STUDIES ON THE ALLOSTERIC ENZYMES PHOSPHOENOLPYRUVATE CARBOXYLASE FROM *SALMONELLA TYPHIMURIUM* AND MALIC ENZYME FROM *ESCHERICHIA COLI*

by

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ABSTRACT

The enzyme phosphoenolpyruvate carboxylase [orthophosphate: oxalacetate carboxylase (phosphorylating) EC 4.11.31] from Salmonella typhimurium LT_2 was purified to homogeneity as determined by polyacrylamide gel electrophoresis.

Earlier molecular weight studies on phosphoenolpyruvate carboxylase from S. typhimurium indicated that this protein consisted of 4 identical subunits, the tetrameric oligomer having a molecular weight of 193,000 $\pm$ 7,000 (Maeba, 1968; Maeba and Sanwal, 1969). A thorough investigation of this enzyme by equilibrium centrifugation studies indicates that the molecular weight of the native enzyme is heterogeneous, exhibiting molecular weights ranging anywhere from 100,000 to 400,000. The smallest major species observed in SDS polyacrylamide gels displayed a molecular weight of 100,000, whereas the smallest species observed in equilibrium centrifugation runs performed in guanidine hydrochloride was 50,000, suggesting a possible 8 subunit structure of the native enzyme. Electron microscopy studies of the protein shows the presence of tetrameric structures along with higher molecular weight aggregates.

According to the terminology of Monod, Changeux and Jacob (1963) the enzyme was shown to be allosteric, and as such, was subject to control by a number of modulators. It was shown to be activated by acetyl CoA (Cánovas and Kornberg, 1965), fructose diphosphate (Sanwal and Maeba, 1966b), activated in a compensatory manner by nucleoside di- and triphosphate pools (Sanwal and Maeba, 1966a), and controlled by feedback inhibition by aspartate (Maeba and Sanwal, 1965).

It was also shown earlier that a cooperative type of activation was present between the activator pairs acetyl-CoA and fructose 1,6-di-P (Sanwal, 1970; Maeba and Sanwal, 1969), acetyl-CoA and GTP or fructose 1,6-di-P and GTP (Sanwal and Maeba, 1966a). This cooperative type of interaction by regulatory pairs has been known for some time (Caskey, Ashton and Wyngaarden, 1964) but the mechanism was not known. We, therefore, decided to investigate these problems by studying the characteristics of binding of the various regulatory ligands at equilibrium to phosphoenolpyruvate carboxylase from S. typhimurium to throw some light on the mode of action of allosteric enzymes in general. The binding of Mn^{2+} to the enzyme was also studied. A model for this mode of action was thus postulated.

The TPN^+-specific malic enzyme from Escherichia coli has been purified 100 fold from malate grown cells. The enzyme is inhibited by acetyl-CoA, oxalacetate, TPNH, DPNH and cyclic AMP in an allosteric manner. Glycine at concentration ranges above 0.5 M was shown to activate the enzyme as well as to desensitize it reversibly to the effect of the various inhibitors.

A kinetic study was made of the purified malic enzyme. The double reciprocal initial velocity plots of both of the substrates, TPN^+ and malate, were linear in the absence of allosteric inhibitors. Product inhibition studies with bicarbonate and pyruvate also gave linear inhibitions with either TPN^+ or malate as varied substrates. In the presence of each of the allosteric inhibitors, the initial velocity plots for TPN^+ remained linear but the plots for malate became non-linear. This non-linearity vanished when assays were perfomed

in the presence of 0.72 M glycine. Inhibition by acetyl-CoA and oxalacetate was competitive with malate, but non-competitive with TPN^+. In the presence of glycine TPNH gave linear non-competitive inhibition against TPN^+ as the varied substrate.

From these studies it was postulated that malic enzyme followed an ordered mechanism with isomerization of the free enzyme as an obligate step of the reaction pathway. The kinetic behaviour of malic enzyme in the presence of allosteric inhibitors was analyzed in terms of a two conformational state model and the conclusions reached were, (a) that the E-TPN^+ complex existed in the absence of allosteric ligands in a form which had a high affinity for malate (state R), and (b) that the binding of any one of the several inhibitors to the enzyme resulted in its conversion to a state (T) which had very little affinity for malate.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF PLATES

LIST OF TABLES

ABBREVIATIONS

Acetyl-CoA	Acetyl coenzyme A
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
DEAE	Diethylaminoethyl acid
DNS	1-dimethylamino-naphthalene-5-sulfonyl chloride (dansyl chloride)
DPN^+	Oxidized diphosphopyridine nucleotide
DPNH	Reduced diphosphopyridine nucleotide
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
FDP	Fructose-1,6-diphosphate
GTP	Guanosine triphosphate
Hepes	N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
OAA	Oxalacetate
PEP	Phosphoenolpyruvate
Pi	Inorganic phosphate
SDS	Sodium dodecyl sulfate
TPN^+	Oxidized triphosphopyridine nucleotide
TPNH	Reduced triphosphopyridine nucleotide
Tris	Tris (hydroxymethyl) aminomethane

HISTORICAL INTRODUCTION

GENERAL

The myriad chemical processes occurring within living cells indicate to some extent the bewildering complexities associated with biological organisms. In bacteria specific control mechanisms have evolved at certain positions along many metabolic routes which ensure a correct, coordinated flow of carbon fragments into the biosynthetic channels and into the energy-generating pathways. One of the commonly utilized control mechanisms, namely the induction of enzymes, was reported as early as 1899 by Duclaux and was named "enzymatic" adaptation by Karstrom (1938). Roberts, Abelson, Cowie, Bolton and Britten in 1955 showed that the presence of exogenously supplied amino acids in the growth medium prevented the de novo synthesis of amino acids from glucose. Thus, when the organisms were placed in a medium containing certain amino acids they were able to selectively utilize the amino acids that were supplied and at the same time stop the synthesis ("repression") of the enzymes responsible for the synthesis of these amino acids. Monod and Cohen-Bazire (1953) showed that the presence of tryptophan and certain analogues of tryptophan could selectively repress the formation of the enzyme tryptophan synthetase. The importance of the induction and repression phenomenon was first outlined in the classic paper of Jacob and Monod (1961). A second control mechanism, viz., feedback inhibition, was reported as far back as 1941 in simple biosynthetic anabolic pathways. Dische, for instance, observed that the phosphorylation of glucose in erythrocyte hemolysates was specifically inhibited by phosphoglycerate, and that the inhibition did not occur through competition for the active site; therefore he postulated that

phosphoglycerate played a regulatory role in glucose metabolism. It was Umbarger (1956) and Yates and Pardee (1956) who pointed out the real significance of this type of control. Umbarger, for example, demonstrated in vitro that isoleucine, the end product of the threonine-isoleucine pathway, inhibited the first enzyme (threonine deaminase) of that pathway. This he interpreted as a negative feedback type of control mechanism. Yates and Pardee came to a similar conclusion following their observations that cytidine triphosphate inhibited aspartate transcarbamylase. At present there are a large number of enzymes in biosynthetic pathways that are susceptible to a negative type of feedback control. Witness, for example, some of the sophisticated variations of this basic type of mechanism that have evolved in branched metabolic pathways:

1. Enzyme multiplicity (Stadtman, Cohen, Le Bras and Robinchon-Schulzmaster, 1961; Smith, Rauel, Lax and Shive, 1962; Umbarger and Brown, 1957; 1958)---multiple enzymes catalyzing the same reaction are produced, each form being susceptible to inhibition by a different end product;

2. Concerted or multivalent inhibition (Datta and Gest, 1964; Paulus and Gray, 1964)---all end products of the branched pathway must be present to cause inhibition of the first common step;

3. Cooperative feedback inhibition (Caskey, Ashton and Wyngarden, 1964; Nierlich and Magasanik, 1965)---individual end products alone cause partial inhibition of the first enzyme but the simultaneous presence of two or more end products result in a greater inhibition than the sum of the fractional inhibitions caused by each independently;

4. Cumulative feedback inhibition (Woolfolk,and Stadtman, 1964)---each end product acts independently of the others in causing partial

inhibition. In this case when two or more of the end products are present at the same time the total activity is equal to the product of the individual activities when each end product is present alone.

Another control mechanism symmetrically opposite to the feedback systems described above has been termed "precursor activation" by Sanwal, Zink and Stachow (1963). Here, the precursor of a given sequence activates the last enzyme of that pathway. A number of such "feed-forward" loops have been described mostly in amphibolic pathways (Sanwal, 1970a).

PHOSPHOENOLPYRUVATE CARBOXYLASE

1. Distribution — Bandurski and Griener (1953) were the first to report a PEP carboxylase in extracts of spinach leaves. Bandurski (1955) later purified the enzyme some ten fold and established the basic properties of the reaction, and the enzyme. The distribution of the enzyme in various common plants was then reported by Vennesland and her associates (1955; 1958) and Jackson and Coleman (1959). However, the presence of PEP carboxylase in bacteria was not established until 1958 when Suzuki and Werkman (1958) noted its presence in the autotroph, Thiobacillus thioparus. The following year Amarsingham (1959) reported that the enzyme could also be found in Escherichia coli. Since then there have been a number of reports of its presence in many bacterial genera. It has been reported for example, in Pseudomonas (Large, Peel and Quayle, 1962), Salmonella (Theodore and Englesberg, 1961), Nitrosomonas (Rao and Nicholas, 1966), Streptococcus (Lachica and Hartman, 1969), Acetobacter (Claus, Orcutt and Belly, 1969; Benziman, 1969), Ferrobacillus (Din, Suzuki and Lees, 1967) and Brevibacterium (Ozaki

and Shiio, 1969). PEP carboxylase has also been observed in algae (Kates and Jones, 1965) and protozoa (Siu, 1967; Ohmann and Plhák, 1969). Based on the reports thus far, PEP carboxylase appears to be absent from animal tissues, yeasts and fungi.

2. Regulatory Properties — On the basis of their regulatory properties, PEP carboxylases may be classified into at least three groups. The first group consists of enzymes from E. coli (Canovas and Kornberg, 1966; Canovas and Kornberg, 1965; Izui, 1970) and Salmonella typhimurium (Maeba and Sanwal, 1969; Sanwal and Maeba, 1966a; Sanwal and Maeba, 1966b). These are activated by both acetyl-CoA and fructose-diphosphate, and inhibited by aspartate. The evidence strongly suggests control sites that are non-identical to the substrate binding sites. The literature suggests that the enzymes from Brevibacterium flavum (Ozaki and Shiio, 1969) and Ferrobacillus ferroxidans (Din, Suzuki and Lees, 1967), may also be classified in this group. Another group consists of PEP carboxylases from plant roots, protozoa and some bacteria. These enzymes have been reported to be inhibited by di- or tricarboxylic acids but are not activated by acetyl-CoA, fructose-diphosphate, etc. The enzyme from corn roots (Ting, 1968) is inhibited by L-malate. Other members of this group are the enzymes from Euglena gracilis, which is inhibited by citrate and oxalacetate (Ohmann and Flhák, 1969), that from Acetobacter xylinum, which is inhibited by succinate and ADP (Benziman, 1969) and the enzyme from Acetobacter suboxydans which is inhibited by aspartate (Claus, Orcutt and Belly, 1969). The third group includes the enzyme extracted from spinach which is neither activated nor inhibited by the above mentioned compounds (Izui, Nishikido, Ishihara and Katsuki, 1970).

Others in this group may be the wheat and potato enzyme. The enzyme from Thiobacillus thiooxidans could perhaps be placed in a fourth group as it is activated by acetyl-CoA, but is not inhibited by aspartate (Iszui, Nishikido, Ishihara and Katsuki, 1970) or other dicarboxylic acids.

3. Physico-Chemical Properties — Of the many varieties of PEP carboxylases detected in various organisms the enzyme has been purified to a reasonable extent and studied only from five main sources. Included in the five are peanut (Maruyama, Esterday, Chang and Lane, 1966), potato (Smith, 1968), spinach (Nowak, Miziorko, Bayer and Mildvan, private communication, 1970), E. coli (Smith, 1968) and Salmonella typhimurium (Maeba and Sanwal, 1969). Maruyama et al (1966) did sedimentation studies on purified PEP carboxylase from germinating peanut cotyledon, and showed the presence of a major peak with an $S_{20,w}$ of 13.9, and a faster moving minor peak. The molecular weight of the 13.9 s peak was estimated at 350,000 using gel filtration through Sephadex G-200. Proton relaxation rate studies suggested the presence of six Mn^{2+} binding sites on the enzyme surface (Miller, Mildvan, Chang, Esterday, Maruyama and Lane, 1968). Nowak et al (1970) purified spinach PEP carboxylase and found the enzyme had a molecular weight of 750,000, and 12 tight binding sites for Mn^{2+}. The morphology of the enzyme was found to be similar to glutamine synthetase from E. coli (Valentine, Shapiro and Stadtman, 1968). The $S_{20,w}$ of potato PEP carboxylase was 10 s and the molecular weight was found to be 265,000 g/mole (Smith, 1968). PEP carboxylase from E. coli (Smith, 1971) and S. typhimurium (Maeba and Sanwal, 1969) has been purified to

homogeneity. The molecular weights of both of the enzymes have been determined by the method of Martin and Ames (1961). By this method three forms of the E. coli enzyme with $S_{20,w}$ values of 5.8, 8.4 and 12.2 → 13.2, were found to have respective molecular weights of 94,000 for the monomer, 188,000 for the dimeric form, and 376,000 for the tetramer. Urea (1 M) was found to favour dissociation of the enzyme to a monomeric form (molecular weight 94,000) and Mg^{2+} or aspartate were found to favour association to a tetrameric form (Smith, 1968). In the absence of effectors or cations the dimer was found to be the predominant species. Sucrose gradient centrifugation analysis indicated that the Salmonella enzyme had a tetrameric molecular weight of 198,000. Also, the monomeric molecular weight was determined by SDS gel electrophoresis and amino acid analysis and found to be 49,200 and 49,980 respectively (Maeba and Sanwal, 1969). The E. coli enzyme was further characterized by sedimentation equilibrium studies yielding molecular weights of 402,000 for the tetrameric form, and 99,590 (guanidine hydrochloride) and 107,000 (SDS gel electrophoresis) for the monomeric form of the enzyme (Smith, 1971). The sedimentation coefficient for the E. coli enzyme was 12.2 (Smith, 1971) and 11.8 s - 53 s for the Salmonella enzyme (depending on length of storage and the presence of reducing agents; Maeba and Sanwal, 1969). A tetrameric structure was therefore predicted for both enzymes except that the tetramer from Salmonella was considered to have exactly half of the molecular weight of the Escherichia enzyme (Smith, 1968; 1971; Maeba and Sanwal, 1969).

The Salmonella enzyme has been partially desensitized (Monod, Changeux and Jacob, 1963) with dioxane. In the presence of 10% dioxane,

but not in other solvents such as 2-propanol, ethanol, dimethyl sulfoxide and propylene glycol, the enzyme gets desensitized to the effect of aspartate and fructose diphosphate (Sanwal, Maeba and Cook, 1966). However, Corwin and Fanning (1968) suggested that at saturating concentrations of phosphoenolpyruvate (10 mM), the desensitization effect of dioxane for aspartate was overcome by higher aspartate concentrations. These authors also postulate from their results a stable intermediate form of the E. coli enzyme at half saturating concentrations suggesting the "sequential model" of enzyme action described by Koshland, Nemethy and Filmer (1966). The E. coli enzyme has been genetically desensitized to the effectors dioxane, fructose diphosphate, aspartate and acetyl-CoA (Morikawa, Izui and Katsuki, 1971).

The direct binding of the effectors to the enzyme from either E. coli or Salmonella has not been studied. Part of this thesis is an attempt to clarify the earlier molecular weight assignments of the enzyme from S. typhimurium, and to explain its mode of action by studying the equilibrium binding of acetyl-CoA, aspartate and Mn^{2+} to the enzyme.

MALIC ENZYME

Early investigations of pigeon liver extracts led to the discovery of an enzyme which catalyzed two major reactions (Ochoa, Mehler and Kornberg, 1947; Ochoa, Mehler and Kornberg, 1949; Evans, Vennesland and Slotin, 1943):

1. L-malate + TPN^+ $\xrightleftharpoons{Mg^{2+} \text{ or } Mn^{2+}}$ CO_2 + pyruvate + TPNH

2. oxalacetate $\xrightarrow{Mg^{2+} \text{ or } Mn^{2+}}$ CO_2 + pyruvate

In the course of further work on its diverse catalytic function purified enzyme preparations were found to catalyze three additional reactions (Hsu and Lardy, 1967a; Hsu and Lardy, 1967b; Hsu, 1970):

3. Pyruvate + TPNH $\xrightarrow{Mg^{2+} \text{ or } Mn^{2+}}$ L-lactate + TPN^+

4. oxalacetate + TPNH $\xrightarrow{Mg^{2+}}$ L-malate + TPN^+

5. L-malate $\xrightarrow{Mg^{2+}}$ L-lactate + CO_2

Besides being found in pigeon liver, malic enzyme has been found in turkey liver (Ochoa, Mehler, Blanchard, Jukes, Hoffman and Regan, 1947), rat liver (Shrago, Lardy, Nordlie and Foster, 1963; Mehlman, 1970), mammalian kidney (Green, Loomis and Auerback, 1948), ox brain (Ochoa and Weisz-Tabori, 1948), other animal tissues (Wood, Lifson and Lorber, 1945; Topper and Hastings, 1949), plant sources such as wheat germ, beets, spinach, carrots, parsley root, parsnip and peas (Conn, Vennesland and Kraemer, 1949), the green algae Scendesmus (Bassham, Benson and Calvin, 1950), malate adapted Lactobacillus arabinosus (Korkes, del Campillo and Ochoa, 1950) Hela cells (Barban and Schultze, 1956), Phaseolus vulgaris (Anderson and Evans, 1956), Acaris lumbricoides var suis (Saz and Hubbard, 1957), Lactobacillus plantorum (Nathan, 1960), mammalian arterial tissues (Kirk, 1960), molluscan muscle (Shibata Takeshi, Tadashi Kitahara and Katsuji Yoshimura, 1965), apple fruit (Dilley, 1966), Escherichia coli (Katsuki, Takeo, Kameda and Tanaka, 1967; Stern, 1966), Neurospora crassa (Zink, 1967), in the mitochondria and cytosol of bovine adrenal cortex (Simpson, Crammer and Estabrook, 1968), in a Pseudomonas spp. (Hopper, Chapman and Dagley, 1970), Halobacterium cutirubrum (Cazzulo and Vidal, 1972), maize (Johnson,

1970) and in tubers of Jerusalem artichoke Helianthus tuberosus (Coleman, 1972).

As early as 1958, the purification and some of the properties of the malic enzyme from pigeon liver were described (Rutter and Lardy, 1958). Both the malic oxidative decarboxylation and oxalacetate decarboxylase activities were exhibited with this preparation, TPN^+ analogues were found to be only slightly inhibitory, and the concentration of malate greatly influenced the pH-optimum of malic oxidative decarboxylase activity. These authors also showed the various metal requirements for optimal activity. Later, in 1967, Hsu and Lardy (1967a) described the isolation and crystallization of the same enzyme from pigeon liver. The crystalline protein was found to be homogeneous in the ultracentrifuge, in gradient centrifugation, and in gradient chromatography. An $S_{20,w}$ value of 10.0 was determined for this preparation, and a corresponding molecular weight of 2.8×10^5 assigned to the protein. The binding of reduced triphosphopyridine nucleotides by the pigeon liver enzyme was studied by the fluorescence technique (Hsu and Lardy, 1967c). The purified enzyme was reported to bind TPNH strongly yielding a binding molecular weight of 76,000 g of protein per mole of TPNH indicating 4 binding sites per molecular weight of 2.8×10^5. The dissociation constant for TPNH was found to be 7.5×10^{-7} M. L-malate was demonstrated to bind to the enzyme -TPNH complex in the presence of Mn^{2+} but at a site other than the TPNH binding site. Also, TPN^+ exhibited binding (calculated binding constant 9.7×10^{-7} M) to the malic enzyme in competition with TPNH suggesting the binding of TPN^+ and TPNH at the same site.

Two malic enzymes have been reported in E. coli, a TPN^+specific (Parvin, Pande and Venditasubramanian, 1964; Ashworth, Kornberg and Ward, 1965), and a DPN^+specific (Katsuki, Takeo, Kameda and Tanaka, 1967; Sanwal, 1970b). The TPN^+specific enzyme has been known to occur widely in animals and in plants, whereas the DPN^+specific enzyme has only been found in some lactic acid bacteria grown on malate as the carbon source (Korkes and Ochoa, 1948; Duerre and Lichstein, 1961) and in Shizosaccharomyces pombe grown on grape juice medium (Temperli, Kiinsch, Mayer and Busch, 1965). Also it was reported by Saz and Hubbard (1957) that the enzyme from Acaris lumbricoides responded to TPN^+as well as DPN^+but both activities were probably due to the same enzyme. The enzymes in E. coli, however, occurs as two separate proteins whose activities are differentially affected by the growth conditions of the cells (Katsuki et al, 1967; Murai, Tokushige, Nagai and Katsuki, 1971).

Spina, Bright and Rosenbloom (1970) have more recently purified the TPN^+-linked enzyme from E. coli to a nearly homogeneous state as determined by gel electrophoresis. The molecular weight of the native enzyme as determined by sedimentation equilibrium measurements was found to be 550,000 g/mole having an $S_{20,w}$ value of 17.5. The subunit molecular weight as determined by sedimentation equilibrium measurements was 67,000 g/mole. These results, combined with the amino acid analyses of the protein have resulted in a postulation of an octameric structure for the enzyme. The DPN^+-linked enzyme (Katsuki, Takeo, Kameda and Tanaka, 1967) has been reported to be activated by aspartate (Takeo, Murai, Nagai and Katsuki, 1967) and inhibited by CoA and ATP (Sanwal, 1969). This enzyme has been partially purified from Escherichia coli

and its regulatory properties have been studied (Sanwal, 1969). The results indicated that the inhibitory sites for CoA and ATP were different from each other and that the presence of aspartate reversed the inhibition caused by CoA but not by ATP.

Several theories have been proposed to explain the metabolic function of the TPN^+ specific malic enzyme. According to the more recent reports the possibility of its functioning in the CO_2 fixation process was excluded (Theodore and Englesburg, 1964; Ashworth and Kornberg, 1966; Brice and Kornberg, 1967). The degradation of malate to form acetyl-CoA *via* pyruvate seems like a logical postulation (Jacobson, Bartholomaus and Gunsalus, 1966; Fernández, Medrano, Ruiz-Amil and Lasada, 1967; Zink, 1967). In accord with this idea is the demonstration in this thesis that acetyl-CoA along with TPNH serves as an allosteric inhibitor for the TPN^+-specific enzyme in *E. coli* (Sanwal, Wright and Smando, 1968; Sanwal and Smando, 1969). The physiological role for TPNH production for lipogenesis and other biosynthetic reactions has been postulated elsewhere (Kornacker and Ball, 1965; Young, Shrago and Lardy, 1964). Both the acetyl-CoA and TPNH inhibition may seem as negative type of feedback inhibitions.

In *E. coli*, the existence of a DPN^+-linked enzyme (Katsuki, Takeo, Kameda and Tanaka, 1967) along with a TPN^+-linked enzyme complicates matters somewhat. The fact that the DPN^+-linked enzyme is activated by aspartate (Takeo, Murai, Nagai and Katsuki, 1967) and PEP carboxylase is inhibited by aspartate (Maeba and Sanwal, 1965) and activated by acetyl-CoA (Canovas and Kornberg, 1965) seems to indicate that the DPN^+-linked enzyme plays a role in the decomposition of malate when

aspartate concentrations are high (Sanwal, 1970b; Murai, Tokushige, Nagai and Katsuki, 1971).

The second part of this thesis is concerned with the kinetic studies of the malic enzyme. The TPN^+-specific malic enzyme from Escherichia coli has been partially purified from malate-grown cells and the effect of the inhibitors acetyl-CoA, oxalacetate, TPNH and DPNH studied. From these studies, an ordered mechanism with isomerization of free enzyme was postulated.

MATERIALS AND METHODS

PHOSPHOENOLPYRUVATE CARBOXYLASE

Organism — The bacterial species used during the course of this work was Salmonella typhimurium strain LT_2. Stock cultures were maintained in trypticase soy agar at 4°.

Growth Conditions — When required cells were grown at 37° in a basic minimal salts medium which consisted of

$K_2 H PO_4$	10.5 g
$K H_2 PO_4$	4.5 g
$(NH_4)_2SO_4$	1.5 g
$Mg SO_4$	0.05 g

and glass distilled water to a fianl volume of 1000 ml. This medium was routinely made up as a 20 x concentrated solution (the $Mg SO_4$ was added last, as a solution, after all other solids had been dissolved) and stored at room temperature in the presence of a small amount of chloroform. The medium after dilution was sterilized by autoclaving at 121° for 20 minutes. The carbon source (usually glucose) was prepared and autoclaved separately as a 20% solution and then added to the above to a final concentration of 0.4%.

When larger quantities of cells were required 1.0 liter of an overnight culture was used to inoculate a 20 liter carboy containing 15 liters of the minimal salts — glucose medium. Aeration was achieved by forcing air through sintered glass spargers submerged in the medium. After 12-14 hours of growth this carboy in turn was used as an inoculum for a 200 liter New Brunswick Fermacell Fermentor. After growth in the fermentor at 37° the cells were harvested with an attached Sharples centrifuge, generally in the late log phase of growth. On the average,

1.5 kg of cells were obtained from 200 liters of media. The cells were washed once in 0.05 M Tris-Cl (pH 8.0) and resuspended in the same buffer at a final concentration of 50% wet weight cells. The entire batch of cells was stored at -20° until used.

Assay Procedures — Phosphoenolpyruvate carboxylase was routinely assayed by a coupled spectrophotometric method (assay 1) and occasionally by a procedure which depends on the incorporation of ^{14}C-bicarbonate into oxalacetate (assay 2).

Assay 1: The standard mixture contained 3.3 mM PEP, 10.0 mM $MgCl_2$, 10.0 mM $NaHCO_3$, 0.133 mM DPNH, 18 µg pig heart malate dehydrogenase (specific activity, 720 i.u.) and 0.1 M Tris-Cl (pH 9.0) in a final volume of 3.0 ml. The reagents were added to silica cuvettes of a 1 cm light path and the reaction was started by the addition of a properly diluted sample of phosphoenolpyruvate carboxylase. The rate of the oxidation of DPNH was measured in a Gilford 2400 recording spectrophotometer at 340 nanometers. A summary of the reaction system is given below:

(i) P-enolpyruvate + HCO_3^- $\rightleftharpoons$ Oxalacetate + Pi

(ii) Oxalacetate + DPNH + H^+ $\rightleftharpoons$ Malate + DPN^+

P-enolpyruvate + HCO_3^- + DPNH + H^+ $\rightleftharpoons$ Malate + Pi + DPN^+

Assay 2: The standard mixture contained 10.0 mM $NaHC^{14}O_3$ (0.1 µc/mmole) 10.0 mM $MgCl_2$, 5.0 mM PEP and 0.1 M Tris-Cl, pH 9.0 in a volume of 1.0 ml. The reaction was started at 30°, by the addition of enzyme and terminated after five minutes by the addition of 1.0 ml of 2.0 N H_2SO_4 containing 10 mg/ml carrier oxalacetate. The mixture was then gassed for five minutes with 95% carbon dioxide and aliquots

transferred to scintillation fluid for counting in an Intertechnique scintillation counter. The scintillation fluid (Bray, 1960) contained 7.0 g 2,5-diphenyloxazole, 0.3 g 1,4-bis-[2-(4-methyl-5-phenyloxazole)]-benzene and 100 g naphthalene, in 1 liter of scintillation grade dioxane.

Protein Concentration — This was measured by the absorbancy of solutions at 280 nm using the extinction coefficient $E_{cm}^{1\%} = 14.5$. The coefficient was determined by taking a concentrated sample of the enzyme (10 mg/ml) and dialyzing it against 0.005 M Tris-Cl, pH 8.0 overnight. The next day aliquots from the dialyzed sample were diluted out to various concentrations and pipetted into pre-dried and pre-weighed 6 x 50 mm culture tubes and taken down to dryness over phosphorus pentoxide for a few days. The vials containing the precipitate were again weighed, the precipitates dissolved in a small volume of 0.1 N NaOH, and the absorbance determined at 280 nm for a given weight of protein.

In crude extracts protein was measured by the method of Lowry et al (1951). The purified protein gave almost identical values when its concentration was measured by either the Lowry method, Biuret method (Gornall, Bardawill and David, 1949) or by using the value of the extinction coefficient.

N-Terminal Analysis — The dansyl method (Gray, 1967) for the determination of N-terminal amino acids has been modified for application to long polypeptide chains by Gros and Labouesse (1969). Free amino groups react with 1-dimethyl amino-naphthalene-5-sulfonyl chloride (dansyl chloride), and after acid hydrolysis, the amino acids comprising the protein are released, together with the N-terminal dansyl-amino acid. Thin layer chromatography in two dimensions serves to separate the

dansyl-amino acids which may then be identified by reference to standard dansyl amino acids.

In a thick-walled hydrolysis tube, a sample of the enzyme containing 5 to 10 mμmole protein was taken to dryness in a Buchler rotary evaporator. To the protein, 0.5 ml of an 8 M urea solution (from which cyanate and ammonia had been removed by passage of the solution through a mixed-bed resin, Rexyn 300, Fisher Scientific Co.), 0.15 ml of 0.4 M sodium phosphate buffer, pH 8.2, and 0.25 ml of dimethyl formamide were added with thorough mixing after each addition. A suspension of 5 mg dansyl chloride in 0.1 M acetonitrile was added next, and after mixing, the tube and contents were allowed to stand at room temperature for 30 min. To precipitate the dansylated protein, 10 ml of 10% trichloracetic acid was added, and the fine white precipitate was recovered by centrifugation at 8000 g for 10 min. The residue also contained the excess dansyl chloride, and this was removed by washing with two 3 ml portions of acetone. The protein precipitate aggregated while the dansyl chloride dissolved in the acetone to give a yellow solution. The dansylated protein was dried in a vacuum oven for a few minutes. The protein was then hydrolyzed at 105° in 0.5 ml constant-boiling HCl (under vacuum) for 4 hours. This time was sufficient to release the dansyl-amino acid almost completely, while avoiding degradation which might occur during longer treatment. The hydrolyzate was reduced to dryness and about 30 μl ethyl acetate added to extract the dansyl-amino acid.

Thin layer chromatography was done most conveniently on a sheet of polyamide (Cheng Chin, Trading Co., Taiwan; Woods and Wang, 1967).

A 20-30 µl sample of the ethyl acetate extract was transferred by a microcapillary tube to one corner of the sheet, which was then placed in a glass tank for ascending chromatography. Solvent 1 was a 1.5% formic acid solution, and after drying the sheet for 15-20 min in a stream of air, the sheet was subjected to chromatography at right angles to the first dimension in solvent 2: benzene-glacial acetic acid (9:1). The two solvents were found sufficient to separate the dansyl-amino acids in all samples, so that further chromatography with either solvents was unnecessary. The spot of the dansylated amino acid was visualized in ultraviolet light as a greenish fluorescing compound, the relative position of which on the polyamide sheet was sufficient to identify it by reference to the migration of standard dansyl-amino acids run in the same way. Confirmation was obtained by mixing in a known standard with the sample, and subjecting the total to the first two solvent systems plus a third one (ethyl acetate-methanol-glacial acetic acid (20:1:1)). Thus the spot was positively identified if it was observed to have travelled the same distance as the marker.

Digestion and Separation of Tryptic PEP Carboxylase Peptides — Ten milligrams of PEP carboxylase were dissolved in 0.05 M Tris-Cl buffer containing 8 M urea and 40 mM 2-mercaptoethanol (pH 8.6) and dialyzed against the same buffer overnight to get rid of the small amount of ammonium sulfate in the preparation. This enzyme preparation was then diluted to 2 mg/ml with the urea-containing buffer and iodoacetic acid was added to a final concentration of 0.1 M. The pH was adjusted to 8.6 with sodium hydroxide and kept at this value by periodic additions of alkali during the reaction. The total time of

incubation was 30 minutes. At the end of this period 2-mercaptoethanol was added to a final concentration of 150 mM. The preparation was then dialyzed extensively against distilled water and the carboxymethylated precipitate dried, and subsequently dissolved in 1.0 ml of 0.2 M ammonium bicarbonate buffer, pH 8.5. A small amount of trypsin (0.15 mg) was then added. The pH was maintained by the addition of NaOH. Under these conditions the reaction was completed in one hour when no further change in pH occurred. The pH was adjusted to 6.5 and 2.0 milligram samples chromatographed on Whatmann 3 MM filter paper in n-butanol-acetic acid-water (4:1:5) followed by electrophoresis in pyridine-acetic acid-water (1:10:289) cooled with varsol. The chromatogram was run over night in a descending manner, and the electrophoresis carried out at 2000 volts (drawing 150 ma). At the end of the electrophoresis run a glass rod was passed under the top of the paper, the paper clipped to the rod with stainless steel clips, and then dried in an oven at 70°. A ninhydrin reagent grade spray was used to detect the peptides.

Polyacrylamide Gel Electrophoresis — For monitoring the degree of purity of the enzyme during purification electrophoresis was performed according to the method described by Ornstein (1964) and Davis (1964). Protein samples, 50-200 µg, were used and the gels electrophoresed for about 45 minutes. The gels were normally stained with coomasie blue and destained electrophoretically for about 2 hours in acetic acid-methanol-water (75 ml: 50 ml: 875 ml).

To determine the molecular weight of the protein the method of Shapiro, Vinuela and Maizel (1967) was used. The enzyme sample (20-40 µg) was layered on top of either 5% or 10% acrylamide gels as a 10%

sucrose solution containing a small amount of 2-mercaptoethanol along with enough bromophenol blue or cytochrome c to act as a tracer. Before application, all protein samples were treated at pH 7.0 with 1% SDS and 1.0% 2-mercaptoethanol for 4 hours and then dialyzed overnight against 0.01 M phosphate containing 0.1% SDS and 0.1% 2-mercaptoethanol. Electrophoresis buffer contained 0.1 M phosphate and 0.1% SDS. Current was applied at 8 ma per tube for approximately 2 hours until the marker band had reached the bottom of the tube, at which time the gels were syringed out of the tubes with water and the relative distances of the colored zones and gel lengths measured. The proteins were stained for 2 hours in 0.05% coomasie Blue (made up in 454 ml of 50% methanol and 46 ml of glacial acetic acid) and destained either by passive diffusion or electrophoretically in a solution made up of 75 ml glacial acetic acid, 50 ml 100% methanol and 875 ml water. The protein bands and gel lengths were again measured after destaining (Weber and Osborn, 1969) and the mobility calculated as M:

$$\text{Where } M = \left[\frac{\text{Distance protein migration}}{\text{Distance dye migration}}\right] \left[\frac{\text{Length of gel before staining}}{\text{Length of gel after staining}}\right]$$

To determine the subunit structure of phosphoenolpyruvate carboxylase, the enzyme was cross-linked with dimethyl suberimidate as described by Davies and Stark (1970) and subsequently analyzed on dodecyl sulfate gels as previously described.

Determination of Sedimentation Coefficient — Unless otherwise stated the samples to be analyzed were in 0.05 M Tris-Cl, pH 8.0 buffer and had been dialyzed against the same buffer overnight before use.

The concentration of protein in various samples varied from 1.55 mg/ml to 9.3 mg/ml.

The sedimentation runs were performed in 12 mm double-sector cells in the AnD rotor at 20° with a Beckman model E ultracentrifuge using the Schlieren optical system. Most of the runs were performed at a rotor speed of 44,000 rpms after which approximately 15 photographs were taken at 4 min intervals. The photographic plates were then aligned and analyzed on a Nikon shadowgraph microcomparator and the distance from the outer reference hole to the top of the Schlieren peak was measured at different times. The total distance (x) in cm from the center of the rotor to the peak was then calculated and the sedimentation coefficient (S_{20} obs) computed from the equation (Schachman, 1959a):

$$S_{obs} = \frac{1}{W^2 x}\left[\frac{dx}{dt}\right] = \frac{2.303}{60\omega^2}\left[\frac{d \log x}{dt}\right]$$

where w is the rotor speed in radians per second, t, the time in minutes when the picture was taken, and $\frac{d \log x}{dt}$ is the slope of the line obtained through points plotted as the log x versus time.

