chapter 6). It is significant that, in all reports concerning mammalian PEP carboxykinase, coupled carboxylation assays have been used.

E. coli PEP carboxylase has been classed by Levitzki and Koshland (1969) as an enzyme showing negative cooperativity. This was based on the data of Corwin and Fanning (1968) obtained with an uncoupled assay. The interactions were shown with Mn^{2+} at pH 7.2 but not with Mg^{2+} at pH 7.2 or 9.0. An intermediary plateau region was obtained when acetyl CoA was varied at 10 mM PEP but at 2 mM PEP sigmoidal kinetics resulted. The saturation kinetics of PEP at pH 7.2 with Mn^{2+} were sigmoidal but, in the region of 6 - 9 mM, there was an intermediary plateau region whereas in the presence of acetyl CoA, an allosteric activator, hyperbolic saturation kinetics were found. Sigmoidal saturation kinetics for PEP at pH 7.2 with Mg^{2+} were reported in the presence and absence of acetyl CoA. Therefore, with PEP carboxylase, the appearance of the plateau regions characteristic of negative cooperativity also seems to be dependent on the combination of pH, metal ion and

substrate concentration used similar to PEP carboxykinase reported here and is probably the result of the assay used rather than a property of the enzyme.

The significance of this proposed product inhibition by OAA can be appreciated when it is realised that the OAA concentrations in these assays are only of the order of 10^{-6} M to 10^{-5} M. The mitochondrial levels of OAA, based on NAD/$NADH_2$ ratios, are below the 1 - 10 μM range reported for whole tissues (Williamson, Lund and Krebs, 1967; Williamson, 1969; Loffler and Wieland, 1963; Baird et al., 1968; Ballard, Hanson and Kronfeld, 1968) while the intramitochondrial PEP concentration may be as high as 4 mM (Garber and Ballard, 1969). This level is considerably higher than the 0.1 - 0.2 mM range reported for whole liver (Ray, Foster and Lardy, 1966; Rolleston and Newsholme, 1967; Weidemann, Hems and Krebs, 1969; Baird and Heitzman, 1970). Therefore, it would appear that the in vivo conditions resemble quite closely the conditions of the uncoupled PEP carboxykinase assay system. Furthermore, the true K_m for total PEP is about 2×10^{-4} M as determined in a coupled assay system while that for OAA is as high as 4.5×10^{-4} M as determined with the CO_2:OAA exchange reaction (Chapter 6) in good agreement with values reported by other workers (Holten and Nordlie, 1965; Chang et al., 1966; Foster et al., 1967). Consequently, the carboxylation reaction of

mitochondrial PEP carboxykinase could be important *in vivo* in view of the relative OAA and PEP levels in which case the relative contributions of carboxylation and decarboxylation would be controlled by the GDP/GTP ratios. The recent findings of Johnson, Ebert and Ray (1970) that isolated rabbit liver mitochondria incubated with pyruvate and HCO_3^- liberate large amounts of malate and aspartate (if given NH_4^+) to the medium but only small amounts of PEP would further emphasise that PEP synthesis is probably not, in fact, the predominant function of mitochondrial PEP carboxykinase. A significant "anaplerotic" role is therefore indicated for this activity whereby the interactions set up by OAA would then be significant in the control of its own synthesis.

LeJohn (1968) reported a unidirectional control of the reversible glutamate dehydrogenase from *Blastocladiella emersonii*. In this case, the metabolites, citrate, isocitrate, fructose-1,6-diphosphate and fumarate, and the non-metabolite, EDTA, completely inhibited the oxidative deamination of glutamate but were without effect on the reductive amination of α-oxoglutarate. The fact that OAA is such a potent inhibitor of the carboxylation of PEP carboxykinase might suggest that this enzyme is an ideal candidate for such a control especially in view of the complex relationship between nucleotide specificity, metal ion specificity and pH.

Because of the similarity between the *in vivo* conditions and the uncoupled assay with respect to OAA and PEP concentrations and the appearance of non-Michaelis-Menten kinetics using this assay, it is suggested that this type of assay should be used to investigate possible effectors of PEP carboxykinase. These effectors could quite feasibly act by increasing or decreasing the apparent interaction between OAA and IDP or PEP. Such interactions could not be detected with a coupled assay although they have the capability of markedly altering the balance between carboxylation and decarboxylation.

CHAPTER 6. MECHANISM OF PEP CARBOXYKINASE

6.1. INTRODUCTION

In Chapter 4, the nature of the complexes formed between enzyme, Mn^{2+} and substrates were discussed in the light of the N.M.R. studies. In this chapter, the interaction of these complexes to give the central complex consisting of enzyme, Mn^{2+}, PEP, IDP and CO_2 was investigated by kinetic analysis and a mechanism is proposed for the reaction.

Lane and his coworkers studied the mechanism of action of pig liver mitochondrial PEP carboxykinase using N.M.R., gel filtration and equilibrium dialysis binding studies and a limited kinetic analysis (Miller *et al.*, 1968; Miller and Lane, 1968). They proposed the formation of a central complex consisting of enzyme, Mn^{2+}, PEP, IDP and CO_2 by a mixed ordered-random addition, *i.e.*, when Mn^{2+} was bound, either PEP, IDP or CO_2 might bind next, but if IDP or CO_2 bound prior to PEP, the PEP site was unavailable and a dead-end complex (Cleland, 1963b) was formed. Although the binding of PEP proceeded in a random fashion with respect to Mn^{2+}, the affinity of the enzyme for PEP was greatly enhanced by the presence of Mn^{2+}. The ternary enzyme.Mn^{2+}.PEP complex may bind either IDP or CO_2 and this complex can then bind the third substrate to form the central complex. An alternative route to the enzyme.Mn^{2+}.PEP.IDP complex involves the binding of $Mn.IDP^-$ to the enzyme.PEP complex. The central complex then undergoes conversion by a concerted mechanism to a new complex consisting of enzyme,

Mn^{2+}, OAA and ITP. However, the conditions used for the kinetic analysis were not ideal because often three substrates were varied simultaneously at non-saturating levels making interpretation of the results difficult and subject to error. This criticism does not invalidate the proposed concerted mechanism but introduces an element of doubt and caution regarding the proposed order of substrate addition.

Felicioli, Barsacchi and Ipata (1970) have recently presented evidence for an ordered Bi Ter mechanism (Cleland, 1963a) for the decarboxylation reaction catalysed by chicken liver mitochondrial PEP carboxykinase. However, the binding characteristics of the sheep kidney mitochondrial enzyme (this thesis) and the pig liver mitochondrial enzyme (Miller et al. (1968)) show random addition of substrates which would preclude any such mechanism.

The results of the kinetic analysis of the sheep kidney mitochondrial PEP carboxykinase suggest an ordered mechanism in contrast to the random binding of substrates. It is therefore proposed that this enzyme possesses a sequential mechanism but with a preferred pathway where IDP is bound first followed by the random addition of PEP and CO_2. This mechanism is in general agreement with that proposed by Miller and Lane (1968) for the pig liver mitochondrial enzyme.

Chang et al. (1966) reported that the only exchange reaction catalysed by pig liver mitochondrial PEP carboxy-

kinase was a Mn^{2+}- and ITP-dependent CO_2:OAA exchange reaction consistent with a sequential mechanism with the dissociation of IDP or PEP the rate limiting step. In addition, Miller and Lane (1968) demonstrated that ^{18}O of $H_2{}^{18}O$ was not incorporated into the phosphoryl group of PEP and IDP during decarboxylation. These observations also suggest a concerted mechanism for the phosphoryl transfer and eliminate any mechanism involving hydrolysis of a phosphate bond of an intermediate by water of the solvent. However, studies on the partial reactions catalysed by sheep kidney mitochondrial PEP carboxykinase show an ITP-independent exchange of CO_2 into OAA although the presence of nucleotide (either ITP or IDP) stimulates this activity. These results suggest that the carboxylation of PEP catalysed by sheep kidney mitochondrial PEP carboxykinase is a two step process with the phosphoryl transfer preceding carboxylation.

6.2. METHODS

The PEP carboxykinase used in these experiments was prepared as described in Chapter 3 and had a specific activity of 2.5 units per mg of protein (Section 2.8).

(a) Initial velocity studies on the carboxylation reaction

The carboxylation of PEP was followed by the incorporation of $H^{14}CO_3^-$ into OAA whichwas converted to aspartate by aspartate transaminase. The 0.5 ml reaction mixtures contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50;

GSH, 0.8; sodium glutamate, 10; aspartate transaminase, 4.5 units; PEP carboxykinase, 0.01 units; $MnCl_2$, PEP, IDP and $NaHC^{14}CO_3$ (3×10^5 cpm per μmole), as described in the legends to Figures 6.1 - 7. All solutions were adjusted to pH 6.5. The reaction was initiated by the addition of enzyme, incubated for 5 min at 30° and stopped with 0.2 ml of a 10%(w/v) trichloroacetic acid solution. Each assay solution was processed in triplicate as described in Section 2.7(i).

Although the ideal situation for initial velocity studies would be to use an uncoupled assay system, this was unsatisfactory with this enzyme because non-linear double reciprocal plots made interpretation impossible (see Figs. 5.12 - 13). McClure (1969) has analysed the use of coupled systems for kinetic studies and reached the conclusion that they give an accurate estimate of the enzymic activity under the appropriate conditions.

(b) Kinetic studies on the CO_2:OAA exchange reaction

PEP carboxykinase catalyses a Mn^{2+}-dependent CO_2:OAA exchange reaction which is stimulated by either ITP, IDP or IMP (Table 3.3). The exchange reaction assay (total volume 0.5 ml) contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; GSH, 0.8; PEP carboxykinase, 0.01 - 0.02 units; and $MnCl_2$, ITP, OAA and $NaH^{14}CO_3^-$ (6×10^5 cpm per μmole) in amounts as indicated in the legends to Figures 6.8 - 10. The reaction was initiated by the addition of OAA, incubated for 2 or 4 min. (as detailed) at 30° and stopped by the addition

of 0.05 ml of 6 N HCl saturated with 2,4,-dinitrophenylhydrazine. The assay solutions were processed in triplicate as detailed in Section 2.7(ii). With the exchange studies, the turnover of substrates was small and in no case would it amount to more than 5% of the initial levels.

(c) Product inhibition studies

IDP, ITP, $NaHCO_3$ and PEP, at the concentrations indicated in the legends to Figs. 6.11 and 6.12, and $MnCl_2$ at a constant concentration of 4.0 mM were added to the standard coupled assay system.

(d) Processing of data

Reaction velocity data were processed on a CDC 6400 digital computer using the Fortran programs of Cleland (1963d). Although the initial velocity data are presented in the double reciprocal form, the computer analyses were performed on a best fit to the hyperbolic form of rate equations:

$$v = \frac{VS}{K + S} \qquad \ldots (6.1)$$

assuming equal variance for the velocities.

The data were analysed in subsets consisting of velocities obtained when the concentrations of two substrates were varied at some constant saturating level of the third substrate. The data in each subset could thus be treated as a bireactant system and compared with Equation 6.2 if parallel lines were obtained

or with Equation 6.3 if intersecting lines were obtained.

$$v = \frac{VAB}{K_b A + K_a B + AB} \qquad \ldots (6.2)$$

$$v = \frac{VAB}{K_{ia}K_b + K_b A + K_a B + AB} \qquad \ldots (6.3)$$

The nomenclature of the kinetic constants follows the definitions and notation of Cleland (1963a). The initial velocity patterns were analysed by inspection as outlined by Cleland (1963c).

Concentrations of free and complexed forms of PEP and IDP as well as free Mn^{2+} were calculated using the following dissociation constants for the Mn^{2+}.substrate complexes: $K_{Mn.PEP}$ = 1.79 mM (Wold and Ballou, 1957); $K_{Mn.IDP}$ = 0.1 mM (rounded off from that obtained by O'Sullivan and Cohn (1966) and corrected for pH 6.5). However, the initial velocity patterns were the same whether the reciprocal velocity was plotted as a function of the reciprocal of free substrate, Mn.substrate or total substrate.

(e) Presence of an enzyme-bound CO_2 intermediate

PEP carboxykinase (1 - 3 units) was incubated at 0° and pH 6.5 with $NaH^{14}CO_3$ (6×10^5 cpm per μmole) in the presence of various combinations of substrates and products including conditions where the CO_2:OAA exchange, the PEP carboxylation or the OAA decarboxylation reactions were active.

The enzyme protein was then isolated at 0 - 4° by one of the following methods:

(i) the protein was precipitated with trichloroacetic acid and recovered on a Whatman glass-fibre filter (GF/C) supported on a Millipore suction apparatus. The filter was washed with acetone and examined for radioactivity;

(ii) the protein was isolated by the method of Kaziro and Ochoa (1961). A Dowex 1 (X8, 200 - 400 mesh) column (0.8 x 4 cm) was equilibrated with 0.02 M tris (Cl^-), pH 8.0. Immediately before use, 3 ml of a solution of 0.02 M tris (Cl^-) pH 8.0 containing 10^{-4} M GSH were run through the column. The reaction mixture (0.5 ml) was loaded onto the column, washed in with 0.6 ml of buffer and the protein was eluted with three additions of 1 ml of buffer. Corresponding volumes of eluate (0.5, 0.6 and 3 x 1 ml) were collected and 0.2 ml aliquots were added to 1.8 ml of Triton X100 scintillation fluid (Section 5.2(c)) and assessed for radioactivity;

(iii) a Sephadex G25 column (1.4 x 10 cm) was equilibrated with a solution of 0.05 M phosphate (K^+), pH 6.8 containing 10^{-4} M EDTA and 10^{-4} M GSH and calibrated with Blue Dextran and potassium ferricyanide. The reaction mixtures (0.5 ml) were applied to the column and the protein eluted with 20 ml of the equilibrating buffer at a flow rate of 1 ml per min. Eluant (0.5 ml fractions) was collected manually and 0.2 ml aliquots were assessed for radioactivity as described in (ii) above.

(f) Utilisation of pyruvate by PEP carboxykinase

Utilisation of pyruvate was investigated by its carboxylation or its exchange into OAA. The ability of both IDP and ITP to facilitate a utilisation of pyruvate was examined.

(a) The carboxylation of pyruvate was assessed during a reaction mixture (0.5 ml) containing (in μmoles): imidazole (Cl^-), pH 6.5, 50; $MnCl_2$, 2.5; IDP (or ITP), 2; $NaH^{14}CO_3$ (6×10^5 cpm per μmole), 20; pyruvate, 10; $NADH_2$, 1; malate dehydrogenase, 5 units; PEP carboxykinase, 0.2 units. Incubation was carried out at 30° for various intervals up to 30 min. The reaction was stopped by the addition of 0.25 ml of a 10% (w/v) trichloroacetic acid solution. Aliquots of the protein-free supernatant were assessed for radioactivity as described in Section 2.7(i).

(b) The exchange of pyruvate into OAA was determined using a reaction mixture (0.5 ml) containing (in μmoles); imidazole (Cl^-), pH 6.5, 50; $MnCl_2$, 1.5; IDP or ITP (if present), 1; OAA, 1; $NaHCO_3$, 10; ^{14}C-2-pyruvate, 5; PEP carboxykinase, 0.05 units. Incubation was carried out at 30° for various intervals up to 10 min and the reaction was stopped with trichloroacetic acid (to 3%, w/v). Precipitated protein was removed by centrifuging. The supernatant was neutralised with KOH and the ^{14}C-2-OAA was converted to aspartate by the addition of 10 μmoles of glutamate, 0.02 μmoles of pyridoxal phosphate and

1 unit of aspartate transaminase. Transamination was stopped with trichloroacetic acid (to 3%, w/v) after 5 min. incubation at 22°. Aspartate (10 μmoles) was added as carrier and precipitated protein was removed by centrifuging.

The aspartate was separated from pyruvate using the method of Scrutton, Keech and Utter (1965). An Amberlite IRC-120 column (1 x 9 cm) was washed with 10 ml of 1 M HCl, and then water until the eluant was about pH 5.0. The supernatant solution from the transamination reaction was washed into the column with 30 ml of water, 15 ml of 0.15 M NH_4OH and finally 0.3 M NH_4OH until the eluant was alkaline. The subsequent 35 ml of eluant was collected and a 1 ml aliquot of this solution was added to 9 ml of Triton K100 scintillation fluid (Section 5.2(c)) and assessed for radioactivity.

6.3. RESULTS

(a) Initial velocity studies on the carboxylation reaction

The initial velocity patterns shown in Figure 6.1 were obtained when PEP and IDP were the variable and fixed variable substrates. The family of linear parallel lines indicated an irreversible connection between the enzyme forms binding these to substrates. Secondary plots of intercepts from the primary plots with respect to reciprocal concentrations of the fixed substrates were also linear. These are presented in Fig. 6.1 shown as dashed lines, and represent standard Lineweaver-Burk (1934) plots at infinite concentrations of cosubstrates.

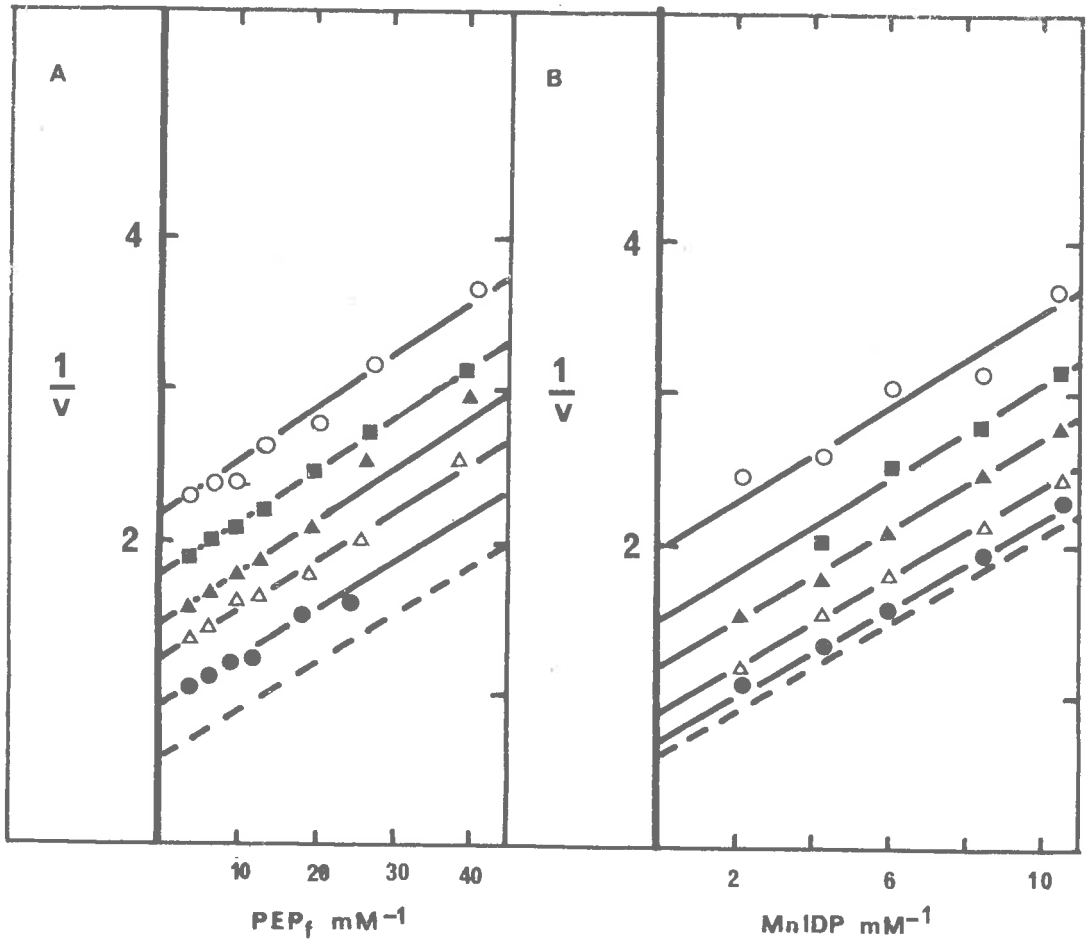


FIG. 6.1. Double reciprocal plots of initial velocity against free PEP (PEP_f) (A) and $Mn.IDP^-$ (B) concentrations. Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): $NaH^{14}CO_3$, 10mM; $MnCl_2$, 2mM; PEP and IDP as indicated. (A) PEP_f was varied at the constant $Mn.IDP^-$ levels: (●——●), 0.465mM; (△——△), 0.236mM; (▲——▲), 0.165mM; (■——■), 0.118mM; (○——○), 0.095mM. (B) Replots of the data in (A). $Mn.IDP^-$ varied at constant PEP_f levels: (●——●), 0.26mM; (△——△), 0.1mM; (▲——▲), 0.049mM; (■——■), 0.037mM; (○——○), 0.024mM. The dashed lines are secondary plots of intercepts from the related primary plots.

Figure 6.2 shows the initial velocity patterns obtained when PEP and HCO_3^- were varied. The family of linear intersecting lines indicated a reversible connection between the enzyme forms binding these two substrates. The secondary plots of intercepts and slopes from the primary data with respect to reciprocal concentrations of the fixed substrates were all linear (Insets, Fig. 6.2).

When IDP and HCO_3^- were varied, the initial velocity patterns indicated an irreversible connection between the enzyme forms binding these two substrates as shown by the families of linear parallel lines obtained (Fig. 6.3). The dashed lines are the secondary plots of intercepts, obtained from the primary data, with respect to reciprocal concentrations of the fixed substrates. These were linear.

The initial velocity patterns obtained when Mn^{2+} and HCO_3^- were varied were families of linear intersecting lines suggesting a reversible connection between the enzyme forms binding these two ligands (Fig. 6.4). The secondary plots of intercepts and slopes obtained from the primary data with respect to the reciprocal concentrations of the fixed substrates were linear (Insets, Fig. 6.4).

With Mn^{2+} and PEP as the covariables, the initial velocity patterns obtained were families of linear intersecting lines indicating a reversible connection between the enzyme forms

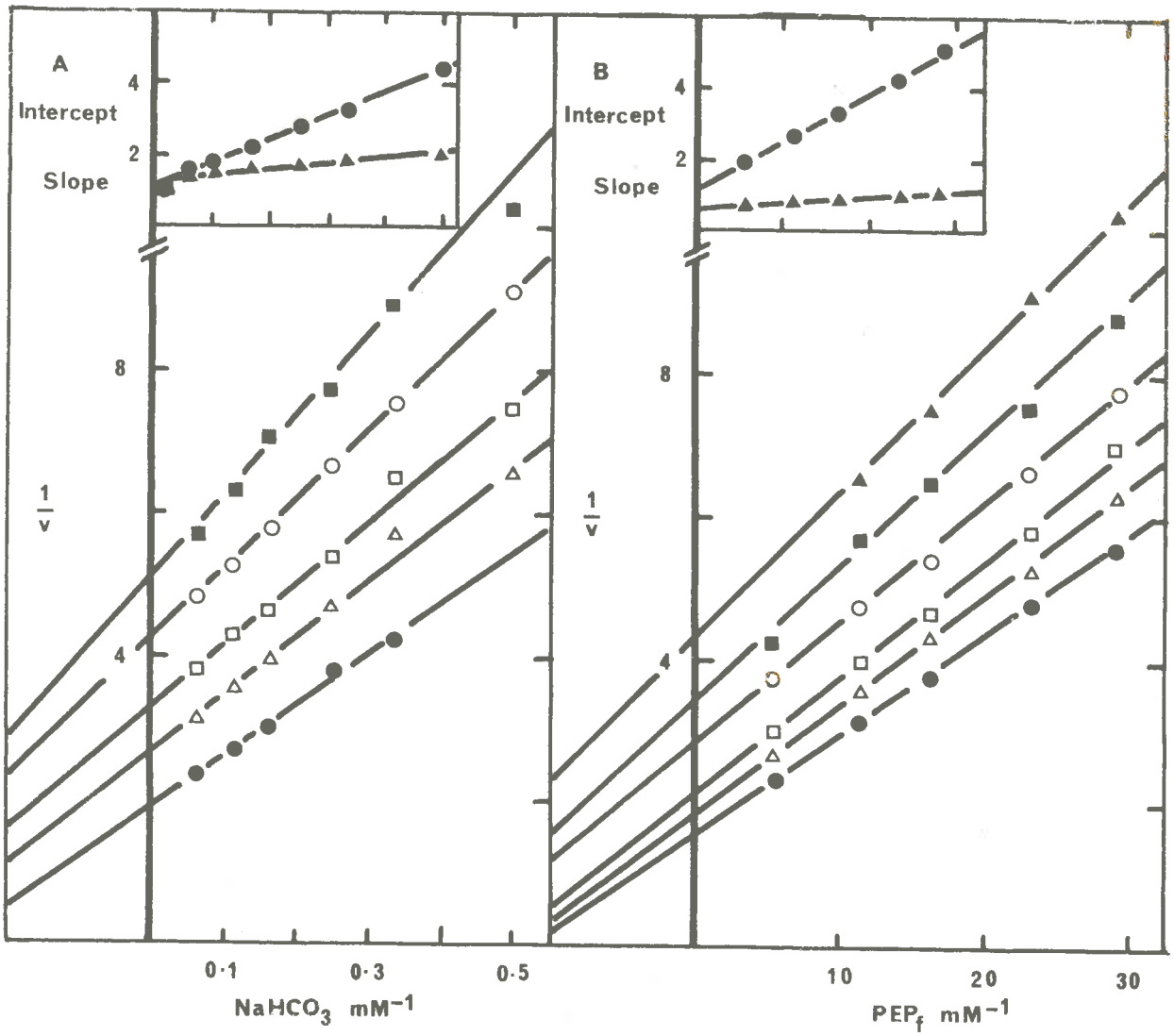


FIG. 6.2. Double reciprocal plots of initial velocity against $NaHCO_3$ (A) and free PEP (PEP_f) (B). Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): $MnCl_2$, 4mM; Mn.IDP$^-$, 0.485mM; $NaHCO_3$ and PEP_f as indicated. (A) $NaHCO_3$ was varied at the constant PEP_f levels: (●——●), 0.18mM; (△——△), 0.087mM; (□——□), 0.06mM; (○——○), 0.043mM; (■——■), 0.034mM. (B) Replots of the data in (A). PEP_f varied at the constant $NaHCO_3$ levels: (●——●), 16mM; (△——△), 9mM; (□——□), 6mM; (○——○), 4mM; (■——■), 3mM; (▲——▲), 2mM. Insets: Secondary plots of intercepts (●——●) and slopes (▲——▲) from the related primary plots versus the reciprocal substrate concentration.

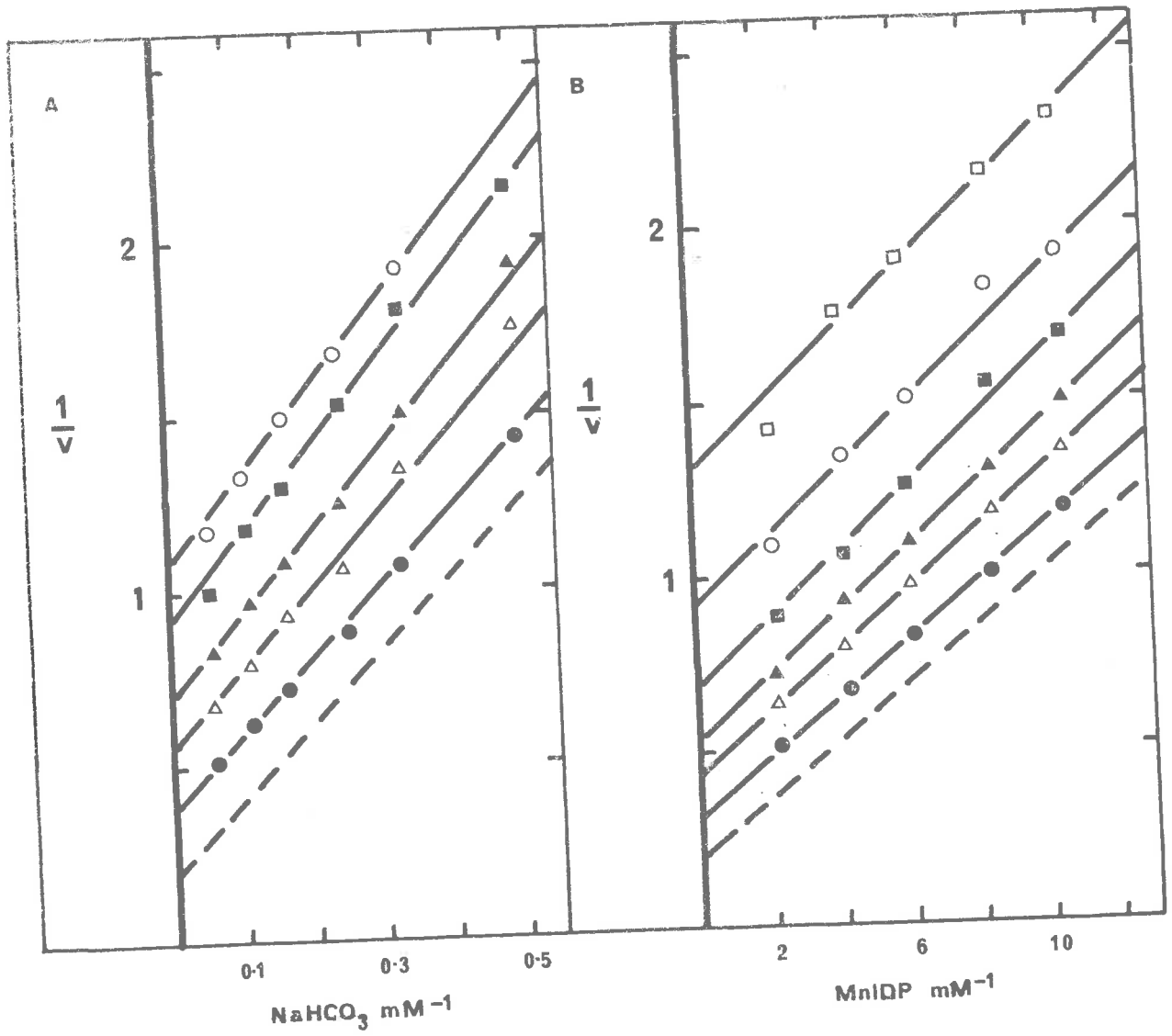


FIG. 6.3. Double reciprocal plots of initial velocity against $NaHCO_3$ (A) and $Mn.IDP^-$ (B). Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): $MnCl_2$, 2mM; PEP, 0.5mM; $NaHCO_3$ and $Mn.IDP^-$ as indicated. (A) $NaHCO_3$ was varied at the constant $Mn.IDP^-$ levels: (●——●), 0.465mM; (△——△), 0.235mM; (▲——▲), 0.165mM; (■——■), 0.118mM; (○——○), 0.094mM. (B) Replot of the data of (A). $Mn.IDP^-$ varied at the constant $NaHCO_3$ levels: (●——●), 16mM; (△——△), 9mM; (▲——▲), 6mM; (■——■), 4mM; (○——○), 3mM; (□——□), 2mM. The dashed lines are secondary plots of intercepts from the related primary plots.

