THE ALLOSTERIC PHOSPHOENOLPYRUVATE CARBOXYLASE OF SALMONELLA: ITS PROPERTIES AND PHYSIOLOGICAL ROLE

BY

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ABSTRACT

THE ALLOSTERIC PHOSPHOENOLPYRUVATE CARBOXYLASE OF SALMONELLA: ITS PROPERTIES AND PHYSIOLOGICAL ROLE

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Under the supervision of Professor B. D. Sanwal

The enzyme phosphoenolpyruvate carboxylase (orthophosphate: oxalacetate carboxylase (phosphorylating) EC 4.1.1.31) from Salmonella typhimurium LT2 was highly purified, and by the criteria used, appeared to be a homogeneous preparation. Physico-chemical studies indicated that this protein was composed of 4 identical subunits, the tetrameric oligomer having a molecular weight of 193,000 ± 7,000. Evidence is given for the presence of accessible hydrophobic regions in the enzyme as well as exposed areas of negatively charged groups. Four moles of sulfhydryl groups per mole of enzyme were found.

The enzyme was allosteric, according to the terminology of Monod, Changeux and Jacob (1963), and as such, was subject to control by a number of modulators. In addition to acetyl CoA (Cánovas and Kornberg, 1965), FDP stimulated activity. The activation by acetyl CoA and FDP appeared to be a mechanism ensuring a supply of oxalacetate for citrate formation. Certain nucleotides (CMP, CDP and GTP) also activated PEP carboxylase. This effect is believed to be a possible compensatory feedback mechanism which offsets conditions that inhibit the enzyme to allow continued synthesis of oxalacetate. A strong feedback inhibition was exerted on PEP carboxylase by two effectors, aspartate

and malate. The significance of these control mechanisms are discussed and, from kinetic data, a model for the mode of action of the enzyme is presented.

An investigation of pyruvate kinase showed that the enzyme had a molecular weight of 100,800 ± 2,700 and was "dimeric", the "monomeric" form being inactive. AMP and FDP were found to activate this enzyme; the activation being cumulative in the presence of both activators.

The effect of modulators on the activity on PEP carboxylase and pyruvate kinase are discussed in terms of a coordinated mechanism which controls the diversion of PEP formed through glycolysis into catabolic and anaplerotic pathways.

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ABBREVIATIONS

ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
CDP	cytidine diphosphate
CMP	cytidine monophosphate
CTP	cytidine triphosphate
CoA	coenzyme A
DEAE	diethylaminoethane
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
EDTA	ethylenediaminetetraacetic acid
F-6-P	fructose 6-phosphate
FDP	fructose 1,6-diphosphate
G-1-P	glucose 1-phosphate
G-6-P	glucose 6-phosphate
GMP	guanosine monophosphate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
IDP	inosine diphosphate
NAD	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate

OAA	oxalacetate
PEP	phosphoenolpyruvate
PMB	p-hydroxymercuribenzoic acid
P_i	inorganic phosphate
PP_i	inorganic pyrophosphate
SDS	sodium dodecyl sulfate
TCA	tricarboxylic acid
TDP	thymidine diphosphate
Tris	Tris(hydroxymethyl)aminomethane
UDPG	uridine diphosphoglucose
UMP	uridine monophosphate
V_m or V_{max}	maximum velocity

I. INTRODUCTION

Ever since the discovery of feedback inhibition by Umbarger (1958) and Yates and Pardee (1956) in amino acid and pyrimidine biosynthetic pathways, a large number of enzymes have been shown to be susceptible to end product control (Atkinson, 1966; Stadtman, 1966). However, much of this work has been done with enzyme sequences which are biosynthetic, i.e., which require energy. With the broad principles of control already enunciated as a result of these studies (see Historical), the time is ripe for the investigation of control mechanisms in biochemical pathways which perform more than one function. Such is the case, for instance, with the well known glycolytic sequence which serves to supply the carbon skeletons not only for biosynthetic purposes but also for the generation of energy either directly or via the citric acid cycle. The fundamental question that arises here from the point of view of control is, what mechanism determines how the carbon skeletons will be partitioned into the biosynthetic and the catabolic channels? This question is much more intriguing when applied to microorganisms because rigid, compartmentation controls, such as are available to higher organisms due to the presence of mitochondria, are largely lacking in them. Once the question is formulated, it is easy to see that the most promising answers would be obtained from the study of any one of the branch points of the glycolytic channel. One such interesting branch point

is at the level of phosphoenolpyruvate which (in enteric bacteria) is diverted into oxalacetate by the anaplerotic arm (catalyzed by phosphoenolpyruvate carboxylase) and into pyruvate by the catabolic arm (catalyzed by pyruvate kinase).

The questions that we have asked, therefore, are (1) what is the nature of these enzymes in the enteric bacteria, (2) what are the allosteric effectors of these enzymes, and finally (3) how are the two channels coordinated?

This thesis is an attempt to answer these questions. It presents the results of purifications, some physico-chemical properties, enumeration of the various activators and inhibitors, and the kinetics of phosphoenolpyruvate carboxylase and pyruvate kinase of the enteric bacteria (Escherichia coli and Salmonella typhimurium).

II. HISTORICAL

The metabolic processes occurring within a living cell are a bewildering complex of chemical reactions. Most of these reactions have now been studied and the pathways which degrade or synthesize various compounds have been elucidated. The major emphasis at present is the study of the regulatory mechanisms which control the rate at which these pathways operate and the coordination of their operations.

In the course of their detailed studies of Escherichia coli, the results of Roberts, Abelson, Cowie, Bolton and Britten (1955) showed that the presence of exogenous amino acids in the growth medium prevented the de novo synthesis of amino acids from glucose. The organisms were able to selectively utilize the amino acids that were supplied and at the same time, stop syntheses of these amino acids, that is, the end products regulated their own syntheses. The cessation of de novo synthesis of amino acids was due in part to the specific inhibition of the synthesis of enzymes required for their formation. Monod and Cohen-Bazire (1953) showed that the presence of tryptophan and certain tryptophan analogues could selectively prevent the formation of tryptophan synthetase. This repression effect (Vogel, 1957) was subsequently observed for a variety of biosynthetic systems. Symmetrically opposed to repression is the induction effect in which enzymes are formed only in the presence of their substrates. This

phenomenon reported as early as 1899 by Duclaux was named "enzymatic adaptation" by Karstrom (1938) and was the subject of much experimentation.

That induction and repression of enzyme synthesis were manifestations of a common mechanism was revealed in the already classic paper of Jacob and Monod (1961). The importance of their work in elucidating this regulatory mechanism is emphasized by the fact that these two workers with André Lwoff were awarded the Nobel Prize in 1965. Such a mechanism by its very nature is sluggish since time for enzyme synthesis is required before the substrate-inducer can be utilized, and in the case of repression of enzyme synthesis, time is required for the existing enzymes to be degraded or diluted (by cell division) before biosynthesis can effectively be halted.

Umbarger (1956) demonstrated another regulatory mechanism which was independent of control over the synthesis of enzyme. He found that the presence of isoleucine markedly reduced the activity of the enzyme threonine deaminase when assayed in vitro. Threonine deaminase catalyzes the first reaction of a series which produces isoleucine as its end product. To emphasize the functional importance of the mechanism it was called feedback or end product inhibition. As in the case of genetic control, a symmetrically opposite phenomenon which resulted in the specific enhancement of enzyme activity by metabolites, termed activation, was reported (Umbarger and Brown, 1957; 1958). The terms inhibition and activation of enzyme activity have their counterparts in repression and induction of enzyme synthesis. The observation of Umbarger was substantiated in a large number of systems and opened the

door to the field of allosteric or regulatory enzymes. This study has been the subject of several recent reviews (Moyed and Umbarger, 1962; Stadtman, 1963; 1966; Cohen, 1965; Atkinson, 1966).

Although it was Umbarger's work that launched the study of allosteric enzymes, the phenomenon had been reported as far back as 1941 by Dische. He reported that the capability of erythrocyte hemolysates to phosphorylate glucose was specifically inhibited by phosphoglycerate. He deduced that since the inhibitor did not participate directly in the enzyme catalyzed reaction, and since it was structurally dissimilar to either substrate or product, the inhibition did not occur through competition for a reactive site. On this basis he postulated that phosphoglycerate played a regulatory role in glucose metabolism through its ability to inhibit glucose phosphorylation. It is surprising that such a novel observation and fundamental concept passed unnoticed and played no role in what is now a major field of study.

Regulatory enzymes differ from the normal enzymes in several respects. The most notable difference is that the enzyme possesses a second site for binding of an effector molecule, the binding of which leads to either activation or inhibition of enzyme activity. In view of the fact that the effector is sterically unrelated to the substrate molecule, that is, allosteric rather than isosteric, a second specific site necessarily should exist. That an allosteric site is present is shown by the fact that it can be rendered inoperable by mutation or chemical modification of the protein without altering the catalytic activity. Such modified enzymes are said to be desensitized. The rate-concentration curves for these enzymes show a

marked deviation from the normal Michaelis-Menten kinetic plot in many, but not all, cases; the curves being S-shaped or sigmoid rather than a rectangular hyperbola. The present concepts of the nature and function of allosteric proteins have been derived through the observations of a number of these enzymes as discussed by Monod, Changeux and Jacob (1963). Although in speaking of allosteric enzymes this broad concept of enzyme activity susceptible to the control of effector molecules will be used, one should bear in mind that the term allosterism has come to denote cooperative subunit interactions according to the theory postulated by Monod, Wyman and Changeux (1965).

Despite the fact that the controls over enzyme synthesis and enzyme activity are genetically independent of each other, both types of control may function in a single metabolic pathway. The control over enzyme synthesis is slower; it would have an all-or-none effect whereas the control over activity would have a quick effect and be more amenable to precise modulation of rates of reaction. It should not be thought, however, that all enzymes are subject to modulating effect of metabolites, but that this feature is possessed by only certain enzymes which play key roles in metabolic patterns. A generalization that arose from the observations of many regulatory enzymes was that in biosynthetic pathways, the end product inhibits the enzyme or enzymes concerned with the earlier, usually first, reactions in the pathway.

In branched metabolic pathways a common precursor may be required for several end products. If one of these end products is produced in sufficient quantities to exert a feedback inhibition such that the

formation of the common precursor is stopped, then formation of the other products that may be required for growth will also be hindered. To circumvent this obstacle a variety of feedback mechanisms have been evolved. Briefly these are: (1) Enzyme multiplicity (Stadtman, Cohen, LeBras and Robinchon-Shulzmaster, 1962; Smith, Ravel, 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 (Casky, Ashton and Wyngaarden, 1964; Nierlick 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 simultaneously in saturating amounts, the total residual activity is equal to the product of the residual activities observed when each end product is present individually at saturating levels. Although different mechanisms have evolved to accommodate the problem of branched biosynthetic pathways in various organisms, they are in effect variations and refinements of the inhibitory control over simple biosynthetic sequences. Specific examples of these controls have been reviewed by Cohen (1965) and Stadtman (1966).

The metabolism of carbohydrates presents a problem of increasing complexity in terms of regulation. Glucose metabolism involves several reaction sequences, namely, gluconeogenesis, glycogenesis, glycolysis, glycogenolysis and glucose oxidation. These pathways utilize the same intermediates as well as common enzymes for many of the reactions, such that glycolysis and gluconeogenesis are, as are glycogenesis and glycogenolysis, in essence, the same sequence of reactions operating in opposite directions. Not only does the direction of operation of these sequences present problems in control but the interlocking of carbohydrate degradation with the tricarboxylic acid cycle creates additional regulatory difficulties for the cell. This arises due to the bifunctional nature of the tricarboxylic acid cycle, for it is responsible for the production of energy in the form of ATP via terminal respiration, as well as that of precursors of the amino acids aspartate, glutamate, lysine and methionine and their derivatives. Thus the products of carbohydrate degradation must be channelled into both catabolic or energy-yielding reactions and biosynthetic sequences.

Although glycolysis and gluconeogenesis involve similar reactions in opposite directions, the sequences are in effect unidirectional in the sense that one is not the faithful reversal of the other. Two reactions in glycolysis provide thermodynamic barriers ensuring that the pathway can function in only one direction. These are (1) the formation of G-6-P from glucose and ATP catalyzed by the enzyme hexokinase, and (2) the formation of FDP and F-6-P catalyzed by phosphofructokinase; both being highly exergonic with $\Delta G'$ of - 5.1 and - 4.2 kcal/mole, respectively (see Krebs and Kornberg, 1957).

In gluconeogenesis, these energy blocks are bypassed by substituting two hydolytic reactions which imposes unidirectionality in the opposite direction to that of glycolysis. These are (1) the hydrolysis of G-6-P to glucose and inorganic phosphate catalyzed by glucose-6-phosphatase and (2) the hydrolysis of FDP to F-6-P and inorganic phosphate catalyzed by fructose diphospatase. The $\Delta G'$ are - 4.02 and - 4.0 Kcal/mole, respectively (Krebs and Kornberg, 1957). Although the presence of energy barriers allow intermediates to flow in one direction, Stadtman (1966) has pointed out the problems arising from such control mechanisms. If one considers the reactions catalyzed by fructose diphosphatase and phospofructokinase, i.e.,

$$\text{FDP} + H_2O \longrightarrow \text{F-6-P} + P_i \qquad (1)$$

$$\text{F-6-P} + \text{ATP} \longrightarrow \text{FDP} + \text{ADP} \qquad (2)$$

then the presence of both reactions occurring simultaneously produces the result:

$$\text{ATP} + H_2O \longrightarrow \text{ADP} + P_i$$

In effect, a short circuit would be produced which could deplete the energy reserve of the cell.

To prevent this eventuality and to allow the required flow to proceed the activity of the enzyme catalyzing these reactions are closely modulated by metabolites or effectors. These effectors serve as signals, and indeed it is the level of these metabolites rather than the enzymes themselves, which determine the direction that the glycolytic sequences should operate. Generally, it is found that metabolites that activate the enzyme responsible for the reaction in one direction are strong inhibitors of the enzyme catalyzing the

reverse reaction. In such cases, regulatory enzymes not only respond to the level of metabolites present but are able to divert metabolic products along different pathways.

The interconversion of FDP and F-6-P illustrates this concept. Lardy and Parks (1959) showed that ATP strongly inhibits phosphofructokinase of muscle. This has been verified in subsequent studies with phosphofructokinase from skeletal muscle, heart, liver and brain tissues as well as plants and bacteria (Lowry and Passonneau, 1964; Passonneau and Lowry, 1964) and yeast (Ramaiah, Hathaway and Atkinson, 1964; Viñuela, Salas and Sols, 1963). In addition, the mammalian systems are strongly inhibited by citrate (Ramaiah et al., 1964; Passonneau and Lowry, 1963; Parmeggiani and Bowman, 1963; Garland, Randle and Newsholme, 1963) which provides an interlocking control between glycolysis and the tricarboxylic acid cycle. The effectors which inhibit the enzyme are those signalling a high level of energy-rich compounds. Although ATP inhibition has been reported for E. coli phosphofructokinase (Lowry et al., 1964; Atkinson and Walton, 1965), this was due to ATP chelation of magnesium, a cofactor for the enzyme (Blangy, 1967). Rather, Blangy (1967) has demonstrated that ADP and GDP are activators and PEP an inhibitor for this enzyme. However, the resulting effect produced by ATP inhibition or ADP activation would essentially be the same, that is, the activity of the enzyme responds to the energy level within the cell.

The allosteric inhibition of phosphofructokinase by ATP can be relieved by 3',5'-AMP in liver fluke (Mansour et al., 1962; 1963), 5'-AMP in yeast and by both 3'- and 5'-AMP in mammalian tissues

(Lowry et al., 1964). It is readily seen that such a mechanism allows the precise modulation of the rate of flow of metabolites through the glycolytic pathway in response to the energy level with the cell.

As mentioned earlier, the presence of fructose diphosphatase in the cell will negate any effective control over FDP formation by short circuiting the reaction at this point. It is not surprising then that there should be allosteric controls over the reverse step. Several laboratories have demonstrated that the mammalian fructose diphosphatase is strongly inhibited by 5'-AMP (Taketa and Pogell, 1963; 1965; Newsholme, 1963; Mendicino and Vasarhily, 1963; Salas, Vinuela and Sols, 1964; Krebs and Woodford, 1965). In such a system, the simultaneous presence of phosphofructokinase and fructose diphosphatase will not lead to short circuiting since the effectors which regulate their activities operate in a reciprocal manner.

While the control mechanism at the site of interconversion of F-6-P and FDP illustrates the role of allosteric enzymes in controlling glucose metabolism, several other key points exist which further exemplify the importance of these regulatory enzyme. These have been summarized by Mahler and Cordes (1966):

Process		Activator	Inhibitor
Breakdown (Catabolism)	Glycogen ⟶ G-1-P	AMP	—
	F-6-P ⟶ FDP	ADP, AMP, F-6-P	ATP, citrate
	PEP ⟶ pyruvate	—	ATP
Synthesis	pyruvate ⟶ OAA ⟶ PEP	AcCoA	—
	FDP ⟶ F-6-P	—	AMP, FDP
	G-6-P ⟶ Glucose	—	PP_i, glucose
	G-1-P ⟶ UDPG ⟶ glycogen	G-6-P	—

Recent reviews by Stadtman (1966), Atkinson (1966), and Wood (1966) give detailed summaries of the work done in this field.

Although the pathways of glucose degradation have been documented under a variety of fermentative pathways (Axelrod, 1960; Mahler and Cordes, 1966), the main advantage of glucose metabolism lies not only in the energy derived through glycolysis, but also in the availability of biosynthetic intermediates and the energy that can be extracted by degradation through terminal respiration *via* the tricarboxylic acid cycle. The "end products" of glycolysis, that is, PEP and pyruvate, are diverted into pathways that serve different functions and the regulation of this junction is necessarily quite complex. Pyruvate arises in glycolysis through the conversion of PEP mediated by the enzyme pyruvate kinase. The conversion is essentially irreversible since the $\Delta G'$ of the reaction is - 6.1 kcal/mole (Burton, 1955); this formidable thermodynamic block ensures against the use of this reaction for carbohydrate synthesis. Allosteric activation by FDP has been reported for the enzyme from yeast (Hess, Haeckel and Brand, 1966; Gancedo, Gancedo and Sols, 1967), various animal tissues (Taylor and Baile, 1967; Tanaka, Sue and Morimura, 1967), trout (Hochachka and Somero, 1968), and developing loach embryos (Milman and Yurowitzki, 1967). In loach embryos the enzyme is activated by 3',5'-cyclic AMP. Hess *et al*. (1966) have termed this "feed forward" activation and in essence is similar to "precursor activation" reported earlier by other authors (Hilz, Tarnowski and Arend, 1963; Sanwal, Zink and Stachow, 1963).

The rat liver enzyme is also inhibited by ATP, the half inhibitory concentration of ATP being 0.16 mM (Tanaka et al., 1967), whereas Milman et al. (1967) show that the loach embryo enzyme is not affected by 3 to 6 mM ATP although the activating effect of FDP was blocked by its presence. The regulation of pyruvate kinase activity controls the rate at which pyruvate is formed in response to the fluctuating levels of FDP and ATP within the cell.

In gluconeogenesis, the pyruvate kinase reaction imposes an energy barrier which cannot be readily reversed. Recently, Cooper and Kornberg (1965) have demonstrated the existence of an enzyme PEP synthase which catalyzes the direct phosphorylation of pyruvate with ATP to give PEP, AMP and 2 molecules of inorganic phosphate. The enzyme has been demonstrated in the photosynthetic bacterium, Chlorobium thiosulfatophilum (Evans and Buchanan, 1965), and is implicated in the carbon reduction cycle postulated by Evans, Buchanan and Arnon (1966). It has been demonstrated by both groups that the equilibrium lies far in the direction of PEP synthesis. Benziman (1966) also reported the presence of this enzyme in Acetobacter xylinum. No effector has been reported for this enzyme, but one is tempted to predict that some activator and/or inhibitor of physiological importance must be present to prevent coupling of this reaction with the pyruvate kinase reaction.

The predominant fate of pyruvate arising from the pyruvate kinase reaction in most animal cells and in those of aerobic microorganisms is its conversion to acetyl-CoA mediated by the multienzyme pyruvate dehydrogenase complex. This complex has been isolated from pig heart (Jagannathan and Schweet) and from E. coli (Koike, Reed and

Carrol, 1964; Hayakawa et al., 1964). The E. coli enzyme is a massive complex consisting of aggregates of pyruvate decarboxylase, lipoic reductase, transacetylase and dihydrolipoate dehydrogenase; all of which are required for the conversion of pyruvate to acetyl-CoA. The overall reaction represents another energy barrier for gluconeogenesis, the $\Delta G'$ being - 9.38 kcal/mole.

Hansen and Henning (1966) reported that NADH is a strong inhibitor of the dihydrolipoate dehydrogenase moiety of the complex from E. coli. Although NADH is an end product of the enzyme, an allosteric site is implicated since extremely low concentrations are required for inhibition--so low that the K_i could not be determined with accuracy. However, at NAD concentrations saturating the enzyme, the reaction is inhibited 50% at a NADH/NAD ratio as low as 0.02. Similarly, lipoate causes 50% inhibition of the reaction at a concentration of 0.005 mM. Since the NADH/NAD ratio is much higher in anaerobically grown cells, the authors (Hansen and Henning, 1966) imply that pyruvate dehydrogenase does not function under anaerobic conditions; pyruvate, presumably, is then metabolised by the different fermentative pathways. It should be stated that the enzyme, dihydrolipoate dehydrogenase was inhibited both in the free state independent of the complex and in the bound state.

The nucleotides, CMP, AMP, UMP, GMP and GDP at a concentration of 5×10^{-4} M were able to stimulate by a factor of 2 the activity of the pyruvate decarboxylase portion of the complex both in the free and bound state (Schwartz and Reed, 1968). However, the authors did not postulate any physiological function for this effect.

The acetyl CoA formed can be utilized directly for the synthesis of fatty acids required for structural purposes as in membrances or energy yielding reserves, or alternatively, acetyl CoA can condense with oxalacetate to produce citrate, which enters into the citric acid cycle (tricarboxylic acid cycle, Krebs cycle). This represents a bifurcation in the flow of metabolites, and in accordance with the concepts of biological regulation, is a site where enzyme control mechanisms are applicable. The steps involved in fatty acid synthesis are catalyzed by a complex of enzymes, like pyruvate dehydrogenase, with the involvement of an acyl carrier protein. The details have been extensively reviewed recently by Lennarz (1966), Olson (1966) and Marjerus and Vagelos (1967). The first step in the synthesis is catalyzed by the enzyme acetyl CoA carboxylase resulting in the formation of malonyl CoA from acetyl CoA, bicarbonate and ATP.

The enzyme from mammalian systems is activated by citrate in rat adipose tissue (Martin and Vagelos, 1962; Vagelos, Alberts and Martin, 1963) and rat liver (Lynen, Matsuhashi, Numa and Schweizer, 1963) whereas isocitrate is the most potent activator in avian liver systems (Waite and Wakil, 1962). Recently Wakil, Goldman, Williamson and Toomey (1966) have reported that phosphorylated sugars activate acetyl CoA carboxylase both from bacterial and mammalian sources; FDP being the most potent. Such activations would prevent accumulation of precursor compounds and allow for the storage of energy in the form of fatty acids.

A number of workers using cell free systems have shown that palmityl CoA is the end product of fatty acid synthesis (see Wakil,

Pugh and Sauer, 1964), and it is not surprising that palmityl CoA should inhibit acetyl CoA carboxylase (Numa, Bortyz and Lynen, 1965). Although functionally an end product inhibition by palmityl CoA seems reasonable, its significance has been questioned by Taketa and Pogell (1966) who have shown that this reagent inhibits a large number of unrelated enzymes, possibly through its activity as a detergent. At any rate it would seem reasonable that the synthesis of fatty acids should be controlled both by the metabolites feeding into the pathway and the end products of it.

An alternative fate for the acetyl CoA is degradation through the tricarboxylic acid cycle. This cycle occupies a central position in the metabolic pattern of most cells. Not only are various substrates degraded by the cycle to yield energy, but as important is its role as a precursor for the formation of a host of metabolites, that is, the cycle is amphibolic in nature (Davis, 1961). In a catabolic role the degradation of acetyl CoA is coupled with the electron transport system and oxidative phosphorylation and is responsible for ATP formation. In biosynthesis the intermediates give rise to an array of compounds - citrate to fatty acids; α-ketoglutarate to glutamate and glutamic acid family of amino acids; succinyl CoA to porphyrins; and oxalacetate to pyrimidines, PEP, aspartate and the aspartic acid family of amino acids. A recent review of this subject has been published (Lowenstein, 1967). Allosteric control mechanisms known at present which play a part in the cycle proper are limited to the enzymes citrate synthetase and isocitric dehydrogenase.

The initiating step of the tricarboxylic acid cycle is the condensation of acetyl CoA with oxalacetate to form citrate. Acetyl CoA, as shown earlier, can arise from pyruvate by the pyruvate dehydrogenase reaction or alternatively via the acetate thiokinase reactions (Lynen and Reichart, 1951), or the reaction catalyzed by the citrate cleavage enzyme (Srere and Lipmann, 1953). Other entries are through formation of the intermediates of the cycle such as α-ketoglutarate, oxalacetate and succinate. The reaction of acetyl CoA and oxalacetate to form citrate is catalyzed by citrate synthetase; sometimes referred to as the citrate condensing enzyme. The reaction is essentially unidirectional with a $\Delta G'$ of - 9.08 kcal/mole (Johnson, 1960).

Tubbs (1963) and Weiland and Weiss (1963) reported that palmityl CoA was able to inhibit the citrate synthetase from pig heart. The effect was specific in that free CoA and palmitate were unable to produce this effect. Hathaway and Atkinson (1965) verified these results with a relatively crude enzyme preparation from yeast and also reported the inhibitory action of ATP. ATP inhibition has been subsequently reported for plants also (Bogin and Wallace, 1966; Shephard and Garland, 1966). The E. coli enzyme is not inhibited by ATP but rather by NADH (Weitzmann, 1966) and α- ketoglutarate (Wright, Maeba and Sanwal, 1967). The NADH inhibition could be relieved by low concentrations of AMP and ADP when the inhibitor was present in saturating amounts (Weitzmann, 1967).

It is postulated that the effectors signalling high energy levels within the cell (ATP and NADH) will tend to direct the flow of acetyl

CoA towards fatty acid synthesis. Under such conditions the citrate concentration will tend to increase (Hathaway and Atkinson, 1963a; 1963b) resulting in acetyl CoA carboxylase activation mentioned earlier, which will reinforce the diversion of acetyl CoA to fatty acids. The inhibition by ∝- ketoglutarate is explained as a feedback inhibition based on the premise that the tricarboxylic acid cycle can be divided into three parts (Amarsingham and Davis, 1965; Gray, Wimpenny and Mossman, 1966; Hanson and Cox, 1967) of which ∝- ketoglutarate is the end product of the first unit.

Most organisms possess two types of isocitrate dehydrogenases, one specific for the coenzyme NAD and the other for NADP. The former is generally found intramitochondrially and functions in catabolic reactions while the other is extramitochondrial and is reserved for bioxynthetic functions. Bacteria lack the NAD-specific enzyme, notable exceptions being Acetobacter peroxydans (Hathaway and Atkinson, 1963a) and Xanthomonas pruni (Ragland, Kawasaki and Lowenstein, 1966). The enzyme has been best studied from lower fungi and animal sources (see Lowenstein, 1967). It appears that only the NAD-specific enzyme functions as a regulatory enzyme. In yeast (Kornberg and Pricer, 1951; Hathaway et al., 1963a), Neurospora (Sanwal, Zink and Stachow, 1963) citrate (or isocitrate) activate the enzyme. The NAD-specific isocitrate dehydrogenase from rat heart (Goebbel and Klingenberg, 1964) behaves similarly to the fungal enzymes but ADP rather than AMP functions as the activator. Similarly beef heart NAD-specific isocitrate dehydrogenase is activated by ADP (Chen and Plaut, 1963), but in this case isocitrate plays no activating role.

One inhibitor, NADH, has been reported for the beef heart enzyme (Chen, Brown and Plaut, 1964). Although NADH is a product of the reaction, in view of its very low K_i (39 μM at saturating substrate concentrations), the effect is probably a specific one serving a specific function. AMP and NADH are indicators of energy levels within the cell and these signals will determine the rate of operation of isocitrate dehydrogenase. The role of citrate activation is that of "precursor activation" (Sanwal, Zink and Stachow, 1963) or as others have termed this type of phenomenon "feedback activation" (Atkinson, 1966), or "feedforward activation" (Hess et al., 1966). It should be noted also that citrate accumulation will tend to activate acetyl CoA carboxylase as mentioned earlier such that accumulation of citrate is prevented.

Although these controls may serve physiological functions in organisms containing mitochondria it is important to remember that the bacteria contain only one isocitrate dehydrogenase that is dependent on NADP as a cofactor. As in higher organisms no allosteric effectors have been reported for this type of enzyme and the control of this reaction in bacteria remains an enigma.

Controls at the point of citrate formation and isocitrate oxidation appear to be the main points of control within the TCA cycle proper. Subsidiary to these are the controls at the level of fatty acid biosynthesis which utilizes an intermediate of the cycle. Another subsidiary reaction which branches off the cycle that is under allosteric control is the glutamate dehydrogenase reaction. This enzyme resembles isocitrate dehydrogenase in many respects: (1) multiple

forms are present, one specific for NAD and the other for NADP in Neurospora (Stachow and Sanwal, 1964); and Thiobacillus (Léjohn, 1967); (2) the NAD-specific enzyme is susceptible to control; (3) bacteria generally produce only the NADP-specific enzyme.

The NAD-specific enzyme from beef liver has been the subject of much work mainly by Tomkins, Yielding and their coworkers (see Tomkins, Yielding, Talal and Curran, 1963) and Frieden (1963). These workers have reported the inhibition of glutamate oxidation by ATP, GTP and NADH and activation of the same reaction by ADP, leucine and methionine. The GTP inhibition has been confirmed for the Neurospora enzyme (Stachow and Sanwal, 1963) and AMP and ADP activation in Thiobacillus (Léjohn, 1967; Léjohn, Suzuki and Wright, 1968) and in a water mold, Blastocladiella emorsonii (Léjohn and Jackson, 1968).

In view of the fact that NAD coenzymes participate in catabolic reactions whereas NADP is used for bioxynthesis, the effectors for the NAD-specific glutamate dehydrogenase are, not surprisingly, signals indicating energy levels within the cell, and control the rate of glutamate entry into the TCA cycle via α- ketoglutarate. The fact that most bacteria have neither the NAD-specific isocitrate dehydrogenase nor glutamate dehydrogenase but possess only the TPN-specific enzymes which so far appear to be unregulated remains to be explained.

The major controls so far discussed appear to operate in response to the energy levels within the cell. Since the TCA cycle is responsible for the high yield of energy from catabolized compounds, this is not surprising. But the intermediates of the cycle

not only function as carriers for acetyl CoA but, as previously mentioned, are precursors for a variety of metabolic products. The intermediates of the cycle then are constantly being drained away for these biosynthetic purposes and the cycle would be in danger of running down if these are not replenished. There appear to be several mechanisms by which replenishment occurs. Kornberg, the proponent of these vitally important reactions, has called such reactions anaplerotic sequences, the word being derived from the Greek, to replenish.

The well known glyoxylate cycle (or Krebs-Kornberg cycle) is such a cycle, and is the means by which higher plants and micro-organisms convert fats or two carbon metabolites into carbohydrates and other cell constituents. Two key enzymes, isocitratase (Campbell, Smith and Eagles, 1953) responsible for the cleavage of isocitrate to glyoxylate and succinate, and malate synthase (Wong and Ajl, 1956) catalyzing the synthesis of malate from glyoxylate and acetyl CoA participate in this scheme. Also participating in this cycle are three enzymes of the TCA cycle, namely, citrate synthetase, aconitase and malate dehydrogenase. The net result of one turn of this cycle is the formation on one molecule of succinate from two molecules of acetyl CoA. The succinate formed can be utilized for biosynthetic purposes or serve as an intermediate in the TCA cycle; at any rate, there would be no need for the TCA cycle to be depleted of its intermediates since the glyoxylate shunt ensures the supply of succinate.

Of the various enzymes involved, isocitratase, is under the inhibitory control of PEP. In a system such as this, PEP plays a key

role in that the four carbon compounds formed through the glyoxylate cycle must first be converted to PEP before taking part in gluconeogenesis; PEP, as well, can form acetyl CoA required both for energy and fatty acid synthesis. The inhibition of isocitratase by PEP then represents a feedback inhibition to prevent its overproduction. Since the acetate thiokinase reaction by which acetate is converted to acetyl CoA would still be functional under such conditions, it appears that the energy yielding reactions would still be able to proceed even if inhibition of this enzyme did occur.

A similar role is played by the enzyme PEP carboxylase of Enterobacteriaceae (Kornberg, 1966). The enzyme was originally reported by Bandurski and Griener (1953) in spinach leaves and subsequently in wheat germ (Tchen and Vennesland, 1955), peanut cotyledons (Maruyama and Lane, 1963), Thiobacillus thiooxidans (Suzuki and Werkman, 1958), E. coli (Cánovas and Kornberg, 1965) and Ferrobacillus ferrioxidans (Din, 1967).

Studies with mutants of Salmonella typhimurium (Theordore and Englesberg, 1964) and of E. coli (Ashworth, Kornberg and Ward, 1965; Ashworth and Kornberg, 1966) which fail to grow in media containing glucose, glycerol or pyruvate as a carbon source (unless utilizable intermediates of the tricarboxylic acid cycle are also supplied) have shown that the carboxylation of PEP is necessarily involved in the maintenance of the TCA cycle. Such mutants were shown to be deficient in PEP carboxylase, and pointed to the now accepted proposal that it is this enzyme which is responsible for the anaplerotic function. Other enzymes implicated because of their ability to catalyze CO_2

fixation of PEP or pyruvate were the NADP-dependent malic enzyme, PEP carboxykinase and PEP phosphotransferase.

The K_m of the E. coli PEP carboxylase for PEP is rather high (5.5 mM) as reported by Cánovas and Kornberg (1965), but decreases to 6.5×10^{-4} M in the presence of 0.5 mM acetyl CoA, a potent activator. The authors postulate that since the enzyme would be virtually inactive in the absence of acetyl CoA, that the activator potentiates the action of the enzyme which catalyzes the formation of oxalacetate required for oxidation of acetyl CoA. Under conditions where the supply of acetyl CoA is limited PEP would not be carboxylated but would be diverted into its catabolic route, which would, by supplying acetyl CoA, now activate PEP carboxylase. The operation of such a regulatory system would ensure that during growth in glucose or other precursor of three carbon acids, the necessary balance between catabolic and anaplerotic reactions may be maintained.

In mammalian tissues, yeasts and pseudomonads, PEP carboxylase is not present. In these cases, it appears that pyruvate carboxylase which catalyzes the carboxylation of pyruvate to oxalacetate and the simultaneous conversion of ATP to ADP fulfills the anaplerotic function (Keech and Utter, 1963; Utter, Keech and Scrutton, 1964). In this respect the pyruvate carboxylase from different sources is also activated by acetyl CoA. The mammalian pyruvate carboxylase has an absolute requirement for acetyl CoA (Scrutton and Utter, 1965); the same enzyme from pseudomonads and yeasts wholly or partially retaining their maximum enzymic activities in the absence of acetyl CoA (Seubert and Remberger, 1961; Losada, Cánovas and Ruiz-Amil, 1964). The role of pyruvate carboxylase and the functional aspects of

regulation by acetyl CoA has been reviewed by Utter, Keech and Scrutton (1964).

With the elucidation of anaplerotic function of PEP carboxylase and pyruvate carboxylase, it has become increasingly clear that other enzymes which could, in theory, effect the net synthesis of four carbon acids by carbon dioxide fixation, are anabolic rather than anaplerotic (Kornberg, 1966). These enzymes, the malic enzyme, PEP carboxykinase and PEP carboxytransphosphorylase, in an anabolic role would act as decarboxylases and serve to produce PEP, the precursors for gluconeogenesis, pentoses, aromatic amino acids, histidine and serine, or through the formation of pyruvate would serve for biosynthesis of alanine, valine, and oxalacetate; the NADPH formed being utilized for reductive biosynthesis. Support for such postulates comes from the finding that mutants devoid of PEP carboxylase grow on four carbon but not three carbon compounds, whereas mutants lacking PEP carboxykinase grow well on three carbon but not four carbon acids (Kornberg, 1965).

In this respect, the PEP synthase reaction (Cooper and Kornberg, 1965) should be mentioned again. Organisms that possess PEP carboxylase are able to grow on pyruvate or compounds that give rise to pyruvate eg., lactate, alanine. In such cases, when the glyoxylate cycle cannot function and PEP must be supplied for anaplerosis, the only means by which PEP can be synthesized is the PEP synthase reaction. In this capacity, PEP synthase would be playing an anaplerotic role. Evidence for this comes from the finding that mutants lacking PEP synthase are able to grow on glucose, glycerol or acetate but not on lactate or pyruvate (Cooper and Kornberg, 1965).

Although the regulation of metabolic patterns reported here deal solely with allosteric control mechanisms it should be borne in mind that the complexity of metabolism, growth, and morphogenesis require that other controls should exist. Not mentioned are the controls over enzyme levels, hormonal controls, effects of substrate and coenzyme levels, the presence of enzymes which inactivate or activate regulatory enzymes nor the observation that metabolites can cause the aggregation or disaggregation of the subunit structure of enzyme.