The value obtained for S_{obs} is corrected to standard conditions, that is, water at 20° to give a value $S_{20,w}$ by the formula (Schachman, 1959b):

$$S_{20,w} = S_{obs}\left[\frac{Nt}{N_{20}}\right]\left[\frac{Nsol}{N_w}\right]\left[\frac{1 - \bar{v}\,\rho_{20,w}}{1 - \bar{v}\,\rho_{t,sol}}\right]$$

where $\frac{Nt}{N_{20}}$ is the correction of the viscosity of water with temperature

(in this case $\frac{Nt}{N_{20}} = \frac{N_{20}}{N_{20}} = 1$ as runs were performed at 20°), $\frac{Nsol}{N_w}$ is the relative viscosity of solvent to water at temperature t, $\rho_{20,w}$ and $\rho_{20,sol}$ are the densities at 20° of water and solvent respectively in g/ml, and $\bar{v}$ is the apparent partial specific volume of the protein in ml/g. The viscosities were determined in an Ostwald viscometer by measuring the times of outflow of a 10 ml volume of the solution and the densities were determined in a pycnometer at 20°.

Sedimentation Equilibrium — The molecular weight of PEP carboxylase was determined by the meniscus depletion sedimentation equilibrium method, first suggested by Wales, Adler and Van Holde (1951) and treated extensively by Yphantis (1964). This method requires a speed of two to three times that used for conventional sedimentation equilibrium, and as a result, the centrifuge must be operated at a speed sufficiently high that all macromolecular solute is sedimented out of the region of cell near the meniscus. The advantages of this method are the small quantities of material needed (about 30 μg protein/ml solvent) for the run, shorter transient times, and the relative ease in computations of molecular weights. This method eliminates the necessity, due to the higher rotor speeds, for the measurement of the concentration of the solute at the meniscus, the value of which is needed for other methods.

The solutions in the six channel Yphantis type cell used were examined by Rayleigh interference optics. The interference fringes recorded in a photographic plate were measured on a two-dimensional microcomparator in the conventional way. The apparent molecular weight, M_{app}, was calculated from:

$$M_{app} = \frac{RT\ (dc/dx)}{(1 - \bar{v}\rho)\ w^2 xc}$$

where R equals the gas constant, 8.314×10^7 ergs per mole per degree, T equals temperature in absolute degrees, $\bar{v}$ equals partial specific volume of the solute, ρ equals density of the solvent at temperature T, w equals angular velocity in radians per sec., c equals concentration of solution at distance x cm from the center of rotation.

Calculation of the Partial Specific Volume — The partial specific volume, $\bar{v}$ of a solute is defined as the volume change caused by the addition of an infinitesimal amount of the solute to the solution where,

$$\bar{v} = \frac{\delta\ v}{\delta\ m\ \ T\ \rho}$$

The physical counterpart of this is $\bar{v} = \frac{v}{m}$, where m is the mass which causes a volume change, v when added to a large volume of solvent. The partial specific volume of PEP carboxylase was calculated using the relationship (Cohn and Edsall, 1965):

$$v = \frac{\varepsilon\ \ Wi\ \ Vi}{\varepsilon\ \ Wi}$$

where Wi is the weight percent of each amino acid residue in the protein and Vi is the specific volume of an amino acid residue. From the results of the amino acid analysis of PEP carboxylase $\bar{v}$ = 0.730 cc/g.

Electron Microscopy — The electron micrographs were obtained

on a Philips EM 300 electron microscope. The enzyme was first diluted to 50 µg protein per ml in 0.05 M Tris-Cl (pH 8.0) at 23°. One drop of the diluted enzyme was then applied with a platinum loop to a carbon-coated nitrocellulose film on an electron microscope grid which was blotted at the edge with filter paper to give a thin film which was permitted to evaporate for a few seconds. Just before the film dried a drop of the negative stain, 4% phosphotungstate pH 7.0, was added and blotted off. The grids were then examined in the microscope at a magnification of 31,900 - 39,900.

Amino Acid Analysis — The amino acid content of the enzyme was determined by a Beckman 121 C automatic amino acid analyzer. One-half milligram samples of PEP carboxylase were hydrolyzed in vacuo in 6 M HCl for 24, 48 and 72 hours to correct for the destruction of threonine and serine and for the partial hydrolysis of isoleucine and valine. At the end of the hydrolysis times the tubes were opened, the samples dried on a flash evaporator, and subsequently redissolved in 0.55 ml of 0.2 M citrate buffer, pH 2.2. Samples of 0.25 ml were applied to the short and long columns packed and handled according to standard procedures of Spackman, Stein and Moore (1958). The values obtained from the 24, 48 and 72 hour hydrolysates were averaged for each amino acid. For serine and threonine the time of hydrolysis was plotted against corresponding amounts of amino acid and the plot extrapolated to zero time. This value was taken as the content of serine and threonine. For isoleucine and valine the value obtained from the 72 hour hydrolysate was used.

Tyrosine and tryptophan were determined spectrophotometrically by the method of Edelhoch (1967).

G-200 Sephadex Chromatography — A 2.5 x 100 cm column was packed with G-200 Sephadex and was calibrated according to Andrews (1964) for a molecular weight estimation. The void volume was measured by blue dextran and the final elution volume by dinitrophenylalanine. The column was equilibrated with 0.05 M phosphate buffer pH 7.5. The protein standards and PEP carboxylase (each 2 mg) were chromatographed at 4°.

Binding Studies — The binding of radioactive ligands by PEP carboxylase was measured by using dialysis microcells consisting of 2 chambers (capacity, 150 µl per chamber) separated from each other by Visking dialysis membrane (Myer and Schellman, 1962). The membranes were washed at 100° before use once in 0.05 M sodium bicarbonate, once in double distilled water, once in 1 mM ethylenediaminetetracetic acid, and two times in distilled water. The membranes were stored in water at 4° until use. After the assembly of the dialysis cells 100 µl aliquots of the ligand were placed in one compartment and 100 µl of enzyme solution in the other chamber with the help of Hamilton syringes. The entry port-holes were sealed off with Scotch Tape and the cells equilibrated at 4° for 18-30 hours with gentle shaking on a rotary Cole Parmer rotator. At the end of the equilibration times 50 µl samples were withdrawn from each chamber and counted in Bray's (1960) scintillation mixture. Protein quenching, activity and equilibrium controls were set up at the same time. It was found that the protein in the amounts used in our experiments quenched very little if at all in this scintillation fluid and the enzyme remained at least 97% active at the end of the dialysis procedure.

The binding ^{35}S-SDS was measured at room temperature by placing aliquots of the enzyme (0.5 ml samples) in small dialysis bags and dialyzing the contents against 25 ml of radioactive 0.1% SDS buffered with 0.01 M sodium phosphate at pH 7.0 for the required length of time. Because of the slight volume changes during the dialysis procedure the protein concentration inside the bag was determined before and after equilibration by the method of Lowry et al (1951).

Electron Spin Resonance — Before use the enzyme (0.1 mM; calculated on the basis of a molecular weight of 100,000) was dialyzed against 10 mM EDTA for two days followed by a 48 hour dialysis against 2.0 liters of 0.05 M Tris-HCl, pH 8.0 with about 8 changes of buffer during this final dialysis period.

The E.S.R. spectra were obtained at room temperature (24°) on a Varian E-6 spectrophotometer operating at 9.1 GHz (X-band). Each sample (~ 50 µl) was taken up in glass disposable micro-sampling pipets (Corning), which was then inserted into the microwave cavity in a reproducible position.

MALIC ENZYME

Organism — Escherichia coli wild type strain 3,000 was used for the studies unless stated otherwise.

Growth Conditions — For extraction of the malic enzyme the cells were previously grown in 500 ml of the previously mentioned minimal medium supplemented with 1% DL-malate as the sole carbon source. These adapted cells were then inoculated into 15 liter carboys containing minimal medium and 1% DL-malate and aerated at 37° through sintered glass filters for 48 to 72 hours. The cells were harvested by means

of a Sharples centrifuge and washed once with 0.9% NaCl solution and once with 0.05 M sodium phosphate buffer, pH 7.0 containing 10^{-4} M EDTA and 10^{-4} dithiothreitol. A 30% suspension of the cells in phosphate buffer was frozen at -20°. When needed, the cells were thawed at room temperature.

Extracts — Freshly harvested (suspended in 3 volumes of phosphate buffer) or thawed cells were disrupted by sonic oscillation for 15 min at 8-10°. The disrupted cells were centrifuged at 27,000 x g for 30 min and the supernatant solution was used as an enzyme source. To measure the specific activities of malic enzyme under different growth conditions, the extract before use was dialyzed overnight against 0.05 M sodium phosphate buffer, pH 7.0. All operations after the sonic oscillation step were performed at 3-7°. Protein content of the extracts was measured by the method of Lowry et al (1951) with bovine serum albumin as a standard.

Enzyme Assays — Malic enzyme was assayed in a standard reaction mixture containing 1 mM $MnCl_2$, 10 mM sodium L-malate, 0.077 mM TPN^+ and 0.1 M Tris-Cl buffer, pH 7.5. The total volume of the reaction mixture was 3.0 ml and the reduction of TPN^+ measured at 340 mμ in silica cuvettes of 1 cm light path with the use of a Gilford 2400 recording spectrophotometer at 22-24°. One unit of enzyme is defined as the change in absorbance of 1.0 per min.

ANALYTICAL REAGENTS

The chemicals used in this study were standard reagent grade compounds. Calcium phosphate gel, fructose diphosphate, protamine sulfate, streptomycin sulfate and phosphoenolpyruvate used in routine enzyme assays (the trichlorohexylammonium salt) were supplied by Calbiochem. Phosphoenolpyruvate (sodium salt), pig heart malate dehydrogenase, acetyl-CoA and oxalacetate were obtained through Boehringer and Soehne, DEAE-cellulose, DPNH, TPNH, TPN^+ and GTP were purchased from Sigma and cold acetyl-CoA was obtained from P-L Biochemicals Inc., Milwaukee, Wisconsin. Dimethyl suberimidate was prepared according to the method of Davies and Stark (1970). The radioactive compounds ^{14}C-L-aspartate, ^{14}C-acetyl-CoA and ^{14}C-ethylenediaminetetracetic acid were supplied by Amersham/Searle, Des Plaines, Illinois. The purity of acetyl-CoA was checked by chromatography before use and found to be 85% pure. The standard proteins used in the determination of the molecular weight of PEP carboxylase were obtained from the following sources: Ribonuclease, ovalbumin, aldolase and carboxypeptidase were obtained from Worthington; catalase, lysozyme and trypsin from Sigma; glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle pyruvate kinase and yeast alcohol dehydrogenase from Boehringer; bovine serum albumin, from Calbiochem. The molecular weights used for the proteins were those listed in the paper of Klotz and Darnall (1969).

RESULTS ON THE STUDY OF

PHOSPHOENOLPYRUVATE CARBOXYLASE

PHOSPHOENOLPYRUVATE CARBOXYLASE

Purification of PEP Carboxylase — The following procedure adopted for the purification of the enzyme after several trials is a modification of the method of Maeba (1968) and is designed to give consistent results with about 1500 g, wet weight, of cells. Unless stated otherwise, all of the steps given below were carried out at 4°. All buffers and solutions were stored at 4° and contained 1.0 mM EDTA and 40 mM 2-mercaptoethanol (freshly diluted), pH 8.0. In a typical purification run cells of S. typhimurium frozen at -20° (1500 g, wet weight) were thawed out overnight at 4°. The thawed suspension of cells, in 80 ml batches was subjected to sonic oscillation for 15 min using a Bronwill Biosonik III oscillator and the broken cells allowed to stand onvernight at 4°.

The next morning the suspension was centrifuged at 48,000 x g for 30 min to remove the insoluble cell debris. To the cell-free supernatant solution was added with continuous agitation 1/5 of the original extract volume of a 5% streptomycin sulfate (pH 7.0) solution followed immediately by 1/20 volume of 2% protamine sulfate (pH 6.5) suspension. The mixture was stirred for 30 min, and then centrifuged at 48,000 x g for 30 min. The sticky brownish precipitate was discarded and the supernatant fractionated with solid ammonium sulfate. The precipitate obtained in the 36-50% saturation fraction was dissolved in a small volume (1/15 the initial volume) of 0.05 M Tris buffer, stirred for one hour with 2 volumes of 2% protamine sulfate (pH 6.5), and then brought to 40% saturation with solid ammonium sulfate. The creamy colored precipitate was collected by

centrifugation at 48,000 x g and dissolved in 0.01 M Tris-Cl to give a volume equal to 1/15 of the original.

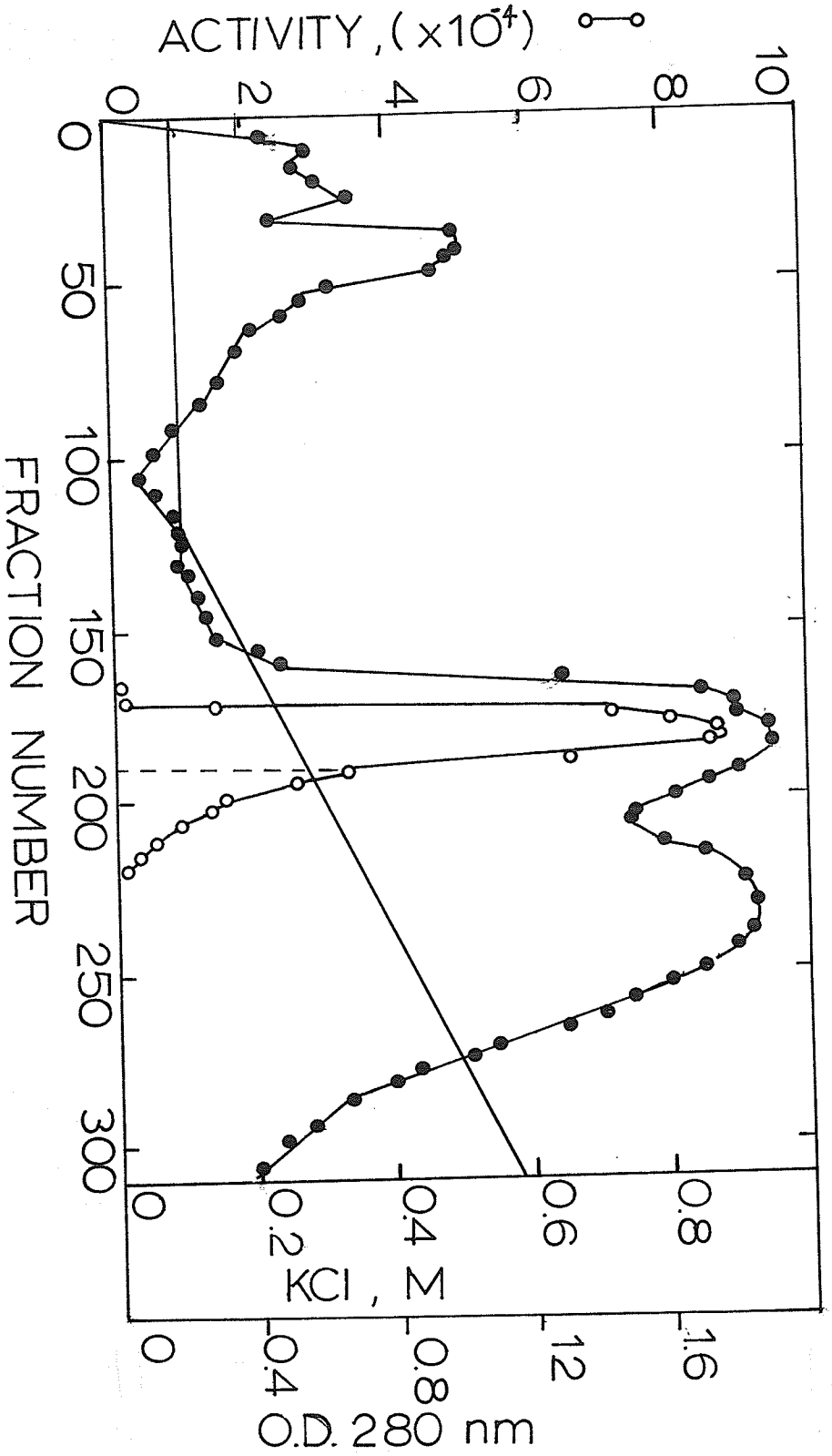
An additional centrifugation was required to remove some insoluble material which precipitates out on standing.

With stirring, a 2:1 (mg:mg) ratio of calcium phosphate gel was added to the enzyme preparation, and the mixture centrifuged at 5,000 x g for 10 min. The yellow supernatant containing no activity was discarded and the gel was washed three times with 0.3 M ammonium sulfate (made up in 0.01 M Tris-Cl) and the enzyme finally eluted out with 0.7 M ammonium sulfate made up in the same buffer. Usually, three elutions were required to recover most of the enzymic activity. The eluted fractions were pooled and then brought to 60% saturation with ammonium sulfate to precipitate the enzyme.

The precipitate from the last step was dissolved in a small amount of buffer and was dialysed overnight in 0.005 M Tris-Cl. It was then applied to a 5.0 x 100 cm DEAE-cellulose column which had previously been equilibrated with the same buffer used in the dialysis step. The column was washed with about 500 ml of 0.1 M potassium chloride made up in the equilibrating buffer and then eluted with a total 3000 ml of a linear gradient of 0.1 → 0.7 M potassium chloride. The enzyme activity appears in fractions from 0.25 → 0.35 M potassium chloride. The elution profile is shown in Fig. 1. The active fractions were pooled and brought to 60% saturation with ammonium sulfate.

The precipitate was then extracted successively with 26%, 21.5% and 18% ammonium sulfate solutions (in 0.05 M Tris-Cl).

Figure 1. Elution profile of PEP carboxylase from a DEAE cellulose column. The optical density of the fractions $\left[\frac{10 \text{ ml}}{\text{tube}}\right]$ is shown by solid circles and enzyme activity by open circles. The solid line indicates the gradient of KCl used to elute the enzyme from the column. The vertical dotted line describes the portion of protein pooled for the next purification step.


ACTIVITY, (x10⁻⁴)
0
2
4
6
8
10
FRACTION NUMBER
0
50
100
150
200
250
300
KCl, M
0
0.2
0.4
0.6
0.8
O.D. 280 nm
0
0.4
0.8
1.2
1.6

Extraction of the enzyme was achieved by suspension of the precipitate at each concentration of ammonium sulfate and centrifugation at 15,000 x g. Three extractions in each of the first two concentrations of ammonium sulfate and about five of the last were required to recover most of the activity. The various extracts were pooled together, and the enzyme was precipitated by adjusting the concentration of ammonium sulfate in the pooled fraction to 60%. The precipitate was dissolved in a minimal quantity of 0.02 M Tris-Cl, pH 8.0.

The enzyme solution (about 20 ml) was then applied to a 5.0 x 100 cm G-150 column that had previously been equilibrated with 0.02 M Tris-Cl buffer. Figure 2 shows the elution pattern of the enzyme from the column. The enzyme from the desired fractions was precipitated with 60% ammonium sulfate. The combined precipitates were extracted with 26, 21.5 and 18% solutions of ammonium sulfate in the same manner as described previously. The eluates were again pooled and the enzyme precipitated with ammonium sulfate as before.

The precipitate was dissolved in the smallest quantity possible of a 10% ammonium sulfate solution (made up in 0.05 M Tris-Cl) and enough of a 26% ammonium sulfate solution was added to bring the final concentration to 16%. The solution was warmed to room temperature and allowed to sit at this temperature for two hours, and then placed in the cold at 4° until required. The purification scheme is shown in Table 1.

Criteria of Purity — The enzyme was judged to be in a high state of purity as revealed by polyacrylamide gel electrophoresis (Plate 1). Only one band was observed in a freshly purified enzyme

Figure 2. Elution pattern of PEP carboxylase from a Sephadex G-150 column. The optical density of the fractions (3.0 ml) is shown by open circles and the enzyme activity shown by solid circles. The pooled fractions are indicated by the vertical dotted lines.

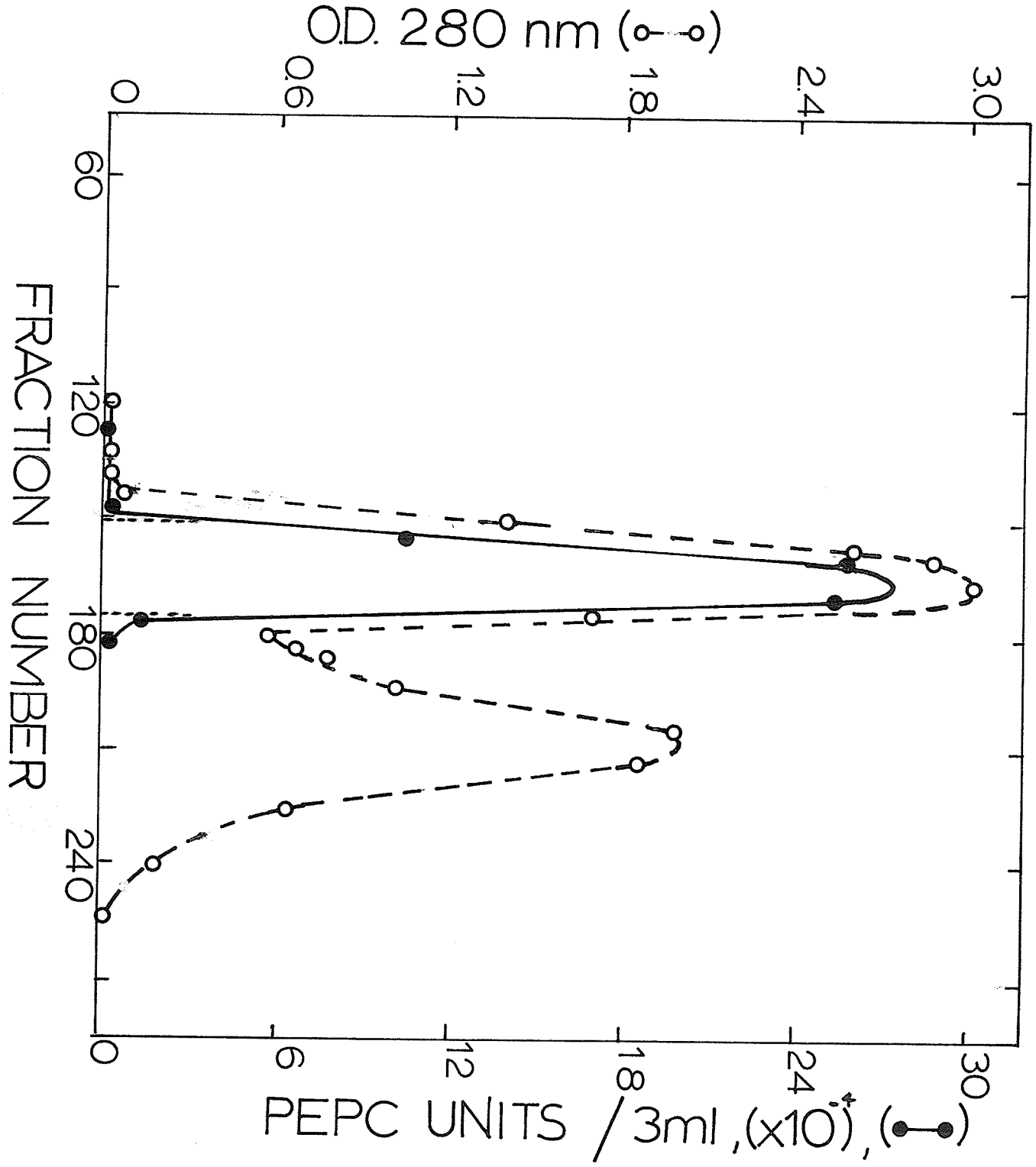

O.D. 280 nm (o—o)
0
0.6
1.2
1.8
2.4
3.0
60
120
180
240
FRACTION NUMBER
0
6
12
18
24
30
PEPC UNITS / 3ml , (x10^{-4}) , (●—●)

TABLE 1

Purification of PEP carboxylase

Step	Volume (ml)	Total Units	Total Protein (mg)	Specific Activity	Purity	Yield (%)
1. Streptomycin Sulfate-Protamine Sulfate	2,280	19,152,000	127,680	149	1.00	100
2. Ammonium Sulfate 0.36 - 0.50% Saturation	208	14,641,600	22,464	650	4.36	76.5
3. Protamine Sulfate-0.4% Ammonium Sulfate	180	11,808,000	14,040	844	5.66	62.0
4. Calcium Phosphate-Dialysis	200	7,440,000	3,900	1,910	12.80	38.9
5. DEAE Cellulose	160	4,608,000	880	5,240	35.20	24.0
6. Ammonium Sulfate Extraction	120	7,040,000	690	10,200	68.50	36.7
7. Sephadex G-150	46	3,280,000	280	11,700	78.60	17.1
8. Ammonium Sulfate Extraction	64	1,520,000	60	25,300	170.00	7.5

Plate 1. Appearance of PEP carboxylase in 0.1% SDS polyacrylamide gels after electrophoresis and staining with coomasie blue ["Methods"]. Approximately 30 μg of the enzyme was applied to the gel. The direction of migration was from top to bottom. The molecular weight of the species in the gel was 100,000 g/mole^{-} (Fig. 16). The smaller component (50,000 g/mole^{-}) cannot be seen in this gel.

preparation on gels, with or without the inclusion of SDS, or with the polarity switched either in the normal or reversed direction. The enzyme also showed only one peak in the Spinco model E analytical ultracentrifuge when viewed by the Schlieren optical system (Plate 2).

Stability — The enzyme was routinely stored as a 16% ammonium sulfate suspension in the presence of 1.0 mM EDTA and 40 mM 2-mercaptoethanol at 4° and was stable for periods up to one month. At this time the loss in activity could be restored by the addition of a freshly prepared solution of 2-mercaptoethanol to the enzyme preparation (Fig. 3).

CHEMICAL PROPERTIES OF THE ENZYME

1. Amino Acid Analyses — The results of the amino acid analysis are given in Table 2 for 24 hr, 48 hr and 72 hr hydrolysis. The protein is rich in aspartic and glutamic acids as well as in amino acids with hydrophobic side chains. This property possibly explains the behaviour of the enzyme reported by others (Sanwal, Maeba and Cook, 1966), viz., that it is activated by hydrophobic reagents such as dioxane as well as carboxyl binding reagents such as polylysine.

2. Amino-Terminal Residue — In order to decide whether the enzyme consisted of identical subunits, PEP carboxylase was treated with dansyl chloride as described in "Methods". A two dimensional chromatogram of the hydrolyzed dansylated protein gave methionine as the only dansylated residue (Fig. 4). It would seem from these results that the enzyme consists of identical subunits. All attempts to determine the carboxyl terminal of the enzyme by using

Plate 2. Schlieren pattern of PEP carboxylase during sedimentation velocity centrifugation in the Spinco Model E ultracentrifuge. The buffer system used was 0.05 M Hepes buffer, pH 8.0, and the concentration of protein was 4.25 mg/ml. The pictures were taken at 4 minute intervals after the rotor had reached the speed of 44,000 rpm. The $S_{20,w}$ of the protein was 11.95. Sedimentation was from left to right.

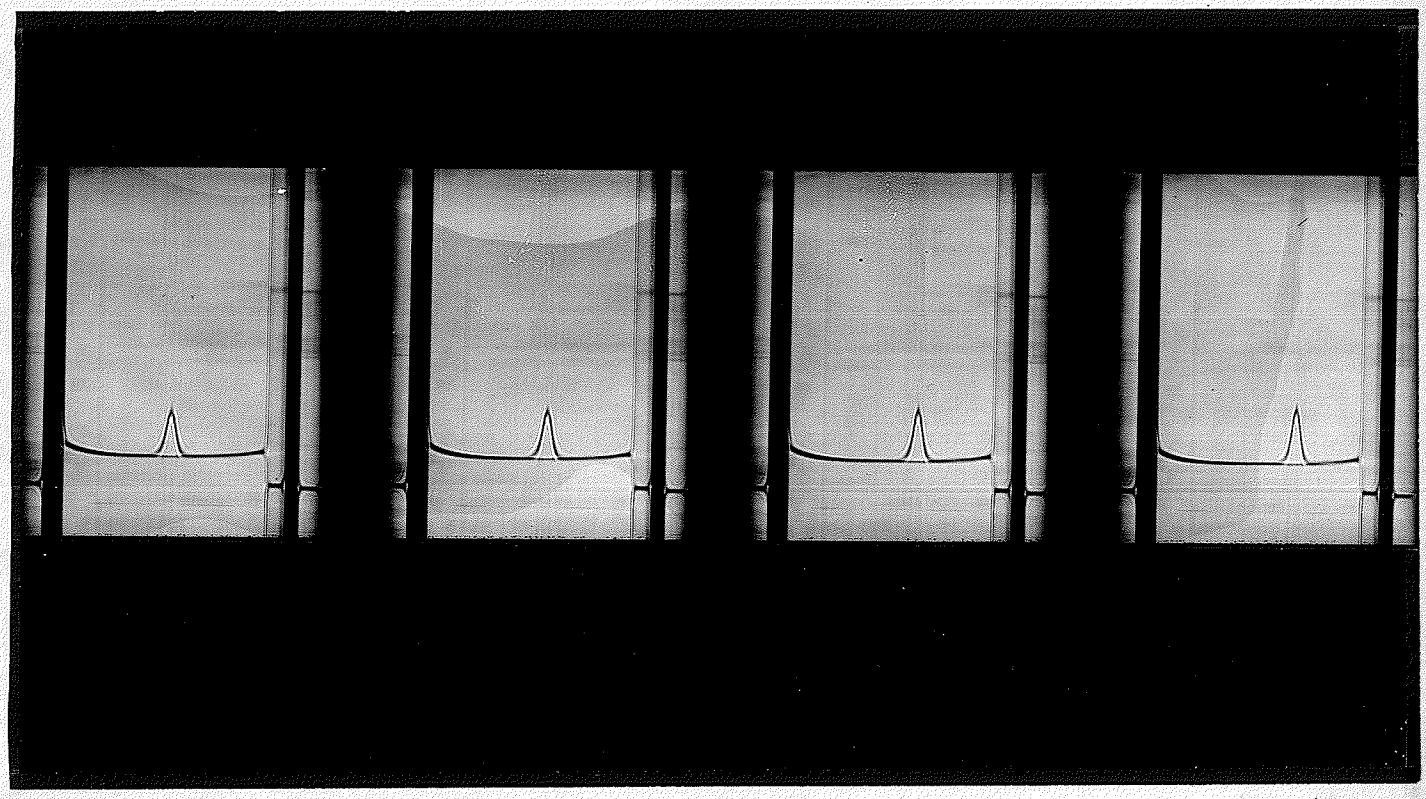


Figure 3. Recovery of PEP carboxylase activity. An aged enzyme preparation (5.0 mg/ml) was allowed to incubate in small plastic Eppendorf centrifuge tubes at 5° in the presence and absence of either 2-mercaptoethanol (ME) or dithiothreitol (DTT). Aliquots of 10 λ were taken out at the indicated times and assayed for activity in the Gilford 2400 spectrophotometer as described in "Methods". The concentrations of reducing agent used are shown above the lines. The bottom solid line represents the control activity found in the absence of reducing agent.

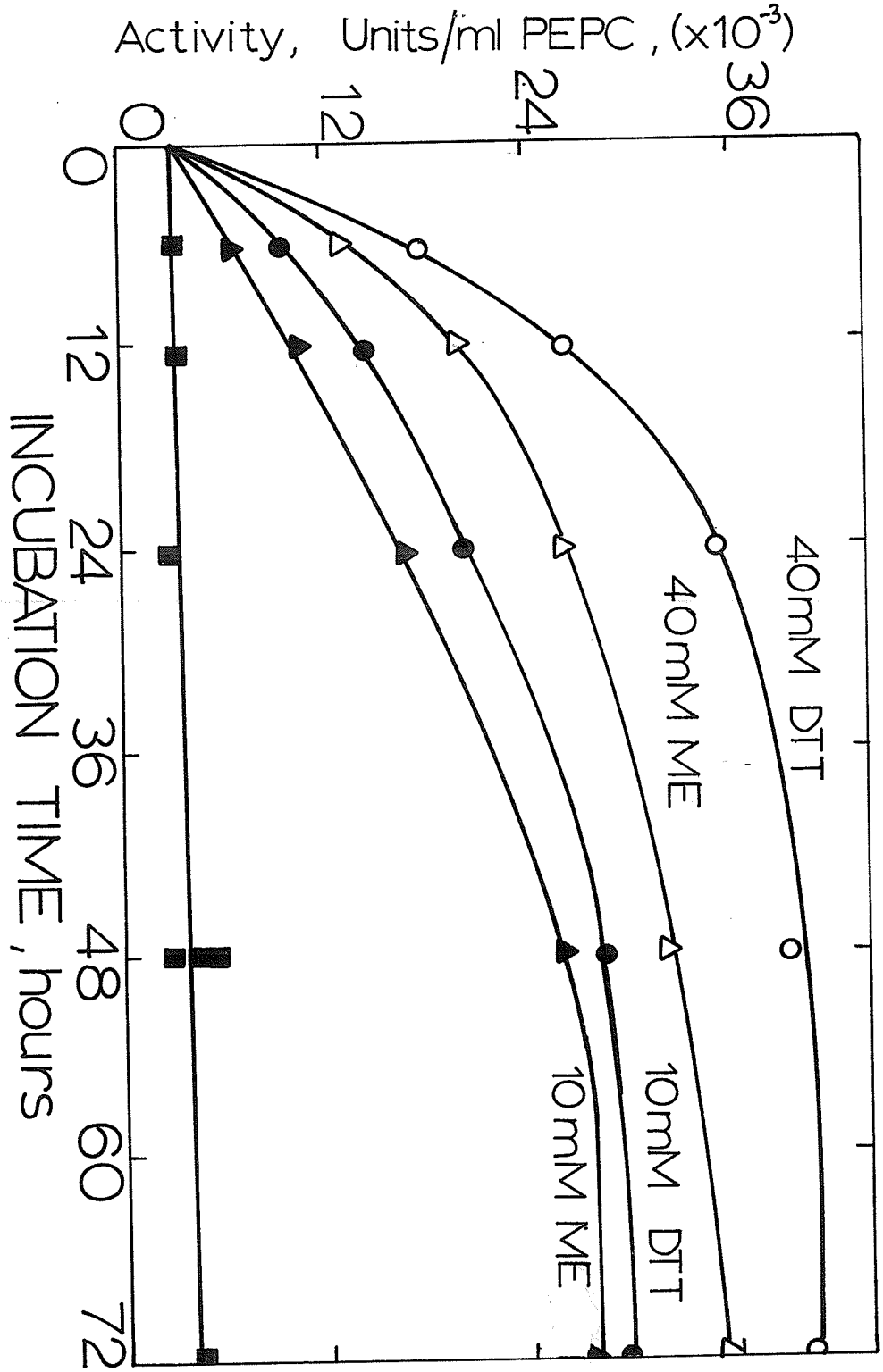

Activity, Units/ml PEPC, (x10⁻³)
0
12
24
36
INCUBATION TIME, hours
0
12
24
36
48
60
72
40mM DTT
40mM ME
10mM DTT
10mM ME

TABLE 2

Amino acid analysis of PEP carboxylase

Residue	Relative Molar Quantities			Average Integer/ 49,000
	Hydrolysis Time			
	24 hr.	48 hr.	72 hr.	
Lysine	103	103	95.19	26
Histidine	17.2	18.7	17.2	5
Ammonia	466	507	403	121
Arginine	90.5	112	102	27
Aspartate	151	148	160	40
Threonine[a]	80.2	78.2	76.2	22
Serine	92.6	86	84.5	25
Glutamate	151	179	173	44
Proline	79.8	79.3	79.3	21
Glycine	102	100	97.0	26
Alanine	131	131	126	34
Valine	85.6	86.9	93.1	25
Methionine	27.2	25.3	44.9	12
Isoleucine[b]	68.6	67.5	76.7	20
Leucine	214	206	214	56
Tyrosine	42.3	43.1	43.1	11
Phenylalanine	33.2	34.8	34.5	9
Cysteine[c]	-	-	-	5
Tryptophan[d]	-	-	-	10
Subunit Molecular Weight				49,348.6

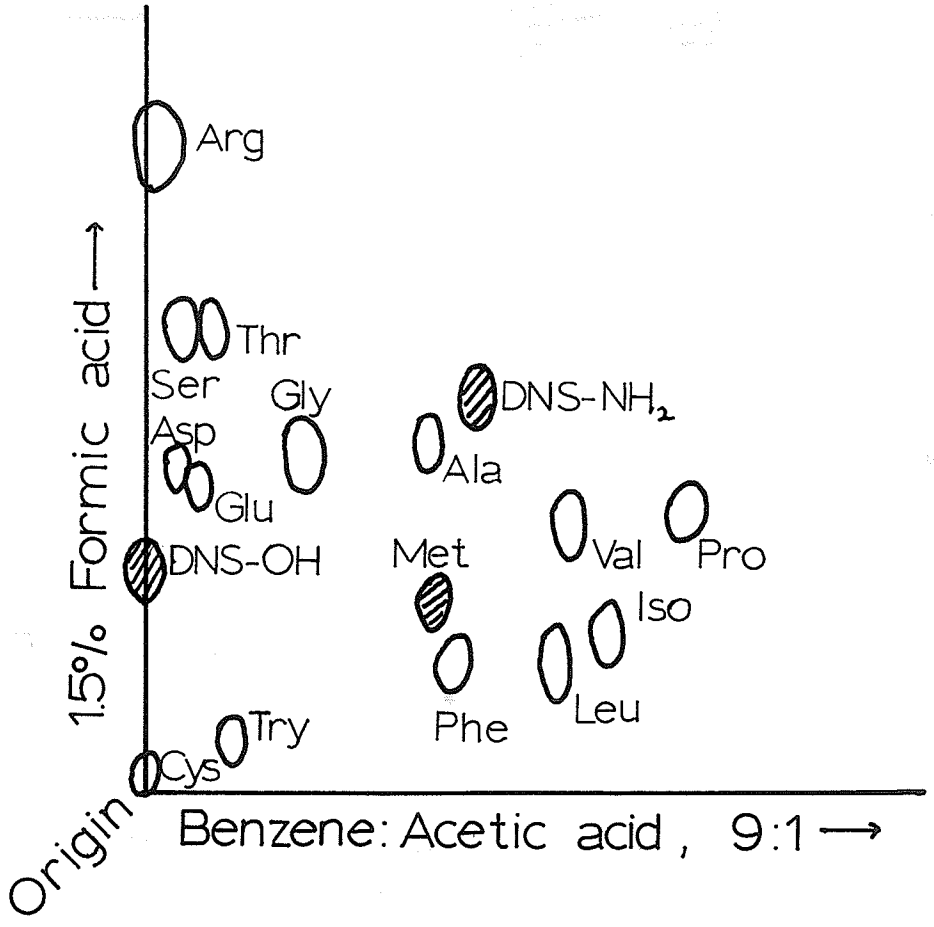
[a] Extrapolated to zero time for average.