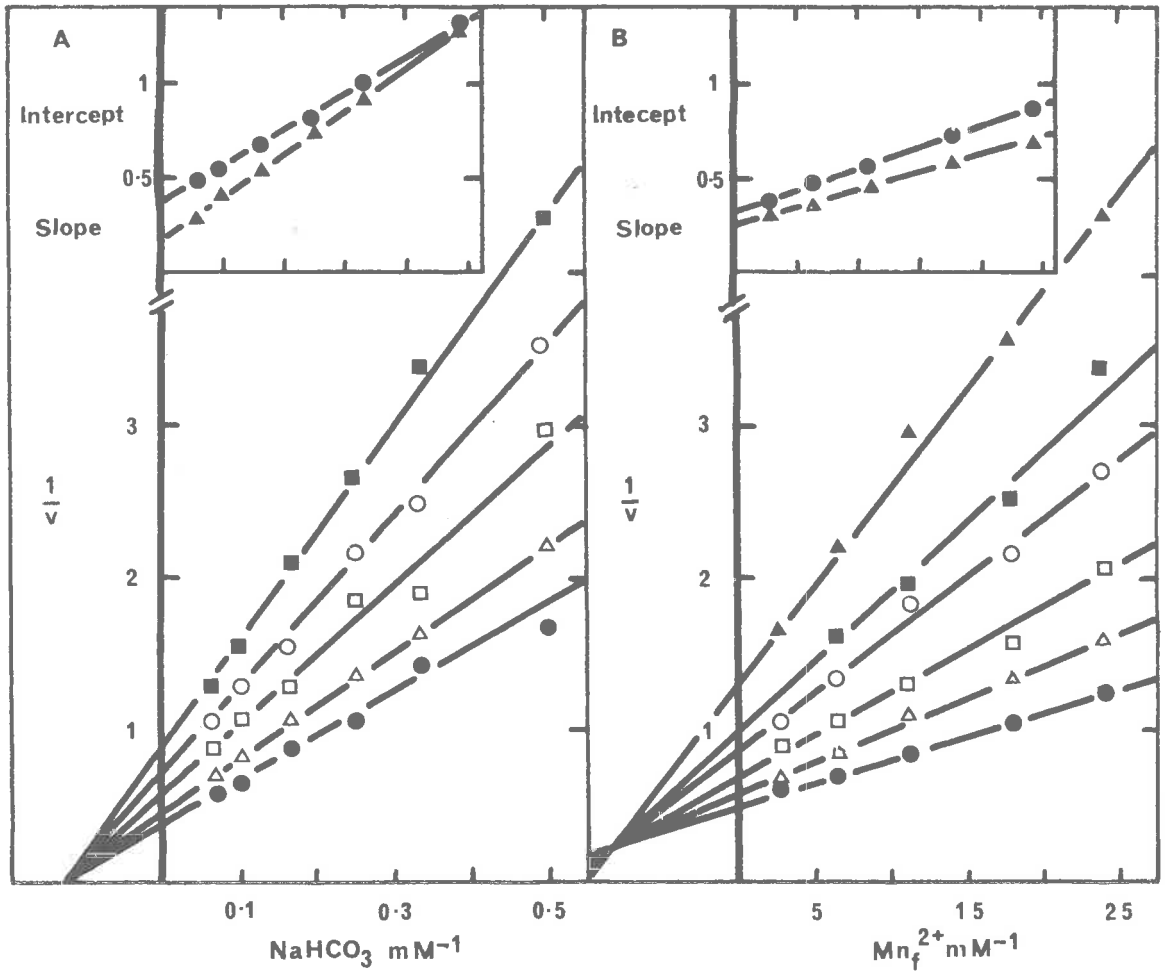


FIG. 6.4. Double reciprocal plots of initial velocity against $NaHCO_3$ (A) and free Mn^{2+} (Mn^{2+}_f) (B). Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): IDP, 0.5mM; PEP, 0.5mM; $NaHCO_3$ and Mn^{2+}_f as indicated. (A) $NaHCO_3$ was varied at the constant Mn^{2+}_f levels: (●——●), 0.48mM; (△——△), 0.156mM; (□——□), 0.09mM; (○——○), 0.056mM; (■——■), 0.042mM. (B) Replots of the data of (A). Mn^{2+}_f was varied at the constant $NaHCO_3$ levels: (●——●), 16mM; (△——△), 9mM; (□——□), 6mM; (○——○), 4mM; (■——■), 3mM; (▲——▲), 2mM. Insets: Secondary plots of intercepts (●——●) and slopes (▲——▲) from the related primary plots versus the reciprocal substrate concentration.

binding these two ligands (Fig. 6.5). The secondary plots of slopes and intercepts obtained from the primary data with respect to the reciprocal concentrations of the fixed substrates were linear (Insets, Fig. 6.5).

This same data, presented as S_t and M_t profiles where velocity is plotted against total substrate (S_t) or total metal (M_t) (London and Steck, 1969), are shown in Figure 6.6. For this treatment, M_t was taken as (Mn^{2+}_{free} + Mn.PEP), i.e., total Mn^{2+} less that complexed with IDP. The symmetry between the two profiles suggests we are dealing with a system where free metal, free substrate and the metal.substrate complex may all bind to the enzyme but to different degrees with no metal activation of the enzyme. These profiles do not suggest any essential requirement for complex formation.

The S_t and M_t profiles (London and Steck, 1969) showing the relationship between Mn^{2+} and IDP are shown in Figure 6.7. There appears a definite relationship between the maximum velocity and the stage where IDP_{total} equals Mn^{2+}_{total} (here again Mn^{2+}_{total} was taken as Mn^{2+}_{free} plus Mn.IDP). Such behaviour is characteristic of a system where the Mn. nucleotide complex is the true substrate.

(b) Initial velocity studies on the CO_2:OAA exchange reaction

The exchange reactions were used to determine the interactions of OAA with the components of the decarboxylation

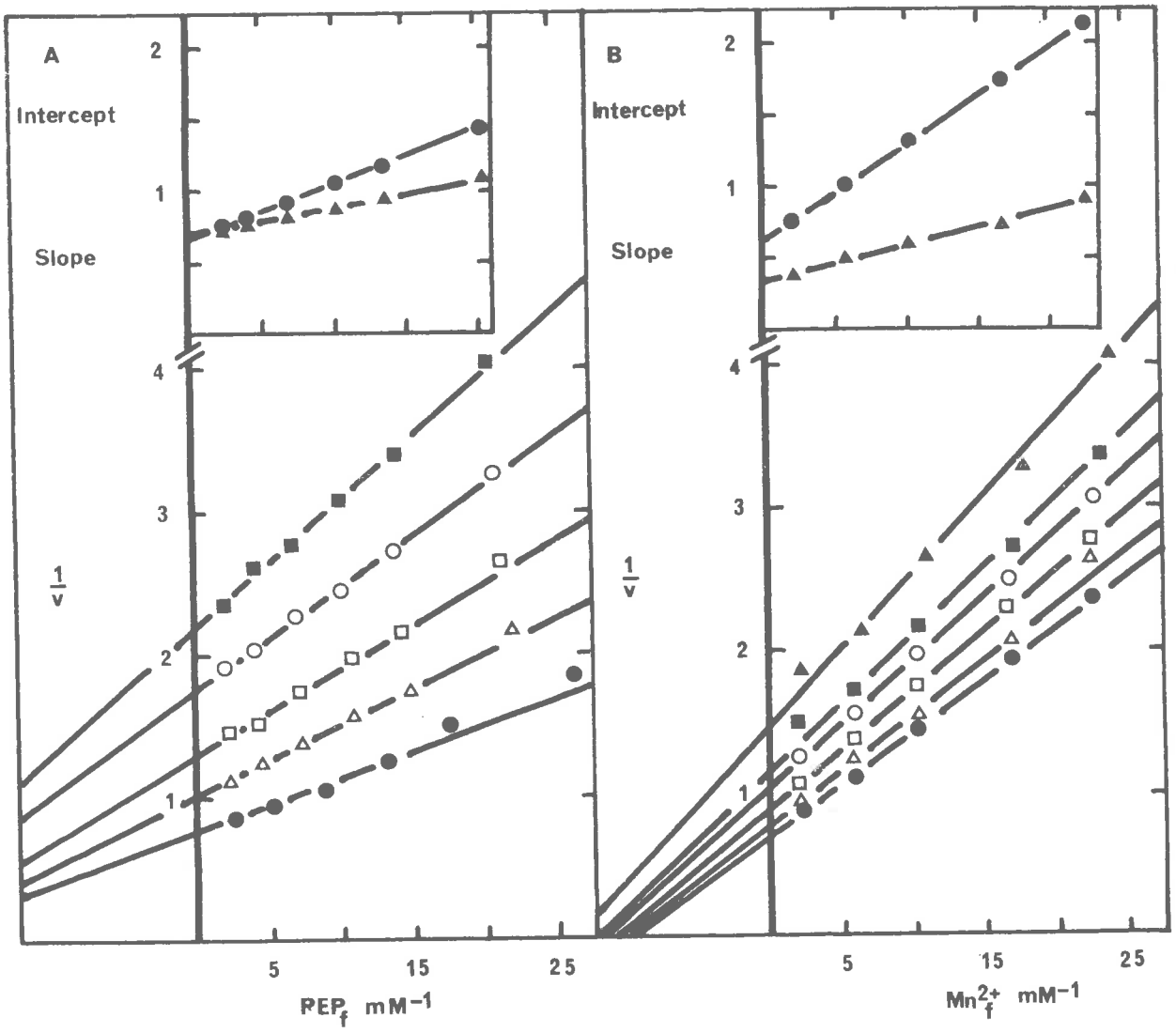


FIG. 6.5. Double reciprocal plots of initial velocity against free PEP (PEP_f) (A) and free Mn^{2+} (Mn^{2+}_f) (B). Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): IDP, 0.5mM; $NaHCO_3$, 12mM; PEP_f and Mn^{2+}_f as indicated. (A) PEP_f was varied at the constant Mn^{2+}_f levels: (●——●), 0.48mM; (△——△), 0.156mM; (□——□), 0.09mM; (○——○), 0.056mM; (■——■), 0.042mM. (B) Replot of the data of (A). Mn^{2+}_f was varied at the constant levels of PEP_f: (●——●), 0.48mM; (△——△), 0.242mM; (□——□), 0,144mM; (○——○), 0.096mM; (■——■), 0.071mM; (▲——▲), 0.047mM. Insets: Secondary plots of intercepts (●——●) and slopes (▲——▲) from the related primary plots versus the reciprocal substrate concentration.

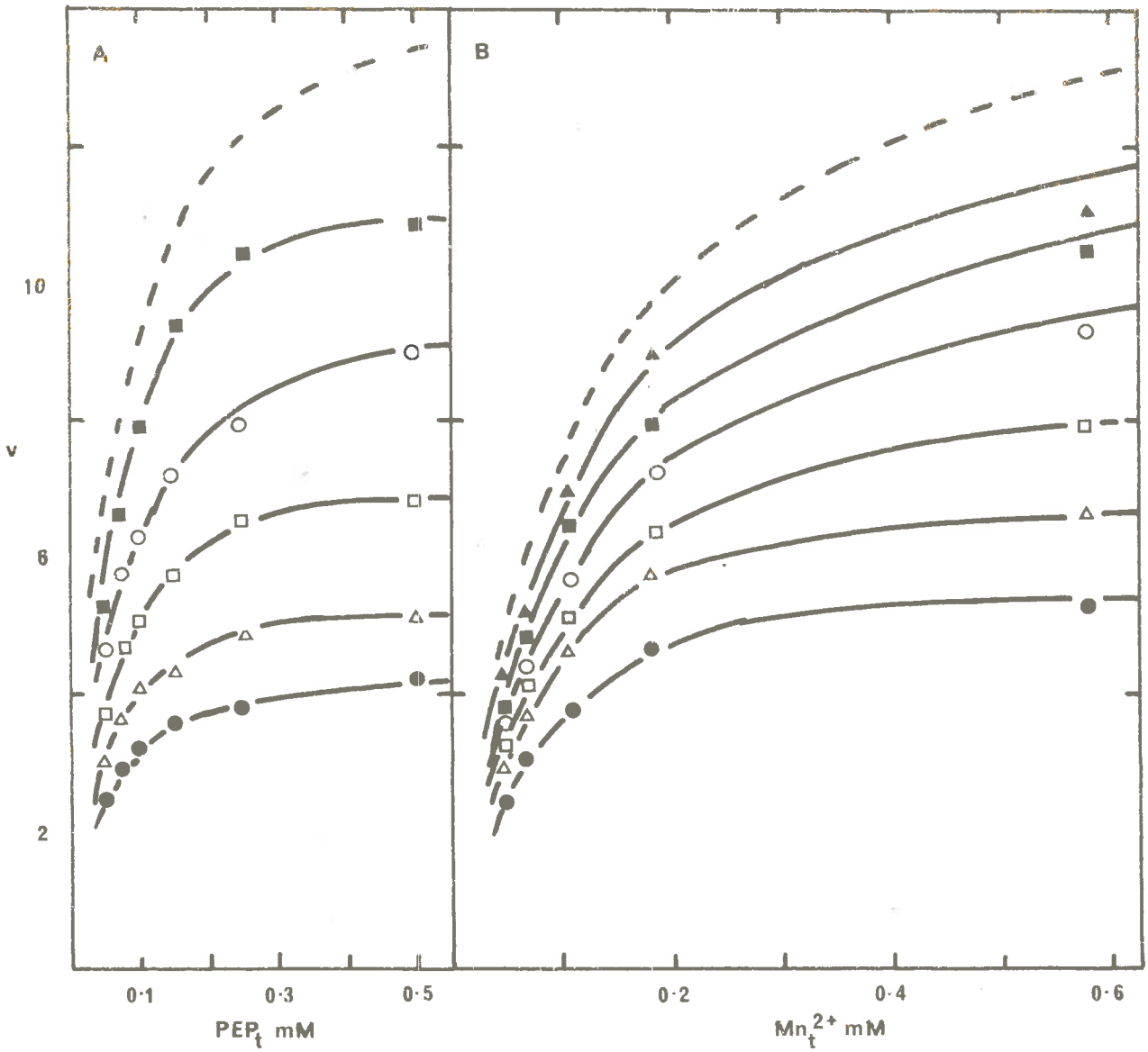


FIG. 6.6. S_t and M_t profiles for PEP (PEP_t) (A) and Mn^{2+} (Mn^{2+}_t) (B). The data of Fig. 6.5. is replotted according to the method of London and Steck (1969). M_t was calculated as free Mn^{2+} + Mn.PEP. (A) S_t profile for PEP_t at the constant Mn^{2+}_t levels: (●——●), 0.047mM; (△——△), 0.066mM; (□——□), 0.105mM; (○——○), 0.184mM; (■——■), 0.58mM; (– – – – –), ∞. (B) M_t profile for Mn^{2+}_t at the constant PEP_t levels: (●——●), 0.05mM; (△——△), 0.075mM; (□——□), 0.1mM; (○——○), 0.15mM; (■——■), 0.25mM; (▲——▲), 0.5mM; (– – – – –), ∞.

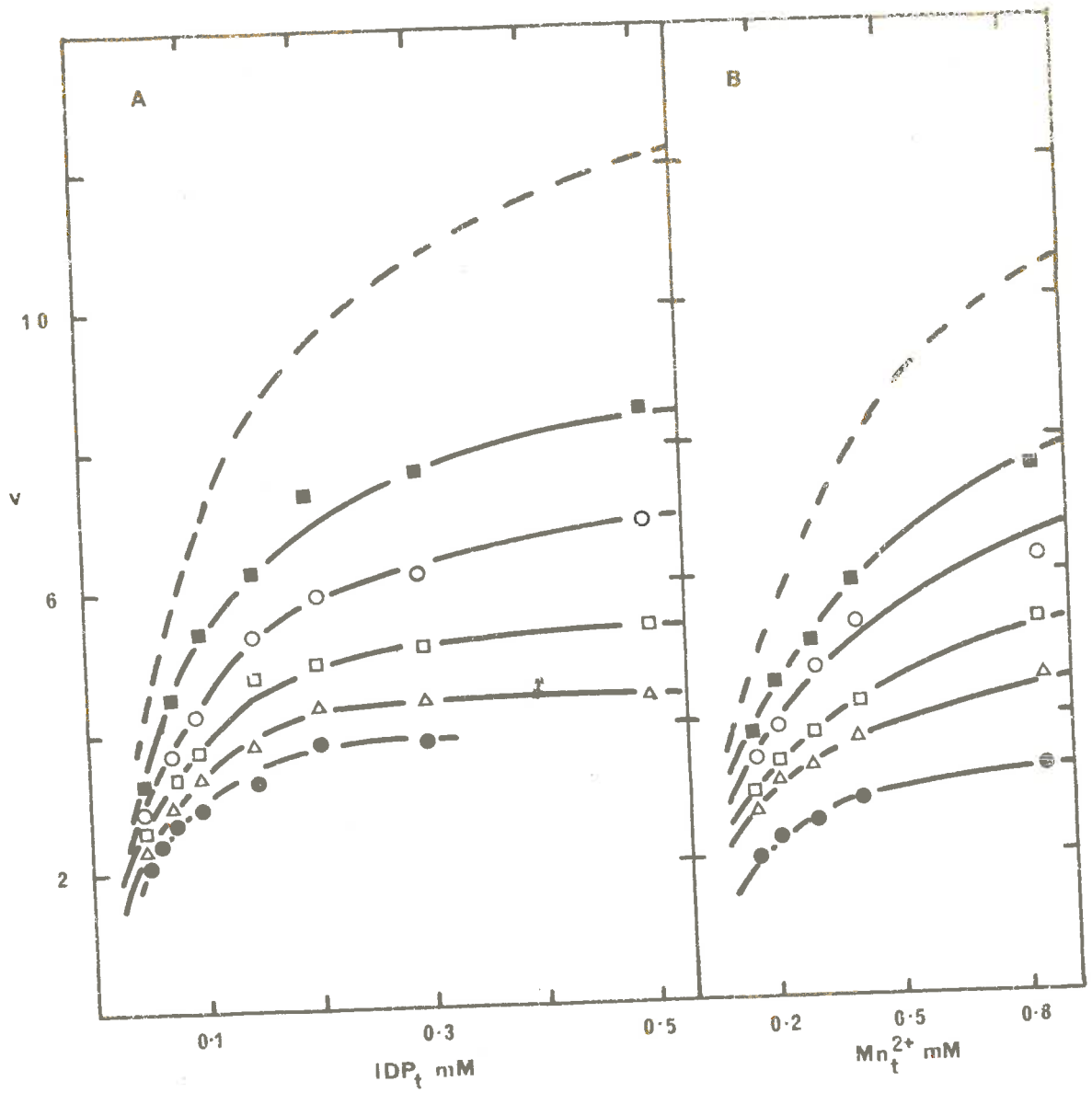


FIG. 6.7. S_t and M_t profiles for IDP (IDP_t) (A) and Mn^{2+} (Mn^{2+}_t) (B). Initial velocity data obtained when IDP and Mn^{2+} were varied was plotted according to the method of London and Steck (1969). M_t was calculated as free Mn^{2+} + Mn.IDP$^-$. Reaction mixtures contained (in addition to standard components listed in Section 6.2.(a)): $NaHCO_3$, 10mM; PEP, 0.5mM; IDP_t and Mn^{2+}_t as indicated. (A) S_t profile for IDP_t at the constant Mn^{2+}_t levels: (●——●), 0.175mM; (△——△), 0.22mM; (□——□), 0.31mM; (○——○), 0.43mM; (■——■) 0.86mM; (– – – – –), ∞ . (B) M_t profile for Mn^{2+}_t at the constant IDP_t levels: (●——●), 0.05mM; (△——△), 0.075mM; (□——□), 0.1mM; (■——■), 0.2mM; (– – – – –), ∞ .

reaction. Studying the decarboxylation reaction using a coupled system consisting of pyruvate kinase and lactate dehydrogenase was unsatisfactory because of the cation and nucleotide requirements of the coupling enzymes. Also, experience with the uncoupled carboxylation reaction threw doubt on the use of an uncoupled decarbocylation reaction.

Varying $NaHCO_3$ and OAA gave a family of linear intersecting lines for the initial velocity patterns of the exchange reaction. Figure 6.8A shows the pattern obtained in the absence of ITP while that obtained in the presence of 0.25 mM ITP is shown in Fig. 6.8B. Thus, ITP did not alter the interactions in HCO_3^- and OAA binding.

When Mn^{2+} and OAA were the variable and fixed variable ligands, ITP was omitted to eliminate unnecessary interactions in the system. Figure 6.9 shows that the initial velocity pattern, obtained when varying these substrates, was a family of linear intersecting lines. The secondary plots of slopes andintercepts, obtained from the primary data, with respect to the reciprocal of the concentrations of the fixed substrates were linear (Inset, Fig. 6.9). These results suggest a reversible connection exists between the enzyme forms binding Mn^{2+} and OAA.

The initial velocity pattern of the exchange reaction obtained when ITP and OAA were the varied substrates was a family of linear parallel lines indicating an irreversible

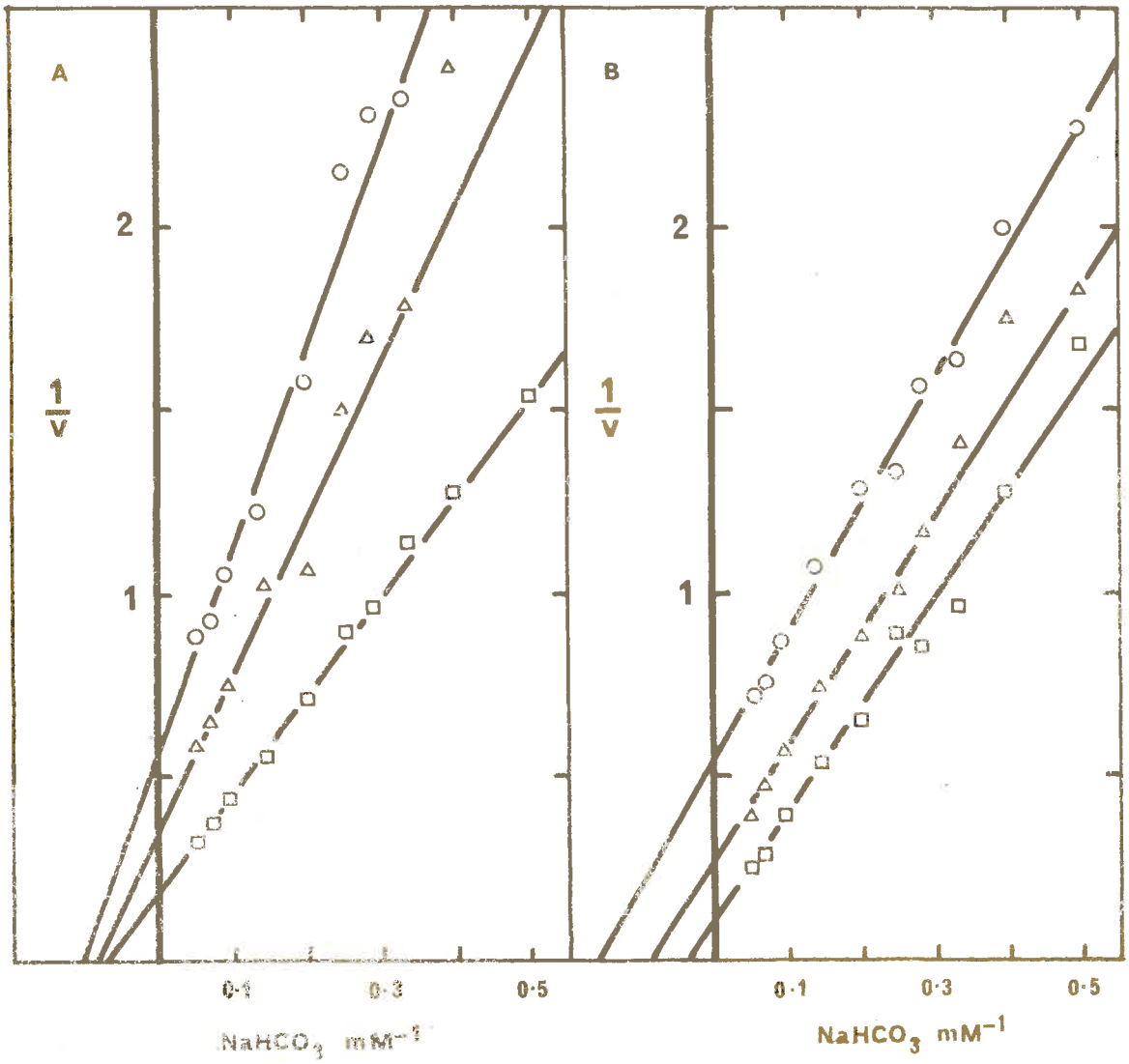


FIG. 6.8. Double reciprocal plots of the initial velocity of the CO_2:OAA exchange reaction against $NaHCO_3$ concentration in the absence(A) and presence (B) of 0.25mM ITP. Reaction mixtures contained (in addition to standard components listed in Section 6.2.(b)): $MnCl_2$, 4mM; ITP (in B), 0.25mM. $NaHCO_3$ was varied at the constant OAA levels: (□——□), 2mM; (△——△), 0.6mM; (○——○), 0.4mM.

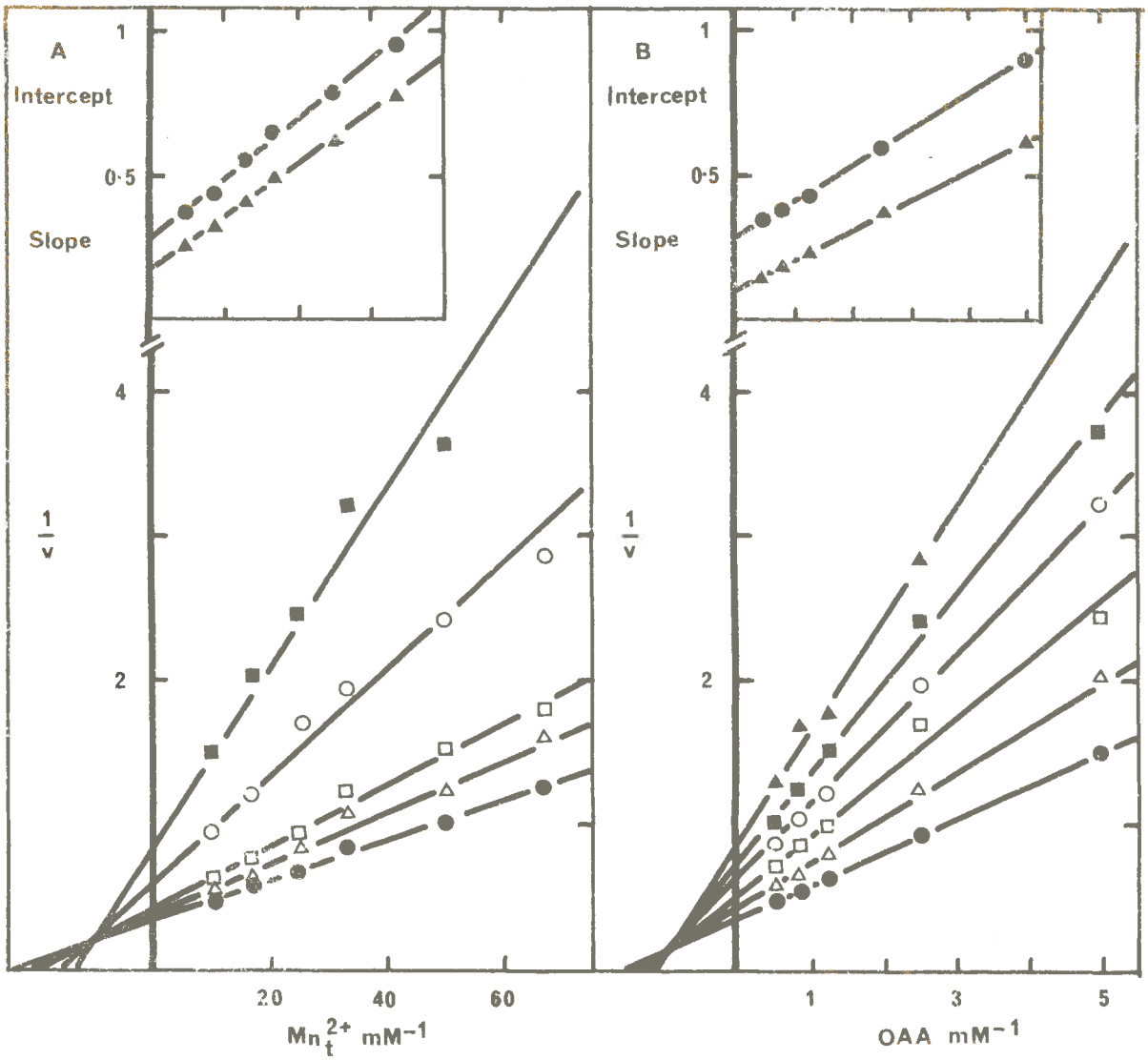


FIG. 6.9. Double reciprocal plots of the initial velocity of the CO_2:OAA exchange reaction against $MnCl_2$ (A) and OAA (B). Reaction mixtures contained the standard components listed in Section 6.2.(b) plus $MnCl_2$ and OAA as indicated. (A) Mn^{2+} was varied at the constant OAA levels: (●——●), 2mM; (△——△), 1.2mM; (□——□), 0.8mM; (○——○), 0.4mM; (■——■), 0.2mM. (B) Replot of the data of (A). OAA was varied at the constant $MnCl_2$ levels: (●——●), 0.1mM; (△——△), 0.06mM; (□——□), 0.04mM; (○——○), 0.03mM; (■——■), 0.02mM; (▲——▲), 0.015mM. Insets: Secondary plots of the intercepts (●——●) and slopes (▲——▲) from the primary plots versus the reciprocal substrate concentration.

connection between the enzyme forms binding these substrates (Fig. 6.10).

(c) Product inhibition studies

Product inhibition studies with OAA as product inhibitor were not possible with the carboxylation system because of interference from the CO_2:OAA exchange reaction which is stimulated by IDP. Thus, product inhibition studies were restricted to ITP as the product inhibitor of the carboxylation reaction.

ITP was shown to be a linear non-competitive inhibitor with respect to both PEP (Fig. 6.11A) and HCO_3^- (Fig. 6.11B). In both cases, linear replots of slopes and intercepts obtained from the primary data were observed with respect to ITP concentration (Insets, Fig. 6.11A and B).

With IDP as the variable substrate, ITP showed non-linear inhibition of enzymic activity apparently stimulating at low IDP levels but subsequently inhibiting at high IDP concentrations (Fig. 6.12).

(d) Summary of the K_m values for substrates

In Table 6.1 are listed the K_m values for all substrates obtained from the secondary plots of the data in Figs. 6.1 - 5; 6.9. In all cases, there is good agreement between similar values obtained from separate plots.