With the recognition and acceptance that allosteric or regulatory enzymes possess distinct sites at which effector molecules can bind, the classical concept of a protein molecule capable of catalyzing specific reactions was considerably altered. The observations of non-Michaelian kinetics, the steric non-identity between substrate or product and the effector molecules, desensitization and the alteration of reaction rate presented problems in postulating a mechanism by which these proteins functioned.

The comparison of the properties of a number of regulatory enzymes, e.g., threonine deaminase (Umbarger, 1956; Changeux, 1961), aspartate transcarbamylase (Gerhart and Pardee, 1962), phosphoribosyl-ATP pyrophosphorylase (Martin, 1962), aspartokinase (Stadtman et al., 1961), homoserine dehydrogenase (Patte, LeBras,Loviny and Cohen, 1963) has led to the present day concepts of the mode of action of allosteric enzymes (Monod, Changeux and Jacob, 1963). Monod et al. (1963) postulated that allosteric proteins possess two or at least two, distinct, non-overlapping receptor sites. One of these, the

active site binds the substrate and is responsible for the activity of the protein. The other, or allosteric, site is complementary to the structure of another metabolite, the allosteric effector, which it binds specifically and reversibly. The formation of the enzyme-allosteric effector complex does not activate a reaction involving the effector itself; it is assumed only to bring about a discrete reversible molecular transition of the protein (allosteric transition) which modifies the properties of the active site, changing one or several of the kinetic parameters which characterize the biological activity of the protein (Monod, Changeux and Jacob, 1963).

The similarity of the sigmoid rate-concentration curves of allosteric enzymes to that of oxygen binding to hemoglobin led Davis (1961) to suggest a similar mechanism for binding of substrate (Davis, 1961) and effectors (Wieland and Weiss, 1963) to these enzymes. With hemoglobin it has been proposed that combination of one molecule of ligand with the macromolecule can influence the binding of the same or different ligands; this being exerted through conformational changes in the macromolecule (Wyman, 1948; Wyman and Allan, 1951). Monod, Wyman and Changeux (1965) proposed an elegant model to account for the characteristic properties based on conformational changes in the quaternary structure of the enzyme induced by association with ligand.

The basic assumption in this model is that allosteric proteins are composed of identical subunits or protomers arranged in a symmetrical manner in the polymeric (oligomeric) molecule. Associated with each protomer is one, and only one, stereospecific site for each ligand, i.e., substrate or effector; symmetry of sites being necessitated by the symmetry of the oligomeric structure. The oligomeric

protein has reversible access to at least two states which differ by the distribution and/or energy of the inter-protomer bonds (conformational restraints). When the protein passes from one state to another, the molecular symmetry is preserved, although the affinity of a site for its ligand is altered. Therefore, although the sites are independent, there will be at least two dissociation constants for each site and its ligand, corresponding to the state the protein is in.

In such a model the enzyme is envisaged to exist in two states which are in equilibrium, the R (relaxed) and T (tight) states. When the substrate, which has a higher affinity for the R state, is added to the system, the equilibrium is shifted to favor the R state such that the fraction of R conformer increases in the total population of R and T conformers. Since these proteins are composed of identical subunits and have more than one substrate site, the increase in the relative concentrations of R makes the binding subsequent substrate molecules more favorable. The cooperative nature of this homotropic interaction, i.e., interaction between like ligands, becomes more pronounced if the initial equilibrium favors the T state and if the affinity of the substrate is much higher for the R state.

The role of effectors in such a model is to shift the equilibrium to favor one of the two states depending upon which state the effector binds; an activator is exclusively limited to the R state and the inhibitor to the T state. The binding of effector and substrate to an allosteric protein will induce heterotropic interactions, i.e., between unlike ligands, and leads to an activating (cooperative) effect or an inhibitory (antagonistic) effect depending upon the nature of the

effector. In the presence of inhibitor the substrate saturation curve will show greater cooperativity while the converse is true in the case with activator.

In this model the ligands have preferential affinity for one of two states. A modification of the model in which a ligand may have significant affinity for both states, i.e., non-exclusive binding has recently been formulated (Rubin and Changeux, 1966). Allosteric transition models have been applied to real enzyme systems,e.g., phosphorylase b (Buc, 1967) and notably aspartate transcarbamylase (Changeux, Gerhart and Schachman, 1968; Gerhart and Schachman, 1968; Changeux and Rubin, 1968). Such models are amenable to treatment by methods for multiple equilibria studies and mathematical formulations of the models have been made (Monod, Wyman and Changeux, 1965; Rubin and Changeux, 1966).

Another model has been put forth by Koshland and his coworkers. It deviates from the Monod-Wyman-Changeux model in that symmetry of an allosteric protein is not obligatory. As a consequence of this modification the number of possible arrangements of subunits within the polymeric protein is increased. Also without the restriction imposed by symmetry, each subunit can exist in two states, i.e., bound and unbound, independent of the other subunits such that the number of ways that subunits in each state can exist in a molecule is also increased; that is, sequential alteration of subunits can occur. The introduction of these parameters makes the mathematical model necessarily bulky and unwieldy (Koshland, Nemethy and Filmer, 1966; Kirtley and Koshland, 1967; Haber and Koshland, 1967).

Both models were derived on the assumption that deviation of behavior of allosteric enzymes from Michaelis-Menten kinetics renders a kinetic approach impracticable. The models consequently depend on thermodynamic equilibrium considerations rather than that of steady state used in kinetic studies. However, non-linear double reciprocal (1/velocity vs. 1/substrate) plots which are S-shaped in rate concentration plots have been analyzed by classical kinetic techniques (Reiner, 1959; Frieden, 1964). With the methods introduced by Cleland (1963a; b; c) such approaches have provided alternate mechanisms for allosteric enzymes. In a comprehensive kinetic analysis of the allosteric isocitrate dehydrogenase of Neurospora, Sanwal and coworkers (Sanwal, Zink and Stachow, 1964; Sanwal, Stachow and Cook, 1965; Sanwal and Cook, 1966) have shown that in the absence of the activator AMP, an ordered reaction becomes random resulting in non-linear plots. In the presence of AMP, sigmoidal rate-concentration plots could be interpreted as a result of two site sequential binding of substrate, one at an allosteric site and the other at the active site. In such a model there is no need to invoke the concept of subunit interaction. This same group has demonstrated that the allosteric threonine deaminase of S. typhimurium can exhibit non-linear kinetics in the presence of the inhibitor, isoleucine, due to alternate pathways by which substrate can bind enzyme (Maeba and Sanwal, 1965a). Other multisite models have been proposed by Worcel, Goldman and Cleland (1965) for NADH oxidase. Recently, Sweeny and Fisher (1968) have constructed various kinetic models based on steady state assumptions.

III. MATERIALS AND METHODS

Organisms: The bacterial species used throughout the course of the work were Salmonella typhimurium, strain LT 2, and Escherichia coli, strain B. These organisms, belonging to the family Enterobacteriaceae, are closely related genetically and exhibit similar growth patterns. Stock cultures were maintained on trypticase soy agar slants at 4°.

Growth: When required the bacteria were grown in a minimal salts medium, the composition which is given below:

10.5 g	K_2HPO_4
4.5 g	KH_2PO_4
0.05 g	$MgSO_4$
1.5 g	$(NH_4)_2SO_4$
1.0 liter	distilled water.

This medium was sterilized by autoclaving at 121° for 20 minutes. The carbon source, glucose, was prepared and autoclaved separately as a 20% solution and was added to the sterile medium to give a final concentration of 0.4%.

When large quantities were required, cells were grown at 30° in 20 liter carboys containing 15 liters of the minimal salts medium; aeration being provided by forcing air through glass spargers submerged in the medium. One liter of an overnight culture grown in the same medium served as an inoculum. When growth reached the late log phase, usually 12 to 14 hours, the cells were harvested with a Sharples

centrifuge. The cells were washed once with 0.14 M NaCl, suspended in 0.05 M Tris-HCl, pH 8.0, to a concentration of 0.5 g (wet weight) per ml and stored in 100 - 200 ml lots at -20°.

Assay Procedures:

Phosphoenolpyruvate carboxylase: This enzyme was assayed by two methods; assay 1 depending on the incorporation of radioactive bicarbonate-C^{14} into oxalacetate and the other (assay 2), a coupled spectrophotometric method.

Assay 1. The standard mixture contained 10.0 mM $NaHC^{14}O_3$ (0.1 μc/mM), 5.0 mM PEP, 10.0 mM $MgCl_2$ and 0.1 M Tris-HCl in a volume of 1.0 ml. The reaction run at 24 - 25° was started by the addition of enzyme and was terminated after five minutes with 1.0 ml 2.0 N H_2SO_4 containing five mg per ml carrier oxalacetate. The mixture was gassed for five minutes with 100% carbon dioxide and aliquots were transferred to scintillation fluid for counting in a Tricarb scintillation counter. The scintillation fluid 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 dioxane.

Assay 2. The standard mixture contained 5.0 mM PEP, 10.0 mM $NaHCO_3$, o.15 mM DPNH, 15 μg pig heart malate dehydrogenase and 0.1 M Tris-HCl, pH 9.0, in a volume of 3.0 ml. The mixture was made in silica cuvettes of 1 cm light path. The oxidation of DPNH was followed at 340 mμ with a Gilford 2000 recording spectrophotometer attached to a Beckman DU monochromater.

Pyruvate kinase: The enzyme was assayed in a coupled system in which the pyruvate formed was reduced by DPNH in the presence of

lactate dehydrogenase. The standard assay was run in silica cuvettes of 1 cm light path containing 1.0 mM PEP, 1.5 mM ADP, 10.0 mM $MgCl_2$, 0.15 mM DPNH, 100 µg beef heart lactate dehydrogenase and 0.02 M HEPES buffer, pH 7.0 in a total volume of 3.0 ml. HEPES is N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid. The reaction rate was followed at 340 mµ with a Beckman DU monochromater attached to a Gilford 2000 recording unit.

All assays described above were established to be linear with regards both to time and enzyme concentration. The velocities were measured at 24 - 25°.

Molecular Weight Determinations: The molecular weights of the enzymes were estimated by ultracentrifugation in a linear sucrose gradient (4 to 20%, w/v) according to the method of Martin and Ames (1961). The gradient was prepared by connecting two chambers filled with 2.4 ml of sucrose solution; the mixing chamber containing 20% (w/v) sucrose and the reservoir 4% (w/v) sucrose. Both solutions were made in the appropriate buffer. The solutions were stirred in the mixing chamber with a bent wire mounted on a rheostated motor. The mixed solution was then allowed to flow through a capillary tube down the side of a centrifuge tube; the time for the chambers to empty being regulated to 15 to 20 minutes.

The enzyme solution was layered on top of the gradient and the tubes centrifuged in a Spinco Model L ultracentrifuge with a SW 39L swinging bucket rotor. Centrifugation was done at 39,000 rpm for 12 to 14 hours at 5°. After the run was completed, the rotor was allowed to stop without applying the brake. The tubes were removed, punctured

at the bottom with a 26 1/2 gauge hypodermic needle and two drop fractions were collected and assayed.

Sedimentation Velocity Studies: Sedimentation studies were performed with a Spinco Model E analytical ultracentrifuge equipped with a schlieren optical system. Unless otherwise stated, the runs were made at 5 to 10° in 0.05 M Tris-HCl, pH 8.0, with the AND 20h1 rotor. All S values reported have been corrected for viscosity to $S_{20,w}$.

Amino Acid Analysis: Amino acid content of protein preparations was determined with a Technicon amino acid analyzer. A 72 cm column packed with Technicon Type C-2 resin (8% cross-linked) was used to resolve the amino acids. Protein samples were hydrolyzed in 6 N HCl for 18 hours and 24 hours at 121° prior to analysis. Performic acid oxidations were performed at 0° (95% performic acid) for 2 hours. The protein sample was then hydrolyzed as above. The results given in Table 8 (see later) are the averaged values obtained from treated and untreated protein samples. Tryptophan content of the untreated protein was measured in 0.1 N NaOH spectrophotometrically by the method of Bencze and Schmid (1957).

Polyacrylamide Gel Electrophoresis: Disc electrophoresis was used to study the purity of enzyme preparations and the effect of some reagents on enzyme structure. Electrophoresis was performed after the method described by Ornstein (1964) and Davis (1964). The runs were made in an apparatus modelled after those made commercially by Canalco (Bethesda).

Protein samples of 50 to 100 µg were used. After the run (approx. 30 minutes), the gels were stained with amido black and destained with 20% acetic acid. After destaining the gels were stored in water. An alternative method for staining was sometimes employed. In this procedure described by Chambrach, Reisfield, Wycoff and Zacari (1967), the gel is fixed for two hours in 12.5% trichloracetic acid, stained in 0.025% Coomasie Blue in 12.5% trichloracetic acid and destained in 10.0% trichloracetic acid.

Analytical Procedures: Protein assays were regularly made according to the colorimetric method described by Lowry et al. (1951). When precise protein content was required the Biuret colorimetric method (Gornall, Bardawill and David, 1949) was used. In both cases crystalline bovine serum albumin, fraction V, was used as the standard.

Enzymatic methods were used to determine PEP (Czok and Eckert, 1963), FDP (Bücher and Hohorst, 1963), cytidine and guanosine diphosphates (Adam, 1963), and malate (Hohorst, 1957).

Inorganic phosphate was determined by the method of Fiske and SubbaRow as modified by Leloir and Cardini (1957).

Reagents: Phosphoenolpyruvate (sodium salt), pig heart malate dehydrogenase, and oxalacetic acid were obtained from Boehringer und Soehne. Calcium phosphate gel, protamine sulfate and streptomycin sulfate were supplied by Calbiochem. Lactate dehydrogenase (beef heart), NADH (DPNH), DEAE-cellulose, acetyl coenzyme A, fructose diphosphate, nucleoside mono-, di-, and triphosphates, and amino acids were purchased from Sigma. All reagents used were reagent grade and were checked for purity by paper chromatography before using.

RESULTS

PEP Carboxylase

Purification of PEP Carboxylase: Frozen batches of S. typhimurium, LT 2, stored as described in "Methods" were allowed to thaw overnight at 4°. The thawed suspension of cells was subjected to sonic oscillation for 30 minutes in 50 - 75 ml batches in a Raytheon 10 kc sonic oscillator fitted with a cooling jacket. Broken cells were allowed to stand overnight at 4°. All following steps were carried out at 0 - 4°; all buffers and solution contained 1.0 mM EDTA and 20 mM 2-mercaptoethanol, and were adjusted to pH 8.0 unless otherwise stated.

The following morning the sonicated cells were centrifuged at 27,000 x g for 30 minutes to remove cell debris and some material which precipitated on standing. With constant stirring 1/5 volume 5% streptomycin (pH 7.0) was added to the supernatant followed immediately by the addition of 1/20 volume 2% protamine sulfate (pH 6.5). The suspension was stirred for 15 minutes, then centrifuged at 10,000 x g for 30 minutes. The precipitate was discarded and the supernatant fractionated with solid ammonium sulfate; the fraction precipitating between 40 and 55% saturation with ammonium sulfate being dissolved in 0.05 M Tris-HCl. The volume was made 1/10 that of the original extract with the same buffer.

This fraction was stirred with 2 volumes of 2% protamine sulfate (pH 6.5) for one hour, then brought to 40% saturation with ammonium

sulfate. After stirring 20 minutes, the precipitate was collected by centrifugation and dissolved in 0.01 M Tris-HCl to give a volume equal to 1/10 of the original extract. A further centrifugation at 15,000 x g for ten minutes was required to remove insoluble material.

With stirring a suspension of calcium phosphate gel (40 mg dry weight per ml) was added to the enzyme preparation such that the gel to protein ratio was 2:1. The mixture was stirred for 20 minutes, then centrifuged to pack the gel (5,000 x g, five minutes), the yellow supernatant being discarded. The gel was washed three times with 0.3 M ammonium sulfate in 0.01 M Tris-HCl. After each wash the gel was centrifuged and the supernatant discarded. The enzyme was eluted from the gel with 0.7 M ammonium sulfate in 0.01 M Tris-HCl; a total of three elutions being required. Each wash and elution step consisted in suspending the gel in the appropriate solution using 1/2 the volume adsorbed and stirring for 10 to 15 minutes. The eluted fractions were pooled, brought to 55% saturation with ammonium sulfate and the precipitated enzyme after centrifugation was dissolved in a small amount of 5.0 mM Tris-HCl.

The enzyme solution was dialyzed overnight against 5.0 mM Tris-HCl then applied to a DEAE-cellulose column (2.5 x 40 cm) which had previously been equilibrated with 5.0 mM Tris-HCl. The adsorbed enzyme was washed with 200 ml 0.1 M KCl in equilibrating buffer, then eluted with a linear gradient of KCl. The mixing chamber contained 500 ml 0.1 M KCl and the reservoir 500 ml 0.75 M KCl, both solutions being made in equilibrating buffer. The enzyme was consistently recovered in the fractions corresponding to a KCl concentration of

0.25 - 0.35 M. An elution profile is shown in Figure 1. The active fractions were pooled and concentrated by adding ammonium sulfate to 75% saturation, stirring gently for one hour and centrifuging. The pH at this step was adjusted to 8.0 with concentrated NaOH.

The precipitate obtained in the previous step was extracted by stirring for 15 minutes successively with 26.0%, 21.5% and 18.0% ammonium sulfate solutions (w/v) made in 0.01 M Tris-HCl. After each extraction the mixture was centrifuged at 15,000 x g for 15 minutes and supernatant was assayed for enzyme activity. The enzyme was recovered in the 18.0% ammonium sulfate extraction; a total of three extractions was required to recover most of the enzyme. The enzyme was concentrated by 75% saturation with ammonium sulfate, the precipitate being dissolved in a minimal quantity of 0.02 M Tris-HCl.

The fraction from the previous step was applied to a column (2.5 x 100 cm) packed with G-150 Sephadex (Pharmacia) under a hydrostatis pressure of 10 - 15 cm. The enzyme was eluted from the column with 0.02 M Tris-HCl in 50 drop fractions and was recovered in the first void volume as shown in Figure 2. The active fractions were pooled and the enzyme was precipitated by adding solid ammonium sulfate to give 75% saturation.

The pellet was then extracted with 26.0%, 21.5% and 18.0% ammonium sulfate solutions (w/v). The procedure is a repetition of the extraction of the ammonium sulfate precipitate recovered after passage through DEAE-cellulose. The extracts containing enzyme were pooled and brought to 60% saturation to precipitate the enzyme. The precipitate was collected by centrifugation and dissolved in a minimal

FIGURE 1. Elution pattern of PEP carboxylase from DEAE cellulose column. Optical density of fractions is shown by circles, enzyme activity by crosses and the molarity of the KCL gradient by the unmarked line. Five ml fractions were collected at 4^{o}.

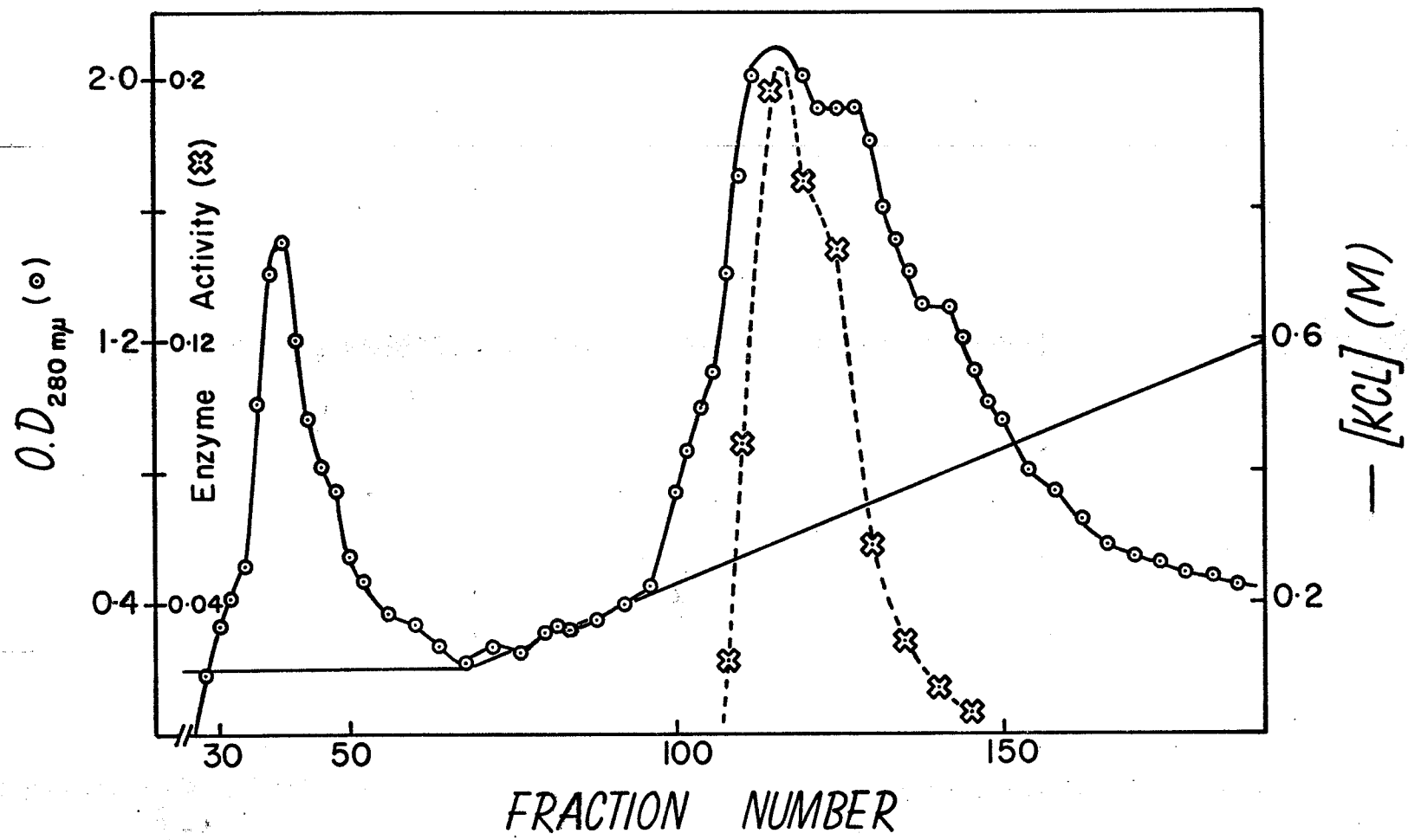
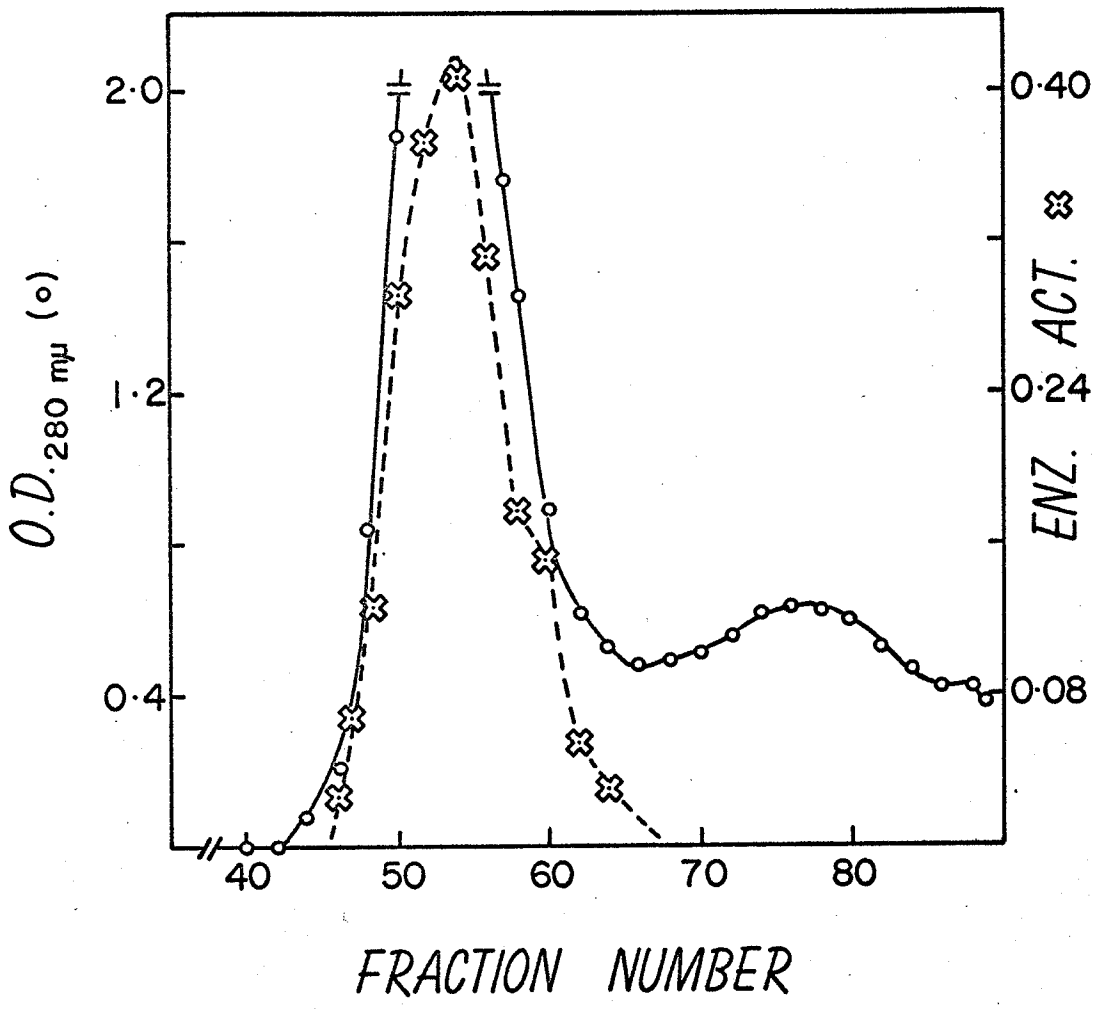

2·0
1·2
0·4
0·2
0·12
0·04
0·6
0·2
O.D 280 mμ (○)
Enzyme Activity (⊠)
— [KCL] (M)
30
50
100
150
FRACTION NUMBER

FIGURE 2. Elution of PEP carboxylase from G-150 Sephadex column. Optical density was measured at 280 mμ and enzyme activity in the fractions was measured spectrophotometrically as described in "Methods". Three ml fractions were collected at 4°.


2·0
1·2
0·4
O.D.280 mμ (○)
0·40
0·24
0·08
ENZ. ACT. ✕
40
50
60
70
80
FRACTION NUMBER

volume of 10.0% (w/v) ammonium sulfate. With stirring, enough 26.0% (w/v) ammonium sulfate was added to bring the final concentration of the salt to 16.0%. After warming to room temperature, the solution was allowed to stand at 4° until precipitation of the enzyme occurred, usually two to three days.

An outline of the purification scheme is given in Table 1.

Criteria of Purity: The enzyme preparation was judged to be near a state of complete purity by polyacrylamide gel electrophoresis. Only one band was visible after staining with Coomasie Blue (Plate 1) as described in "Methods". In one experiment the gel was sliced in half longitudinally, only one half being stained. The other half of the gel was sliced into 1/16 inch cross sections and placed in test tubes containing 0.5 ml of 0.1 M Tris-HCl, pH 8.0 and 10.0 mM 2-mercapto-ethanol. After the enzyme had been eluted from the gel, each eluate was tested for enzyme activity. It was found that eluate possessing enzyme activity coincided in position to the major staining band in the stained half of the gel.

When centrifuged in the Spinco Model E analytical ultracentrifuge, only one peak was visible by the schlieren optical system. The results shown in Plate 2 indicate that the enzyme was nearly homogeneous.

Stability: The enzyme in the precipitated state was stable for periods up to one month when stored in the presence of 10.0 mM 2-mercaptoethanol at 0 - 4°.

Molecular Weight: As will be seen later, PEP carboxylase shows a concentration-dependent aggregation phenomenon. It was, therefore, impossible to determine its molecular weight by sedimentation

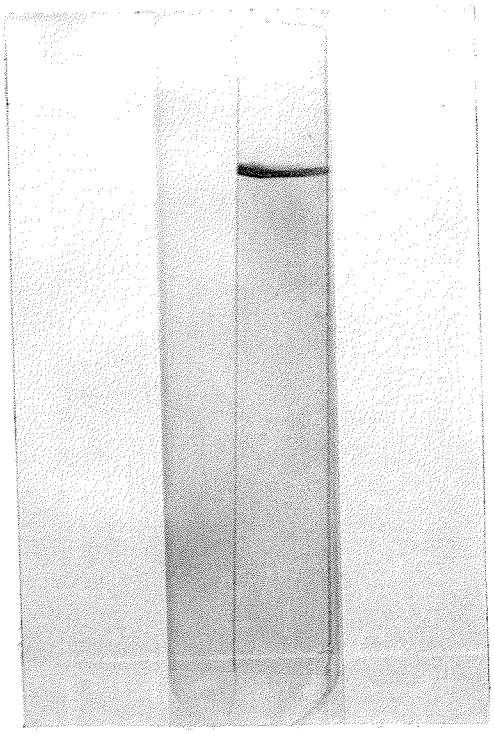
TABLE 1

A SUMMARY OF THE PURIFICATION PROCEDURE FOR PEP CARBOXYLASE

Step	Volume ml	Units*	Protein mg	Specific Activity	Purification	Yield %
1. Protamine sulfate	2,960	6,990,000	77,000	90.8	1	100
2. Ammonium sulfate (0.4 - 0.55%)	255	5,030,000	13,000	384.0	4.2	72
3. Protamine sulfate + ammonium sulfate	227	4,655,000	8,200	567.7	6.3	66.6
4. Calcium phosphate gel treatment	42	3,500,000	670	5,225	57.5	50.1
5. DEAE-Cellulose	120	3,020,000	288	10,400	114.5	42.9
6. Ammonium sulfate extract (18.5%) I	8	2,500,000	105	23,800	262.1	35.8
7. Sephadex G-150	42	2,100,000	72	27,800	306.2	28.6
8. Ammonium sulfate extract II	17	1,400,000	46	30,400	334.8	20.0

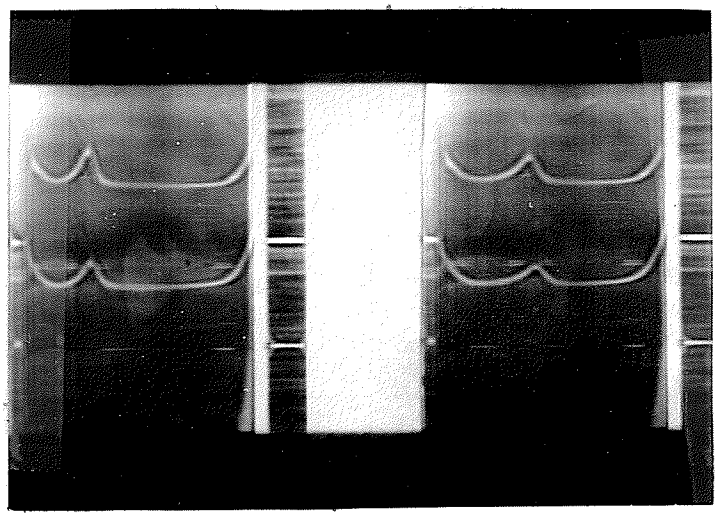
*By spectrophotometric assay.

PLATE 1



Appearance of PEP carboxylase in polyacrylamide gels after electrophoresis and staining with Coomasie blue ("Methods"). Approximately 50 µg of the enzyme was applied to the gel.

PLATE 2



Schlieren pattern of PEP carboxylase during sedimentation velocity centrifugation in Spinco Model E ultracentrifuge. The conditions are described in "Methods". The upper curve was made with an enzyme concentration of 3 mg/ml and the lower with 6 mg/ml. Pictures were taken at 6 (left) and 10 (right) minutes after rotor had reached speed. The $\underline{S}_{20,w}$ calculated were 34.6 and 39.8 for 3 mg/ml and 6 mg/ml, respectively.

equilibrium. An estimate of the molecular weight of the enzyme in dilute solution, however, could be made by zone centrifugation in sucrose density gradients (as described in "Methods"). Using pig heart malate dehydrogenase as an internal standard, the molecular weight of PEP carboxylase was estimated to be 193,000 ± 7,000. This corresponds to a sedimentation coefficient of 11.3.

Initial Velocity Studies: The enzyme catalyzes the reaction:

$$\text{Phosphoenolpyruvate} + HCO_3^- \longrightarrow \text{Oxalacetate} + P_i$$

The reaction was essentially irreversible and can only be measured in the direction of oxalacetate formation. Magnesium ion was required as a cofactor, the K_m for magnesium being 1.0 mM when assays were carried out with 5.0 mM PEP and 10.0 mM HCO_3^-. The Lineweaver-Burke (1934) double reciprocal plot from which the K_m was calculated is shown in Figure 3. In all subsequent assays the magnesium concentration was maintained at 10.0 mM, a value 10 x K_m. At higher concentrations magnesium caused pronounced inhibition of enzyme activity.

The reaction does not follow the classic Michaelis-Menten (1913) kinetics when PEP is used as the variable substrate. In the presence of 10.0 mM magnesium and 10.0 mM HCO_3^-, the resultant velocity versus PEP plot deviated markedly from the classical rectangular hyperbola (Figure 4). In the inset to Figure 4, the data are plotted in the double reciprocal form. Instead of a straight line indicative of a Michaelis-Menten type reaction, the plot is non-linear. The data suggest that more than one molecule of PEP participates in the reaction sequence.

FIGURE 3. Double reciprocal plot of PEP carboxylase assays with varied magnesium concentrations. The spectrophotometric assay (see "Methods") was used with PEP and bicarbonate concentrations held constant at 10.0 mM and with 80 units of enzyme.

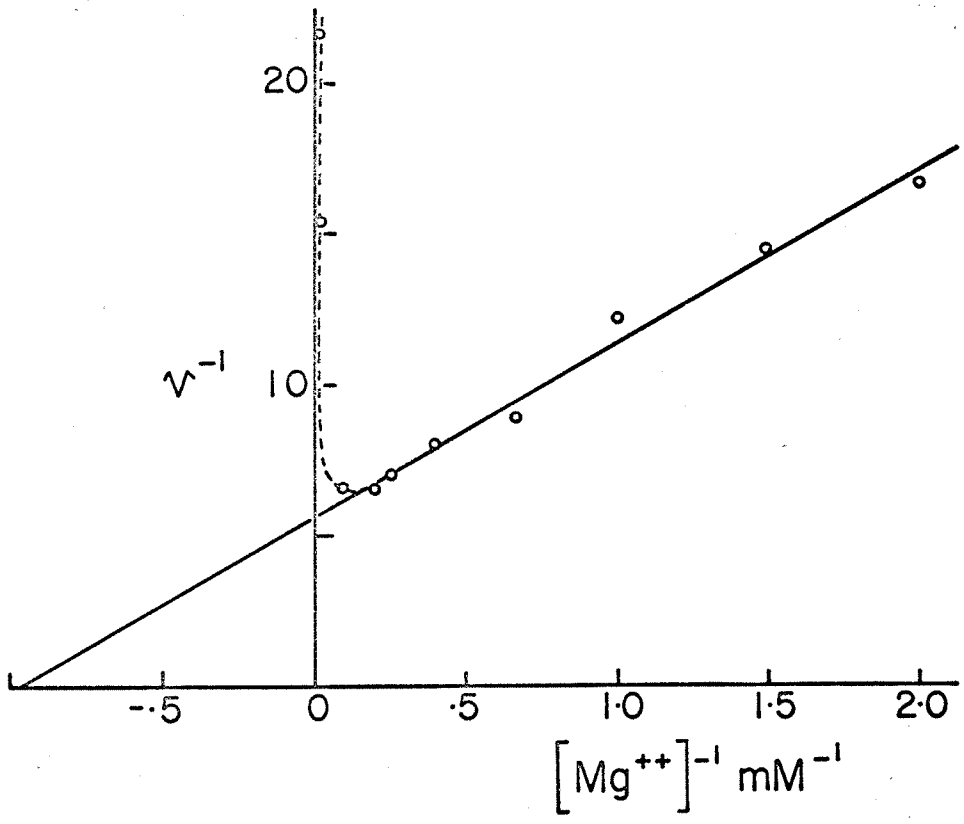
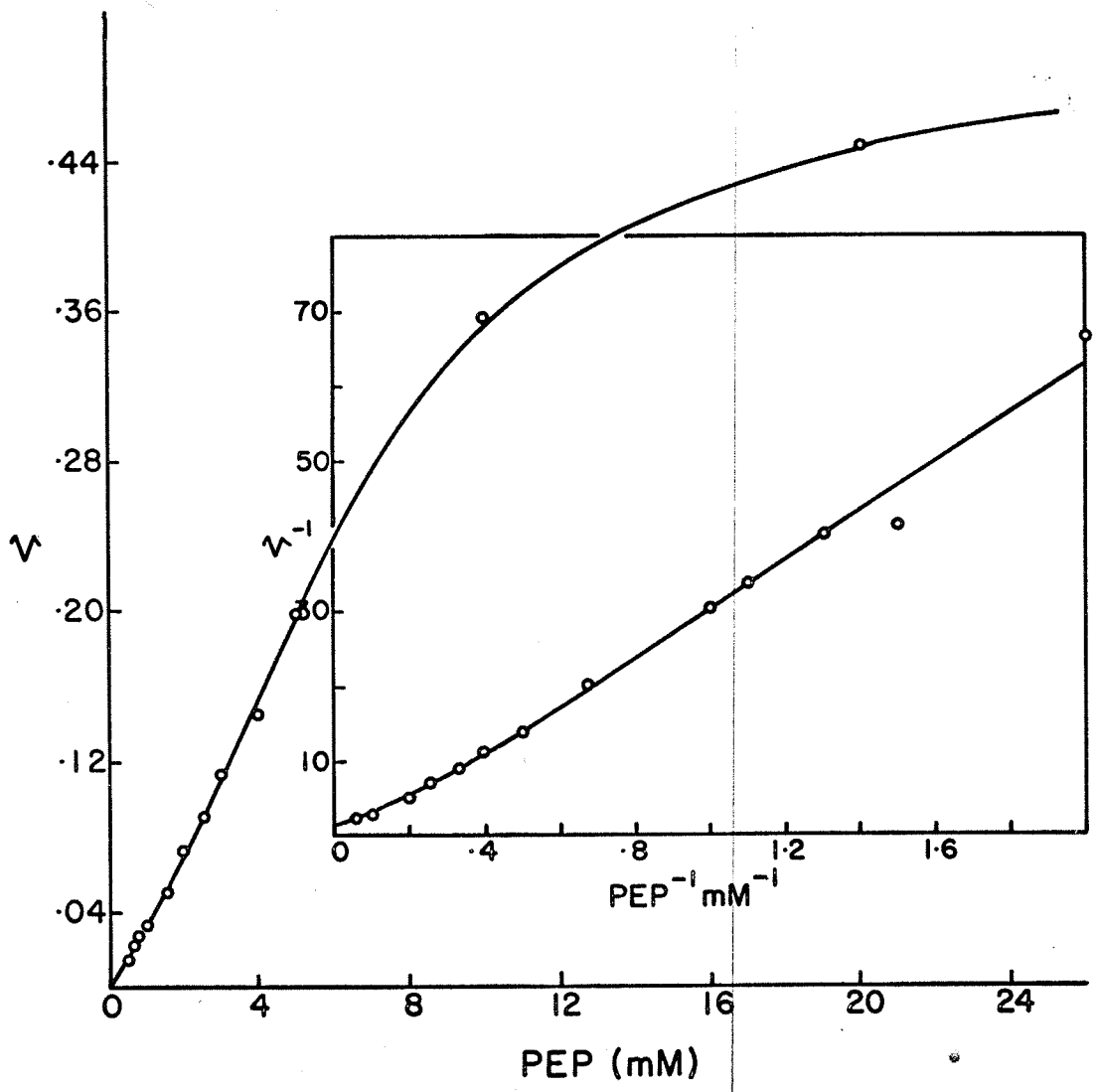

20
10
v^{-1}
-·5
0
·5
1·0
1·5
2·0
$[Mg^{++}]^{-1}$ mM^{-1}

FIGURE 4. Initial velocity data from PEP carboxylase assays measured spectrophotometrically (see "Methods") with varied PEP concentrations and with concentrations of magnesium and bicarbonate both fixed at 10.0 mM. Two hundred enzyme units were used for each assay. The inset shows the data plotted in the double reciprocal form.