[b] Highest value taken for average.

[c] Determined by the method of Spencer and Wold (1969).

[d] Determined by the method of Edelhoch (1967).

Figure 4. N-terminal determination of PEP carboxylase by the dansyl chloride procedure ["Methods"]. Dansyl chloride (DNS-Cl) is 1-dimethylaminonaphthalene-5-sulfonyl chloride. The standard amino acid markers are represented as DNS-amino acids. DNS-OH is 1-dimethylaminonaphthalene-5-sulfonic acid and DNS-NH_2 is 1-dimethylaminonaphthalene-5-sulfonamide. Chromatography was first performed in solvent 1 (formic acid) and after drying, the chromatogram was subjected to chromatography in solvent 2 (benzene-acidic acid) at right angles to the first dimension. The shaded areas are the dansylated products obtained upon completion of the experiment.


Arg
Thr
Ser
Gly
Asp
Glu
DNS-NH2
Ala
DNS-OH
Met
Val
Pro
Iso
Phe
Leu
Try
Cys
Origin
15% Formic acid ⟶
Benzene: Acetic acid , 9:1 ⟶

carboxypeptidase A and B (Neurath, 1960; Folk and Schirmer, 1963; Folk, Piez, Carroll and Gladner, 1960) were in vain.

Peptide Fingerprints — PEP carboxylase was treated as described by Katz, Dreyer and Antinsen (1959). Figure 5 shows the peptide map obtained by this procedure. The relative intensities of the peptides are not indicated in the Figure, but a total of 33 peptides were located on the chromatograms (Fig. 5). This result indicates identity of the subunits, since according to the amino acid analysis 53 moles of lysine plus arginine are obtained per 50,000 gm of protein. If non-identical subunits were present about twice the number of peptides actually found should have been obtained.

PHYSICAL PROPERTIES AND SUBUNIT COMPOSITION

1. Sedimentation Rates — Different concentrations of a freshly purified preparation of PEP carboxylase were used to determine the S value at zero protein concentration. These experiments were performed in order to find whether the enzyme shows an aggregation phenomenon at high protein concentrations such as those used in equilibrium binding studies (described later). It has been reported that aged preparations of PEP carboxylase do show aggregation (Maeba and Sanwal, 1969). Under the conditions used in our experiments, preparations stored for more than a month also at 4° in 16% ammonium sulfate turn gradually turbid due to the formation of large, insoluble aggregates. In fresh preparations of the enzyme, however, the $S_{20,w}$ value is independent of protein concentration (Fig. 6).

The average $S_{20,w}$ value of about 12.25 remains unaltered when centrifugations are performed in the presence of the various

Figure 5. Peptide pattern of a tryptic digest of PEP carboxylase. Descending chromatography was performed first (the direction indicated by arrow) followed by electrophoresis at right angles to the first dimension. (The polarity of the electrodes indicated by + and -). The spots were developed by a reagent grade ninhydrin spray.

♀

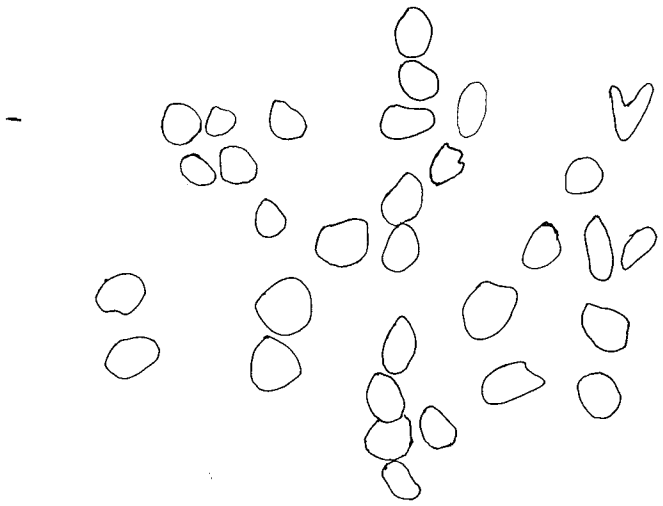
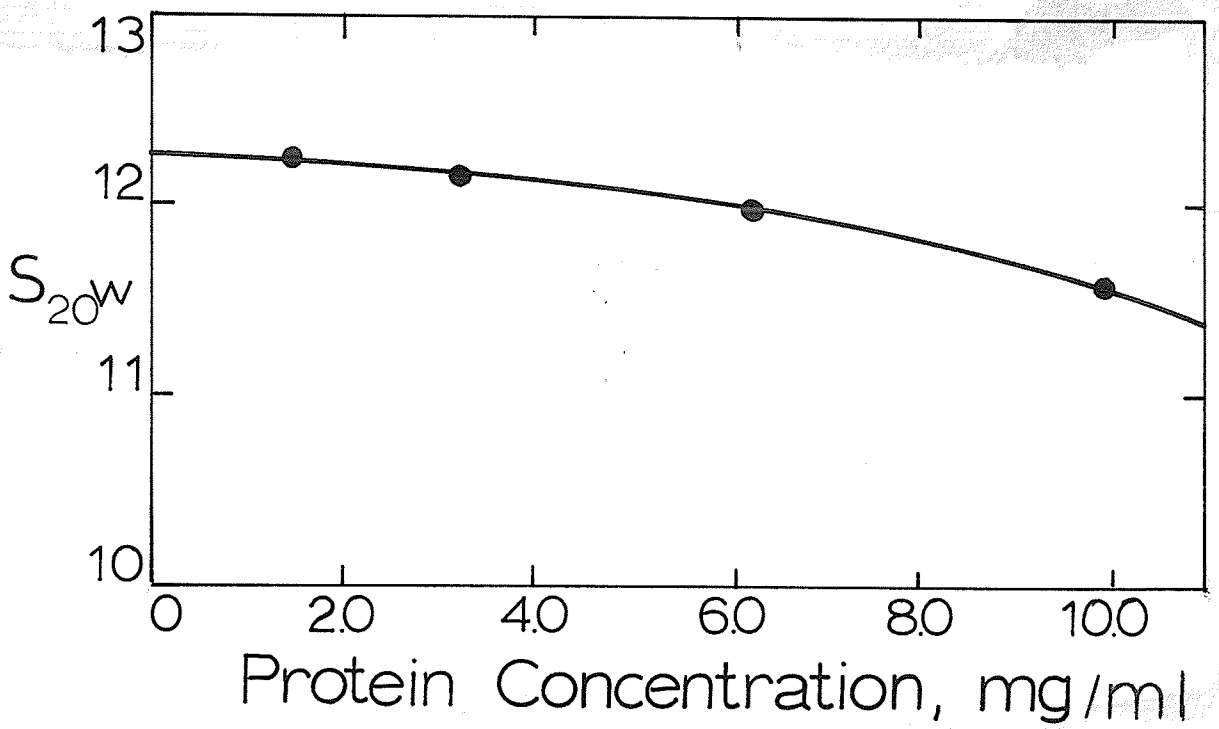


Figure 6. Determination of $S_{20,w}$ at zero protein concentration. The sedimentation run was performed in the Model E ultracentrifuge at 44,000 rpm. The buffer used was 0.05 M Tris-Cl (pH 8.0). The extrapolation to zero protein concentration was done by eye.


13
12
11
10
S_{20w}
0
2.0
4.0
6.0
8.0
10.0
Protein Concentration, mg/ml

effectors of the enzyme (Table 3). These results also militate against the possibility that equilibrium binding curves (presented later) obtained in the presence of various effectors are due to their gross physical effects on the enzyme itself.

2. Molecular Weight of the Enzyme

(i) Sedimentation Equilibrium — Low speed sedimentation equilibrium studies of freshly purified enzyme were performed on PEP carboxylase. Figure 7 shows the Calcomp plots of the molecular weight averages of enzyme samples in the presence (A) and absence (B) of 1% mercaptoethanol. The results in Fig. 7 (A and B) are compatible with the assignment of molecular weight of 380,000 for a homogeneous protein.

Figure 8 shows the Calcomp plots of concentration versus molecular weight data and an ideal two species plot of a run performed at a higher speed (11,299 rpm) than that of Fig. 7 (9,174 rpm) in the presence of 1% 2-mercaptoethanol. It can be seen from Fig. 8 A that there is a considerable heterogeneity in molecular weight species present in contrast to the results of Fig. 7. The two species plot in Fig. 8 B yields intercepts with the hyperbola at molecular weight values of 200,000 and 400,000 for the enzyme.

Figure 9 shows the Calcomp plots of the data obtained from a centrifuge run at 11,299 rpm (same as Fig. 8) without the inclusion of 2-mercaptoethanol. Figure 9 A shows a significant deviation from 380,000 (Fig. 7, A and B) to a possible 100,000 molecular weight species. The x ($\bar{M}_z$) and Δ ($\bar{M}_{z+1}$) values indicate a possible 400,000 molecular weight species, while the * ($\bar{M}_w$) and

TABLE 3

Effect of the various effectors on the sedimentation of PEP carboxylase

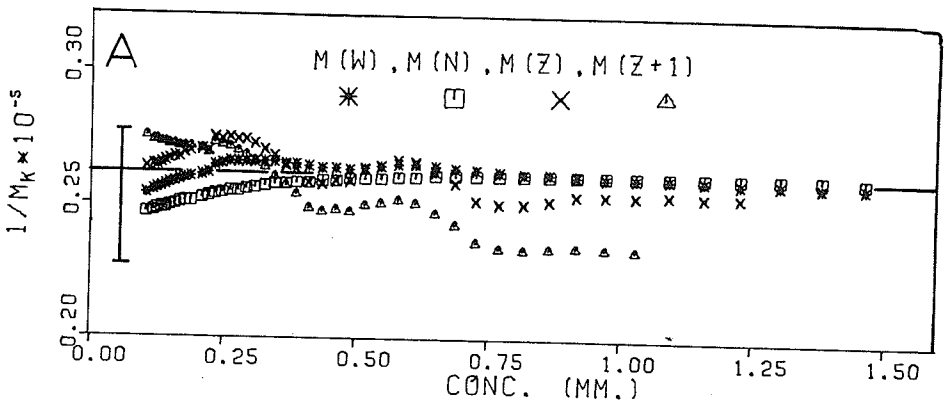
Additions	$S_{20,w}$
-	11.8
20 mM FDP	11.8
1.0 mM Acetyl-CoA	11.7
5 mM Aspartate	11.9
1.0 mM Acetyl-CoA + 1.0 mM GTP	11.9
1.0 mM Acetyl-CoA + 1.0 mM FDP	11.8
10.0 mM $(NH_4)_2SO_4$	12.1

The enzyme at a concentration of 5.0 mg/ml was pre-incubated with the various ligands indicated for 30 minutes, and the runs performed in the model E ultra-centrifuge at 44,000 rpm. The buffer used was 0.05 M Tris-Cl (pH 8.0).

Figure 7. Calcomp plots of the molecular weight averages obtained from the computer program of Roark and Yphantis (1968). The samples (0.5 mg/ml) had been spun at 9174 rpm at 20°. The buffer system used was 0.1 M Tris-Cl, pH 8.0. The molecular weight data are: *, $\bar{M}_w$; □ , $\bar{M}n$; x, $\bar{M}_z$; and Δ , $\bar{M}_{z+1}$.

A. Is with 1% 2-mercaptoethanol.

B. Is without mercaptoethanol. The error bar near the left represents the error on the $\bar{M}n$ value at the lowest protein concentration. (MM = millimeters fringe displacement).


A
M(W), M(N), M(Z), M(Z+1)
1/M_K * 10^-5
0.30
0.25
0.20
0.00
0.25
0.50
0.75
1.00
1.25
1.50
CONC. (MM.)

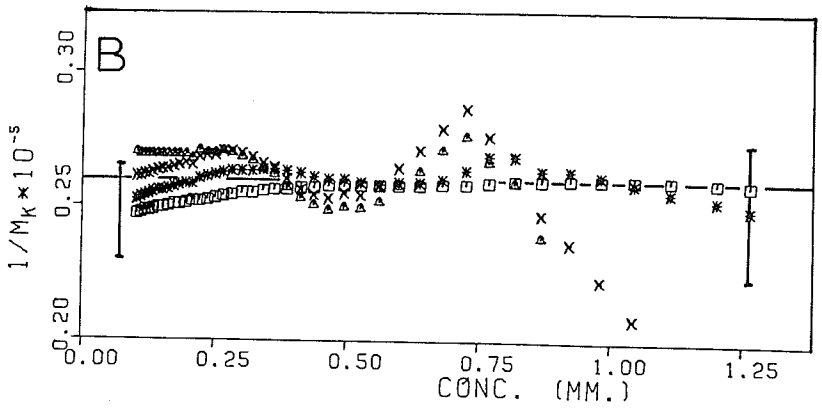

B
1/M_K * 10^-5
0.30
0.25
0.20
0.00
0.25
0.50
0.75
1.00
1.25
CONC. (MM.)

Figure 8. Calcomp plots of the molecular weight averages and the ideal two species data obtained from the computer program of Roark and Yphantis (1968). The samples (0.5 mg/ml) were spun at 11,299 rpm at 20° in 0.1 M Tris-Cl, pH 8.0 buffer containing 1% 2-mercaptoethanol. The molecular weight data are: *, $\bar{M}_w$; □, $\bar{M}_n$; x, $\bar{M}_z$; and △, $\bar{M}_{z+1}$. (MM = millimeters of fringe displacement).

A. The data shows a significant trend away from the molecular weight of 380,000.

B. Shows the two species data: x, $\frac{1}{\bar{M}_n}$ vs $\bar{M}_w$; □, $\frac{1}{\bar{M}_w}$ vs $\bar{M}_z$; *, $\frac{1}{\bar{M}_z}$ vs $\bar{M}_{z+1}$. The two species data is plotted assuming the behaviour of the system is ideal. The curved line is the theoretical hyperbola for a $\frac{1}{M_k}$ vs M_k plot. The straight line gives intercepts with the hyperbola at 400,000 and 200,000.

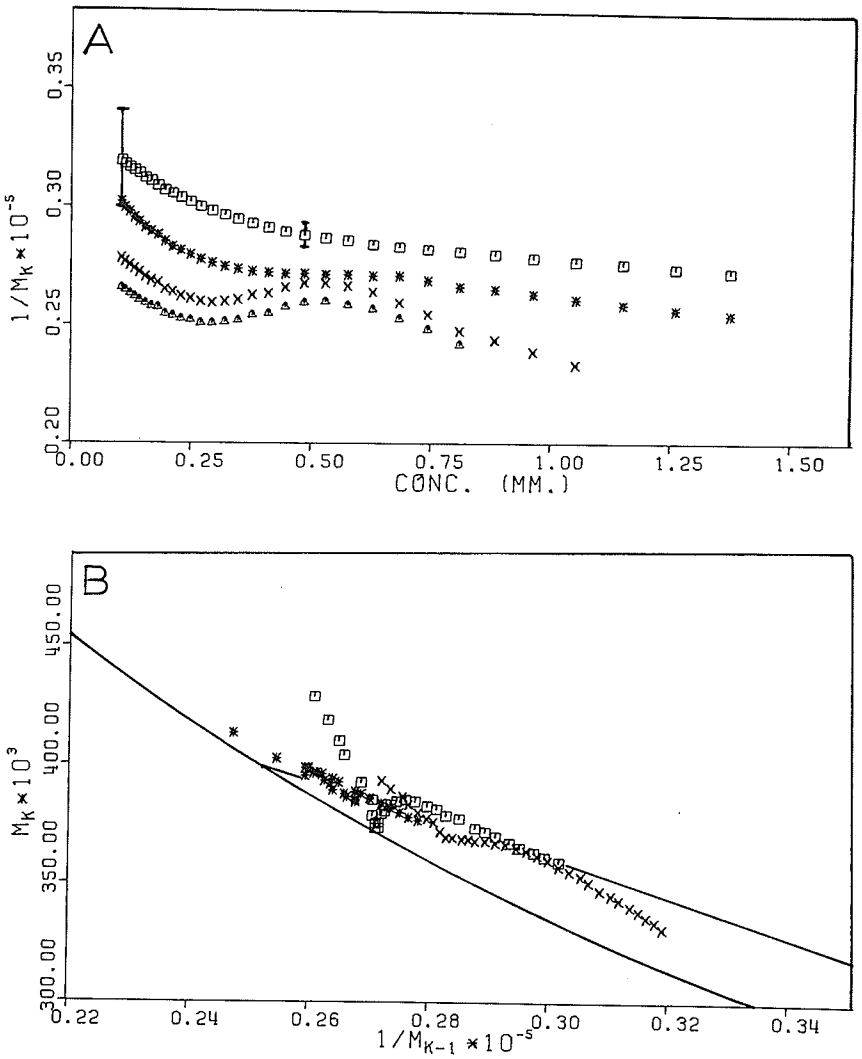
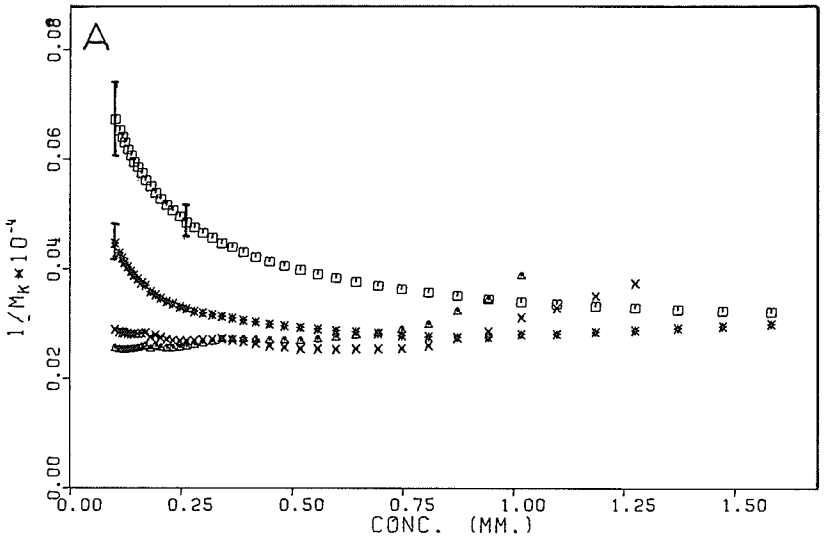
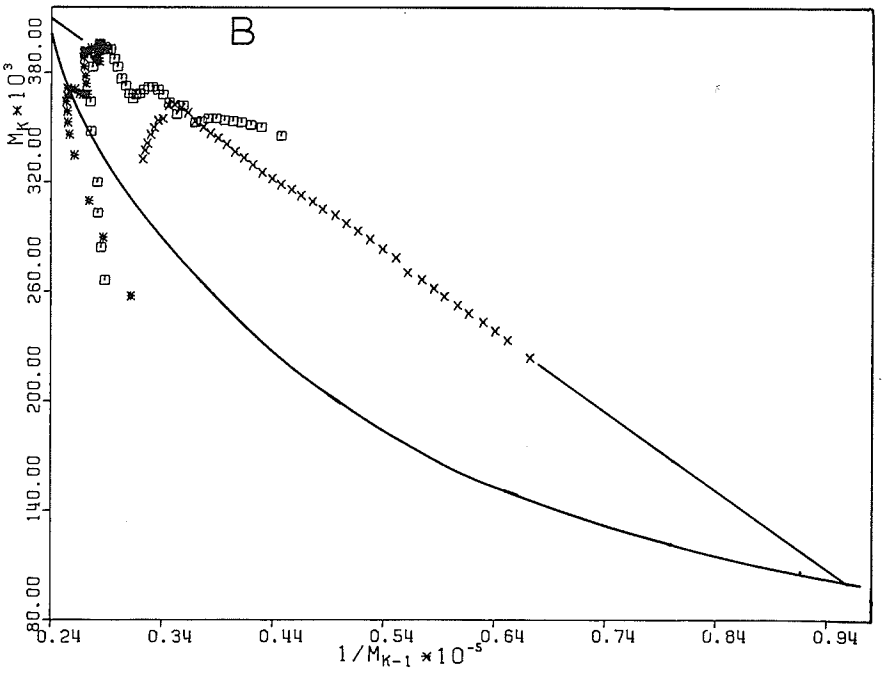

A
0.35
0.30
0.25
0.20
$1/M_K * 10^{-5}$
0.00
0.25
0.50
0.75
1.00
1.25
1.50
CONC. (MM.)
B
450.00
400.00
350.00
300.00
$M_K * 10^3$
0.22
0.24
0.26
0.28
0.30
0.32
0.34
$1/M_{K-1} * 10^{-5}$

Figure 9. Calcomp plots of the molecular weight averages and the ideal two species data obtained from the method of Roark and Yphantis (1968). The sample and speed was the same as that indicated in Fig. 8, only without the inclusion of 2-mercaptoethanol. The symbols are also the same as that indicated in Fig. 8.

A. Concentration (MM = millimeter fringe displacement versus $\frac{1}{M_w}$).

B. Ideal two species plot (same as in Fig. 8).


A
0.08
0.06
0.04
0.02
0.00
$1/M_K \times 10^{-4}$
0.00
0.25
0.50
0.75
1.00
1.25
1.50
CONC. (MM.)


B
380.00
320.00
260.00
200.00
140.00
80.00
$M_K \times 10^3$
0.24
0.34
0.44
0.54
0.64
0.74
0.84
0.94
$1/M_{K-1} \times 10^{-5}$

□ ($\bar{M}_n$) values indicate the smallest molecular weight species (100,000) present in the system. Figure 9 B shows the ideal two species plot for the system. The straight line intersects with the theoretical hyperbola at 420,000 and 104,000 g/mole. This ideal two species plot shows the trend of "hooking" which is a sign of non-ideality. The non-ideal two species plots calculated from the data of Fig. 9 are given in Fig. 10. Although considerable scatter is apparent the data are still compatible with the presence of molecular weight species of 420,000 and 104,000 as observed in Fig. 9.

Figure 11 shows the results of a centrifugation run of PEP carboxylase that was previously treated with 6.3 M guanidine hydrochloride containing 1% 2-mercaptoethanol. Extrapolation to zero concentration gives values of weight-average molecular weights between 47,000 to 53,000 g/mole (Fig. 11 A). These values, however, may be considered an upper limit because guanidinium ion is known to bind to some proteins and thus change the partial specific volume of a protein. For the calculation of data in Fig. 11 we have assumed a partial specific volume of 0.73. The two species plot (Fig. 11 B) of the data intersects with the hyperbola (for $\bar{M}_w = \bar{M}_n$) at two points corresponding to molecular weights of about 46,000 and 103,000 which suggests that the carboxymethylated protein is a two component interacting system.

Figure 12 contains data for PEP carboxylase that had previously been treated with 7.5 M guanidine hydrochloride in 0.1 M Tris-Cl, pH 8.0. The plot in Fig. 12 A again shows considerable heterogeneity and indicates presence of at least two species of the

Figure 10. Calcomp plots of the non-ideal two species plot for data described in Fig. 9. The data are:

$$x,\ (2\bar{M}_w - \bar{M}_z)\ \underline{vs}\ \frac{1}{\bar{M}_n};\ \square,\ \left[\frac{\bar{M}_z}{\bar{M}_w}\ (\bar{M}_z + \bar{M}_w - \bar{M}_z + 1)\right]$$

$$\underline{vs}\ \frac{1}{\bar{M}_{y2}};\ *,\ (2\bar{M}_z - \bar{M}_z + 1)\ \underline{vs}\ \left[\frac{2}{\bar{M}_w} - \frac{1}{\bar{M}_z}\right].$$

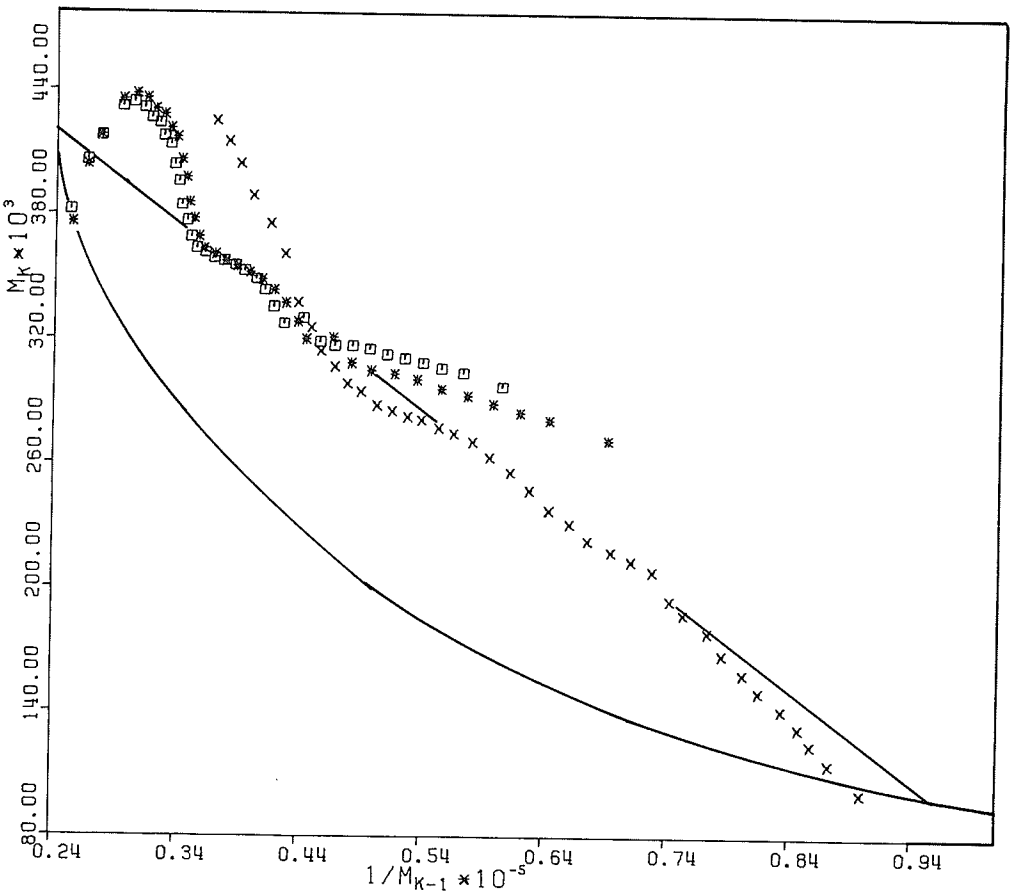
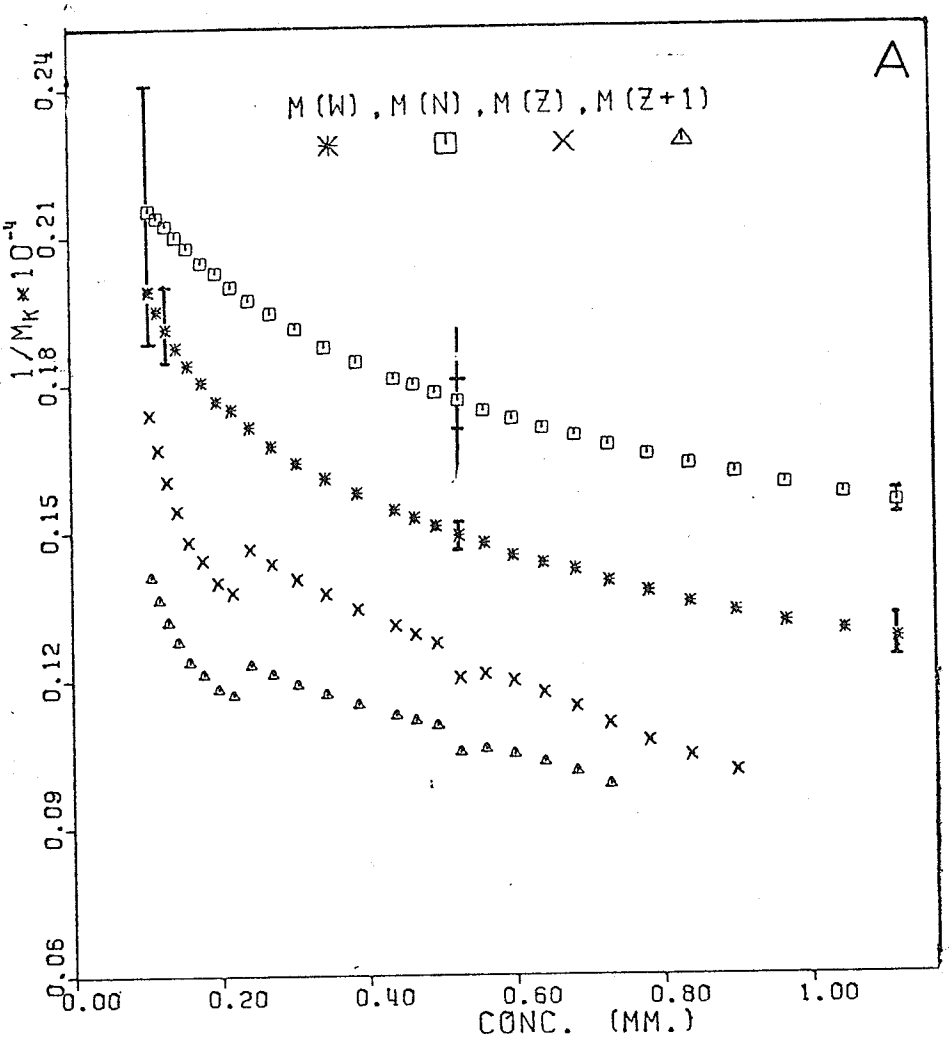

440.00
380.00
320.00
260.00
200.00
140.00
80.00
$M_K * 10^3$
0.24
0.34
0.44
0.54
0.64
0.74
0.84
0.94
$1/M_{K-1} * 10^{-5}$

Figure 11. Calcomp plots of sedimentation equilibrium results as computed by the method of Roark and Yphantis (1968). The native protein was carboxymethylated and dissociated in 6.3 M guanidine-HCl.

A. $\frac{1}{M_k}$ versus concentration plots (MM = millimeters of fringe displacement).

B. A two-species plot according to Roark and Yphantis (1968) for an ideal interacting two species system. The hyperbola is calculated for $M_{(N)} = M_{(W)} = M_{(Z)}$. The protein samples (0.4 mg/ml) were spun at 29,851 rpm at 20°.


A
M(W), M(N), M(Z), M(Z+1)
$1/M_K * 10^{-4}$
0.24
0.21
0.18
0.15
0.12
0.09
0.06
0.00
0.20
0.40
0.60
0.80
1.00
CONC. (MM.)

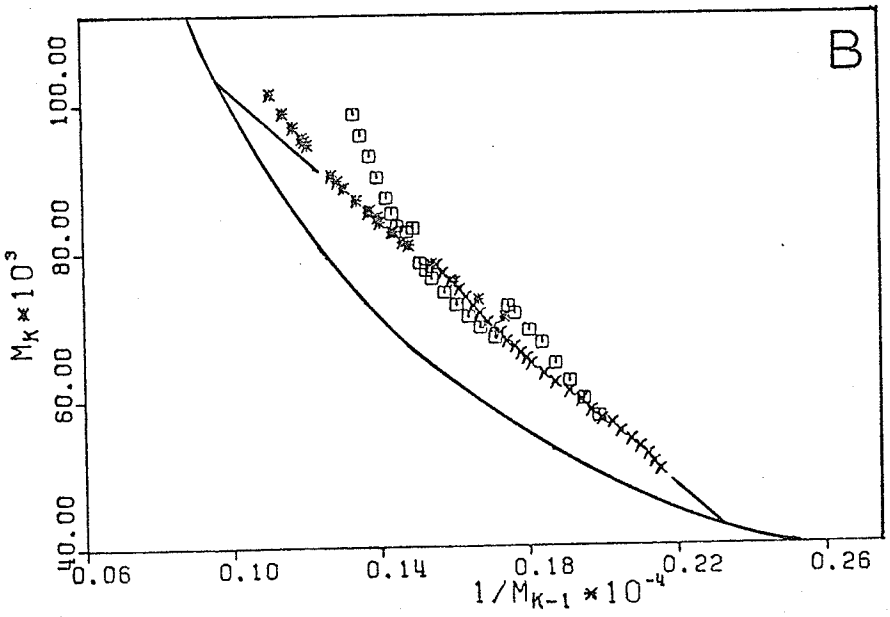
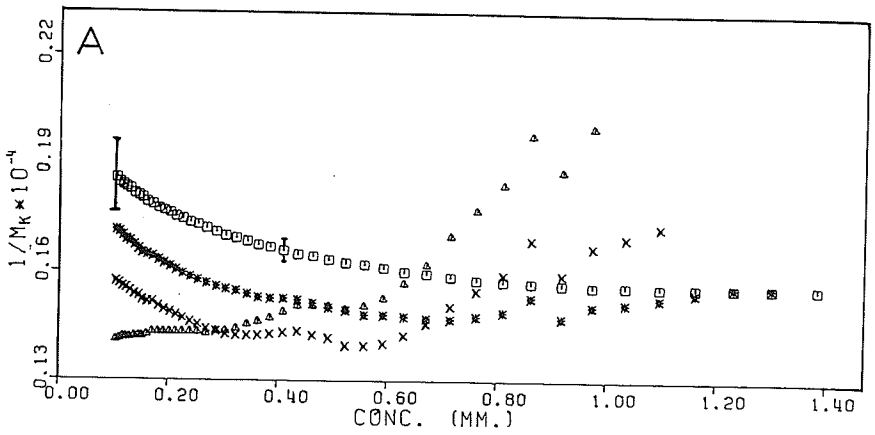
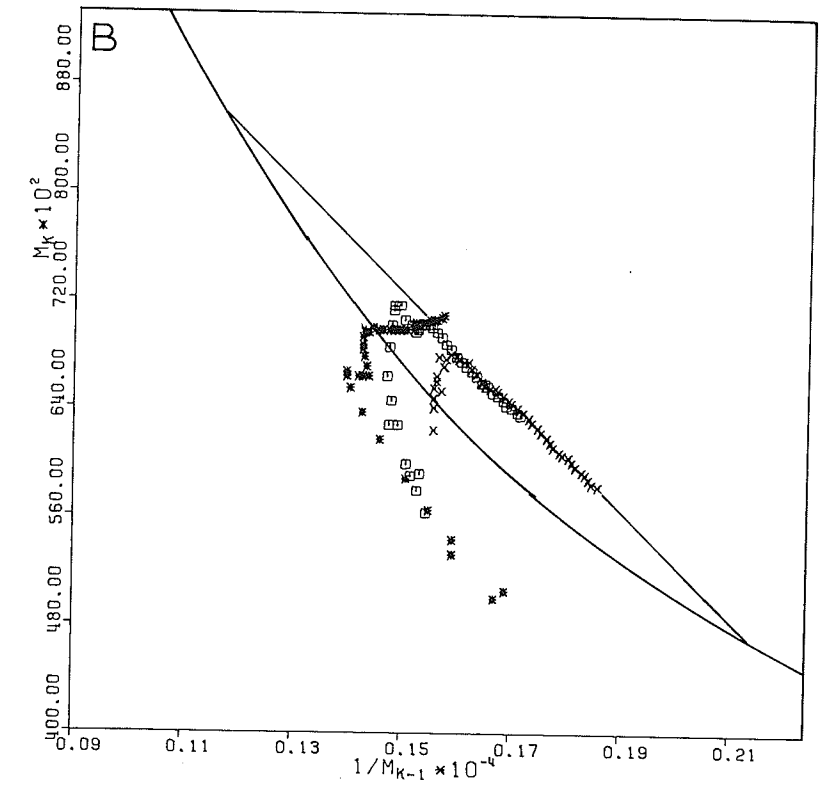

B
$M_K * 10^3$
100.00
80.00
60.00
40.00
0.06
0.10
0.14
0.18
0.22
0.26
$1/M_{K-1} * 10^{-4}$

Figure 12. Calcomp plots of the molecular weight data obtained by the method and computer program of Roark and Yphantis (1968). The sample (0.4 mg/ml) had been dialyzed against 7.5 M guanidine hydrochloride in 0.1 M Tris-Cl, pH 8.0. The symbols are the same as in Figure 8.