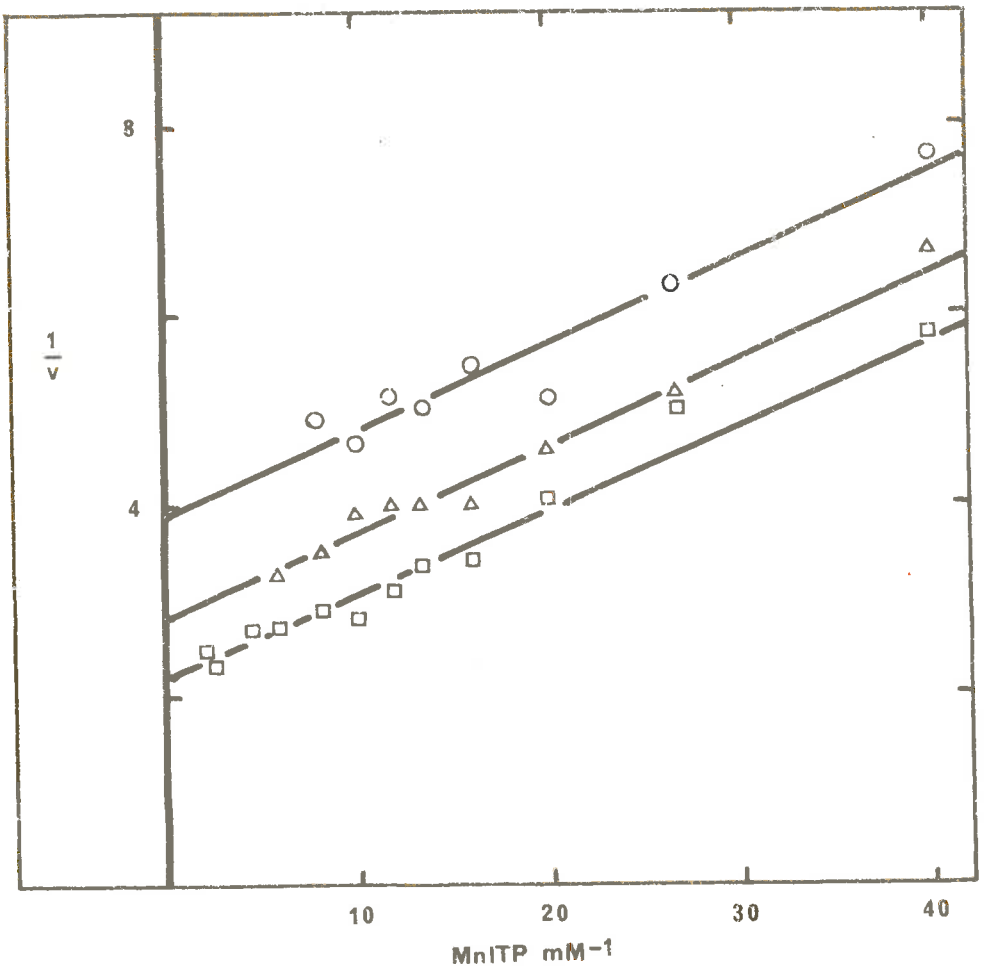


FIG. 6.10. Double reciprocal plot of the initial velocity of the CO_2:OAA exchange reaction against $Mn.ITP^{2-}$ concentration. Reaction mixyures contained (in addition to the standard components listed in Section 6.2.(b)): $NaHCO_3$, 20mM; $MnCl_2$, 2mM; OAA and $Mn.ITP^{2-}$ as indicated. OAA concentrations were: (□——□), 2mM; (△——△), 0.6mM; (○——○), 0.4mM.

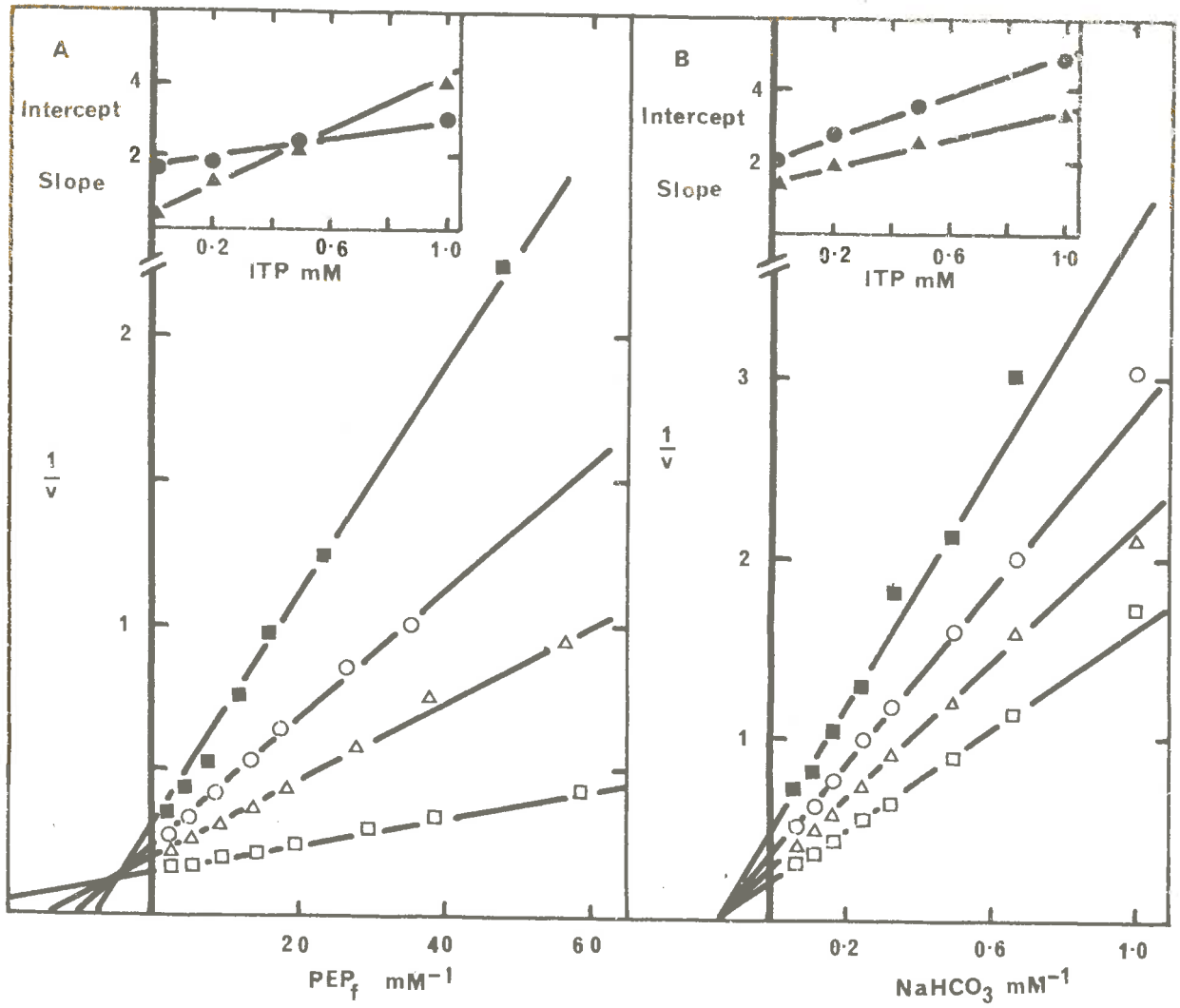


FIG. 6.11. Inhibition patterns caused by the presence of product. Reaction mixtures contained (in addition to standard components listed in Section 6.2.(c)) $MnCl_2$, 4mM; $Mn.IDP^-$, 0.485mM; $NaHCO_3$, 12mM (in A); PEP, 0.5mM (in B). (A) Free PEP (PEP_f) was varied at the constant $Mn.ITP^{2-}$ levels: (□——□), 0.0mM; (△——△), 0.2mM; (○——○), 0.5mM; (■——■), 1mM. (B) $NaHCO_3$ was varied at the constant $Mn.ITP^{2-}$ levels: (□——□), 0.0mM; (△——△), 0.2mM; (○——○), 0.5mM; (■——■), 1mM. Insets: Secondary plots of intercepts (●——●) and slopes (▲——▲) from the primary plots versus $Mn.ITP^{2-}$ concentration.

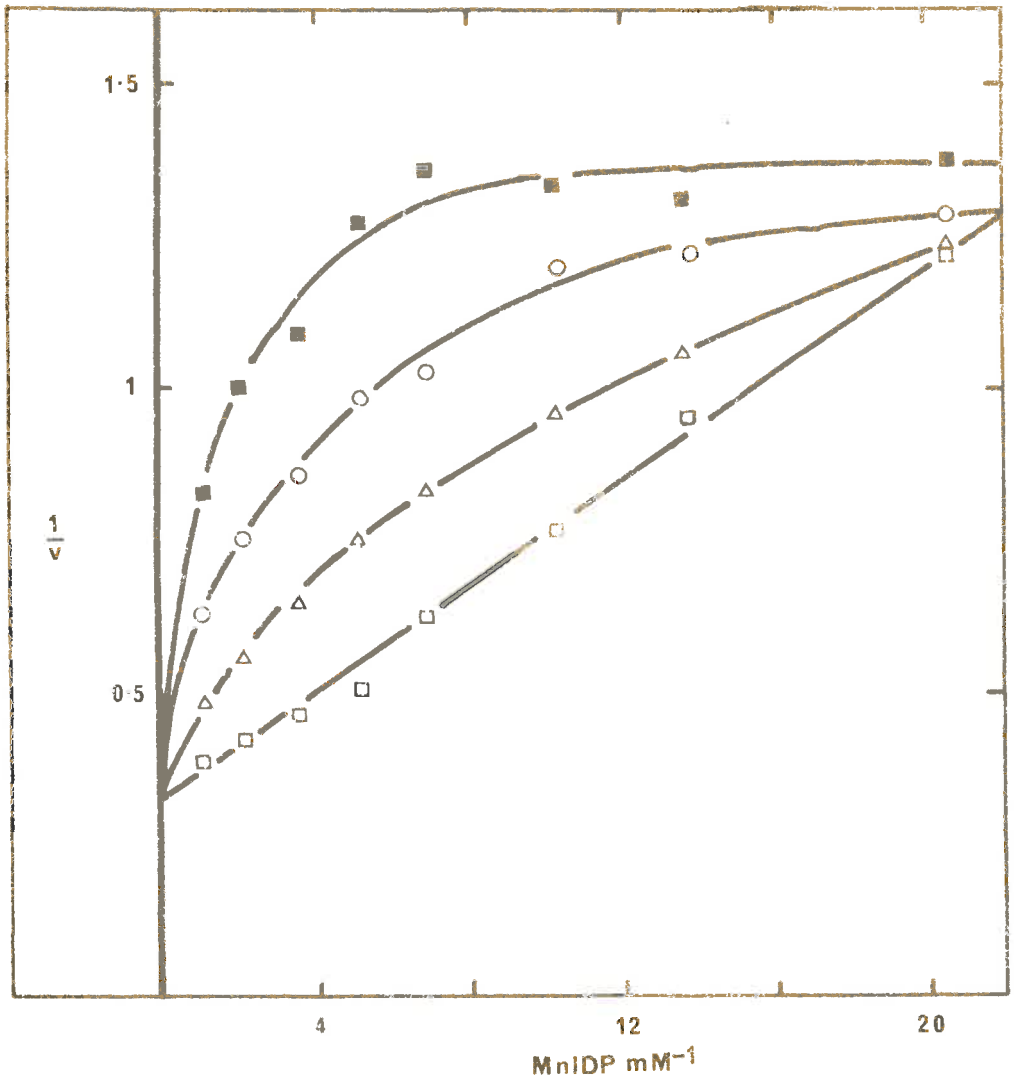


FIG. 6.12. Product inhibition pattern caused by the presence of $Mn.ITP^{2-}$. Reaction mixtures contained (in addition to standard components listed in Section 6.2.(c)): $MnCl_2$, 4mM; $NaHCO_3$, 12mM; PEP, 0.5mM. $Mn.IDP^-$ was varied at the constant $Mn.ITP^{2-}$ levels: (□——□), 0.0mM; (△——△), 0.2mM; (○——○), 0.5mM; (■——■), 1mM.

TABLE 6.1: K_m VALUES FOR THE SUBSTRATES OF PEP CARBOXYKINASE

Substrate	Co-substrate	K_m (M)[a]	Average (M)[a]	Computed K_m (M)
PEP_f	$NaHCO_3$	$1.183 \times 10^{-4} \pm 0.11$	8.19×10^{-5}	7.76×10^{-5}
	IDP.Mn	$6.38 \times 10^{-5} \pm 0.54$	± 0.58	± 0.73[b]
	Mn^{2+}_f	$6.35 \times 10^{-5} \pm 0.09$		
$NaHCO_3$	PEP_f	$5.76 \times 10^{-3} \pm 0.61$	5.64×10^{-3}	8.78×10^{-3}
	Mn^{2+}_f	$5.1 \times 10^{-3} \pm 0.21$	± 0.53	± 0.86[b]
	IDP.Mn	$6.05 \times 10^{-3} \pm 0.77$		
IDP.Mn^-	PEP_f	$2.98 \times 10^{-4} \pm 0.49$	3.56×10^{-4}	3.63×10^{-4}
	$NaHCO_3$	$4.14 \times 10^{-4} \pm 0.21$	± 0.35	± 0.31[c]
Mn^{2+}_f	$NaHCO_3$	$7.63 \times 10^{-5} \pm 0.46$	8.82×10^{-5}	7.56×10^{-5}
	PEP_f	$1.0 \times 10^{-4} \pm 0.02$	± 0.24	± 0.75[d]
	OAA	$3.37 \times 10^{-5} \pm 0.42$	$3.37 \times 10^{-5} \pm 0.42$	----
OAA	Mn^{2+}	$4.45 \times 10^{-4} \pm 0.02$	----	----

a, determined from the replots of the data of Figs. 6.1 - 5, 6.9;

b, average of values from Equation 6.2 and 6.3;

c, from Equation 6.2;

d, from Equation 6.3.

(e) Inhibition by Ca^{2+}

Cohn (1963) has classified metal-activated enzymes into two groups based on their response to Ca^{2+}. This cation could activate only those enzymes where the activating metal ion was not bound directly to the enzyme, e.g., creatine kinase, but was inhibitory in those cases where the activating metal ion was bound by the enzyme, e.g., enolase of pyruvate kinase. The results of the N.M.R. studies presented in Chapter 4 demonstrate that sheep kidney mitochondrial PEP carboxykinase binds Mn^{2+} directly.

Although Ca^{2+} failed to activate PEP carboxykinase, it was only a weak inhibitor in the presence of Mn^{2+} (Table 6.2). A possible explanation of this phenomenon could be the involvement of two metal ions with Ca^{2+} activating via a $Ca.IDP^{-}$ complex and Mn^{2+} activating via the enzyme.Mn^{2+} complex. This hypothesis can be tested by evaluating the nature of the inhibition by Ca^{2+} with respect to Mn^{2+}. With pyruvate kinase, where the evidence indicates that only one metal ion is required for catalysis, Ca^{2+} is strictly a competitive inhibitor with respect to Mn^{2+} and it competes for Mn^{2+} in the formation of the enzyme.Mn^{2+} complex as shown by a diminution of the PRR enhancement of the binary complex (Mildvan and Cohn, 1965). However, with sheep kidney mitochondrial PEP carboxykinase, Ca^{2+} was a non-competitive inhibitor with respect to Mn^{2+} consistent with the involvement of two metal ions in catalysis (Fig. 6.13).

TABLE 6.2: INHIBITION OF PEP-CARBOXYKINASE BY Ca^{2+}.

Ca^{2+} (mM)	Mn^{2+} (mM)	Activity (μmoles)	% Inhibition
0.0	3.0	0.052	--
0.8	3.0	0.049	6
3.0	3.0	0.047	10
3.0	0.0	0.00	100

$CaCl_2$ (A.R., B.D.H.) was dried at 200° to constant weight before the stock solution was made. Assay solutions (0.5 ml) contained (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; metal ion as indicated; IDP, 0.5; PEP, 0.4; $NaH^{14}CO_3$, 5; GSH, 0.8; sodium glutamate, 5; pyridoxal phosphate, 0.02; aspartate transaminase, approx. 4.5 units. Incubation was for 5 min at 30°. The reaction was stopped with 0.25 ml of a 10% (w/v) trichloroacetic acid solution.

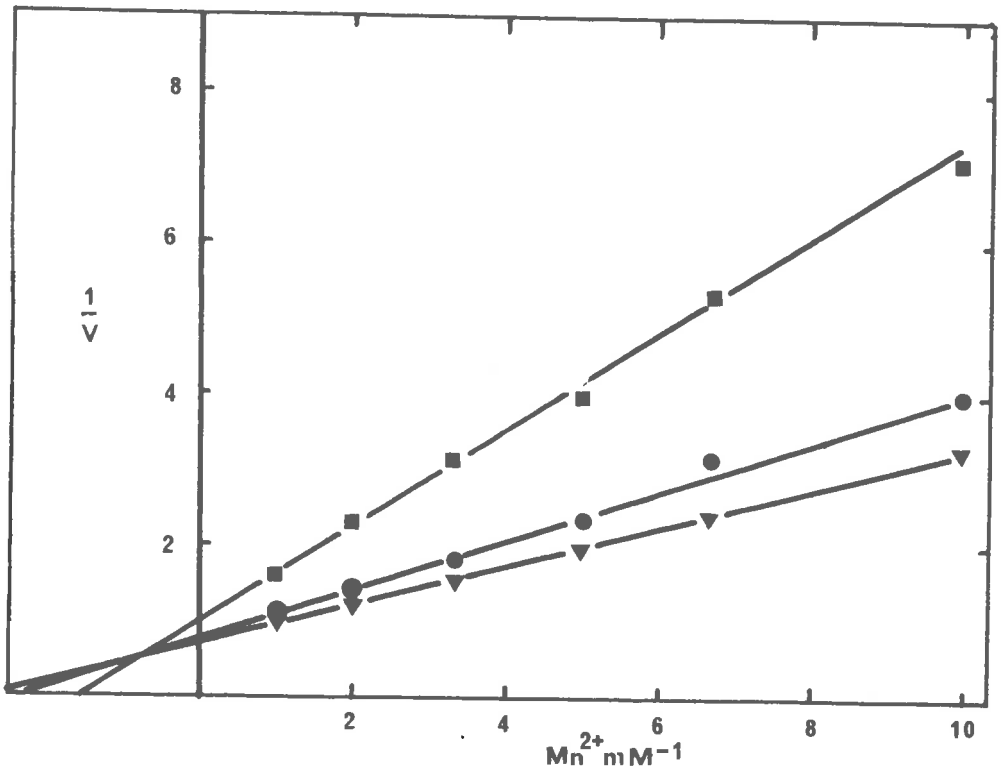


FIG. 6.13. Product inhibition pattern caused by the presence of Ca^{2+}. Reaction mixtures contained (in addition to the standard components listed in Section 6.2.(a)): PEP, 0.5mM; IDP, 0.5mM; $NaHCO_3$, 12mM; Mn^{2+} and Ca^{2+} as indicated. Mn^{2+} was varied at the constant Ca^{2+} levels: (▼——▼), 0.0mM; (●——●), 1mM; (■——■), 6mM.

(f) Presence of an enzyme-bound CO_2 intermediate

All biotin enzymes depend for carboxylation on the obligatory formation of an enzyme.biotin.CO_2 complex (Kaziro and Ochoa, 1961; Ochoa and Kaziro, 1961). Furthermore, ribulose diphosphate carboxylase also forms an enzyme-bound CO_2 complex in the presence of Mg^{2+} and this can be isolated. In the presence of ribulose-1,5-diphosphate, the complex will form two moles of 3-phosphoglycerate (Akoyunoglou and Calvin, 1963).

The formation of an enzyme-bound CO_2 intermediate with PEP carboxykinase was investigated by incubating the enzyme with $NaH^{14}CO_3$ in the presence of various combinations of substrates and products followed by isolation of the protein by one of the following ways: (a) trichloroacetic acid precipitation, (b) ion-exchange chromatography (Kaziro and Ochoa, 1961), (c) gel filtration on Sephadex G25 (see Section 6.2(e) for details of the methods). In no case was evidence obtained for the formation of such an intermediate. However, this result is in agreement with the CO_2:OAA exchange reaction being the only detectable exchange reaction (Chang, et al., 1966).

(g) Studies on the CO_2:OAA exchange reaction

Contrary to the results of Chang et al. (1966), sheep kidney mitochondrial PEP carboxykinase catalyses a Mn^{2+}-dependent CO_2:OAA exchange reaction (Fig. 6.9) and this

activity is markedly stimulated by the addition of ITP or IDP (Table 3.3). However, with the addition of ITP or IDP, the decarboxylation system would be complete giving PEP or pyruvate respectively (Section 3.3).

The marked stimulation of the exchange activity in the presence of the nucleotides immediately raises two questions, viz.:

(i) Could the reversal of the decarboxylation reaction be a significant factor in the observed stimulation? This possibility is negated by the results of Table 6.3 where the decarboxylation products are continuously removed by coupling systems without any significant effect on activity.

(ii) Is the enhanced activity energy-dependent (cf. Chang et al., 1966) in which case the low levels of exchange activity observed in the absence of added nucleotide could result from an abortive pathway? The results shown in Table 6.4 demonstrate that the CO_2:OAA exchange reaction is independent of phosphoryl transfer in both cases. In fact, there was no turnover of nucleoside diphosphate despite an enhanced exchange activity.

These results are consistent with the carboxylation of PEP catalysed by sheep kidney mitochondrial PEP carboxykinase being a two step process with the phosphoryl transfer preceding carboxylation.

TABLE 6.3: DEPENDENCE OF THE CO_2:OAA EXCHANGE REACTION ON THE REVERSAL OF THE PEP-CARBOXYKINASE REACTION.

Components	Activity (cpm incorporated)
(a) $MnCl_2$ + OAA + ITP + $NaH^{14}CO_3$ + KCl	1460
(b) $MnCl_2$ + OAA + IDP + $NaH^{14}CO_3$	880
(a) + pyruvate kinase + ADP	1500
(b) + lactate dehydrogenase + $NADH_2$	850

Components common to all assay mixtures (0.5 ml) were: 0.1 M imidazole (Cl^-), pH 6.5, (adjusted at 30°); 4 mM $MnCl_2$; 2 mM OAA; 2 mM ITP (or IDP); 20 mM $NaH^{14}CO_3$ (6×10^5 cpm per μmole); 1.6 mM GSH. In addition, 0.5 mM ADP, 1 mM $NADH_2$, 50 mM KCl, 5 units pyruvate kinase and 4.5 units lactate dehydrogenase were added as indicated. The reaction was initiated by the addition of OAA and incubation was for two minutes at 30°. The reaction was stopped by the addition of 0.05 ml of 6 N HCl saturated with 2,4-dinitrophenylhydrazine and the radioactivity was determined in 0.05 ml aliquots as detailed in Section 2.7(i).

TABLE 6.4: COMPARISON OF CO_2 AND NUCLEOTIDE TURNOVER IN THE CO_2/OAA EXCHANGE REACTION

Activating Nucleotide	Turnover (μmoles/min.)	
	$^{14}CO_2$ [a]	^{32}P-Nucleotide [b]
GTP	0.0236	0.0152
GDP	0.0159	0.0

[a] 0.012 units enzyme were added to a reaction mixture (total volume 0.5 ml) containing (in μmoles): imidazole (Cl^-), pH 6.5 (adjusted at 30°), 50; $MnCl_2$, 2: GTP (or GDP), 1; OAA, 1; $NaH^{14}CO_3$, 20; GSH, 0.8. Reaction was started by the addition of OAA and incubated at 30° for 2.25 and 4.5 min. The reaction was stopped by the addition of 0.05 ml of 6 N HCl saturated with 2,4-dinitrophenylhydrazine and analysed for radioactivity as described in Section 2.7 (ii).

[b] Conditions were as above except that non-radioactive $NaHCO_3$ was used as well as α-^{32}P-GTP (or β-^{32}P-GDP). The reaction was started with OAA and incubation, at 30°, was stopped after 0, 2.25 and 4.5 min. with 0.05 ml of 5 N formic acid. Denatured protein was removed by centrifuging and aliquots of the supernatant were chromatographed on PEI-paper (Gilliland, Langman and Symons (1966) using 0.3 N NH_4HCO_3. The U.V. absorbing regions were cut out and counted in a Packard-Tricarb scintillation spectrometer.

(h) Pyruvate as an intermediate in the PEP carboxykinase reaction

Enzyme-bound pyruvate as an intermediate of the PEP carboxykinase reaction is indicated by its formation in the IDP-stimulated CO_2:OAA exchange reaction and decarboxylation of OAA. However, attempts to demonstrate a utilisation of pyruvate by its carboxylation to OAA or its exchange into OAA were unsuccessful (see Section 6.2(f) for experimental detail). This negative result does not necessarily exclude pyruvate as an intermediate but rather could simply indicate an inability of the enzyme to bind or utilise pyruvate without prior activation to the enol form.

6.4. DISCUSSION

Analysis of the initial velocity data presented here indicates an irreversible connection between the enzyme forms binding the substrate pairs, IDP and PEP, and IDP and HCO_3^-, but a reversible sequence between those binding Mn^{2+} and HCO_3^-, Mn^{2+} and PEP, and PEP and HCO_3^-. In addition to these restrictions, any proposed mechanistic scheme must account for the following observations:

(a) Both IDP_{free} and ITP_{free} bind tightly to the enzyme in the absence of other substrates (Miller et al., 1968; Chapter 7 and Barns and Keech, 1968).

(b) Mn^{2+} binds strongly in the absence of other substrates ($K_D = 5.9 \times 10^{-5}$ M at pH 6.5, Chapter 4).

(c) ITP, IDP, PEP and OAA bind to the enzyme in the presence of Mn^{2+} (Chapter 4; Miller *et al.*, 1968).

(d) The enzyme catalyses a CO_2:OAA exchange reaction in the presence of Mn^{2+}. This reaction is stimulated by both IDP and ITP while Mg^{2+} can only partially replace Mn^{2+} (Table 3.3).

(e) In the decarboxylation reaction, phosphoryl transfer apparently occurs by a concerted mechanism without hydrolysis of a phosphate bond by solvent water as indicated by the lack of incorporation of ^{18}O from $H_2{}^{18}O$ in the phosphate groups of PEP and IDP (Miller and Lane, 1968). Thus, at least PEP and IDP in the carboxylation reaction and ITP and OAA in the decarboxylation direction must form ternary complexes.

Considering only the organic components of this system, since Mn^{2+} was always at saturating levels and need not be considered, the system becomes one of three substrates and two products. The initial velocity data suggested a Bi Uni Uni Uni Ping Pong mechanism (Cleland, 1963a) as described in Figure 6.14. However, such a scheme fails to incorporate the random binding of substrates and the nucleotide-independent CO_2:OAA exchange reaction. Furthermore, this mechanism, apart from its other limitations, would necessarily incorporate a phospho-enzyme intermediate, contrary to (e) above, and attempts to detect a GDP:GTP exchange reaction in the presence of various combinations

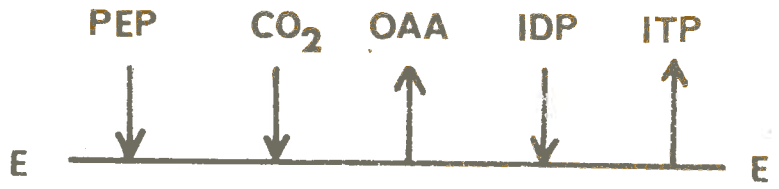


FIG. 6.14. The Bi Uni Uni Uni Ping Pong mechanism applied to PEP carboxykinase.

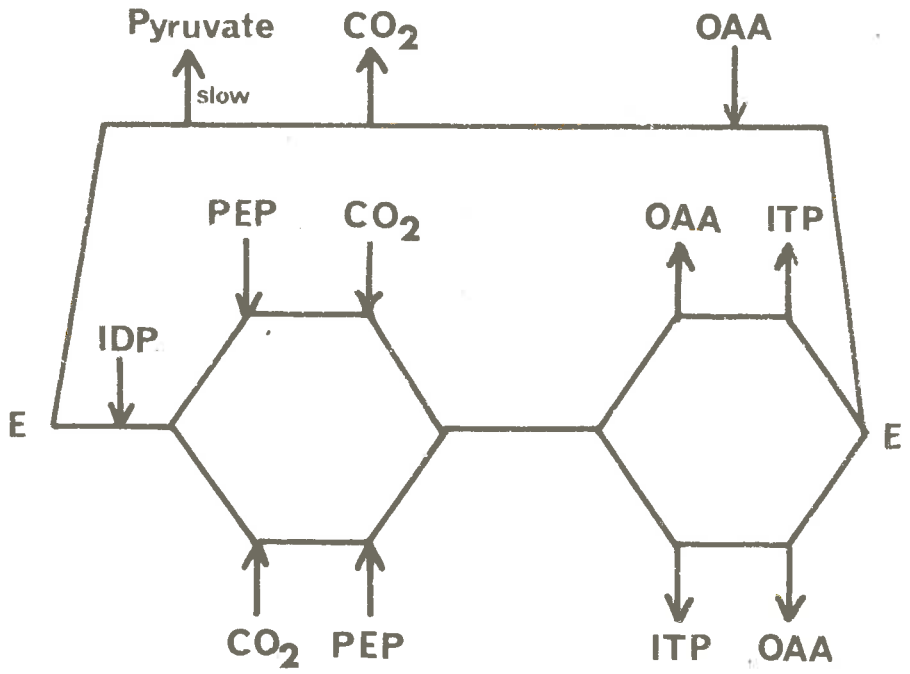


FIG. 6.15. The reaction mechanism of sheep kidney mitochondrial PEP carboxykinase showing the preferred pathway to the central complex. IDP binds first followed by the random addition of PEP and CO_2. The outer sequence shows the mechanism whereby the nucleotide-independent CO_2: OAA exchange reaction occurs.

of substrates have been unsuccessful (cf., Chang et al., 1966).

To incorporate all the kinetic and binding properties, a scheme is proposed where there exists a random addition of substrates. However, to explain the appearance of kinetic patterns characteristic of an ordered sequence, one must invoke a preferred order of addition of substrates with IDP binding first followed by the random addition of PEP and CO_2 (Fig. 6.15). It would therefore follow that, under the conditions of the kinetic analysis reported here, when IDP was a variable substrate the second substrate to bind in this scheme would be that substrate which is held at a fixed saturating level. Finally, the second variable substrate would be bound. Therefore, this scheme would predict parallel initial velocity patterns when IDP is a variable substrate because the addition of the second component which is at saturating levels would create an irreversible connection between the addition of IDP and the third substrate (cf., Figs. 6.1 and 6.3). On the other hand, the enzyme forms which bind CO_2 and PEP would always be reversibly connected because of the random addition of these two substrates and so intersecting initial velocity patterns are predicted (cf. Fig. 6.2).

Furthermore, one would predict that with such a random scheme, ITP would be a non-competitive inhibitor with respect to PEP and CO_2. This was in fact observed (Fig. 6.11). It is interesting in the case of ITP inhibition with respect to IDP that non-linear plots are observed in view of the non-linear initial velocity plots obtained with an uncoupled assay system

(Chapter 5). Such an assay system which allows the accumulation of OAA and ITP, admittedly to a small extent, does not show hyperbolic saturation kinetics. Removal of these low levels of OAA with a coupling system shows a reversion to Michaelis-Menten saturation kinetics. Furthermore, the inflection point in the inhibition plot ($\approx$ 0.2 mM IDP_{total}) is the same as that shown with the uncoupled system (Fig. 5.6). Thus, it can be argued that the interactions observed with the uncoupled assay and induced by OAA have now been introduced into the product inhibition system by ITP, although much higher levels are needed assuming $Mn.ITP^{2-}$ to be the active species. Thus, the scheme depicted in Fig. 6.15 is in accord with all the observed binding properties and the initial velocity and product inhibition studies. This scheme also shows the sequence whereby the nucleotide-independent CO_2:OAA exchange reaction occurs. In this alternate pathway, the presence of nucleotide is not essential but rather an activator of this pathway (see below). If IDP binds rather than the normal decarboxylation substrate (ITP), phosphoryl transfer can not occur so that pyruvate is formed rather than PEP (see Section 3.2.B(a)) but at a faster rate than in the absence of IDP.