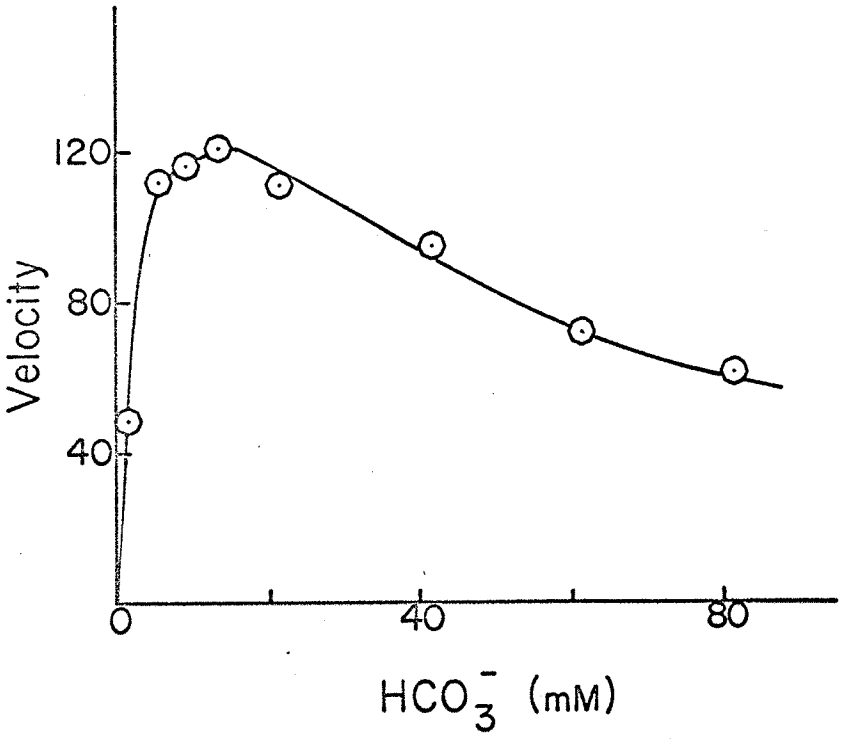

·44
·36
·28
·20
·12
·04
v
0
4
8
12
16
20
24
PEP (mM)
70
50
30
10
v⁻¹
0
·4
·8
1·2
1·6
PEP⁻¹ mM⁻¹

This type of curve remained unchanged when magnesium concentration was increased to 20 mM or when the concentration of the ion was used at a constant ratio of 10:1 in excess of PEP. Nor did the use of buffers other than Tris-HCl alter the shape of the curve.

Since it was impossible to drive off dissolved carbon dioxide at the pH of the assay system (pH 9.0), the carbon dioxide in the assay mixture was estimated in Warburg monometers. The value obtained (1.7 mM) was taken into account when initial velocities were obtained at varied bicarbonate concentrations. The velocity versus HCO_3^- concentration plot shown in Figure 5 was obtained using a constant PEP and magnesium concentration (both of 10.0 mM). A rough K_m of 2.0 mM was calculated from this graph. Since at higher HCO_3^- concentrations enzyme activity was inhibited, the HCO_3^- concentration was kept at 10.0 mM, a value 5 x K_m, in all assays unless otherwise stated. As can be seen from the graph it is impossible to decide whether at low HCO_3^- concentrations the curve follows a hyperbolic function or whether it is S-shaped.

In summary, initial velocity studies of enzyme activity with substrate showed non-linearity of the double reciprocal plots for PEP. The K_m's for the reactants were 1.0 mM for magnesium, 10.0 mM for PEP and 2.0 mM for HCO_3^-.

Effectors of PEP Carboxylase: The enzyme occupies a key position in the metabolic pattern within the bacterial cell and it is not surprising that its activity is subject to a variety of controls. In the course of this work it has been found that aspartate (Maeba and


120
80
40
Velocity
0
40
80
HCO_3^- (mM)

Sanwal, 1965b) and malate serve as inhibitors and that acetyl CoA, FDP (Sanwal and Maeba, 1966a) and a variety of nucleotides (Sanwal and Maeba, 1966b) and able to activate the enzyme.

Inhibitors: L-aspartate and L-malate were strong inhibitors of enzyme activity, the K_i's being 0.1 mM and 0.2 mM, respectively. The K_i is defined as the concentration of inhibitor causing half-maximal inhibition. These constants were found by measuring enzyme activity at varied concentrations of inhibitor in the presence of fixed concentrations of PEP (2.5 mM), HCO_3^- (10.0 mM) and magnesium (10.0 mM). The results are shown in Figure 6. The spectrophotometric assay method as well as the radioactive HCO_3^- incorporation method was used for measuring aspartate inhibition and only the latter method was used for measuring malate inhibition.

Specificity of Inhibition: A variety of compounds are able to produce some inhibitory effect on PEP carboxylase activity. Using the standard $HC^{14}O_3$ incorporation assay in the presence of 2.0 mM concentrations of a variety of compounds tested for inhibitory effect, the results tabulated in Table 2 were obtained. It is evident that of all the reagents tested that L-aspartate and L-malate were the strongest inhibitors.

Since several compounds were known to activate the enzyme it was of interest to see the effect of the presence of activators on inhibition. Consequently, the experiment described in the previous paragraph was repeated with either 0.5 mM acetyl-CoA, 5.0 mM FDP or 5.0 mM CDP included in each assay. These results are described in Table 2.

FIGURE 6. Inhibition of PEP carboxylase by aspartate (crosses) and malate (circles). The radioactive C^{14}-bicarbonate incorporation assay was used ("Methods") with 2.5 mM PEP, 10.0 mM bicarbonate (0.1 μc/mM) and 10.0 mM magnesium. The inset shows the same data plotted in the log-log form.

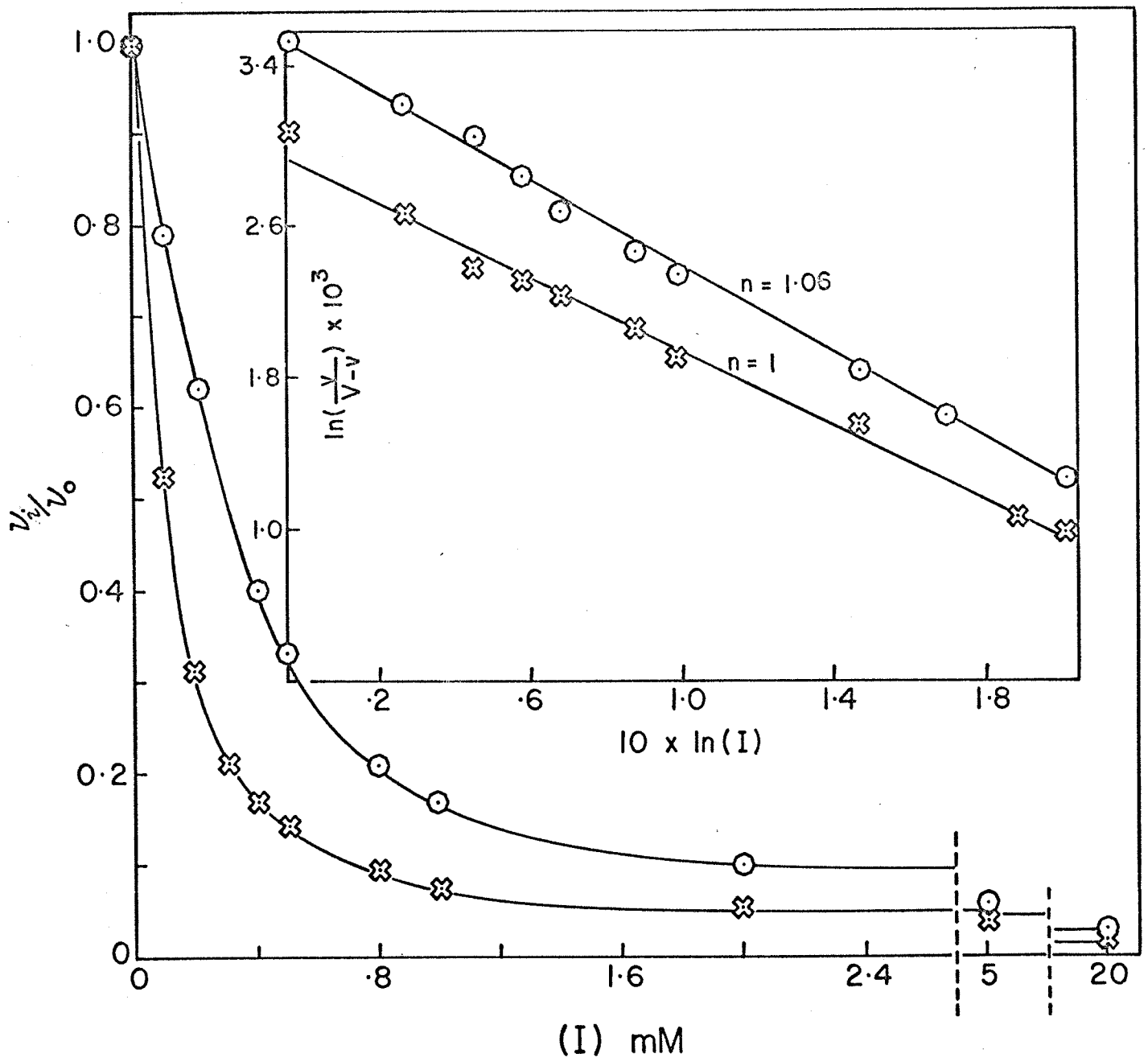

1·0
0·8
0·6
0·4
0·2
0
V_i/V_o
0
·8
1·6
2·4
5
20
(I) mM
3·4
2·6
1·8
1·0
$\ln(\frac{v}{V-v}) \times 10^3$
n = 1·06
n = 1
·2
·6
1·0
1·4
1·8
10 x ln(I)

TABLE 2

THE EFFECT OF CARBOXYLIC ACIDS AND RELATED COMPOUNDS ON PEP CARBOXYLASE ACTIVITY

Additions to Assay Mixture*	C^{14} counts incorporated into oxalacetate			
	Complete	0.5 mM Acetyl CoA#	5.0 mM FDP #	5.0 mM CDP #
None	1,149	20,372	9,901	17,800
Oxalate	743	23,116	5,840	8,850
Malonate	1,070	21,746	9,312	14,850
L-aspartate	86	5,960	414	755
D-aspartate	570	--	--	--
DL-β-methylaspartate	383	--	--	--
L-malate	161	12,875	958	7,072
Fumarate	467	20,222	5,360	9,955
Succinate	804	21,004	7,396	8,932
Maleate	918	17,710	8,404	10,445
Tartarate	809	20,057	7,231	13,433
Isocitrate	810	17,019	9,160	10,658
Citrate	744	23,249	9,209	14,542
α-ketoglutarate	886	19,961	11,019	14,962
L-glutamate	1,344	22,129	10,878	15,944
L-alanine	1,122	21,793	10,858	16,609
L-homoserine	1,223	20,223	10,160	16,796
L-threonine	1,354	22,254	11,033	17,142

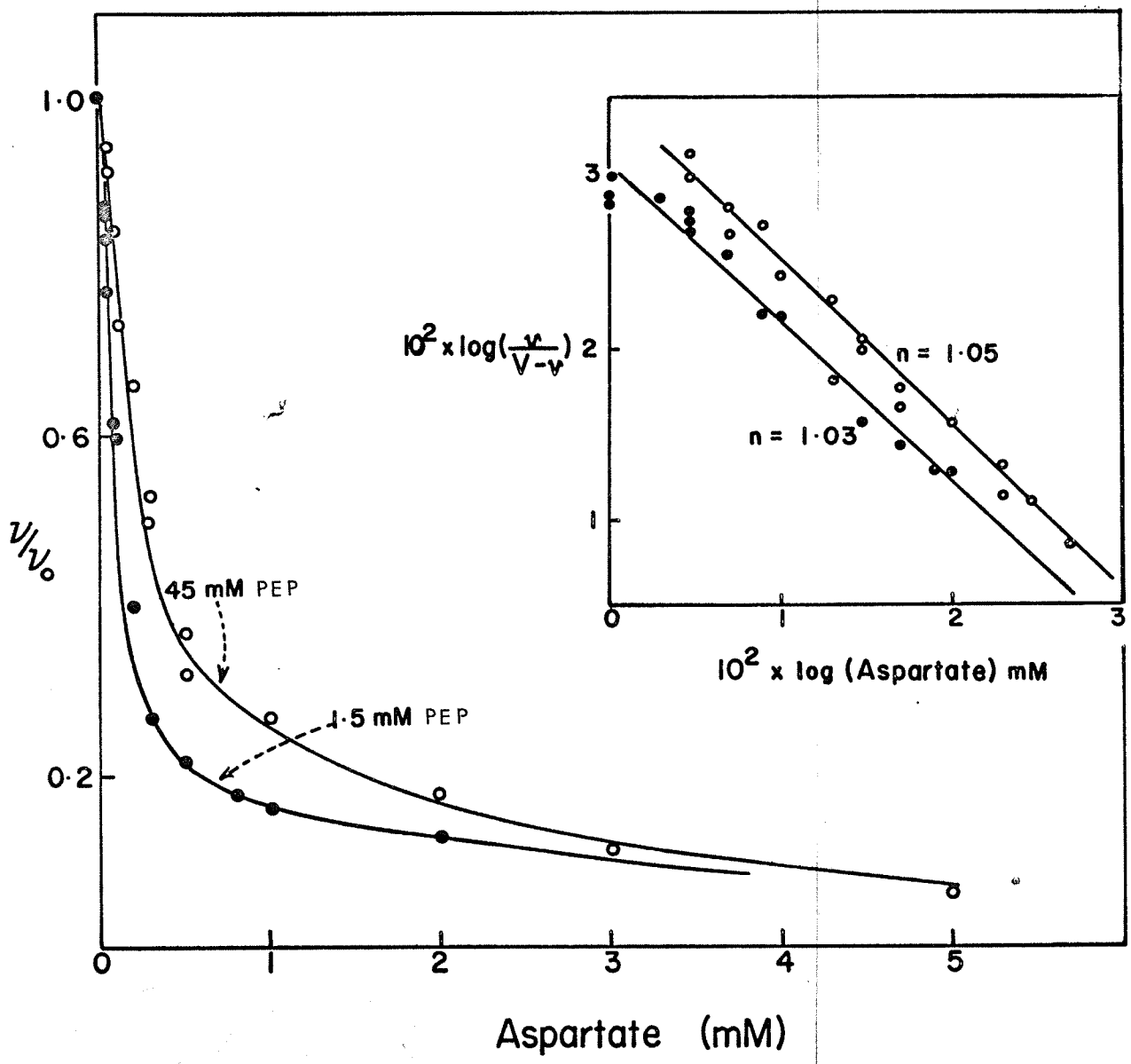
*Additions to the assay mixture were at a concentration of 2.0 mM. Assay mixtures also contained 5.0 mM PEP, 10 mM magnesium and 10 mM C^{14}-bicarbonate (0.1 uc/mM) and the reactions were measured as described in "Methods".

#In addition to the complete assay system, these assays contained activators as indicated.

Although the pattern of inhibition remained the same in the presence of FDP and CDP, it is seen that in the presence of acetyl CoA the effect of all the compounds except malate and aspartate are abolished. Of all the reagents tested malate and aspartate were the most potent inhibitors and were consistently so under the different conditions described.

Kinetics of Inhibition: When the data for the inhibition curves described earlier (Figure 6) are plotted in the log-log or Hill plot form, straight lines are obtained with slopes of -1.0 (inset Fig. 6). This indicates the absence of interaction between inhibitor molecules in producing inhibition. As stated earlier, the PEP concentration for these studies was kept constant at 2.5 mM. If the mechanism for aspartate inhibition conforms to the allosteric transition model proposed by Monod, Wyman and Changeux (1965), an S-shaped curve should result at high PEP concentrations when all the enzyme should theoretically be in the R state. However, with aspartate as the inhibitor, the same type of curve was obtained regardless of the PEP concentration. Thus, as shown in Figure 7, the shape of the curve does not vary when the PEP concentration is increased from 1.4 mM to 45.0 mM. The inset to Figure 7 shows that the slopes of the lines remain close to -1.0. It may be noted that the highest concentration of PEP tried is only 4.5 x K_m, a concentration at which the enzyme is not more than 75% saturated. Owing to the non-specific ionic effects expected to be caused by concentrations higher than 45 mM, no attempts were made to find inhibition patterns under conditions where the enzyme would be saturated with PEP.

FIGURE 7. Inhibition of PEP carboxylase by varying aspartate concentrations. The standard spectrophotometric assay as described in "Methods" was used. The inset shows the data plotted in the log-log form with "n" representing the slope of the lines. Open circles indicate assays with 45 mM PEP and the closed circles are those with 1.5 mM PEP.


1·0
0·6
0·2
V/Vo
45 mM PEP
1·5 mM PEP
0
1
2
3
4
5
Aspartate (mM)
10² x log(v/V-v)
3
2
1
n = 1·05
n = 1·03
0
1
2
3
10² x log (Aspartate) mM

It was of interest to see whether aspartate would alter the sigmoidity of the velocity versus PEP concentration curve (see Figure 4). When velocity measurements were made with varying PEP concentrations in the presence of 3.0 aspartate, a value 30 x K_i, and in its absence, the velocity versus PEP curves remained sigmoidal. The conditions for the test and the results are shown in Figure 8. When these data were plotted in the log-log form, the degree of sigmoidity could be evaluated from the slope of the curves. As shown in Figure 9, a non-linear curve was obtained with more than one slope. Such curves indicate that the velocity curves do not conform to a straight line nor to a parabola when plotted in the double reciprocal form.

Activation by Acetyl CoA: Cánovas and Kornberg (1965) have shown that PEP carboxylase of E. coli was strongly activated by acetyl CoA. Therefore it was not surprising that acetyl CoA should also activate the same enzyme from S. typhimurium. Using assay 2 as described in "Methods" with varied concentrations of acetyl CoA, the concentration of activator required to produce half-maximal activation, or K_a, was found to be 0.25 mM (Figure 10). This value depended upon the amount of PEP used in the assay system; the K_a increasing as the concentration of PEP was decreased. This is shown in Figure 11. The K_a for acetyl CoA was 0.2 mM when the system contained 45.0 mM PEP and increased to 0.8 mM at a PEP concentration of 1.5 mM. The inset to Figure 11 shows an expanded view of the saturation curve in the presence of 45.0 mM PEP at low acetyl CoA concentrations and indicates the deviation from a hyperbolic function. The deviation is more readily seen when the data are plotted in the Hill plot (Figure 12). Such plots

FIGURE 8. Initial velocity studies of PEP carboxylase with varied PEP concentrations using the spectrophotometric assay system ("Methods") in the absence (upper curve) and in the presence (lower curve) of aspartate.

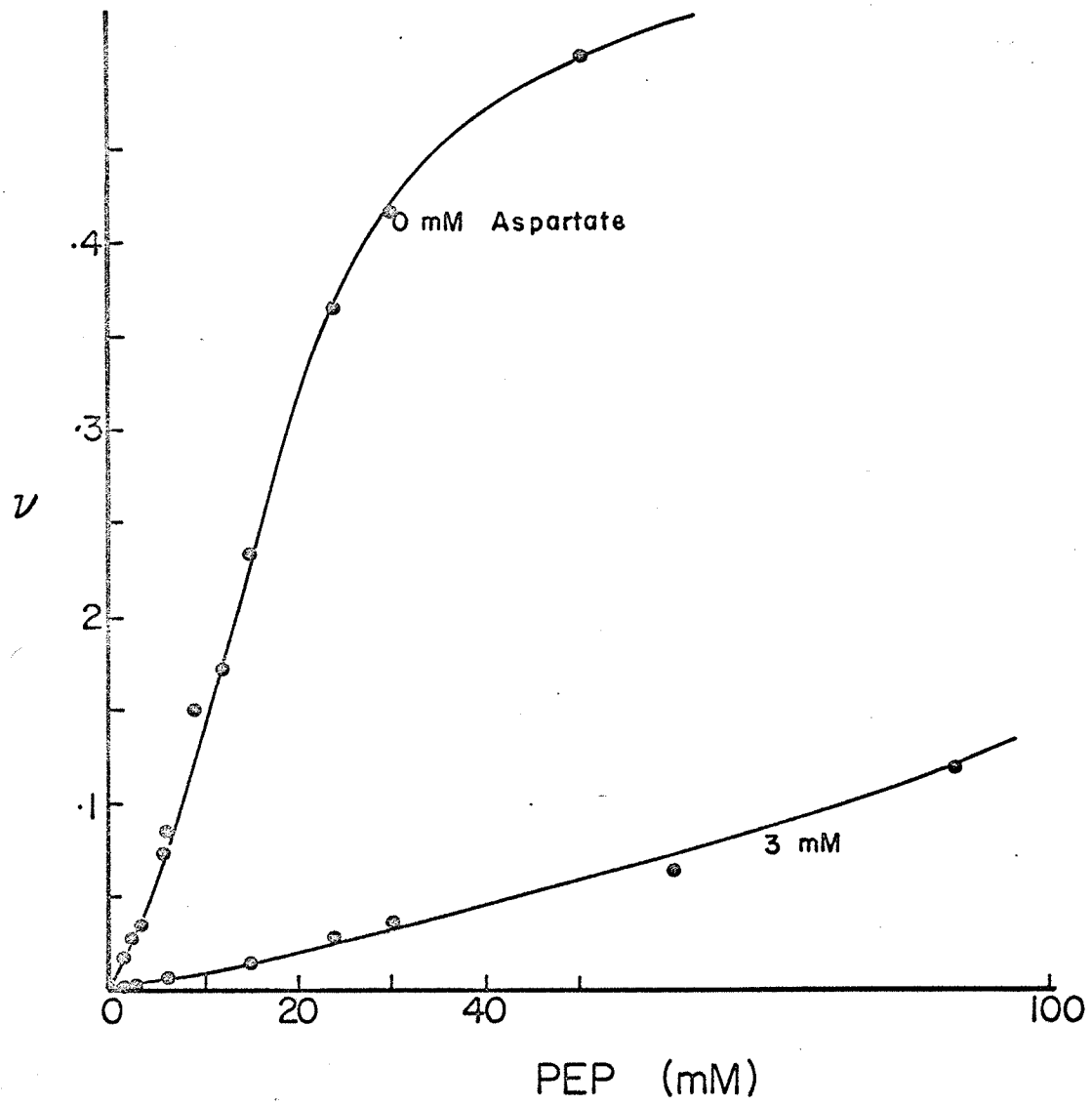

0 mM Aspartate
3 mM
ν
.4
.3
2
.1
0
20
40
100
PEP (mM)

FIGURE 9. The data from Figure 8 plotted in the log-log form.

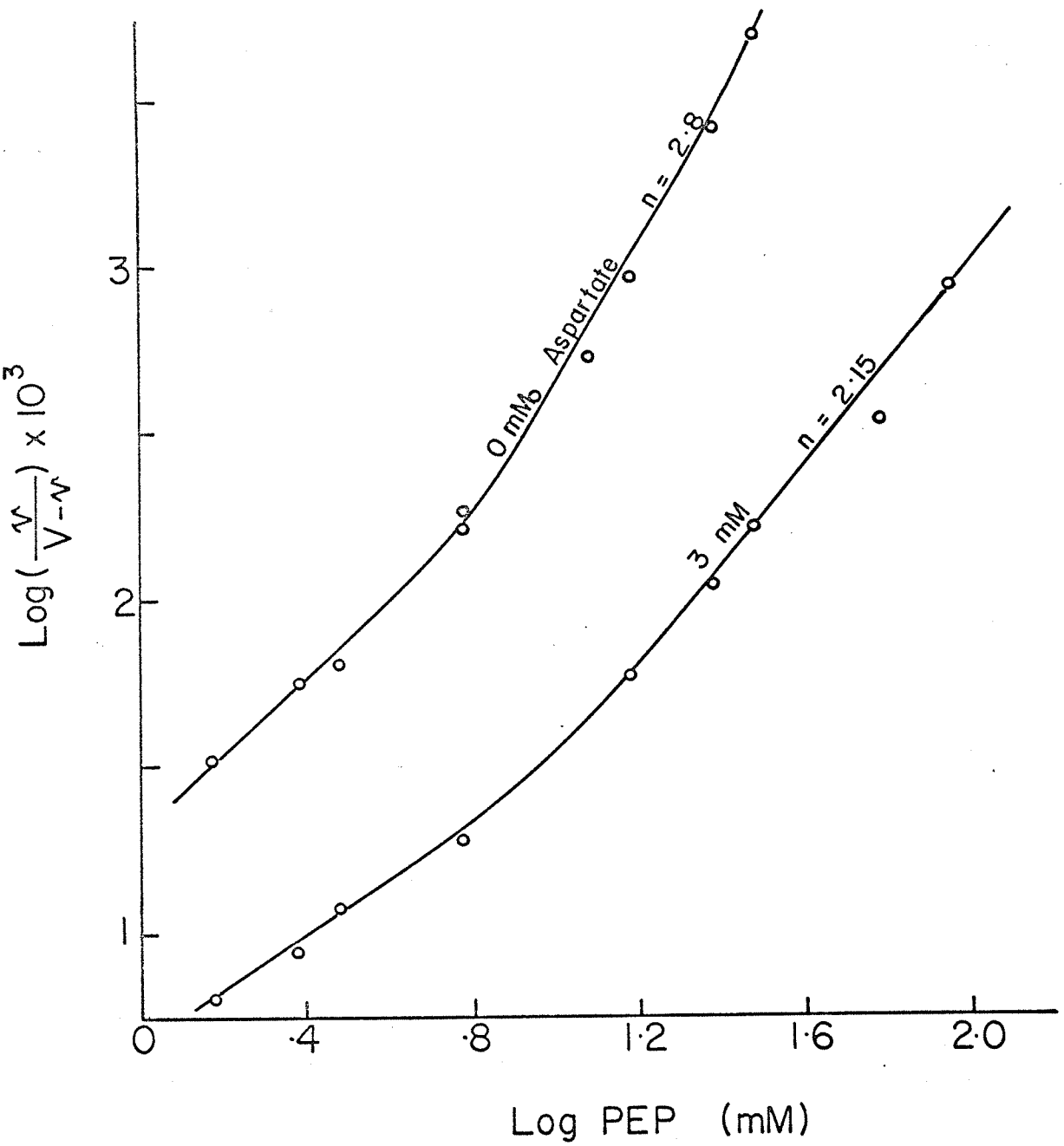

Log $(\frac{v}{V-v}) \times 10^3$
3
2
1
0
·4
·8
1·2
1·6
2·0
Log PEP (mM)
0 mM Aspartate
n = 2·8
3 mM
n = 2·15

FIGURE 10. Activation curve showing the effect of acetyl CoA on PEP carboxylase activity. The standard spectrophotometric assay ("Methods") was used with 15 enzyme units. The inset shows the same data plotted in the log-log form.

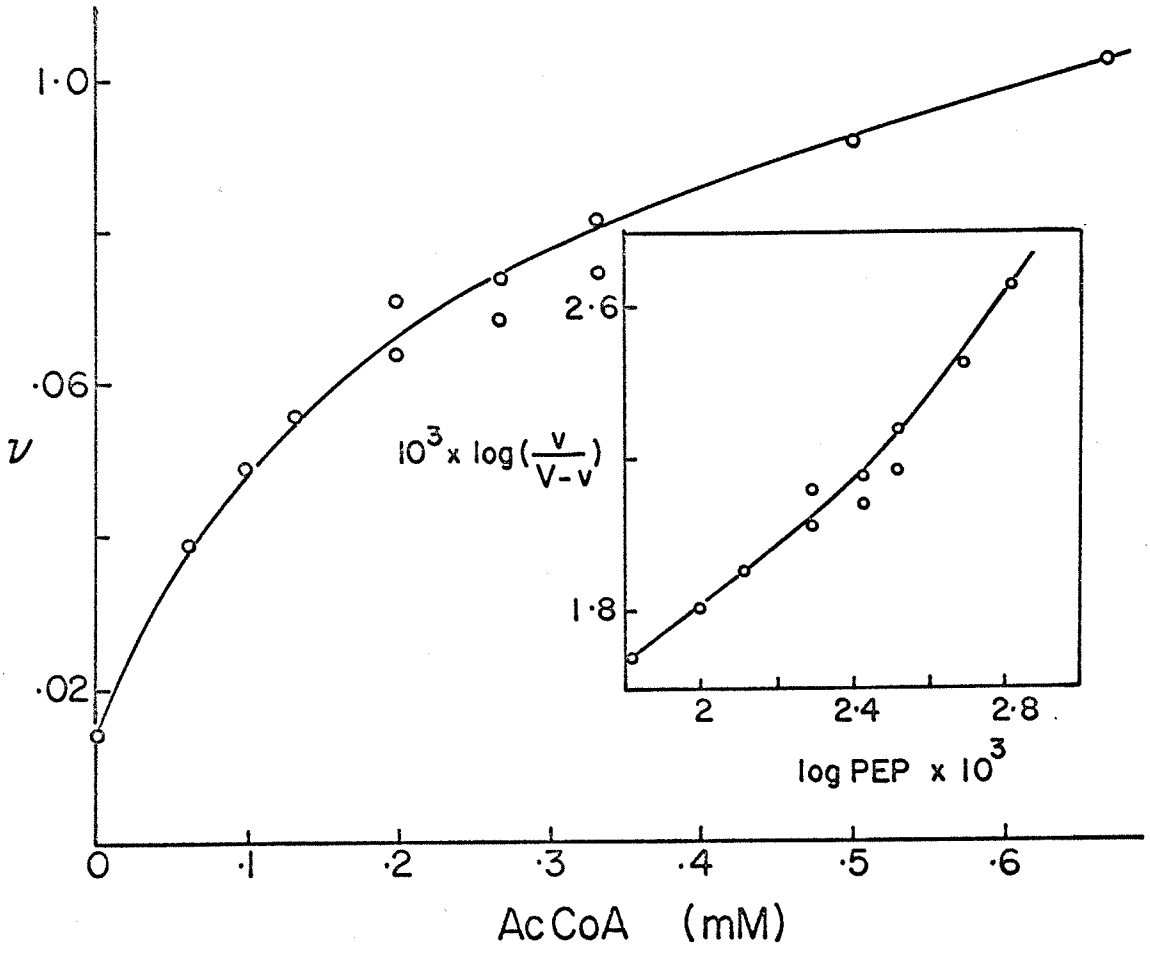

1·0
·06
·02
v
0
·1
·2
·3
·4
·5
·6
AcCoA (mM)
2·6
1·8
$10^3 \times \log(\frac{v}{V-v})$
2
2·4
2·8
log PEP x 10^3

FIGURE 11. Activation of PEP carboxylase by varying concentrations of acetyl CoA in the presence of 45 mM PEP (upper curve) and 1.5 mM PEP (lower curve). The inset shows the upper curve (45 mM PEP) in an expanded form at low acetyl CoA concentrations. The assays were done spectrophotometrically as described in "Methods".

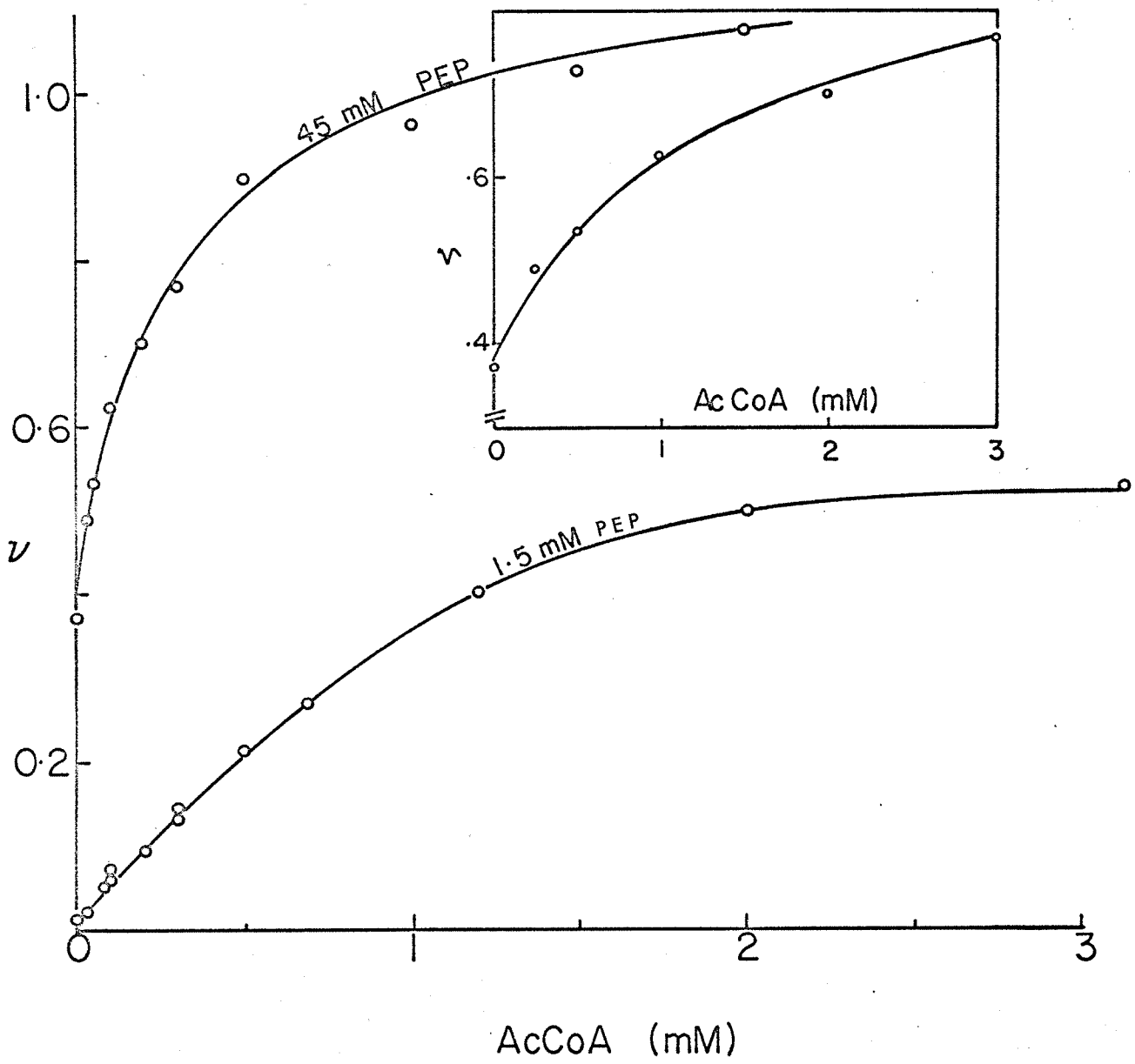
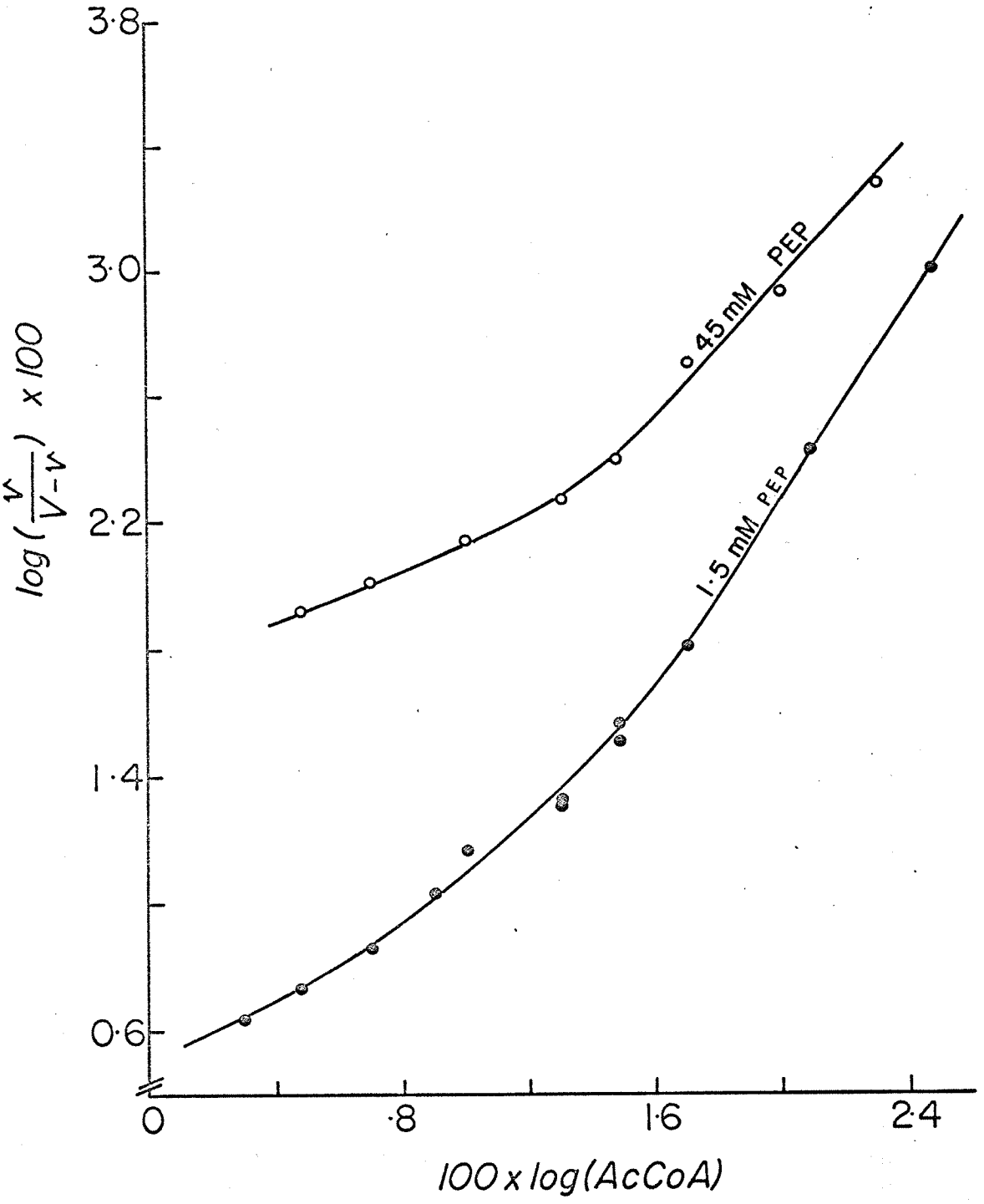

1·0
0·6
0·2
ν
45 mM PEP
1·5 mM PEP
0
1
2
3
AcCoA (mM)
·6
·4
ν
AcCoA (mM)
0
1
2
3

FIGURE 12. The data from Figure 11 plotted in the log-log form.