A. The concentration plot shows that there is non-ideality and probably two species present in the system.

B. Two species plot of the data shown in Fig. 12 A.


A
0.22
0.19
0.16
0.13
$1/M_K * 10^{-4}$
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
CONC. (MM.)


B
880.00
800.00
720.00
640.00
560.00
480.00
400.00
$M_K * 10^2$
0.09
0.11
0.13
0.15
0.17
0.19
0.21
$1/M_{K-1} * 10^{-4}$

enzyme. The two species plot shown in Fig. 12 B also indicates non-ideality in the system. The straight line of the data intersects with the theoretical hyperbola (M_K vs $\frac{1}{M_K}$) at two points yielding molecular weights for the two interacting species of 87,200 and 47,500.

Figure 13 shows the non-ideal Calcomp plot of the data described in Fig. 12. The concave down plots again indicate a non-ideal interacting system in 7.5 M guanidine hydrochloride. The intersecting line yields species with molecular weights of 87,200 and 47,500 in addition to molecular weight species of 147,000 and 52,000.

Figure 14 shows the Calcomp plots for the data obtained for a PEP carboxylase sample that was dialyzed against 0.1% SDS in 0.1 M sodium phosphate buffer, pH 8.0. Figure 14 A indicates the presence of a two species, interacting system and Fig. 14 B clearly shows the presence of two species with molecular weights of 45,000 and 130,000.

(ii) G-200 Sephadex Chromatography — Using a column calibrated according to the method of Andrews (1964) the estimated molecular weight of native, freshly prepared PEP carboxylase was found to be about 400,000. Using the method of Ackers (1964) the Stoke's radius was calculated to be 73 Å. Results of these experiments are summarized in Fig. 15. A smaller molecular weight species evident in ultracentrifugation experiments (Figs. 8, 9 and 10) is not discernible here.

(iii) Polyacrylamide Gel Electrophoresis — Electrophoresis was performed in SDS-containing gels at 5% and 10%

Figure 13. Calcomp plot of the non-ideal two species data from the sample described in Fig. 12. The symbols are as in Fig. 10.

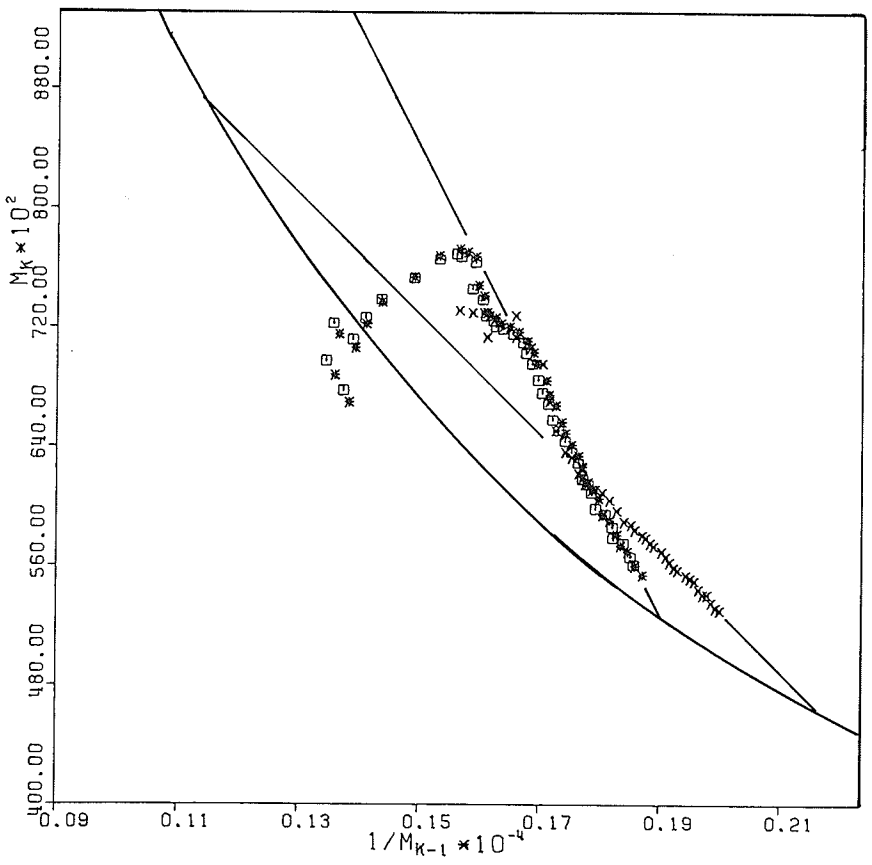

400.00
480.00
560.00
640.00
720.00
800.00
880.00
$M_K * 10^2$
0.09
0.11
0.13
0.15
0.17
0.19
0.21
$1/M_{K-1} * 10^{-4}$

Figure 14. Calcomp plots for molecular weight data obtained by the method of Roark and Yphantis (1968). The sample was dialyzed against 0.1% SDS in 0.1 M sodium phosphate buffer, pH 8.0 and was spun at 26,000 rpm at 20°. The symbols are as in Fig. 7 for (A) and Fig. 10 for (B).

A. Shows the concentration plots of the data.

B. Shows the non-ideal two species plot.

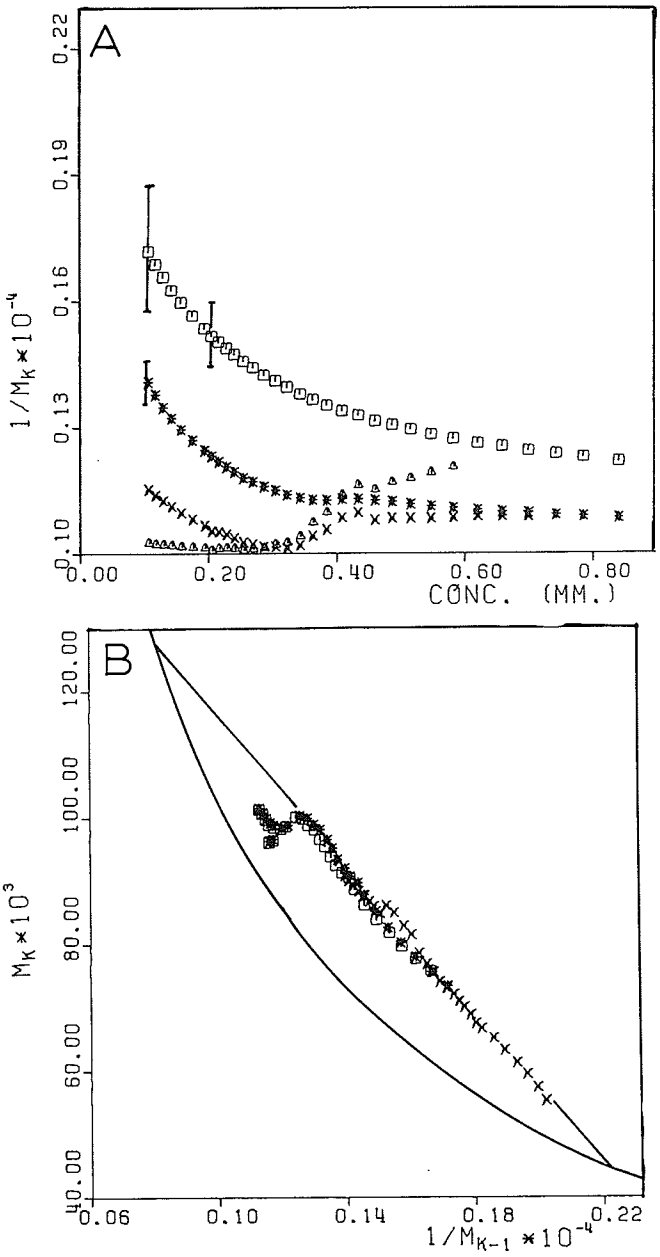
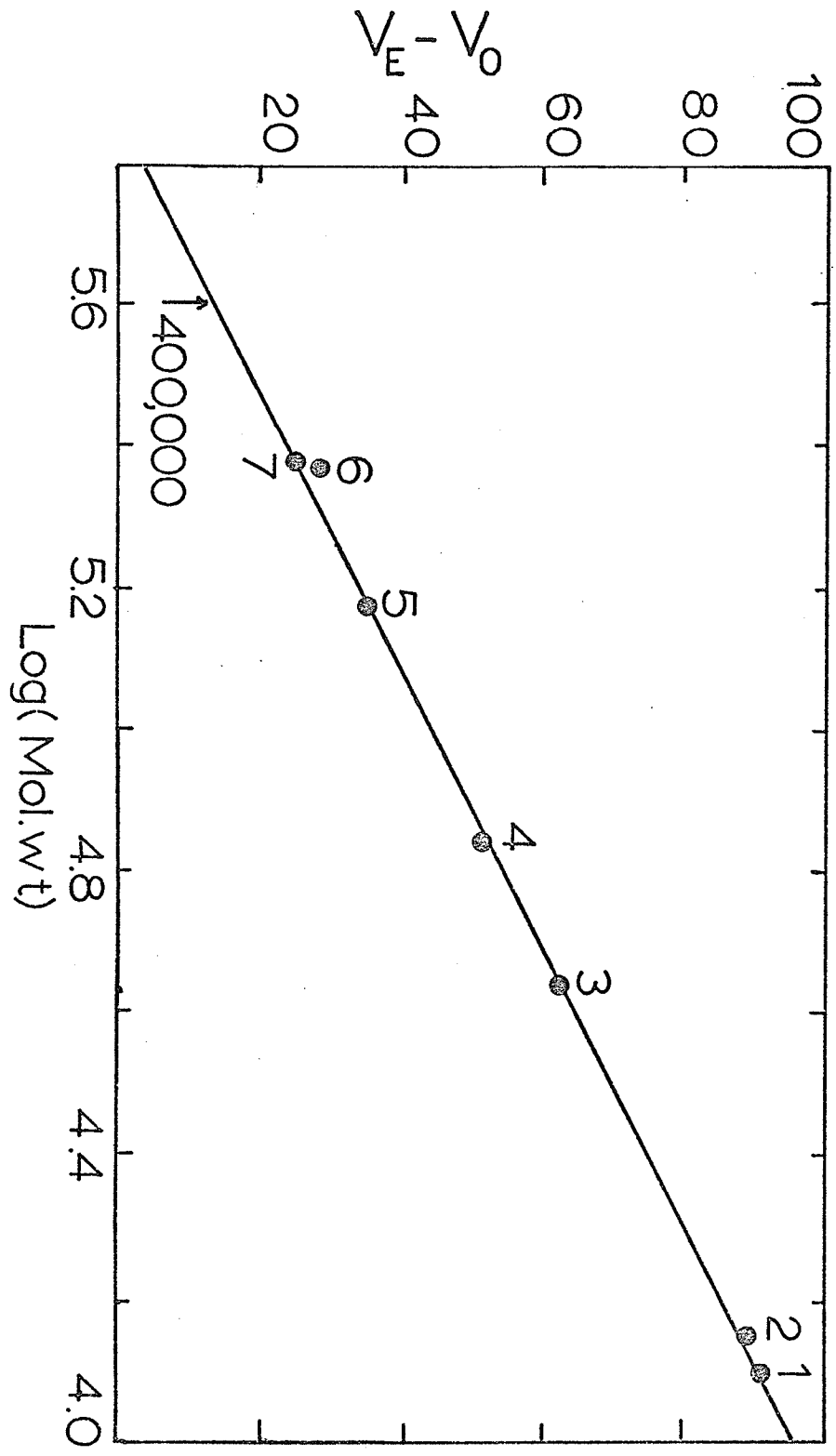

A
0.22
0.19
0.16
0.13
0.10
$1/M_K * 10^{-4}$
0.00
0.20
0.40
0.60
0.80
CONC. (MM.)
B
120.00
100.00
80.00
60.00
40.00
$M_K * 10^3$
0.06
0.10
0.14
0.18
0.22
$1/M_{K-1} * 10^{-4}$

Figure 15. Gel filtration of PEP carboxylase on Sephadex G-200. The arrow indicates the PEP carboxylase peak. Details are described in "Methods". The numbers over the points denote: 1, cytochrome c; 2, ribonuclease; 3, ovalbumin; 4, bovine serum albumin; 5, alcohol dehydrogenase; 6, catalase; and 7, rabbit muscle pyruvate kinase.


$V_E - V_0$
20
40
60
80
100
5.6
5.2
4.8
4.4
4.0
Log(Mol.wt)
400,000
7
6
5
4
3
2
1

polyacrylamide (Fig. 16) concentrations, as described under "Methods". PEP carboxylase exhibited a major band with a molecular weight of 100,000 and a fainter, minor band with a molecular weight of 50,000. This suggests, in conformity with the results obtained in sedimentation equilibrium experiments, that the native enzyme breaks down only partially in the detergent. Carboxymethylated PEP carboxylase behaved in the same way in SDS gels as the untreated enzyme.

3. Subunit Composition — Results described before suggest that PEP carboxylase in a native form is an octamer of a basic subunit whose molecular weight is around 50,000. Judging from the results of NH_2-terminal analysis the monomers seem to be identical. To confirm conclusions drawn from hydrodynamic studies, the cross-linking procedure of Davies and Stark (1970) was utilized to determine the subunit structure. In 5% SDS-polyacrylamide gels, samples of PEP carboxylase (0.33 mg/ml) previously cross-linked with three concentrations of dimethylsuberimidate (0.025, 0.25 and 1.25 mg/ml) showed, after dissociation and electrophoresis, 4 major bands and a few minor ones (Plate 3). The three ratios of protein to cross-linker did not appear to alter the principal species detected. The approximate molecular weight of the fastest moving major band (Plate 3) was 100,000 as judged by its mobility relative to standard protein markers (Fig. 16). The molecular weights of the other three major bands could not be calculated from the standards used because of their high molecular weights. In general, the results obtained here are consistent with results obtained with unlinked PEP carboxylase (Plate 1; Fig. 16). It does seem that it is hard to dissociate the dimer of 100,000 molecular weight in SDS.

Figure 16. Semilog plot of the monomer molecular weights (obtained by the dodecyl sulfate-polyacrylamide gel electrophoresis procedure) against distance of migration relative to the marker dye. The distances migrated by PEP carboxylase are marked by arrows. The concentration of polyacrylamide in the upper line was 5% and in the lower 10%. The numbers over the points denote: 1, phosphorylase; 2, bovine serum albumin; 3, catalase; 4, ovalbumin; 5, aldolase; 6, glyceraldehyde-3-phosphate dehydrogenase; 7, trypsin; and 8, lysozyme.

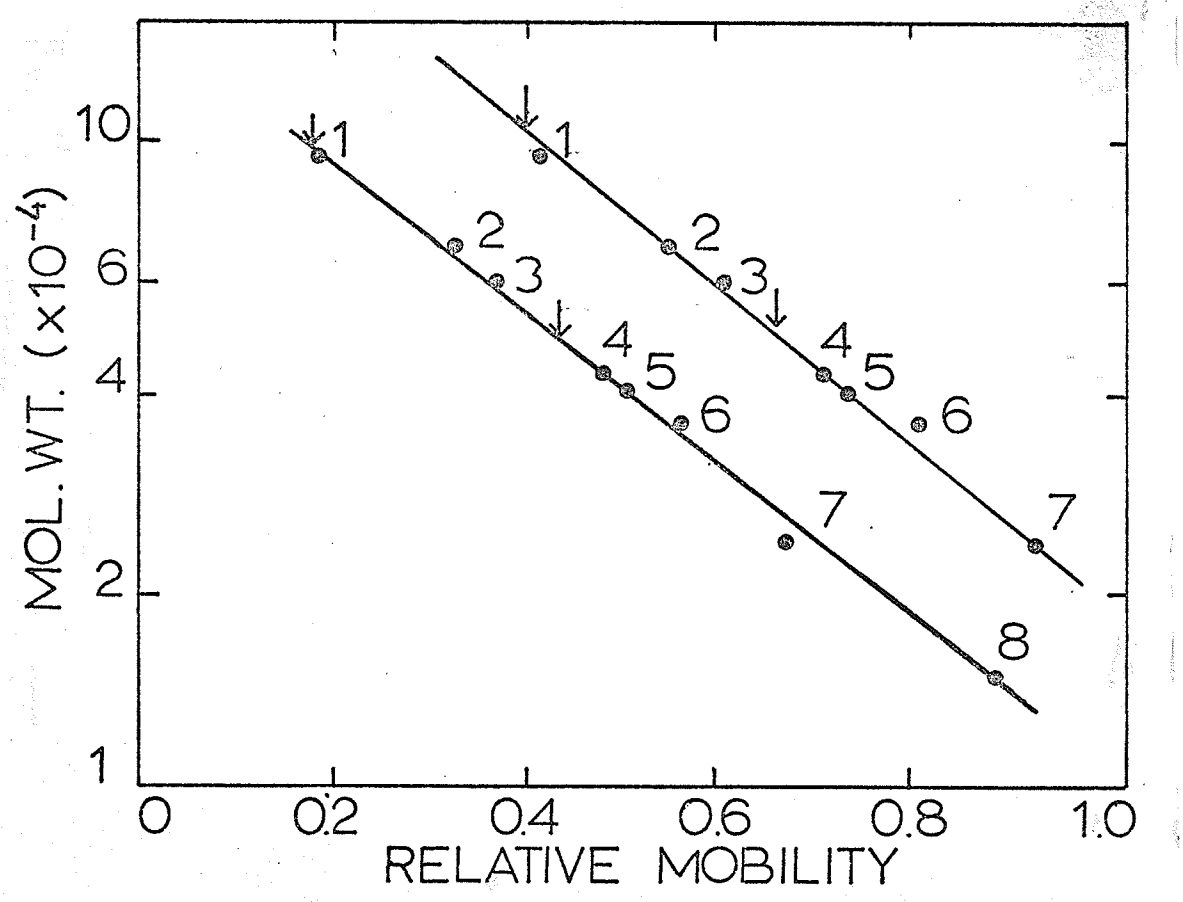
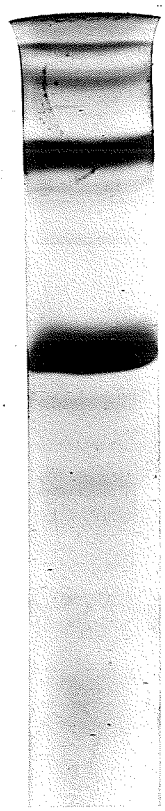

MOL. WT. (x10⁻⁴)
10
6
4
2
1
0
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY
1
2
3
4
5
6
7
8

Plate 3. The location of the subunits of PEP carboxylase in sodium dodecyl sulfate-polyacrylamide (5.0%) after cross-linking with dimethyl suberimidate. The concentration of dimethyl suberimidate used here was 0.25 mg/ml and the concentration of PEP carboxylase was 0.33 mg/ml.



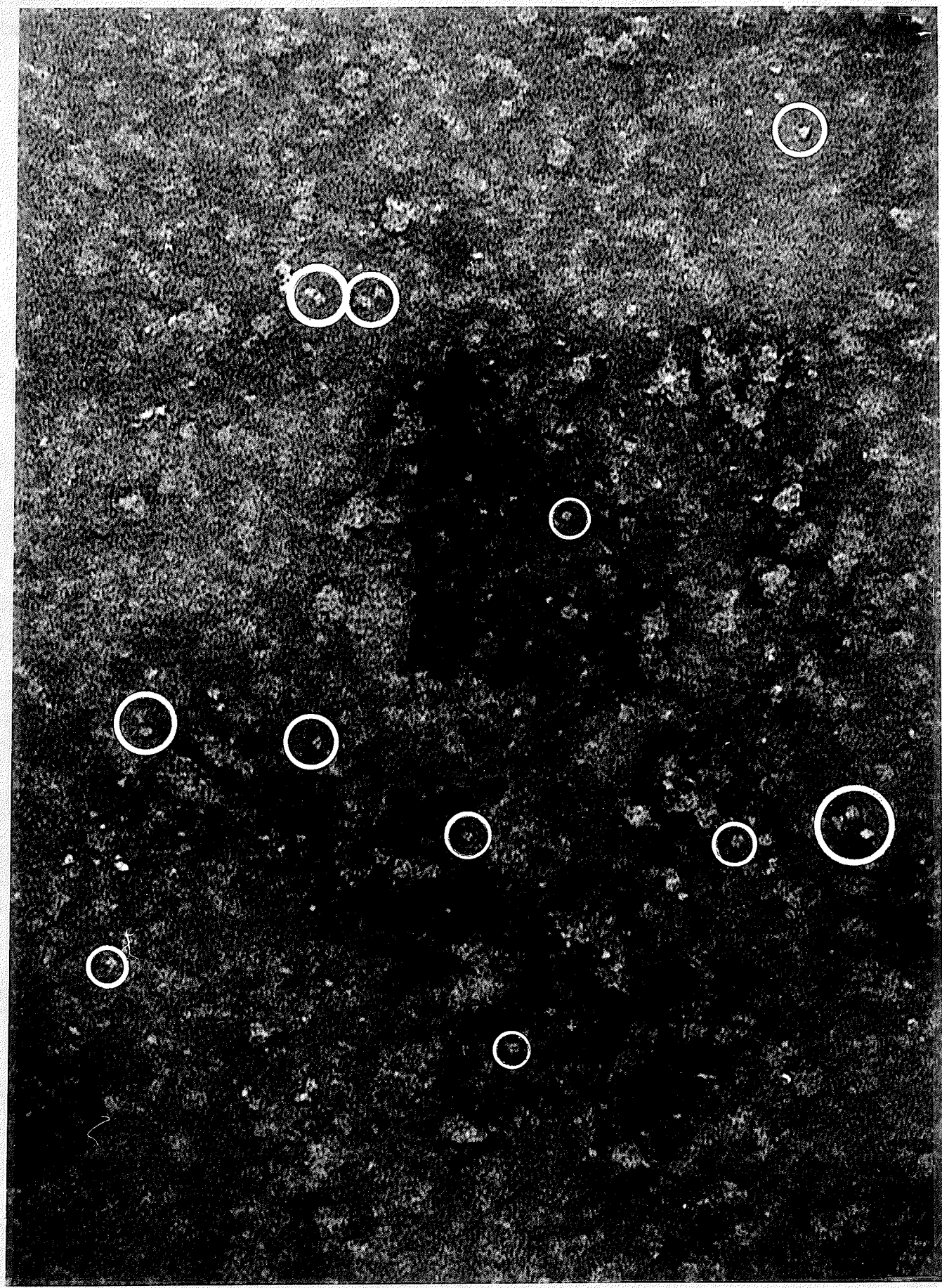
Electron Microscopy — Preparations of PEP carboxylase for examination in the electron microscope (as described in Methods) always showed amorphous debris in the photographs suggesting that considerable denaturation of the protein had occurred during the stain and drying procedures employed. The molecules look similar to those examined by Valentine et al (1966) of pyruvate carboxylase. The few intact molecules observed appear as tetramers with the four subunits arranged at the corners of squares (Plate 4).

Desensitization of the Enzyme — The enzyme PEP carboxylase was found to be reversibly desensitized (Monod, Changeux and Jacob, 1963) to the allosteric effects of aspartate and fructose-1,6-diphosphase, by the compound dioxane (Sanwal, Maeba and Cook, 1966). Various additional attempts were made to permanently desensitize the enzyme using the various treatments such as heat or mercurials, trypsin, acetic anhydride, pyridoxal phosphate, or dinitrophenylation. In all cases only the substrate activity was lost during the reaction and the per cent inhibition or activation by the allosteric ligands remained constant. Efforts were also made to protect the enzyme activity by the inclusion of the substrate(s) in the reaction mixture, but were unsuccessful. Since these desensitization attempts, the enzyme from E. coli has now been genetically desensitized (Morikawa, Izui and Katsuki, 1971).

KINETIC EXPERIMENTS

Initial Velocity Studies with Substrates — Maeba (1968) and Maeba and Sanwal (1969) have extensively studied the kinetic

Plate 4. Electron micrograph of PEP carboxylase prepared as described in "Methods". The few intact molecules (circled areas) observed show four subunits arranged at the corners of a square; magnification, 160,000 x.



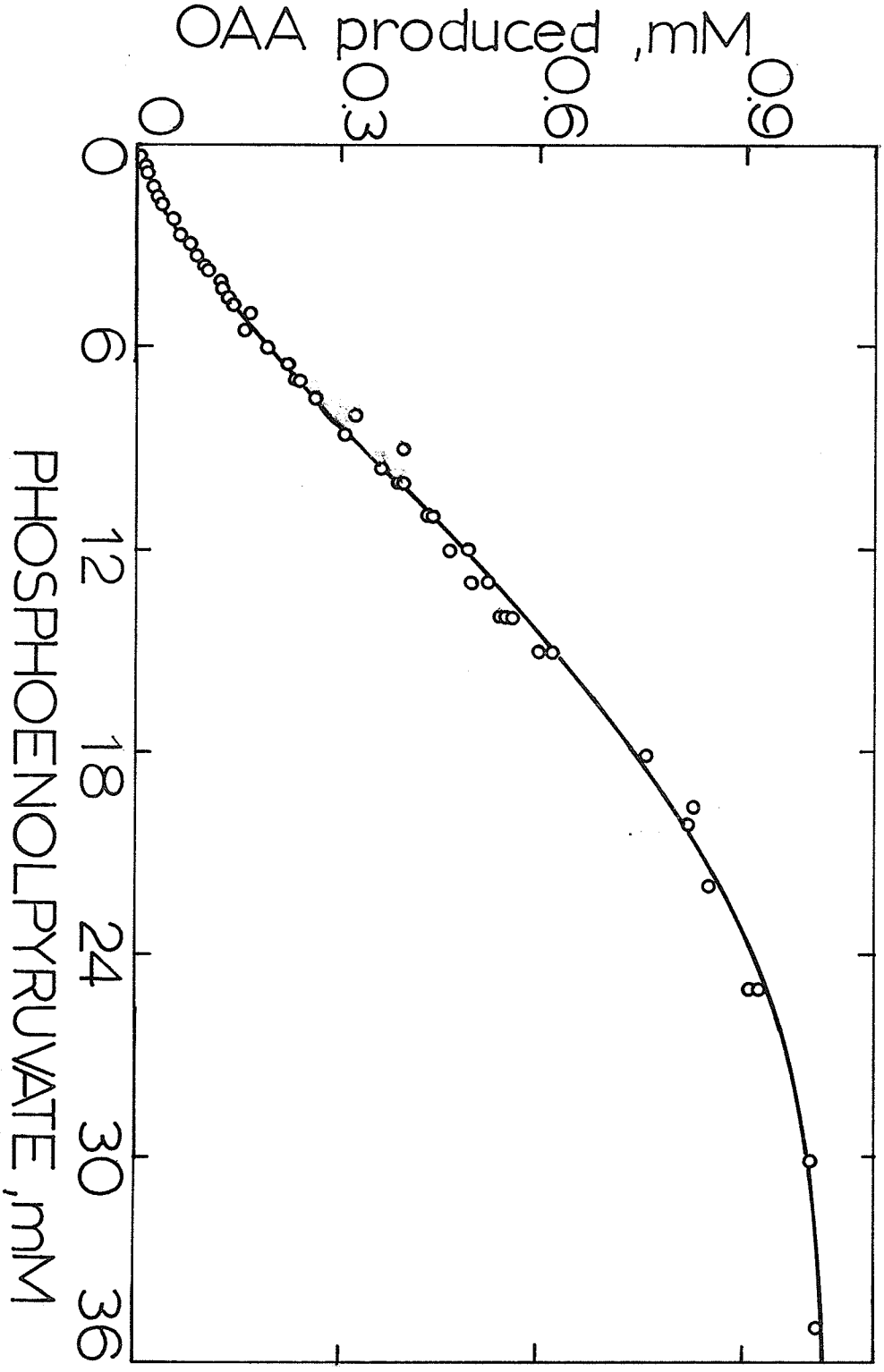
parameters of the enzyme using a coupled spectrophotometric assay. Corwin and Fanning (1968) using a radioisotopic assay presented some initial velocity results which were somewhat at variance with the results obtained by Maeba (1968). Since kinetic experiments have been used extensively in support of one or the other of several allosteric models proposed by diverse authors, initial velocity patterns using PEP as a variable substrate were re-examined with an isotopic assay.

The results of this study are presented in Fig. 17. With PEP as substrate a smooth sigmoid curve is obtained without any breaks or plateaus as described by Corwin and Fanning (1968). The cause for this discrepancy between their results and those presented here is not known. It has been suggested by Kirschner (1971) that under certain conditions aged enzyme preparations may produce artefactual curves suggesting negative cooperativity (Levitzki and Koshland, 1968; Koshland, Nemethy and Filmer, 1966; Conway and Koshland, 1968; Cook and Koshland, 1970). It is therefore possible that Corwin and Fanning used an "aged" enzyme. Exactly as with the spectrophotometric assay (Maeba, 1968) the data in Fig. 17 yield a K_m of about 12.0 mM for PEP.

Indeed, we have repeated several of the kinetic experiments of Maeba and Sanwal (1969) using the isotopic procedure instead of the spectrophotometric assay they have used and the results are quite comparable. We have, therefore, not presented these results here.

While investigating the cation specificity of the enzyme

Figure 17. Plot of oxalacetate (mM) formed versus PEP concentration (mM). The radioactive assay was performed as outlined in "Methods". Thirty μg of enzyme was used per assay. The incubation time was five minutes at 30°.

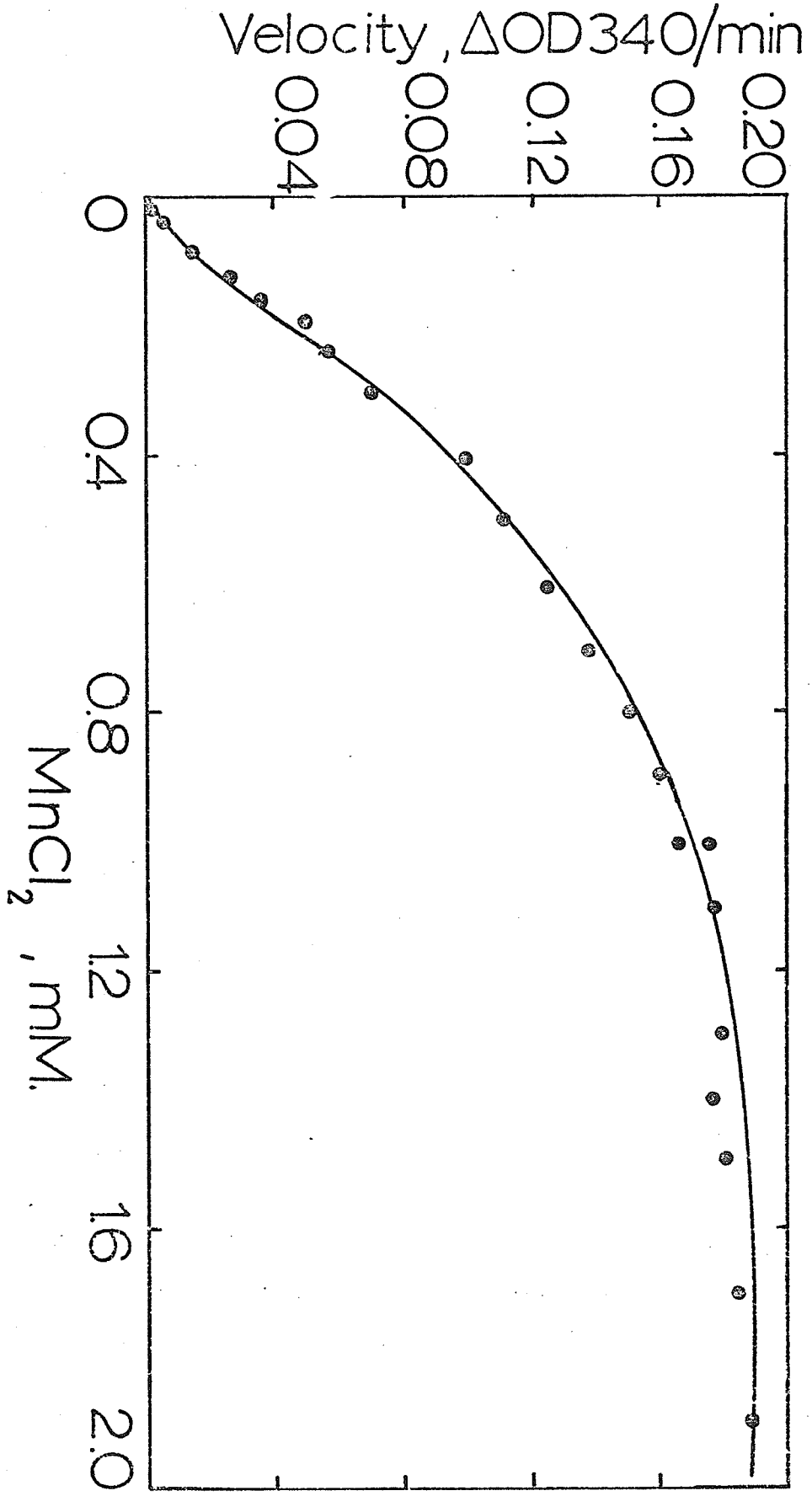

OAA produced ,mM
0
0.3
0.6
0.9
PHOSPHOENOLPYRUVATE ,mM
0
6
12
18
24
30
36

it was found that manganese could replace magnesium in the reaction catalyzed by PEP carboxylase. When manganese was used as the variable substrate (with magnesium omitted from the reaction mixtures), a sigmoid saturation curve was obtained (Fig. 18), suggesting that the binding of manganese to the enzyme was a cooperative process. The K_m value for Mn^{2+} evaluated from Fig. 18 is 0.37 mM. This value may be compared with 1.0 mM which is the K_m for magnesium under identical assay conditions (5 mM PEP and 10 mM HCO_3^-).

BINDING OF LIGANDS AT EQUILIBRIUM

It has become increasingly clear in recent years that initial velocity kinetic studies by themselves are not suitable to an understanding of the allosteric phenomenon. It is, for instance, well known that sigmoid rate-concentration plots can be generated (e.g., Sanwal and Cook, 1966) if alternate pathways for product release are available in the reaction pathway. Biphasic kinetic curves can similarly be generated if certain critical rate constants are modified in the reaction sequence. In order to formulate a mechanism for allosteric inhibition and activation of PEP carboxylase, therefore, ligand binding to the enzyme at equilibrium was studied. Owing to technical problems, however, it has not been possible to study this facet of the problem in any great detail. The binding of the substrates of the enzyme, such as PEP could not be studied because of its extremely small affinity (K_m = .083 mM) for the protein. Similarly, the binding of certain effectors like FDP and GTP could not be studied because of their low affinities. A consistent difficulty also has been the relatively low yields of

Figure 18. Plot of velocity (ΔOD/min) versus mM $MnCl_2$. The spectrophotometric assay was used to follow the change in optical density/min. Fifty µg enzyme was used per assay.


Velocity, ΔOD340/min
0.04
0.08
0.12
0.16
0.20
0
0.4
0.8
1.2
1.6
2.0
MnCl2, mM.

the enzyme. For instance, on the average, only about 60 mg of the pure enzyme could not be obtained from about 1500 gm, wet weight of cells.