Considering now the role of the metal ion in the reaction sequence, the S_t and H_t profiles (London and Steck, 1969) for PEP and Mn^{2+} shown in Fig. 6.6 do not suggest any requirement for complex formation. In fact, they suggest almost independent binding of the two species because, as the metal ion concentration increases, maximum velocity is still achieved at

essentially the same PEP concentration, even when Mn^{2+} concentration is infinite. Furthermore, free PEP does not appear to be a potent inhibitor of the enzyme. Thus, it would appear that the preferred species of PEP is the uncomplexed form although this does not exclude the possibility that Mn.PEP can be bound as a catalytically active species.

Fig. 6.4 shows that Mn^{2+} has little or no effect on HCO_3^- binding because of the small differences in the horizontal intercepts although, with PEP, Mn^{2+} does appear to influence its binding. However, the binding of PEP, HCO_3^- and Mn^{2+} are reversibly connected. Thus, a random addition of Mn^{2+} and PEP followed by HCO_3^- would explain the kinetic data. Lack of curvature in the kinetic plots (Fig. 6.5) would suggest an approach to rapid equilibrium or perhaps a preferred pathway with Mn^{2+} binding first. It would seem doubtful that Mn.PEP would be the preferred substrate as suggested by Miller and Lane (1968) for the pig liver mitochondrial enzyme firstly because of the high dissociation constant for this complex (Wold and Ballou, 1957; Mildvan and Colm, 1966) and secondly because the dissociation constant at pH 7.5 for the enzyme and the Mn.PEP complex is an order of magnitude higher than that for PEP and enzyme.Mn^{2+} (Table 4.2). The data of Miller *et al.* (1968) suggest that Mn^{2+} and PEP have a mutual stabilising effect on the ternary enzyme.Mn^{2+}.PEP complex, or in other words, PEP and Mn^{2+} each provide part of the binding site for the other in accord with the "metal-bridge" structure (Mildvan and Colm, 1970) proposed for this enzyme (Miller *et al.*, 1968). The

N.M.R. data for the sheep kidney mitochondrial enzyme can also be interpreted in terms of such a structure. In this case, PEP would provide one ligand for the enzyme-bound Mn^{2+} in the ternary enzyme.Mn^{2+}.PEP complex (see Chapter 4).

Contrary to the case with PEP, the S_t and M_t profiles showing the relationship between Mn^{2+} and IDP do suggest a definite relationship between the maximum velocity and the stage when IDP_{total} equals Mn^{2+}_{total} (Fig. 6.7). Such behaviour is characteristic of a system where the Mn-nucleotide complex is the true substrate. Furthermore, free IDP is a potent inhibitor. Therefore, it is proposed that Mn.IDP^- is the substrate for the enzyme. However, in this case, K_D for Mn.IDP^- is low (O'Sullivan and Cohn, 1966) but the K_D for enzyme. IDP is even lower (8.06×10^{-6} M at pH 7.5; Barns and Keech, 1968). Thus, IDP must bind in such a manner that Mn^{2+} can still complex with the IDP in the binary enzyme.IDP complex., i.e., a role of charge neutralisation is suggested.

Figure 6.9 shows there is a reversible association between enzyme forms binding OAA and Mn^{2+} similar to that shown for PEP. Although there is no information available concerning the order of release of OAA and the metal ion, it is presumed to be similar to that with PEP.

The involvement of two metal ions is therefore proposed for sheep kidney mitochondrial PEP carboxykinase based on the initial velocity studies. One metal ion would facilitate PEP binding while the role of the second ion would be that of

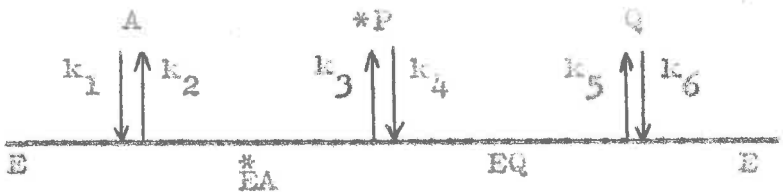
neutralising the negative charge carried by IDP (cf., creatine kinase (Cohn and Leigh, 1962; O'Sullivan and Cohn, 1966) and adenylate kinase (O'Sullivan and Noda, 1968)). Other evidence in favour of such a scheme includes (i) the specific Mn^{2+} effect indicated in the pH profile for the carboxylation of PEP (Chapter 3), (ii) Mn^{2+} protects against DNFB inactivation while Mg^{2+} is ineffective (Table 7.4), (iii) the low degree of inhibition by Ca^{2+} (Table 6.2), (iv) the synergistic effect of Mn^{2+} and Mg^{2+} shown for the decarboxylation reaction by both mitochondrial and cytosol PEP carboxykinase from guinea pig liver (Holten and Nordlie, 1965) and by the rat cytosol enzyme (Foster et al., 1967), (v) the involvement of two metal ions in the related enzyme, PEP carboxytransphosphorylase (Wood, Davis and Willard, 1969), (vi) complexes of the type enzyme.Mn^{2+}.substrate.Mn^{2+} were detected for GDP and GTP with the pig liver enzyme (Miller et al., 1968). However, the fact that Ca^{2+} is a non-competitive inhibitor of the sheep kidney enzyme with respect to Mn^{2+} provides the most convincing evidence for a dual role of the metal ion.

No GDP:GTP or ^{14}C-PEP:OAA exchange reaction could be detected with pig liver mitochondrial PEP carboxykinase (Chang, et al., 1966) eliminating the involvement of a phosphoryl-enzyme intermediate. This also appears to be the case for the sheep kidney mitochondrial enzyme since attempts to detect a GDP:GTP exchange reaction under a variety of conditions have failed.

A concerted mechanism is therefore indicated for the phosphoryl transfer. In support of this, Miller and Lane (1968) demonstrated that ^{18}O of $H_2{}^{18}O$ was not incorporated into the phosphoryl group of PEP or IDP during decarboxylation. This result would eliminate any mechanism involving hydrolysis of a phosphate bond of an intermediate although an inherent assumption in such an experiment is that the enzyme-bound water is in equilibrium with the water of the solvent and that hydrolysis is not effected by water generated during the reaction and which is immediately used without equilibrium.

Chang et al. (1966) showed that the CO_2:OAA exchange reaction catalysed by pig liver mitochondrial PEP carboxykinase was apparently dependent on the presence of ITP or GTP. However, studies with the sheep kidney mitochondrial enzyme showed a nucleotide-independent CO_2:OAA exchange activity which required Mn^{2+}. This activity was markedly stimulated by the presence of ITP or IDP (Table 3.3). If this exchange were in fact a true partial reaction, the enzymic mechanism would not be a fully concerted one as proposed by Miller and Lane (1968) but rather a two step one with enzyme-bound pyruvate as an intermediate.

Consider Reaction 6.1 which is the sequence whereby $^{14}CO_2$ is incorporated



... (6.1)

into OAA (where A = OAA; P = $^{14}CO_2$; Q = pyruvate and * represents a ^{14}C component).

From steady state kinetics,

$$\frac{d\,\overset{*}{EA}}{dt} = k_4 \overset{*}{P}\,EQ - (k_2 + k_3)\,\overset{*}{EA} = 0 \qquad \ldots (6.4)$$

$$\therefore \quad \overset{*}{EA} = \frac{k_4 \overset{*}{P}\,EQ}{(k_2 + k_3)} \qquad \ldots (6.5)$$

$$\overset{*}{v} = \frac{d\,\overset{*}{A}}{dt} = k_2 \overset{*}{EA}$$

$$= \frac{k_2 k_4 \overset{*}{P}\,EQ}{(k_2 + k_3)} \qquad \ldots (6.6)$$

where $\overset{*}{v}$ is the initial velocity of the isotope exchange reaction. Using the distribution equation derived by the method of King and Altman (1956) and putting Q = 0

$$\frac{EQ}{E_t} = \frac{k_1 k_3 A}{k_5(k_2 + k_3) + k_1(k_3 + k_5)A + k_1 k_4 AP + k_2 k_4 P} \qquad \ldots (6.7)$$

Substituting for EQ in Equation 6.6

$$\overset{*}{v} = \frac{k_1 k_2 k_3 k_4 A \overset{*}{P} E_t}{(k_2 + k_3)(k_5(k_2+k_3) + k_1(k_3+k_5)A + k_1 k_4 AP + k_2 k_4 P)}$$

$$\ldots (6.8)$$

which is an equation for intersecting lines. In fact, the expected intersecting initial velocity patterns are obtained

when CO_2 and OAA are the variable substrates (Fig. 6.8A) confirming that the nucleotide-independent CO_2:OAA exchange activity is a true partial reaction. Furthermore, the results of Table 6.4 indicate that the exchange is independent of phosphoryl transfer, i.e., the nucleotide becomes an activator of the exchange activity (cf., Bridger, Millen and Boyer, 1968). If the nucleotide is an activator rather than an obligatory reactant, the presence of ITP should not alter the initial velocity patterns obtained when CO_2 and OAA are the covariables. This is indeed found (Fig. 6.8B).

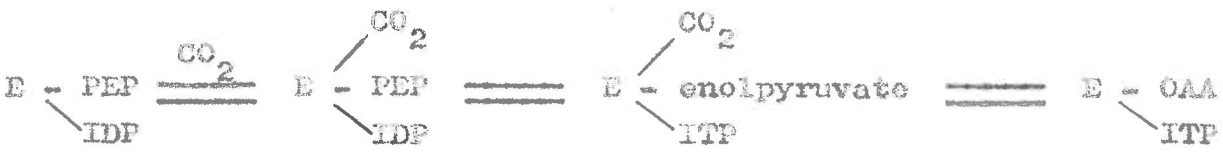
The evidence presented in Tables 6.3-4 confirms that the CO_2:OAA exchange reaction is a true partial reaction independent of phosphoryl transfer, i.e., the reaction is not in fact a fully concerted one as proposed by Miller and Lane (1968) but rather a two-step process where, in the carboxylation of PEP, phosphoryl transfer precedes carboxylation. Such a mechanism would require enzyme-bound pyruvate as an intermediate. Attempts to show this by carboxylation of pyruvate or by a pyruvate exchange into OAA were unsuccessful. However, this failure does not necessarily exclude enzyme-bound pyruvate as an intermediate but rather could simply indicate an inability of the enzyme to bind or utilise pyruvate without prior activation to the enol form. Energy from the phosphoryl transfer would appear to be required to stabilise the enzyme-bound pyruvate in the enol form with the result that PEP carboxykinase would

appear to be incapable of activating pyruvate to the enol form, i.e., the substrate must be supplied in this form. The related enzyme, PEP carboxytransphosphorylase, also forms pyruvate from PEP in the absence of CO_2 and also appears to catalyse a Mn^{2+}-dependent CO_2:OAA exchange reaction although pyruvate can not exchange with PEP or OAA (Wood, Davis and Willard, 1969).

The catalysis of the CO_2:OAA exchange reaction in the absence of nucleotide (Fig. 6.9) implies that nucleotide is not essential for the exchange reaction. Furthermore, the stimulation of the exchange activity in the presence of nucleotides is independent of phosphoryl transfer (Table 6.4). In fact, there was no turnover of nucleoside diphosphate despite an enhanced exchange activity. Rather, the nucleotide becomes an activator and its turnover, in the case of the triphosphate, becomes secondary to its primary activating effect (cf., Bridger, Millen and Boyer, 1968). Therefore, phosphoryl transfer or the splitting of a high energy phosphate bond would not be essential for carboxylation or decarboxylation per se.

The properties of the CO_2:OAA exchange reaction are consistent with the carboxylation of PEP catalysed by sheep kidney mitochondrial PEP carboxykinase being a two-step process with the phosphoryl transfer from PEP to IDP (or GDP) preceding carboxylation of the enzyme-bound enolpyruvate to OAA (Reaction 6.2).

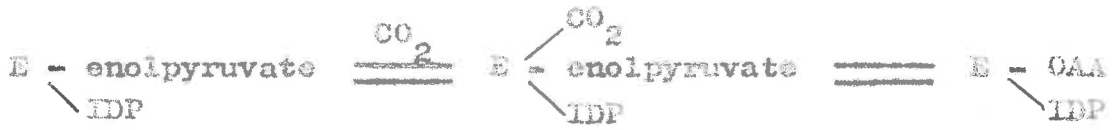
$$E + PEP + IDP \rightleftharpoons$$



$$\rightleftharpoons E + ITP + OAA \qquad \ldots (6.2)$$

The IDP-stimulated CO_2:OAA exchange reaction and OAA decarboxylation would occur as shown in Reaction 6.3.

$$E + IDP + \text{pyruvate} \leftarrow$$



$$\rightleftharpoons E + IDP + OAA$$

$$\ldots (6.3)$$

As the exchange of CO_2 into OAA is more rapid than the decarboxylation of OAA, the dissociation of CO_2 in both Reactions 6.2 and 6.3 must be more rapid than the dissociation of the other substrates such that CO_2 is rebound to the enzyme before the decarboxylation process is complete, i.e., a coupled system where PEP in Reaction 6.2 or pyruvate in Reaction 6.3 are continuously removed should have little effect on the CO_2:OAA exchange activity as shown (Table 6.3).

A tentative mechanism, proposed as a working hypothesis for future experimentation, is detailed in Fig. 6.16. This scheme takes into account the two step reaction sequence and the requirement for two metal ions in the catalysis although the role of the second metal ion is probably charge neutralisation as appears to be the case for creatine kinase (Cohn and Leigh, 1962; O'Sullivan and Cohn, 1966) and adenylate kinase (O'Sullivan and Noda, 1968). The essential feature of this mechanism is the involvement of Mn^{2+} - bound hydroxyl ions which are continuously utilised and reformed without any net uptake consistent with the lack of incorporation of ^{18}O of $H_2{}^{18}O$ (Miller and Lane, 1968). Also, the carboxylation-decarboxylation process is not necessarily stoichiometric with the phosphoryl transfer process such that the CO_2:OAA exchange reaction is independent of phosphoryl transfer with enzyme-bound pyruvate (or more strictly the enolate ion) as an intermediate. Furthermore, the possibility does exist that the enzyme-bound enolpyruvate intermediate is stablised by the formation of a hemi-thioketal with the active centre sulphydryl residue (see Chapter 7) in a manner similar to that proposed for ribulose diphosphate carboxylase by Rabin and Trown (1964). Carboxylation of the enzyme-bound enolpyruvate intermediate would yield the keto form of OAA, the primary carboxylation product of PEP carboxykinase (Graves et al., 1956).

This mechanism is similar to that proposed by Wood,

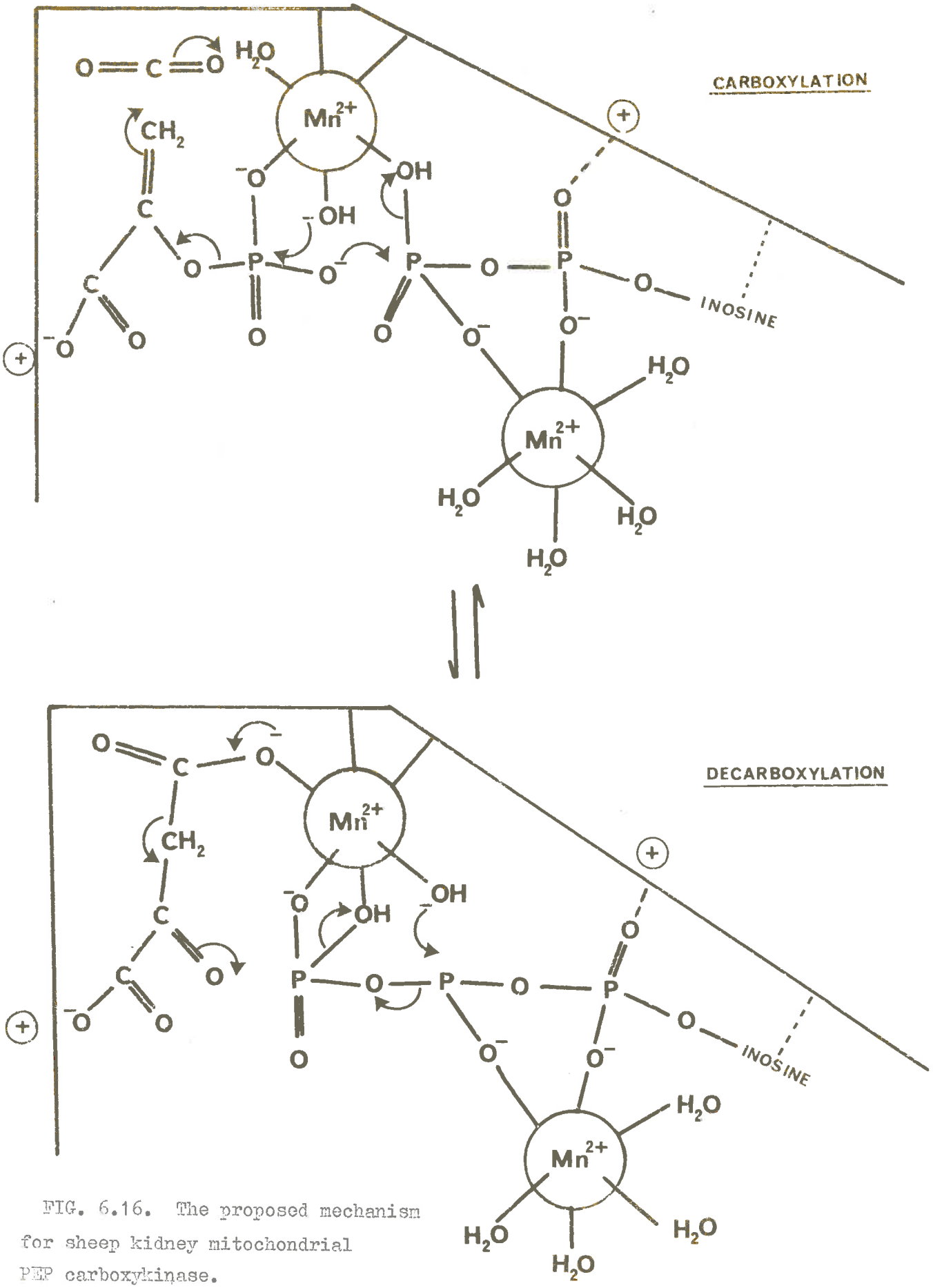


FIG. 6.16. The proposed mechanism for sheep kidney mitochondrial PEP carboxykinase.

Davis and Willard (1969) for PEP carboxytransphosphorylase (Fig. 1.2) except that these workers proposed an enzyme-bound pyrophosphoenolpyruvate intermediate whereas the equivalent intermediate for PEP carboxykinase, ITP-enolpyruvate, could not exist in accord with the CO_2:OAA exchange reaction being independent of phosphoryl transfer. In fact, a PP_i-independent exchange reaction does appear to exist for PEP carboxytransphosphorylase so that very similar mechanisms probably exist for these two enzymes.

CHAPTER 7. CHEMICAL STUDIES ON THE ACTIVE CENTRE

7.1. INTRODUCTION

A detailed molecular description of enzymic catalysis demands the characteristisation of the role of the amino acid residues in the active centre, their geometry and the micro-environment in which each particular active centre residue is located. It is this local environment which endows each amino acid functional group with its peculiar reactivity. At present, the most valuable information concerning the relationship between such functional groups and enzymic function can be obtained by means of chemical modification. This may be achieved by the chemical modification of a limted number of reactive groups and evaluation of the consequent alteration in kinetic and/or physical properties of the enzyme. Such an approach would establish whether an essential group in the active centre has been blocked or whether the modification of a group or groups elsewhere in the protein has caused non-specific changes leading to an inactive or desensitised enzyme.

This approach has its limitations because of the lack of specificity of the reagents for any one type of group and the difficulty in assigning an unambiguous role to a modified group since any modification may have multiple effects. However, there are instances where confidence in the analysis of chemical modification data is greatly enhanced. One such case is the situation where chemical modification differentially affects the enzymic activity towards different substrates.

Examples of this case are the differential effects of modification on the peptidase and esterase activity of carboxypeptidase A, (Simpson, Riordan and Vallee, 1963) and the modification of methionine-192 in α-chymotrypsin where th binding of aromatic and aliphatic substrates is differentially affected (Knowles, 1965). In such cases, full retention of one type of activity or activity towards one group of substrates clearly indicates that those features essential to the functional integrity of the active centre still persist.

A second case where confidence in chemical modification results is justified is the negative result where modification of residues causes no detectable alteration in enzymic properties. This would delineate those groups which are not involved in the catalytic processes.

A third such case would be the use of substrate analogues (Baker, 1967) where the specificity requirements of t he enzymes are invoked to achieve a specific orientation within the active centre. Correct design of the modifier can then achieve unique directional reaction with adjacent groups. An example of this category would be the active-site directed reagents of the type introduced by Schoellmann and Shaw (1962, 1963), tosylphenylalanine chloromethylketone (TPCK) and tosyllysine chloromethylketone (TLCK), which enabled the detection of histidine in the active centre of α-chymotrypsin and trypsin respectively. An extension

of this approach would permit the gaining of some knowledge of the topography of the active centre by the use of appropriately designed bifunctional reagents.

X-ray crystallographic studies permit a topographical description of the active centre using competitive inhibitors or substrates with low activity as active centre markers. Consequently, confirmation of the conclusions based on modification studies is now becoming available. In the cases of ribonuclease A, α-chymotrypsin and carboxypeptidase A, all of which have been the subject of extensive modification studies, X-ray crystallographic studies have confirmed all the observations obtained using the chemical approach (Koshland and Neet, 1968). On the other hand, the three-dimensional structure of lysozyme was established prior to extensive modification studies (Blake et al., 1967). Certain predictions regarding functional residues in the active centre were made from this structure. These have subsequently been confirmed by chemical studies (Koshland and Neet, 1968). These two techniques thus become complimentary tools for the enzymologist exploring the active centre of enzymes.

Both chemical and X-ray crystallographic studies also allow predictions regarding the micro-environment of particular amino acid residues. Whether such groups are present in a relatively hydrophobic, hydrophilic or ionic environment may be deduced from their reactivity or their position in the

active centre relative to the other amino acid side chains. An extension of the modification studies leads to the use of reporter groups to monitor the micro-environment (Hille and Koshland, 1967). In most cases, these are chromophoric compounds usually covalently bound at or near the active centre. Their spectra thus become powerful indices of their environment both in the free enzyme and in the enzyme-substrate complexes.

PEP-carboxykinase from chicken liver (Utter and Kurahashi, 1954) and pig liver mitochondria (Chang and Lane, 1966) required the presence of reducing compounds such as GSH or cysteine to exhibit maximum activity while each was inactivated by sulphydryl group reagents such as NEM and p-hydroxymercuribenzoate. The sheep kidney mitochondrial enzyme also shows a similar dependence for maximum activity on the presence of GSH and a similar sensitivity to sulphydryl group reagents. This would suggest the dependence of catalytic activity on at least one sulphydryl group. This chapter provides evidence that in sheep kidney mitochondrial PEP carboxykinase the integrity of a cysteine residue is, in fact, essential for enzymic activity and that its function is associated with some aspect of the catalytic process as distinct from binding. Evidence is also presented that the reactivity of this residue is under the influence of a group of $pK_a \approx 6.5$, presumably a histidine group. In addition, there is a second sulphydryl group reactive towards DNFB (and TNBS)

7.2. METHODS

The PEP carboxykinase used in these experiments was prepared as described in Chapter 3 and had a specific activity of 1.5 - 4.0 units per mg of protein (Section 2.8).

(a) Identification of the 1-^{14}C-NEM amino acid derivative from the 1-^{14}C-NEM-labelled enzyme

Labelling of the enzyme was achieved by the addition of 0.75 mg of enzyme to a solution of 50 µmoles of phosphate (K^+), pH 7.0, and 0.18 µmoles of 1-^{14}C-NEM (0.5 µC) at 20°. The reaction was stopped by the addition of 50 µmoles GSH, pH 7.0, and the protein precipitated with 10% (w/v) trichloroacetic acid. The denatured protein was collected on an oxoid membrane held in a Millipore suction apparatus. Washing was effected with several aliquots of 10% (w/v) trichloroacetic acid containing 1×10^{-3} M NEM and finally 1% (v/v) acetic acid. The protein was dissolved in 0.1 M NH_4OH and the pH adjusted with formic acid to pH 7.5. The modified protein was hydrolysed with pronase for 72 hr at 37° after which time hydrolysis had not gone to completion as shown by electrophoretic analysis. The hydrolysate was further incubated with 6 M HCl at 106° for 16 hr and 70 hr. The HCl was removed under vacuum and the residue dissolved in a minimum quantity of glass-distilled water (0.15 ml) and used for electrophoretic analysis.

Electrophoresis was performed on Whatman 3 MM paper with pyridine/acetic acid/water (400/16/3600, v/v/v) buffer, pH 6.5,

on a "flat-bed" apparatus at 40 volts per cm. The papers were dried in an oven at 80° for 5 min. and the radioactivity was detected in 1 cm strips of the sample lane of the electrophoretogram by scintillation counting. The standard amino acid derivatives were detected on the electrophoretograms by spraying with ninhydrin-acetone (0.01%, w/v) followed by heating at 80° for 5 min. (Toennies and Kolbe, 1951).

(b) Identification of the DNP-amino acid derivative from the ^{14}C-DNFB-labelled enzyme

Labelling of the enzyme with ^{14}C-DNFB was achieved by incubating 10 mg of enzyme with 100 µmoles of N-ethylmorpholine (Cl^-), pH 7.5, and 0.5 µmoles of ^{14}C-DNFB for 10 min. at 20°. (Total volume 2 ml.) The reaction was stopped by the addition of 400 µmoles of lysine, pH 7.5, and the protein was precipitated with 10% (w/v) trichloroacetic acid. The donatured protein was collected on a Whatman Glass Fibre Filter (GF/C) held in a Millipore suction apparatus. Washing was effected with several aliquots of 10% (w/v) trichloroacetic acid containing 1 x 10^{-3} M ε-DNP-lysine and finally 1% (v/v) acetic acid. The modified protein was hydrolysed with 6 N HCl at 106° for 32 hr. The HCl was removed under vacuum and the residue dissolved in 2 ml glass-distilled water. This solution was filtered to remove glass fibre pieces from the GF/C filter and the filtrate was lyophilised. The residue was dissolved in 1 ml of glass-distilled water and used for chromatographic analysis.

Analysis of the ^{14}C-DNP-amino acids was achieved by

descending chromatography in the solvent systems: 1 -butanol/ acetic acid/water (4/1/1, v/v/v) or 1-butanol/water/conc. ammonia (87/12/1, v/v/v). The papers were dried in an oven at 60° for 10 min. and the radioactivity was detected in 1 cm strips of the sample lane of the chromatogram by scintillation counting. The standard DNP-amino acids and their decomposition products, 2,4-dinitroaniline, 2,4-dinitrophenol and 2,4-dinitrothiophenol, were detected by their absorption under UV light. S-DNP-cysteine and 2,4-dinitrothiophenol were found to oxidise slowly in air. Thus, it was found necessary to add 2-mercaptoethanol (10 mM) to both the standard solutions and the chromatographic solvents.

7.3 RESULTS

A. MODIFICATION STUDIES USING NEM

(a) Inhibition using typical sulphydryl reagents

Sulphydryl-modifying reagents may broadly be divided into three groups (i) alkylating agents where the sulphydryl is covalently and irreversibly modified, (ii) mercaptide-forming agents which are reversible by incubation with an excess of a second sulphydryl compound, e.g., GSH, and (iii) oxidising agents where a disulphide bond is formed between two protein molecules or between the protein and the modifier. Inactivation of PEP carboxykinase was investigated with two reagents from each of the above three groups (Table 7.1). The enzymic activity was susceptible to reagents from each of

TABLE 7.1: INHIBITION OF PEP CARBOXYKINASE BY SULPHYDRYL INHIBITORS

Reagent	Concentration (M)	Time of Incubation (min.)	% Initial Activity
N-ethylmaleimide	1×10^{-4}.	10	22.8
Iodoacetamide	5×10^{-4}	20	70.5
	1×10^{-4}	30	77.4
Iodosobenzoate	5×10^{-4}	20	29.0
	1×10^{-4}	30	49.1
dithiobis (2-nitrobenzoate)	5×10^{-4}	2	70.7
	1×10^{-4}	5	73.2
p-hydroxymercuri-phenylsulphonate	5×10^{-4}	2	13.2
	1×10^{-4}	5	41.3
p-hydroxymercuri-benzoate	1×10^{-4}	2	5.3
	2×10^{-5}	2	61.8

The enzyme was preincubated in a solution (final volume 0.1 ml) containing 5.0 μmoles phosphate, pH 7.0, 0.1 units PEP carboxykinase and sulphydryl inhibitor as indicated below. Incubation was at 20° for the indicated times when the reaction was halted by the addition of 0.025 ml 1% (w/v) B.S.A. containing 10^{-1} M glutathione, pH 7.0. Aliquots were withdrawn and analysed for residual activity. In the case of p-hydroxymercuribenzoate, incubation was at 30° in a solution containing 5.0 μmoles tris, pH 8.0.

these groups.

NEM has been shown by Smyth et al. (1964) and Brewer and Rheim (1967) to react with both histidine and lysine but at a much reduced rate as compared with sulphydryl groups. Under appropriate conditions, iodoacetamide (and iodoacetic acid) will react with histidine, tryptophan, the ϵ -NH_2 group of lysine, the -OH group of tyrosine and the -SCH_3 group of methionine as well as -SH groups (Cecil, 1963; Vallee and Riordan, 1969). However, coupled with the almost complete specificity of the mercaptide and oxidising agents for sulphydryl groups, we may conclude that the inactivation by these modifiers indicates the presence of a cysteine residue whose integrity is essential for catalytic activity.

In the further experiments described in this section, the alkylating agent NEM was used because modification was rapid and irreversible.