3·8
3·0
2·2
1·4
0·6
0
·8
1·6
2·4
log (v / V-v) x 100
100 x log (AcCoA)
45 mM PEP
1·5 mM PEP

produced curved lines. The saturation curves point to a mechanism involving more than one molecule of this effector in the activation process.

Using assay 2 (see "Methods"), rate versus PEP concentration curves were constructed at several fixed concentrations of acetyl CoA. As shown in Figure 13, such curves were sigmoidal at 0.2 mM (non-saturating) acetyl CoA, but became hyperbolic or "normalized" at 1.0 mM acetyl CoA, a value 5 x K_a. When the data were plotted in the double reciprocal form (Figure 14) this alteration was seen as a change from a curved plot to that of a straight line. In the Hill plot form (Figure 15), the normalization of the rate plots was indicated by a slope change to a value near 1.

Acetyl CoA was able to alter two kinetic parameters of the enzyme, namely, the V_m and K_m for PEP. The V_m was increased at least ten-fold and the K_m decreased ten-fold. It should be stated that the effect of acetyl CoA was quite specific, and related compounds such as acetyl phosphate and coenzyme A had no effect on the enzyme.

Effect of FDP: That FDP functions as an activator of PEP carboxylase has been reported (Sanwal and Maeba, 1966a). The effect was specific, and as shown in Table 3, related compounds did not stimulate enzymic activity. Of the numerous glycolytic intermediates tested, only glycerate and fructose-1-phosphate, in addition to FDP, activated the enzyme, and the amount of activation was not comparable to that obtained by FDP.

The K_a for FDP was found to be 2.2 mM when assayed in the presence of 5.0 mM PEP, 10.0 mM HCO_3^- and 10.0 mM $MgCl_2$ (Figure 16).

FIGURE 13. Rate curves of PEP carboxylase activity with varied PEP in the presence of different fixed concentrations of acetyl CoA (as indicated on curves). The spectrophotometric assay as described in "Methods" was used.

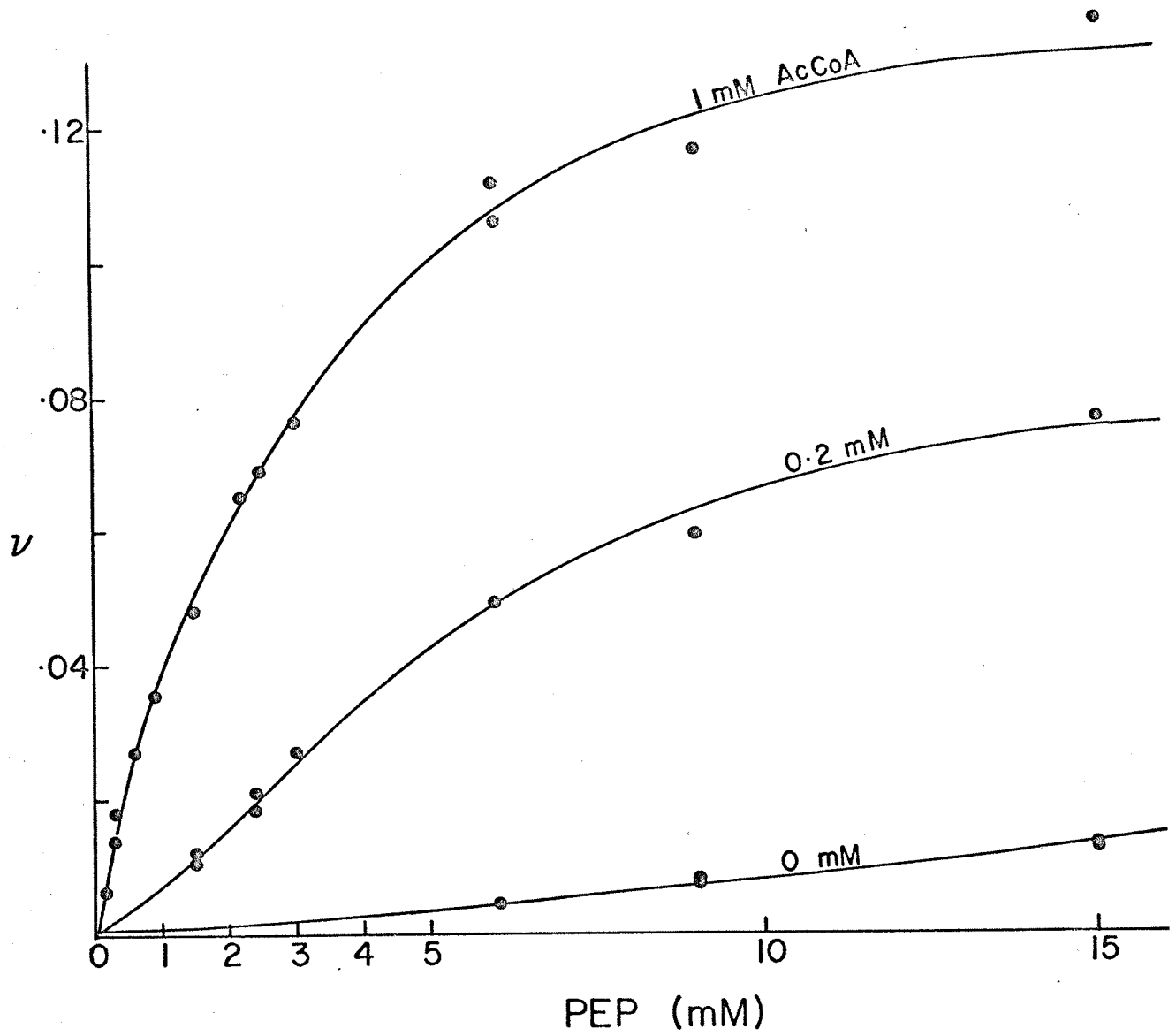

1 mM AcCoA
0·2 mM
0 mM
·12
·08
·04
ν
0
1
2
3
4
5
10
15
PEP (mM)

FIGURE 14. The data from Figure 13 plotted in the double reciprocal form.

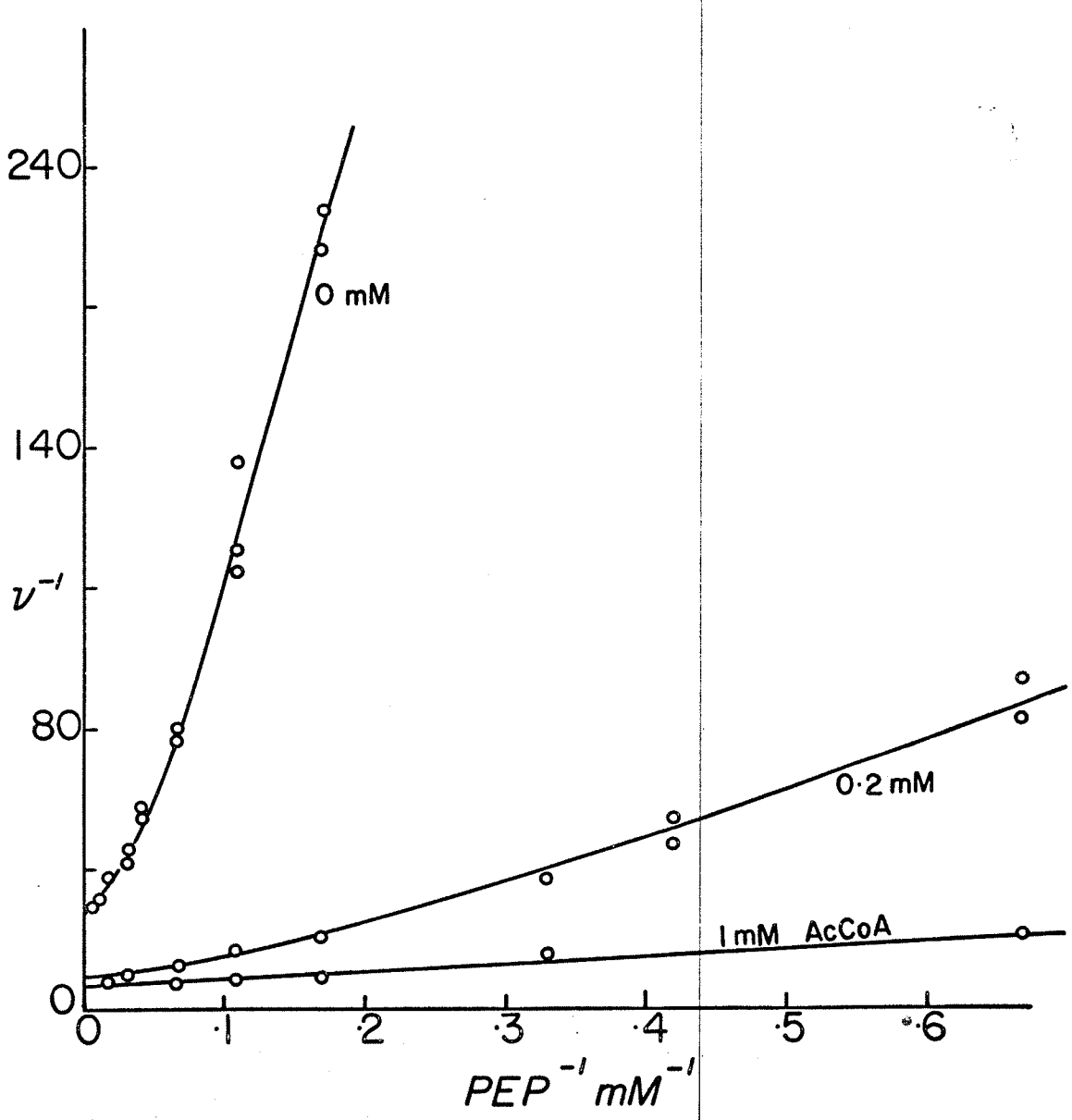

240
140
80
0
v^{-1}
0 mM
0·2 mM
1 mM AcCoA
0
·1
·2
·3
·4
·5
·6
$PEP^{-1}\ mM^{-1}$

FIGURE 15. The data from Figure 13 plotted in the log-log form.

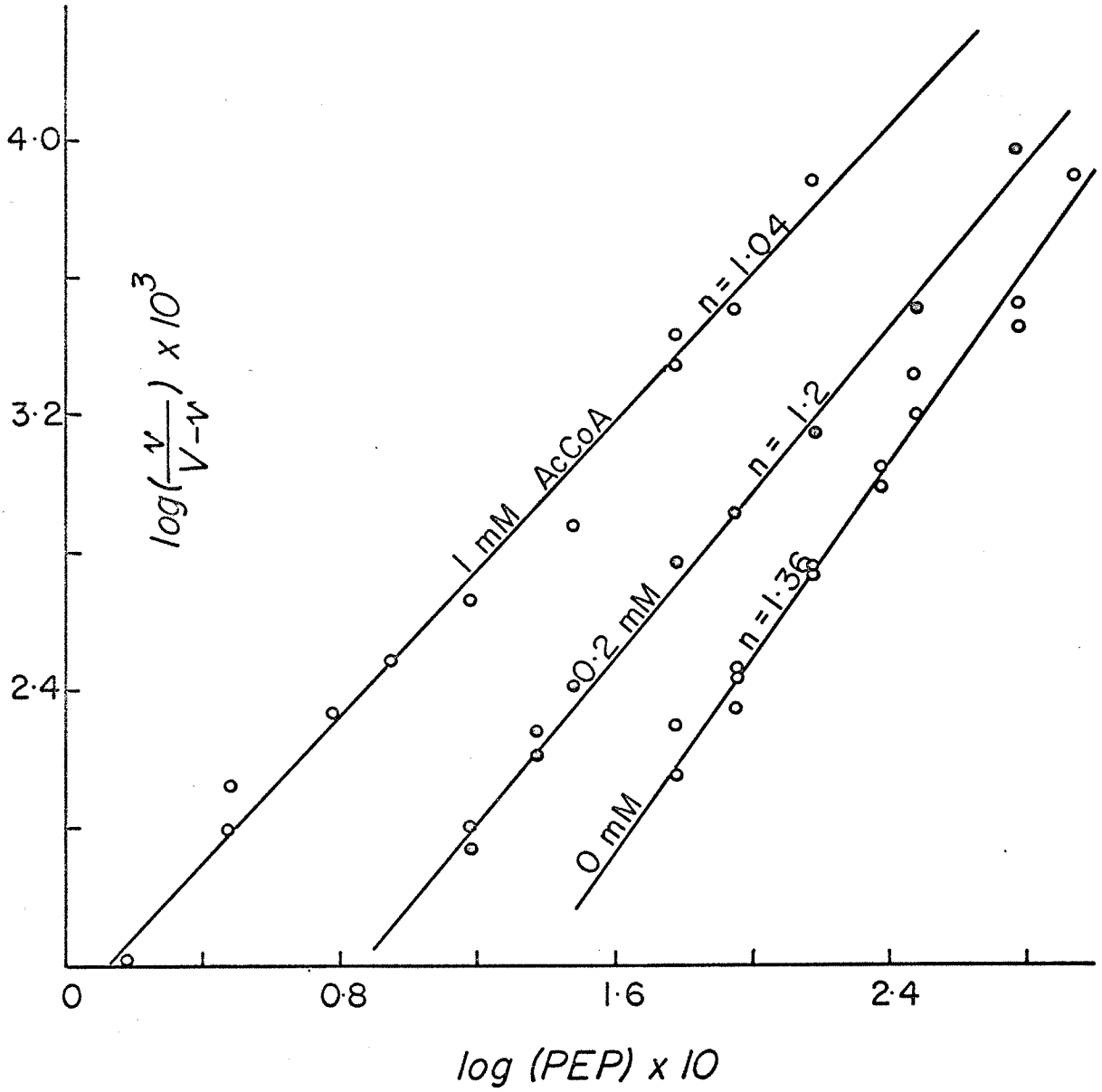

4·0
3·2
2·4
0
0·8
1·6
2·4
log (PEP) x 10
log(v/V−v) x 10³
1 mM AcCoA
n=1·04
0·2 mM
n=1·2
0 mM
n=1·36

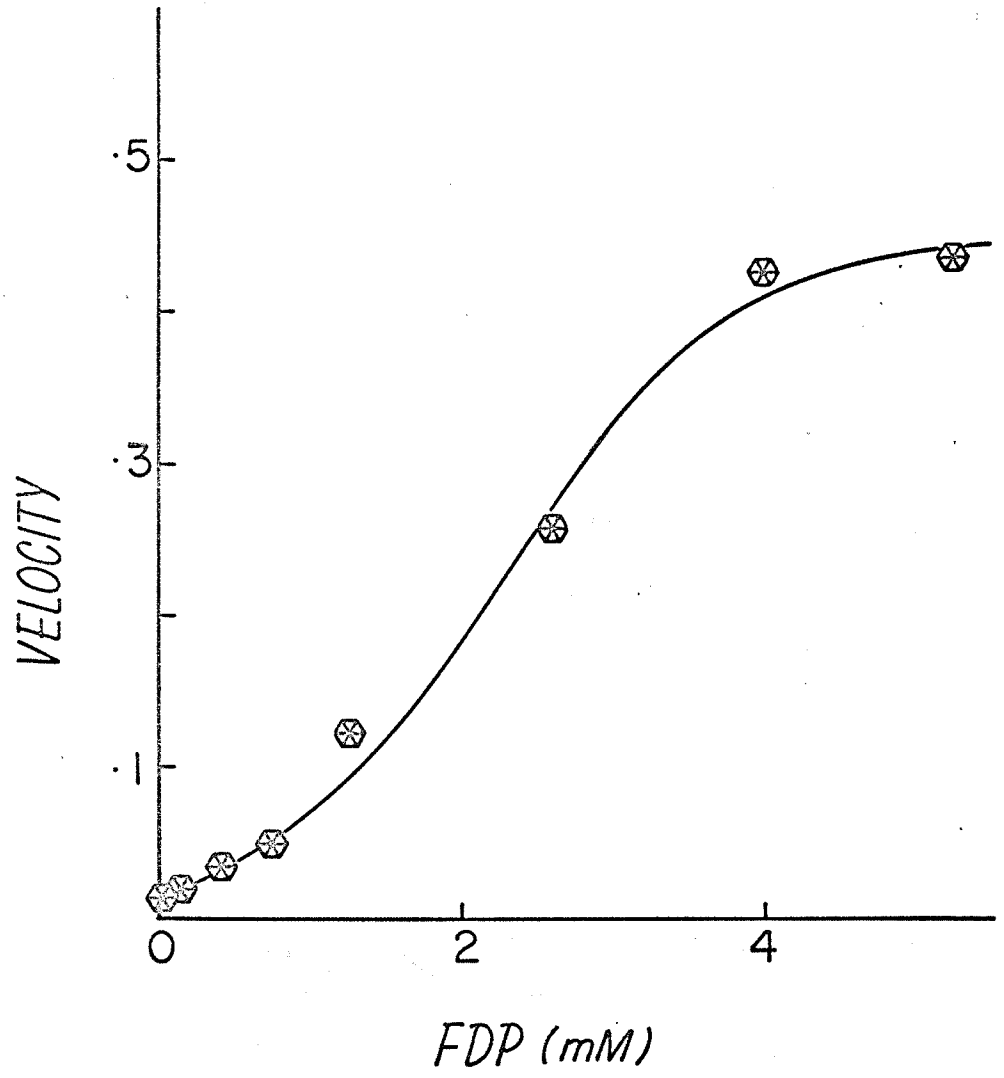
TABLE 3

THE EFFECT OF VARIOUS INTERMEDIATES OF THE GLYCOLYTIC CYCLE ON THE ACTIVITY OF PEP CARBOXYLASE

Additions to Assay Mixture*	Concentration (mM)	Velocity
None	--	.036
Glycerol	5.0	.036
D-glucose	5.0	.038
2-phosphoglyceric acid	5.0	.037
3-phosphoglyceric acid	5.0	.040
Dihydroxyacetone phosphate	5.0	.040
Fructose-6-phosphate	5.0	.044
Glucose-1-phosphate	5.0	.036
Glucose-6-phosphate	3.3	.044
DL-glyceric acid	1.67	.053
Fructose-1-phosphate	5.0	.090
Fructose-1,6-diphosphate	1.3	.288

*Velocities were measured spectrophotometrically ("Methods") at a PEP concentration of 1.66 mM PEP and 100 enzyme units.

FIGURE 16. Activation of PEP carboxylase by FDP. The standard spectrophotometric assay ("Methods") with 20 enzyme units was used.


VELOCITY
.5
.3
.1
0
2
4
FDP (mM)

This value was constant as shown in Figure 17, and did not change when the PEP concentration was increased from 1.5 mM to 45.0 mM. Similar to the saturation curve for acetyl CoA, the saturation curve for FDP remained sigmoidal regardless of the amount of PEP used in the assay system. This indicated that FDP participated more than once in the activation process. In the log-log plot the saturation curves yield curved lines from which no slope value could be obtained (see Figure 18).

The effect of FDP on the rate-concentration plot for PEP is shown in Figure 19. Unlike acetyl CoA, the activator FDP was unable to normalize the rate curve even at 4 x K_a values of the activator. The fact that acetyl CoA and FDP affect the rate plot in different manners may be indicative of distinct sites for the two effectors. Also it can be seen from the graph (Figure 19) that in the presence of FDP, the K_m for PEP is decreased and the V_m of the enzyme is increased.

Activation by Nucleotides: Activation of PEP carboxylase by nucleotides as a possible compensatory feedback effect has been previously described (Sanwal and Maeba, 1966b). As shown in Table 4, various nucleoside mono-, di- and triphosphates are able to produce a stimulatory effect. Not included in the table are the following compounds which were tested, each at a concentration of 3.0 mM, but failed to activate the enzyme; uridine, UMP, UDP, UTP, AMP, 3', 5'-cyclic AMP, ADP, ATP and deoxy ATP. As can be seen from the Table 4, the most potent activators of the enzyme are CMP, CDP and GTP.

FIGURE 17. Activation of PEP carboxylase by varying levels of FDP in the presence of 45 mM PEP (upper curve) and 1.5 mM PEP (lower curve). The spectrophotometric assay system ("Methods") was used.

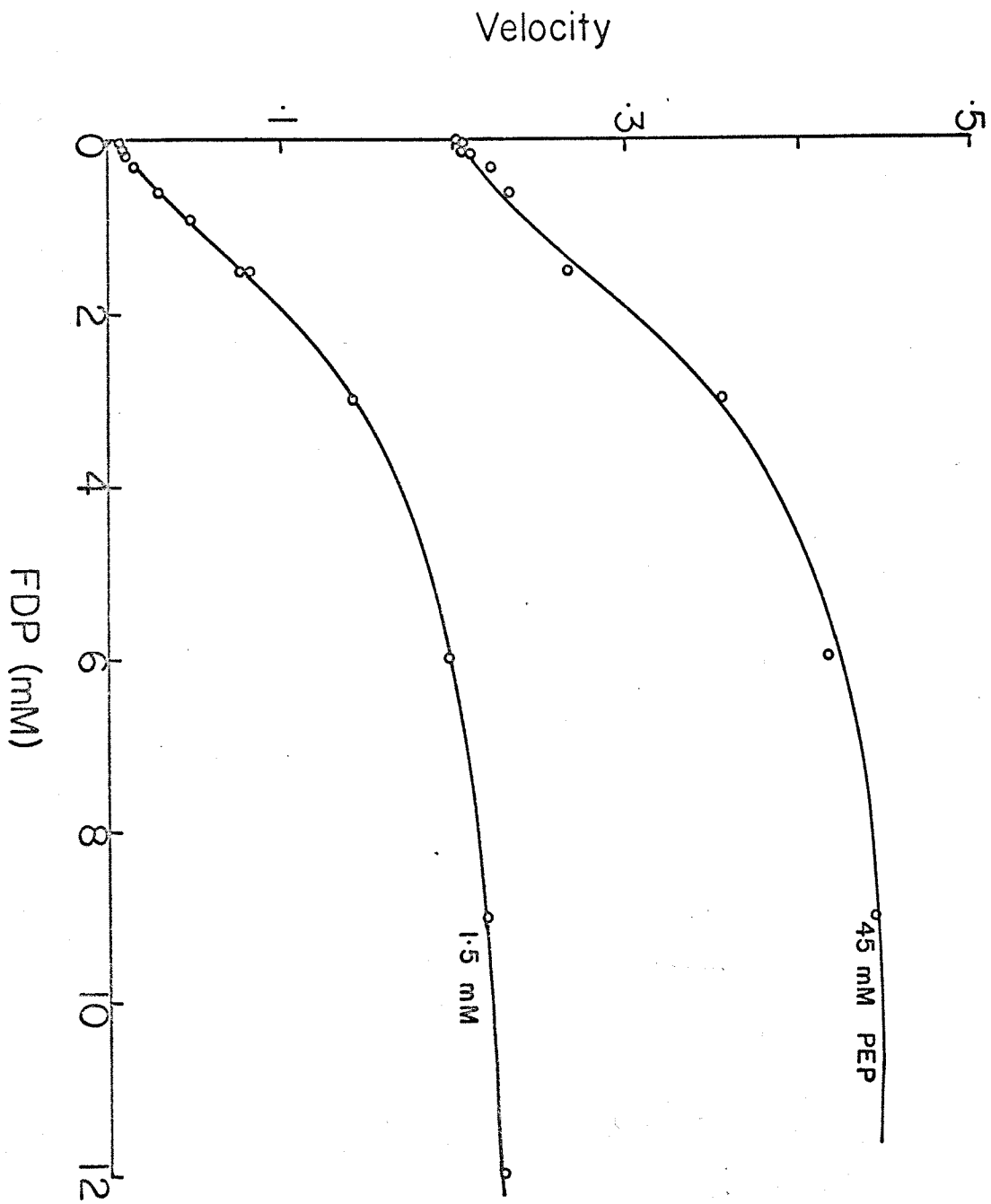

Velocity
.1
.3
.5
0
2
4
6
8
10
12
FDP (mM)
1.5 mM
.45 mM PEP

FIGURE 18. The data from Figure 17 plotted in the log-log form.

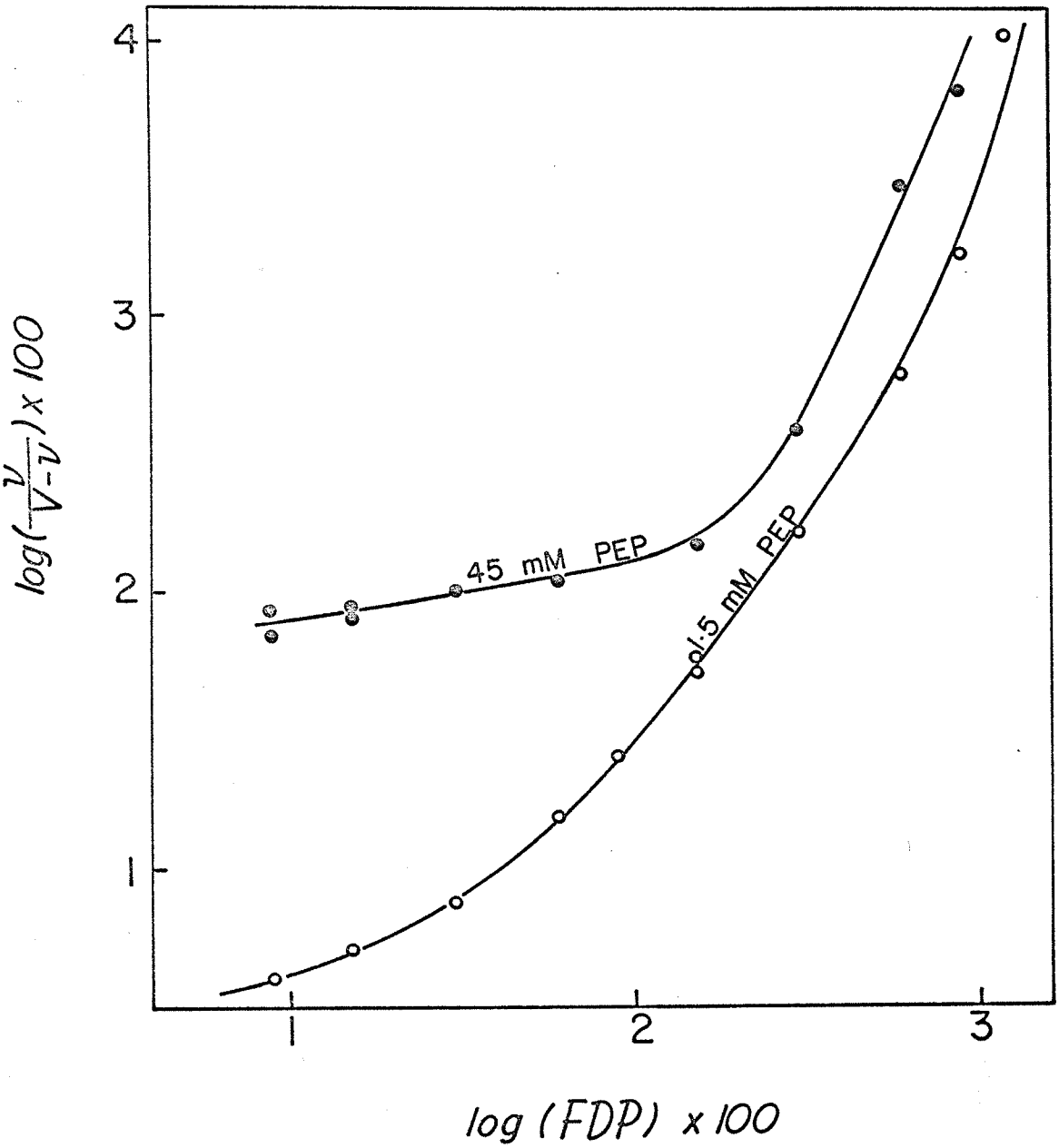

4
3
2
1
1
2
3
45 mM PEP
1.5 mM PEP
log (FDP) x 100
log(v/V-v) x 100

FIGURE 19. The effect of 7.0 mM FDP on the rate curve of PEP carboxylase with PEP as varied substrate. The lower curve is the control in the absence of FDP. Assays were done spectrophotometrically as described in "Methods".

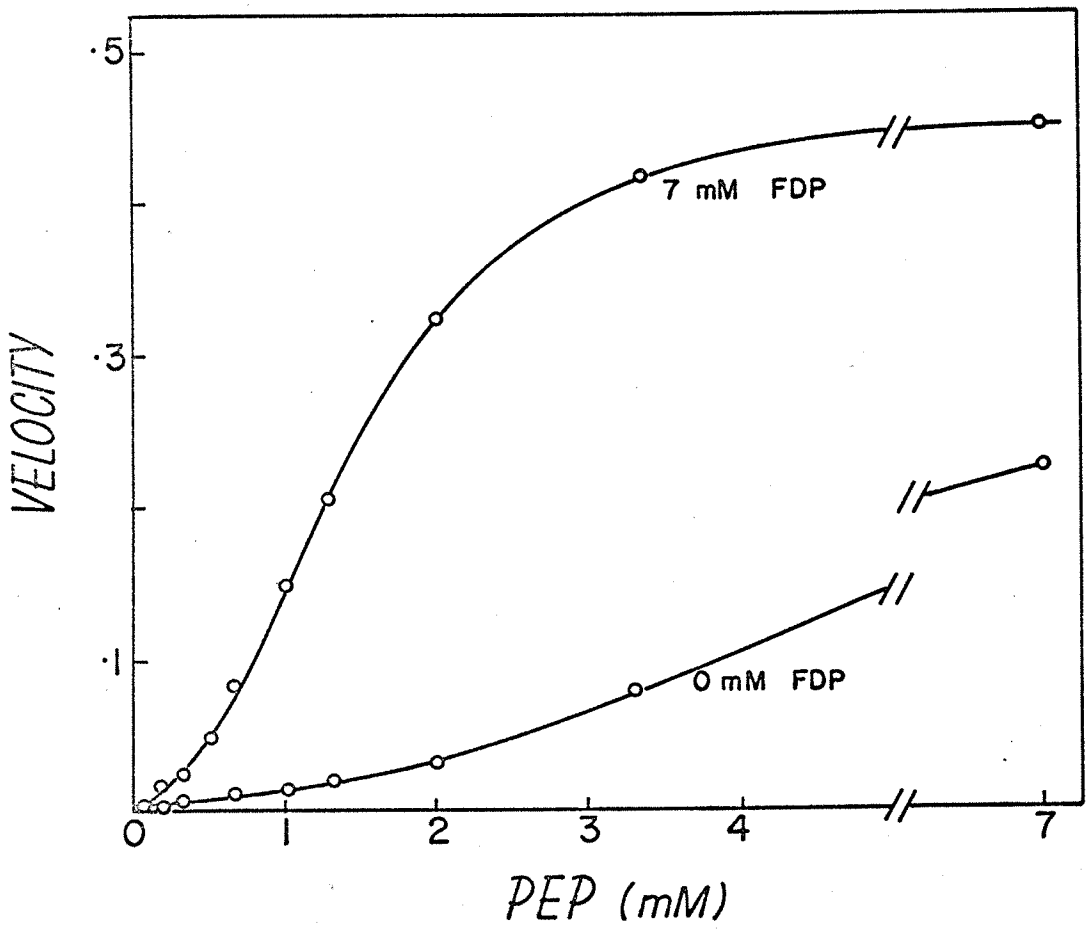

·5
·3
·1
VELOCITY
7 mM FDP
0 mM FDP
0
1
2
3
4
7
PEP (mM)

TABLE 4

ACTIVATION OF PEP CARBOXYLASE BY VARIOUS PYRIMIDINE AND PURINE NUCLEOTIDES

Additions to Assay Mixture*	Concentration (mM)	Velocity
None	--	.028
Cytidine	3.3	.036
Cytidine monophosphate	3.0	.108
Cytidine diphosphate	2.4	.124
Cytidine triphosphate	2.4	.072
Guanosine monophosphate	3.3	.042
Guanosine diphosphate	2.6	.070
Guanosine triphosphate	2.0	.116
Inosine monophosphate	2.8	.028
Inosine diphosphate	2.6	.060
Inosine triphosphate	2.2	.072
Deoxycytidine triphosphate	2.4	.070

*The standard spectrophotometric assay system with PEP at a concentration of 1.66 mM and enzyme concentration of 80 units ("Methods") was used to measure velocities.

The nucleotides play only a catalytic role in the reaction. Attempts to show $C^{14}O_2$ - oxalacetate exchange according to the method of Utter and Kurahashi (1954) in the presence of the nucleotides were negative, indicating the absence of a carboxykinase type reaction. The results and conditions of these tests are outlined in Table 5. Also stoichiometric experiments in the presence of the effectors, FDP, GDP and CDP show that these compounds are not depleted when the assay system for PEP carboxylase include them (see Table 5).

Saturation curves for two representative nucleotides, GTP and CDP are presented in Figure 20. Under the assay conditions described in the legend to Figure 20, the K_a's for GTP and CDP were found to be nearly the same, around 1.1 mM. However, the amount of activation differed in that the maximum velocity was two-fold greater in the presence of CDP than with GTP. The variation in K_a's and V_m's of the different nucleotides which activate PEP carboxylase is outlined in Table 6. The saturation curve for both CDP and GTP show deviation from a hyperbolic function indicating that these nucleotides participate more than once in the activation process.

The form of the saturation curve for CDP remained the same regardless of the amount of PEP in the assay system. This is shown in Figure 21 where the data for saturation curves for CDP in the presence of 1.5 mM PEP and 45.0 mM PEP are presented. The inset to this figure shows the ezpanded view of the upper curve (45.0 mM PEP) and emphasizes the sigmoidity of the curve at lower CDP concentrations. Similar curves were obtained for GTP but are now shown.