1. Effect of Allosteric Ligands on the Sedimentation Properties of the Enzyme — In order to draw valid conclusions regarding the nature of binding of the allosteric ligands to PEP carboxylase, it had to be ascertained whether these substances had any effect on the aggregation-deaggregation properties of the enzyme. Several allosteric systems are now known where binding of certain effectors on the enzyme surface causes an aggregation (Maley and Maley, 1964; Chen, Brown and Plant, 1964) or deaggregation (Frieden, 1959; Wolff, 1962; Frieden, 1062; Yielding and Tomkins, 1960; Yielding and Tomkins, 1961; Frieden, 1963; Tomkins et al, 1963) of the enzyme subunits. It will be seen from Table 3 that various effectors of PEP carboxylase singly, or in various combinations had no significant effect on the sedimentation velocity of the enzyme. The $S_{20,w}$ values remained substantially around 12 even in the presence of saturating concentrations of various allosteric ligands.

2. Binding of L-Aspartate — Earlier kinetic experiments of Maeba and Sanwal (1968) had shown that the inhibition curve for L-aspartate was hyperbolic. In the present work we have been able to confirm this result. In view of the kinetic results the equilibrium binding curve for L-aspartate was expected to yield a Langmuir isotherm. This expectation was borne out by the binding experiment presented in Fig. 19. A Scatchard plot of the data in the absence of allosteric ligands (Fig. 20) yielded one binding site

Figure 19. Plot of $\bar{v}$ (number of moles of L-[^{14}C]-aspartate bound per mole of enzyme) as a function of free aspartate in the absence (○) and presence of acetyl-CoA (●), FDP (△), and GTP (▲). The enzyme concentration used was 0.2 mM (calculated on the basis of a molecular weight of 100,000), and the concentration of the ligands were 1.0 mM acetyl-CoA, 10 mM fructose-1,6-di-P, and 10 mM GTP. The experiment was performed in 0.05 M Tris-Cl, pH 8.0 at 5°.

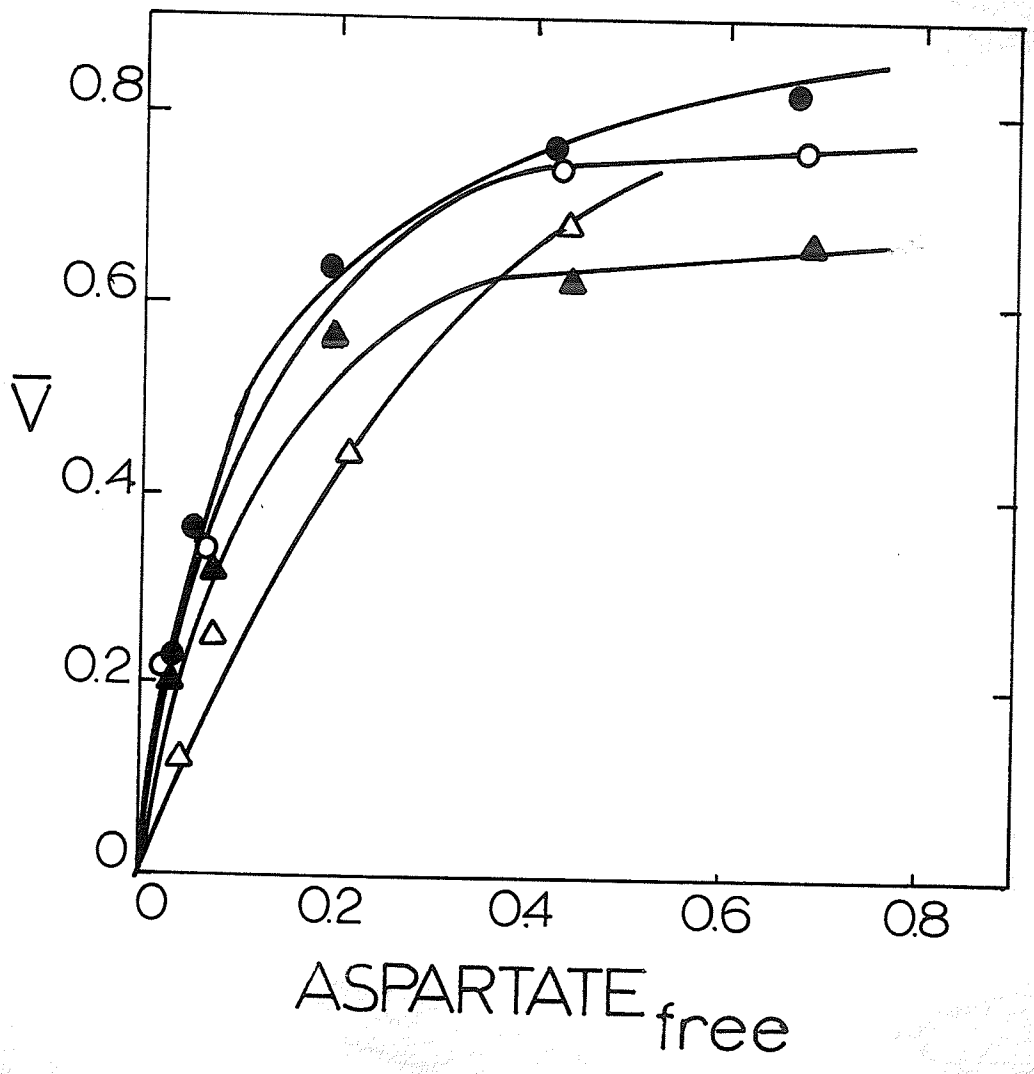
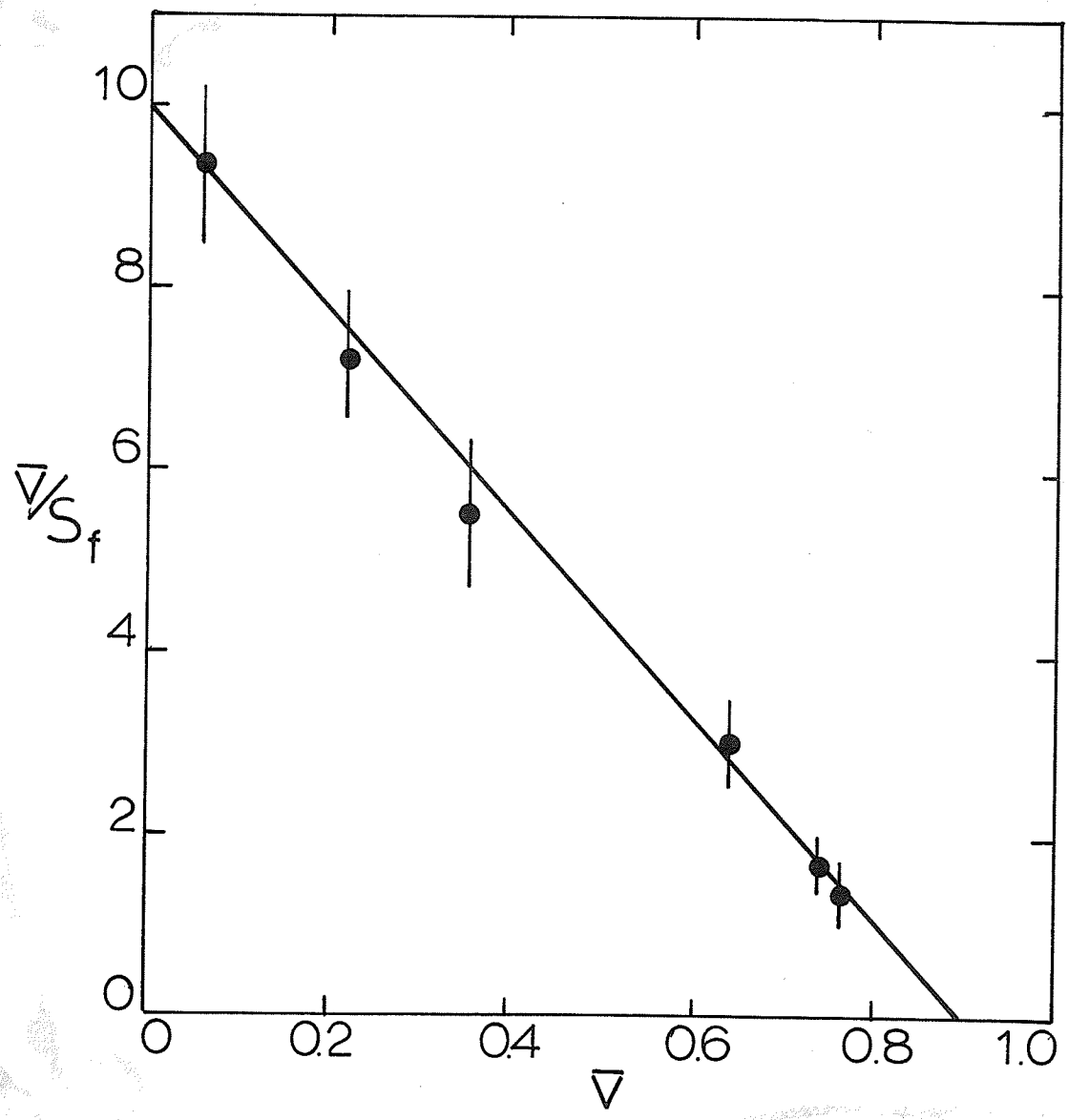

0.8
0.6
0.4
0.2
0
$\bar{V}$
0
0.2
0.4
0.6
0.8
ASPARTATE free

Figure 20. Scatchard plot of the binding of L-[^{14}C]-aspartate to PEP carboxylase in the absence of allosteric ligands. The results presented in Fig. 19 was used to generate this curve. $\bar{v}$ is the number of moles of aspartate bound per mole of enzyme and S_f is the free aspartate concentration.

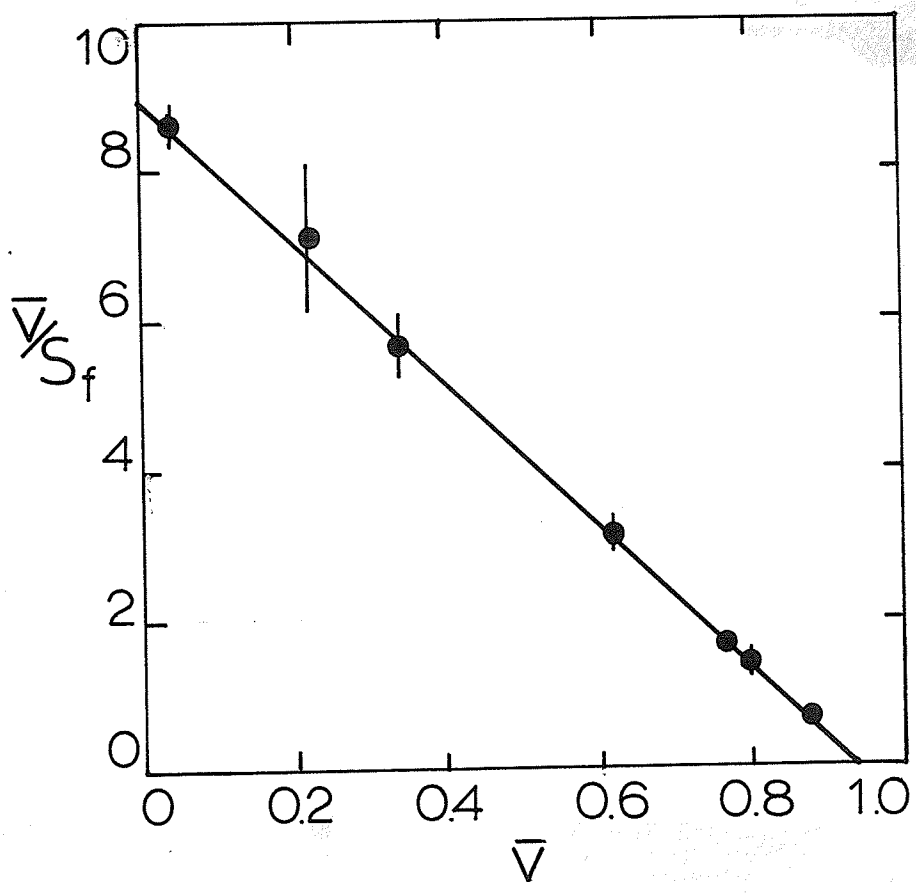

10
8
6
4
2
0
$\bar{V}/S_f$
0
0.2
0.4
0.6
0.8
1.0
$\bar{V}$

for aspartate per 100,000 molecular weight subunit of the enzyme. If our surmise regarding the monomer molecular weight is correct this result suggests that a dimer may actually be the protomer or the functional unit of PEP carboxylase. This result is unexpected but not surprising. In recent years several enzyme systems have been described which have less than one site per monomer (Kuehn, Barnes and Atkinson, 1971; Harvey, Giorgio and Plant, 1970).

The aspartate binding curve presented in Fig. 19 remains substantially unaltered when binding of this ligand is done in the presence of 10 mM Mg^{2+} or 4 mM Mn^{2+}. It also suggests that Mg^{2+} is not involved in the binding of aspartate. The dissociation constant (K_D) for aspartate obtained from the data of Fig. 20 can be calculated to be 0.090 mM. This value is approximately the K_i value obtained in kinetic experiments.

3. Effect of Allosteric Activators on the Binding of L-Aspartate — To formulate a molecular model for the mode of action of PEP carboxylase it was essential to know the binding behaviour of aspartate in the presence of various substrates of the enzyme and allosteric activators. Owing to the high K_m of the substrate, PEP, as indicated before, it could not be used for binding experiments. The effect of three activators of the enzyme, acetyl-CoA, fructose diphosphate and GTP, however, could be tested separately and in various combinations. As shown in Fig. 21 constant presence of a saturating amount (1.0 mM) of cold acetyl-CoA during dialysis had no effect on the binding of radioactive L-aspartate to the enzyme. Neither the dissociation constant nor the number of sites binding

Figure 21. Scatchard plot of the binding of L-[^{14}C]-aspartate to PEP carboxylase in the presence of 1.0 mM acetyl-CoA. The curve presented here was obtained from the binding curve presented in Fig. 19. $\bar{v}$ is the number of moles of aspartate bound per mole of enzyme and S_f is the free aspartate concentration.


10
8
6
4
2
0
$\bar{V}/S_f$
0
0.2
0.4
0.6
0.8
1.0
$\bar{V}$

aspartate changed in the presence of this activator. The K_D value in the presence of 1.0 mM acetyl-CoA is 0.105 mM (Fig. 21). In the presence of 10 mM cold FDP (Fig. 22), again, one molecule of aspartate is bound to 1 mole of 100,000 molecular weight subunit. As shown in the Scatchard plot of Fig. 22 the K_D value in presence of FDP is 0.094 mM to be compared with 0.090 mM in its absence. The third activator, GTP, tested at a concentration of 10 mM (Fig. 23) changed the dissociation constant of aspartate to 0.25 mM. It is noteworthy that the binding curve for aspartate in the presence of the various effectors does not change shape.

It had been shown earlier by Maeba and Sanwal (1969) that the activators of PEP carboxylase show a cooperative effect, i.e., tested singly they cause only slight activation but when tested at the same concentration <u>together</u> the effect on the velocity of the reaction is more than additive. This finding suggested to us that combinations of various activators may be able to affect the binding of L-aspartate on the enzyme surface. Accordingly, we tested the binding of radioactive aspartate in the presence of 1 mM acetyl-CoA plus 10 mM FDP and 1 mM acetyl-CoA plus 10 mM GTP. The results of this investigation are given in Fig. 24. It is clear that in both of these cases the binding of aspartate is drastically reduced. The more effective combination in this regard was found to be acetyl-CoA and GTP. Barely perceptible binding occurred in this case at around 1.0 mM aspartate. With the second combination of activators (acetyl-CoA plus FDP), however, only 20% of the available sites for aspartate were occupied at 1.0 mM aspartate. Also

Figure 22. Scatchard plot of the binding of L-[^{14}C]-aspartate to PEP carboxylase in the presence of 10 mM FDP. The curve presented here was obtained from the binding data presented in Fig. 19. $\bar{v}$ is the number of moles of aspartate bound per mole of enzyme and S_f is the free aspartate concentration.

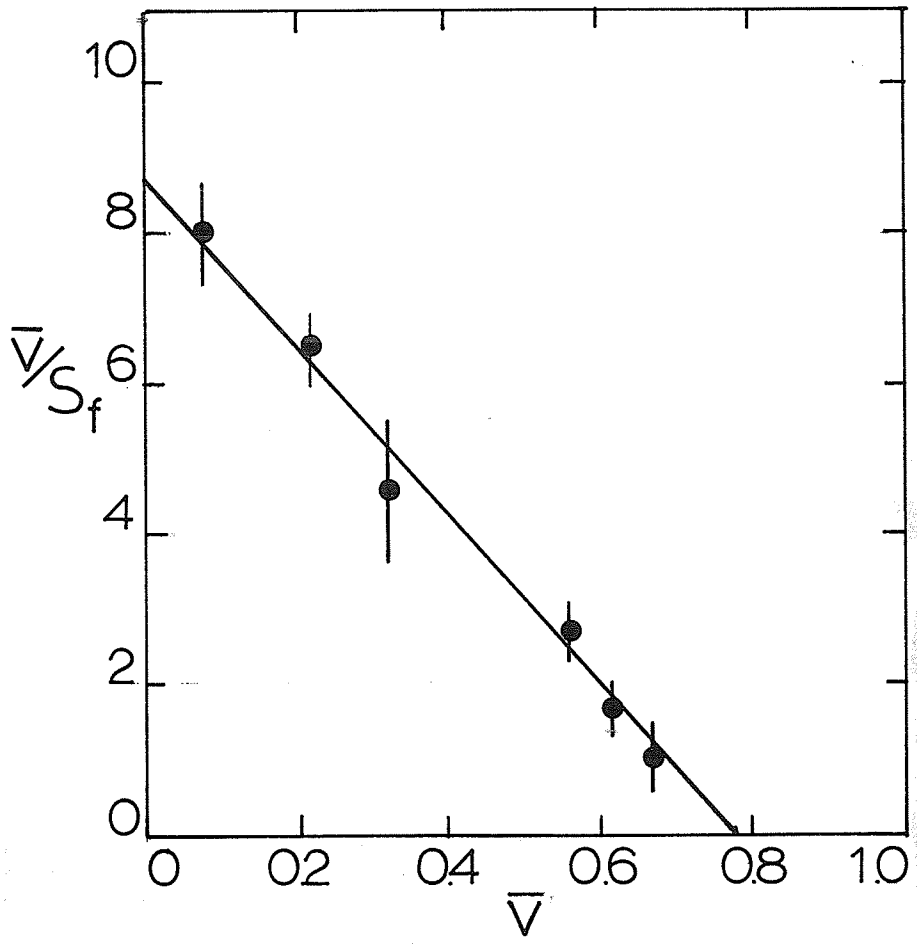

10
8
6
4
2
0
$\bar{V}/S_f$
0
0.2
0.4
0.6
0.8
1.0
$\bar{V}$

Figure 23. Scatchard plot of the binding of L-[^{14}C]-aspartate to PEP carboxylase in the presence of 10 mM GTP. The curve presented here was generated from the binding data presented in Fig. 19. $\bar{v}$ is the number of moles of aspartate bound per mole of enzyme and S_f is the free aspartate concentration.

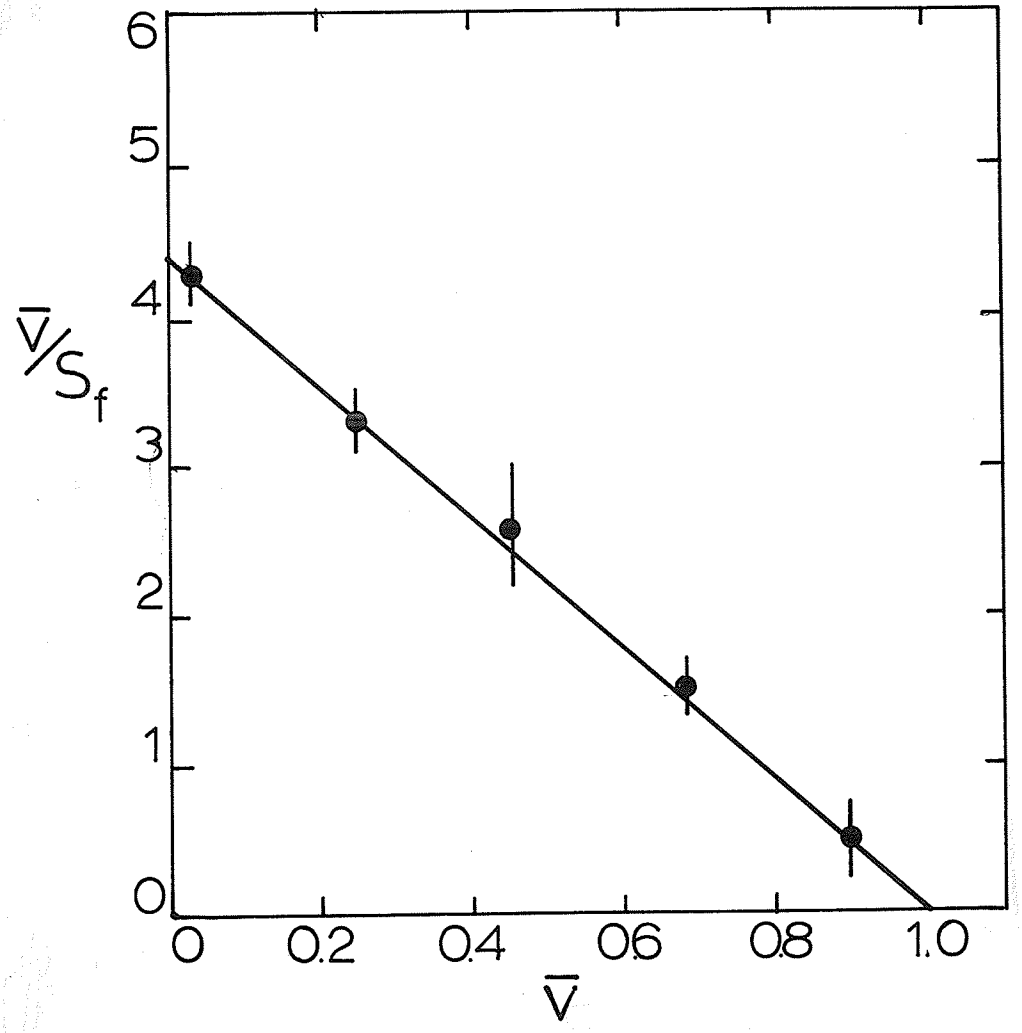
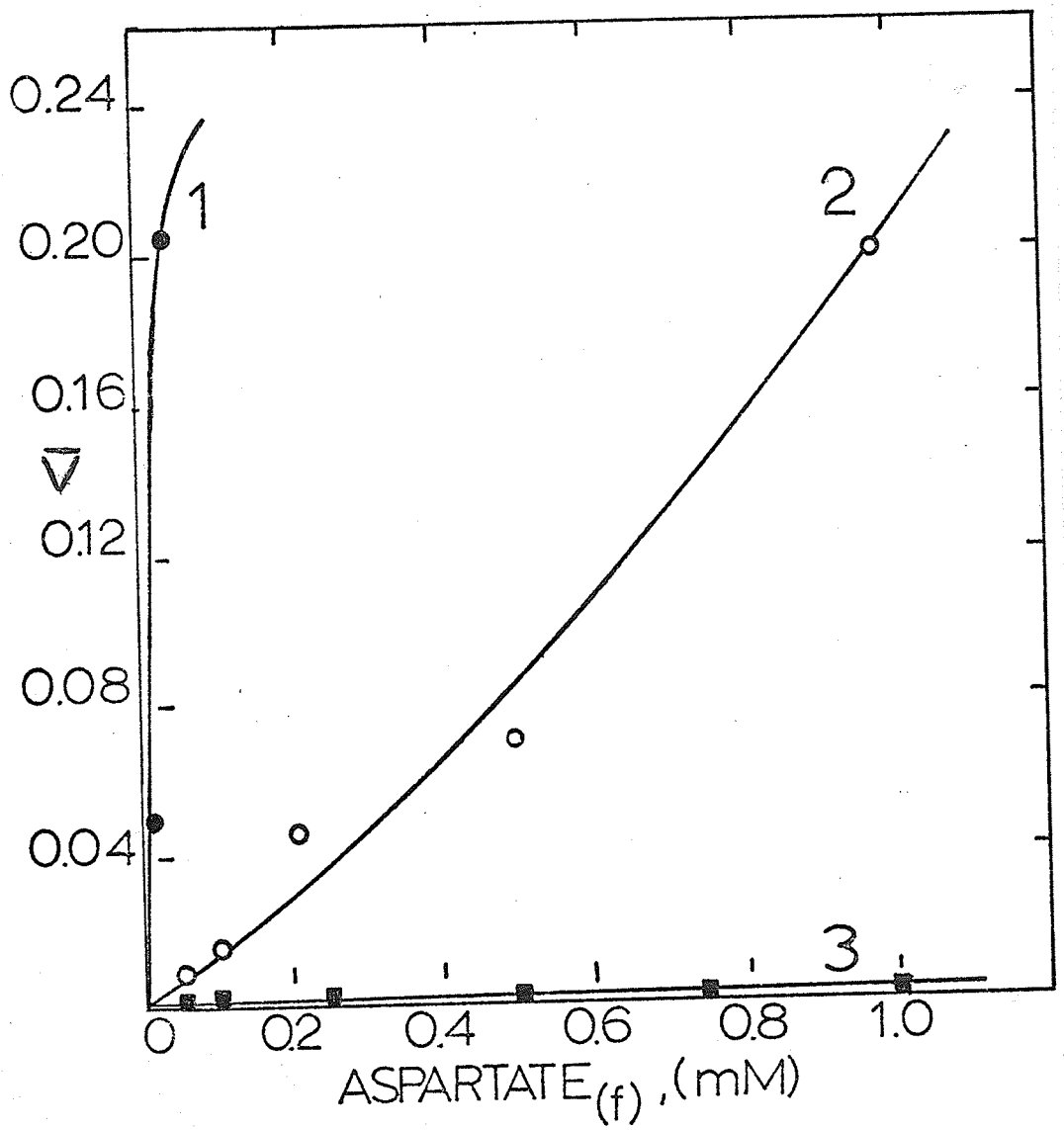

6
5
4
3
2
1
0
$\bar{V}/S_f$
0
0.2
0.4
0.6
0.8
1.0
$\bar{V}$

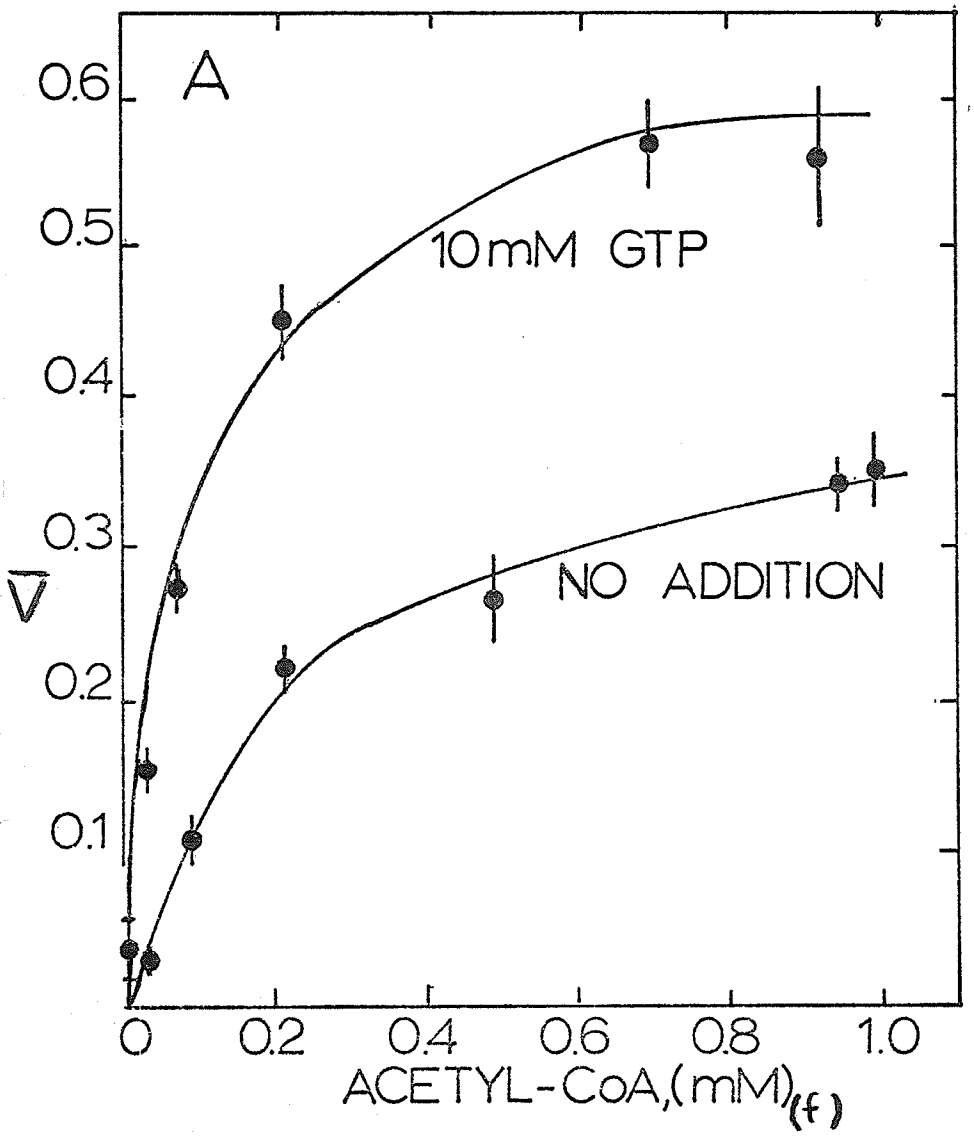
Figure 24. Cooperative effect of two allosteric activators on the binding of L-[^{14}C]-aspartate to PEP carboxylase. The protein concentration used throughout was 0.2 mM (calculated on the basis of a molecular weight of 100,000 g/mole). The numbers above the lines represent: 1, no effectors; 2, in the presence of cold 1 mM acetyl-CoA and 10 mM fructose-1,6-di-P; and 3, in the presence of cold 1 mM acetyl-CoA and 10 mM GTP. Only two points for aspartate binding are shown in line 1 for comparative purposes. $\bar{v}$ is the number of moles of aspartate bound per mole of enzyme and aspartate$_f$ is the free concentration of aspartate.


0.24
0.20
0.16
$\bar{V}$
0.12
0.08
0.04
1
2
3
0
0.2
0.4
0.6
0.8
1.0
ASPARTATE$_{(f)}$, (mM)

noteworthy here is the fact that the binding curve becomes sigmoid, or cooperative in the presence of the activators. Owing to the limited availability of the enzyme and technical difficulties attendant on the use of high concentrations of aspartate, we were unable to obtain binding points beyond 1 mM aspartate. We could not, therefore, decide whether the interaction between the inhibitor and the activators is competitive.

4. Binding of Acetyl-CoA — As has been indicated before, acetyl-CoA is the most powerful activator of PEP carboxylase. Indeed, in the absence of acetyl-CoA the enzyme is hardly active at all. It has already been demonstrated by Maeba and Sanwal (1969) that acetyl-CoA changes the K_m of the PEP from 12.0 mM to about 1.0 mM. In view of this kinetic effect of acetyl-CoA and also because this compound has a reasonable affinity for the enzyme (Maeba and Sanwal, 1969) compared to other known activators (GTP and FDP) of the enzyme, we decided to test its binding behaviour under equilibrium conditions. A representative curve for the binding of acetyl-CoA to PEP carboxylase is shown in Fig. 25. It can be seen that binding of acetyl-CoA on the enzyme is extremely complex. The initial part of the curve, at low concentrations of acetyl-CoA, is positively cooperative while at higher concentrations of the effector, the curve becomes negatively cooperative, i.e., it continues slowly sloping upwards. Despite several attempts to obtain reliable binding data above 1.0 mM acetyl-CoA we did not succeed in getting consistent results. The points gave too much scatter. The behaviour of the curve did not substantially change when binding of acetyl-CoA was

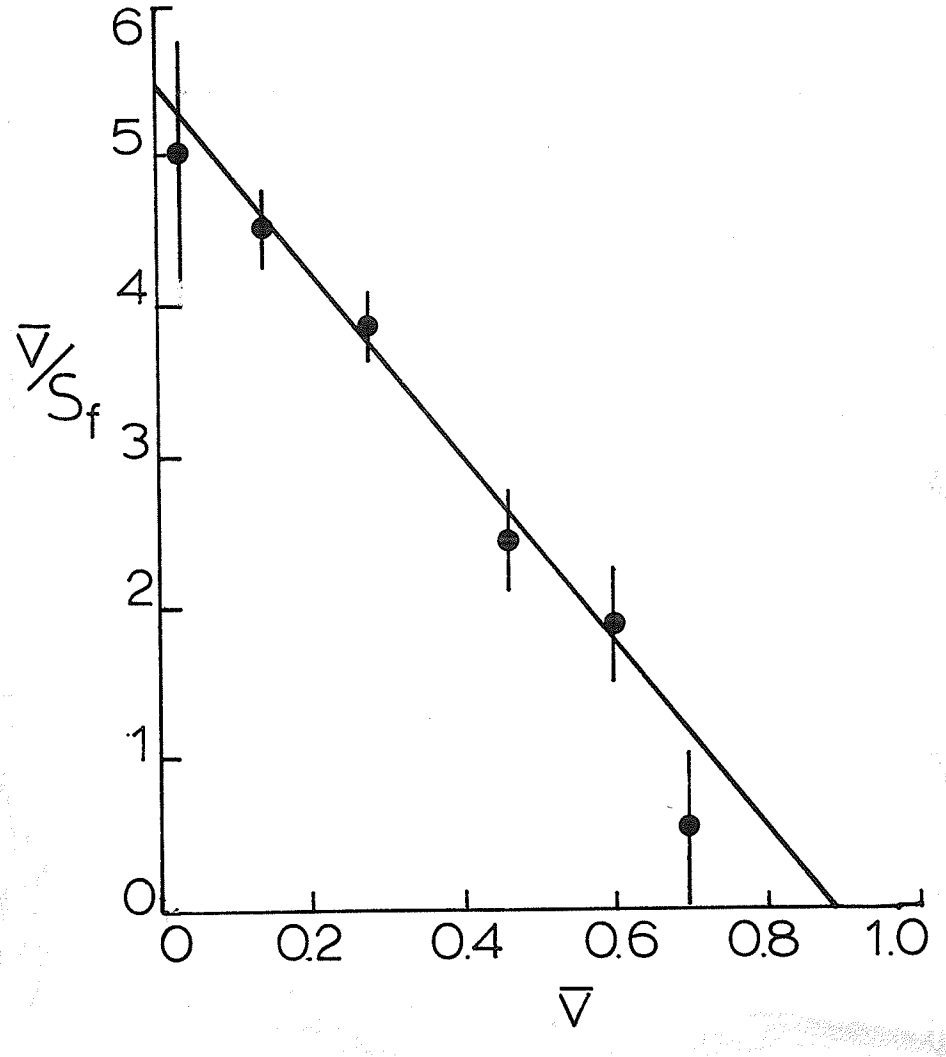
Figure 25. Binding of [^{14}C]-acetyl-CoA to PEP carboxylase in the absence and presence of 10 mM GTP. The protein concentration used was 0.2 mM (calculated on the basis of a molecular weight of 100,000). Acetyl-CoA was varied between 0.01 to 1.0 mM. $\bar{v}$ is the number of moles of acetyl-CoA bound per mole of enzyme and acetyl-CoA_f is the free concentration of acetyl-CoA.


A
0.6
0.5
0.4
0.3
0.2
0.1
$\bar{V}$
10mM GTP
NO ADDITION
0
0.2
0.4
0.6
0.8
1.0
ACETYL-CoA,(mM)(f)

done in the constant presence of 10 mM Mg^{2+}. Kirschner (1971) in a recent review suggested that complicated curves of the kind shown in Fig. 25 could arise if "aged" enzyme preparations are used in binding. Conceivably aged enzyme preparations would contain heterogenous molecules of protein, some of whose binding sites may be inactivated. This explanation is not applicable in our case, because the same protein preparation used to generate the acetyl-CoA binding curve given in Fig. 25, yielded an isotherm when binding of acetyl-CoA was done in the presence of 10 mM cold FDP or 10 mM cold GTP (Fig. 26). A Scatchard plot of the binding date in the presence of GTP shows (Fig. 26) very little positive cooperativity at low concentrations of acetyl-CoA. However, it is still difficult to saturate the enzyme with acetyl-CoA. This difficulty is reflected to some degree in the value of n obtained by extrapolation of the data. This value comes to about 0.88 per 100,000 subunit molecular weight. More likely, one site for acetyl-CoA per dimer is present on the enzyme surface. In the presence of either 10 mM FDP or 10 mM GTP the approximate dissociation constant for acetyl-CoA is about 0.16 mM.