(b) Order of inactivation with respect to time and NEM concentration

Plots of log percentage activity as a function of time of NEM inactivation were linear to the loss of 100% activity (Fig. 7.1) indicating that the inactivation process approximated first-order kinetics with respect to time at fixed concentrations of this inhibitor. The same data, when the rate of inactivation, expressed as the reciprocal of the

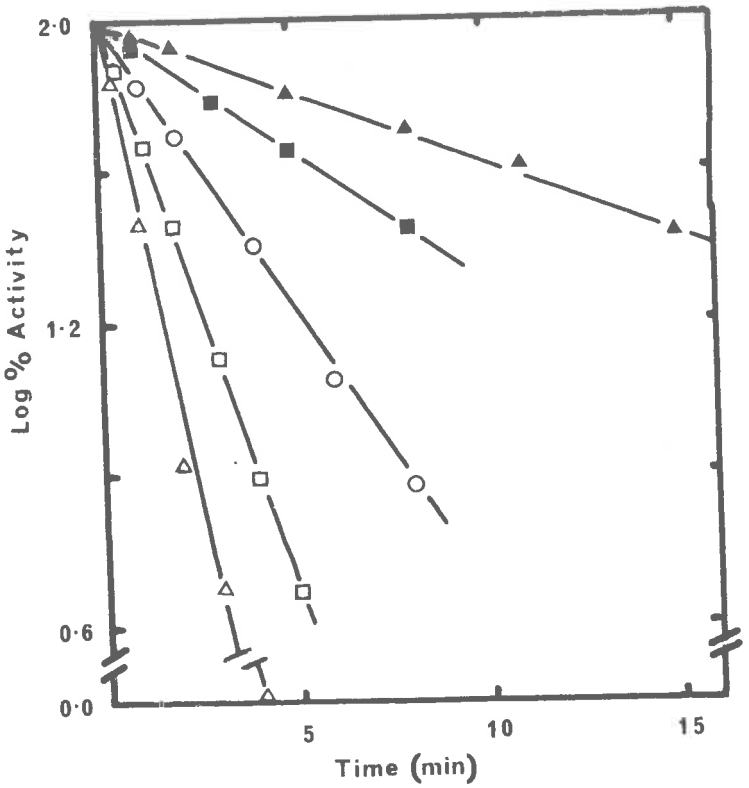


FIG. 7.1. The rate of inactivation of PEP carboxykinase with varying concentrations of NEM. Enzyme was incubated at 20° in a solution containing 10 μmoles of phosphate (K^+), pH 7.0, 0.3 units of enzyme and varying amounts of NEM as indicated. Aliquots of 0.025 ml were diluted with 0.05 ml of 1% (w/v) B.S.A. containing 10^{-1}M GSH, pH 7.0, and analysed for residual activity as detailed in Section 2.7.(i). Log_{10} % activity was plotted against time. The concentrations of NEM were: (△——△), 2 x 10^{-3}M; (□——□), 1 x 10^{-3}; (○——○), 5 x 10^{-4}M; (■——■), 2.5 x 10^{-4}M; (▲——▲), 1.25 x 10^{-4}M.

half-time ($^1/t_{0.5}$), were replotted as a function of NEM concentration (Fig. 7.2), showed that the inactivation process was pseudo first-order with respect to inhibitor concentration.

If one assumes the inactivation process to be,

$$E + nI \xrightarrow{k_1} EI_n \qquad \ldots (7.1)$$

where E, I, EI_n, n and k_1 are the free enzyme, inhibitor, enzyme-inhibitor complex, the number of inhibitor molecules reacting per active site and the second order rate constant respectively, the rate of the inactivation process, v, becomes

$$v = k'[I]^n \qquad \ldots (7.2)$$

where $k' = k_1[E]$, the pseudo first-order rate constant.

Expressing the rate of inactivation (v) as the reciprocal of the half-time ($^1/t_{0.5}$) we obtain

$$\log_{10}(^1/t_{0.5}) = \log_{10}k' + n \log_{10}[I] \qquad \ldots (7.3)$$

such that when $\log (^1/t_{0.5})$ is plotted against $\log[I]$ a straight line is obtained of slope n, i.e., the apparent number of molecules of inhibitor reacting per intact catalytic site to produce an inactive enzyme-inhibitor enzyme.

When the data of Fig. 7.1 are replotted in this manner (Fig. 7.3), the slope, n = 1.0, indicated that an average of one molecule of NEM reacts with each catalytic site of the enzyme.

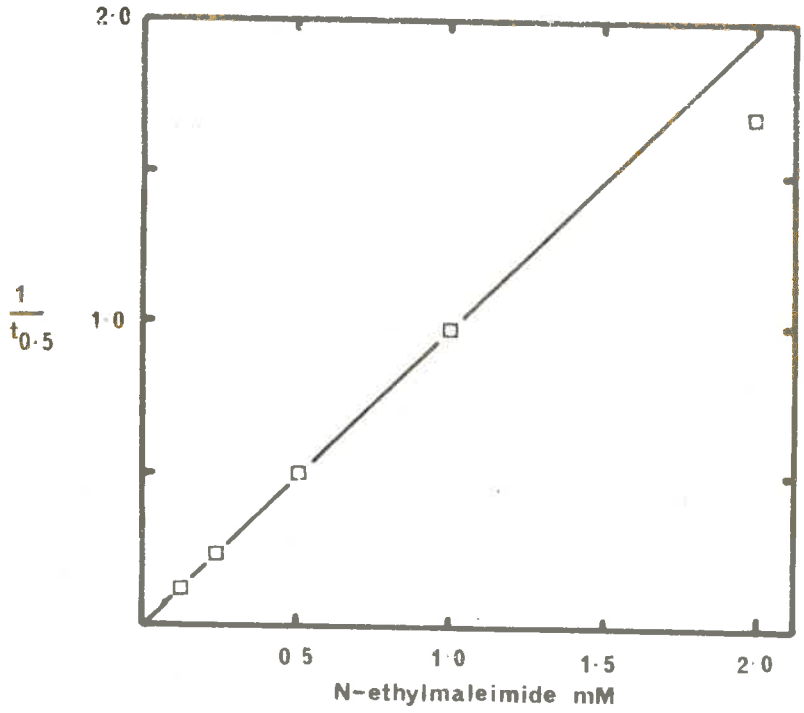


FIG. 7.2. The pseudo first-order kinetics of inactivation with respect to NEM. The half-time, $^{1}/t_{0.5}$, was obtained from Fig. 7.1. The reciprocal of the half-time was plotted against NEM concentration.

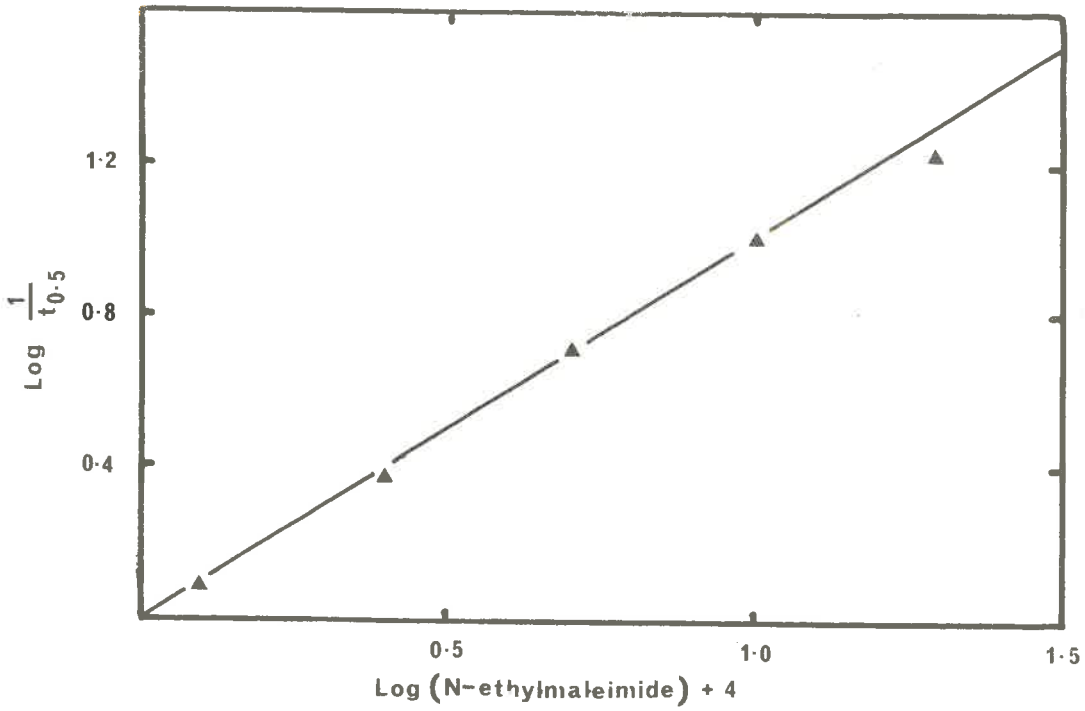


FIG. 7.3. The data of Fig. 7.1. were replotted as $\log_{10}$ of the reciprocal of the half-time of inactivation against $\log_{10}$ NEM concentration.

(c) Effect of pH on the rate of inactivation by NEM

It was found that NEM inactivation was markedly dependent on the H^+ ion concentration. The results of this investigation are shown in Fig. 7.4. The rate of inactivation remained essentially constant below pH 7.0 but increased rapidly above this pH showing the ionised form of cysteine to be the reactive species. From this data, the pK_a of the reactive residue would be about 8.0 which is low in comparison with the normal range of 8.5 - 9.2 for cysteine in an electrostatically neutral environment, and would suggest the close proximity of a positively charged group (Webb, 1963).

A replot of the data of Fig. 7.4 in terms of log rate of inactivation against pH gives a straight line region from pH 6.5 - 8.0 with a slope of 0.9. This confirms the data of Fig. 7.3 and indicates that one ionisable group was reacting with NEM over this range of pH (Fig. 7.5).

(d) IDP protection against NEM inactivation

In an attempt to assign a role for the reactive cysteine residue in the enzymic process, various combinations of components of the assay were tested for their ability to protect the enzyme against NEM inactivation. Table 7.2 shows that almost complete protection was afforded by IDP, or any combination of components containing IDP, and the presence of Mn^{2+} was not essential for IDP protection. HCO_3^- alone was the only component not to show any protection, but in the

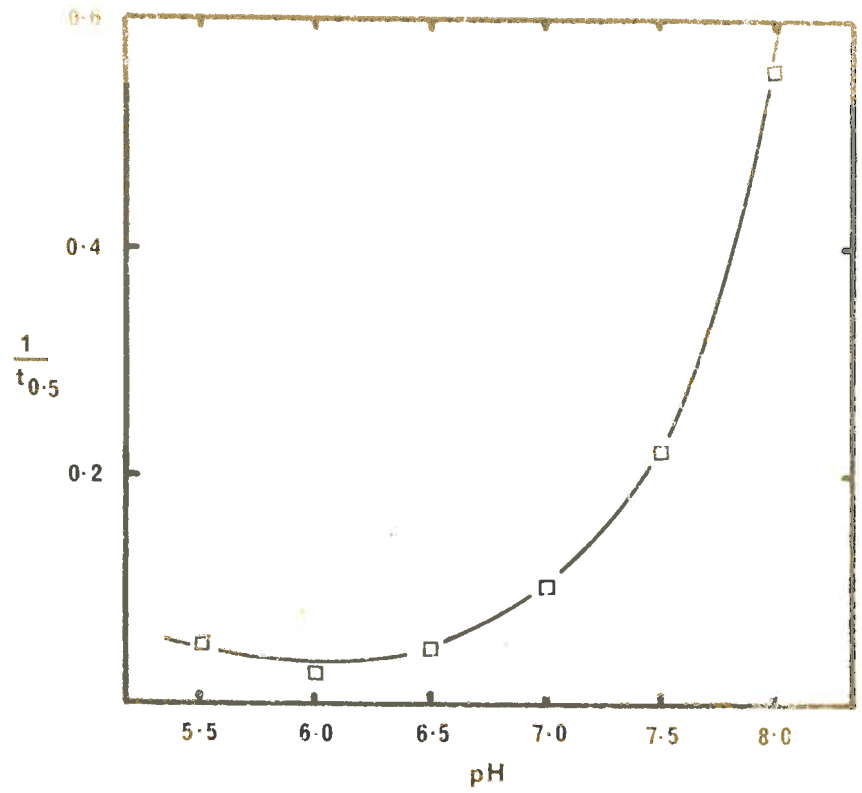


FIG. 7.4. Effect of pH on the rate of inactivation by NEM. Conditions were as in Fig. 7.1. using 5 x 10^{-2}M tris.phosphate buffer (pH 5.5 - 8.0) and 0.02 μmoles of NEM. The reciprocal of the half-time of inactivation was plotted against pH.

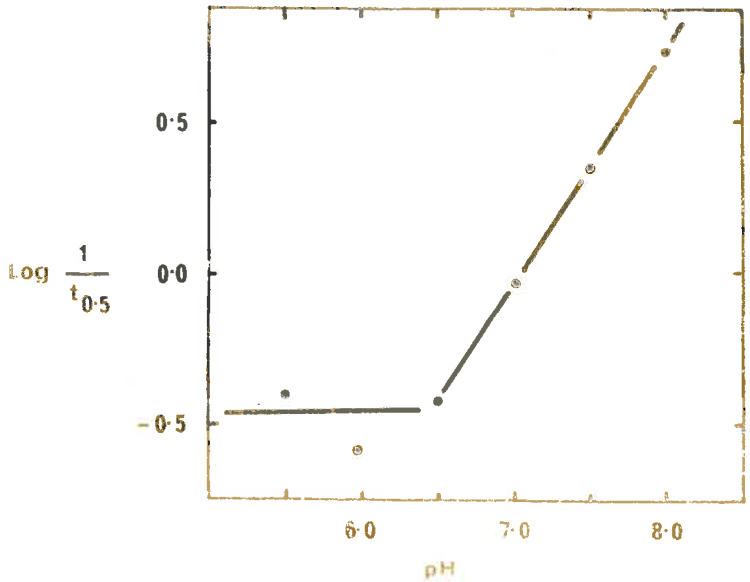


FIG. 7.5. The number of ionising groups reacting with NEM. The data of Fig. 7.4. were replotted as $\log_{10} {}^{1}/t_{0.5}$ against pH. The slope of the line gives the number of ionising groups reacting with NEM.

TABLE 7.2: PROTECTION OF ENZYMIC ACTIVITY BY REACTION MIXTURE COMPONENTS

	Addition	% Original Activity
A.	None	22.8
	Mn^{2+}	37.2
	PEP	29.1
	IDP	86.9
	Bicarbonate	22.6
	PEP + Mn^{2+}	53.8
	IDP + Mn^{2+}	83.0
	Bicarbonate + Mn^{2+}	55.2
	PEP + IDP	86.0
B.	None	4.8
	ITP	33.5
	IDP	80.8
	IMP	24.6
	Inosine	8.6
	Inosine + PEP + Mn^{2+}	8.8
	IMP + PEP + Mn^{2+}	27.0
	ITP + PEP + Mn^{2+}	35.3
	ITP + OAA + Mn^{2+}	39.7
	Mn^{2+}	6.3
	PEP	6.2
	PEP + Mn^{2+}	5.1
	Bicarbonate + Mn^{2+}	3.7

Conditions of preincubation were as in Table 7.1 with 0.01 µmoles NEM and components of the assay mixture were added as indicated. A. Mn^{2+} : 2×10^{-3} M, PEP 2×10^{-3} M, IDP: 2.5×10^{-3} M, Bicarbonate: 2×10^{-2} M, Incubation, 10 min. B. Bicarbonate: 2×10^{-3} M, all others were at 2×10^{-4} M. Incubation, 19 min.

presence of Mn^{2+} some protection was evident. If the substrates were added at concentrations approximating their $K_{m(app)}$ value, there is an absolute specificity for the nucleotide with the preferred order ITP < IDP > IMP > inosine.

The protection afforded by IDP was found to be concentration dependent. (Fig. 7.6) These data may be plotted according to the method of Scrutton and Utter (1965). These workers derived the equation

$$\frac{V_a}{V_o} = \frac{k_2}{k_1} + K_D \frac{1 - V_a/V_o}{A} \qquad \ldots (7.4)$$

where V_a and V_o represent, respectively, the psuedo first-order rate constants for inactivation in the presence and absence of A, i.e., IDP; k_1 and k_2 are the fractional-order-rate constants for inactivation of free enzyme and enzyme-IDP complex and A is the concentration of protecting agent, i.e., IDP. When the ratios (V_a/V_o), of the pseudo first-order rate constants for inactivation in the presence and absence of IDP are plotted against $(1 - V_a/V_o)/A$ the intercept represents k_2/k_1, the ratio of the fractional-order rate constants for the reaction of enzyme-IDP complex and free enzyme with the modifying agent. The slope of the line represents K_D, the dissociation constant. The data of Fig. 7.6 plotted in this manner are shown in Fig. 7.7.

The line has an intercept on the y-axis of 0.0165 $\pm$ 0.0118 such tht the frequency with which this line would pass through the origin is small. Thus, it is concluded that IDP

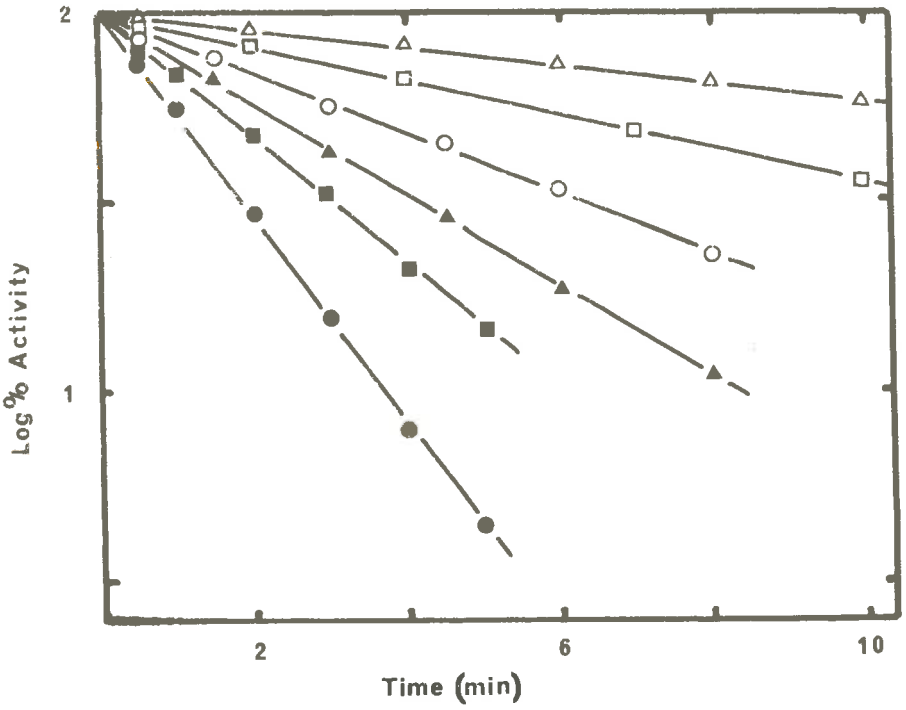


FIG. 7.6. Protection of NEM inactivation by IDP. Conditions were as in Fig. 7.1. with 0.1 μmoles of NEM and varying concentrations of IDP as indicated. The reciprocal of the half-time of inactivation was plotted against time of inactivation. The IDP concentrations were: (●——●), 0.0mM; (■——■), 0.1mM; (▲——▲), 0.04mM; (○——○), 0.02mM; (□——□), 0.01mM; (△——△), 0.005mM.

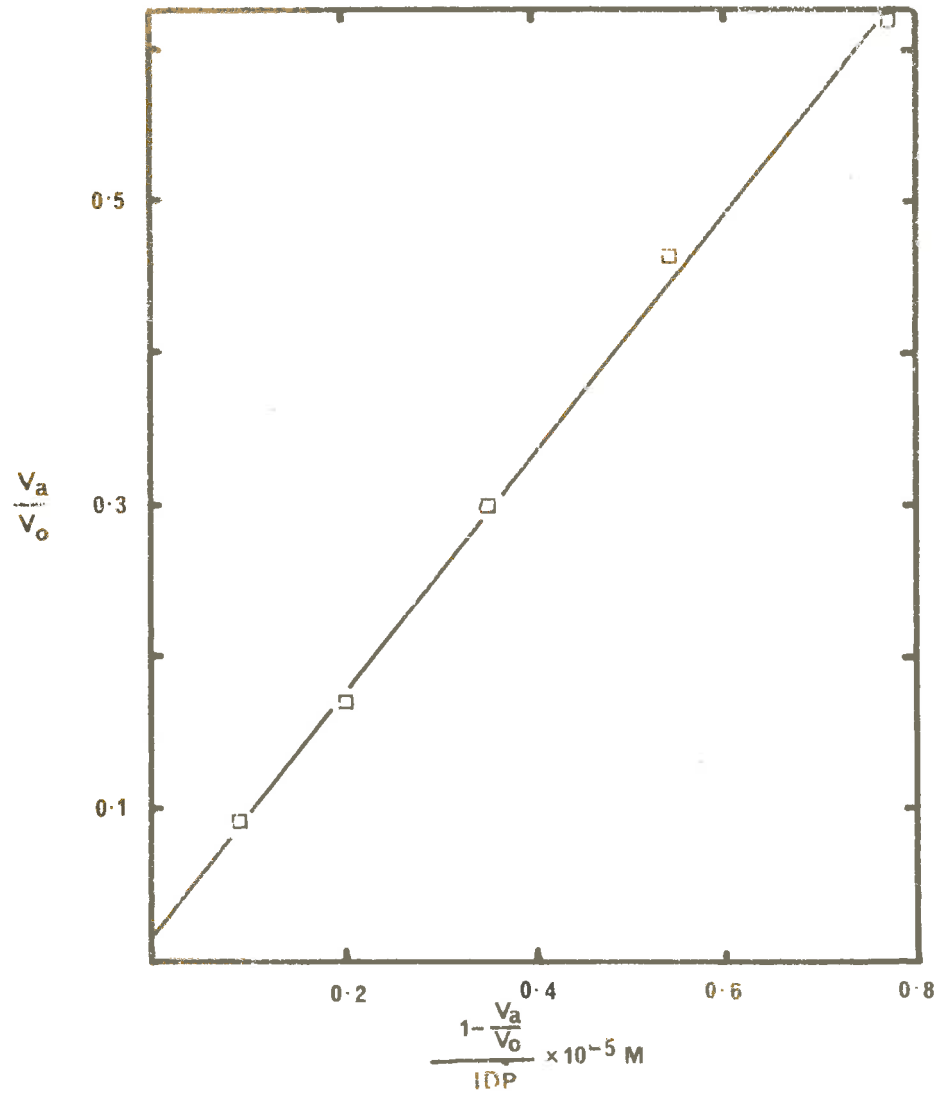


FIG. 7.7. Determination of the dissociation constant, K_D, for IDP. The rate of inactivation of the enzyme in the presence (V_a) and absence (V_o) of the protecting agent, IDP, was obtained from Fig. 7.6. and the ratio, V_a/V_o, was plotted against $(1 - V_a/V_o)/IDP$. The slope of the line gives K_D.

does not afford complete protection but that NEM is able to react with the enzyme-IDP complex although at a much reduced rate, i.e., k_2 is small compared with k_1. The dissociation constant for the enzyme-IDP complex was calculated at 8.04 x 10^{-6} M $\pm$ 0.26.

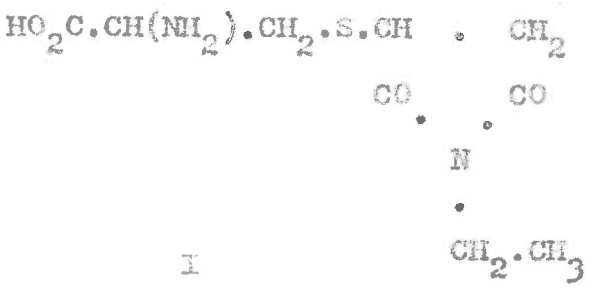
Scrutton and Utter (1965) demonstrated the complete protection by ATP against avidin inactivation of avian liver pyruvate carboxylase, while Edwards and Keech (1967) showed complete protection of pig heart propionyl-CoA carboxylase by propionyl-CoA against NEM inhibition and acetyl-CoA affords complete protection of sheep kidney pyruvate carboxylase against inactivation by DNFB (Keech and Farrant (1968)). However, O'Sullivan and Cohn (1966a) demonstrated that nucleotides did not afford complete protection to creatine kinase against iodoacetic acid inactivation.

(e) Identification of the NEM-amino acid derivative from ^{14}C-NEM-labelled enzyme

A 16 hr acid hydrolysate of ^{14}C-NEM-labelled enzyme, following pronase digestion, was subjected to electrophoretic analysis at pH 6.5 (see Section 7.2). Analysis of the electrophoretogram revealed two major radioactive bands which moved in opposite directions. In a similar experiment where the enzyme was protected against alkylation by IDP (5 x 10^{-4} M) the radioactivity of both bands was reduced correspondingly by

40% (Fig. 7.8).

Analysis of the 70 hr acid hydrolysate showed that the radioactive band which moved towards the anode increased while that band which moved slowly towards the cathode was diminished (Fig. 7.9A), indicative of the conversion of the one derivative to the other corresponding to the cleavage of the pyrrolidine ring of S-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine (I)



I

and liberation of ethylamine to give S-(1,2-dicarboxyethyl)-L-cysteine (II)

II

the fast moving component. (Smyth *et al.* (1964)). In all cases, the fast- and slow-moving components were the only bands detected where radioactivity exceeded background.

The sample lane of the electrophoretograms between 1 cm and 3 cm from the origin toward the cathode and between 17 cm and 22 cm from the origin towards the anode was eluted with glass-distilled water, concentrated by freeze-drying and the two samples were subjected to electrophoretic analysis at

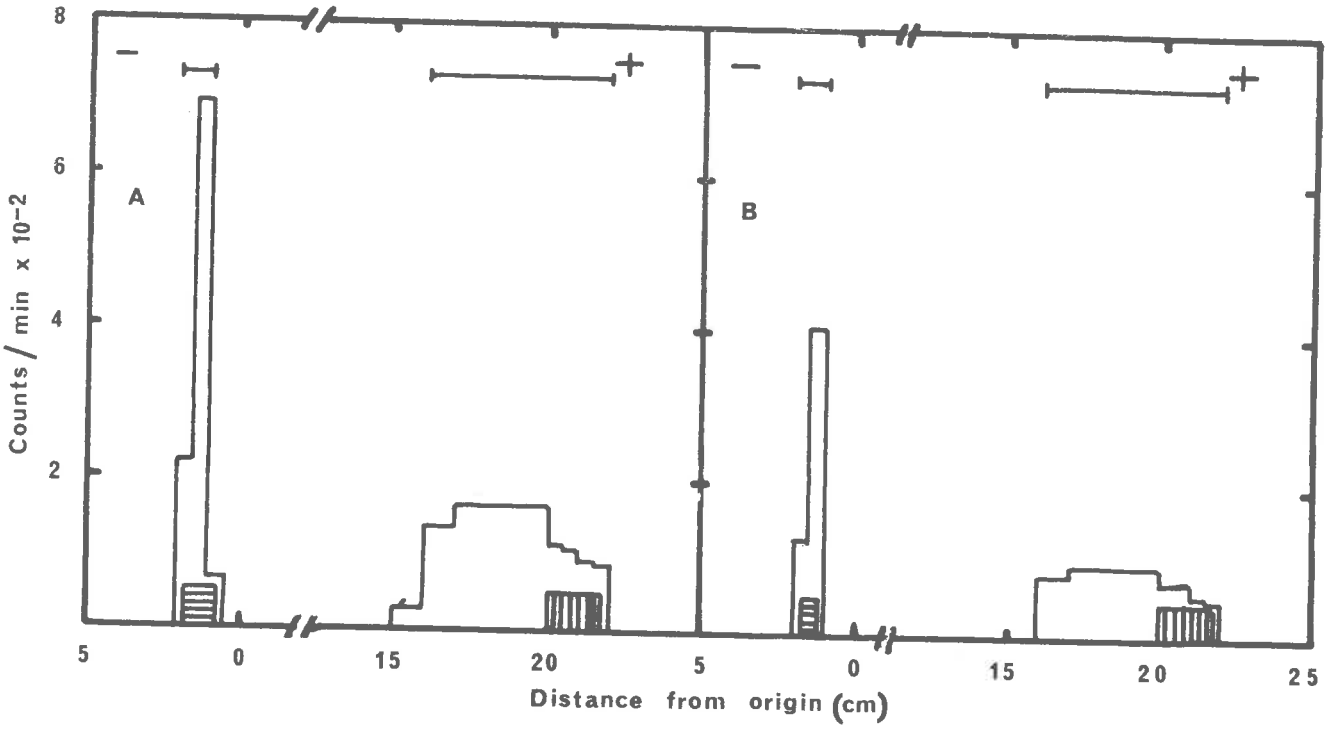


FIG. 7.8. Electrophoresis of 16 hr acid digest of ^{14}C-NEM-labelled enzyme. The labelled enzyme after 16 hr acid hydrolysis was subjected to electrophoresis at pH 6.5 (Section 7.2.(a)). The figure represents the radioactivity profile obtained. (A) Absence of any protecting agent; (B) 0.5 μmoles of IDP added during the labelling process. ▤, S-(1-ethyl-2,5-dioxopyrrolidin-3-yl)-L-cysteine; ▥, S-(1,2-dicarboxyethyl)-L-cysteine.

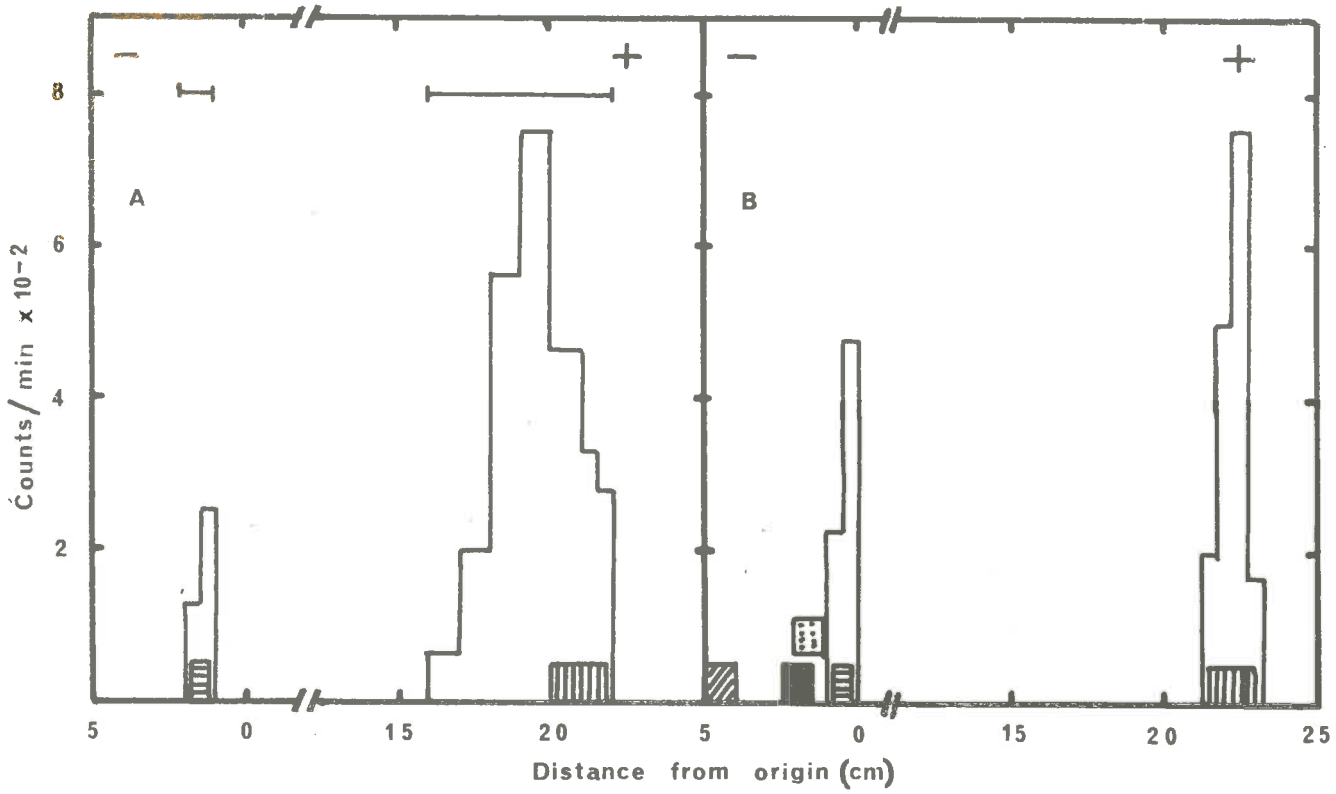


FIG. 7.9A. Electrophoresis of 70 hr acid digest of ^{14}C-NEM-labelled enzyme. The labelled enzyme was subjected to electrophoresis at pH 6.5 (Section 7.2.(a)) after 70 hr acid hydrolysis. ▤ , Compound I; ▥ , Compound II.