TABLE 5

STOICHIOMETRY OF THE PEP CARBOXYLASE REACTION

Activator	Micromoles				
	Initial Conc.	Final Conc.	PEP utilized	Malate formed	P_i formed
None	--	--	0.58	0.56	0.60
FDP	8.0	7.9	0.88	0.82	0.93
GDP	4.40	4.41	0.67	0.67	--
CDP	3.80	3.78	0.72	0.70	--

The reaction mixture contained 6.6 μmoles PEP; 1.7 μmoles DPNH; 6 μg malate dehydrogenase; 7.1 μmoles of bicarbonate; 30 μmoles magnesium sulfate; 32 units of enzyme in mixtures without activator, 6 units in mixtures with FDP, and 8 units in mixtures with CDP or GDP. The final concentration of the buffer was 0.1 M Tris-HCl and the final volume was 3.0 ml. The pH of the reaction mixture was 9.0 and the temperature was 30°. After a 10 minute incubation the reaction was stopped by the addition of perchloric acid to a final concentration of 6%. The pH was then adjusted to 4.0 by adding K_2CO_3 and the precipitate was removed by centrifugation. Aliquots of the supernatant were analyzed for the various compounds listed (see "Methods").

FIGURE 20. Activation of PEP carboxylase by varied concentrations of GTP (lower curve marked with crosses) and CDP (upper curve marked with circles). Assays were made spectrophotometrically ("Methods") with 5.0 mM PEP, 10.0 mM bicarbonate and 10.0 mM magnesium.

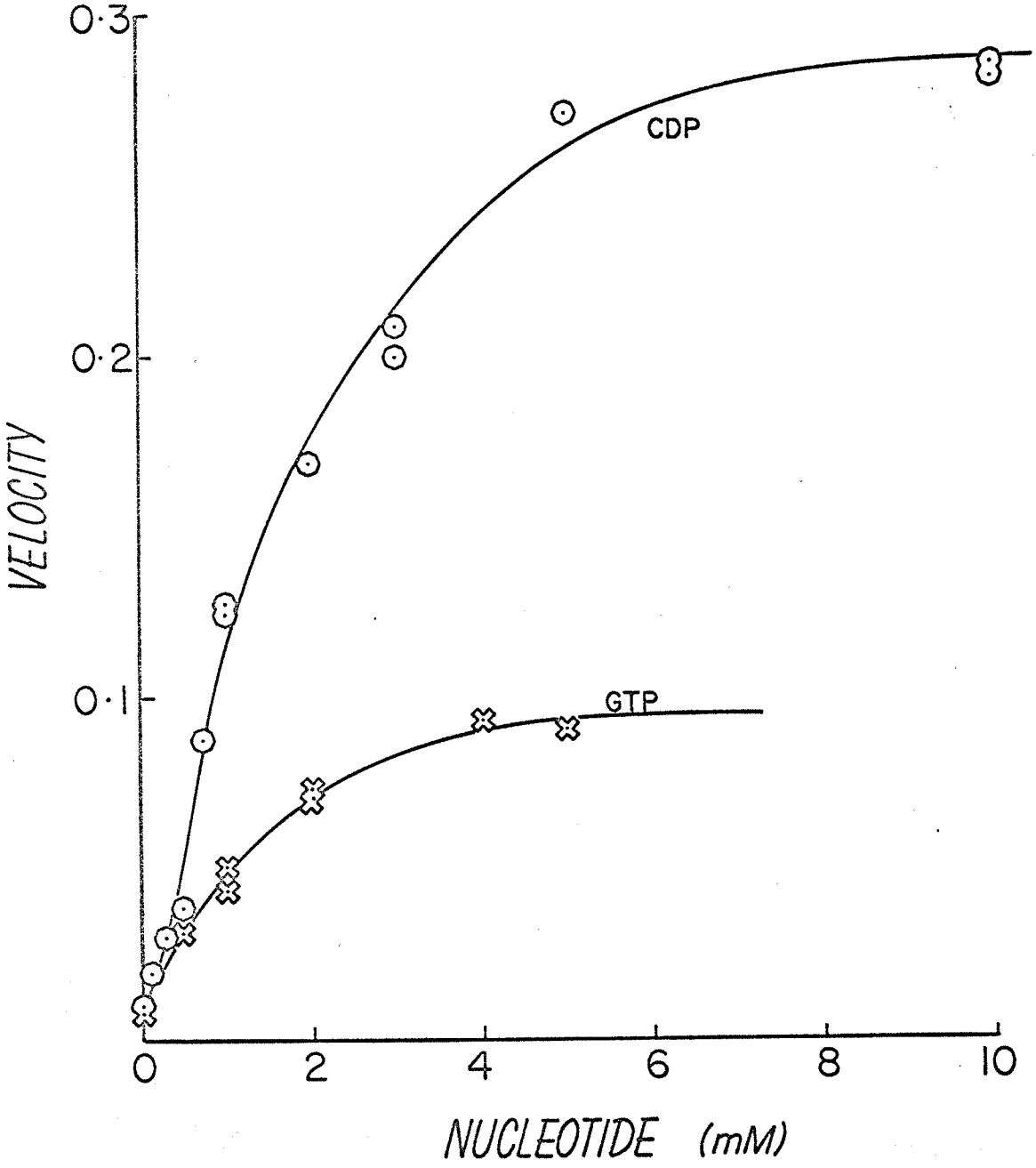

0·3
0·2
0·1
VELOCITY
CDP
GTP
0
2
4
6
8
10
NUCLEOTIDE (mM)

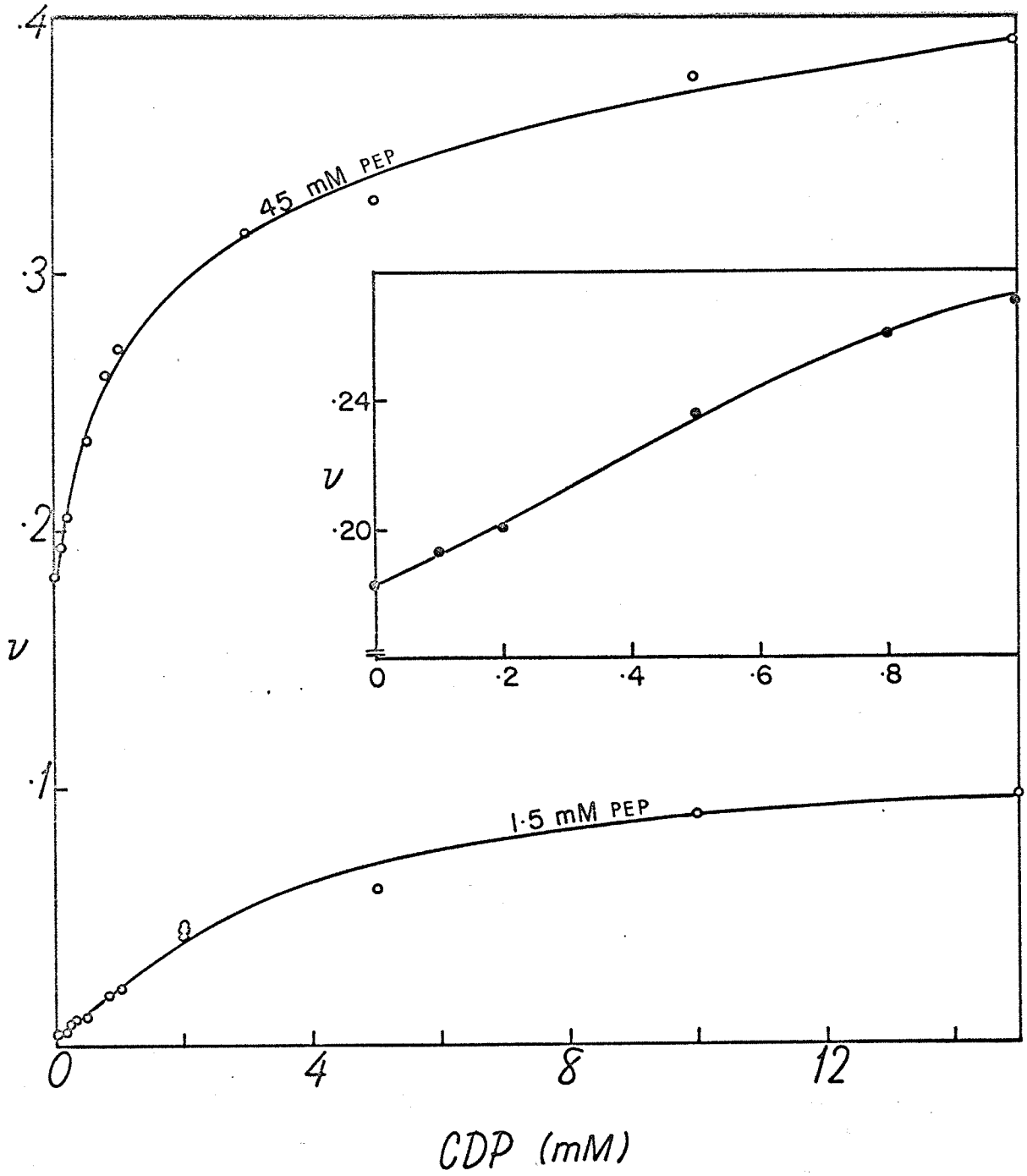
TABLE 6

THE KINETIC PARAMETERS ASSOCIATED WITH THE NUCLEOTIDE ACTIVATORS OF PEP CARBOXYLASE

Activator	$K_{activation}$	V_{max}
CDP	1.3	.275
GTP	1.2	.143
CMP	8.3	.220
CTP	1.2	.066
DCTP	1.4	.065
GDP	42.0	.120

Rate curves for PEP carboxylase with PEP at a concentration of 1.66 mM were constructed in the presence of varied concentrations of the activators listed above. $K_{activation}$ refers to the concentration of activator required to produce half-maximal activation under these conditions, and V_{max} is the maximum velocity at which reaction proceeded at saturating activator concentrations. The assay were done spectrophotometrically ("Methods") and the velocities are given as the change in optical density units at 340 mμ per minute.

FIGURE 21. Activation of PEP carboxylase by varied concentrations of CDP in the presence of 45 mM PEP (upper curve) and 1.5 mM PEP (lower curve). Assays were made spectrophotometrically ("Methods"). The inset shows the upper curve in an expanded form at low CDP concentrations.


.4
.3
.2
.1
v
45 mM PEP
1·5 mM PEP
.24
.20
v
0
·2
·4
·6
·8
0
4
8
12
CDP (mM)

Both GTP and CDP are able to normalize the kinetics of the enzyme with respect to its substrate, PEP. Figure 22 shows that at a concentration of 2.5 mM CDP (approx. 2 x K_a for CDP) the PEP rate plots become hyperbolic. When plotted in the double reciprocal form (Figure 23) the normalization of the kinetics is seen as a conversion of the curved lines in the absence of and low concentrations of CDP to straight lines at higher concentrations. Similar curves for GTP were constructed but are not shown. The nucleotide activators exhibit the same characteristics as acetyl CoA (see previous section).

Interaction Between Activators:

GTP and Acetyl CoA: Since acetyl CoA and the nucleotides, GTP and CDP, affect the rate concentration plot for PEP in the same manner, i.e., to normalize the plot, it was of interest to see whether the binding site for these activators were identical. The experiment outlines in Figure 24 argues against identical sites. In this experiment GTP was varied against several fixed concentrations of acetyl CoA in the presence of non-saturating amounts of PEP (0.83 mM). As shown in Figure 24 at each level of acetyl CoA and GTP together, the velocity is more than the sums of the velocities obtained in the presence of each activator alone. Furthermore, the saturation curve for GTP which was sigmoid at lower concentrations of acetyl CoA became hyperbolic when the acetyl CoA concentration became saturating. Under these conditions the K_a for acetyl CoA was 0.5 mM, and for saturating conditions a value 8 x K_a (3.8 mM) was used.

The sigmoid nature of the rate-concentration curve may be due to GTP binding at a distinct nucleotide activation site causing the

FIGURE 22. Rate curves of PEP carboxylase with PEP as variable substrate in the presence of different fixed concentrations of CDP (as indicated on curves). Assays were done spectrophotometrically with a constant amount of enzyme.

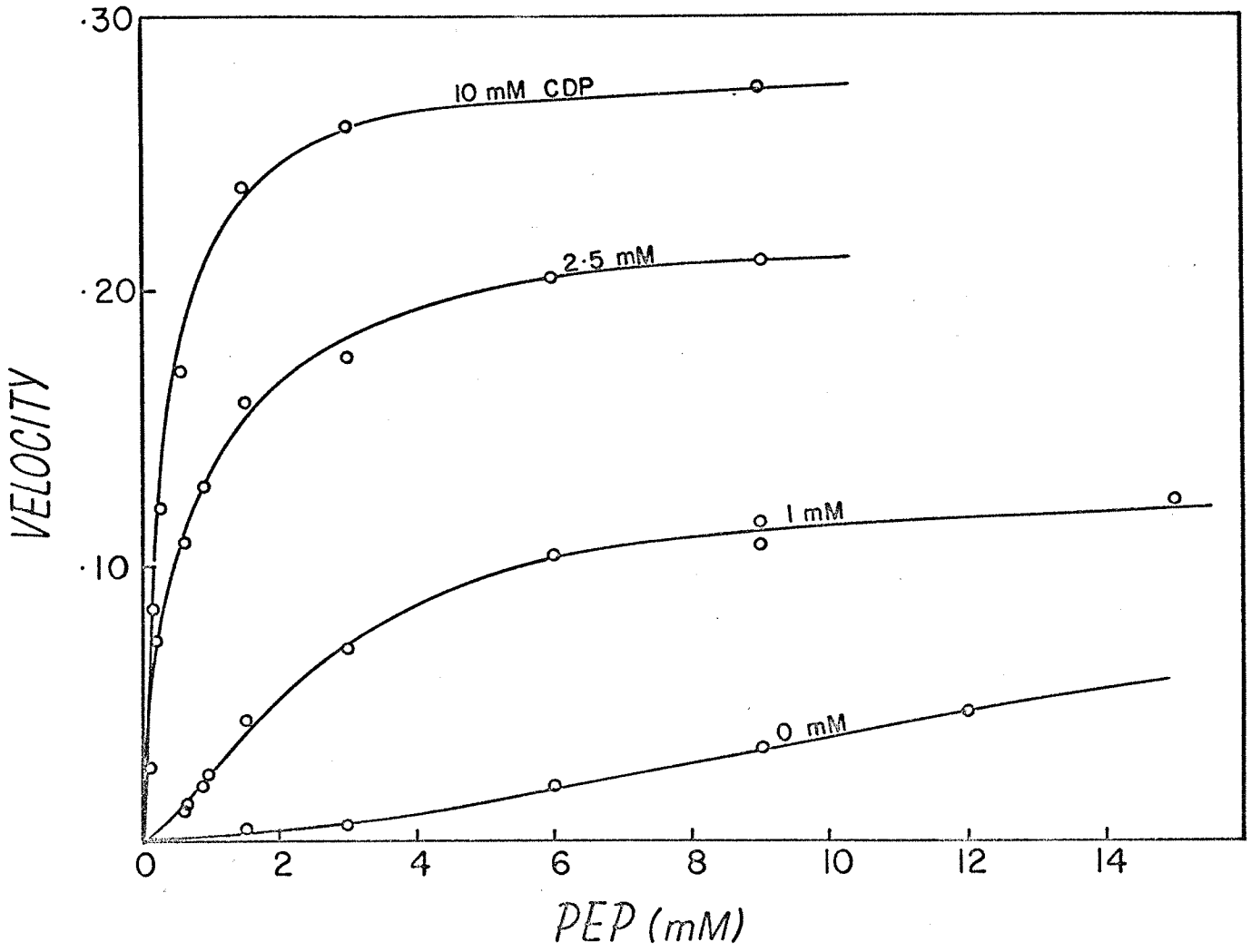

·30
·20
·10
VELOCITY
10 mM CDP
2·5 mM
1 mM
0 mM
0
2
4
6
8
10
12
14
PEP (mM)

FIGURE 23. The data of Figure 22 plotted in the double reciprocal form.

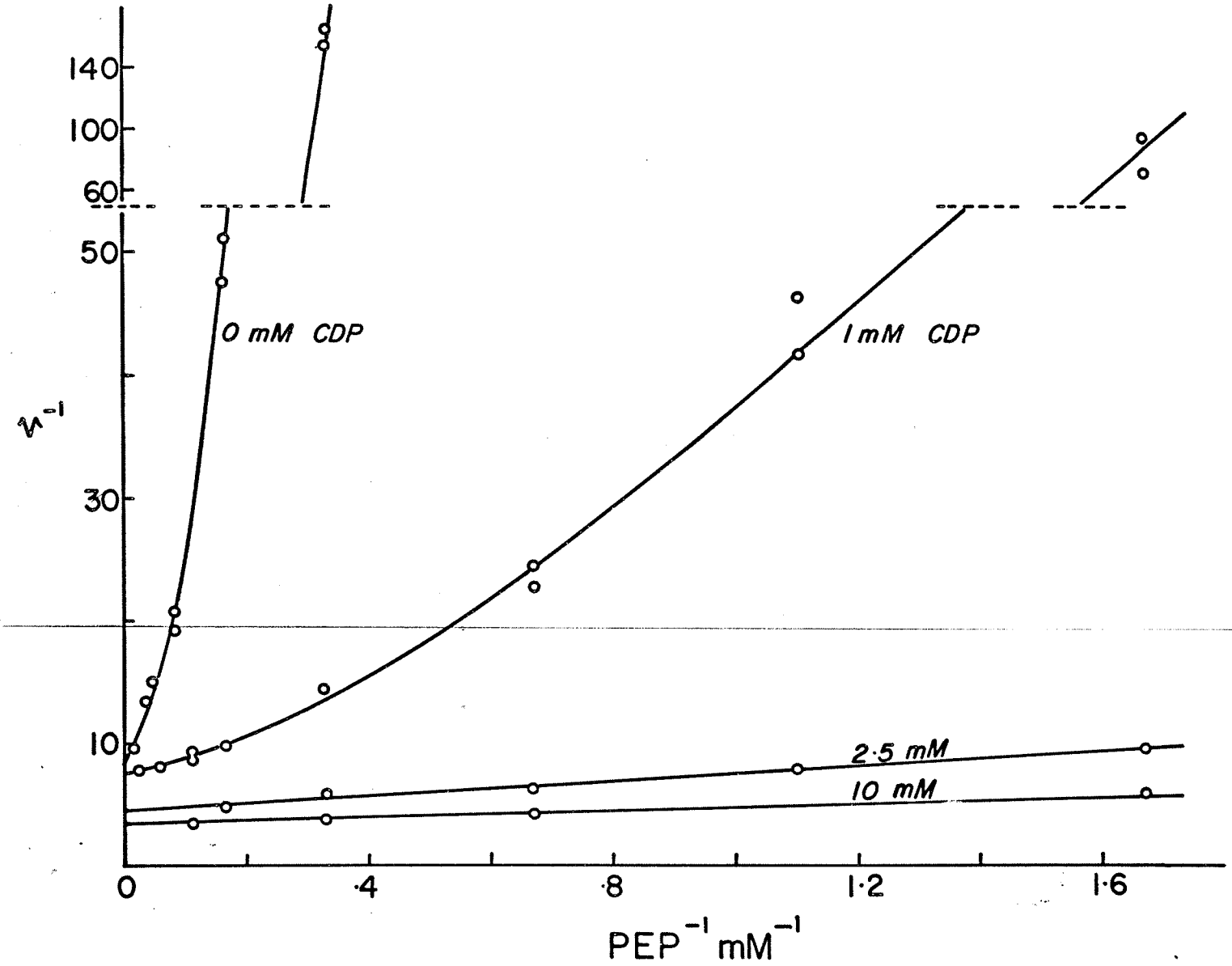
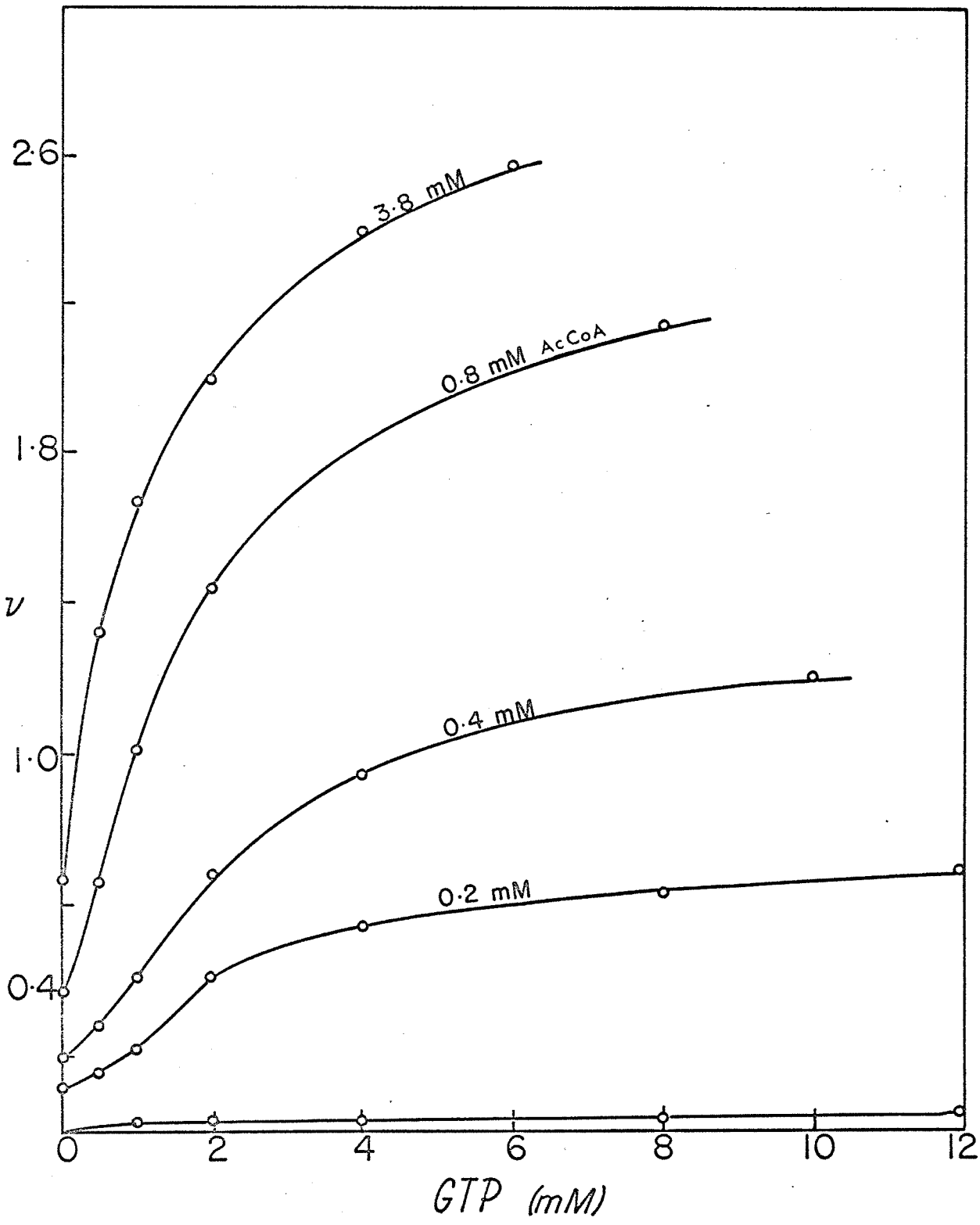

140
100
60
50
30
10
v^{-1}
0 mM CDP
1 mM CDP
2·5 mM
10 mM
0
·4
·8
1·2
1·6
$PEP^{-1}\ mM^{-1}$

FIGURE 24. The effect of GTP on the velocity of the PEP carboxylase reaction in the presence of several fixed concentrations of acetyl CoA. PEP concentration was held constant at 0.83 mM. The numbers above the lines represent the concentrations of acetyl CoA. The assays were performed spectrophotometrically ("Methods") with 6 enzyme units.


2·6
1·8
1·0
0·4
v
3·8 mM
0·8 mM AcCoA
0·4 mM
0·2 mM
0
2
4
6
8
10
12
GTP (mM)

development of alternate reaction pathways. The same type of curve can also be obtained if there is cooperativity in the binding of GTP between two identical GTP sites.

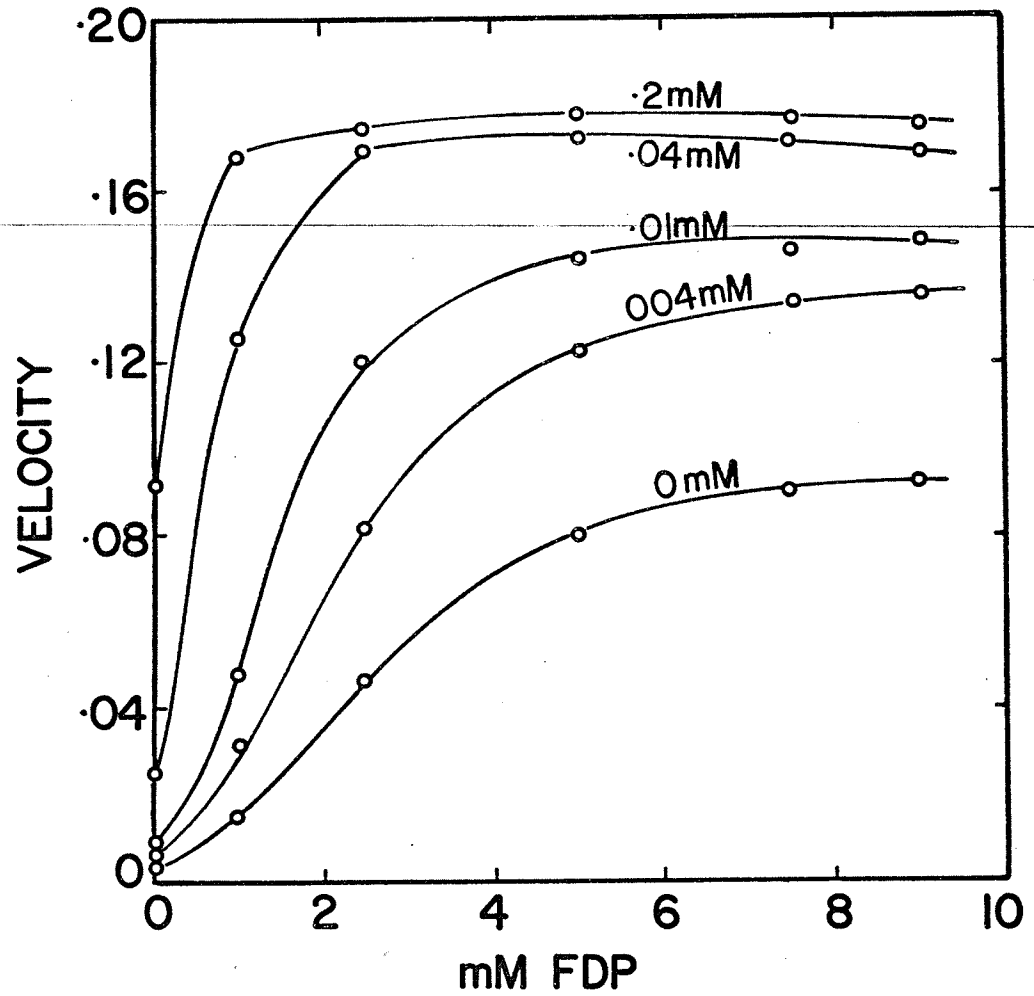
Also in the presence of high concentrations of acetyl CoA the K_m for GTP becomes 0.5 mM. Under identical conditions, i.e., 0.83 mM PEP, 10.0 mM HCO_3^- and 10.0 mM $MgCl_2$, the K_a for GTP is 5.0 mM when acetyl CoA is absent. Thus, in the presence of acetyl CoA, the K_a for GTP was decreased ten-fold.

Although the data are not shown, similar results were obtained when this experiment was performed with CDP instead of GTP. The K_a for CDP in the presence of 3.8 mM acetyl CoA was about 0.35 mM, a decrease of ten-fold compared to the K_a calculated under identical conditions without acetyl CoA.

FDP and Acetyl CoA: The interaction between the activators FDP and acetyl CoA was studied in the same manner (Figure 25). At saturating concentrations of HCO_3^- and 1.0 mM PEP (0.1 x K_m), 2.5 mM FDP causes half-maximal activation. In the presence of 0.2 mM acetyl CoA, the concentration of FDP required to give half-maximal activation was reduced to 0.5 mM. Also similar to the GTP-acetyl CoA interaction was that the sigmoidal saturation curve of FDP becomes hyperbolic in the presence of acetyl CoA.

These curves indicate that the site for the two effectors, FDP and acetyl CoA are distinct since the velocities in the presence of both effectors together is greater than the sum of velocities in the presence of the effectors individually. As an example, the velocity of the reaction in the presence separately of 0.04 mM acetyl CoA and 1.5 mM FDP is 0.025 but changes to 0.15 in the presence of activators together.

FIGURE 25. The effect of FDP on the velocity of the PEP carboxylase reaction in the presence of several fixed concentrations of acetyl CoA. The numbers above the lines represent the concentrations of acetyl CoA. Assays were done spectrophotometrically with a constant amount of enzyme.


·20
·16
·12
·08
·04
0
VELOCITY
·2mM
·04mM
·01mM
004mM
0mM
0
2
4
6
8
10
mM FDP

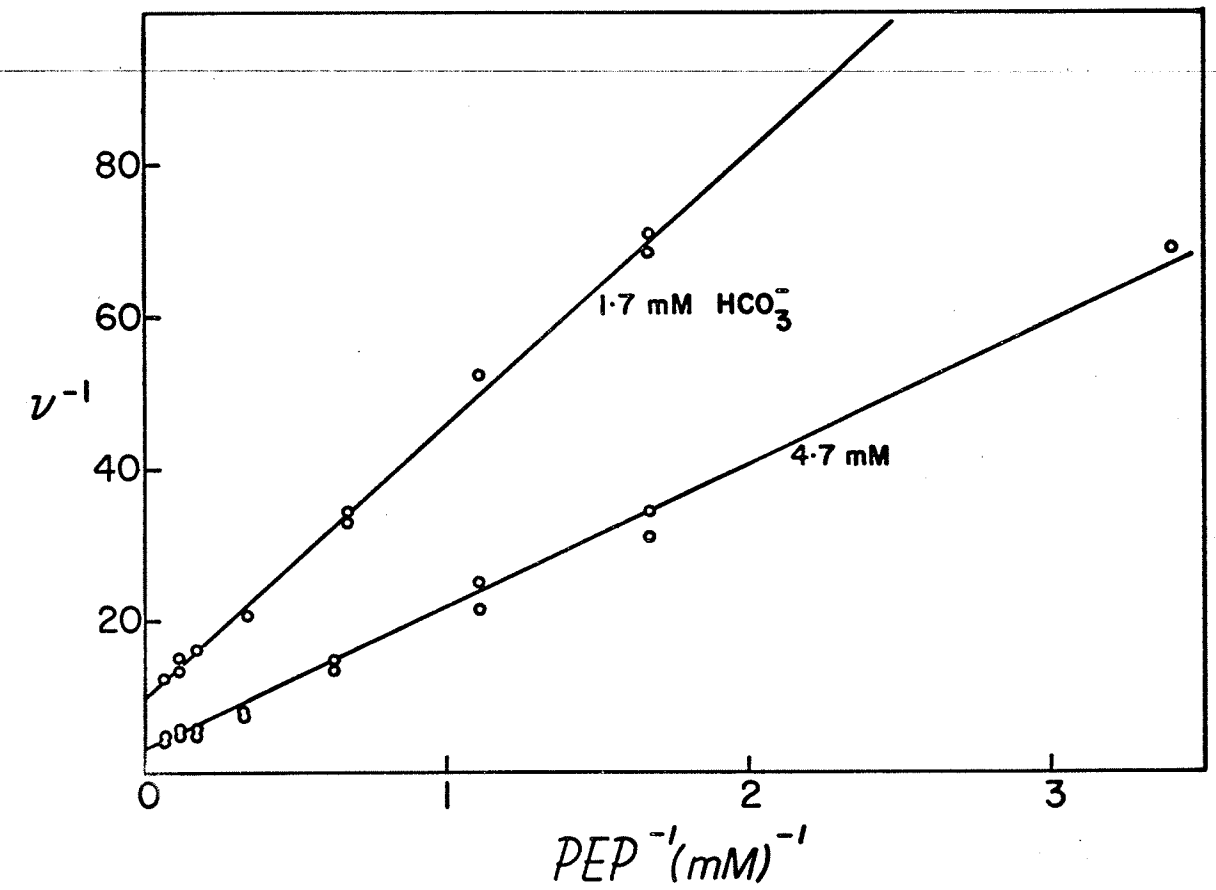
If the result of Figure 25 is plotted with acetyl CoA as the variable activator (not shown), it was seen that the K_a value for acetyl CoA was about 0.1 mM in the absence of FDP, but changes to less than 0.01 mM in the presence of 2.5 mM FDP.

Kinetics of the Activated System: Because of the marked deviation from the normal Michaelis-Menten kinetics it was difficult to evaluate a mechanism for the enzyme by kinetics alone. As noted previously, the activators acetyl CoA, GTP and CDP in saturating concentrations are able to normalize the rate-concentration curves for PEP. Thus, a kinetic analysis in the presence of saturating CDP was attempted to elucidate the nature of enzyme action under conditions of "normality".

In the experiments reported below, CDP was employed in all assays at a concentration of 10.0 mM, a value approximately 10 x K_a. The K_m for PEP at this concentration of CDP is approximately 1.5 mM although this was dependent to some extent on the age of the enzyme. The first experiment shows the effect of fixed concentrations of HCO_3^- (1.7 mM and 4.7 mM), on the rate-concentration curve for PEP. The data are plotted in the double reciprocal form (Figure 26). As seen from this graph, the curves are linear and that the slopes and intercepts of the lines are different. Higher concentrations of HCO_3^- could not be employed since, as previously noted, inhibition by excess of this substrate occurs.

Product Inhibition by Oxalacetate: In the presence of saturating CDP (10.0 mM), and varied PEP concentrations, velocities were measured at several fixed concentrations of oxalacetate, a product of the reaction.

FIGURE 26. Double reciprocal plots of velocity versus PEP concentrations in the presence of different fixed levels of bicarbonate (as indicated on curves) and with saturating concentrations of CDP (10.0 mM or approx. 10 x $K_{(CDP)}$).


80
60
40
20
v^{-1}
1·7 mM HCO_3^-
4·7 mM
0
1
2
3
$PEP^{-1}(mM)^{-1}$

Assay 1 (see "Methods") was used to measure reaction rates. The results shown in Figure 27 indicated that the inhibition by this product was non-competitive.

Phosphate could not be used as a product inhibitor since turbidity due to precipitation occurred in the assay mixture. The reciprocal experiments with HCO_3^- as the variable substrate were omitted since, as stated earlier, it is difficult to control its concentration at the pH of the assay (pH 9.0).

Inhibition by Aspartate: It was of interest to see how aspartate would inhibit the enzyme in the presence of saturating CDP. Using the spectrophotometric assay system with 10.0 mM CDP (constant) and PEP as variable substrate, velocities were measured at several fixed concentrations of aspartate. The results are shown in Figure 28 in the double reciprocal form.

The graph shows that aspartate inhibits with PEP in a competitive manner. When aspartate was included in the assay, the plots became non-linear; the curvature increasing as the concentration as aspartate was increased. When plotted in the log-log form the curves are all linear although the slopes of the lines increases from 1.07 in the absence of aspartate to 1.26 in the presence of 0.8 mM aspartate.

Interaction With Macroions: During purification of PEP carboxylase, it was noted that protamine sulfate activated the enzyme. In order to find whether other polycations were capable of activating the enzyme, a number of compounds listed in Table 7 were tested for their effect on enzyme activity.

FIGURE 27. Double reciprocal plot showing product inhibition of PEP carboxylase by oxalacetate in the presence of saturating CDP. The radioactive C^{14}-bicarbonate assay ("Methods") was used with 10 enzyme units per assay. Numbers above the lines refer to the concentrations of oxalacetate used in the assays.