5. Binding of Mn^{2+} — Kinetic experiments described earlier (Fig. 18) demonstrated that Mn^{2+} can replace Mg^{2+} in the reaction catalyzed by PEP carboxylase. Since the K_m for Mn^{2+} was lower than that of Mg^{2+} and since binding of Mn^{2+} could easily be studied by ESR methods we investigated the binding behaviour of Mn^{2+}. Before use the enzyme which had been prepared in the presence of EDTA was extensively dialyzed to get rid of the chelating agent. In a simulated experiment where ^{14}C-labelled EDTA was used it took about

Figure 26. Scatchard plot of the data for acetyl-CoA binding in the presence of 10 mM GTP (top line, Fig. 25). $\bar{v}$ (limiting value = 0.88) is the number of moles of acetyl-CoA bound per mole of enzyme and S_f is the free acetyl-CoA concentration. The dissociation constant is 0.16 mM.


6
5
4
3
2
1
0
$\bar{V}/S_f$
0
0.2
0.4
0.6
0.8
1.0
$\bar{V}$

36 hours to dialyze out most of the added EDTA (Fig. 27). The binding of Mn^{2+} to the EDTA-free enzyme is shown in the form of a Scatchard plot in Fig. 28. Two remarkable features are immediately obvious from this Figure. One is the positive cooperativity of the sites and the second is the presence of <u>two</u> manganese binding sites per 100,000 subunit molecular weight of the enzyme. This is in marked contrast to the presence of only 1 site per 100,000 molecular weight for the negative (aspartate) as well as the positive (acetyl-CoA) effectors. Although the curve is extrapolated by eye, it is clear from the data that definitely more than 1 site is present for Mn^{2+} per 100,000 molecular weight. Under our experimental conditions 1.5 sites per 100,000 daltons can easily be saturated. Approximate dissociation constants of 0.22 mM and 0.095 mM are obtained at 22-28% saturation and 62-68% saturation by manganese, respectively.

Figure 27. Time dependent dialysis of [^{14}C]-EDTA from a PEP carboxylase solution contained inside a dialysis bag. The concentration of PEP carboxylase used was 0.1 mM (calculated on the basis of a molecular weight of 100,000), and the diameter of the dialysis tubing was 1/4". The contents of the bag were allowed to dialyse against 500 ml of a 0.05 M Tris-Cl, pH 8.0 solution, samples were taken from inside and outside the bag at the times indicated, and checked for both enzymatic activity and amount of radioactivity. The concentration of cold EDTA used at the beginning of the dialysis was 10 mM as was the concentration of EDTA used to initially rid the enzyme of any bound metal. The concentration of cold EDTA at 36 hrs was 2.7 μM. No loss of enzymic activity occurred during this dialysis period.

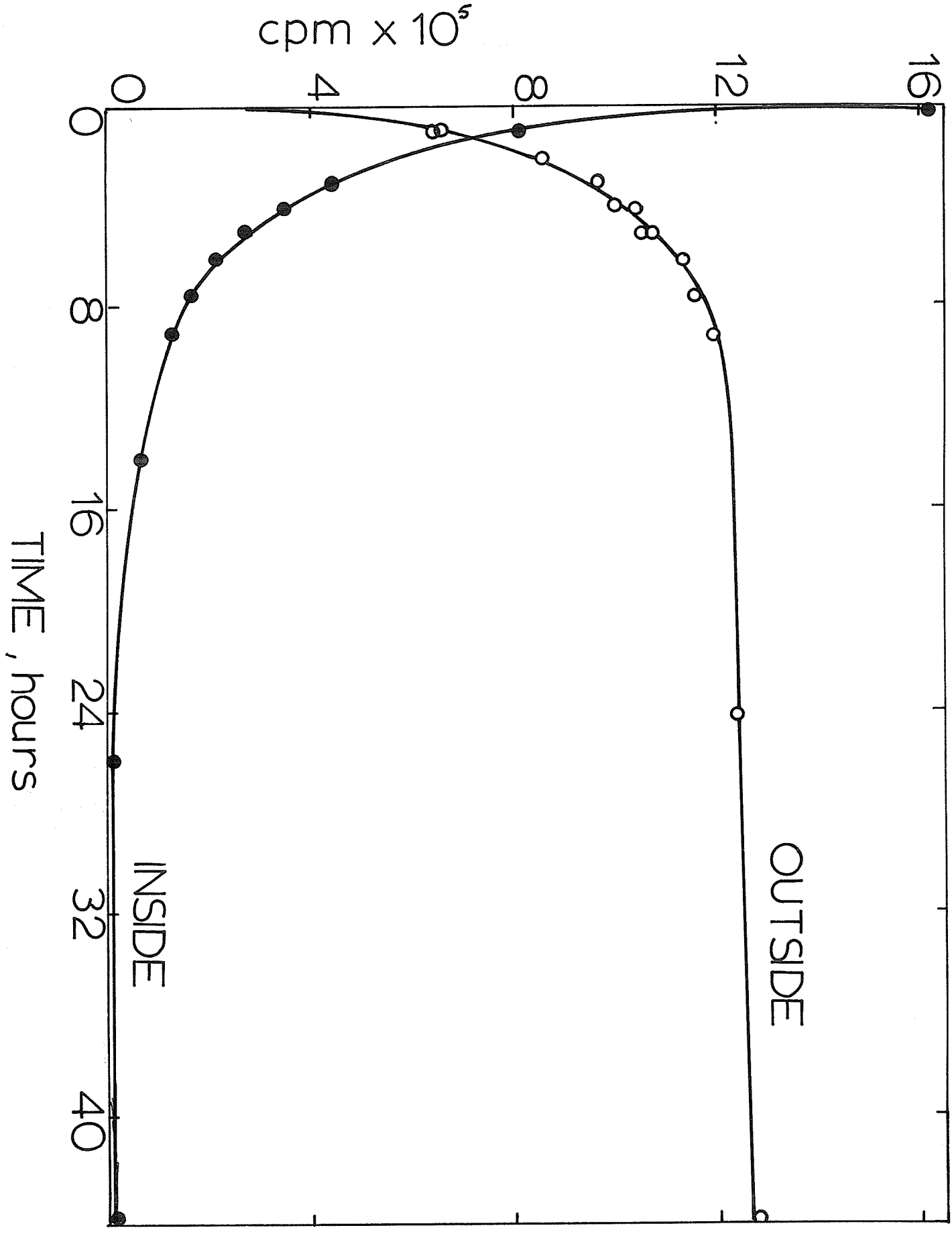
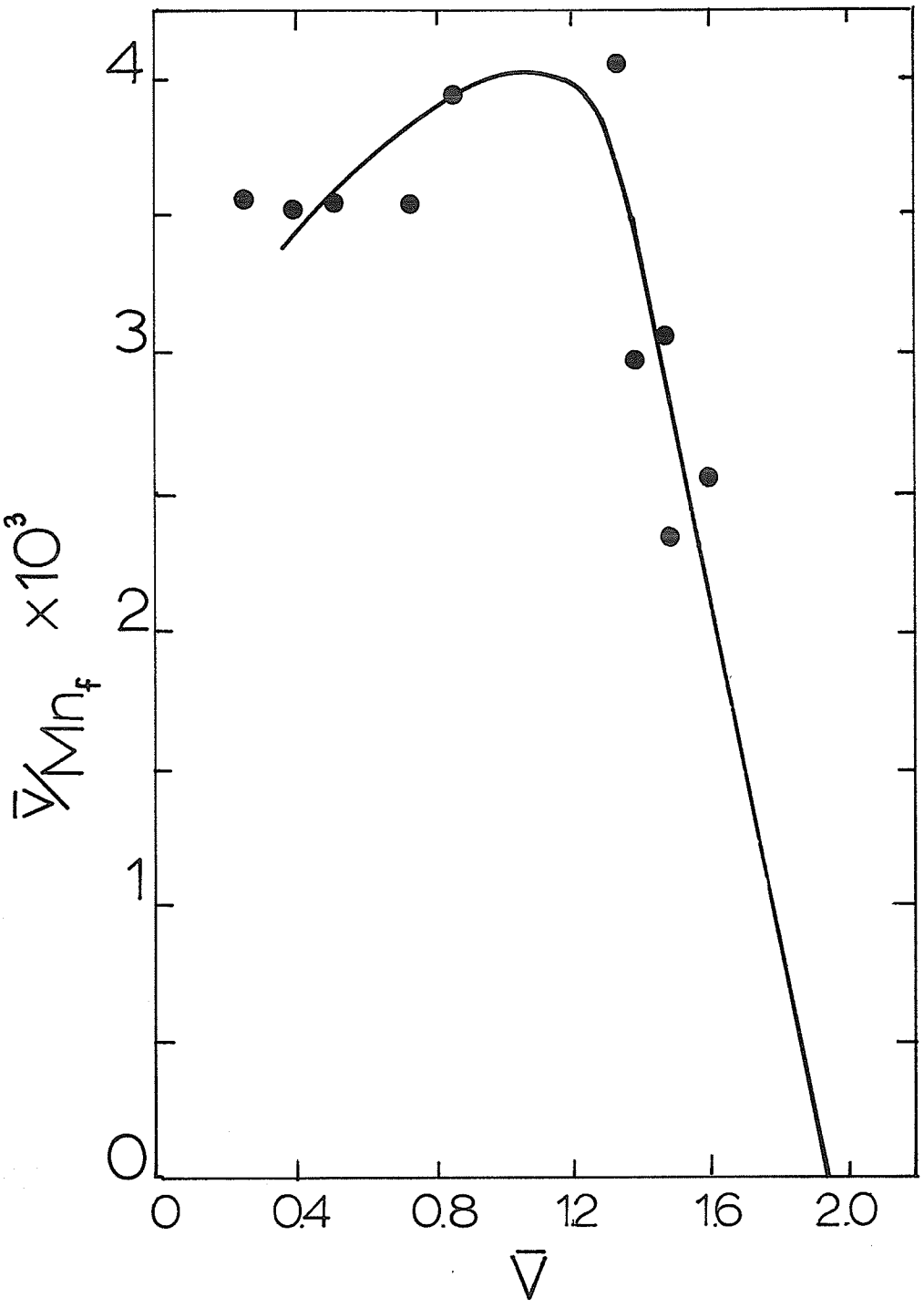

cpm x 10⁵
0
4
8
12
16
TIME, hours
0
8
16
24
32
40
INSIDE
OUTSIDE

Figure 28. Scatchard plot of the binding of Mn^{2+} by the electron spin resonance technique. $\bar{v}$ is the amount, in moles, of Mn^{2+} bound per mole of enzyme (concentration used was 0.1 mM, based on a molecular weight of 100,000), and Mn_f is the free Mn^{2+} concentration. The buffer used in this experiment was the same as that used in the previous binding experiments, which was, 0.05 M Tris-Cl, pH 8.0.


4
3
2
1
0
$\bar{V}/Mn_f \times 10^3$
0
0.4
0.8
1.2
1.6
2.0
$\bar{V}$

RESULTS ON THE STUDY OF MALIC ENZYME

MALIC ENZYME

Specific Activity Under Different Growth Conditions — Data presented in Table 4 show that the specific activity of the enzyme is highest when the cells are grown with malate as the carbon source. Also it is clear that glucose exerts catabolite repression on the formation of malic enzyme.

PURIFICATION

Protamine Sulfate Treatment — To 300 ml of crude extract, 80 ml of a freshly prepared 2% solution of protamine sulfate were added. After the suspension was stirred for 10 min the precipitate was discarded by centrifugation at 11,000 x g for 30 min.

pH and Heat Treatment — The pH of the supernatant solution was adjusted to 5.1 by adding 10% acetic acid. After stirring for 10 min the suspension was centrifuged at 11,000 x g and the precipitate was discarded. The supernatant solution was then quickly brought to 50° and maintained at this temperature for 10 min. The precipitate was discarded again by centrifugation.

First Alcohol Precipitation — The solution from the last step (pH 5.0) was cooled to 0° and 120 ml of ethanol (at -20°) were added gradually while the mixture was being stirred. After 30 min the precipitate was collected by centrifugation and dissolved in 40 ml of 0.05 M phosphate buffer, pH 7.0 containing 10^{-4} M dithiothreitol and 10^{-4} M EDTA. Any insoluble residue appearing at this stage was removed by centrifugation. The pH of the solution was again adjusted to 5 by means of 10% acetic acid and the precipitate discarded after centrifugation.

TABLE 4

Specific activity of the malic enzyme under different conditions of growth

Carbon Source	Specific Activity
Glucose (0.4%)	0.02
Glycerol (0.4%)	0.052
Succinate (1.0%)	0.057
DL-malate (1.0%)	0.120

Second Alcohol Precipitation — To the supernatant solution from the last step (cooled to 0°) 6 ml of cold ethanol were added. After stirring for 30 min the precipitate was recovered by centrifugation and dissolved in 4 ml of 0.01 M phosphate buffer, pH 7.0, containing 10^{-4} M dithiothreitol and 10^{-4} M EDTA.

Chromatography on DEAE-Cellulose — The preparation from the last step was chromatographed on a DEAE-cellulose column (2.5 x 40 cm), equilibrated with 0.01 M phosphate buffer, pH 4.0. Elution was done with a linear gradient of KCl between 0 and 0.5 M. Fractions of 5 ml were collected. The enzyme comes off as a uniform peak at around 0.4 M KCl. The tubes showing specific activity of higher than 23 were pooled. To the pooled solution 70% solid ammonium sulfate was added and the precipitate was recovered by centrifugation. It was dissolved in 3 ml of 0.05 M phosphate buffer, pH 7.0, and dialyzed overnight against the same buffer.

This solution was stored at 4° for further use. At this temperature the enzyme solution does not lose activity for at least 1 week. Freezing at -20° destroys the enzyme. A summary of the purification procedure is given in Table 5.

pH Optimum — TPN^+ malic enzyme tested in 0.1 M Tris-HCl buffer is maximally active at pH 8.2 to 8.4 (Fig. 29).

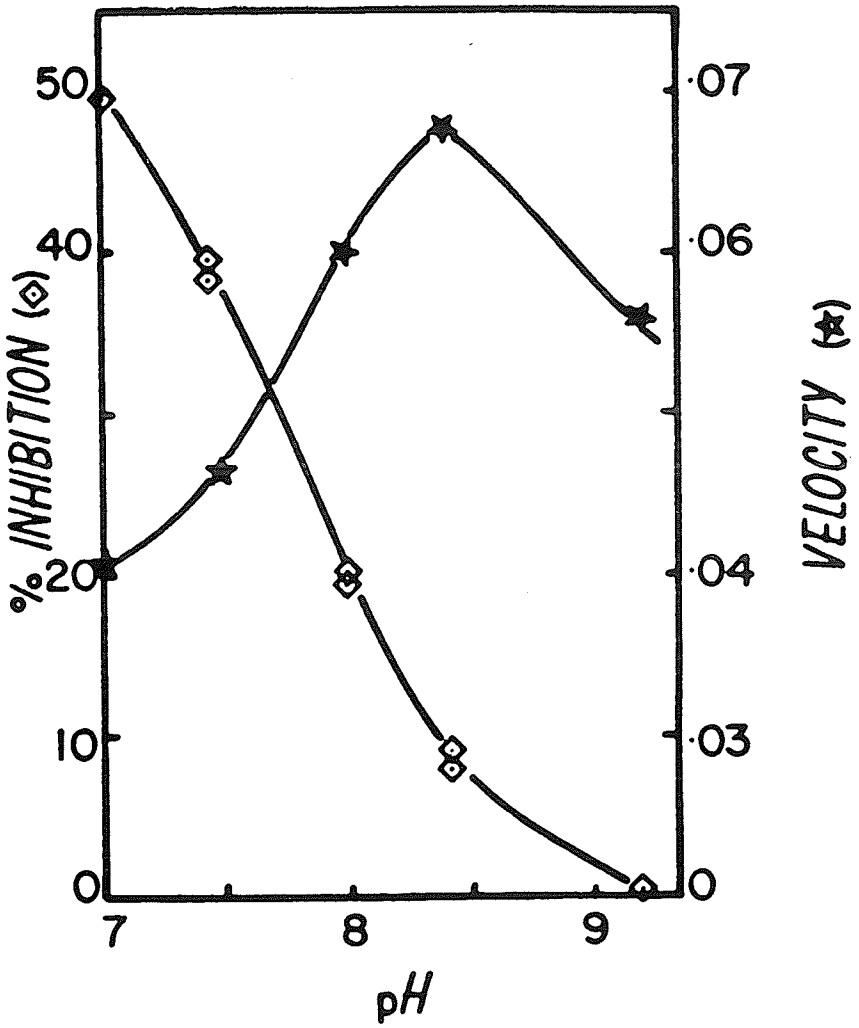
Coenzyme Specificity — The purified enzyme seems to utilize TPN^+ very much better than DPN^+. Thus, in one representative experiment, in the presence of 10 mM malate and 1 mM $MnCl_2$ the velocity of the enzyme was 0.24 at a TPN^+ concentration of 0.077 mM. In the presence of 0.3 mM DPN^+ no activity could be discerned. At 0.52 mM DPN^+, however, the velocity was only 0.014. This activity was strictly dependent upon the presence of Mn^{++} which rules out the possibility

TABLE 5

Purification of malic enzyme from Escherichia coli

Step	Volume	Protein		Specific Activity	% Recovery	Purification
		mg/ml	units/ml			
1. Crude	300	36.5	12	0.32	100	
2. Protamine Sulfate	330	21.0	10	0.48	90	1.5
3. pH and Heat	310	5.2	6.5	1.20	56	4.0
4. Ethanol 1	40	6.0	57	9.5	63	30
5. Ethanol 2	4	17.8	265	15.0	30	44
6. DEAE Cellulose Column	45	0.38	11	30	14	100

Figure 29. Effect of pH on the velocity of the enzyme and inhibition by 0.25 mM acetyl-CoA.


50
40
20
10
0
% INHIBITION (◇)
·07
·06
·04
·03
0
VELOCITY (★)
7
8
9
pH

that the activity in the presence of DPN^+ may be due to contamination by malic dehydrogenase. Also, the DPN^+ dependent activity was not stimulated in the presence of 5 mM aspartate which suggests that the DPN^+ specific malic enzyme (Takeo, Murai, Nagai and Katsuki, 1967) was also absent from our preparations.

Sucrose Density Centrifugation — Zone centrifugation were performed in gradients of 4 to 20% sucrose with the method of Martin and Ames (1961). With hemoglobin and crystalline yeast alcohol dehydrogenase as internal standards the molecular weight of malic enzyme can be calculated to be 345,000 $\pm$ 15,000 g/mole (Fig. 30). This value does not change when centrifugations are performed in the presence of 20 mM NH_4Cl or 1 mM acetyl-CoA in the gradients (Sanwal and Smando, 1969). Molecular weight studies by equilibrium centrifugation techniques have revealed a molecular weight of 550,000 g/mole for malic enzyme from *E. coli* (Spina, Bright, Rosenbloom, 1970). The discrepancy between the two above methods is not apparent at present but indicates that the malic enzyme octameric structure may be unstable with sucrose density runs.

Kinetics in Absence of Allosteric Effectors — When TPN^+ was varied against several fixed concentrations of malate (Fig. 31) the double reciprocal plots were all linear and the lines intersected at one point below the horizontal axis. If it is assumed that TPN^+ is the first substrate to add on the enzyme surface, the data of Fig. 31 can be interpreted to mean that the dissociation constant of TPN^+ is lower (0.009 mM) than the Michaelis constant (Frieden, 1957). A replot of intercepts and slopes from Fig. 31 against the reciprocal of malate concentrations is linear and yields K_m values for TPN^+ and malate of 0.018 mM and 4.0 mM, respectively. The data of Fig. 31 serve to show that the binding of substrates on the enzyme is perhaps not diffusion-limited, and they

Figure 30. The separation of hemoglobin (HL), yeast alcohol dehydrogenase (ADH), and malic enzyme (ME) in sucrose density gradients (4 to 20%).

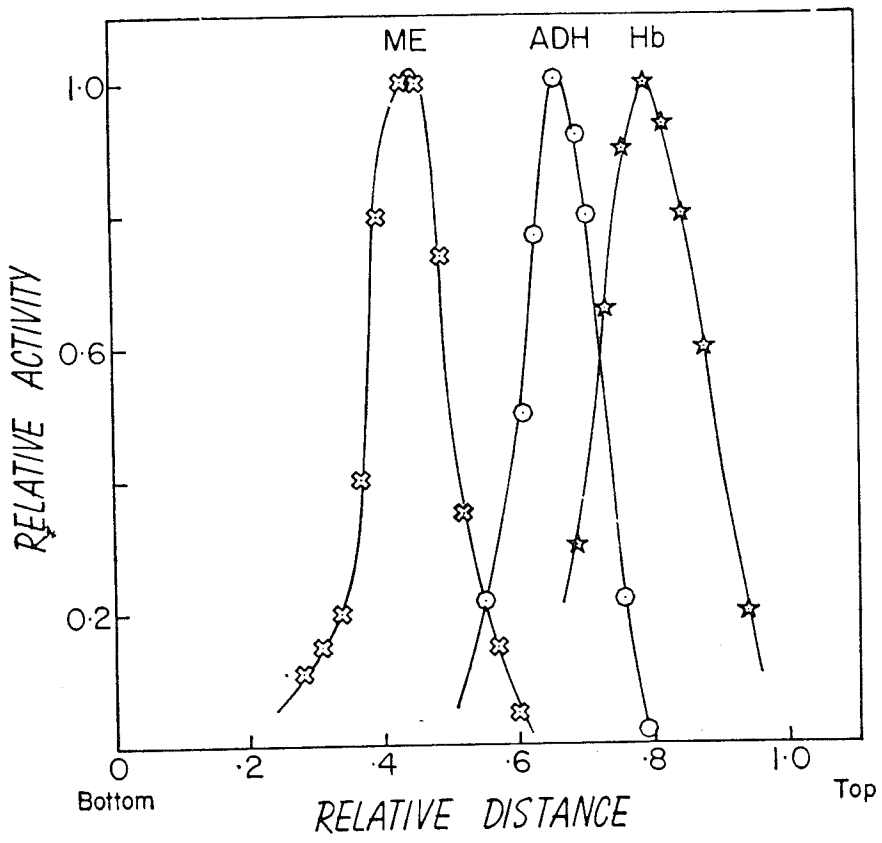
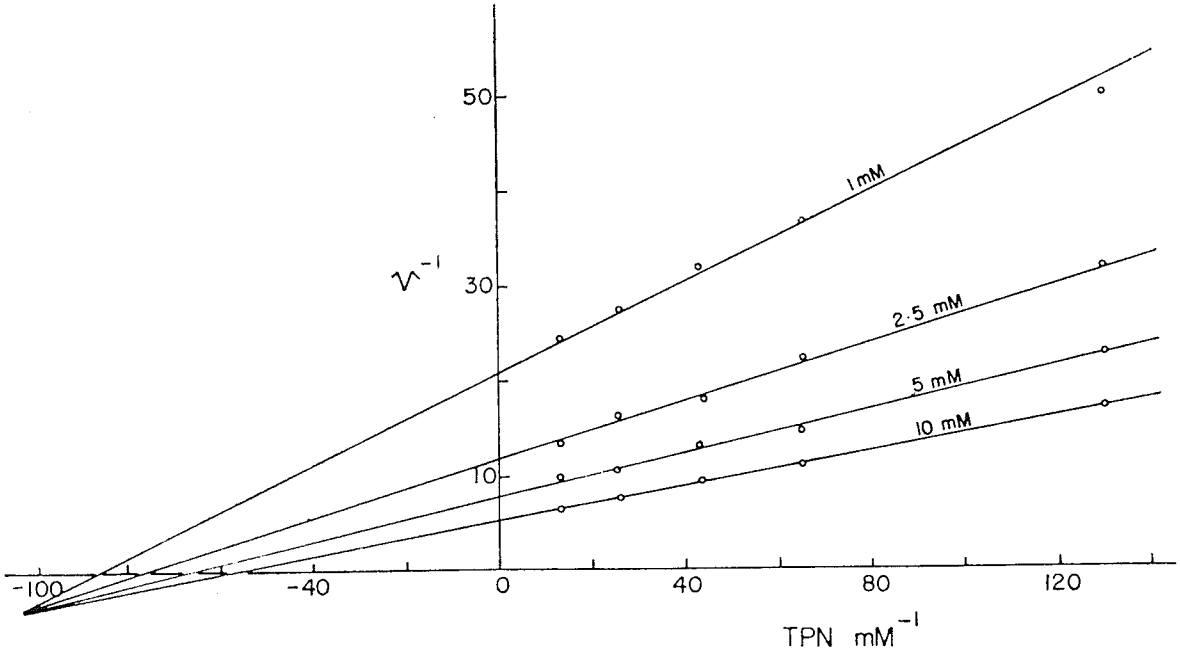

ME
ADH
Hb
1·0
0·6
0·2
RELATIVE ACTIVITY
0
·2
·4
·6
·8
1·0
Bottom
Top
RELATIVE DISTANCE

Figure 31. Double reciprocal plots of 1/velocity versus $1/TPN^+$ at several fixed concentrations of L-malate (indicated above the lines).


50
30
10
v^{-1}
1 mM
2·5 mM
5 mM
10 mM
-100
-40
0
40
80
120
TPN mM^{-1}

give no information about the order, or lack of order, of the addition of substrates (Hsu, Lardy and Cleland, 1967). To ascertain this point, product inhibition studies were undertaken.

Characteristics of Product Inhibition by $NaHCO_3$ and Pyruvate — When these products are used as product inhibitors, the double reciprocal plots were linear with both TPN^+ and malate as variable substrates. Later (Fig. 38) it will be shown that when malate was used as the variable substrate and TPNH as the inhibitor the reciprocal plots, linear in the absence of inhibitor, became markedly curved at low concentrations of TPNH and low concentrations of malate. The data presented in Table 6 however, serves to show that the non-linearity of the double reciprocal plots (Fig. 38) in the presence of TPNH is perhaps not connected with product inhibition of the enzyme per se but results from some other causes. A likely possibility here is that TPNH binds on a regulatory site on the enzyme surface and acts as an allosteric inhibitor in addition to being a product inhibitor. It will also be shown later that glycine desensitizes the enzyme to TPNH inhibition, an indication that at least part of the inhibition is due to allosteric inhibition.

Reversible Desensitization of Enzyme — Before the significance of the inhibitory control of malic enzyme by the diverse inhibitors (acetyl-CoA, oxalacetate, TPNH, DPNH, cyclic 3',5'-AMP) can be evaluated it is desirable to know whether these effectors bind at regulatory or catalytic sites. Earlier in this work (Fig. 29) it was shown that the enzyme could be reversibly desensitized to acetyl-CoA inhibition by a pH change of the assay medium to 8.5 or higher. However, in the present work, while this earlier observation could be reconfirmed for acetyl-CoA we did not find any difference in the percentage

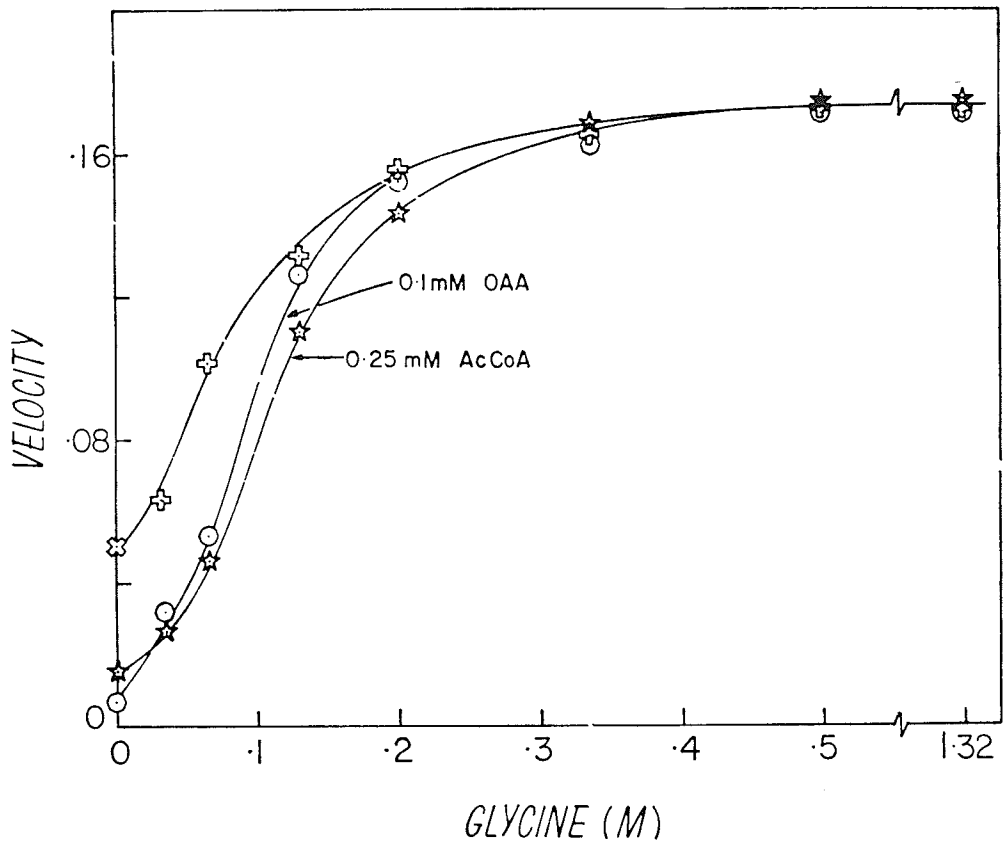
TABLE 6

Varied Substrate	Concentration Of Fixed Substrate mM	Inhibitor	Slope Constant mM	Intercept Constant mM
TPN^+	30	Pyruvate	63	72
Same	30	$NaHCO_3$	138	86
Malate	0.15	Pyruvate		177
Same	0.15	$NaHCO_3$	38	170

inhibition caused by 0.05 mM oxalacetate at pH 7.5 and 8.5 or 9.0 (0.1 M Tris-Cl buffer).

While experimenting with the effect of different kinds of buffers on enzyme activity it was observed that significantly less inhibition was caused by various effectors in 0.1 M glycine-Tris buffer than in the routinely used Tris-Cl buffer. At a pH of 7.3 increasing amounts of glycine in 0.1 M Tris-Cl buffer activated the enzyme (Fig. 32). The glycine activation curve is sigmoidal. It is pertinent to point out here that, whatever physical mechanism may underlie glycine activation, enzyme preparations made at different times vary somewhat in the extent of activation that they show by glycine. We have obtained preparations which show activation anywhere from 1.5 fold to 4 fold at 0.66 M glycine. The cause of this variability is not known. With increasing concentrations of glycine (Fig. 32) there is a progressive desensitization to the inhibitory effect of acetyl-CoA, oxalacetate and DPNH. Complete desensitization is achieved at concentrations of glycine higher than 0.5 M (see Table 7 for DPNH). In one representative experiment shown in Fig. 32, for instance, 0.1 mM oxalacetate causes 84% inhibition, but in the presence of glycine higher than 0.2 M the inhibition is reduced to zero. In another experiment (Fig. 36) in the absence of glycine half-maximal inhibition is caused by about 0.02 mM oxalacetate but in the presence of 1.32 M glycine in the assay medium, concentrations as high as 0.6 mM fail to affect enzyme activity. The continued presence of glycine is required in the assay for desensitization. When concentrated enzyme was previously incubated for 30 min at 25° in 1.32 M glycine, and the mixture was assayed after diluting

Figure 32. The effect of glycine on the activity of malic enzyme in the absence and presence of various effectors. The assay medium contained glycine (pH 7.3) at different concentrations in 0.1 M Tris-Cl (pH 7.3), 0.077 mM TPN^+, 2 mM malate, and 1 mM $MnCl_2$. The various reagents were mixed in simultaneously. OAA, oxalacetate; AcCoA, acetyl-CoA, enzyme A.


VELOCITY
·16
·08
0
0·1mM OAA
0·25 mM AcCoA
0
·1
·2
·3
·4
·5
1·32
GLYCINE (M)

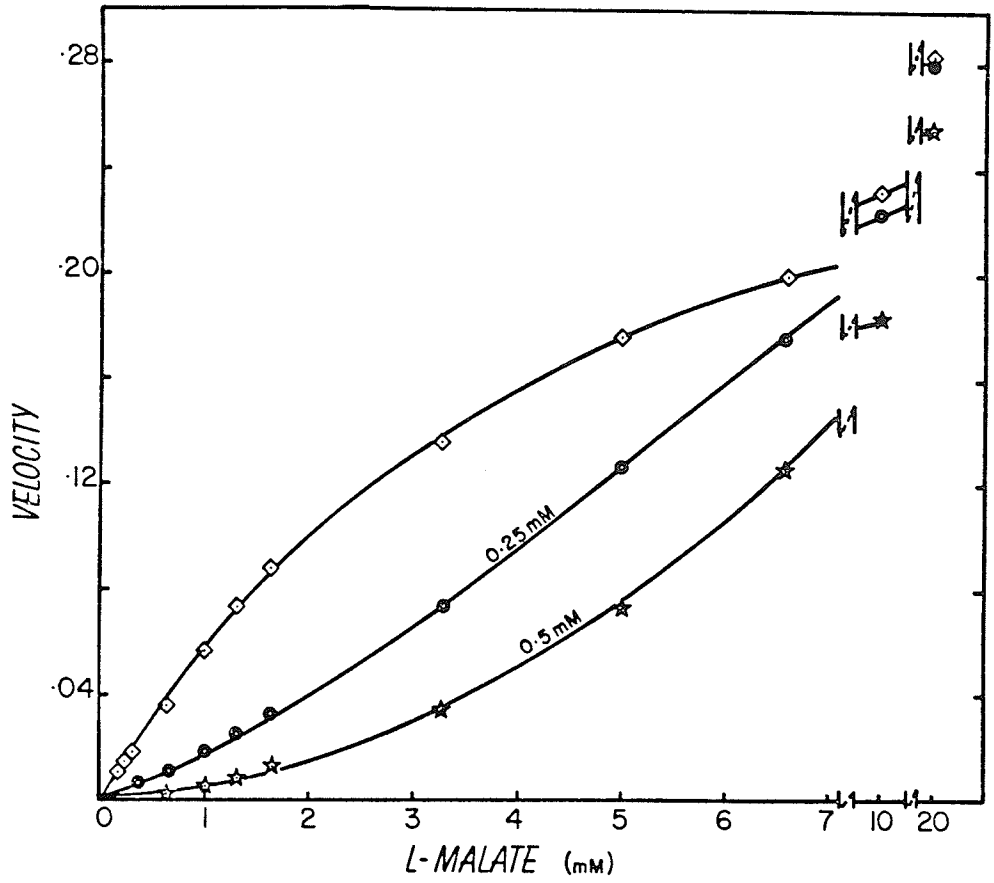
50 fold, no difference was noted in the sensitivity of the previously incubated enzyme to oxalacetate or acetyl-CoA as compared to controls. This interesting effect of glycine on the desensitization of the enzyme is not shared by glycylglycine (0.1 to 0.66 M), NH_4Cl (0.1 to 0.66 M), and urea (0.2 to 0.6 M). All of these compounds inhibit enzyme activity at concentrations higher than 0.3 M. Glycolic acid and glyoxylate were tried as analogues of glycine and were found to inhibit the enzyme at very low concentrations. In one representative experiment, for instance, the velocity in the absence of additions was 0.136 but was reduced to 0.112 in the presence of 0.4 mM glyoxylate and to 0.104 in the presence of 6.6 mM glycolate. At 0.6 M Tris-Cl the same activity was reduced to 0.012.

From the experiments outlined above we reached the conclusion that oxalacetate, acetyl-CoA and DPNH do not bind at the active sites of the enzyme and, therefore, must be classified as allosteric inhibitors (Monod, Changeux and Jacob, 1963).

In the presence of 0.6 M glycine in the assay medium neither the nature of the cyclic 3',5'-AMP inhibition (presented later) curve nor the extent of inhibition by cyclic 3',5'-AMP is altered to any significant extent.

Inhibition by Acetyl-CoA — When malate was used as a variable substrate in the presence of 20 x K_m of TPN^+ (K_m for TPN^+ = 0.018 mM) and 100 x K_m of Mn^{2+} (K_m for Mn^{2+} = 1.3 x 10^{-5} M), the initial velocity plot was hyperbolic (Fig. 33) but it became sigmoidal in the presence of concentrations of acetyl-CoA higher than 0.25 mM. In contrast to malate, the hyperbolic rate concentration curves with TPN^+ as the

Figure 33. Initial velocity plots of the enzyme with malate as the variable substrate in the absence and presence of two concentrations of acetyl-CoA. The concentrations of non-varied substrates were: TPN^+, 0.15 mM and $MnCl_2$, 1.0 mM and Tris-Cl, 0.05 M, pH 7.5, was used as buffer.