FIG. 7.9B. Identification of the residue reactive towards NEM. The sample lane of the electrophoretograms of Fig. 7.8 A and B between 1 and 3 cm from the origin toward the cathode and between 17 and 22 cm from the origin toward the anode were eluted and electrophoresed as detailed in the text. ▤ , Compound I; ▥ , Compound II; ⁘ , NEM-L-histidine; ▨ , α-NEM-L-lysine; ■ , ε-NEM-L-lysine.

pH 6.5. Compound I was included as an internal and external standard with the slow moving component while Compound II was included as an internal and external standard with the fast-moving component. The NEM derivatives with L-histidine and L-lysine were included as external standards. Analysis of the electrophoretograms showed each sample was homogeneous with respect to radioactivity which co-electrophoresed with Compound I in the former case and Compound II in the latter case (Fig. 7.9B).

(f) Methylation of the reactive sulphydryl group

The question which arises is whether the integrity of the sulphydryl group is essential for enzymic activity. Alkylation of the sulphydryl group by NEM causes complete loss of activity. However, this could be the result of (a) structural changes in the enzyme due to chemical modification, (b) steric considerations such that the approach of the sulphur atom toward the appropriate bond in the substrate is prevented, or (c) the electrostatic properties of the sulphur are sufficiently altered as to leave the residue catalytically inert. Therefore, methylation of the enzyme was attempted using methyliodide as the methylating agent. Modification by this reagent was inhibited by IDP suggesting that methylation was occurring at the same sulphydryl group as was alkylated by NEM (Fig. 7.10).

NEM will react only with the free sulphydryl group and not with its methylated derivative such that if NEM were added

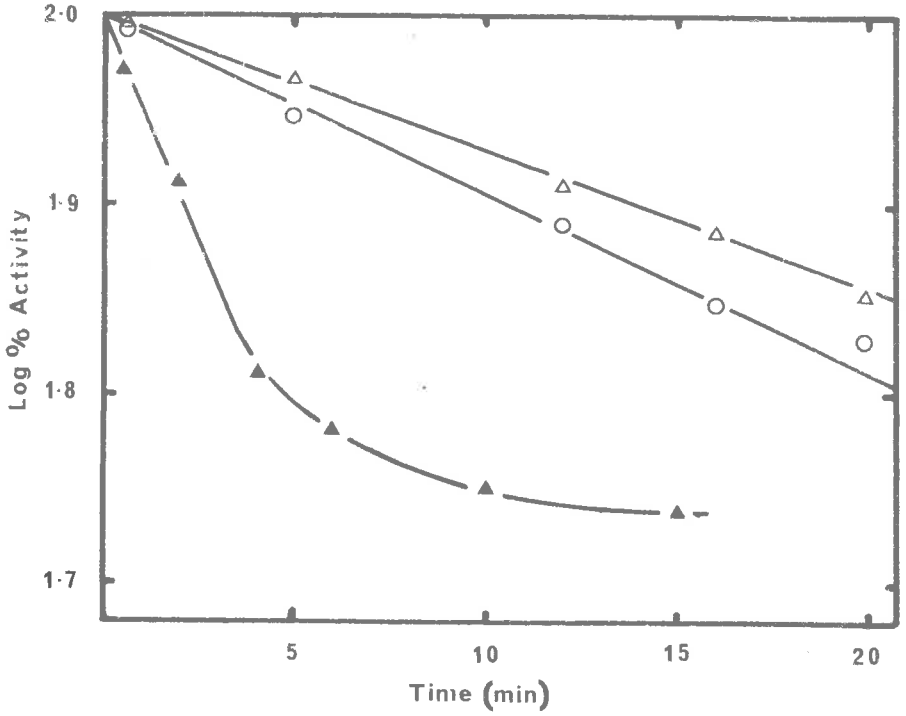


FIG. 7.10. Protection by IDP against modification by methyliodide. Enzyme (0.3 units) were incubated at 30° in 0.2 ml of 5 x 10^{-2}M tris (Cl^-), pH 8.0, containing 2.5 μmoles methyliodide. Varying amounts of IDP were added as indicated. Aliquots of 0.025 ml were diluted with 0.05 ml of 1% (w/v) B.S.A. containing 10^{-1}M dithiothreitol, pH 7.0, and analysed for residual activity (Section 2.7.(i)). Log_{10} % activity was plotted against time. The concentrations of IDP were: (▲——▲), 0.0mM; (Δ——Δ), 0.5mM; (O——O), 0.1mM.

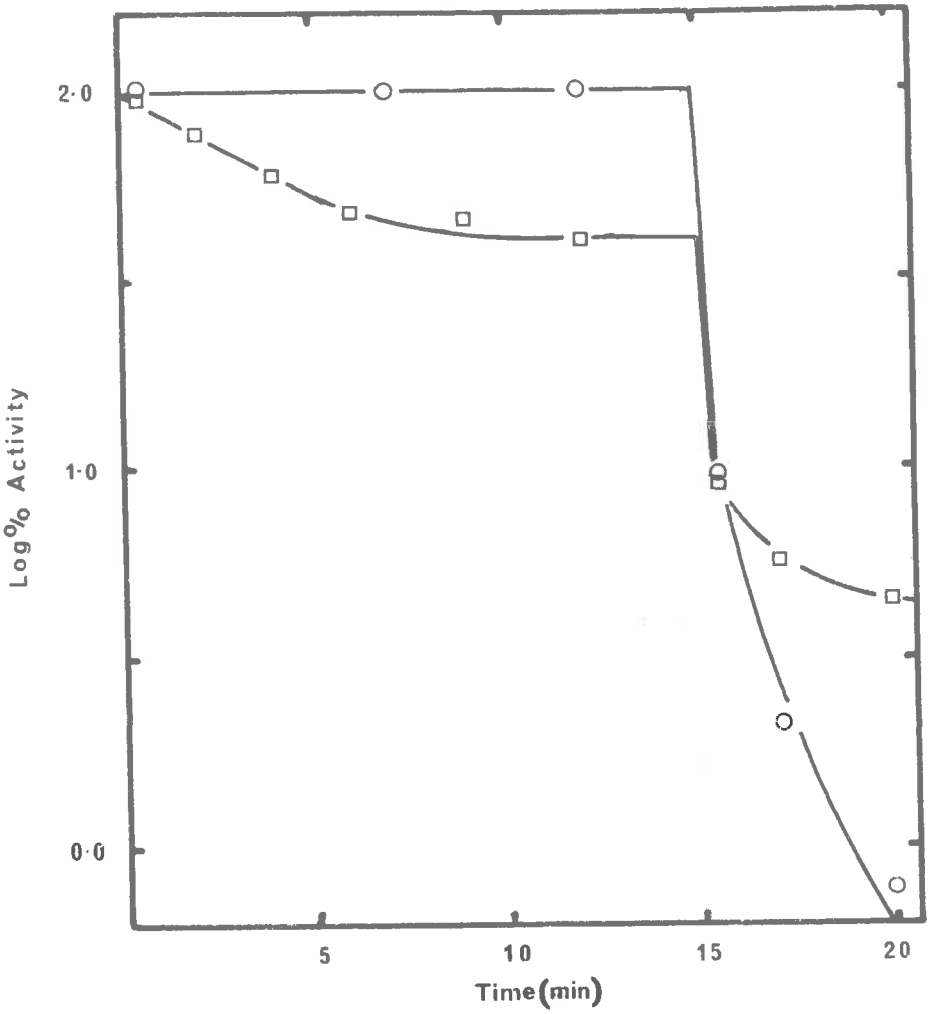


FIG. 7.11. Activity of enzyme modified by methyliodide. Conditions were as in Fig. 7.10. with 10 μmoles of methyliodide. After 15 min incubation, 0.25 μmoles of NEM were added and the incubation was continued at 20°. Log_{10} % activity was plotted against time. (O——O), no methyliodide added; (□——□), in the presence of methyliodide.

after methyliodide modification those enzyme molecules containing the unmodified sulphydryl group would be inactivated by reaction with NEM. Thus, if there were any residual activity after such treatment, it must be due to those enzyme molecules containing the methylated sulphydryl residue. Fig. 7.11 presents the results of such an experiment and does indicate a low residual activity of the methylated enzyme.

(g) Effect of chemical modification on the active centre

Some insight into the role of a particular amino acid residue may be obtained by investigating the kinetics of an enzyme after modification of this group. Such an approach was used by Knowles (1965) to show the involvement of methionine-192 in α-chymotrypsin with the binding of aromatic substrates, while with the aliphatic substrates this group was essentially non-functional.

This approach has been used in the present investigation. Lineweaver and Burk (1934) plots were constructed for two stages of modification (Figs. 7.12 - 14). A series of parallel lines was obtained with decreases in both $K_{m(app)}$ and V_{max} when PEP and IDP were the variable substrates. With HCO_3^- as the variable substrates, no clear-cut result was obtained, but rather a non-competitive type of pattern was approached for the higher degree of modification, while at intermediate degrees of modificatinn, a mixed pattern is

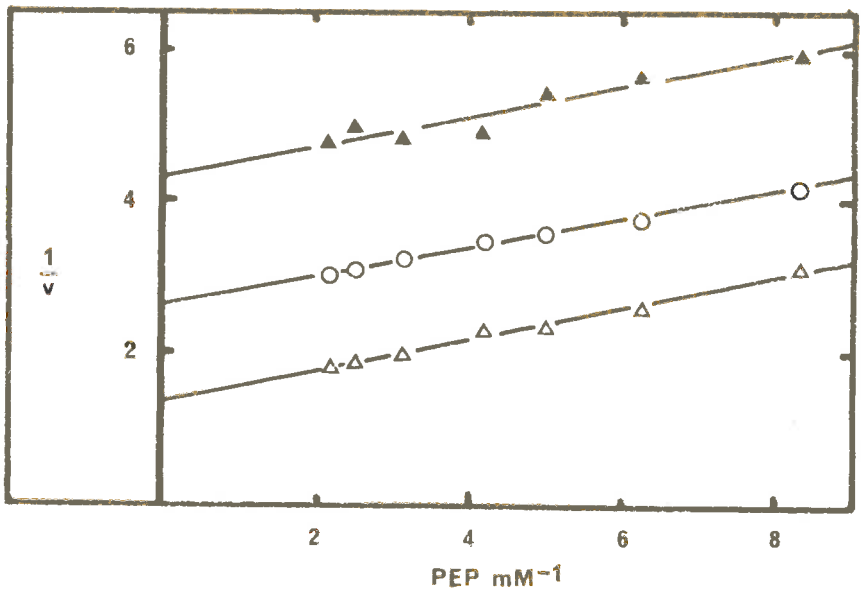


FIG. 7.12. Kinetic constants for PEP in the modified enzyme. Enzyme was incubated in a solution (0.8 ml) containing 40 μmoles of phosphate (K^+), pH 7.0, 0.2 μmoles of NEM and 1.2 units of enzyme. Aliquots of 0.2 ml were removed at 0, 3 and 7 min and diluted with 0.4 ml of 1% (w/v) B.S.A. containing 10^{-1} M GSH. The modified enzyme was assayed using varying levels of PEP. Modification times were: (Δ——Δ), 0 min; (O——O), 3 min; (▲——▲), 7 min.

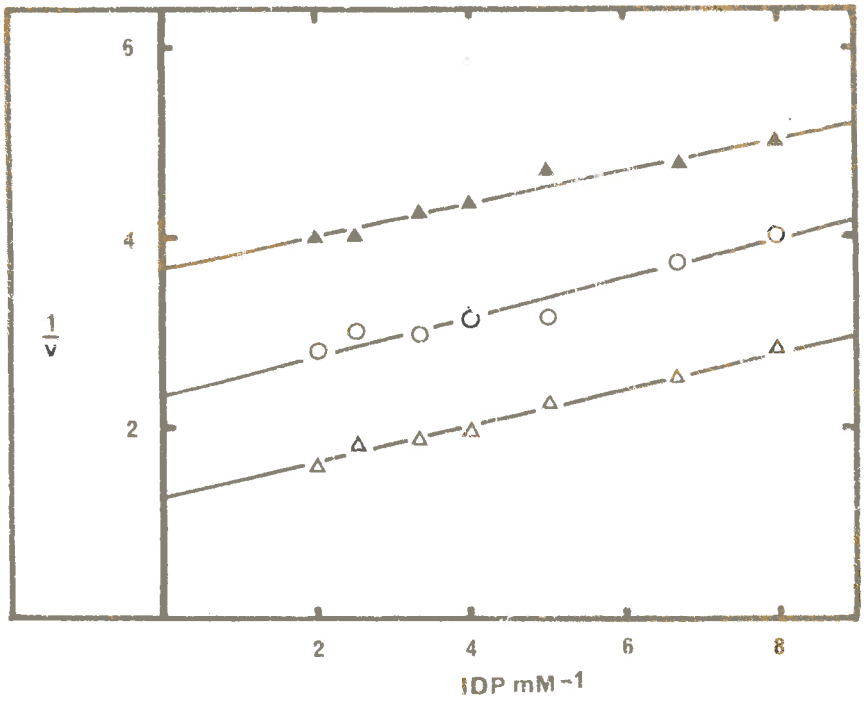


FIG. 7.13. Kinetic constants for IDP in the modified enzyme. The enzyme was modified as in Fig. 7.12. and assayed using varying levels of IDP. Modification times were: (△——△), 0 min; (○——○), 3 min; (▲——▲), 7 min.

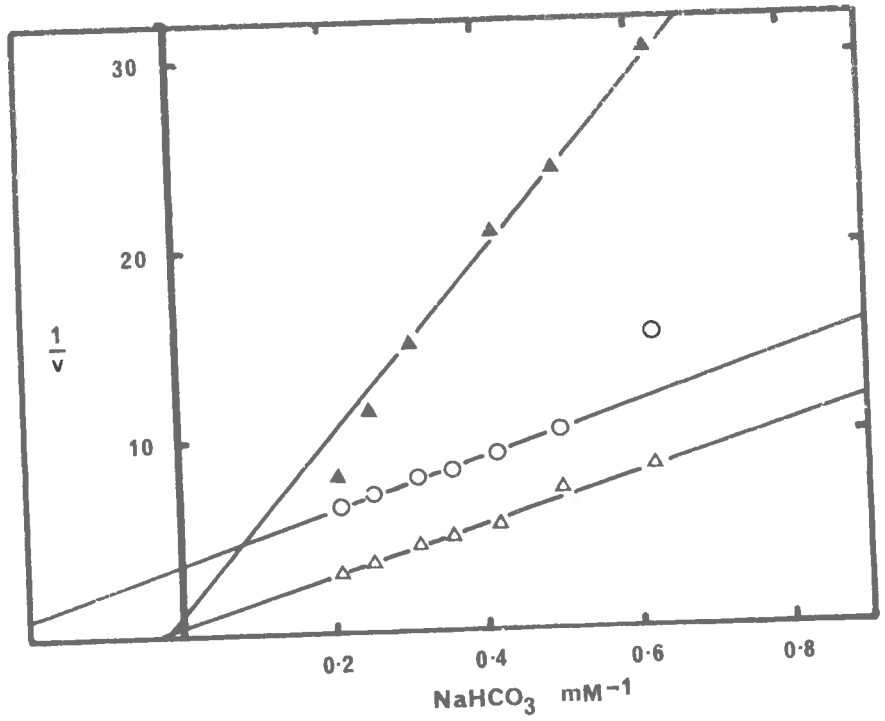


FIG. 7.14. Kinetic constants for $NaHCO_3$ in the modified enzyme. Enzyme was modified as in Fig. 7.12. and assayed with varying $NaHCO_3$ levels. Modification times were: (△——△), 0 min; (○——○), 3 min; (▲——▲), 7 min.

obtained between non-competitive and uncompetitive inhibition. It is apparent that the loss of activity resulting from the alkylation of the sulphydryl group is not due to unsaturation of PEP, IDP or HCO_3^-.

An alternative approach to this question was attempted whereby the rate of inactivation by NEM was assessed by assaying the residual activity six ways, viz., carboxylation, and the CO_2:OAA exchange reaction in the presence of (i) Mn^{2+}, (ii) ITP and Mn^{2+}, (iii) IDP and Mn^{2+}, (iv) ITP and Mg^{2+}, (v) IDP and Mg^{2+}. This approach would differentiate between effects on the carboxylation and phosphoryl transfer processes as well as detecting any specificity changes with respect to metal ion and nucleotide. Table 7.3 shows that the rate of inactivation was the same in all cases.

A third approach to this question is provided by the use of N.M.R. (see Chapter 4) as the effects of chemical modification by NEM may be monitored by the enhancements ($\epsilon *$) of the PRR of the binary enzyme.Mn^{2+} and ternary enzyme.Mn^{2+}.substrate complexes. Here the binding properties of the enzyme are examined directly without complicating the issue with additional possible catalytic changes as in the other two approaches. This method has been used for creatine kinase (O'Sullivan and Cohn, 1966a) which can be inactivated by quantitative reaction of two sulphydryl residues with iodoacetic acid. This inactivation is protected by ADP and ATP

TABLE 7.2: EFFECT OF NEM MODIFICATION OF THE ACTIVE SITE

Method of assay	Half-time of Inactivation (min.)
Carboxylation	5.1
CO_2:OAA Exchange (Mn^{2+}/no nucleotide)	5.04
CO_2:OAA Exchange (Mn^{2+}/ITP)	5.22
CO_2:OAA Exchange (Mn^{2+}/IDP)	5.15
CO_2:OAA Exchange (Mg^{2+}/ITP)	5.17
CO_2:OAA Exchange (Mg^{2+}/IDP)	5.15

The enzyme was preincubated in a solution (final volume 0.4 ml) containing 20 μmoles N-ethylmorpholine (Cl^-), pH 7.5, 0.35 units of enzyme and 0.04 μmoles NEM. Incubation was at 25°. At various times, 0.05 ml aliquots were diluted with 0.1 ml of 1% (w/v) B.S.A. containing 10^{-1}M GSH, pH 7.5 and assayed for residual activity by the methods listed above. Half-times of inactivation were obtained from semi-log plots of residual activity against time. The carboxylation assay was standard as listed in Section 2.7(i). The exchange assay (total volume 0.5 ml) contained (in μmoles): imidazole (Cl^-), pH 6.5, 50.0; Mn^{2+} or Mg^{2+}, 2.0; nucleotide (if present), 1.0; OAA, 1.0; $NaH^{14}CO_3$, 20.0; GSH, 0.8; and incubation was for 4 min. Processing of all assays was as described in Section 2.7(ii).

although the nucleotide is not bound through this thiol. The inactivated enzyme bound $Mn.ADP^-$ with the same dissociation constant as the native enzyme and the same PRR enhancement factor ($\in_t$). This confirmed that the nucleotide in no way bound through the sulphydryl residues but afforded protection against inactivation by inducing a conformational change on binding to the enzyme.

Modification of PEP carboxykinase was achieved with 0.15 mM NEM and 0.076 mM enzyme at pH 7.5 and 20°. Under these conditions, the half-time of inactivation is about 10 min. The reaction was allowed to proceed for two half-times. Beyond this time, precipitation of protein began to occur. Figure 7.15 shows the results of this approach.

There was essentially no alteration in Mn^{2+} binding except after prolonged incubation periods. In Figure 7.15A the reaction was allowed to proceed for too long a period such that the observed enhancement ($\in *$) of the binary enzyme. Mn^{2+} complex was diminished. The PEP effect on $\in *$ was also diminished presumably for the same reason. However, the significant result was the complete abolition of the IDP effect on $\in *$ indicating that either IDP could no longer bind or a conformational change induced by IDP and responsible for the observed change in $\in *$ could no longer occur on IDP binding. Figure 7.15B confirms that the primary effect of NEM modification is not the inhibition of Mn^{2+} or PEP binding as reaction of the enzyme with NEM for two half-times showed

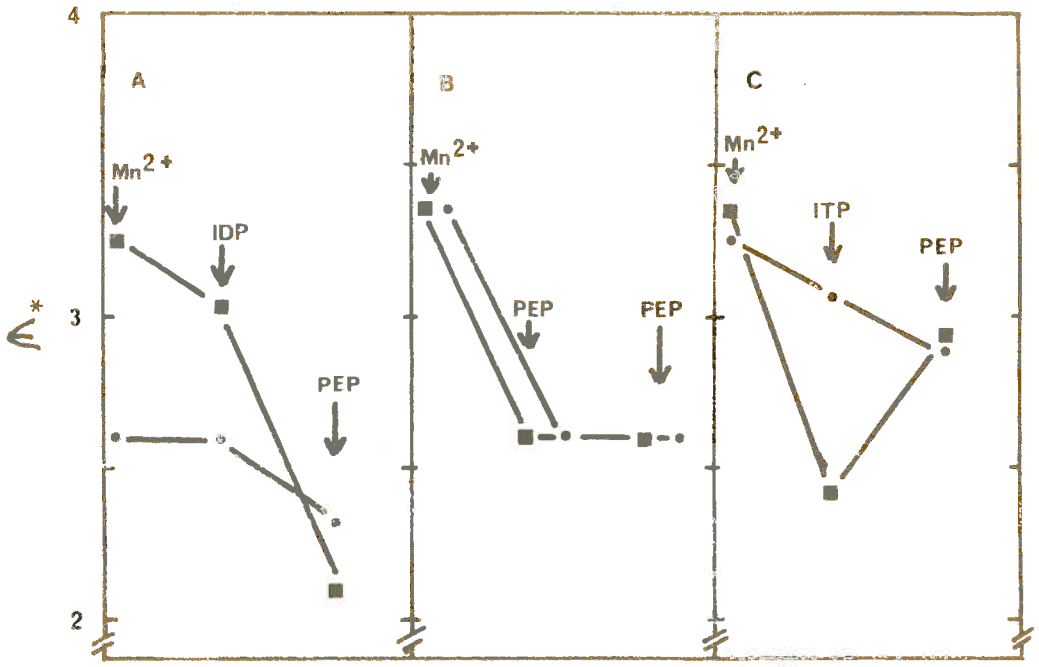


FIG. 7.15. Effect of modification on substrate binding. Enzyme (0.076mM) was incubated at 20° with 0.15mM NEM and 0.07M N-ethylmorpholine (Cl^-), pH 7.5, for two half-times. The enhancement (ϵ^*) of the PRR was estimated in the presence of substrates (see Chapter 4 for methods). Final concentrations of substrates were 0.12mM Mn^{2+}, 0.095mM ITP, 0.09mM PEP (first addition) and 0.55mM (second addition) and 0.06mM IDP. (■——■), control; (●——●), after modification.

no diminution of the effects of Mn^{2+} and PEP on $\in$*.

Figure 7.150 confirms that the nucleotide (ITP) effect on $\in$* is essentially abolished after reaction for two half-times again without diminution of the PEP effect.

The N.M.R. studies could not differentiate between the inhibition of nucleotide binding and the abolition of a nucleotide-induced conformational change. This question was readily resolved by examining the ability of the NEM-inactivated enzyme to bind nucleotide. Modification was achieved with 0.25 mM NEM and 0.15 mM enzyme (volume 0.8 ml) at pH7.5 and 22°. Under these conditions the half-time of inactivation was about 4 min. The reaction was allowed to proceed for 2.5 half-times when the reaction was stopped by the addition of 2μmoles of GSH, pH 7.5. For the control sample, GSH was added before the addition of NEM. The ability of both enzyme samples to bind GTP in the presence of Mn^{2+} (5×10^{-5} M) was examined using the method described in Section 5.2(b) except that 0.05 M tris (Cl^-), pH 7.5, was used. α-^{32}P-GTP was added to the enzyme solution to a concentration of 2×10^{-5} M and the ability of the enzyme to bind GTP was assessed qualitatively by the increase in α-^{32}P-GTP in the dialysate after the addition of 5 μmoles of cold GTP. It was found that the ability of the enzyme to bind GTP was essentially abolished after modification.

(h) Number of sulphydryl residues interacting with IDP

The number of sulphydryl groups interacting with IDP was estimated using Ellman's (1959) reagent. The reaction with

5,5'-dithiobis(2-nitrobenzoic acid) was followed spectrophotometrically at 412 mμ at pH 7.0 and 20°. The initial rapid increase in absorbance, corresponding to two sulphydryl groups per mole of enzyme, was followed by a further increase caused largely by turbidity. In the presence of 2.5 mM IDP, there was a decrease in the rate of reaction with what would appear to be the more reactive of the two groups initially modified (Fig. 7.16).

7.3.D MODIFICATION STUDIES USING DNFB

Preliminary studies had shown that PEP carboxykinase was susceptible to inactivation by DNFB and TNBS. Although DNFB was used routinely in the investigations reported in this section, analysis of the kinetics of the inactivation by both reagents indicated that the reaction with the enzyme was the same in both cases.

(a) Order of inactivation with respect to time and DNFB concentration

Plots of log percentage of PEP carboxykinase activity as a function of time of DNFB inhibition were linear to the loss of at least 80% of the initial activity (Fig. 7.17) indicating that the inactivation process approximated first-order kinetics with respect to time at certain fixed concentrations of inhibitor. The same data when plotted as the rate of inactiva-

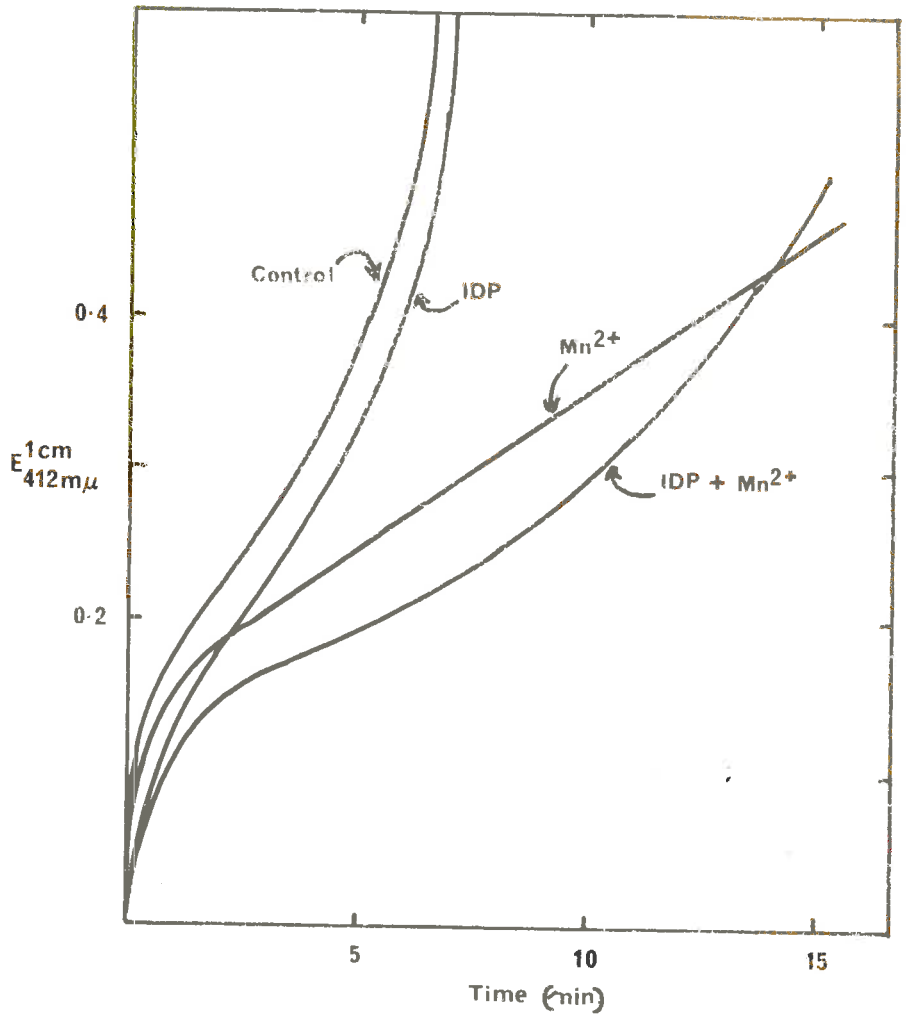


FIG. 7.16. The number of sulphydryl groups interacting with IDP and Mn^{2+}. Enzyme (1.03×10^{-5}M) was incubated at 20° with 1×10^{-4}M 5,5'-dithiobis(2-nitrobenzoic acid) in 4×10^{-2}M N-ethylmorpholine (Cl^-), pH 7.0. The reaction was followed spectrophotometrically at 412 mμ on a Unicam SP800 recording spectrophotometer. Under these conditions, an absorbance change of 0.14 units corresponds to 1 sulphydryl residue per mole of enzyme. Final concentrations of IDP and Mn^{2+} were 2.5mM and 2.0mM respectively except where both IDP and Mn^{2+} were present and the latter then was 4.0mM.

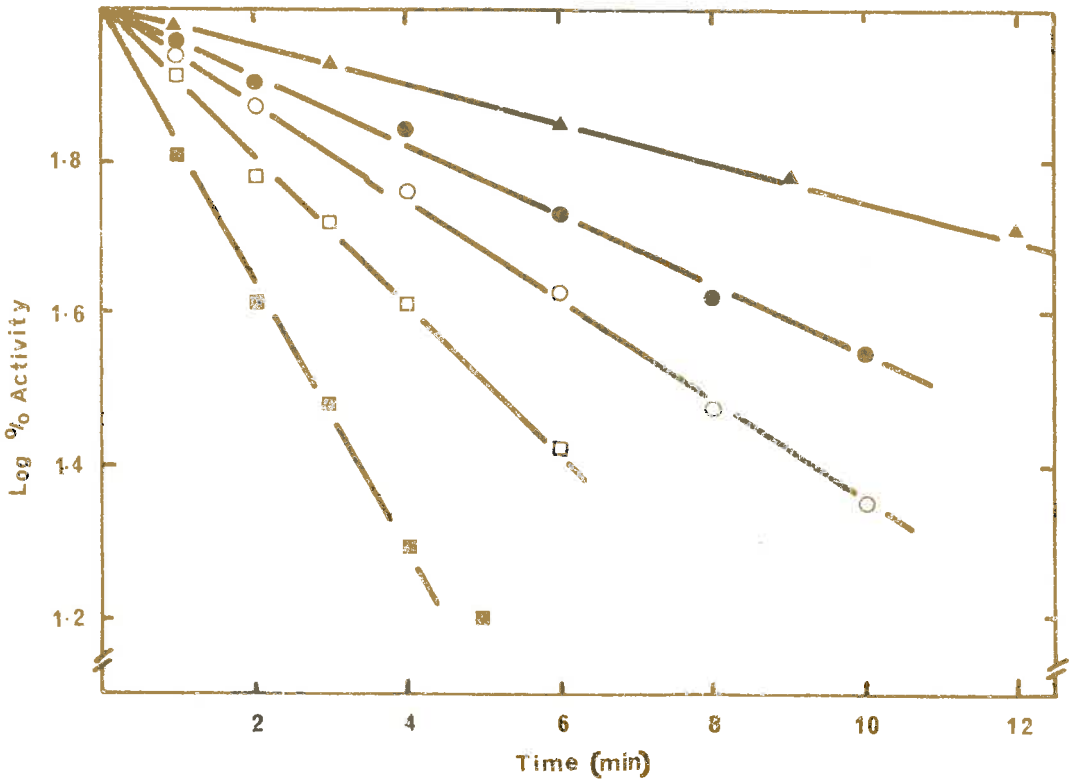


FIG. 7.17. The rate of inactivation of PEP carboxykinase with varying DNFB concentrations. Enzyme was incubated at 25° in a solution (0.4 ml) containing 20 μmoles of N-ethylmorpholine (Cl^-), pH 7.5, 0.4 units of enzyme and DNFB as indicated. Aliquots of 0.05 ml were diluted with 0.325 ml of 1% (w/v) B.S.A. containing 10^{-1}M lysine and 10^{-4}M GSH, pH 7.5, and analysed for residual activity as in Section 2.7.(i). Log_{10} % activity was plotted against time. DNFB concentrations were: (■——■), 0.5mM; (□——□), 0.3mM; (○——○), 0.2mM; (●——●), 0.15mM; (▲——▲), 0.1mM.

tion (expressed as the slope of the primary plot, k') against inhibitor concentration showed that the inactivation process was pseudo first-order with respect to inhibitor concentration (Fig. 7.18). A replot of the data of Fig. 7.17 according to Equation 7.3 gave a straight line of slope 1.25 (Fig. 7.19). Thus, more than one molecule of DNFB was reacting per catalytic unit of the enzyme to cause inactivation suggesting that more than one amino acid residue was being modified by DNFB. A similar experiment with TNBS gave a slope of 1.2 for the double log plot.