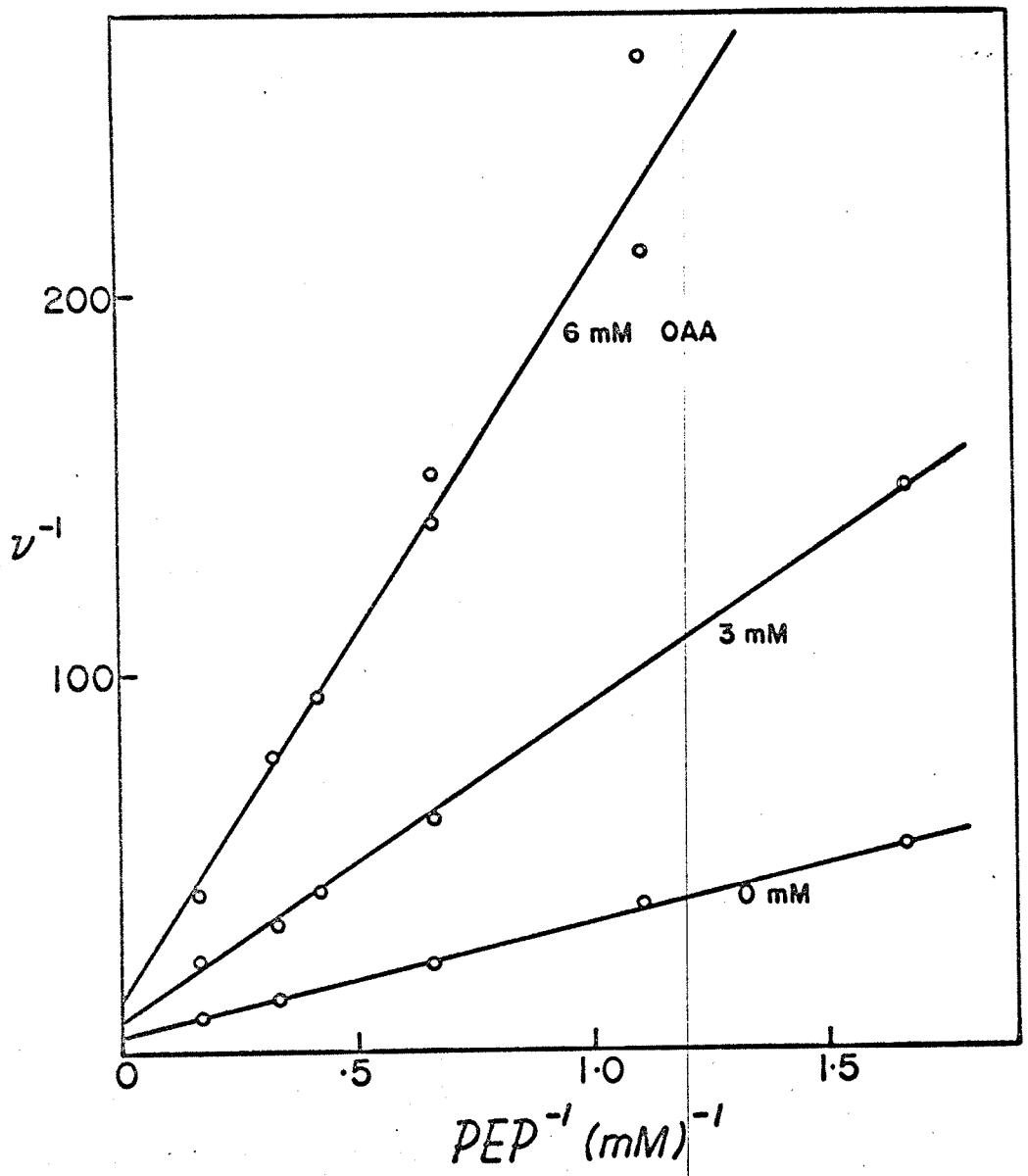

200
100
v^{-1}
6 mM OAA
3 mM
0 mM
0
·5
1·0
1·5
PEP^{-1} $(mM)^{-1}$

FIGURE 28. Double reciprocal plot of velocity versus PEP concentration showing the inhibition of PEP carboxylase by different fixed levels of aspartate (as indicated by numbers above the lines) in the presence of saturating amounts of CDP. The spectrophotometric assay ("Methods") was used with 10 enzyme units for each assay.

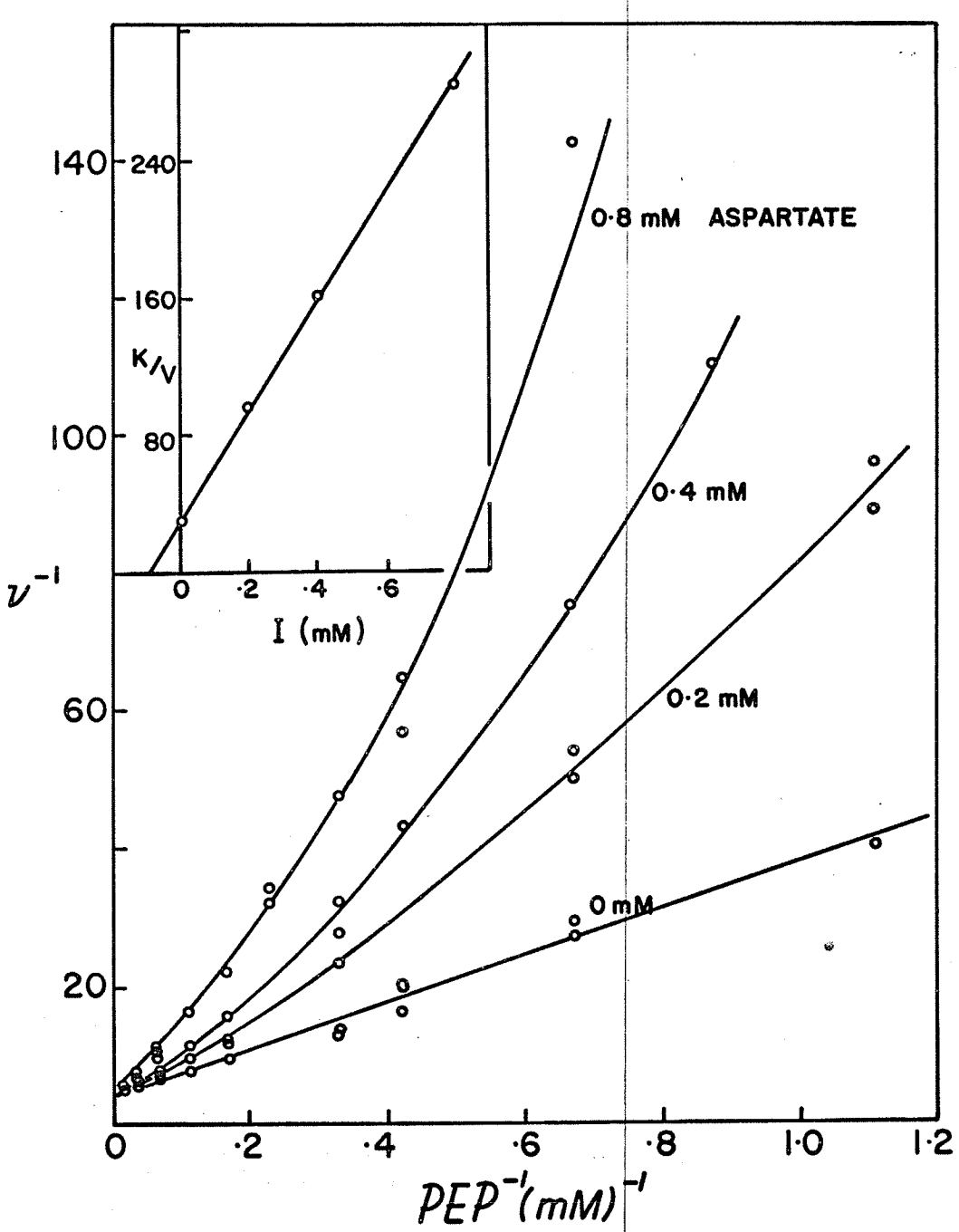

140
100
60
20
v^{-1}
240
160
K/V
80
0
·2
·4
·6
I (mM)
0·8 mM ASPARTATE
0·4 mM
0·2 mM
0 mM
0
·2
·4
·6
·8
1·0
1·2
$PEP^{-1}(mM)^{-1}$

TABLE 7

THE EFFECT OF VARIOUS COMPOUNDS ON THE ACTIVITY OF PEP CARBOXYLASE

Addition	Concentration	Velocity*
None	--	.036
Histone	13 µg/ml	.038
	40 µg/ml	.068
Protamine	30 µg/ml	.039
	100 µg/ml	.060
Poly-L-lysine	1 µg/ml	.248
Polyglucose sulfate	150 µg/ml	.050
	750 µg/ml	.152
Yeast RNA	1000 µg/ml	.037
Spermidine	3.3 mM	.072
L-lysine	5.0 mM	.036
Sodium sulfate	.03 M	.052
Sodium chloride	.03 M	.036

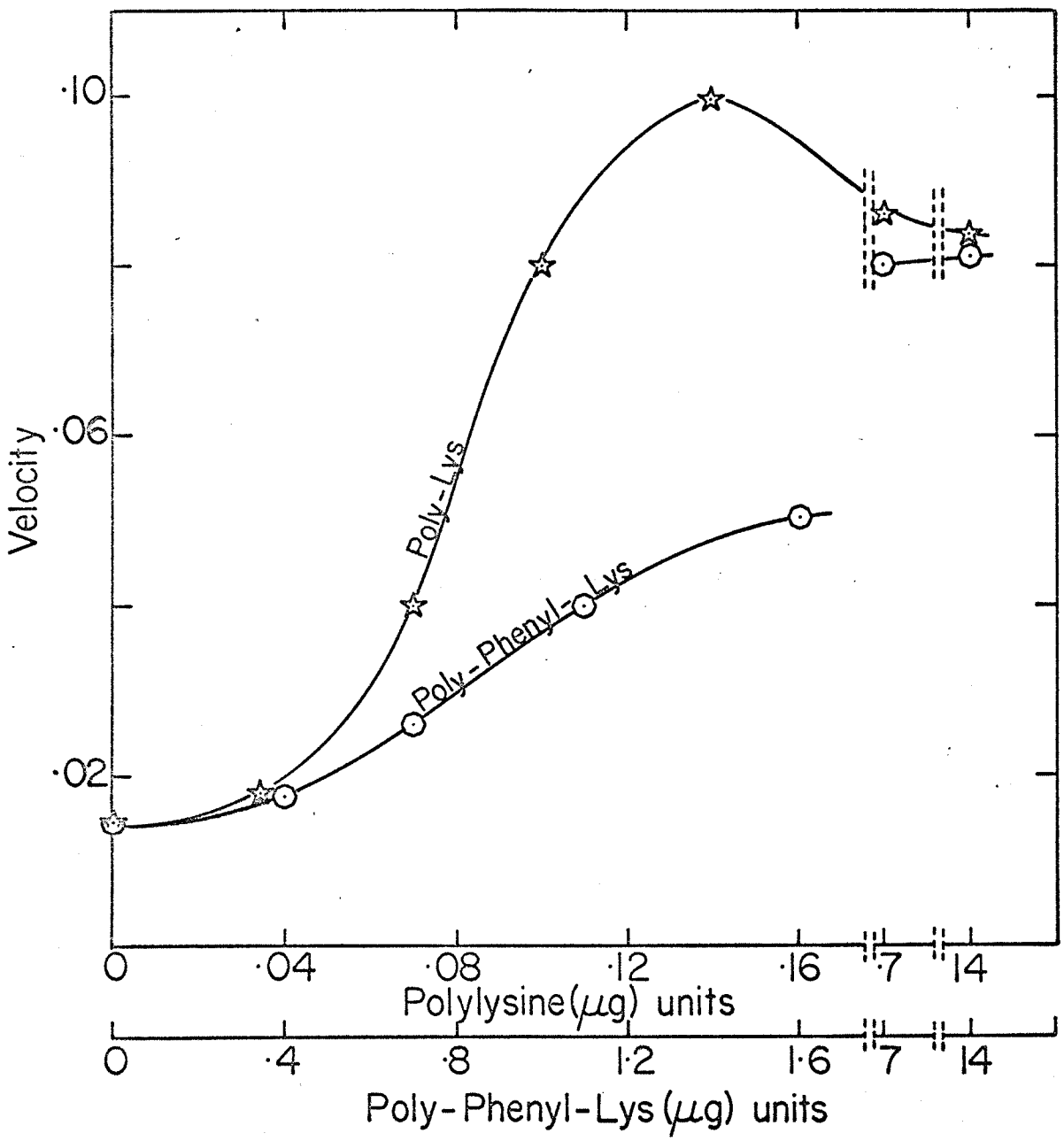
*The standard spectrophotometric assay ("Methods") was used with 1.66 mM PEP and various additives as indicated with 100 enzyme units. Velocity was measured as the change in optical density at 340 mµ per minute.

As shown in the table, basic proteins and spermidine activated the enzyme, the most potent activators being polylysine. One of the polyanions, polyglucose sulfate was able to activate the enzyme when used in high concentrations. Since inorganic sulfate also activates the enzyme, it is not certain whether polyglucose sulfate activation was connected with its polymeric structure or was just due to its sulfate content. In contrast to sulfate, monomeric L-lysine was incapable of activating the enzyme.

Effect of Binding Between PEP polylysine and PEP Carboxylase: Using the standard spectophotometric assay with 1.66 mM PEP, the effect of increasing polylysine concentrations on the velocity of the reaction was studied. As shown in Figure 29, the system became saturated at a polylysine concentration of 0.65 µg/ml. Concentrations higher than this led to a slight decrease in activation. A copolymer, copolyphenylalanyllysine, was able to activate the enzyme, but at concentration ranges many fold higher than polylysine (Figure 29). Thus, to bring about 5.7 fold increase in activity the required concentration of polylysine and the copolymer were 0.5 µg/ml and 36.0 µg/ml, respectively.

Since introduction of nonpolar groups in the polycationic molecule led to a decrease in its properties as an activator, it seemed that the binding between polylysine and PEP carboxylase which produced activation is electrostatic in nature. To pursue this line of study further, the extent of polylysine activation as a function of pH and ionic strength was studied.

FIGURE 29. The effect of polylysine and copolyphenylalanyllysine on the activity of PEP carboxylase. The standard spectrophotometric assay system ("Methods") with 1.66 mM PEP and 15 enzyme units was used.


·10
·06
·02
Velocity
Poly-Lys
Poly-Phenyl-Lys
0
·04
·08
·12
·16
·7
14
Polylysine(μg) units
0
·4
·8
1·2
1·6
7
14
Poly-Phenyl-Lys (μg) units

Keeping the ionic strength constant at 0.084 (0.1 M Tris-HCl), velocities of reactions were measured in an assay mixture containing 1.66 mM PEP and 1 µg/ml polylysine were measured at various pH. The results shown in Figure 30 show that as the pH was decreased the extent of activation was increased. Thus, at pH 9.0 polylysine activated the enzyme seven-fold, but at pH 7.0 this value changed to about 23-fold. As a contrast to the macroionic activation the extent of activation by FDP remained unchanged at all pH values (Figure 30). Other effectors such as acetyl CoA, CDP and aspartate were similar to FDP in this respect in that the extent of activation or inhibition showed no dependence on pH.

The effect of ionic strength was studied. Keeping the pH constant at 9.0, the velocities were measured in a standard assay with 1.66 mM PEP both in the presence of 0.65 µg/ml polylysine, and at various ionic strengths of Tris-HCl. The results summarized in Figure 31 show that the extent of activation decreased as the ionic strength was increased. The activity of the enzyme itself did not change in the absence of polylysine when the ionic strength of the assay medium was varied between 0.1 and 0.5.

Nature of the Binding: As shown previously, the molecular weight of PEP carboxylase was 193,000 as determined by sucrose gradient centrifugation. Similar experiments were conducted in which a mixture of PEP carboxylase and polylysine (2.6 µg/ml) were layered on top of a sucrose gradient for centrifugation. Pig heart malate dehydrogenase was used as a marker and centrifugation was performed as described in "Methods". Under such conditions PEP carboxylase was found as a pellet

FIGURE 30. The effect of pH on the activation of PEP carboxylase by polylysine and FDP. The standard spectrophotometric assay ("Methods") with 1.66 mM PEP was used. V_a and V_o refer to the activities in the presence and absence of activator.

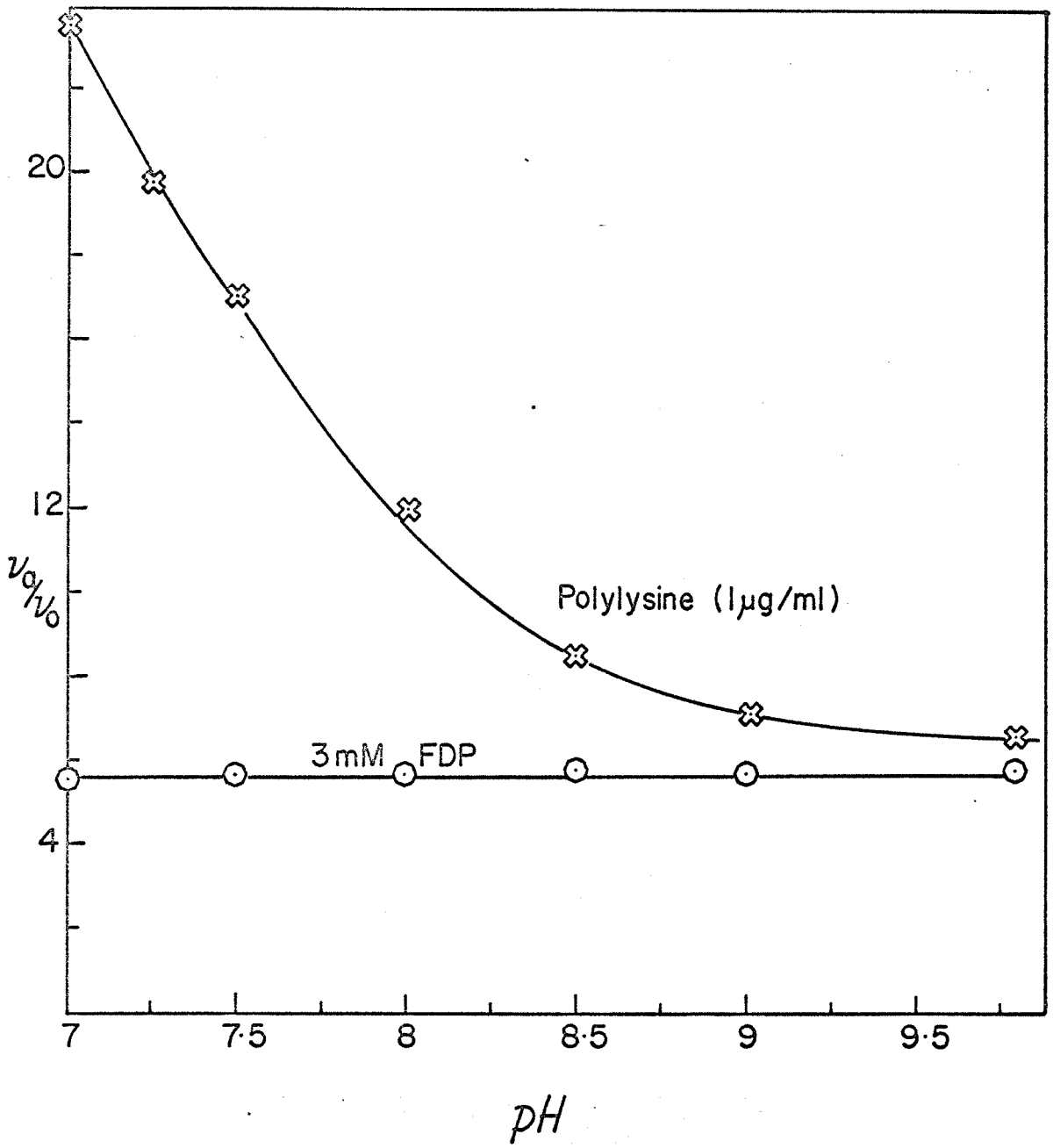
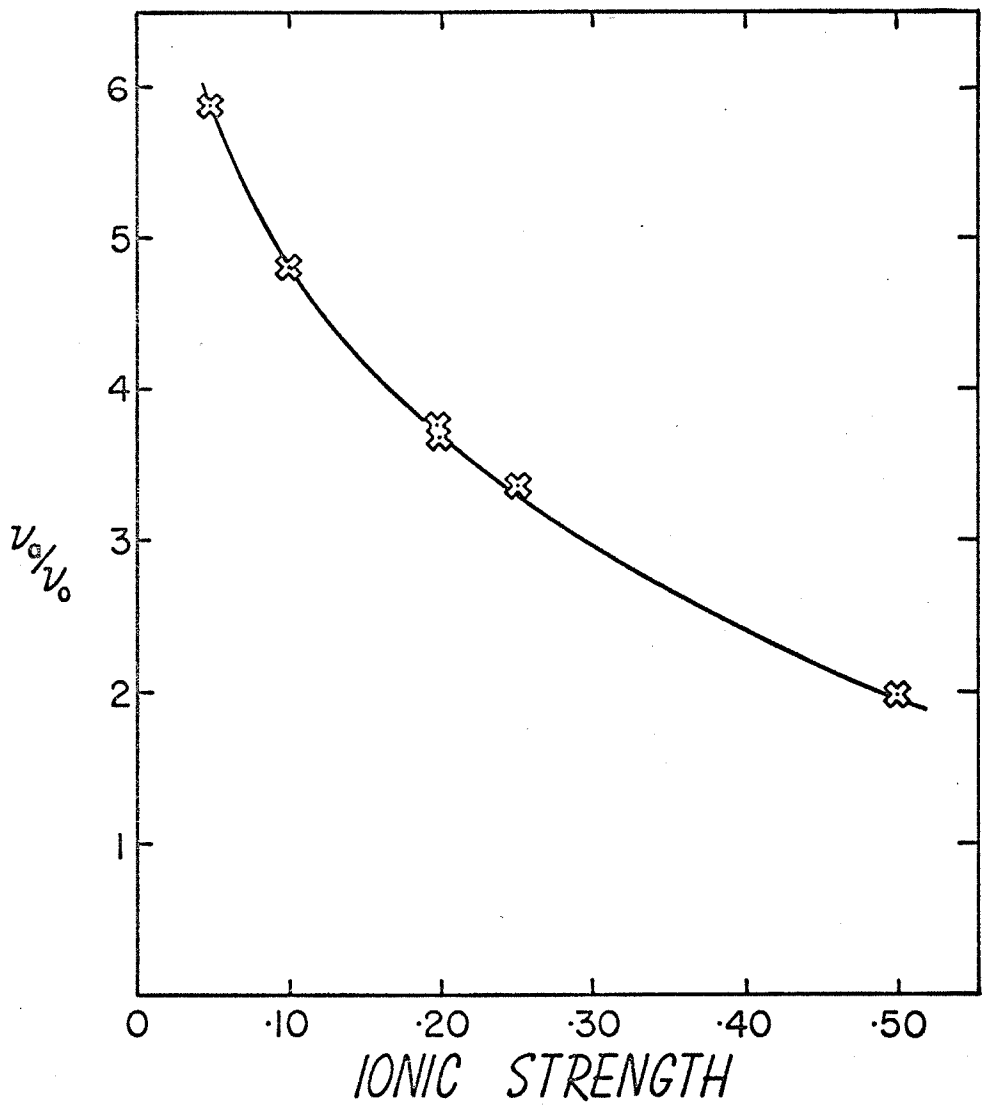

20
12
4
v_o/v_o
Polylysine (1µg/ml)
3mM FDP
7
7·5
8
8·5
9
9·5
pH

FIGURE 31. The effect of ionic strength of the assay medium on the extent of activation of PEP carboxylase by polylysine. Tris-HCl of different ionic strengths at pH 9.0 was used. Each assay contained 0.65 µg/ml polylysine, 1.66 mM PEP and 14 enzyme units in the standard spectrophotometric assay mixture ("Methods"). V_a and V_o refer to the velocities in the presence and absence of polylysine.


6
5
4
3
2
1
v_a/v_o
0
·10
·20
·30
·40
·50
IONIC STRENGTH

at the bottom of the centrifuge tube, with malate dehydrogenase remaining at the expected position. Although this indicated the formation of large aggregates of polylysine-enzyme, it should be mentioned that in assay mixtures with polylysine no turbidity changes could be detected.

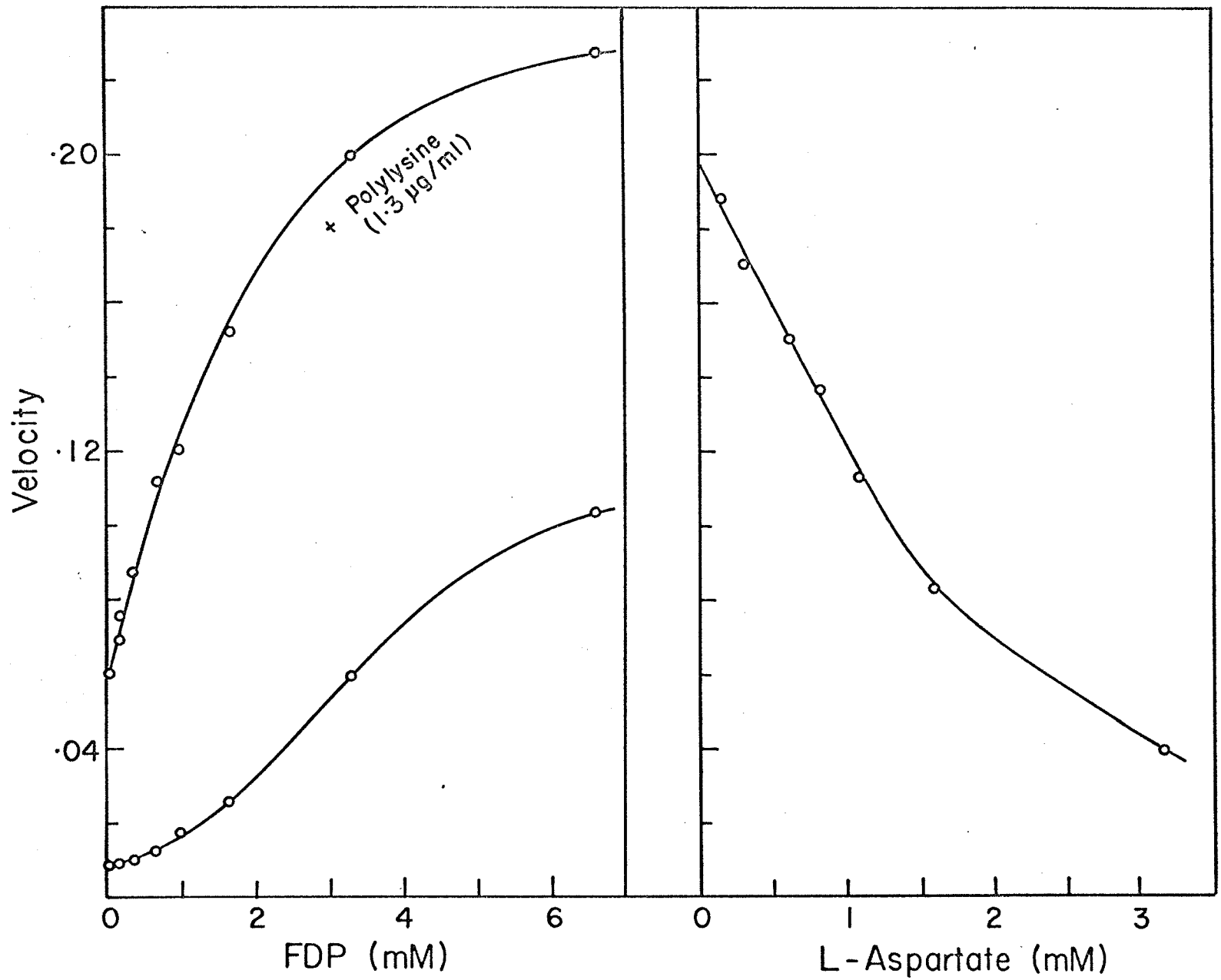
When similar experiments were carried out in the presence of other modifiers such as acetyl CoA (1.0 mM), FDP (12.0 mM) and aspartate (2.0 mM), the molecular weight was the same as when run without these modifiers. In these centrifugations the sucrose solutions also contained the effectors. These results indicate that aggregation was not necessarily a prerequisite for, or a consequence of activation or inhibition.

Effect of Polylysine on Kinetics: Polylysine has a marked effect on the affinity of PEP for its binding site. The K_m value of about 10.0 mM for PEP in the absence of polylysine changes to 1.5 mM in the presence of saturating (1.3 µg/ml) polylysine. Also in its presence, the rate-concentration curve for PEP becomes normalized and appears to follow the Michaelis-Menten type kinetics. In this respect the effect of polylysine on enzyme activity resembles the previously mentioned activators acetyl CoA and the nucleotides.

To see whether polylysine altered the enzyme's sensitivity to its allosteric effectors, velocities of assay mixtures containing both polylysine and effector were measured. Using the standard assay system with 1.66 mM PEP, reaction rates were measured at varied FDP concentrations both in the presence and absence of 1.3 µg/ml polylysine (Figure 32). The results show that the enzyme is still sensitive to activation

FIGURE 32a. (Left) The effect of FDP alone and in combination with polylysine on the activity of PEP carboxylase. PEP concentration was 1.66 mM and 7 enzyme units were used in the spectrophotometric assays ("Methods").

FIGURE 32b. (Right) The inhibition of PEP carboxylase activity by L-aspartate in the presence of 1.3 µg/ml polylysine. Twenty enzyme units and 1.66 mM PEP were used in the spectrophotometric assay mixture ("Methods").


Velocity
·20
·12
·04
+ Polylysine (1·3 µg/ml)
0
2
4
6
FDP (mM)
0
1
2
3
L-Aspartate (mM)

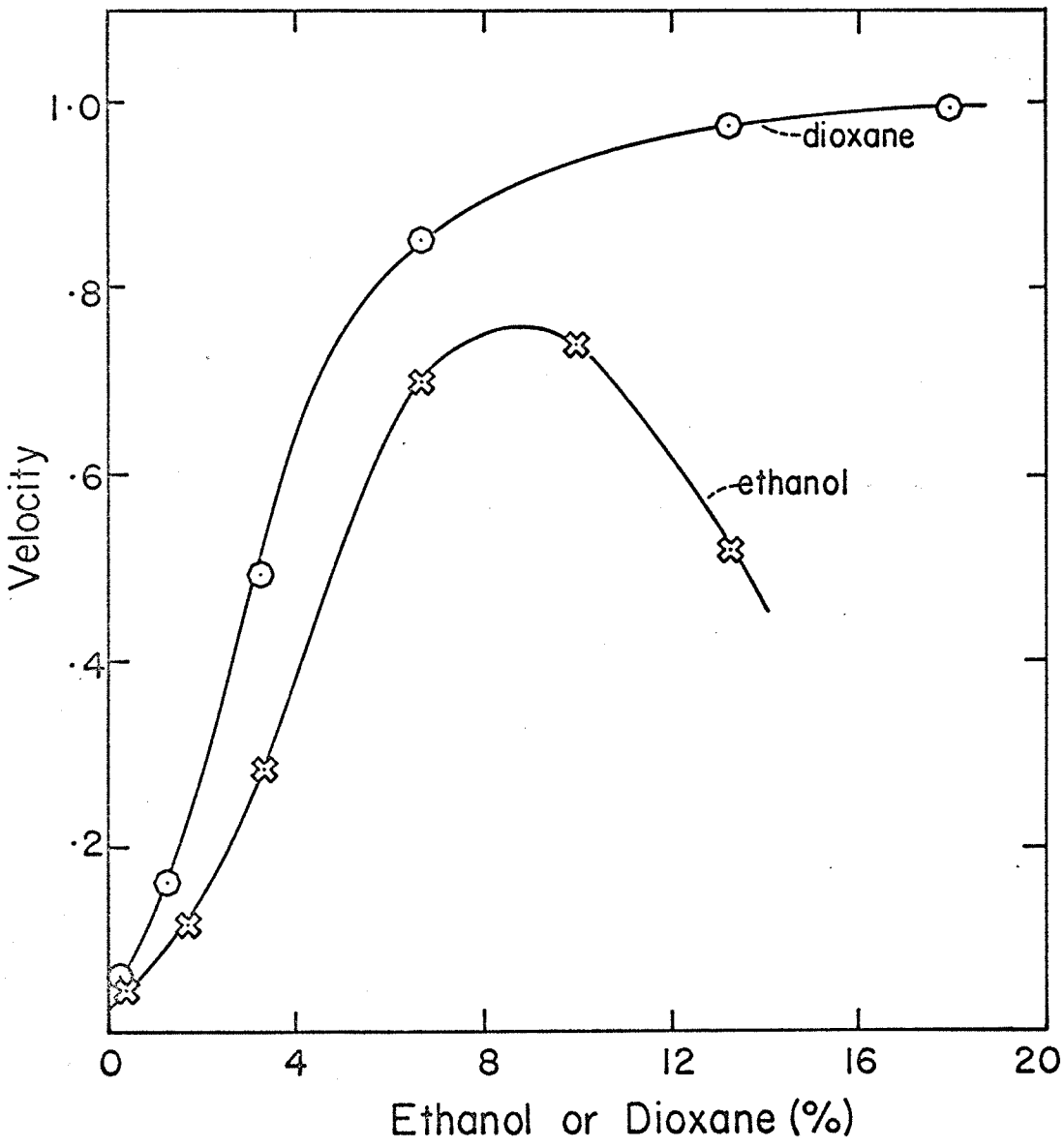
by FDP. Similar experiments with aspartate replacing FDP (Figure 32) showed aspartate was still able to bring about inhibition of the enzyme. It appeared then that the polylysine did not desensitize the enzyme to its activator or inhibitor.

Heat Protection by Polylysine: To test whether other than kinetic parameters were altered by binding of polylysine to the enzyme, heat inactivation studies were conducted. In one experiment the enzyme lost only 23% of its activity at 48° in three hours but in the presence of 2 µg/ml polylysine, no loss of activity occurred. The effectors aspartate (2.5 mM) and FDP (10.0 mM) failed to give any significant protection under similar conditions.

Effect of Ethanol and Dioxane: In the course of the work, the effect of a medium of low dielectric constant on the extent of polylysine activation was attempted. However, in such experiments it was found that ethanol or dioxane alone produced dramatic increases in enzyme activity.

Using assay 2 (see "Methods") with 1.66 mM PEP, the effect of various concentrations of ethanol and dioxane on the reaction velocity was studied. As shown in Figure 33, increasing concentrations of these reagents led to increased enzyme activity. At a concentration of 6% (v/v) ethanol or dioxane, the enzyme activity increased 35 fold with dioxane and 28 fold with ethanol. The degree of activation by ethanol was lower than that obtained with dioxane. Also, the degree of activation was considerably decreased at concentrations of ethanol higher than 10% (v/v).

FIGURE 33. The activation of PEP carboxylase by dioxane and ethanol. The standard assay mixture ("Methods") contained 1.66 mM PEP and 22 enzyme units in each cuvette. The enzyme and activators were added to the enzyme simultaneously.


1·0
·8
·6
·4
·2
Velocity
dioxane
ethanol
0
4
8
12
16
20
Ethanol or Dioxane (%)

Other reagents that lower the dielectric constant were tested for their effect on the reaction velocities. At a concentration of 10% (v/v), 2-propanol, dimethyl sulfoxide, and propylene glycol activated the enzyme 21, 22 and 25 fold, respectively. None of the reagents tested had any effect on malate dehydrogenase which was the other enzymic component in the assay mixture. Control experiments indicated that NADH was not oxidized in the assay mixtures in the presence of these reagents.

The effect of dioxane is irreversible in that prolonged exposure of the enzyme to this compound led to inactivation. It was found that high concentrations (50 mM) 2-mercaptoethanol could protect the enzyme from this inactivation for periods up to 20 hours. The inactivation by dioxane was a time dependent process and for the first ten minutes there was no loss of activity. Thus, the initial velocity data reported here are valid.

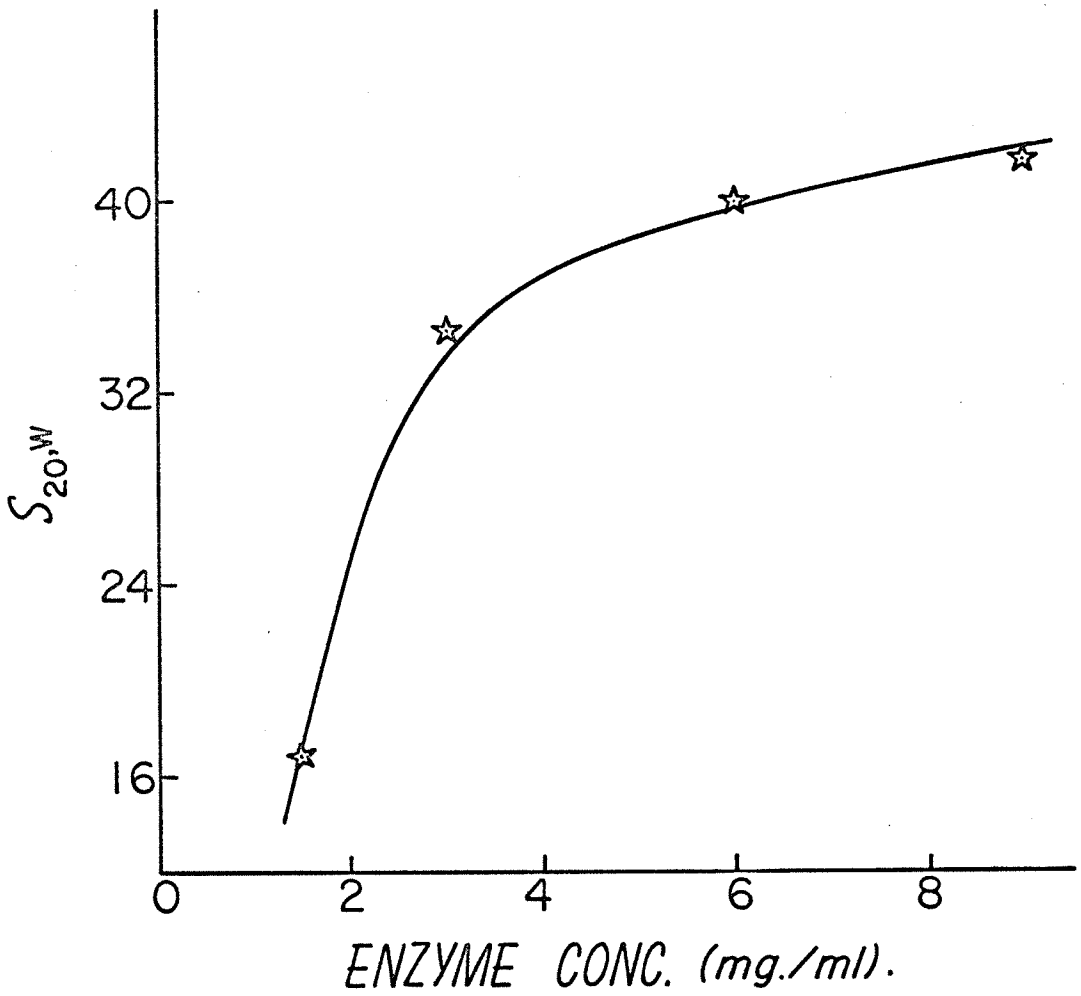
Since dioxane is a known dissociating agent for proteins (Churchich and Wold, 1963; Kauzmann, 1959), it was of interest to see whether activation of the enzyme was brought about by dissociation of the enzyme or other conformational change in structure. Sucrose gradient centrifugations in 6% dioxane in the presence of 50 mM 2-mercaptoethanol were carried out as described in "Methods". No change in sedimentation velocity of PEP carboxylase was detected. The presence of sucrose may have altered the enzyme masking the effect of dioxane so that in sedimentation velcotiy studies no change was visible. However, when assays of the enzyme were conducted in the presence of 10% (w/v) sucrose, dioxane was still found to activate ehe enzyme to the expected degree.