·28
·20
·12
·04
VELOCITY
0·25mM
0·5mM
0
1
2
3
4
5
6
7
10
20
L-MALATE (mM)

substrate do not significantly deviate from a hyperbola in the presence of 0.25 mM acetyl-CoA. Other compounds such as fructose diphosphate, phosphoenolpyruvate, AMP, ATP, CoA, fumarate and succinate failed to affect enzyme activity (Sanwal, Wright and Smando, 1968).

Figure 34 shows the effect of acetyl-CoA on the velocity of the reaction when malate is the variable substrate. The double reciprocal plots in the presence of inhibitor are non-linear and the pattern is the same for oxalacetate (later; Fig. 37). When TPN^+ is the varied substrate, however, acetyl-CoA does not cause any unusual deviations from linearity of the double reciprocal plots (Fig. 35). Replots of slopes from Fig. 34 against acetyl-CoA concentrations are non-linear (curving upward at high inhibitor concentrations).

The question arises as to whether the inhibition by acetyl-CoA is due to its binding on a specific, allosteric site. In an effort to desensitize the enzyme, we therefore tested the effect of pH of the assay medium on the inhibition of acetyl-CoA. These results are presented in Fig. 29. It will be noted from Fig. 29 that 50% inhibition caused by 0.25 mM acetyl-CoA at fixed concentrations of substrates at pH 7.0 is reduced to zero at pH 9.3. Also, the enzyme was shown to be desensitized to acetyl-CoA in the presence of glycine (Fig. 32). It thus seems likely that the inhibitor binding site is quite distinct from the substrate binding site.

Inhibition by Oxalacetate — On testing oxalacetate at low concentrations we noted that the malate oxidation by the malic enzyme was inhibited at pH 7.5. The inhibition by oxalacetate at a fixed concentration of 0.15 mM TPN^+ (20 times dissociation constant) and

Figure 34. Inhibition of the activity of malic enzyme by acetyl-CoA with malate as the variable substrate. The concentration of TPN^+ was 0.077 mM.

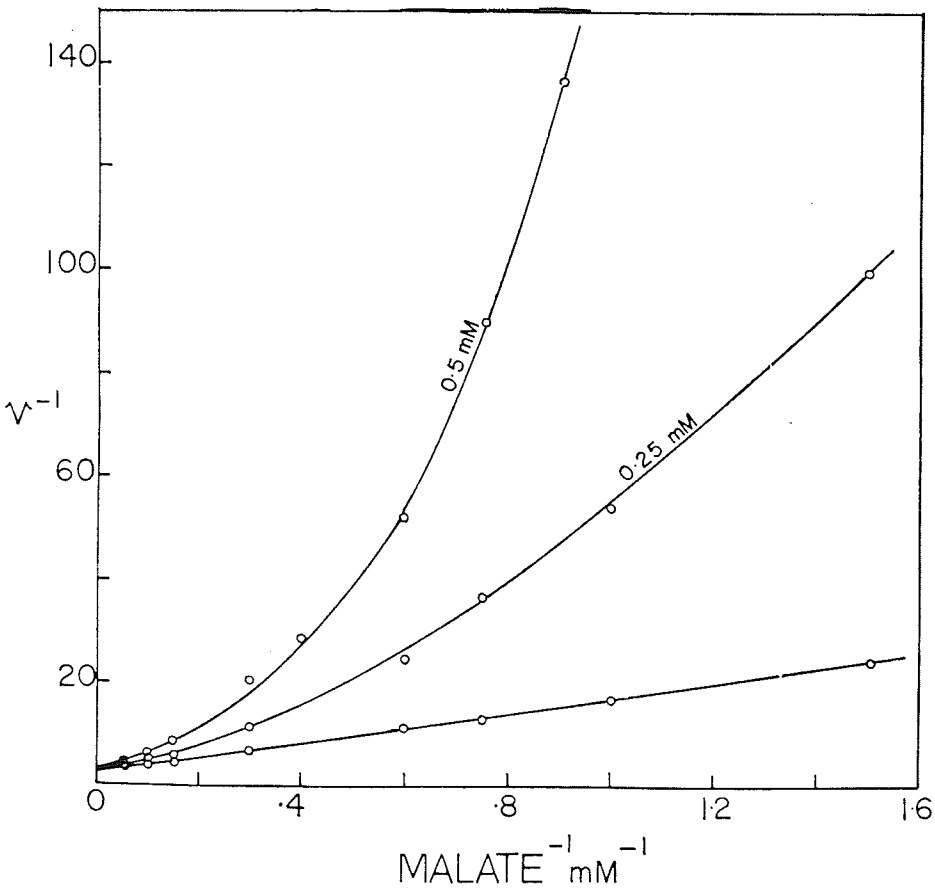
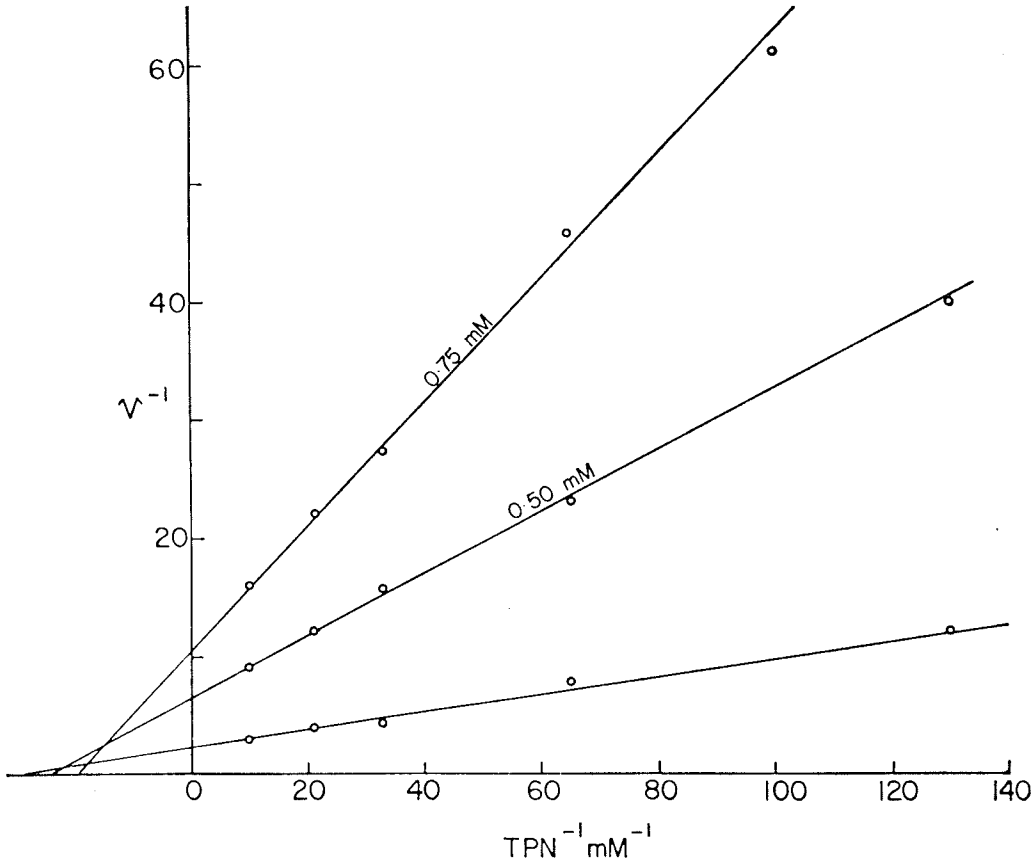

140
100
60
20
0
·4
·8
1·2
1·6
v⁻¹
0·5 mM
0·25 mM
MALATE⁻¹ mM⁻¹

Figure 35. Inhibition of the activity of malic enzyme by acetyl-CoA with TPN^+ as the variable substrate. The malate concentration was 10 mM throughout.


60
40
20
v^{-1}
0.75 mM
0.50 mM
0
20
40
60
80
100
120
140
$TPN^{-1} mM^{-1}$

various levels of malate is shown in Fig. 36. At 0.3 mM and 2 mM malate, the approximate Ki values for oxalacetate are 0.01 mM and 0.02 mM, respectively.

Figure 37 shows the double reciprocal plots with malate as the variable substrate in the presence of fixed concentrations of oxalacetate. The non-linear curves obtained are similar to those seen in Fig. 34 for acetyl-CoA. Although it is not quite accurate to extrapolate these curves by eye to get the V_{max}, when the linear parts only are so manipulated (inset of Fig. 37) the lines meet at approximately the same point on the vertical axis. It is possible, therefore, that oxalacetate changes only the slopes but not the intercepts of the double reciprocal plots. The inhibition pattern with TPN^+ as the variable substrate is quite similar to that shown in Fig. 35 for acetyl-CoA.

Inhibition by Reduced Pyridine Nucleotides — In a study of the specificity of the acetyl-CoA binding site, we found that DPNH and TPNH inhibited the enzyme activity in the presence of nearly saturating concentrations of TPN^+ (0.077 mM; 10 x K_m) in the assay medium. At this level of TPN^+, 0.3 mM DPN^+ neither stimulated nor inhibited the enzyme activity. Results of this investigation are presented in Table 7. Approximately 43% inhibition caused by 0.32 mM DPNH was not relieved by 0.3 mM DPN^+. Under our assay conditions (Table 7) TPNH proved to be a more potent inhibitor than DPNH. Thus, for half-maximal inhibition of the enzyme only about 0.14 mM TPNH was required, while to achieve the same level of inhibition concentrations of DPNH higher than 0.32 mM were needed.

When malate was used as the variable substrate and TPNH as

Figure 36. The effect of oxalacetate (OAA) on the activity of malic enzyme at different levels of malate (concentrations above curves) and 0.15 mM TPN^+. Details are given in the text. The straight line at the top is obtained in the presence of 2 mM malate and 1.32 M glycine at pH 7.5.

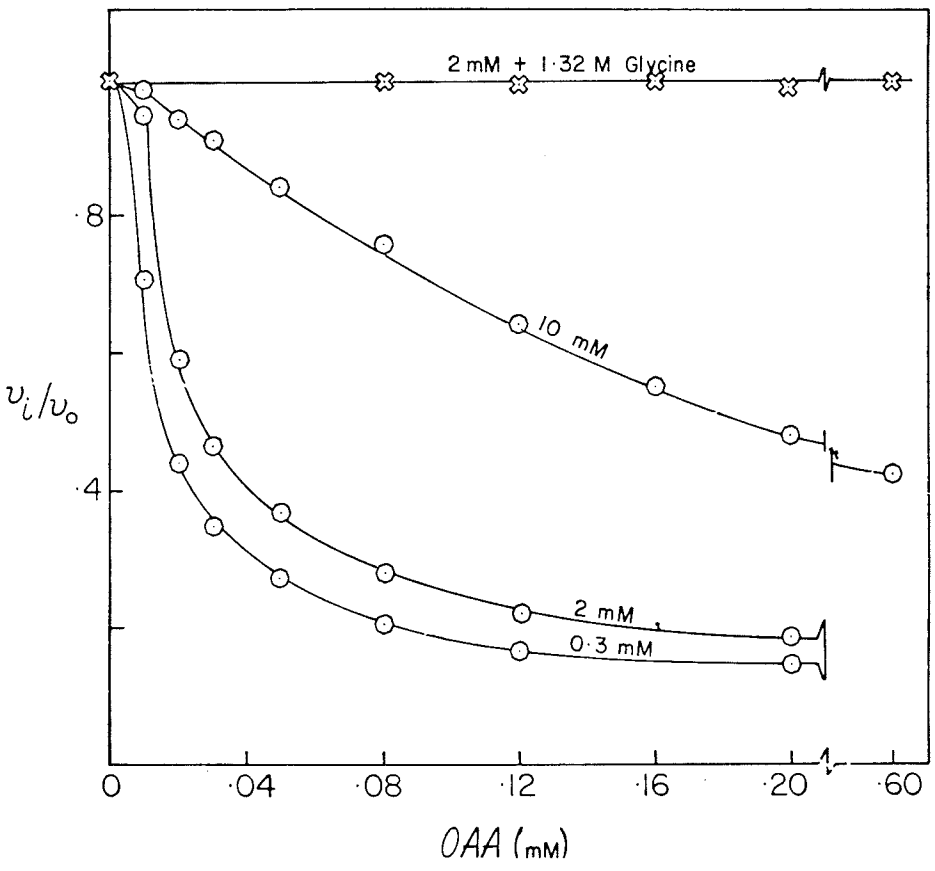

2 mM + 1·32 M Glycine
10 mM
2 mM
0·3 mM
·8
·4
v_i/v_o
0
·04
·08
·12
·16
·20
·60
OAA (mM)

Figure 37. Inhibition of the enzyme activity by various concentrations of oxalacetate (indicated above lines) with malate as the variable substrate. The fixed concentration of TPN^+ was 0.077 mM. The lines have been drawn from fits to equation 4 described in the text; n refers to the slopes of lines when data of this figure are plotted in the log-log form (log $v/v_{max}-v$ versus log substrate). The inset shows an enlargment of the figure below a reciprocal substrate concentration of 0.3 mM.

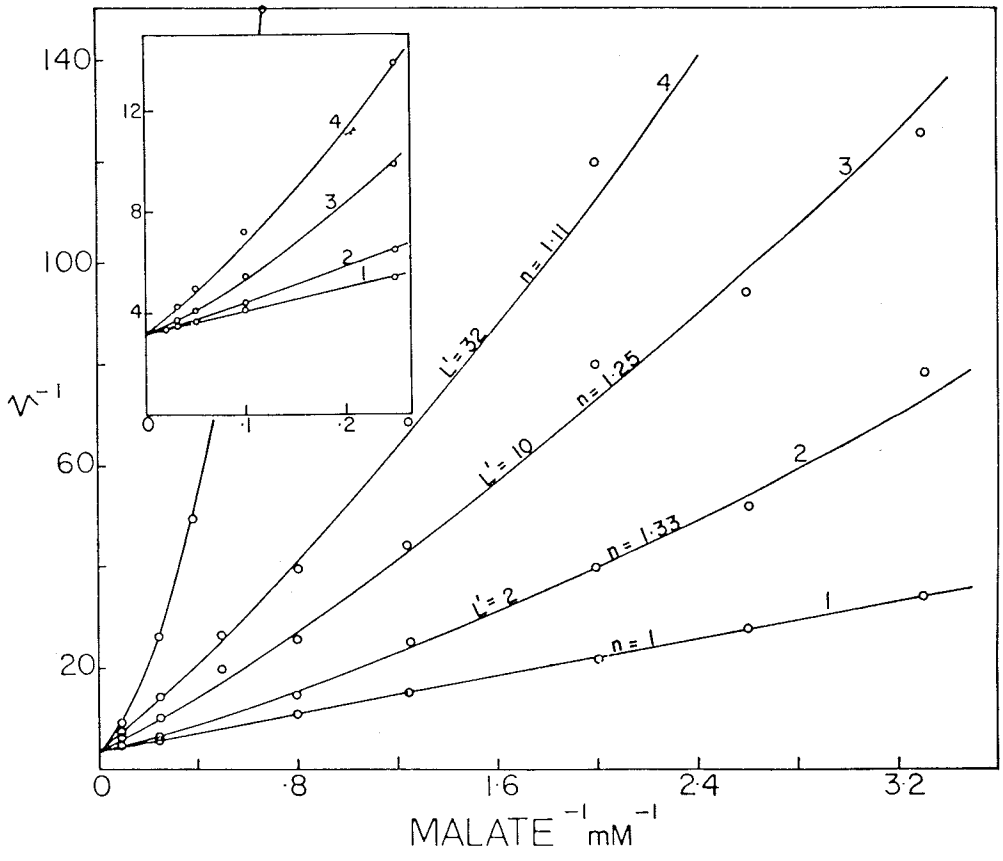

140
100
60
20
v^{-1}
0
·8
1·6
2·4
3·2
MALATE $^{-1}$ mM $^{-1}$
12
8
4
0
·1
·2
4
3
2
1
L' = 32
n = 1·11
L' = 10
n = 1·25
L' = 2
n = 1·33
n = 1

TABLE 7

Effect of reduced coenzymes and related compounds on activity of malic enzyme

Compound Tested	Concentration mM	Velocity
None		0.096
TPNH	0.14	0.047
DPN^+	0.3	0.094
DPNH	0.16	0.087
DPNH	0.32	0.056
DPNH	0.64	0.005
DPNH + DPN^+ (0.3 mM)	0.32	0.058
Glycine (1.32 M)		0.246
Glycine (1.32 M) + DPNH	0.32	0.244

The assay medium consisted of 0.077 mM TPN^+, 2 mM malate, 1 mM $MnCl_2$ and 0.1 M Tris-HCl (pH 7.5). The inhibitors and other reagents were added simultaneously with the substrate.

the inhibitor (Fig. 38), the reciprocal plots, linear in the absence of the inhibitor, became markedly curved at low concentrations of TPNH. At high concentrations of TPNH (0.21 mM in Fig. 38) the plot became linear again. When TPN^+ was used as the variable substrate, however, the double reciprocal plots were all linear (Fig. 39), but the lines did not intersect at a common point on the left of the vertical axis.

We had found earlier that glycine desensitizes malic enzyme to all of the allosteric inhibitors. The mechanism of this desensitization is not known but, as discussed below, glycine presents a possible conformational change triggered by the binding of allosteric ligands. In any case, if TPNH were causing both allosteric as well as product inhibition, glycine desensitization could be used as a tool to dissociate these two inhibitory functions of TPNH. Accordingly, the experiment represented in Fig. 38 was repeated with a constant amount of glycine (0.72 M) in the assay medium. The marked non-linearity obtained in the absence of glycine at malate concentrations lower than 1.0 mM vanished in its presence (Fig. 40). This experiment, thus, lends some support to the idea that part of the inhibition caused by TPNH is due to allosteric inhibition. In the presence of glycine (0.72 M) the pattern of inhibition with TPN^+ as the variable substrate also changes when TPNH is used as the inhibitor. This is clear from a comparison of Figs. 39 and 41. In Fig. 41 the lines when extrapolated intersect at a common point on the left of the vertical axis, and the inhibition clearly is linear noncompetitive. This type of interaction between TPN^+ and TPNH is quite unusual. In most of the pyridine nucleotide-linked dehydrogenases (Hsu, Lardy and Cleland, 1967) a competitive

Figure 38. The effect of TPNH on the velocity of the reaction with malate as the variable substrate. TPN^+ concentration was 0.38 mM. The concentrations of TPNH used are indicated above the lines.

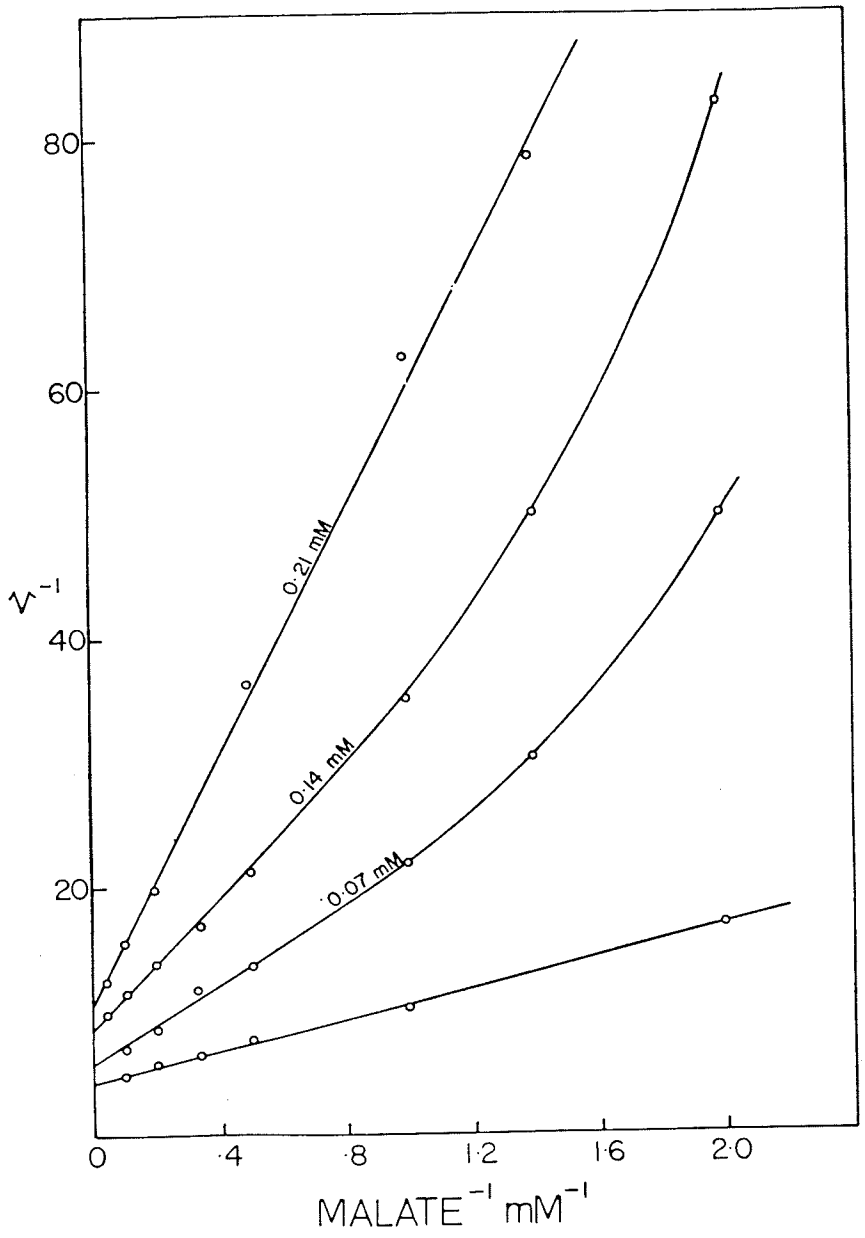

80
60
40
20
v^{-1}
0·21 mM
0·14 mM
0·07 mM
0
·4
·8
1·2
1·6
2·0
MALATE^{-1} mM^{-1}

Figure 39. The effect of TPNH on the velocity of the reaction with TPN^+ as the variable substrate. Malate concentration was 20 mM. Figures above the lines indicate the concentrations of TPNH used.

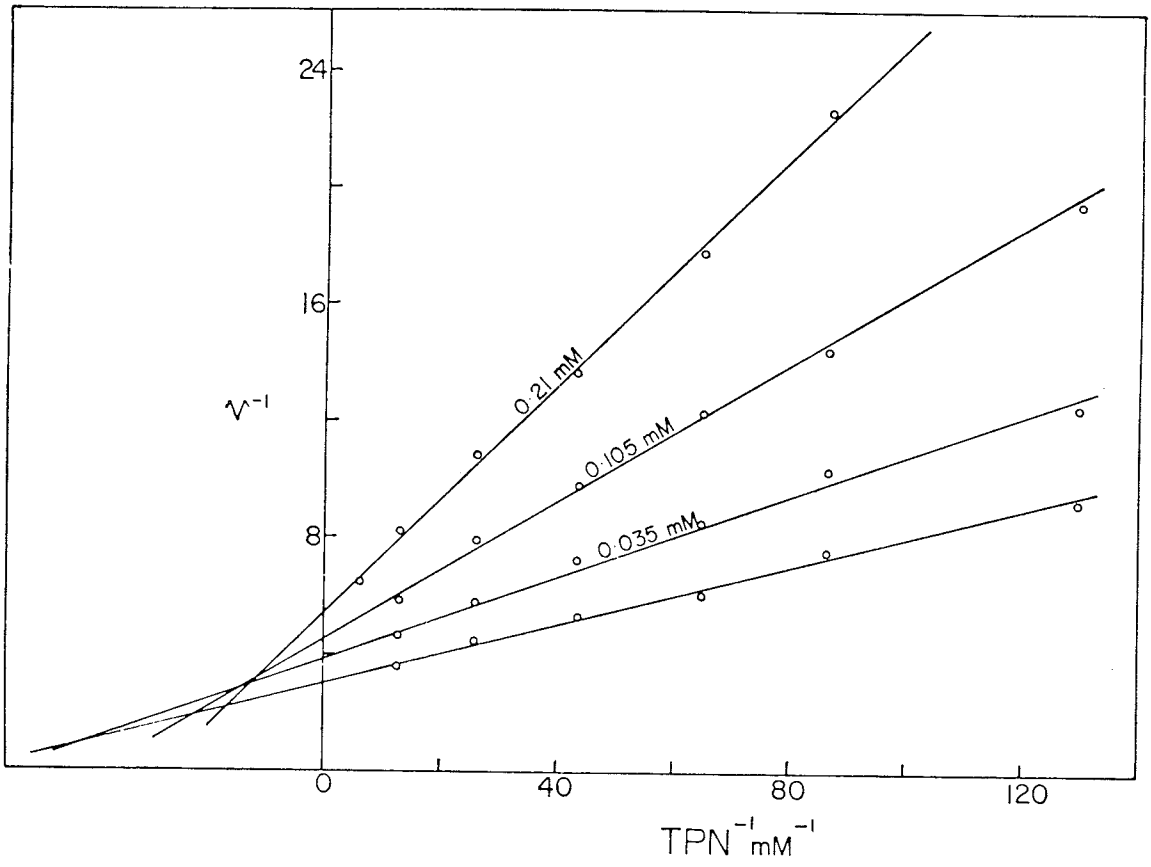

24
16
8
v^{-1}
0·21 mM
0·105 mM
0·035 mM
0
40
80
120
$TPN^{-1}\,mM^{-1}$

Figure 40. The effect of TPNH on the velocity of the reaction with malate as the variable substrate in the presence of 0.72 M glycine. Other conditions are the same as indicated in Fig. 38.

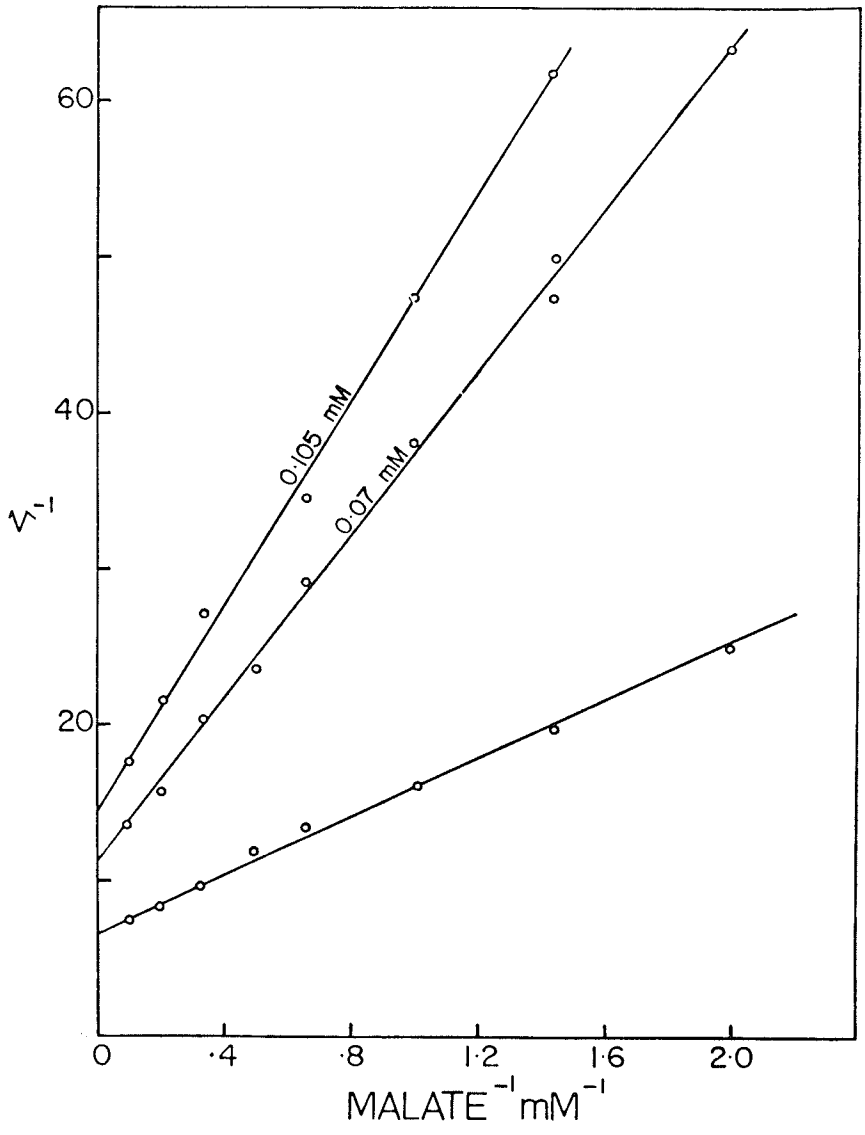
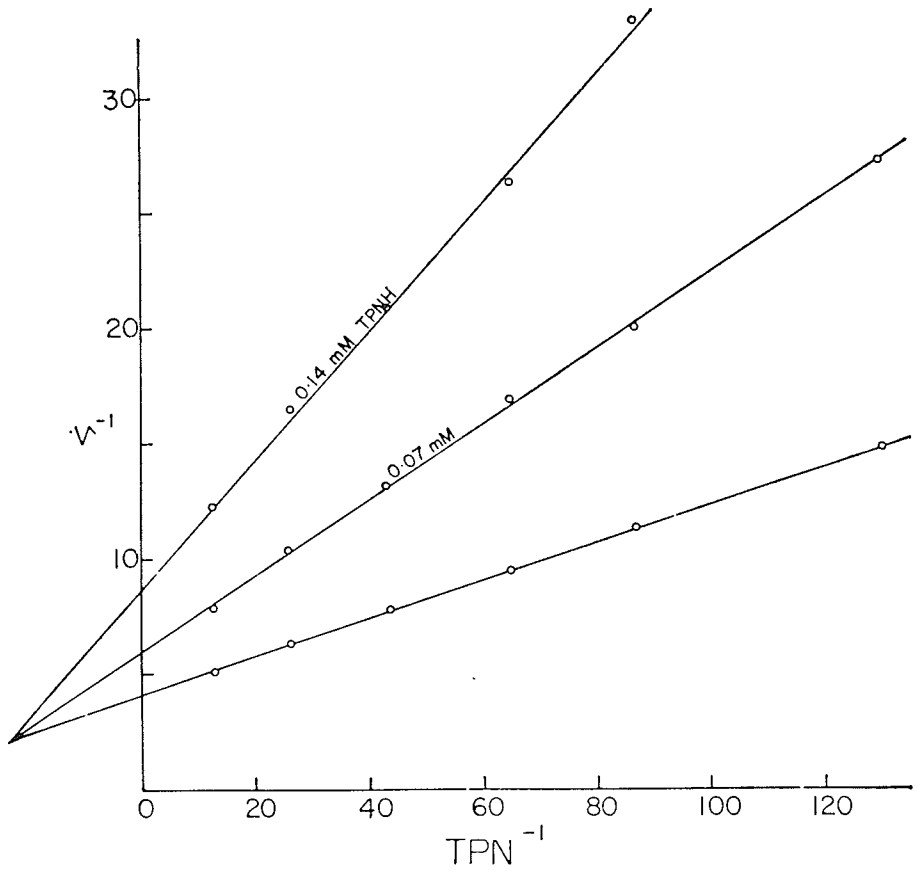

60
40
20
v^{-1}
0·105 mM
0·07 mM
0
·4
·8
1·2
1·6
2·0
MALATE^{-1} mM^{-1}

Figure 41. The effect of TPNH on the velocity of the reaction with TPN^+ as the variable substrate in the presence of 0.72 M glycine. Other conditions were the same as indicated in the legend to Fig. 39.


30
20
10
v^{-1}
0·14 mM TPNH
0·07 mM
0
20
40
60
80
100
120
TPN^{-1}

interaction between the oxidized and reduced coenzyme is the usual finding. A very likely possibility here is that the free enzyme form isomerizes, and TPN^+ and TPNH bind to different forms of the enzyme.

Earlier I had mentioned that DPNH acts as an inhibitor of malic enzyme. This inhibition is possibly of an allosteric nature, because, in the presence of glycine, inhibition by low concentrations is not discernible. This is in contrast to TPNH which inhibits the enzyme both in the absence and presence of glycine (Fig. 38 and 40), possibly, as stated earlier, because it is both a product as well as an allosteric inhibitor. It is possible that DPNH may also act as an alternate product inhibitor (i.e., bind at the active site) at very high concentrations because we had shown earlier that malic enzyme is not absolutely specific with regard to its coenzyme requirement. In any event, DPNH inhibition is markedly similar to that caused by TPNH when malate is the variable substrate. This is shown in Fig. 42. When this same experiment is repeated in the presence of 0.79 M glycine no inhibition is obtained in the constant presence of up to 0.26 mM DPNH and various concentrations of malate. This evidence suggests (assuming that the product inhibition constant is higher than the allosteric site binding constant) that inhibition by DPNH under the conditions of the experiment (Fig. 42) is probably both allosteric as well as alternate product inhibition.

Inhibition by Cyclic AMP — The inhibition of malic enzyme by cyclic 3',5'-AMP is shown in Fig. 43. It will be noted that the amount of the nucleotide required to cause half-maximal inhibition varies depending on the concentration of malate used in the assay medium. Also,

Figure 42. The inhibition of malic enzyme by DPNH with malate as the variable substrate. TPN^+ concentration was 0.077 mM. The concentrations of DPNH are indicated above the curves.

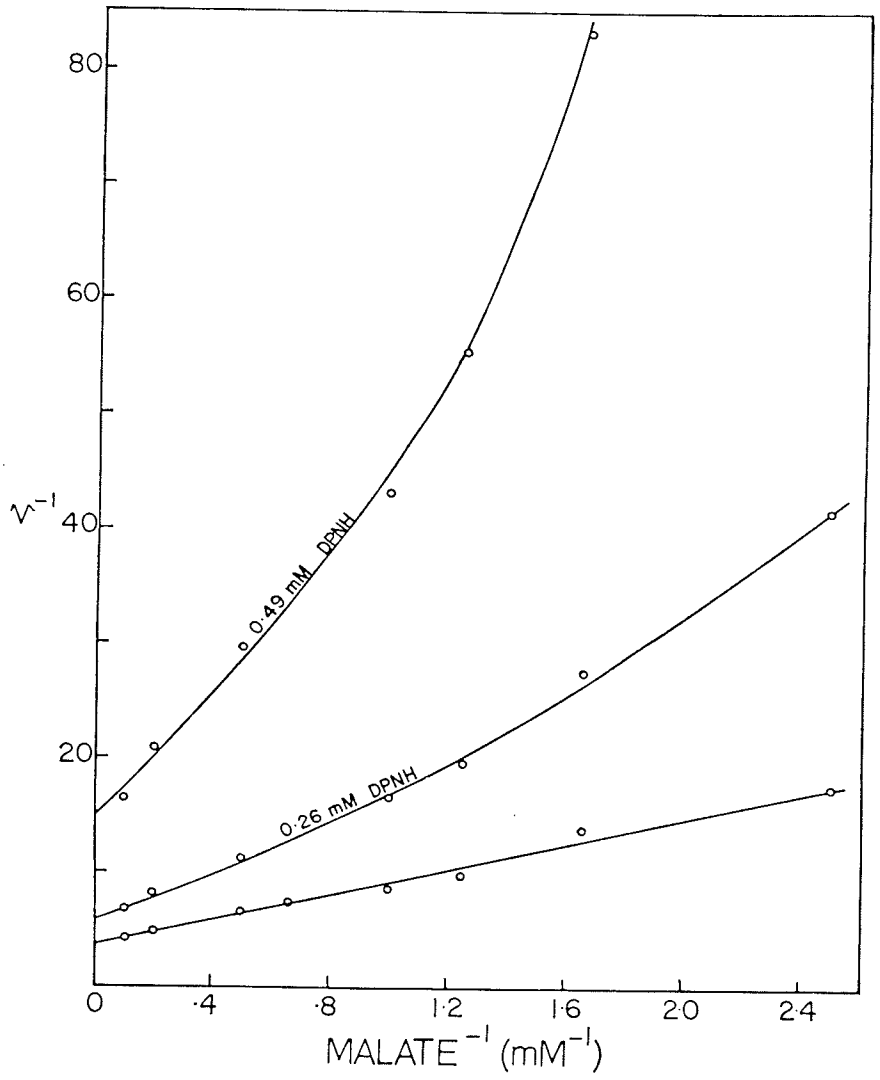
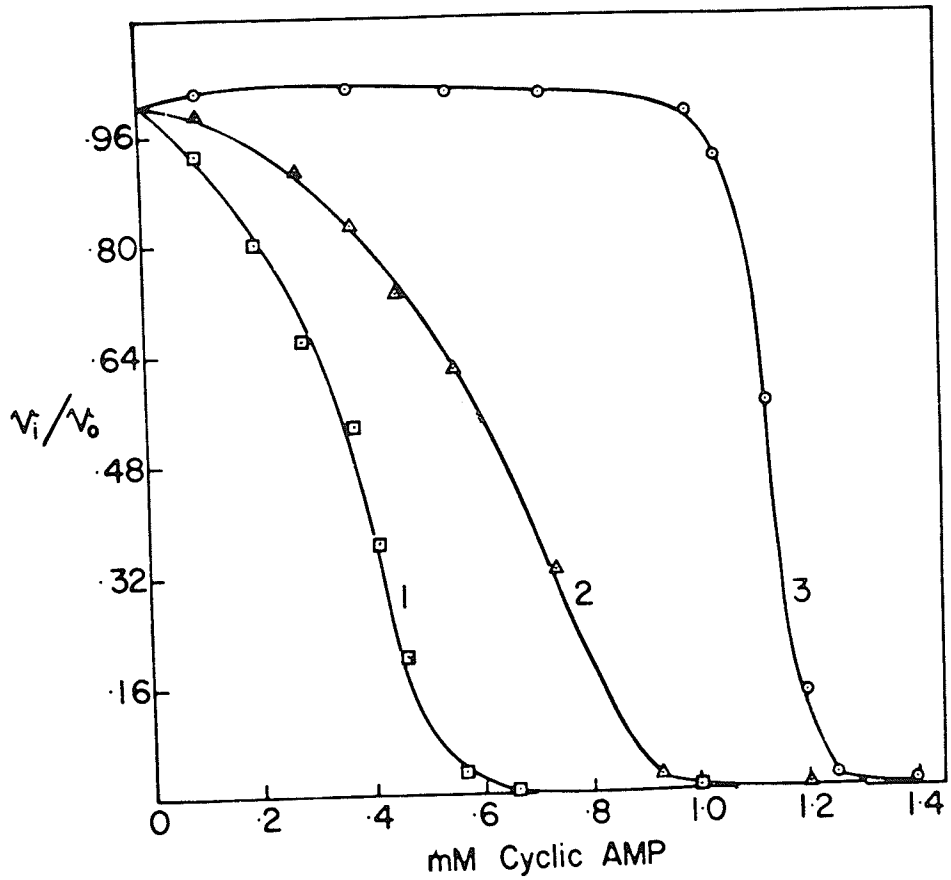

80
60
40
20
v^{-1}
0·49 mM DPNH
0·26 mM DPNH
0
·4
·8
1·2
1·6
2·0
2·4
MALATE^{-1} (mM^{-1})

Figure 43. The effect of cyclic 3',5'-AMP on the velocity of malic enzyme. The reaction mixture contained 0.05 M phosphate, pH 7.2, 0.077 mM TPN^+, 1 mM $MnCl_2$ and L-malate as indicated. 1 = 2 mM malate, 2 = 5 mM malate, and 3 = 11.0 mM malate. Vo and Vi are initial velocities in the absence and presence of the inhibitor respectively.