(b) Effect of pH on the rate of inactivation by DNFB

It was found that DNFB inactivation was markedly dependent on the H^+ ion concentration. The results of this investigation are shown in Figure 7.20, and it is immediately noted the similarity between this plot and that shown in Figure 7.4 for NEM modification. Thus, the pK_a of the group reactive towards DNFB would be around 8.0, the same as for the sulphydryl group reactive towards NEM. A replot of the data of Figure 7.20 in terms of log rate of inactivation (k') against pH gives a straight line region over the pH range 6.5 - 8.0 with a slope of 1.3 (Fig. 7.21) thus confirming the data of Figure 7.19 and indicating that more than one ionisable group was reacting with DNFB to cause inactivation.

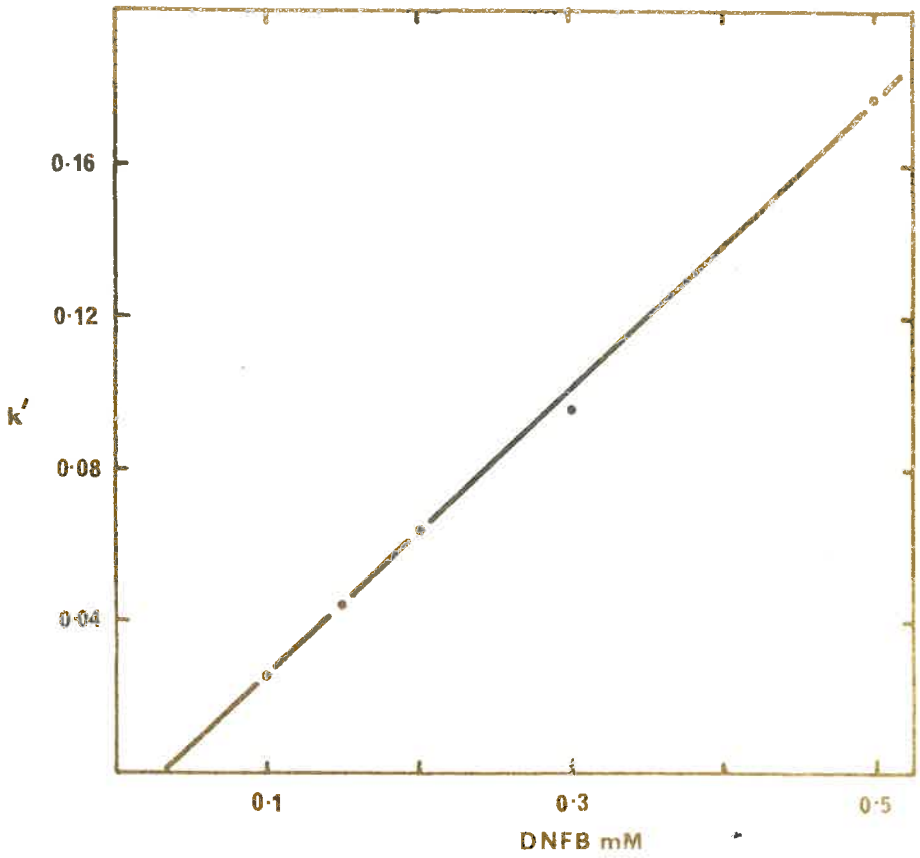


FIG. 7.18. The pseudo first-order kinetics of inactivation with respect to DNFB. The rate of the inactivation, expressed as the slope of the primary plot, k', in Fig. 7.17. was plotted against DNFB concentration.

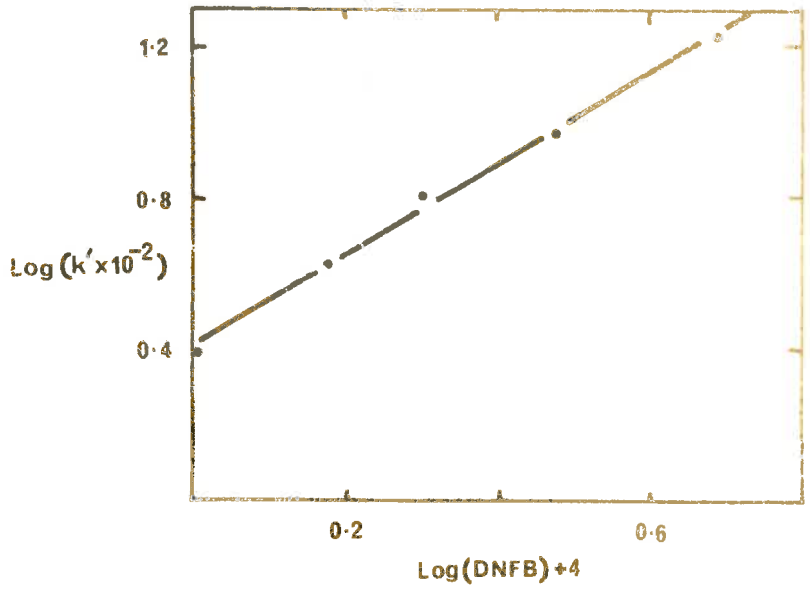


FIG. 7.19. The data of Fig. 7.17. were replotted as $\log_{10}$ k' against $\log_{10}$ DNFB concentration.

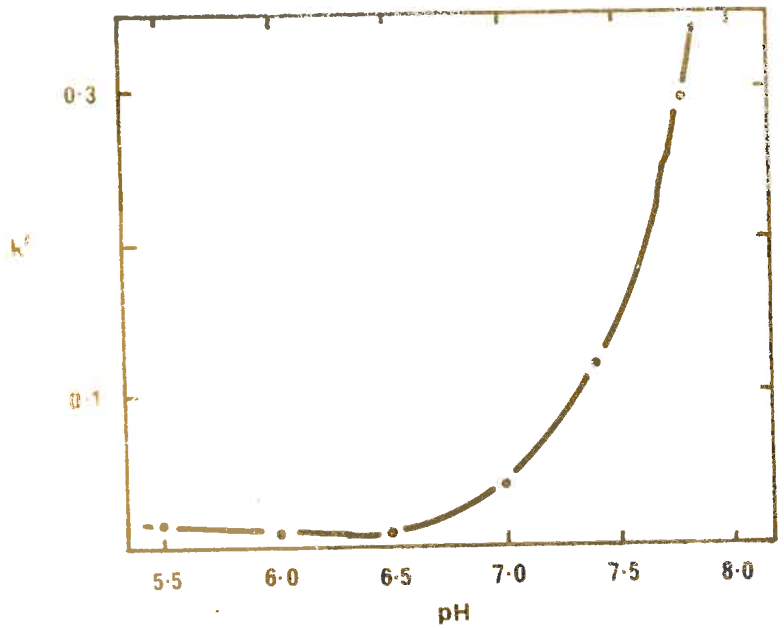


FIG. 7.20. Effect of pH on the rate of inactivation by DNFB. Conditions were as in Fig. 7.17. using 10^{-1}M N-ethylmorpholine.phosphate buffer (pH 5.5 - 7.3) and 0.1 μmoles DNFB. The rate of inactivation (k') was plotted against pH.

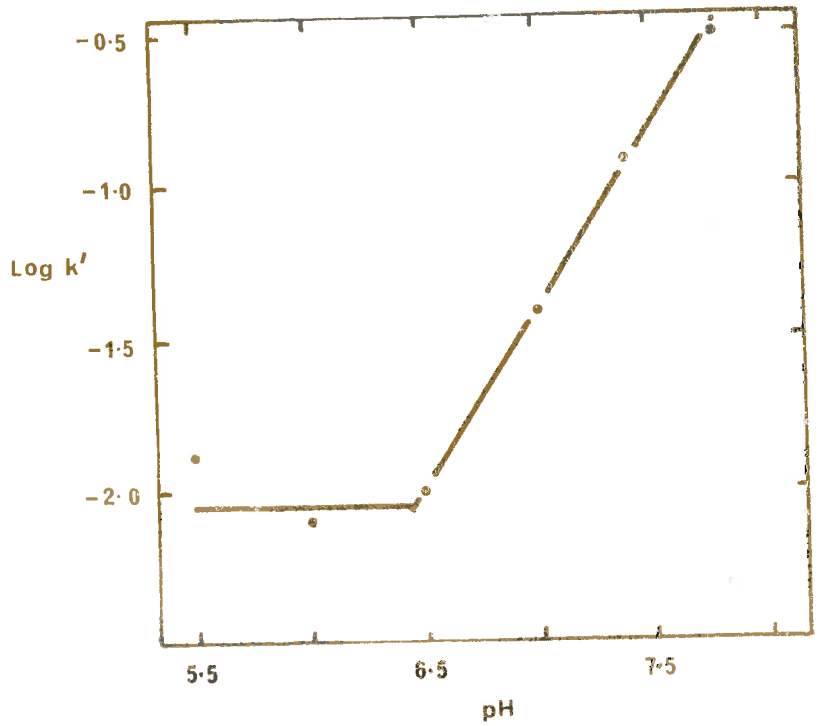


FIG. 7.21. The number of ionising groups reacting with DNFB. The data of Fig. 7.20. were replotted as $\log_{10}$ k' against pH. The slope of the line gives the number of ionising groups reacting with DNFB.

(c) Protection by substrates against DNFB inactivation

The ability of various combinations of substrates to protect against DNFB inactivation was tested (Table 7.4). These results indicated that both IDP (protects against NEM inactivation) and Mn^{2+} separately afforded protection, although, with Mn^{2+}, this protection was almost complete suggesting that a group different from that susceptible to NEM was being modified preferentially by DNFB. It would appear that IDP afforded protection against the NEM-susceptible group which was undergoing slow reaction with DNFB. Mg^{2+} was completely ineffective in replacing Mn^{2+} despite the fact that Mg^{2+} does activate the enzyme at the pH used here (pH7.5).

The protection afforded by Mn^{2+} was found to be concentration dependent (Fig. 7.22). However, attempts to treat this data according to the method of Scrutton and Utter (1965) (Eqn. 7.3) were unsuccessful as non-linear plots were obtained (Fig. 7.23). Non-linearity would be expected to be introduced into this plot if modification also occurred at a residue other than that protected by the component under investigation.

(d) Identification of the DNP-amino acid derivative from ^{14}C-DNFB-labelled enzyme

A 32-hr acid hydrolysate of the ^{14}C-DNFB-labelled enzyme was subjected to chromatographic analysis (Section 7.2(b)). One major radioactive band corresponding to S-DNP-cysteine was

TABLE 7.1: PROTECTION OF ENZYME ACTIVITY BY REACTION MIXTURE COMPONENTS

Additions	% Original Activity
None	34.5
Mn^{2+}	83.7
PEP	35.8
IDP	56.6
$NaHCO_3$	28.0
Mn^{2+} + PEP	82.0
Mn^{2+} + IDP	83.2
Mn^{2+} + $NaHCO_3$	79.0
Mg^{2+}	34.5
Mg^{2+} + PEP	32.8
Mg^{2+} + IDP	61.0
Mg^{2+} + $NaHCO_3$	33.0
Mn^{2+} + Mg^{2+}	72.8

The enzyme was preincubated at 20° in a solution (final volume 0.2 ml) containing 10.0 µmoles N-ethylmorpholine (Cl^-), pH 7.5, 0.2 units enzyme, 0.06 µmoles DNFB and substrates, as indicated, at 2×10^{-4} M except $NaHCO_3$ which was at 2×10^{-3} M. Aliquots of 0.025 ml were withdrawn, diluted with 0.05 ml of 1% (w/v) B.S.A. containing 10^{-1} M lysine and 10^{-4} M GSH, pH 7.5, and analysed for residual activity.

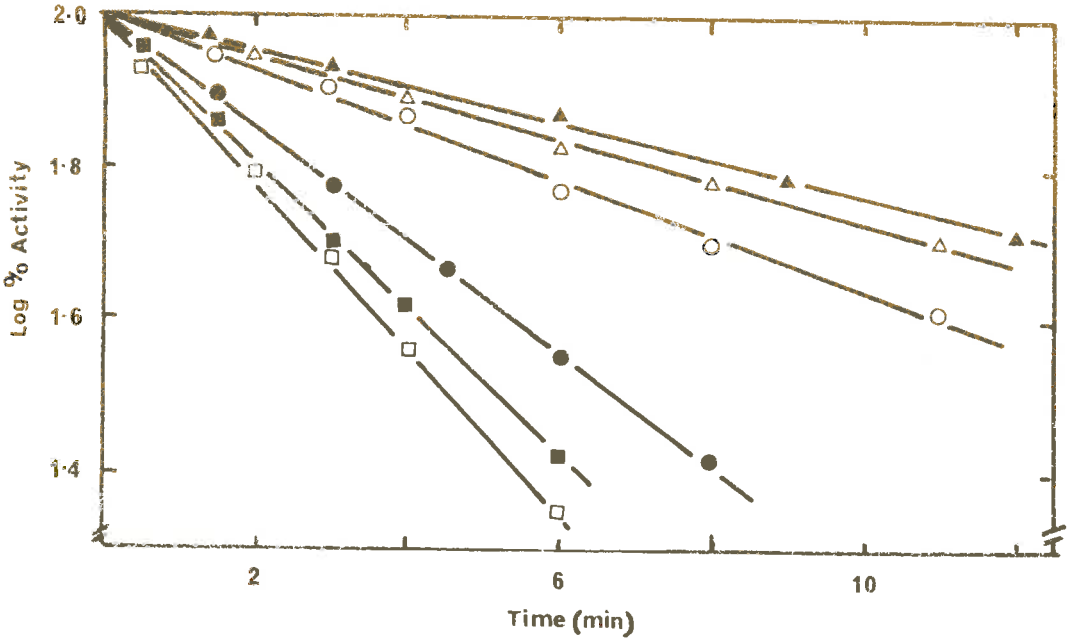


FIG. 7.22. Protection of DNFB inactivation by Mn^{2+}. Conditions were as in Fig. 7.17. with 0.12 μmoles DNFB and varying Mn^{2+} levels as indicated. Log_{10} % activity was plotted against time. Mn^{2+} concentrations were: (□——□), 0.0mM; (■——■), 0.0125mM; (●——●), 0.025mM; (○——○), 0.05mM; (△——△), 0.1mM; (▲——▲), 0.2mM.

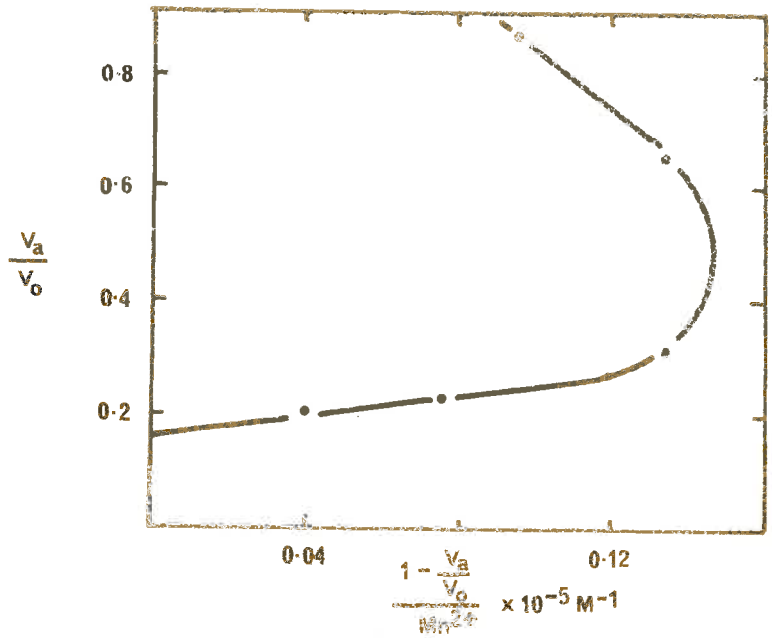
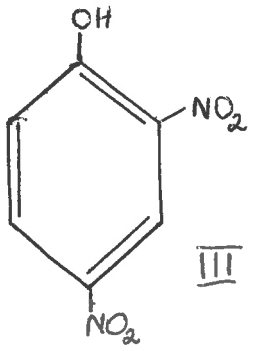


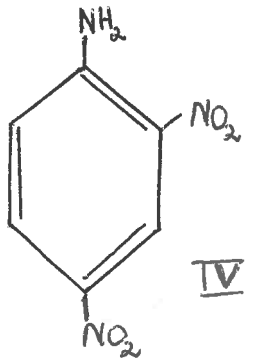
FIG. 7.23. The rate of inactivation in the presence (V_a) and absence (V_o) of Mn^{2+} was obtained from Fig. 7.22. and the ratio V_a/V_o was plotted against $(1 - V_a/V_o)/Mn^{2+}$.

revealed in the acid solvent along with a slower moving compound corresponding to im-DNP-histidine (Fig. 7.24A). A similar result was also obtained with the alkaline solvent. In similar experiments, where the enzyme was protected by 0.5 mM Mn^{2+} or 0.5 mM IDP, the radioactivity was uniformly reduced by 45% and 40% respectively (Fig. 7.24B).

Elution of the sample lane of the chromatogram of Fig. 7.24A from 14 to 22 cm from the origin with glass-distilled water and rechromatography in the same solvent showed that the DNP-amino acid had apparently undergone decomposition as indicated by the appearance of a radioactive band moving almost with the solvent front. It is well known that DNP-amino acids are light-sensitive and decompose to a substituted phenol or aniline, e.g., 2,4-dinitrophenol (III) or 2,4-dinitroaniline (IV). However, if the dinitrophenyl group is bound through a sulphur atom as in S-DNP-cysteine, the decomposition product would be expected to be 2,4-dinitrothiophenol (V). Chromatography in both acid and alkaline solvents confirmed that the decomposition product was 2,4-dinitrothiophenol (Fig. 7.25). Thus, cysteine residues were preferentially labelled by DNFB with a minor reaction at a histidine residue.



III



IV

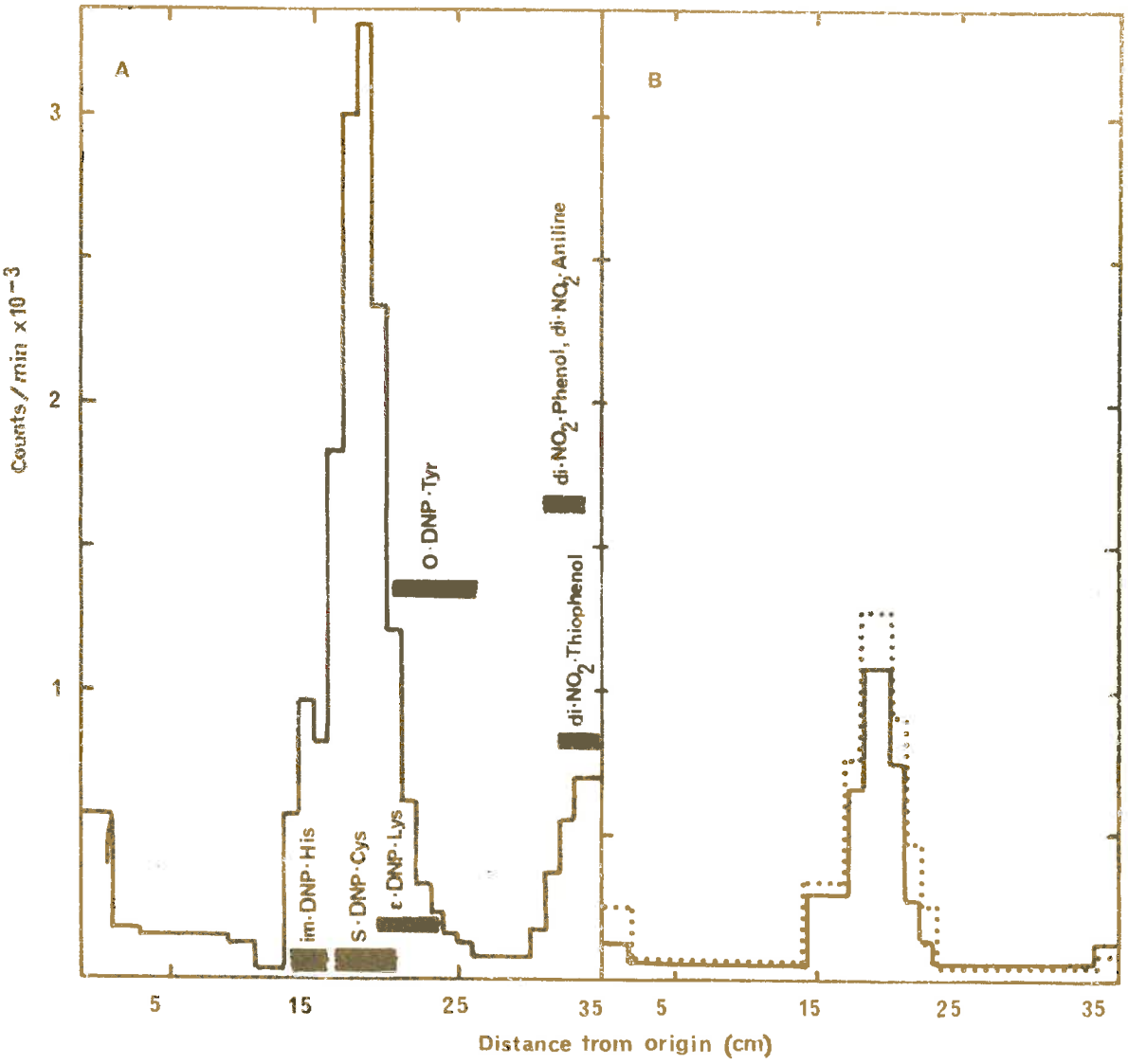


FIG. 7.24. Chromatography of a 32 hr acid digest of ^{14}C-DNFB-labelled enzyme. The labelled enzyme after 32 hr acid hydrolysis was subjected to chromatography in 1-butanol/acetic acid/water (4/1/1, v/v/v) on Whatman No 4 paper for 4.5 hr and analysed for radioactivity as described in Section 7.2.(ii). The standard compounds were detected as dark spots under U.V. light. (A) absence of any protecting agent; (B) 0.5mM $MnCl_2$ (————) or 0.5mM IDP (.....) added during the labelling process.

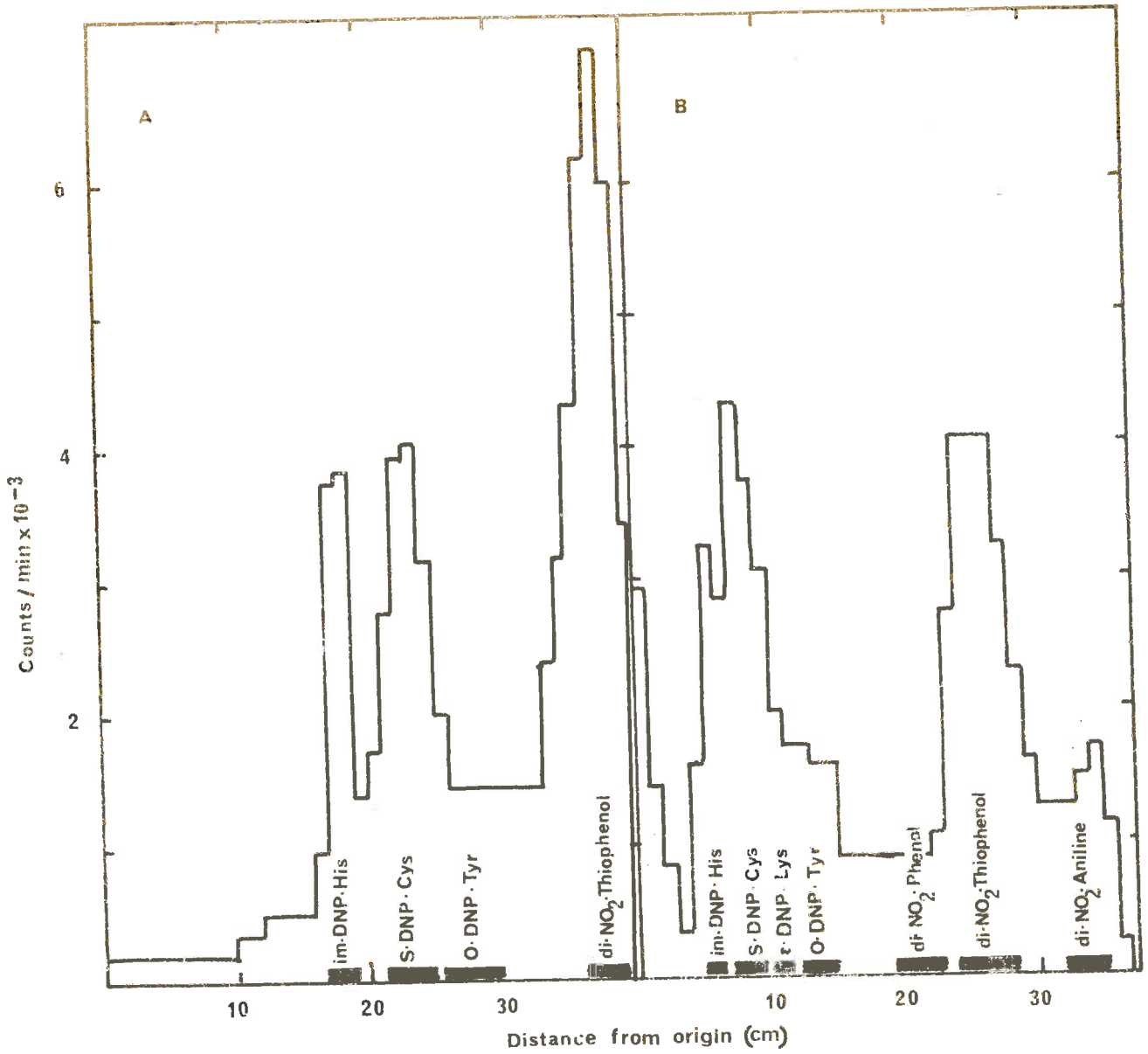
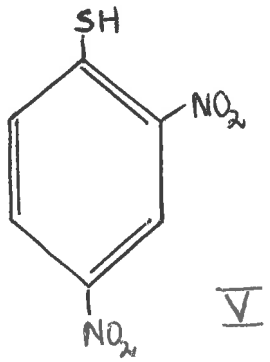


FIG. 7.25. Identification of the residue reactive towards DNFB. The sample lane of the chromatogram of Fig. 7.24 A. between 14 and 22 cm from the origin was eluted with glass-distilled water and rechromatographed on Whatman No 4 paper for 7.5 hr with the two solvents: 1-butanol/water/acetic acid (4/1/1, v/v/v) (A) and 1-butanol/water/ammonia (87/12/1, v/v/v) (B). The chromatograms were processed as in Fig. 7.24.



V

(e) Number of sulphydryl residues interacting with Mn^{2+}

In Section 7.3A(h), two sulphydryl groups were shown to react rapidly with 5,5'-dithiobis (2-nitrobenzoic acid) at pH 7.0 and 20°. That group which appeared to be the more reactive of the two was protected by the presence of IDP. In the presence of 2 mM Mn^{2+}, there was a decrease in the rate of reaction with the other reactive group (Fig. 7.16). The presence of both 2.5 mM IDP, and 4 mM Mn^{2+} produced an additive effect corresponding to the protection of both of the groups initially modified. Mn^{2+} also showed the added effect of inhibiting the precipitation of protein which followed reaction of Ellman's reagent.

(f) Effect of chemical modification on the active site

In the investigations on the NEM-susceptible sulphydryl group, the kinetics of the enzyme after modification was used to help determine the role of the reactive group. However, in the case of DNFB modification, the lack of specificity of its reaction with PEP carboxykinase precluded the use of this

approach as it would be impossible to differentiate between the effects produced by modification of the two groups. Therefore, the alternative approach was used whereby the rate of inactivation by DNFB was assessed by assaying the residual activity six ways. Table 7.5 shows that the rate of inactivation by DNFB was the same in all cases.

Monitoring the effects of DNFB modification by N.M.R. studies (see Chapter 4) yield results identical to those obtained with NEM (Section 7.3A(g)). After reaction of 0.076 mM enzyme with 0.15 mM DNFB at pH 7.5 and 20° in the presence of 0.12 mM Mn^{2+} for 30 min (about two half-times), the binding of Mn^{2+} and PEP was essentially unimpaired (no change in ϵ^*) while the nucleotide (ITP) effect on ϵ^* was abolished (Fig. 7.26). Therefore, modification by DNFB either inhibits IDP (and ITP) binding or a conformational change induced by the nucleotide can no longer occur. However, by analogy with the results on GTP binding to the NEM-modified enzyme, it is concluded that binding of the nucleotide is inhibited.

7.3.C MODIFICATION STUDIES WITH DIAZOBENZE SULPHONIC ACID (DBS)

Maruyama *et al.* (1966) reported that peanut cotyledon PEP carboxylase was susceptible to inhibition by DBS at pH 7.5 and 0°. Sheep kidney mitochondrial PEP carboxykinase was also susceptible to this reagent at pH 7.0 although higher temperatures were needed, *viz.*, 20 - 30°. However, the elevated temperatures

TABLE 7.5: EFFECT OF DNFB MODIFICATION OF THE ACTIVE SITE

Method of Assay	Half-time of Inactivation (min.)
Carboxylation	3.1
CO_2:OAA Exchange (Mn^{2+}/no nucleotide)	3.1
CO_2:OAA Exchange (Mn^{2+}/ITP)	3.05
CO_2:OAA Exchange (Mn^{2+}/IDP)	3.0
CO_2:OAA Exchange (Mg^{2+}/ITP)	3.15
CO_2:OAA Exchange (Mg^{2+}/IDP)	3.15

The enzyme was preincubated in a solution (final volume 0.4 ml) containing 20 μmoles N-ethylmorpholine (Cl^-), pH 7.5, 0.35 units enzyme and 0.05 μmoles DNFB. Incubation was at 25°. At various times, 0.05 ml aliquots were diluted with 0.1 ml of 1% B.S.A. containing 10^{-1} M lysine and 10^{-4} M GSH, pH 7.5 and analysed for residual activity by the methods listed above. Half-times of inactivation were obtained from semi-log plots of residual activity against time. The carboxylation assay was standard as listed in Section 2.7(i). The exchange assay (total volume 0.5 ml) contained (in μmoles): imidazole (Cl^-), pH 6.5, 50.0; Mn^{2+} or Mg^{2+}, 2.0; nucleotide (if present), 1.0; OAA, 1.0; $NaH^{14}CO_3$, 20.0; GSH, 0.8 and incubation was for 4 min. Processing of all assays was as described in Section 2.7(ii).