To verify the above results, sedimentation velocity studies were performed on the Spinco Model E analytical untracentrifuge. The runs were made in 0.05 M Tris-HCl, pH 8.0, at 5° at a protein concentration of 5 mg/ml. Only one peak was observed with the schlieren optical system. The sedimentation coefficients $S_{20,w}$ were 53.0 and 56.2 in the absence and presence of 10% (v/v) dioxane. The high sedimentation coefficients found are discussed in a following section. There was no evidence to show, however, that dioxane had any effect on the gross structure of the enzyme.

Sedimentation Velocity Studies: These studies were conducted with the Model E ultracentrifuge according to the procedure described in "Methods". The sedimentation coefficients were variable in that they were dependent upon the concentration of enzyme used in the run and upon the age of the enzyme. At higher protein concentrations the $S_{20,w}$ increased, and as the age of the enzyme preparation increased the sedimentation coefficients also increased. Under such conditions it was difficult to give an absolute value for the sedimentation coefficient.

In one series of ultracentrifugations at 44,000 rpm using one enzyme preparation, the $S_{20,w}$ increased from 16.9 at a concentration of 1.5 mg/ml to 41.6 at 9.0 mg/ml. It did not appear that 41.6 was the limiting value, since older enzyme preparations at a concentration of 5 mg/ml were shown to have a $S_{20,w}$ of 53.0. Nor does $S_{20,w}$ of 16.9 represent the lower limit since relatively fresh enzyme preparations at a concentration of 2 mg/ml have values of 11.8. The results of one series of ultracentrifugations with one protein preparation are shown in Figure 34.

FIGURE 34. The effect of PEP carboxylase concentration on the sedimentation coefficient of the enzyme. Centrifugations were done at 44,000 rpm at 10° with a Spinco Model E ultracentrifuge as described in "Methods".


40
32
24
16
$S_{20,w}$
0
2
4
6
8
ENZYME CONC. (mg./ml).

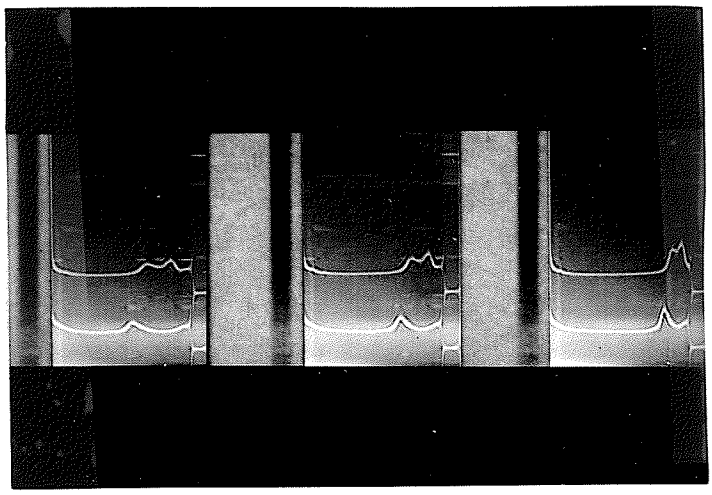
In one experiment, a protein preparation judged to be completely pure by gel electrophoresis showed two peaks in the schlieren pattern (Plate 3). The faster sedimenting peak had an S value of 28.1 and the slower peak one of 12.9. When 2-mercaptoethanol was added to this preparation, one homogeneous peak sedimenting with an S value of 34.7 was observed. Since 2-mercaptoethanol is a known reducing agent and thiol group protectant, attempts were made to investigate the effect of reagents which specifically attack thiol groups. However, when a centrifugation was made in the presence of 5×10^{-5} M parahydroxymercuribenzoate (PMB) the two peaks remained unchanged, sedimenting with S values of 11.8 and 27.6.

The Effect of PMB: Since 2-mercaptoethanol protected the enzyme against dioxane and was involved in reassociating the enzyme, it seemed possible that thiol groups were involved in enzyme structure. Although PMB had no effect on the sedimentation velocity of the enzyme, the possibility that it would affect the kinetic behavior was investigated.

In the standard assay (assay 2), various PMB concentrations were added and velocities measured. The results summarized in Figure 35 show the inhibition of enzyme activity with increasing PMB concentrations. The concentration of PMB required to produce half-maximal inhibition was 0.05 mM PMB. At concentrations 20 x K_i, approximately 8% activity still remained. The residual activity remained after exposures of one hour to this amount of PMB.

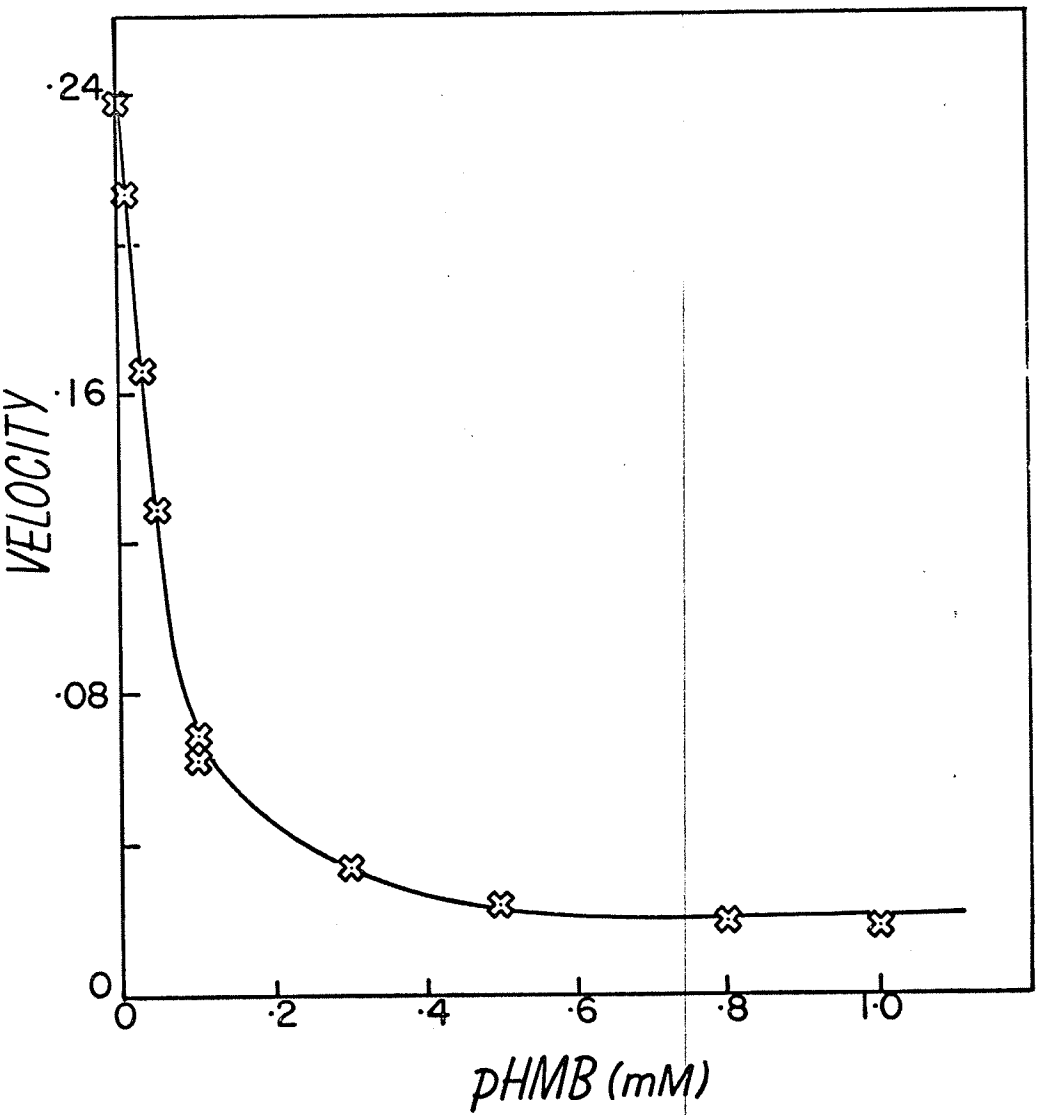
Incubation of the enzyme with 10 x K concentrations of the effectors; malate, aspartate, acetyl CoA, CDP and FDP, and the substrates; HCO_3^-, $MgCl_2$ and PEP did not prevent inactivation of the

PLATE 3



Schlieren pattern of PEP carboxylase during sedimentation velocity runs with Model E ultracentrifuge ("Methods"). The upper curve shows dissociation of the enzyme after storage of enzyme in 0.05 M Tris-HCl (pH 8.0) for 20 hours and the lower curve shows the re-association in the presence of 50 mM mercaptoethanol. Pictures were taken at 8, 16 and 24 minutes (left to right) after rotor had reached 44,000 rpm.

FIGURE 35. Inactivation of PEP carboxylase by different concentrations of PMB. Reaction mixtures contained 5.0 mM PEP, 10 mM magnesium, 10 mM bicarbonate and PMB as indicated. Reactions were started by addition of 240 units of enzyme and were followed spectrophotometrically as described in "Methods".


·24
·16
·08
0
VELOCITY
0
·2
·4
·6
·8
1·0
pHMB (mM)

enzyme by 0.1 mM PMB. Incubated mixtures of enzyme with modifier or substrate in the presence of PMB were tested for activity at 5, 15 and 30 minutes. The activities were compared with that of control mixtures which lacked PMB. The temperature at which these mixtures were incubated was 23°. It should also be stated that although the presence of PMB led to marked decrease in activity, the residual activity was still susceptible to modification by the presence of the effector.

In another set of experiments, the enzyme was incubated with 0.5 mM PMB at 23° for two hours, after which the residual activity was assayed in the presence of malate, aspartate, acetyl CoA, CDP and FDP. It was found that these modifiers were still able to inhibit or activate the enzyme to the expected degree. In these assays the concentration of the effectors was twice their K value. This showed that the enzyme could not be desensitized by PMB under these conditions.

Earlier it was stated that 0.05 mM PMB had no effect on the sedimentation velocity of the enzyme. To see whether other changes could be induced by PMB, the PMB treated enzyme was analyzed by polyacrylamide gel disc electrophoresis. An enzyme preparation was dialyzed overnight against 0.05 M Tris-HCl, pH 8.0 and 1.0 mM EDTA. Then the preparation was dialyzed for three hours against 0.05 M Tris-HCl, pH 8.0 and 0.5 mM PMB. Finally to remove PMB, the enzyme was dialyzed against 0.05 M Tris-HCl, pH 8.0, containing 10 mm 2-mercaptoethanol. Aliquots were taken at each step, that is, after dialysis overnight, after dialysis in PMB and after removal of PMB. The aliquots were assayed for enzyme activity and subjected to gel electrophoresis as described in "Methods".

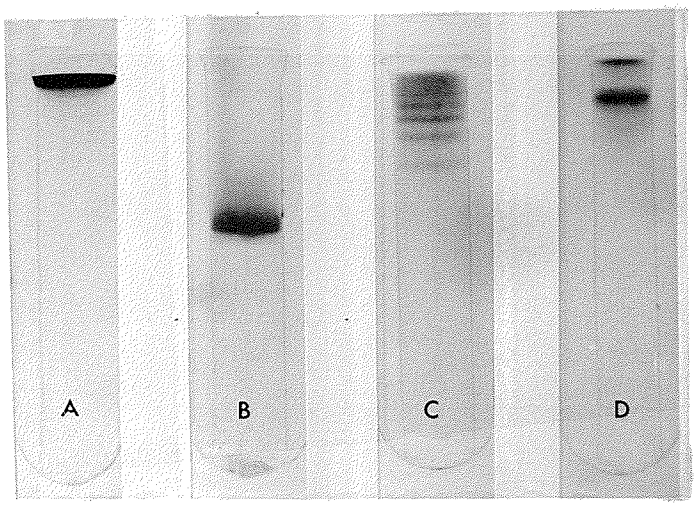
PMB treatment resulted in loss of 80% of the original activity. After dialysis of the PMB treated preparation against the Tris-mercaptoethanol buffer, approximately 85% of the original activity was recovered. By gel electrophoresis, only one major band was visible prior to PMB treatment. After treatment, the major band disappeared and 5-6 fainter bands appear in this region. After redialysis in Tris-mercaptoethanol, one major and possibly two minor, faint bands were seen. The results are shown in Plate 4.

The Effect of Dodecyl Sulfate: At a concentration of 0.1% sodium dodecyl sulfate, the enzyme was completely inactivated. Since the detergent is known to disrupt hydrophobic bonds, it was of interest to see whether the loss of activity due to SDS treatment was related to any gross structural change.

Runs were made in the Model E analytical ultracentrifuge at 60,000 rpm with an enzyme preparation (5 mg/ml) in 0.05 M Tris-HCl, pH 8.0 and 0.1% SDS. When viewed through the schlieren optical system, only one major slowly sedimenting peak was observed. The sedimentation coefficient $S_{20,w}$ was calculated to be 4.1 (Plate 5). This value did not change in the presence of 1.0% SDS.

The effect of SDS treatment was also studied by gel electrophoresis. A sample of the enzyme was treated with 0.1% SDS and applied to the gel as described in "Methods". After staining, only one major band was observed. This band was rather diffuse compared to the untreated sample (see Plate 4), and had migrated in the direction of the anode to a much greater extent than the control.

PLATE 4



Composite picture of PEP carboxylase stained with Coomasie blue after polyacrylamide gel disc electrophoresis ("Methods").

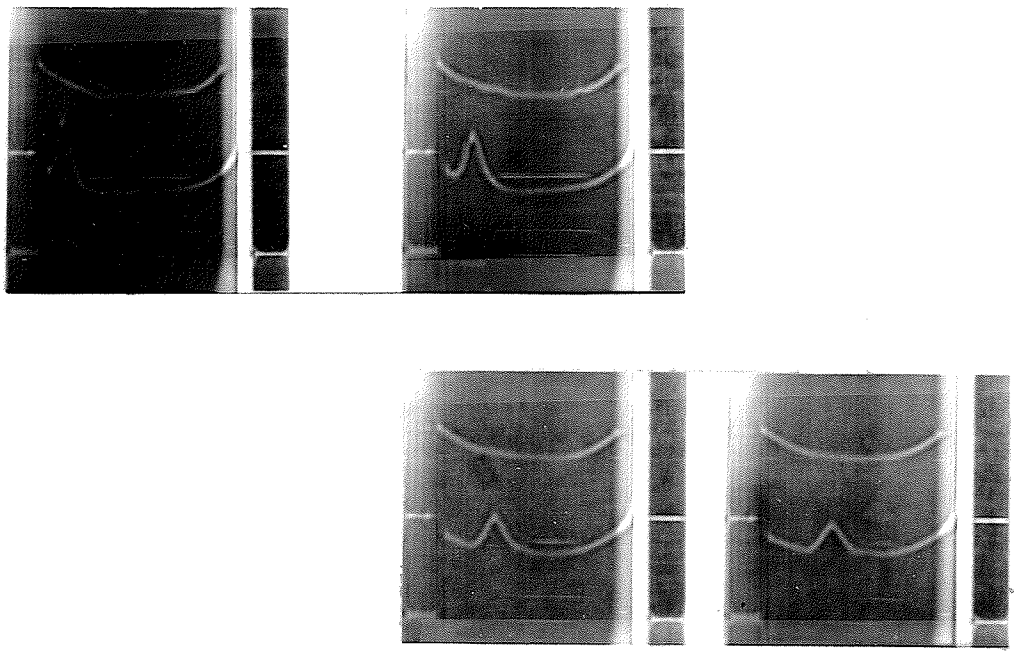
A. Untreated enzyme.

B. Enzyme treated with 0.1% SDS.

C. Enzyme dialyzed 12 hours in 0.5 mM PMB in 0.05 M Tris-HCl (pH 8.0).

D. Same as C, except enzyme has been further dialyzed in 10 mM mercaptoethanol in 0.05 M Tris-HCl (pH 8.0).

PLATE 5



Schlieren pictures during sedimentation velocity centrifugation of PEP carboxylase after dissociation with 0.1% SDS. Pictures were taken at 24, 40, 72 and 90 minutes (top to bottom, and left to right) after rotor had reached 60,000 rpm. Protein concentration was 5 mg/ml.

Amino Acid Analysis: The amino acid content of the enzyme was analyzed by an automatic analyzer (see "Methods"). Five milligrams of enzyme was dialyzed against distilled water adjusted to pH 7.0 - 8.0 for 24 hours and lyophilized. The resulting white powder was either directly hydrolyzed *in vacuo* with HCl or pre-treated with performic acid prior to hydrolysis, then passed through the analyzer. The results of the analysis are given in Table 8. A preponderance of glutamate and aspartyl residues were observed as well as the non-polar amino acids, leucine and valine.

Since the hydrolytic treatment of the enzyme destroys cysteine residues, the method of Ellman (1959) was used to determine the cysteine present. A protein solution (2.35 mg/ml as determined by the Biuret test) was titrated with 5,5'-dithiobis (2-nitrobenzoic acid). This reagent (DTNB) was made to a concentration of 3.96 mg/ml in 0.1 M phosphate buffer, pH 7.0, and was added to the enzyme sample in 10 µl amounts. The optical density of the solution as compared to a control cuvette was recorded at 412 mµ.

The thiol groups present were calculated by the formula (Ellman, 1959): $C_o = A/e \times D$ where C_o is the initial concentration of thiols; A is the absorbance at 412 mu; e is the extinction coefficient (13,600/M/cm); and D is the dilution factor.

Originally there was very little optical density change. The number of cysteine was calculated to be 0.4 moles per mole of enzyme. Since kinetic studies with PMB had previously indicated that the thiol groups were unexposed, the enzyme was treated with 0.1% SDS in an attempt to expose these groups. Further titration with DTNB then

TABLE 8

AMINO ACID ANALYSIS OF P-ENOLPYRUVATE CARBOXYLASE

Details of determinations are given in "Methods"

Amino Acid	eg/100,000 g[c]
Alanine	71
Arginine	62
Aspartate	96
Cysteine[a]	2
Glutamate	125
Glycine	52
Histidine	17
Isoleucine	46
Leucine	125
Lysine	58
Methionine	1.2
Phenylalanine	19
Proline	51
Serine*	22
Threonine*	42
Tryptophan[b]	1.1
Tyrosine	2
Valine	90

*Corrected for 20% decomposition during hydrolysis.

[a]Measured by the DTNB method.

[b]Measured spectrophotometrically.

[c]Assuming a molecular weight of 195,000.

revealed the presence of other cysteine residues. The total cysteine was calculated as 3.9 moles per mole of enzyme.

Taking 4 moles to be the minimal number of cysteine groups present per mole of enzyme, the minimal weight molecular weight of the enzyme was estimated to be 48,980 by the amino acid content data.

Pyruvate Kinase

PEP is channelled into oxalacetate formation for anaplerotic purposes (Kornberg, 1965). On the other hand, PEP can also form pyruvate in a reaction catalyzed by pyruvate kinase for catabolic purposes. In order to study the nature of partitioning of PEP into anaplerotic and catabolic channels, the controls of pyruvate kinase were studied.

Purification of Pyruvate Kinase: *Escherichia coli B* was harvested in the late log phase of growth and disrupted by sonication in 0.05 M Tris-HCl, pH 7.5. Broken cells were centrifuged at 35,000 x g for 30 minutes to remove cell walls and debris. In all following steps the temperature was maintained at 0 - 4° unless otherwise stated and all buffers contained 1.0 mM dithiothreitol.

One-fifth volume 2% protamine sulfate (pH 6.5) was added to the crude extract and stirred for 15 minutes. The precipitate was removed by centrifugation and the supernatant fractionated with solid ammonium sulfate. The fraction that precipitated between 0.4 and 0.5 saturation with ammonium sulfate was dissolved in 0.01 M Tris-HCl, pH 7.5, to a volume 1/4 that of the crude extract.

After dialysis, $CaPO_4$ gel (50 mg/ml) was added to the preparation such that the gel to protein ratio was 2:1 (mg:mg), and stirred for 20

minutes. The gel was collected by centrifugation at 5,000 x g for five minutes and the supernatant discarded. The gel was washed twice with 0.1 M ammonium sulfate, pH 7.5, then the enzyme was eluted with three portions of 0.3 M ammonium sulfate, pH 7.5. The volume of wash and elution solutions was equal to the volume of extract adsorbed.

The elutions from the previous step were pooled and the enzyme precipitated by adding ammonium sulfate to 0.45 saturation. The precipitate was extracted with 24.5% (w/v) and 21.5% (w/v) ammonium sulfate solutions. The latter extract contained pyruvate kinase; the preparation being about 12 fold purified. A summary of the purification is given in Table 9.

Properties of the Enzyme: Adenylate kinase, PEP carboxylase, PEP carboxykinase, DPNH oxidase and lactate dehydrogenase were absent from the preparation. In the presence of 1.0 mM dithiothreitol the enzyme was stable at 0 - 4° for several weeks. Without this protectant the enzyme lost most of its activity over 18-24 hours.

The enzyme showed more activity in HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid) buffer than in a variety of other buffers tested, e.g., TES (N-Tris(hydroxymethyl)methyl-2-amino-ethane sulfonic acid), Tris-HCl, Tris-maleate, etc. Consequently, HEPES buffer was used throughout the course of the work for all assays of pyruvate kinase. The pH optimum lay between 6.5 and 7.5, as shown in Figure 36, and all later assays were conducted at pH 7.0.

Unlike the enzyme of yeast, animals and plants this preparation was not activated by potassium ions. The velocity increased linearly with time and with enzyme concentration.

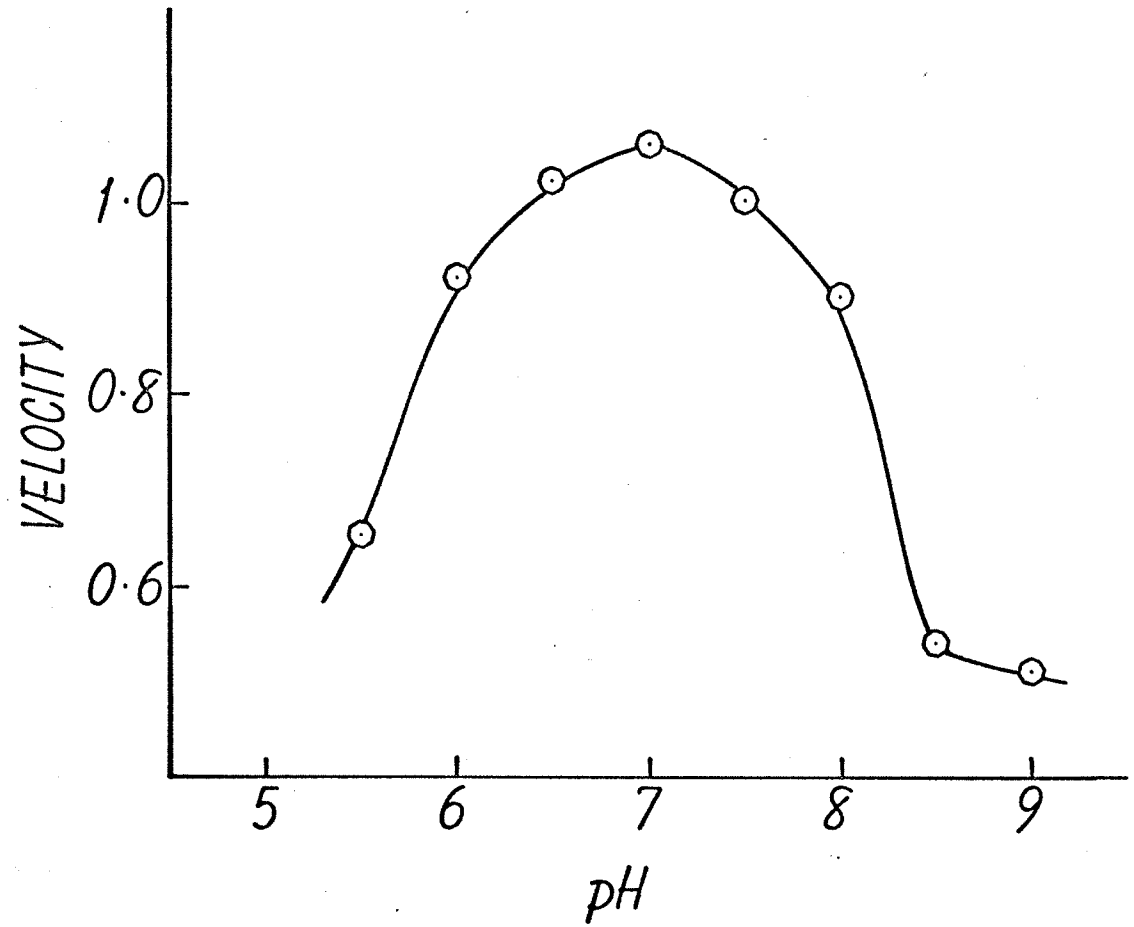
TABLE 9

SUMMARY OF THE PURIFICATION OF PYRUVATE KINASE OF E. COLI B

Step	Protein mg	Units*	Specific Activity	Purification
1. Crude extract	3978	6970	1.8	1.0
2. Protamine sulfate	2590	5760	2.2	1.2
3. Ammonium sulfate (0.4-0.5 saturation)	532	4095	7.7	4.4
4. pH 5.0 treatment	407	3830	9.4	5.2
5. Calcium phosphate gel treatment	227	2775	12.2	6.8
6. Ammonium sulfate extraction	35.5	800	22.5	12.5

*A unit of pyruvate kinase activity is defined as the amount causing a change of 0.1 optical density units per minute when measured with 1.0 mM PEP, 0.15 mM ADP and 10.0 mM $MgCl_2$ as described in "Methods".

FIGURE 36. The effect of pH on the activity of pyruvate kinase. The buffer used was 0.05 M HEPES adjusted to the indicated pH. All assays were done according to the procedure described in "Methods".


VELOCITY
1·0
0·8
0·6
5
6
7
8
9
pH

Substrate Specificity: The substrate ADP can be replaced by other nucleotide diphosphates. As shown in Table 10, CDP, IDP, TDP and GDP tested at a concentration of 0.4 mM were able to produce a noticeable reaction. Judging by the K_m values obtained, ADP remains the best phosphate acceptor and was consequently used for all assays.

The nucleotide monophosphates were unable to serve as phosphate acceptors. The notable exception was AMP which increased the reaction velocity in the presence of ADP, but which by itself does not serve as a substrate (Table 10). Analysis of the reaction products in the presence of AMP showed that its concentration was not altered. AMP must then serve as an activator of pyruvate kinase.

Rate-Concentration Curves: When reaction rates were measured with ADP as the variable substrate in the presence of saturating PEP (10.0 mM), the rate-concentration curve was described by a rectangular hyperbola (Figure 37). In the double reciprocal form (see insert to Figure 37), the plot is a straight line. From this plot the K_m for ADP was calculated to be 0.12 mM.

When PEP was used as the variable substrate with saturating ADP (12.5 mM), the rate-concentration curve was sigmoidal (Figure 38). The inset to Figure 38 shows that the double reciprocal plot for these data is curved. Since K_m can be defined as the concentration of substrate giving half-maximal activity, that for PEP was calculated as 0.1 mM. The sigmoidity of the rate curve indicates a complex reaction mechanism involving participation of more than one PEP molecule in the reaction sequence.

TABLE 10

THE EFFECT OF NUCLEOSIDE MONO- AND DIPHOSPHATES ON THE ACTIVITY OF PYRUVATE KINASE

Additions*	Velocities	
	With ADP	No ADP
None	.066	0
Cytidine diphosphate	.068	.021
Inosine diphosphate	.069	.056
Thymine diphosphate	.075	.027
Guanosine diphosphate	.078	.060
Cytidine monophosphate	.068	0
Inosine monophosphate	.073	0
Thymine monophosphate	.072	0
Adenosine monophosphate	.122	0

*Compound tested were used at a final concentration of 0.4 mM. The reaction system contained 0.1 mM PEP, 0.125 mM ADP (when used), and 10 mM magnesium (see "Methods"). The reaction was started by the addition of 1 unit of pyruvate kinase, and velocities are given as the change in optical density at 340 mμ per minute.

FIGURE 37. Rate curves of pyruvate kinase using ADP as the variable substrate in the presence of saturating PEP (10.0 mM). Assays were done as described in "Methods" using 1.5 enzyme units. The inset shows the data plotted in the double reciprocal form.

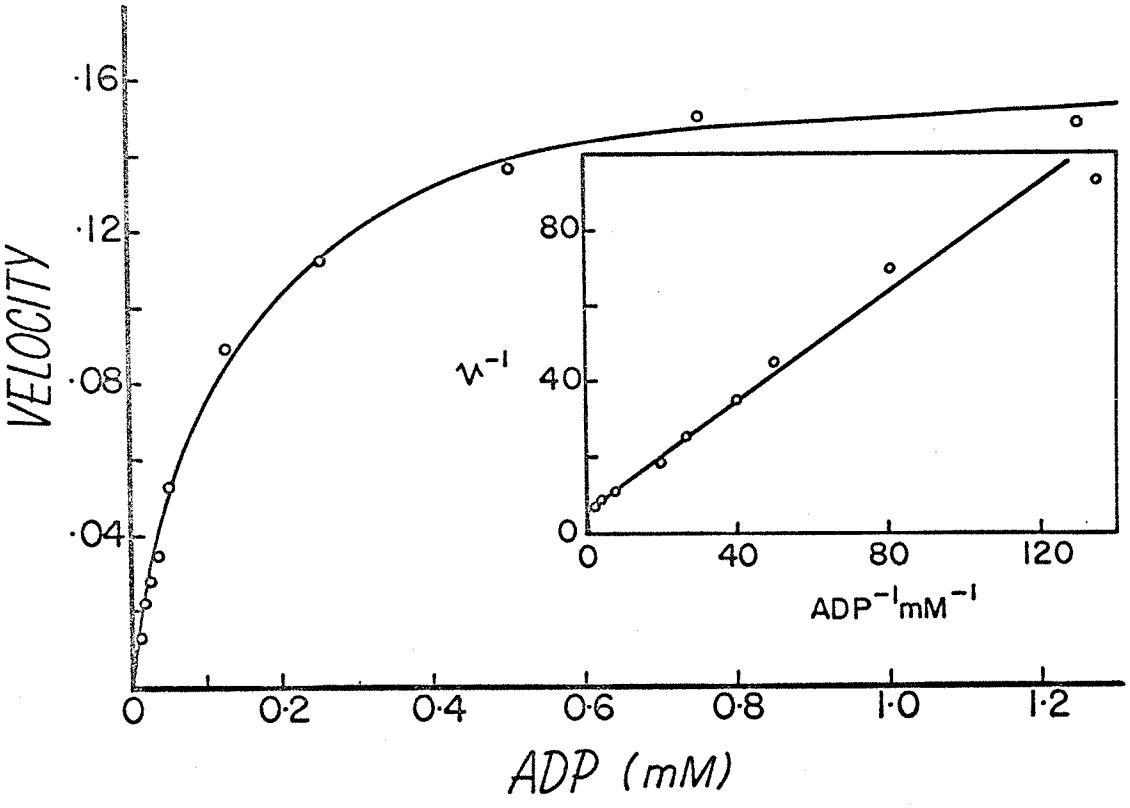
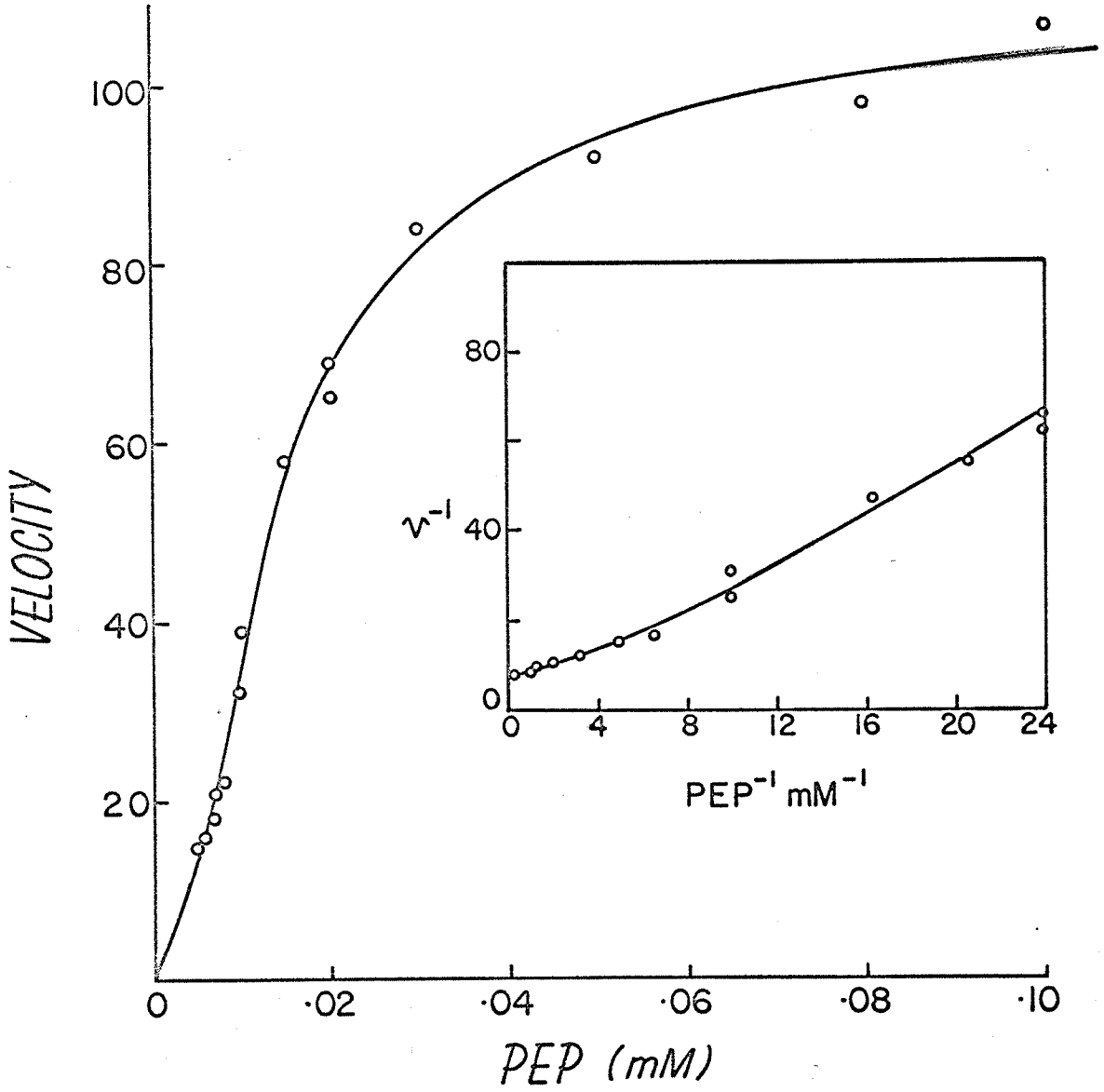

VELOCITY
·16
·12
·08
·04
0
0·2
0·4
0·6
0·8
1·0
1·2
ADP (mM)
v⁻¹
80
40
0
0
40
80
120
ADP⁻¹mM⁻¹

FIGURE 38. Rate curve for pyruvate kinase using PEP as the variable substrate in the presence of 1.25 mM ADP ($12.5 \times K_{(ADP)}$). Each cuvette contained 1.2 enzyme units. The inset shows the data plotted in the double reciprocal form.


VELOCITY
100
80
60
40
20
0
·02
·04
·06
·08
·10
PEP (mM)
v⁻¹
80
40
0
0
4
8
12
16
20
24
PEP⁻¹ mM⁻¹

Activators:

FDP: Since FDP has been reported to be an activator of pyruvate kinase, it was not surprising that the same compound would activate this enzyme from E. coli. The K_a for FDP (concentration giving half-maximal activation), was 0.2 mM when the concentration of PEP and ADP were 0.1 and 0.125 mM, respectively.

The effect of FDP on the rate-concentration curve for PEP is shown in Figure 39a. Although FDP was able to alter the V_m of the enzyme, the initial velocity plots remained sigmoidal. When these data were plotted in the log-log form, the slopes for the curves were both 1.3 both in the presence and absence of FDP.

AMP: As stated earlier, AMP was able to activate pyruvate kinase. Half-maximal activation was caused by 0.1 mM AMP in the presence of 0.05 mM PEP and 0.13 mM ADP. The effect of 5 x K_a values of AMP (0.5 mM) on the rate curve for PEP is shown in Figure 39b. Unlike the activation by FDP, the double reciprocal plot of PEP became linear in its presence, although the V_m was unchanged. To emphasize the different effects that these two activators, FDP and AMP, had on enzyme activity, the two plots were placed together.