·96
·80
·64
·48
·32
·16
V_i/V_o
1
2
3
0
·2
·4
·6
·8
1·0
1·2
1·4
mM Cyclic AMP

the inhibition curves obtained (Fig. 43) at all concentrations of malate are sigmoidal suggesting a multisite binding of cyclic 3',5'-AMP. The inhibition caused by cyclic 3',5'-AMP is specific. At fixed malate concentrations of 2.5 mM the following compounds do not affect the enzyme activity: AMP (2 mM); ATP (2.5 mM); cyclic 2',3'-AMP (2 mM); ADP (1.5 mM); coenzyme A (1.0 mM); acetyl phosphate (4.0 mM).

It was shown earlier that the activity of the enzyme used in the present work is inhibited in an allosteric manner by acetyl-CoA, oxalacetate and DPNH. In conformity with these results the enzyme is desensitized to these inhibitors when assays are performed in 0.6 M glycine, pH 7.0. In this regard, the behaviour of cyclic 3',5'-AMP is completely different. In the presence of 0.6 M glycine in the assay medium neither the nature of the inhibition curve nor the extent of inhibition by cyclic 3',5'-AMP are altered to any significant extent.

It was also shown earlier that the initial velocity plots for both of the substrates of malic enzyme (TPN^+ and malate) are hyperbolic in the absence of allosteric inhibitors but the plots for malate become sigmoidal in their presence (acetyl-CoA, oxalacetate and DPNH). Such is, however, not the case with cyclic 3',5'-AMP. As can be seen from Fig. 44 cyclic 3',5'-AMP causes competitive inhibition when malate is the varied substrate, but the double reciprocal plots, unlike with other inhibitors mentioned earlier, remain linear. With TPN^+ as the varied substrate, cyclic 3',5'-AMP (in common with other inhibitors) produces noncompetitive inhibition (Fig. 45).

Figure 44. Double reciprocal plots of initial velocity data with malate as the variable substrate and cyclic 3',5'-AMP as the inhibitor (concentration indicated above the lines). Concentration of other reactants is the same as in Fig. 43.

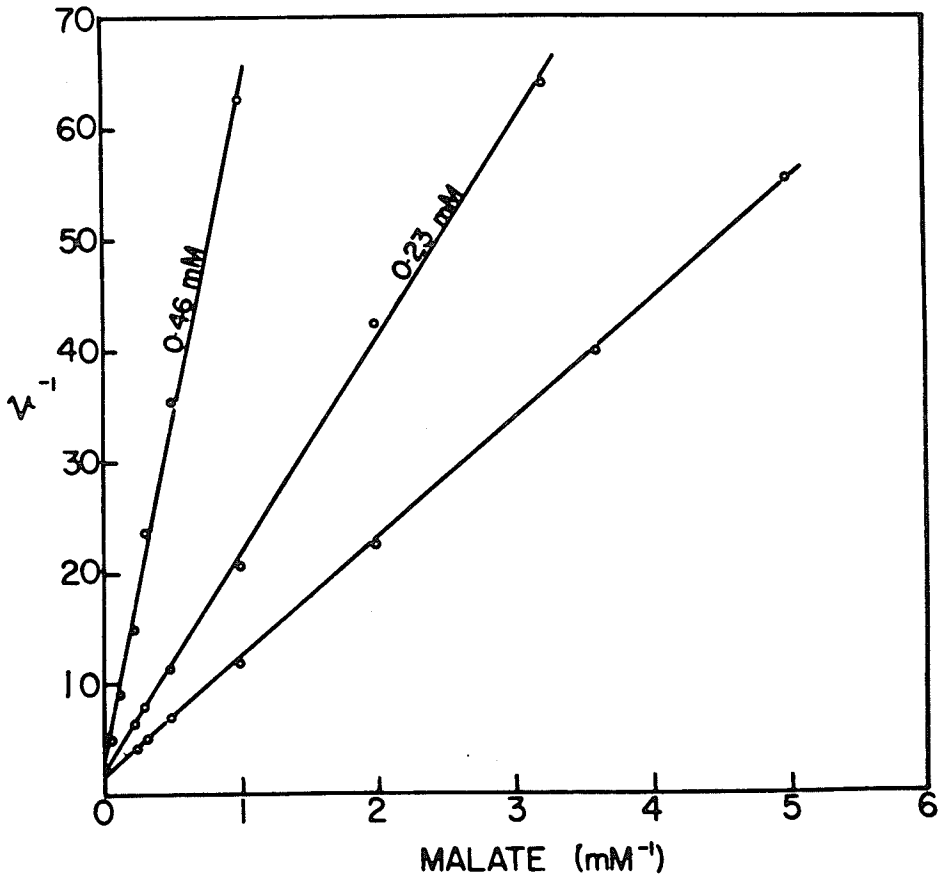
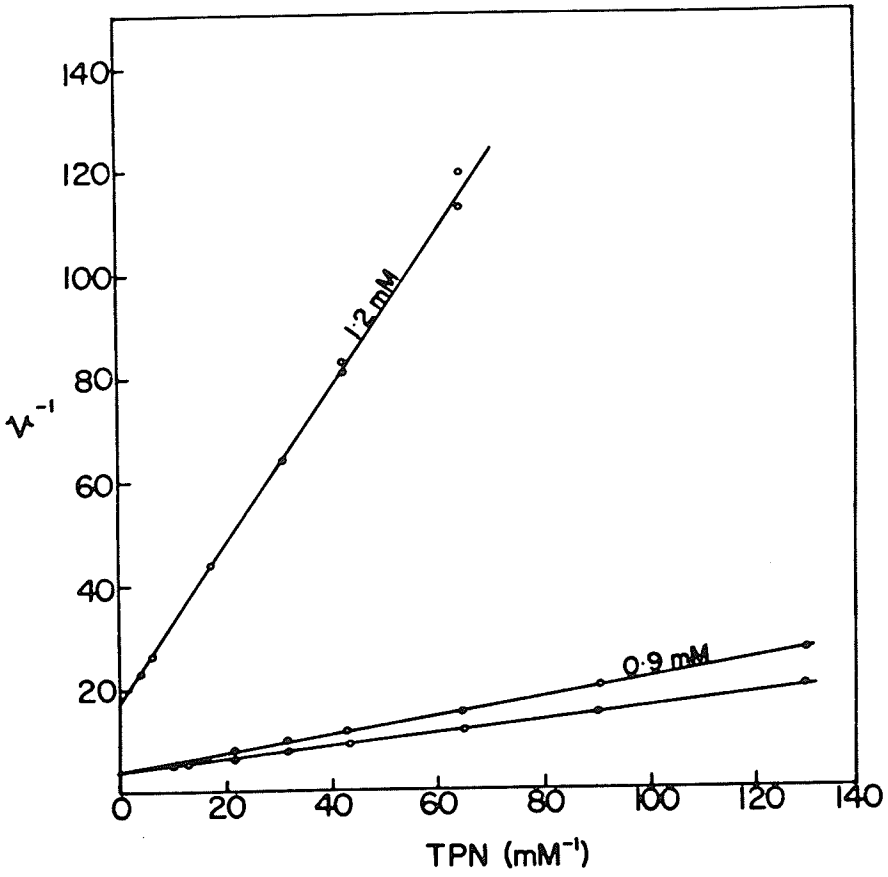

70
60
50
40
30
20
10
0
1
2
3
4
5
6
v^{-1}
0.46 mM
0.23 mM
MALATE (mM^{-1})

Figure 45. Double reciprocal plots of initial velocity data with TPN^+ as the variable substrate and cyclic 3',5'-AMP as the inhibitor. Malate concentration was 10 mM throughout.


140
120
100
80
60
40
20
v^{-1}
1·2 mM
0·9 mM
0
20
40
60
80
100
120
140
TPN (mM^{-1})

DISCUSSION

PHOSPHOENOLPYRUVATE CARBOXYLASE

The work described here was largely undertaken to throw some light on one important aspect of the allosteric control of enzymes, viz., the phenomenon of cooperative regulation. Complementing these efforts molecular weight studies were performed by equilibrium sedimentation centrifugations so that the total number of ligand binding sites on the enzyme surface could be more precisely determined.

The manifestation of cooperative regulation was first described by Caskey, Ashton and Wyngaarden (1964) in the purine biosynthetic pathway of animal tissues. Here, the first enzyme of the pathway, glutamine:phosphoribosyl amidotransferase (EC 2.4.2.14) was found to be inhibited separately by 6-hydroxypurine ribonucleotides, GMP and IMP, and 6-amino purine ribonucleotides AMP and ADP. In the presence of a mixture of these two groups of nucleotides (GMP and AMP or IMP and ADP), however, the inhibition was more than additive. This kind of control was extended by us (Sanwal, 1970; Sanwal and Maeba, 1966; Maeba and Sanwal, 1969) to cover cases where two or more effectors caused a cooperative activation. It has been demonstrated earlier (Sanwal and Maeba, 1966; Maeba and Sanwal, 1969) that PEP carboxylase from Salmonella typhimurium is activated by fructose-1,6-diphosphate, GTP (and several other nucleotides), and acetyl-CoA. When two activator pairs are simultaneously present they cause cooperative activation. It seemed possible that this cooperativity is caused by a reciprocal enhancement of the affinity of one activator for the enzyme in the presence of another. To understand the mode of action of the enzyme, however, we were also interested in finding how the binding

of the inhibitor was affected by the presence of activators separately and together.

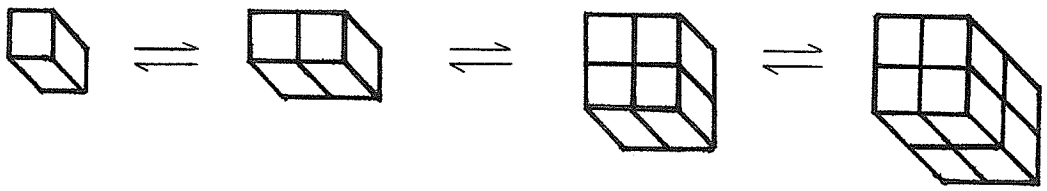
The equilibrium binding results can be interpreted on the basis of a two state model, where the enzyme in the absence of any ligands is assumed to be present predominantly in a state which binds the inhibitor (aspartate) preferentially. Further assumptions of this model would be that the binding sites for all of the activators and the inhibitors are separate, and that there is a direct (i.e., mediated by change in the tertiary structure of each individual subunit) reciprocal interaction of the activator sites on each subunit such that binding of one activator facilitates the binding of the other. It would have to be further assumed that the combination of a substrate and an activator on the enzyme surface or the combination of two activators (in the absence of substrate) converts the enzyme into a high affinity (for substrate) state to which the inhibitor is able to bind only with very low affinity. This model is by no means an original one, having been earlier discussed by several workers (Monod, Wyman and Changeux, 1965; Koshland, Nemethy and Filmer, 1966). However, it is unusual in the proposition that the cooperation of substrate and activator or two activator molecules is required to convert the enzyme to a low affinity state for the inhibitor. The experimental data are consistent with this model. It is expected that in the absence of substrate or activators or in the presence of only one activator the binding curve for aspartate (inhibitor) would be hyperbolic. Our results clearly show that by itself aspartate binds with a dissociation constant of about 0.1 mM and this value either does not change at all, or, changes only

slightly in the presence of each of the activators singly. However, when two activators are simultaneously present, and depending upon the activator pair, the binding curve for aspartate not only becomes sigmoidal but the dissociation constant increases drastically. Very crudely estimated, the dissociation constant for aspartate in the presence of 1.0 mM acetyl-CoA and 10 mM fructose-1,6-di-P is more than 1.5 mM. The heterotropic interaction of aspartate in the presence of activators is reflected in the value of Hill coefficient (n = 1.2) that is obtained in binding.

In keeping with the model above, the binding of acetyl-CoA to the enzyme was found to be sigmoidal at low concentrations. At higher concentrations of the ligand, however, the binding curve shows "tailing" (Yashinaga, Izui and Katsuki, 1970). A very likely cause of this "tailing" is that acetyl-CoA binds non-specifically at higher concentrations to some other site on the enzyme surface. Whatever the cause of this "tailing" may be, it is noteworthy that in the presence of a second activator, GTP, the acetyl-CoA binding curve not only becomes almost hyperbolic but the dissociation constant for acetyl-CoA is also considerably reduced (0.16 mM). A minor point that emerges from the binding studies is that neither Mg^{2+} nor Mn^{2+} is required for the binding of the inhibitor or the activators to the enzyme.

From our results it appears that both aspartate and acetyl-CoA have one binding site per 100,000 g/mole of the enzyme. The total number of sites on the enzyme oligomer depends, of course, on the molecular weight assigned to the native enzyme. Our previous work (Maeba and Sanwal, 1969) with PEP carboxylase from S. typhimurium had shown

that in dilute solutions the molecular weight of the enzyme, obtained from sucrose density centrifugation experiments, was about 200,000. Smith (1971), however, with E. coli enzyme demonstrated that the enzyme dissociates into half molecules on dilution. Our present ultracentrifugation results obtained with freshly prepared enzyme seems to suggest a model which agrees with the following:



50,000 100,000 200,000 400,000

The largest molecular weight species found by either Sephadex G-200 chromatography or equilibrium sedimentation studies would suggest a molecular weight of 400,000 $g/mole^{-1}$ for the native enzyme. Occasionally 200,000 $g/mole^{-1}$ species are found (Fig. 8; Maeba and Sanwal, 1969). The 400,000 $g/mole^{-1}$ unit appears to consist of identical eight smaller species with a molecular weight of 50,000 $g/mole^{-1}$ (or four identical 100,000 $g/mole^{-1}$ species that are difficult to dissociate into the 50,000 units). The presence of the 50,000 $g/mole^{-1}$ species is observed in the denaturants, guanidine hydrochloride and SDS. The existence of the 100,000 MW unit is also indicated in centrifugation experiments performed in the presence and absence of these same denaturants along with the SDS polyacrylamide gel electrophoresis and electron microscopy results. The 100,000

g/mole unit is also confirmed by the cross-linking procedure of Davies and Stark (1970) where the fastest migrating major band was found to have a molecular weight of 100,000. The reason for the incomplete dissociation of the enzyme in these denaturing solvents is not known, but this phenomenon, as pointed out earlier, is certainly not unique to PEP carboxylase (Katzman, 1972; Duckworth and Sanwal, 1972; Kopperschlager, Diezel, Prauche and Hofman, 1972; Coffee, Aaronson and Frieden, 1973). We have recently obtained data to show that unlike other effectors, 2 molecules of Mn^{2+} are bound per 100,000 g/mole^{-1} of the enzyme. This observation would also tend to suggest that the smallest polypeptide unit of PEP carboxylase has a molecular weight of 50,000.

In recent years several examples have come to light where the number of polypeptide chains in an oligomer do not correspond to the number of ligand binding sites, i.e., one to one proportionality does not seem to exist (Kuehn, Barnes and Atkinson, 1971; Kemp and Krebs, 1967; Levitzki, Stallcup and Koshland, 1971). In the case of rabbit muscle phosphofructokinase (Kemp and Krebs, 1967), for example, the protomer (Monod, Wyman and Changeux, 1965) is a subunit of molecular weight 90,000 which binds one mole of fructose 6-phosphate, AMP, ADP and cyclic 3',5'-AMP, but 3 moles of the allosteric inhibitor ATP. This may be very well due to half-site reactivity (Levitzki, Stallcup and Koshland, 1971). Levitzki *et al* (1971) have shown, as an example, that the affinity label 6-diazo-5-oxonorleucine reacts with only one half of the glutamine sites of *E. coli* CTP synthetase, although the subunits of the enzyme all seem to be identical.

MALIC ENZYME

The necessity for the investigation reported here arose from considerations of a fundamental difference between bacteria and higher organisms in the organization of enzyme systems catalyzing reactions of the Krebs cycle and its peripheral channels. We refer here to the absence of mitochondria in E. coli and other bacteria with the consequent lack of rigid compartmentation of certain enzymes and metabolites. This inevitably leads to certain complications in the channeling of metabolites in pathways which share these metabolites as substrates. As an example, and directly pertinent to the work reported here, we can first consider the formation of oxalacetate in mitochondria when acetyl-CoA levels are high. The elegant work of Utter and Keech (1963) has shown that, by activation of pyruvate carboxylase, acetyl-CoA is able to produce that amount of oxalacetate which is necessary for its oxidation. This excess oxalacetate is probably not utilized by mitochondrial malic dehydrogenase owing to its inhibition (Tager and Slater, 1963), and neither is it immediately accessible to the extramitochondrial malic dehydrogenase owing to the problems associated with permeation, etc. (Lardy, Paetkau and Walter, 1965). This sort of compartmentation afforded by the presence of mitochondria reasonably assures that excess oxalacetate will mostly be utilized for condensation with acetyl-CoA. Admittedly, this is an extremely simplified version of the actual and definitely more elaborate controls available in the partitioning of metabolites (Klingenberg, Chance, Estabrook and Williams, 1965; Krebs, Slater, Kaniuga and Wojtezak, 1967) between the mitochondria and the cytoplasm, but illustrates for

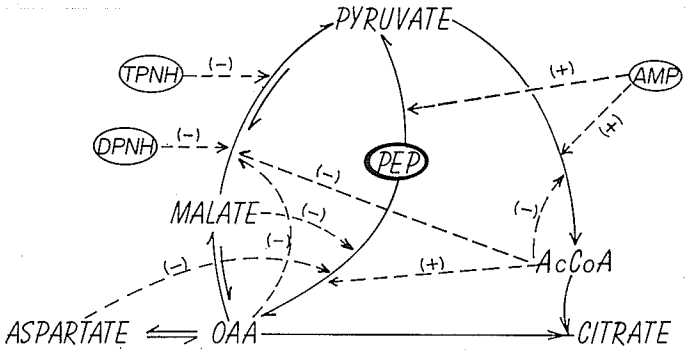


Figure 46. Schematic diagram of the feedback systems affecting malic enzyme and related enzymes utilizing P-enolpyruvate (PEP) and pyruvate in E. coli when carbohydrates are the energy source. (+) and (-) represent activation or inhibition, respectively.

purposes of our hypothesis the well known inference that with compartmentation and effective insulation of metabolites can be achieved from competing enzyme systems.

In bacteria, however, the problems are a little different. Considering an analogous situation, as discussed above, acetyl-CoA in the enterobacteriaceae at least (Canovas and Kornberg, 1965; Maeba and Sanwal, 1965) is capable of powerfully activating P-enolpyruvate carboxylase, and the physiological necessity for this activation seems to be the same as in higher organisms, viz., to generate sufficient oxalacetate so that the oxidation of excess acetyl-CoA can occur. However in the absence of compartmentation, the excess oxalacetate can very easily be diverted to malate and thence (via malic enzyme) to pyruvate which would then be available for the generation of more acetyl-CoA (see Fig. 46). In order to prevent this needless recycling, we argued,

mechanisms should be available which ensure that excess oxalacetate produced in response to a particular need is only utilized to fulfill this need.

This line of argument rewarded us with the finding that malic enzyme is inhibited both by acetyl-CoA and oxalacetate and the importance of this inhibition in view of what has been said above is self-evident for regulation. Complementing this control of malic enzyme is the inhibition by acetyl-CoA of pyruvate dehydrogenase from E. coli discussed by Schwartz, Old and Reed (1968) (Fig. 46). It must also be mentioned that superimposed upon the positive control on P-enolpyruvate carboxylase exerted by acetyl-CoA, there is negative feedback inhibition by aspartate (Maeba and Sanwal, 1965) and malate, two metabolites which are produced directly from oxalacetate (Fig. 46). The level of these compounds must surely determine the amount of oxalacetate formed from P-enolpyruvate in the presence of acetyl-CoA, but so long as extra oxalacetate is produced the inhibition of malic enzyme by this compound would prevent the formation of extra pyruvate which acts as a "deinhibitor" for pyruvate dehydrogenase (Schwartz, Old and Reed, 1968).

The foregoing arguments, following from our assumption that the complex activity controls are necessitated in bacteria as a result of an absence of mitochondria and a consequent lack of rigid compartmentation, presuppose that the enzyme preceding the malic enzyme, viz., malic dehydrogenase (see Fig. 46), must also be controlled in some way under conditions where an excess of acetyl-CoA is available. We have recently found that malic dehydrogenase of E. coli is indeed inhibited at low concentrations of oxalacetate, provided that the DPNH

concentration is high (Sanwal, unpublished observations).

This brings us to the physiological significance of DPNH and TPNH inhibition of the malic enzyme. When one considers that TPNH is an end product of malic enzyme, it is easy to identify this inhibition with a type of negative feedback effect. It is important to mention here that inhibition by TPNH is not only due to product inhibition (in the kinetic sense) but also, like DPNH, due to an allosteric inhibition. This is evidenced by the desensitization experiments noted in Table 7. The reasons for the inhibition of malic enzyme by DPNH are not clear. If one assumes that in E. coli a high DPNH:DPN^+ ratio reflects similarly high ATP:ADP ratios, inhibition of malic enzyme by DPNH may be advantageous because under such conditions there may not be a high demand for pyruvate by the energy generating pathways of the Krebs cycle. This argument finds support from the earlier observations that pyruvate kinase (Maeba and Sanwal, 1968) and pyruvate dehydrogenase (Schwartz and Reed, 1968) are both activated by AMP, a compound whose level is expected to be quite low when the ATP:ADP concentration is high (Krebs, 1964; Atkinson, 1965).

One question of interest regarding the diverse effectors of malic enzyme is whether each of these effectors has a separate site on the enzyme surface. The experimental evidence points to at least two sites, one specific for oxalacetate and another for acetyl-CoA and related pyridine nucleotides. Thus, we had shown that acetyl-CoA is not inhibitory at pH values above 8.5 when, as mentioned above, oxalacetate is fully inhibitory. Despite suggestive evidence that the regulatory sites for the effectors may be different, it is interesting that the

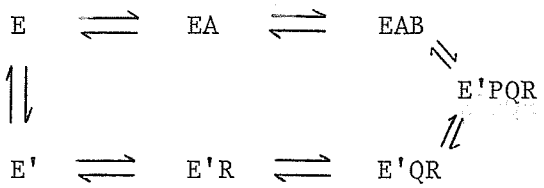
enzyme is desensitized simultaneously to all of the effectors in the presence of glycine. Indeed, we are not aware of any parallel cases in the literature in which glycine or other amino acids cause such effects on proteins, except perhaps an old observation that arginine in the presence of NH_4Cl (Cohn and Edsall, 1943) dissociates serum albumin. The effect caused by glycine seems to be more specific than that caused merely by a change in the ionic strength of the assay medium. If the enzyme is postulated to consist of regulatory and catalytic subunits, glycine by causing a dissociation of these subunits may conceivably bring about a simultaneous desensitization of the enzyme to all of the effectors.

The kinetic behavior of malic enzyme as presented here is of fundamental interest because it seems to differ, at least superficially, from the majority of regulatory enzymes (Atkinson, 1966; Stadtman, 1966), by the lack of cooperativity in the binding of substrates in the absence of allosteric ligands. As is amply clear from the data presented here, substrate cooperativity (or nonlinearity of the conventional double reciprocal plots) is conditional on the presence of any one of the several allosteric inhibitors of the malic enzyme. The question, therefore, arises whether the physical basis of cooperativity in the case of malic enzyme is the same as has been extensively discussed for enzymes which show effector-independent cooperativity in the binding of their substrates (Monod, Wyman and Changeux, 1965; Kirtley and Koshland, 1967; Blangy, Buc and Monod, 1968).

Before this question is discussed, a few characteristics of the reaction mechanism in the absence of allosteric ligands may be

analyzed. Malic enzyme has two substrates (TPN^+ and malate) and three products (CO_2, pyruvate, and TPNH) when the appearance of TPNH is used as the assay in initial velocity studies. From Fig. 31 it can be noted that the binding of one substrate is not independent of the other, as would be expected if the addition of the substrates on the enzyme surface were ordered. Indeed, for most pyridine nucleotide dehydrogenases, including the malic enzyme from pigeon liver (Hsu, Lardy and Cleland, 1967), kinetic and equilibrium binding studies have revealed that the addition of coenzyme on the enzyme is obligatory for the binding of the second substrate to occur. The product inhibition data presented in Table 6 are entirely consistent with the postulation of an ordered mechanism for the malic enzyme of E. coli. In addition, since the product inhibitions are linear (Table 6), they rule out a diffusion-limited random binding of the substrates on the enzyme surface. Such a mechanism, at least in theory (Sanwal and Cook, 1966), can generate non-linear double reciprocal plots.

The unusual feature of the enzymic reaction in the absence of allosteric ligands is the noncompetitive inhibition between TPN^+ and TPNH (Figs. 39 and 41). In an ordered mechanism, since the oxidized and the reduced coenzymes are supposed to bind to the same form of the enzyme (i.e., the free enzyme), the inhibition by reduced coenzyme is always competitive when the oxidized coenzyme is the variable substrate and vice versa. The only reasonable explanation that can be offered for the noncompetitive interaction between TPN^+ and TPNH (Figs. 39 and 41) is that the free enzyme form undergoes isomerization and TPN^+ and TPNH bind to two different forms of the enzyme (Mechanism I).



MECHANISM I

where A = TPN^+, B = malate, R = TPNH, P and Q = CO_2 or pyruvate.

The rate equation for Mechanism I can be written in terms of kinetic constants with the method and terminology of Cleland (1963). Considering TPNH as a product inhibitor only (as, for instance, in the presence of glycine, Fig. 41) the initial velocity equation (setting concentrations of P and Q = 0) is:

$$v = \frac{VAB}{K_{ia}K_b + K_b A + K_a B + AB + \frac{K_p K_{ib} K_{iia} R}{K_{ip} K_{ir}} + \frac{K_a BR}{K_{ir}} + \frac{ABR}{K_{iir}}} \qquad (1)$$

where A, B, and R are TPN^+, malate, and TPNH, respectively; K_a, K_b, and K_p are Michaelis constants for A, B, and P; K_{ia}, K_{ib}, K_{ip}, and K_{ir} are the inhibition constants for A, B, P, and R; K_{iia} and K_{iir} are isoinhibition constants.

Equation 1 predicts noncompetitive inhibition between A and R (Equation 2):

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_b}{B} + \frac{R}{K_{ii}\,R}\right] + \frac{K_a}{V}\left[1 + \frac{K_{ia}\,K_b}{K_a\,B} + \frac{R}{K_{ir}} + \frac{K_p\,K_{ib}\,K_{iia}\,R}{K_a\,K_{ip}\,K_{ir}\,B}\right]\left[\frac{1}{A}\right] \qquad (2)$$

In the absence of products Equation 1 reduces to Equation 3:

$$\frac{1}{v} = \frac{1}{V}\left[1 + \frac{K_b}{B}\right] + \frac{K_a}{V}\left[1 + \frac{K_{ia}\,K_b}{K_a\,B}\right]\left[\frac{1}{A}\right] \qquad (3)$$

which follows the pattern given in Fig. 31.

Mechanism I is consistent not only with the results presented in Fig. 41 but with most of the rate data presented earlier in the absence and presence of products.

With this analysis the mechanism of cooperativity in the binding of malate and the competitive inhibition caused by acetyl-CoA (Fig. 34) and oxalacetate (Fig. 34) is perhaps reasonably explained on the basis of a model which involves the following assumptions. (a) The free enzyme form binds TPN^+ only. The binding causes a specific conformational change (perhaps in a way postulated by the induced fit mechanism of Koshland (Koshland, 1963) in the enzyme which exposes the malate- and inhibitor-binding sites. (b) The $E\text{-}TPN^+$ complex exists predominantly (or even perhaps exclusively) in a conformational state (State R in the terminology of Monod et al (Monod, Wyman and Changeux, 1965) which has high affinity for malate but very low affinity for the allosteric inhibitors. (c) Binding of the allosteric ligands (at regulatory sites)

to the subunits in State R changes their conformation to a form (T) which has very low affinity for malate. In other words, allosteric inhibitors stabilize State T and malate stabilizes State R. (d) Although the binding of both inhibitors and malate is conditional on the initial binding of TPN^+, they do not affect the binding constants of each other. (e) The velocity constants associated with States T and R are the same.

This model is consistent with the experimental data. Thus, since only State R of the enzyme is available in the absence of allosteric inhibitors, the binding of malate will be noncooperative, i.e., follow the Michaelis-Menten equation (Fig. 31). Similarly, since only the $E\text{-}TPN^+$ complex is envisaged to undergo the $R \rightleftharpoons T$ transition in the presence of the inhibitors the binding of TPN^+ will always be noncooperative (Figs. 39, 41 and 35). Also, replots of intercepts and slopes of the double reciprocal plots with TPN^+ as the variable substrate against the inhibitor concentrations (Fig. 35) will be nonlinear. This nonlinearity is simply an indication of the "homotropic" (Monod, Wyman and Changeux, 1965) interactions of the inhibitors, i.e., interactions caused by the binding of the inhibitors to the two states of the protein. The most important support for the model is provided by the competitive nature of interaction between malate and two such structurally dissimilar inhibitors as oxalacetate (Fig. 37) and acetyl-CoA (Fig. 34). As is clear from the postulates of the model, competition must ensue if oxalacetate and acetyl-CoA pull the conformation to State T and malate pulls it to State R. The fact of competition can also be used to rule out any mechanism which would explain cooperativity

of malate plots as resulting from partial inhibitions, i.e., on the basis that the velocity constants for the release of products are different for the enzyme-malate and enzyme-inhibitor-malate complexes. If equations are written for partial inhibition mechanism, with the method of King and Altman (1956), noncompetitive inhibition is predicted between the substrate and the inhibitors (Maeba and Sanwal, 1966).

The postulation of two states of the protein (T and R) is also helpful in understanding the possible mechanism of desensitization by glycine. As has been indicated here, malic enzyme is not inhibited by various allosteric inhibitors when it is assayed in the presence of glycine (concentrations above 0.3 M). A possible reason could be that glycine prevents not the binding of the inhibitors by a localized change at the regulatory sites but the transition of subunits from State R to T. In this way, glycine would desensitize the enzyme to all inhibitors simultaneously.

While the model presented here qualitatively accounts for most of the kinetic properties of the enzyme, it is difficult to give any quantitative estimates of the various rate and thermodynamic parameters. These, of course, would depend upon the model of subunit interaction chosen (Monod, Wyman and Changeux, 1965; Koshland, Némethy and Filmer, 1966; Blangy, Buc and Monod, 1968). Purely as an illustration, if one considers, as in the model of Monod *et al* (1965) that the $R \rightleftharpoons T$ transition of the $E\text{-}TPN^+$ complex is perfectly concerted, Equation 4 should apply.

$$\bar{Y}_{(S)} = \frac{v_0}{V_{max}} = \frac{\alpha(1 + \alpha)^{n-1} + L'c\alpha(1 + c\alpha)^{n-1}}{(1 + \alpha)^n + L'(1 + c\alpha)^n} \qquad (4)$$

where (S) = malate, v_0 = initial velocity, V_{max} = maximal velocity, $\alpha = S/K_{m(S)}$, c = ratio of $K_{m(S)}$ for (R) and (T) states, and L' = equilibrium constant between (R) and (T) states, which is a function of inhibitor concentrations, n = number of protomers or interacting subunits.

The various parameters of Equation 4 can be evaluated for the case of oxalacetate as inhibitor (Fig. 37), for instance, by using the manipulations described by Blangy, Buc and Monod (1968). The fits of Equation 4 to the data of Fig. 37 are quite satisfactory, if one assumes that n = 2 and c = 0.12. For Equation 4 to be valid, it is required, of course, that all or most of the enzyme be present as E-TPN^+ complex, i.e., inhibition data be obtained under conditions in which the enzyme is saturated with TPN^+ (such being the case in Fig. 37).

While Equation 4, based on concerted transition of subunits, fits the data, it is virtually impossible to use it to rule out the sequential model of subunit interactions proposed by Koshland et al (1966). Indeed, the data are also in qualitative agreement with the competitive binding model of Kirtley and Koshland (1967). It is hoped that future equilibrium binding studies will be able to throw more light on this aspect of the problem.

The elucidation of the physical mechanism underlying the inhibitory effect of cyclic 3',5'-AMP will have to await the availability

of malic enzyme in a homogeneous form but the data described in this work are sufficient enough to ascribe a physiological role to the inhibition of the enzyme by cyclic 3',5'-AMP. What this role might be is a matter of conjecture at the present time. Since malic enzyme is significantly inhibited at concentration ranges ($\simeq 10^{-4}$ M) of cyclic 3',5'-AMP which are only found in cells starved of an energy source (Makman and Sutherland, 1965) it is reasonable to suppose that this inhibition is directed towards prevention of wasteful and unnecessary reactions during starvation. Malic enzyme presumably qualifies as one of such dispensable enzymes. This presumption is supported by the observation (Raunio, 1966) that under normal conditions of growth, i.e., in the absence of the limitation of an energy source, E. coli secretes large quantities of pyruvate part of which possibly arises by the withdrawal of malate from the Krebs cycle by malic enzyme. Under normal conditions this diversion of malate to pyruvate may serve a useful purpose; for instance, this conversion may help generate TPNH for fatty acid synthesis. Under starvation conditions, however, when degradative processes take precedence over biosynthetic ones, the demand for reducing power may not be as great and it would help the cell economy if intermediates of Krebs cycle are not drained away unnecessarily. It is possible that one of the roles of cyclic 3',5'-AMP in microorganisms may be connected with mechanisms which help the organism to tide over unfavourable circumstances. This suggestion does not detract from the importance of other regulatory roles that cyclic 3',5'-AMP may have in E. coli such as its demonstrated role as a "derepressor" of catabolite repression (Ullman and Monod, 1968; Perlman and Pastan, 1968; Pastan and Perlman, 1968).

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