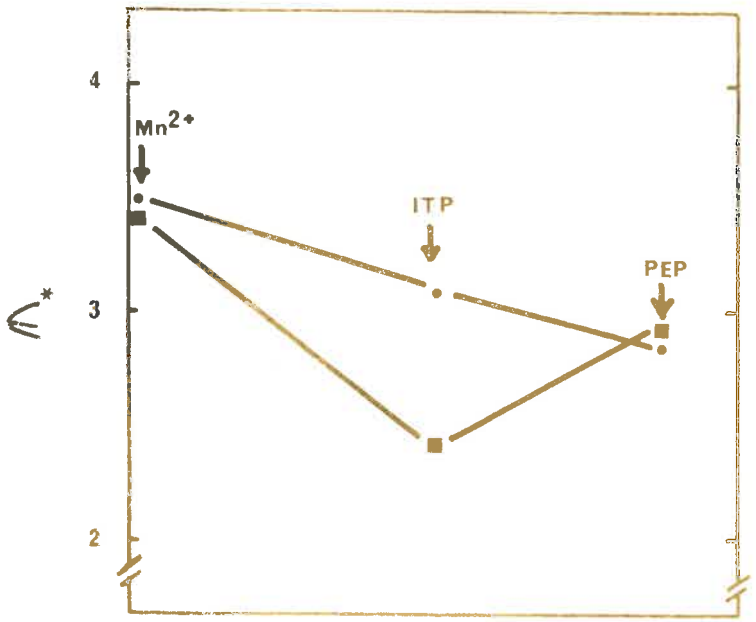


FIG. 7.26. Effect of modification by DNFB on substrate binding. Enzyme (0.076mM) was incubated at 20° with 0.15mM DNFB and 0.07M N-ethylmorpholine (Cl^-), pH 7.5, for two half-times. The enhancement (ϵ^*) of the PRR was estimated in the presence of substrates (see Chapter 4 for methods). Final concentrations of substrates were 0.12mM Mn^{2+}, 0.095mM ITP and 0.09mM PEP. (■——■), control; (●——●), after modification.

make it more difficult to analyse the inhibition in a manner similar to that applied to NEM and DNFB modification because of the hydrolysis of the reagent under these conditions.

(a) Order of inactivation with respect to time and DBS concentration

Plots of log percentage activity as a function of time of DBS inactivation were linear for about 4 min modification (Fig. 7.27) indicating that modification was a pseudo first-order process at certain fixed concentrations of inhibitor. This same data yields a straight line (Fig. 7.28) when log rate of inactivation, expressed as the slope of the primary plot (k') was plotted against log inhibitor concentration (see Eqn. 7.3). Fig. 7.28 also shows a set of data obtained at 20° instead of 30°. However, the slopes of the plots were 2.29 (at 30°) and 1.72 (at 20°). These high values would most likely reflect the rate of hydrolysis of the reagent rather than the number of amino acid residues reacting with DBS, i.e., at the higher temperature, water competes more favourably for the reagent than does the enzyme.

(b) Effect of pH on the rate of inactivation by DBS

The concentration dependence of inactivation gives the number of reagent molecules reacting to cause inactivation and this figure can not necessarily be equated to the number of protein residues being modified especially where the reagent suffers rapid hydrolysis. However, the pH dependence of a

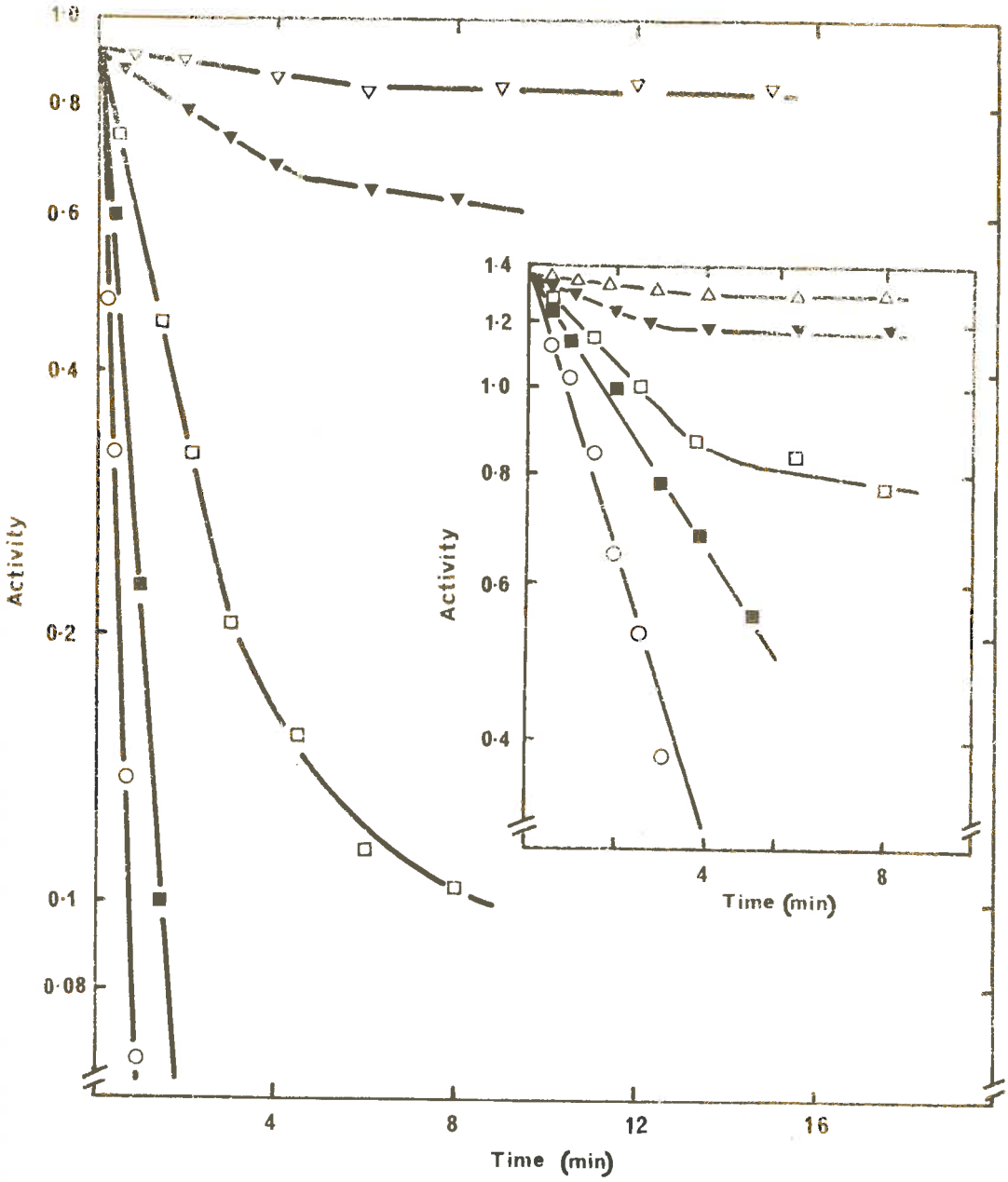


FIG. 7.27. The rate of inactivation of PEP carboxykinase with varying concentrations of DBS. Enzyme was incubated at 30° in a solution (0.2 ml) containing 10 μmoles phosphate (K^+), pH 7.0, 0.3 units of enzyme and varying amounts of DBS as indicated. Aliquots of 0.025 ml were diluted with 0.05 ml of 1% (w/v) B.S.A. containing 10^{-1}M imidazole and 10^{-4}M GSH, pH 6.5, and analysed of activity (Section 2.7.(i)). Activity was plotted against time on a semi-log scale. DBS concentrations were: (○——○), 2mM; (■——■), 1mM; (□——□), 0.5mM; (▼——▼), 0.25mM; (▽——▽), 0.125mM. Inset: Data from a similar incubation at 20° with DBS concentrations: (○——○), 1mM; (■——■), 0.75mM; (□——□), 0.5mM; (▼——▼), 0.3mM; (△——△), 0.2mM.

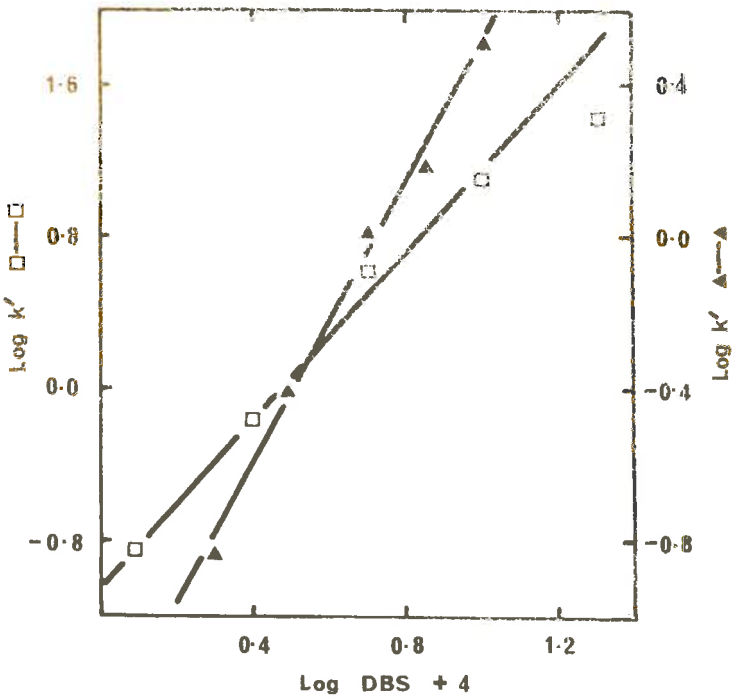


FIG. 7.28. The data of Fig. 7.27. were replotted as $\log_{10}$ k' (the rate of the inactivation process) against $\log_{10}$ DBS concentration. The data was obtained at 20° (▲——▲) and 30° (□——□).

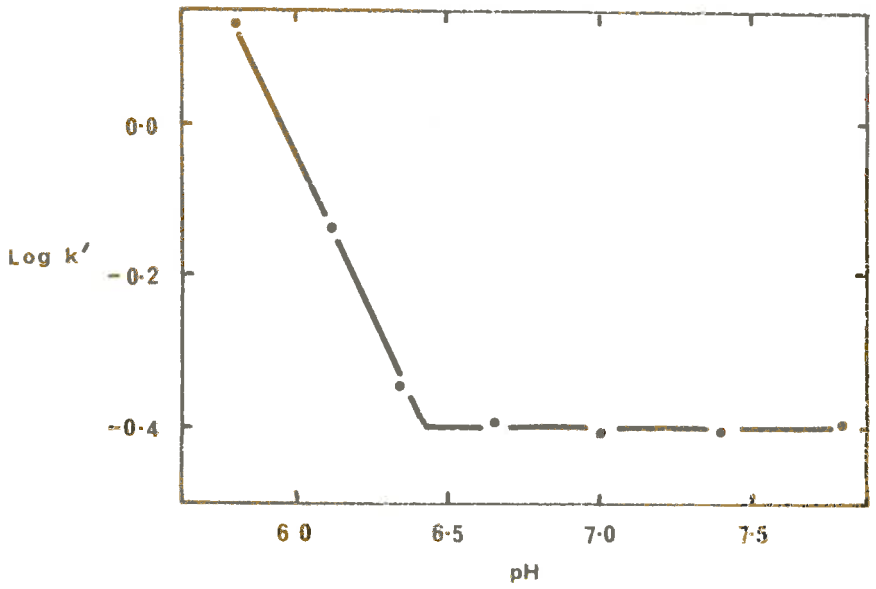


FIG. 7.29. Effect of pH on the rate of inactivation by DBS. Conditions were as in Fig. 7.27. using 10^{-1} M N-ethylmorpholine.phosphate buffer (pH 5.5 - 7.8) and 0.05 μmoles of DBS. Log_{10} k' was plotted against pH. The slope of the line gives the number of ionising groups reacting.

reaction can be used to estimate the number of ionising groups undergoing modification. The slope of the plot of log rate of inactivation against pH gives an estimate of the number of ionising groups which influence the rate of the reaction. Figure 7.29 shows the results of such an investigation. There is a marked increase in the rate of the reaction below pH 6.5 corresponding to the ionisation of only one group (Slope = 0.89). A pK_a of about 6.5 is indicated. It is significant that this same inflection point has appeared in the pH studies on both NEM and DNFB inactivation and most likely represents the ionisation of a histidine group.

(c) Protection by substrates against DBS inactivation

Table 7.6 shows the results of an investigation to establish which substrates could protect against DBS inactivation. It is readily seen that Mn^{2+} was the only substrate capable of protection.

7.4 DISCUSSION

The sulphydryl group of cysteine has all too readily been assigned a role in the enzymic processes because of its ready susceptibility to modifying reagents (Cecil, 1963). However, only a few cases have been documented where a sulphydryl group has been demonstrated to be intimately involved in the catalytic process forming an S-acyl intermediate, e.g., fatty acid synthetase (Lynen, 1961), glyceraldehyde-3-phosphate dehydrogenase

TABLE 7.6: PROTECTION OF ENZYME ACTIVITY BY REACTION MIXTURE COMPONENTS

Additions	% Original Activity	
	Expt. 1	Expt. 2
None	57.1	36.2
Mn^{2+}	76.9	71.1
PEP	69.1	35.1
IDP	69.1	34.1
$NaHCO_3$	61.5	30.9
Mn^{2+} + PEP	77.7	71.8
Mn^{2+} + IDP	75.9	71.2
Mn^{2+} + $NaHCO_3$	74.9	70.4

The enzyme was preincubated at 30° in a solution (final volume 0.1 ml) containing 5 μmoles phosphate (K^+), pH 7.0, 0.1 units of enzyme plus DBS and substrates. For Expt. 1, substrates were 0.5 mM except $NaHCO_3$ which was 5.0 mM, DBS was 0.25 mM and incubation was for 7 min. For Expt. 2, substrates were 2 mM except $NaHCO_3$ which was 20 mM, DBS was 0.5 mM and incubation was for 4 min. Aliquots of 0.025 ml were withdrawn, diluted with 0.05 ml of 1% (w/v) B.S.A. containing 10^{-1} M imidazole (Cl^-), pH 6.5, and 10^{-4} M GSH and analysed for residual activity as described in Section 2.7(1).

(Krimsky and Racker, 1955; Kooppe et al., 1956), glyoxalase (Cliffe and Waley, 1961), papain (Smith, 1958), ficin (Gutfreund (1955) and transglutaminase (Folk and Cole, 1966a,b). On the other hand, a sulphydryl group apparently facilitates the binding of propionyl CoA to propionyl CoA carboxylase but plays no further role as V_{max} is unaffected by complete blocking of this group (Edwards and Keech, 1967). In other cases, the role of the reactive sulphydryl is less readily defined. Modification of the thiol groups of phosphofructokinase (Froede et al., 1968; Forest and Kemp, 1968) and glutamate dehydrogenase (di Prisco, 1967) results in the loss of the kinetic expression of cooperative interactions. The two thiol groups of creatine kinase can be modified by iodoacetate to give a completely inactive enzyme but, although the nucleotide substrate protects against this modification, the binding of the nucleotide is in no way affected (O'Sullivan and Cohn, 1966a). At the other end of the scale, the sulphydryl groups of fumarase have been shown not to be associated with the active site but to be buried in a hydrophobic environment in the interior of the enzyme (Robinson et al., 1967).

Number of groups reacting with NEM, DNFB and DBS

Evidence has been presented here that sulphydryl groups are modified by NEM and DNFB causing loss of activity. Analysis of the kinetics of the inactivation by these two reagents indicate that only one group is modified by NEM (Figs. 7.3 and 7.5) and by DBS (Fig. 7.29) but that more than

one react with DNFB (Figs. 7.19 and 7.21). Using this kinetic approach, Levy, Leber and Ryan (1963) concluded that 3 moles of 2,4-dinitrophenol bind to one molecule of myosin while Scrutton and Utter (1965) assumed at least two moles of avidin were involved in the inactivation of avian liver pyruvate carboxylase. Edwards and Keech (1967) reported that one molecule of NEM bound to pig heart propionyl CoA carboxylase causing inactivation and Keech and Farrant (1968) showed the involvement of one molecule of DNFB in the inactivation of sheep kidney pyruvate carboxylase. However, the data with DBS illustrate the limitations of this approach (Fig. 7.28). It is not always valid to assume that the molecularity of the inactivation process with respect to the chemical modifier is the same as for the reactive residue on the enzyme, especially if the reagent is unstable under the conditions used. In such a case, a more reliable estimate for the molecularity with respect to the enzyme functional group can be obtained from a pH study of the inactivation. If the functional group ionises and the reagent reacts preferentially with one ionised form, the slope of a double log plot of rate of inactivation against H^+ concentration gives the number of ionising groups reacting. This then is a property of the enzymic group and not of the reagent used. This approach confirmed that one group reacted with NEM, more than one with DNFB while only one ionisable group reacted with DBS (Figs. 7.5; 7.21; 7.29).

The presence of two reactive sulphydryl groups was subsequently confirmed with titrations of the enzyme with 5,5'-dithiobis(2-nitrobenzoic acid). It was shown that one of the two groups was protected by IDP, the other was protected by Mn^{2+} (Fig. 7.16). Mn^{2+} also protected the DBS-susceptible group against modification (Table 7.6).

Protection studies

Analysis of the protection afforded by IDP against NEM inactivation according to the method of Scrutton and Utter (1965) showed that IDP does not afford complete protection but that NEM is able to react with the enzyme-IDP complex although at a much reduced rate. Analysis of the protection by Mn^{2+} against DNFB modification was not possible by this means as DNFB reacted with more one residue although Mn^{2+} protected against only one of these. The result with IDP is comparable to the situation with creatine kinase where ADP does not provide complete protection against iodoacetate inactivation. In this case, however, the Mn.ADP complex stimulated inactivation (O'Sullivan and Cohn, 1966a). With PEP carboxykinase, the protection afforded by IDP or Mn^{2+} was in no way altered by the presence of other substrates.

While the opposite effects of ADP and Mn.ADP with creatine kinase provide strong evidence for nucleotide-induced conformational changes influencing the rate of inactivation by iodoacetate, no firm evidence for or against has been found to

suggest that IDP protects against NEM-inactivation of PEP carboxykinase by similar means. However, nucleotide does appear to induce a conformational change in PEP-carboxykinase as IDP and ITP greatly stimulate the CO_2:OAA exchange reaction (see Chapter 6) (cf., Bridger, Millen and Boyer, 1968). Therefore, the possibility does exist that IDP binding to the enzyme results in a decrease in the inherent reactivity or accessibility of the sulphydryl group caused by a conformational change rather than the protection against modification being effected by steric factors as would be the means whereby a substrate would protect its binding groups.

Effect of modification on the active site

Although NEM-modification destroys nucleotide binding by PEP carboxykinase, three observations would argue against the sulphydryl group being strictly a binding group. Firstly, one would expect that inhibition due to modification of a strictly binding group would not be complete since only the efficiency of binding would be impaired and such inhibition as is suffered would be reversed by excess substrate, e.g., the sulphydryl group of propionyl CoA carboxylase (Edwards and Keech, 1967) and the methionine of α-chymotrypsin (Knowles, 1965). This was not observed with PEP carboxykinase (Figs. 7.12 - 14). Secondly, the protection against NEM inactivation showed an absolute specificity for the nucleotide with the preferred order ITP < IDP > IMP > inosine although the protection afforded by IDP was not complete (Fig. 7.7).

Also, one would not expect ITP to be such a poor protecting agent compared with IDP if a binding group common to both these substrates was being modified. Finally, the nucleotide-independent CO_2:OAA exchange activity is inhibited to the same degree as the nucleotide stimulated exchange activities and the carboxylation activity. These findings would not be in accord with the sulphydryl group being a strictly binding group. Although one can not necessarily exclude the possibility that the group plays some part in nucleotide binding.

It is significant that NEM or DNFB modification of PEP carboxykinase destroys the ability to bind only the nucleotide (Figs. 7.15 and 7.26) although the nucleotide-independent CO_2:OAA exchange activity is lost at the same rate as the nucleotide-dependent activities (Tables 7.3 and 7.5). Therefore, reaction with a ctalytic group is suggested.

However, it would be invalid to disregard the possibility that inactivation resulted from either steric effects or consequent structural changes giving a catalytically inert product. Steric effects could hardly be the complete answer as modification by NEM prevents nucleotide binding as well as carboxylation *per se* (*i.e.*, nucleotide-independent CO_2:OAA exchange activity) although these two processes must occur at topographically distinct sites. More likely, such steric effects are, to some extent, instrumental in either of these end results but not both. As discussed previously (Chapter 6), IDP apparently causes a conformational change on binding.

This could well be prevented because of the bulk of the NEM and DNFB groups such that nucleotide binding is prevented or drastically weakened. A role for the thiol residue could be the stabilisation of the enzyme-bound enolpyruvate (Chapter 6) perhaps via a hemi-thioketal formation. In this way, steric effects would inactivate the carboxylation process while structural effects would destroy nucleotide binding. Structural changes are suggested by the observation that inactivation by "reversible" inhibitors such as mercurials was not reversible by incubation with GSH or dithiothreitol. With the less bulky methylated residue, a small residual activity could be possible as the constraints put on the active site by a methyl group would not be as great as those imposed by NEM and DNFB modification.

Microenvironment of the reactive sulphydryl groups

The pH profiles for NEM and DNFB modification are both very similar and indicate a pK_a of about 8.0 for the reactive residues. This value is low compared with the normal range of 8.5 - 9.2 for cysteine in an electrostatically neutral environment and would suggest the close proximity of a positively charged group (Webb, 1963) such as lysine or arginine. However, lysine would be an unlikely contender for this role as DNFB showed no reaction with such a residue. This avenue has not been pursued further because of the lack of a specific arginine reagent. The sulphydryl group protected by Mn^{2+} must be in a

reasonably accessible situation as IDP protects against inactivation by several sulphydryl inhibitors of vastly different nature and mode of action.

Nature of the group reactive towards DBS

In the double log plots of rate of inactivation by both NEM and DNFB against H^+ concentration an inflection at about pH 6.5 was noted (Figs. 7.5 and 7.21). Studies on the relationship between DBS inactivation and pH also show an inflection at pH 6.5 (Fig. 7.29). Such a value would be consistent with the modification of a histidine residue.

The disadvantage of using DBS as a modifying agent is its broad specificity (Landsteiner, 1945) and the difficulty in positively identifying the susceptible group. However, lysine was not modified by DNFB although histidine was slightly, tyrosine could be discounted as N-acetyl imidazole (Riordan et al., 1965) showed no inactivation at pH 8.0 and 30° and attempts to modify carboxyl groups at pH 5.5 and 6.0 at 30° with 1-cyclohexyl-3-(2-morpholinyl(4)-ethyl) carbodiimide metho-p-toluene sulphonate and glycine methyl ester (Hoare and Koshland, 1966; Armstrong and McKenzie, 1967) were unsuccessful. Therefore, it would appear that histidine was most likely being modified by DBS.

Pradel and Kassab (1968) reported the modification by diethylpyrocarbonate of a histidine residue of arginine kinase at pH 6.1 without reaction with the activated sulphydryl group.

Using similar conditions, this reagent did inactivate PEP carboxykinase but Mn^{2+} did not protect. However, a pH study showed the rate of inactivation was independent of pH over the range 5.5 to 8.0, while a spectral analysis of the product showed no evidence for reaction with histidine (carboxyethyl derivatives of imidazole show a λ_{max} at 220 mμ while those of an -SH group have a λ_{max} at about 210 mμ). Rather, there was a progressive increase in turbidity probably indicative of general non-specific reaction as this reagent does have a broad specificity (Wolf, Lesnaw and Reichmann, 1970). Therefore, attempts to detect positively an imidazole residue in the active site were unsuccessful and further studies must await the use of suitably designed substrate analogues.

SUMMARY

It is therefore proposed that the active site of PEP carboxykinase contains two sulphydryl residues and an imidazole group. The sulphydryl group protected by Mn^{2+} must be in a more hydrophobic environment than the other group such that reaction with the more hydrophilic reagent (NEM) is hindered. The two residues protected by Mn^{2+} could well be the ligands for Mn^{2+} provided by the enzyme in the enzyme.Mn^{2+} binary complex. In support of such a structure, Coleman and Vallee (1961) have reported that nitrogen-sulphur bidentate ligands

form more stable complexes with Mn^{2+} than do nitrogen-oxygen or nitrogen-nitrogen bidentate ligands. That sulphydryl residue protected by IDP would appear to have some catalytic function.

CHAPTER 5. GENERAL DISCUSSION

The main conclusions which have been made in this thesis are:

(i) The carboxylation reaction of sheep kidney mitochondrial PEP carboxykinase is controlled by the product of this reaction, OAA.

(ii) PEP carboxykinase has a sequential mechanism and the formation of the central complex occurs by a preferred pathway with IDP binding first followed by the random addition of PEP and CO_2.

(iii) Two metal ions are involved in the catalysis.

(iv) The conversion of substrates to products occurs by a two step process with phosphoryl transfer from PEP to IDP preceding carboxylation of the enzyme-bound enolpyruvate intermediate in the carboxylation reaction.

(v) The integrity of two sulphydryl groups and a histidine residue would appear to be essential for full enzymic activity.

(vi) The sulphydryl group which is protected by IDP against NEM inactivation is not strictly a binding group but would appear to have some catalytic function.

(vii) The sulphydryl and histidine residues which are protected by Mn^{2+} against DNFB and DBS inactivation respectively could serve as the ligands for Mn^{2+} in the binary enzyme.Mn^{2+} complex.

At this stage it would be constructive to consider some of those lines of experimentation which now suggest themselves as an outcome of the investigations reported in this thesis and thereby eliminate those whose application would appear limited or impractical because of the known

properties of the system.

Notwithstanding the recent impetus given to the N.M.R. technique by the introduction of free radical "spin labels" compounds by Hamilton and McConnell (1968), this approach would not appear feasible at the moment with PEP carboxykinase. For this technique, quantitative modification of one residue per active site would be ideal and, needless to say, a soluble modified enzyme is required. With these conditions satisfied, the "spin labels" could yield valuable information about the topography of the active centre with respect to the substrate binding sites. (Cf., Taylor, Leigh and Cohn (1969) who used this approach with creatine kinase.) Unfortunately, PEP carboxykinase does not satisfy the above conditions for using the "spin labelled" sulphydryl reagents despite the ready susceptibility of the sulphydryl residues to modification. If modification is taken beyond about 80% inactivation, precipitation begins to occur such that attempts to isolate PEP carboxykinase with a quantitatively modified sulphydryl residue produce an insoluble protein. However, development of reagents specific for other amino acid residues, such as lysine, histidine or carboxyl groups, could allow the isolation of the desired PEP carboxykinase derivative. Applying this technique with two or more labels specific for different residues in the active centre could possibly elucidate the spatial arrangement of each binding site in relation to each

other. Additional evidence could also be gathered concerning the involvement of two metal ions in the catalysis.

Confirmation of the close proximity of the two sulphydryl residues and the histidine group could be provided by amino acid sequencing and the use of bifunctional reagents or substrate analogues as modifying agents. The former technique suffers from the limitation that the folding of the peptide chain can bring widely separated portions of the peptide backbone into close proximity. The latter technique would appear to be the most rewarding. This approach would most likely severely limit a conventional analysis of the effect of modification since part of the active centrewould be permanently blocked by a bifunctional reagent and even by a substrate analogue especially if that portion of the molecule distant from the reactive centre contains specificity determinants. Instead of this moiety remaining free as with a simple reagent, it would tend to be bound irreversibly to its binding site simply because of its proximity. Therefore, catalysis would most likely be completely destroyed although binding properties could be used as indices of a native conformation.

However, these reagents are more for solving spatial problems than for probing the catalytic functions of residues because of the increased specificity of modification and the unique directional reaction which can be achieved. It can therefore readily be appreciated the information which can be

gathered from this approach both with respect to the residues proximal to each binding site and the spatial arrangement of each site relative to each other. In view of the fact that the common reagents for all amino acids have been tested with PEP carboxykinase and modification was only achieved with the sulphydryl (NEM, DNFB, iodoacetamide etc.) and histidinyl (DES) reagents, further information concerning the active centre of PEP carboxykinase must now await the development of these special reagents.

No attempt has been made to analyse the formation or nature of the proposed enzyme-bound enolpyruvate intermediate. Its existence is suggested by the IDP-stimulated OAA decarboxylation to pyruvate and the CO_2:OAA exchange reaction as IDP undergoes no reaction. Also, the nucleotide-independent CO_2:OAA exchange reaction demands such an intermediate. By analogy with the mechanism proposed by Rabin and Trown (1964) for ribulose diphosphate carboxylase and because of the presence of the reactive sulphydryl residue, it was suggested that this intermediate could be a thiohemiketal. Such a compound would have only a short half-life making its isolation difficult. However, the stability of thioketals is greatly increased under acid conditions so that precipitation of the protein under such conditions could permit the isolation of the intermediate whereupon its nature could then be determined.

However, a covalently-bound intermediate of the type described above would not necessarily be essential. The

requirement for the exchange would simply be that the dissociation and reassociation of CO_2 is more rapid than the dissociation of the pyruvate moiety. The CO_2 can then rebind for subsequent attachment to the pyruvate moiety to reform OAA. As the OAA and PEP are presumably bound to the enzyme via "metal bridge" structures and a salt bridge is also possible through the carboxyl group, the stabilisation of the intermediate could thereby be achieved without a covalent link being formed. The crucial matter would appear to be the maintenance of the enolpyruvate configuration which would be more favourable for carboxylation. Therefore, failure to isolate the enzyme-bound intermediate would not necessarily disprove its existence.

Finally, an apparent nucleotide-induced conformational change is suggested by the marked stimulation of the CO_2:OAA exchange reaction. Attempts to detect the conformational change by enhanced reactivities towards MMTB and DBS were unsuccessful although this approach would only be useful if the particular residue became more accessible where accessibility was a limiting factor in the rate of inactivation or if the inherent reactivity of the residue increased because of consequent electronic effects. Reporter groups (Hille and Koshland, 1967) would be expected to provide evidence either for or against this nucleotide effect as a range of groups could be employed which respond to a variety of environmental perturbations and these could be attached to various residues

at or near the active centre. Although unequivocal interpretation of results obtained with these groups is not always possible, the use of several types of groups overcomes these limitations provided, of course, the presence of the reporter group itself does not destroy the nucleotide-induced effect. Also, the control by OAA of the carboxylation reaction could well be achieved via a conformational change with a consequent effect on the binding constants of the substrates. This could most readily be tested by binding studies in the presence and absence of OAA.

Therefore, despite a reasonably extensive investigation of sheep kidney mitochondrial PEP carboxykinase, much still remains to be elucidated with respect to a more precise delineation of the events during catalysis especially the role played by the amino acid residues in this action. The interesting fact which emerges from this study is the remarkable agreement in physical and kinetic properties between the kidney and liver PEP carboxykinases from widely different species and between the PEP carboxykinases and PEP carboxytransphosphorylase.

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