AMP and FDP: The effect of the two activators together is shown in Figure 40. Using non-saturating substrate concentrations (0.05 mM PEP and 0.13 mM AMP), velocities were measured at various AMP concentrations both in the presence and absence of 0.5 mM FDP. As shown in the graph (Figure 40), the percentage of activation by FDP was constant and the K_a value for AMP did not change in the presence of FDP. It is clear that the activators caused cumulative activation of

FIGURE 39. Double reciprocal plots of initial velocity data using PEP as the variable substrate for pyruvate kinase. Assays were done as described in "Methods".

FIGURE 39a. (Left) The lower curve represents data obtained in the presence of 1.0 mM FDP and the upper in the absence of effectors.

FIGURE 39b. (Right) Same as Figure 39a except that the lower line was obtained in the presence of 0.5 mM AMP.

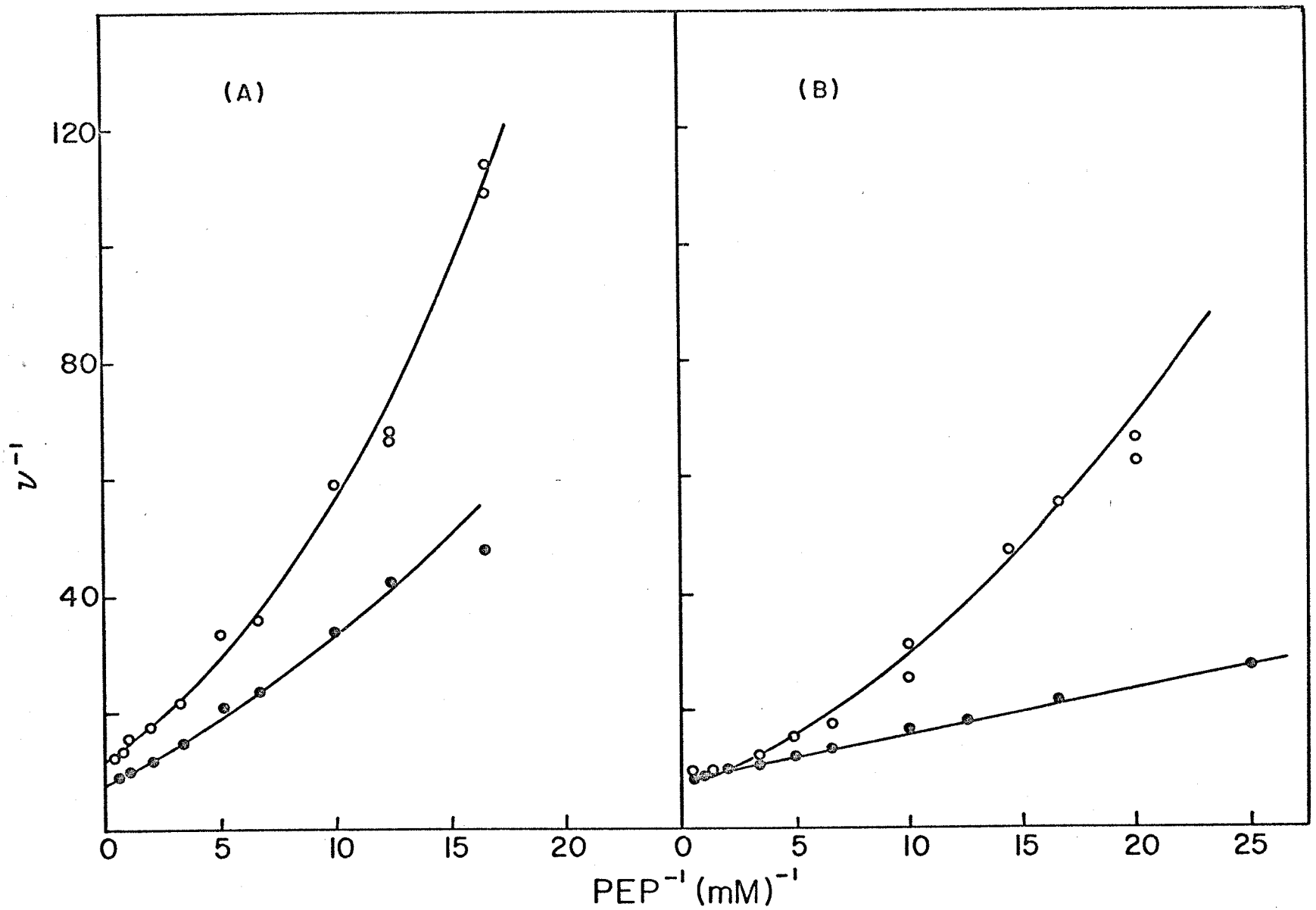
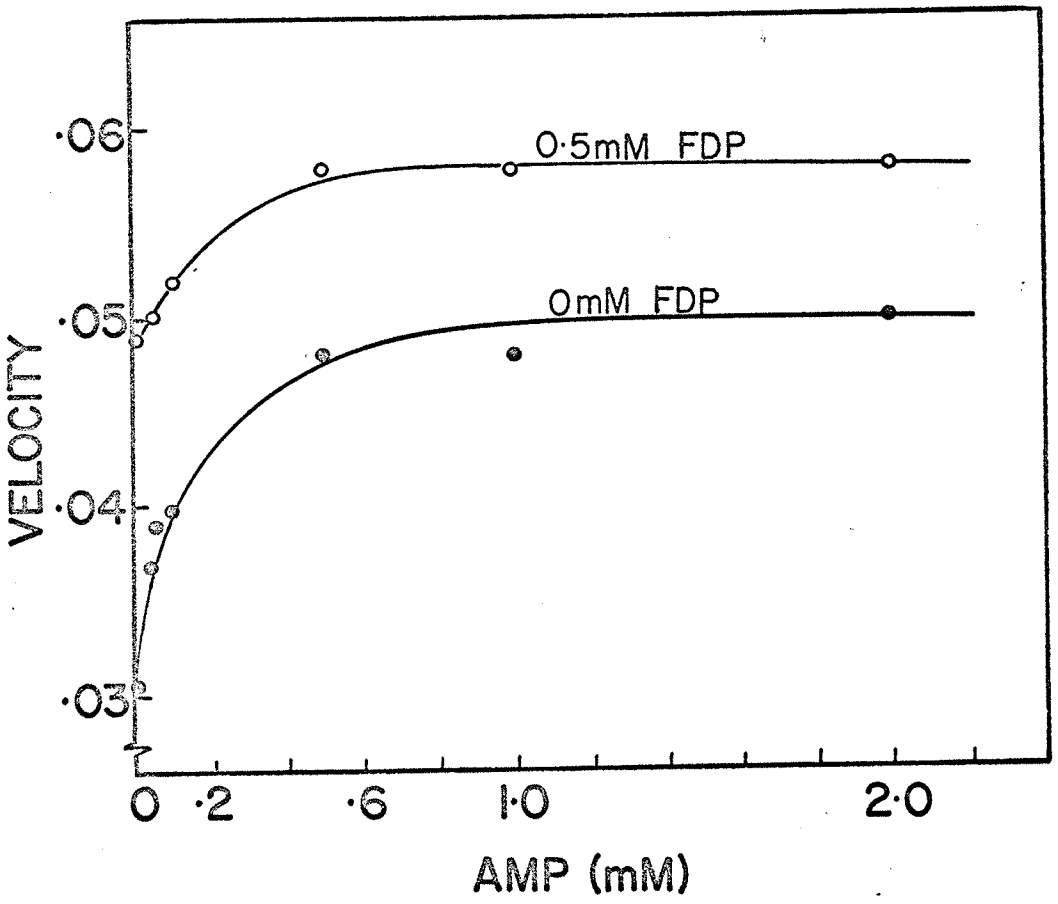

(A)
(B)
120
80
40
v^{-1}
8
0
5
10
15
20
25
PEP^{-1} $(mM)^{-1}$

FIGURE 40. The cumulative activation of pyruvate kinase by FDP and AMP. The standard assay ("Methods") was used with varied concentrations of AMP. The upper line was obtained in the presence of 0.5 mM FDP and the lower line in its absence.

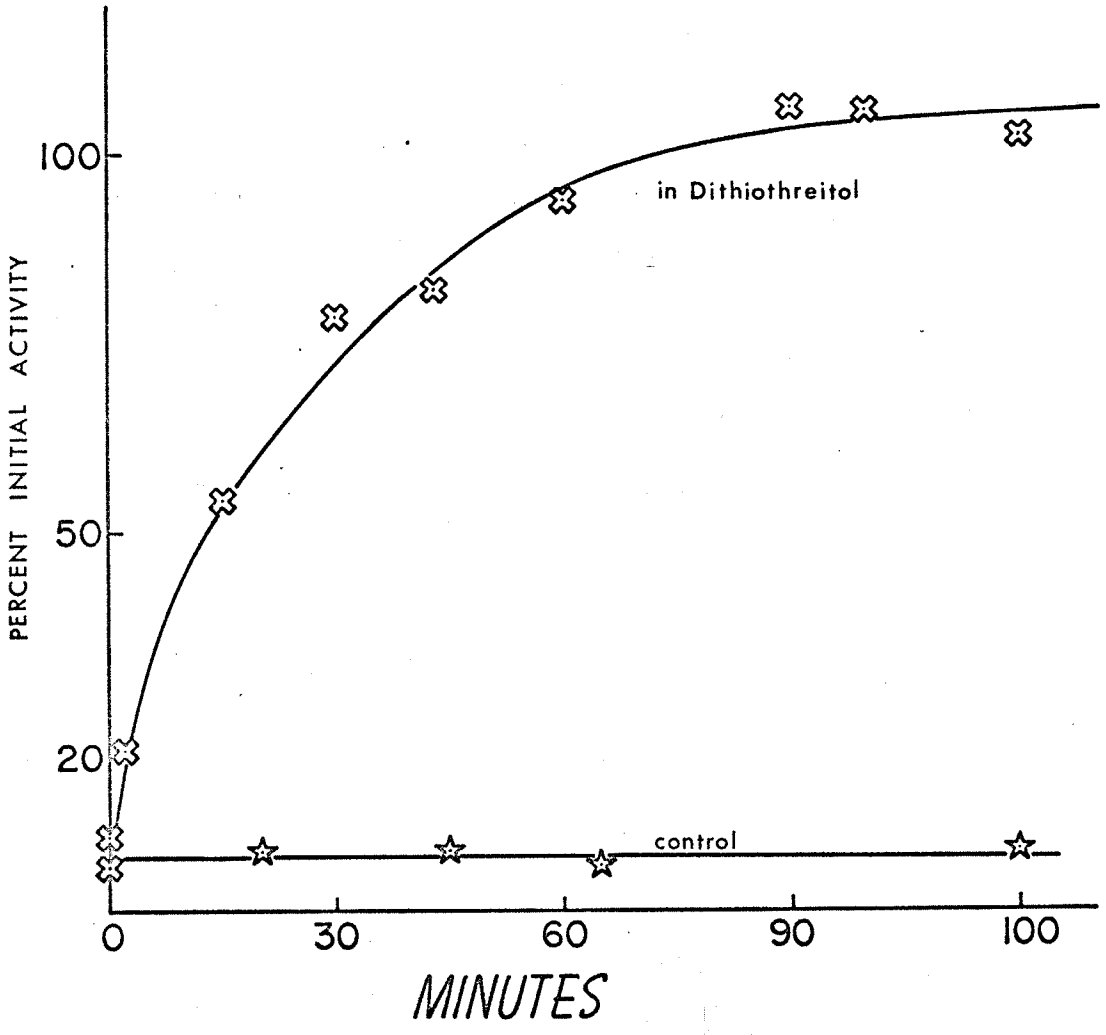

·06
·05
·04
·03
VELOCITY
0·5mM FDP
0mM FDP
0
·2
·6
1·0
2·0
AMP (mM)

enzyme velocities. Higher concentrations of FDP caused no further increase in the activation. This cumulative activation of pyruvate kinase has its counterpart in the cumulative inhibition of glutamine synthetase (Woolfolk and Stadtman, 1964).

Physical Aspects of Pyruvate Kinase: As stated earlier, dithiothreitol has a pronounced stabilizing effect on pyruvate kinase. Also it was able to reactivate the enzyme after it had lost activity. An enzyme preparation (specific activity, 22.5) was dialyzed 24 hours in 0.05 M Tris-HCl, pH 7.5, at 0-4°. After dialysis the specific activity had dropped to 0.9. A portion of the inactivated enzyme was incubated with 2.0 mM dithiothreitol at 4° and aliquots were assayed at various time intervals after addition of the protectant. The other portion of the enzyme served as a control.

The results in Figure 41 show that there was a time-dependent reappearance of activity. In this case a maximum activity of 18.0 was developed after 90 minutes. 2-Mercaptoethanol was also able to reactivate the enzyme. To test whether the inactivation by dialysis and reactivation by dithiothreitol was associated with the physical structure of the enzyme, sucrose density gradient centrifugations were performed according to "Methods". Using malate dehydrogenase of pig heart as a marker, inactive and active enzyme preparations were layered on separate sucrose gradients. The gradient in which the active enzyme was centrifuged contained 2.0 mM dithiothreitol, whereas no protectant was added to the gradient containing the inactive enzyme. One drop of 5.0 mM dithiothreitol was added to each of the fractions obtained after centrifugation of the inactive preparation and incubated two hours at 20° prior to assaying.

FIGURE 41. Time-dependent reactivation of inactive pyruvate kinase by incubation with 2.0 mM dithiothreitol at 25° (upper line). Lower line was made from a control tube containing enzyme with no dithiothreitol. At intervals indicated aliquots from control and incubated mixtures were taken and assayed as described in "Methods".


PERCENT INITIAL ACTIVITY
100
50
20
in Dithiothreitol
control
0
30
60
90
100
MINUTES

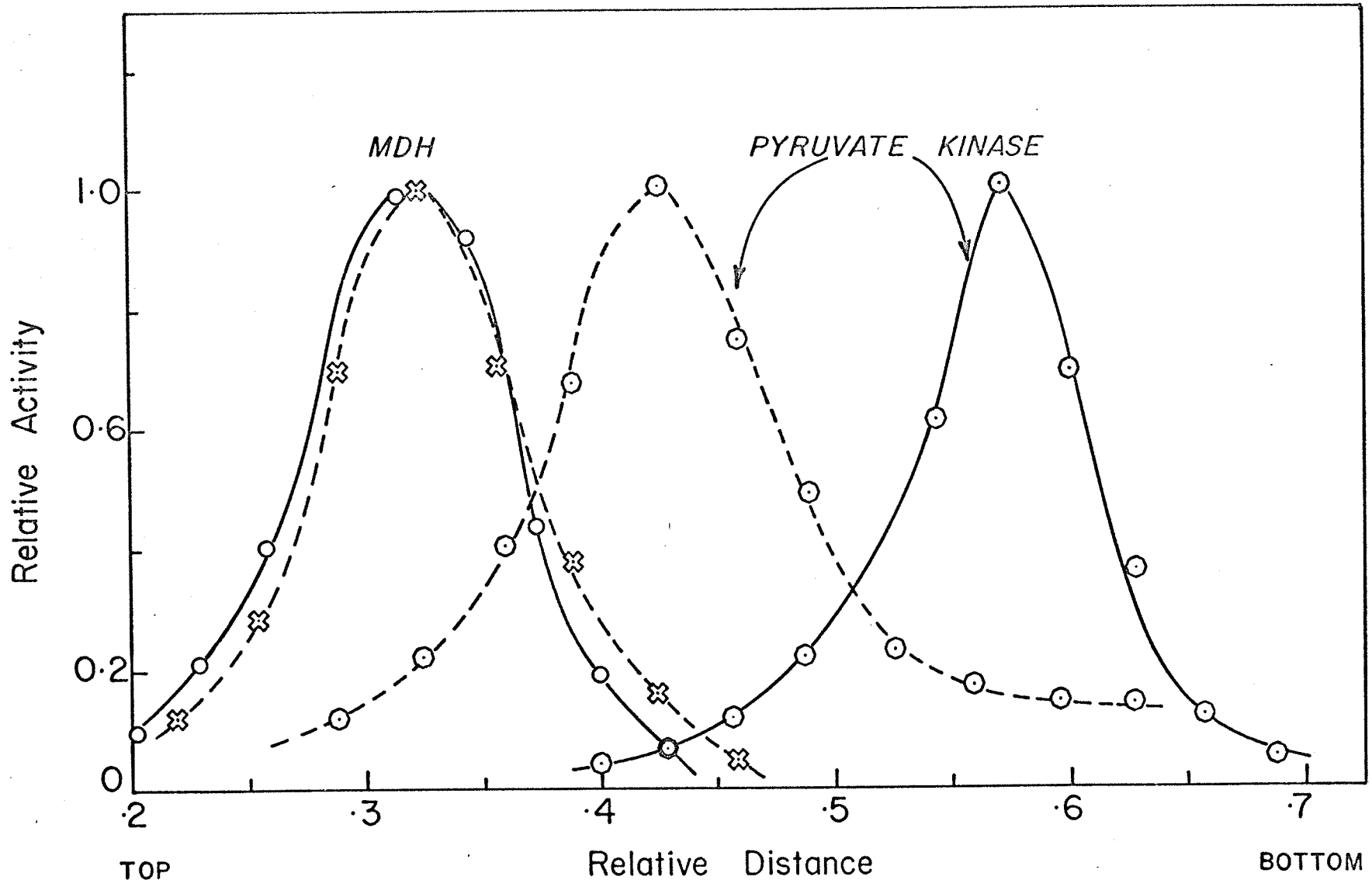
A plot showing the activities of pyruvate kinase and malate dehydrogenase obtained from these centrifugations are shown in Figure 42. The sedimentation velocity of the inactive enzyme preparation was 4.52 ± 0.22 and that of the active enzyme was 6.67 ± 0.11. The latter S value corresponded to a molecular weight of 100,800 ± 2,700. Using the relationship (Durell, Anderson and Cantoni, 1957):

$$S_n = n^{2/3} S_1$$

where S_1 and S_n are sedimentation velocities of the inactive and active species, the n value was found to be 2. This indicates the active enzyme species was dimeric.

AMP and FDP were found to be incapable of activating the inactive enzyme.

FIGURE 42. Plot showing activity of pyruvate kinase and pig heart malate dehydrogenase (internal standard) in 2 drop fractions collected after sucrose gradient centrifugation as described by Martin and Ames (1961). Centrifugation was done at 39,000 rpm in a Spinco 39L rotor for 12 hours at 4° ("Methods"). Inactive pyruvate kinase (see "Results") and malate dehydrogenase were layered on the gradients for centrifugation. The solid line represents the pattern obtained when 2.0 mM dithiothreitol was included in the gradient and the dotted line in its absence. To obtain pyruvate kinase activity when centrifuged without dithiothreitol, 1 drop of 5 mM dithiothreitol was added 2 hours before assaying to each fraction to reactivate the enzyme.


MDH
PYRUVATE KINASE
Relative Activity
1·0
0·6
0·2
0
·2
·3
·4
·5
·6
·7
TOP
Relative Distance
BOTTOM

V. INTERPRETATION AND DISCUSSION OF THE KINETICS OF PEP CARBOXYLASE

Since there are reasons to believe (see "Discussion") that the enzyme in dilute solutions (such as are used in initial velocity studies) is a polymer of four identical protomers, the rationale behind all kinetic experiments was based on the tenet that the enzyme could exist in two (or more) conformational states which were capable of binding the various substrates and effectors with different affinities (Monod, Wyman and Changeux, 1965; Koshland, Nemethy and Filmer, 1966) in each of the two states.

If it is assumed that the enzyme predominantly exists in a state which binds the inhibitors with high affinity and the substrates and activators with low affinity, at least one of the two substrates of PEP carboxylase should show cooperativity. With PEP as the variable substrate, the initial velocity curve is sigmoidal (Figure 4) while the curves obtained with bicarbonate as the variable substrate show no cooperativity as judged by linear double reciprocal plots (Figure 26). It is difficult to get an interaction coefficient (or Hill coefficient) by plotting the data of Figure 4 in log-log form because the resultant curves are never straight. The maximum slope, however, in various experiments, is between the range of n = 1.3 - 2.0. This uncertainty, in part, stems from the high K_m for PEP x ($\sim$ 10 mM) and a consequent difficulty in obtaining the V_m value.

The effect of aspartate and malate (allosteric inhibitors) on the velocity of the reaction at a fixed concentration of the substrates is shown in Figure 6. The data of Figure 6 when plotted in the form of log v/V_m - v versus log of inhibitor concentrations yields straight lines with n values of unity. At the low level of PEP used, therefore, the inhibitors do not show any homotropic interactions. This is the expected result if we assume that the enzyme is present mostly in a state which binds the inhibitors with high affinity. If the enzyme is now converted into a state of low affinity by saturating the enzyme with PEP, one would expect to get cooperative saturation curves for the inhibitor. This experiment is technically difficult to execute because of the high K_m of PEP and a consequent requirement of at least 150 mM PEP for 90% saturation, a concentration at which the enzyme is inhibited slightly owing possibly to non-specific ionic effects. Fortunately, a large number of allosteric activators for PEP carboxylase are known (see "Results") which can be used as tools for this purpose.

The effect of various activators on the velocity of the enzyme have been described in "Results". The effect of different concentrations of CDP and acetyl CoA on the velocity of the enzyme with PEP as the variable substrate is shown in Figures 23 and 14. It will be noted that the non-linear double reciprocal plots in the absence of the activators become linear in the presence of high concentrations of both of the activators. The approximate K_m values for PEP in the presence of 10 mM CDP is 0.3 mM (Figure 23) and in the presence of 1 mM acetyl CoA is approximately 1 mM (Figure 14). The change in V_m of the enzyme in the presence of these activators is also quite noticeable. This

feature (i.e., change in V_m) is contrary to the predictions of the various subunit cooperativity models (Monod et al., 1965; Koshland et al., 1966) which all assume that in both conformational states of the enzyme the velocity constant is the same. While CDP and acetyl CoA both change the shape of the initial velocity plot, the third activator of the enzyme, FDP, only changes the V_m without changing the sigmoidality of the initial velocity plots, even at high concentrations of the activator (Figure 19).

In any case, coming back to the mechanism of aspartate inhibition, it is clear that if the activators CDP and acetyl CoA convert the enzyme to a form which has low affinity for the inhibitor, aspartate should in the presence of saturating concentrations of the activator yield a cooperative inhibition curve. That such is not the case can be seen from an inspection of the double reciprocal plots shown in Figure 28. When the asymptote slopes from Figure 28 are plotted against inhibitor concentrations a straight line results giving a K_i of 0.10 mM (see inset of Fig. 28). Had the inhibitor shown heterotropic interactions (or cooperativity) a parabolic or more complex non-linear curve should have been obtained. The non-linearity of the double reciprocal plots (Fig. 28) must then be ascribed to a direct decrease in the affinity of the PEP or CDP sites (or even both) caused by the binding of aspartate. In contrast to the behavior of aspartate, oxalacetate, a product of PEP carboxylase, causes a linear non-competitive inhibition under conditions where the enzyme is nearly saturated with CDP and PEP is the variable substrate (Fig. 27).

Evidence indicating that the sites for acetyl CoA and CDP (or GTP), and those for acetyl CoA and FDP has been discussed in "Results". Although the curves shown in Figures 24 and 25 cannot be interpreted in any straightforward manner, such behavior of the curves does indicate that the acetyl CoA binding site is possibly different from that of CDP (or GTP) and FDP.

The results of these kinetic studies of PEP carboxylase summarized in this section can be interpreted on the basis of subunit interactions. At least in dilute solutions of the protein the various effectors of the enzyme (aspartate and acetyl CoA) do not change its sedimentation properties in sucrose gradients which rules out the involvement of association-dissociation phenomena during catalysis. A simple model for the mode of action of PEP carboxylase can be constructed with the following assumptions: (1) The subunits of the oligomer exists in at least two conformations, X and Y, with X being the preferred conformation in the absence of ligands. (2) There are as many specific sites as there are substrates and effectors. (3) The activators (CDP, acetyl CoA) and PEP bind with differential affinity to states X and Y, with Y having a higher affinity for these ligands. Occupation of the sites in the X conformation converts the oligomer (or subunits) to the Y conformation. (4) The two conformations have different rate constants for the breakdown of substrates into products, i.e., V_m of the X and Y conformations are different. (5) The inhibitor (aspartate) binds to both conformations X and Y, but does not modify the subunit interactions. It affects the binding constant of PEP (and possibly the activators) directly rather than by changing the equilibrium, $X \rightleftharpoons Y$.

While this qualitative model fits most of the kinetic data presented here, it is extremely difficult to give any quantitative estimates because of the impossibility of finding the various thermodynamic constants from velocity studies alone. The so-called Hill plots (Monod et al., 1965) have generally been utilized in extracting some information from the initial velocity data in the case of other allosteric enzymes. However, all of the sigmoidal plots presented yielded curved Hill plots (see "Results") and it is impossible to obtain any interaction coefficients. The verification of the model would, thus, have to await further information from equilibrium binding studies.

VI. DISCUSSION

The formation of three carbon compounds from catabolism may proceed in a variety of different ways which differ in detail, but produces pyruvate. The pyruvate formed is oxidized to acetyl CoA which in turn is oxidized to CO_2 and water via the tricarboxylic acid cycle. Since the intermediates of the cycle serve two roles; as a "carrier" of acetyl CoA and biosynthesis, the intermediates must be continually replenished so that the catabolic function of the cycle can continue despite the diversion of these compounds for biosynthesis. Reaction sequences responsible for replenishing "amphibolic" cycles (Davis, 1961), are referred to as anaplerotic sequences (Kornberg, 1965). Such sequences as the glyoxylate shunt are anaplerotic (Kornberg, 1966) in organisms able to metabolize two carbon compounds.

As discussed previously (see "Historical"), evidence has accumulated indicating that in enteric bacteria, PEP carboxylase fulfills this role when these organisms utilize three carbon compounds or the precursor of three carbon compounds as the sole carbon source. Cánovas and Kornberg (1965) reported that acetyl CoA exerts a powerful activating effect on enzyme activity, increasing the V_m and decreasing the K_m, when PEP is the varied substrate. The purpose of this activation seems to be to catalyze the formation of oxalacetate required for oxidation of acetyl CoA. The operation of such a regulatory system ensures that during growth on glucose or on other three carbon compounds, the necessary

balance between catabolic and anaplerotic reactions may be maintained (Cánovas and Kornberg, 1965).

As shown in Figures 10 to 15, the acetyl CoA activation was confirmed for the purified Salmonella enzyme. However, unlike the rate-concentration plots of Cánovas and Kornberg (1965), the double reciprocal plots for PEP are decidedly non-linear indicating the involvement of at least two PEP molecules in the reaction mechanism. The source of this discrepancy has not been studied, but it may be that a sufficient range of substrate concentration was not covered for rate curves. These workers report a K_m value of 5.0 mM for PEP, yet according to their data, the highest concentration of PEP used was 4.0 mM. Due to this curvature the K_m's reported are concentrations giving one half-maximal velocity.

Since the TCA cycle functions as a pool for four carbon acids for biosynthesis, and since this function occurs through the glutamate-aspartate transaminase reaction, aspartate can be considered as an end product of a biosynthetic sequence beginning with the enzyme PEP carboxylase. In analogy with other biosynthetic sequences (see Cohen, 1965; Stadtman, 1966), it was not surprising that aspartate should serve as powerful inhibitor of the enzyme (Maeba and Sanwal, 1965b). Figure 6 also shows the strong inhibition of enzyme activity by malate. The degree of inhibition and the fact that some residual activity remains at saturating inhibitor concentrations shows that an allosteric site is involved rather than a competition for an active site. Although other di- and tri-carboxylic acids are able to produce partial inhibition (0 to 50 percent), the same concentrations of aspartate and

malate cause 87 to 93 percent inhibition, respectively (Table 2). This may be analogous to the non-specific inhibition of mammalian pyruvate carboxylase by similar compounds (Freedman and Kohn, 1964). In this respect it is noted that the presence of acetyl CoA abolishes non-specific inhibition whereas this effect is not as marked with the activators FDP and CDP. The fact that these activators are able to modify inhibition by malate and aspartate suggests that the inhibitory control is subject to modulation and is dependent, if not interlocked, with the effect of activators. Aspartate and malate inhibition have been reported to inhibit CO_2 fixation by crude extracts of E. coli (Nishikado, Izui, Iwatani, Katsuki and Tanaka, 1965) in the presence of PEP and is presumably due to inhibition of this enzyme.

Since biosynthetic sequences diverge from the aspartate pool to amino acid and nucleotide biosyntheses, aspartate can be considered a legitimate end product of a biosynthetic sequence. The case of malate inhibition is more difficult to envisage. Since inhibition in the presence of both aspartate and malate is not cooperative (data not shown) but additive, it cannot be assumed a priori that the two inhibition sites are distinct. If the amino group of aspartate is assumed to be important for inhibition as it seems to be (since L-aspartate is a much better inhibitor than the analogue, D-aspartate), then the replacement of a hydroxyl group for the amino group should greatly reduce its inhibitory action. However, such is not the case, indicating different groups may be involved in binding malate to the enzyme surface.

At any rate, either malate and/or aspartate inhibits PEP carboxylase, and in appearance is a redundant control. Since aspartate

and malate are interconvertible *via* the transamination reaction and *via* the combined reactions catalyzed by fumarase and aspartase it may be that inhibition by either compound serves the same purpose. The data of Roberts *et al.* (1954) indicate that malate is a very poor inhibitor of *in vivo* CO_2 fixation in *E. coli*, which suggests that an allosteric inhibition by malate may be physiologically non-functional. However, more research would be required to resolve this question.

The specificity of FDP activation is shown in Table 3. That the site of activation is distinct from that of acetyl CoA can be inferred from Figures 13 and 16 which show that the activators acetyl CoA and FDP have different effects on the rate plots for PEP; acetyl CoA normalizes the curve whereas FDP has no effect on the shape of the curve. If binding of these activators occurred at the same site analogous results would be expected. Also, in the presence of both acetyl CoA and FDP (Fig. 25) a cooperative activating effect occurs indicating distinct sites are responsible for the activation.

Since PEP carboxylase is absolutely necessary for growth on glucose (Kornberg, 1965), it would seem that FDP activation ensures a supply of oxalacetate. However, since this is already assured by acetyl CoA activation (Cánovas and Kornberg, 1965), it seems more reasonable to consider FDP to be related to energy levels within the cell (Atkinson, 1965). From the calculation of Krebs (1964), it is apparent that the ATP/AMP ratio rather than the absolute concentration of ATP or AMP/ADP in a cell is a controlling factor for a large number of enzymes involved in carbohydrate metabolism. When the ATP/AMP ratio is high the level of FDP is expected to drop owing to ATP

inhibition of phosphofructokinase (see "Historical") and that of citrate to increase (Atkinson, 1965) with the resultant removal of acetyl CoA for fatty acid synthesis due to activation by citrate of acetyl CoA carboxylase (see "Historical"). Under such conditions there is neither a necessity (because of already augmented levels of citrate) nor a capability (owing to decreased levels of acetyl CoA and FDP) for increased production of oxalacetate. However, when the ATP/AMP ratio is low FDP concentrations will tend to increase and citrate will tend to decrease (thus increasing the levels of acetyl CoA by slowing down fatty acid synthesis); adequate supplies of oxalacetate would consequently be generated by a concerted activation of PEP carboxylase by FDP and acetyl CoA to increase the levels of citrate.

The activation of enzyme activity by nucleotides is only partially specific (Table 4) suggesting that <u>in vivo</u> the nucleotide pool sizes rather than the concentration of individual nucleotides may be important in controlling PEP carboxylase. A precedent for this occurrence is the feedback inhibition by a number of nucleotides of aspartate transcrabamylase (Gerhard and Pardee, 1962). It may be unimportant which of the two notable activators (CDP or GDP) serves a regulatory function since the presence of nucleotide kinases (Weavers, 1962) with an equilibrium constant near unity will keep such pools in equilibrium. The strength of the activation is a clear indication that they participate in some significant role in the control of intermediary metabolism.

It has been suggested that such activation may be attributed to a compensatory feedback effect (Sanwal and Maeba, 1966b). As a consequence of nucleotide pools inhibiting aspartate transcarbamylase

(Gerhart and Pardee, 1962), the steady state concentration of aspartate will increase, which in turn will inhibit PEP carboxylase resulting in the reduction of oxalacetate concentration. The reduction of oxalacetate may become critical since it is absolutely necessary for the running of the TCA cycle. The activation by nucleotide pools would immediately restore the original levels of oxalacetate compensating for the inhibitory effects mentioned earlier on aspartate transcarbamylase and PEP carboxylase. Compatible with this hypothesis is the fact that acetyl CoA increases the affinity of the nucleotides for the enzyme (Fig. 24). Since the levels of oxalacetate are controlled by acetyl CoA activation of the enzyme, a fine compensatory modulation by the nucleotides will ensure constancy of oxalacetate draining into the tricarboxylic acid cycle. Owing to the high K_a values, the nucleotides in the absence of acetyl CoA are not efficient activators.

The fact that PEP carboxylase is subject to modulation by the inhibitors aspartate and malate as well as the activators acetyl CoA, FDP and the nucleotides indicates that the rate at which the reaction proceeds is critical to the cell. That this should be so is not surprising since the reaction precedes an important bifurcation of sequences--one responsible for biosynthesis and the other for catabolism, both of which are dependent on oxalacetate.

PEP in the cell is itself partitioned into an anaplerotic channel via PEP carboxylase and a catabolic channel via pyruvate kinase. In order to better understand the nature of partitioning the control of pyruvate kinase was studied. Similar to the yeast (Hess, Haeckel and Brand, 1967; Gancedo, Gancedo and Sols, 1967) and mammalian

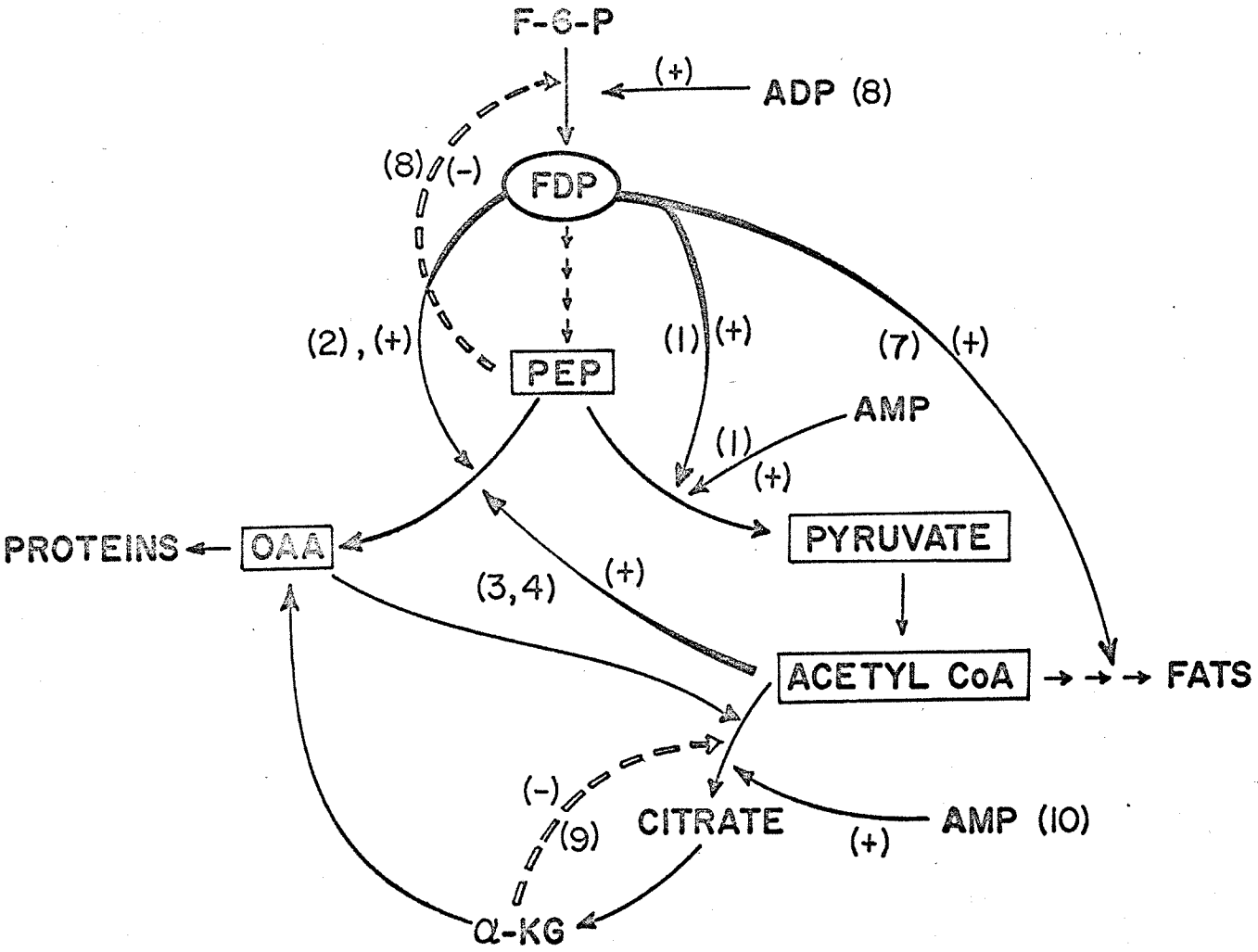
(Taylor and Bailey, 1967) enzymes, the E. coli enzyme was found to be activated by FDP (Fig. 39a). In addition, AMP specifically activates pyruvate kinase (Fig. 39b).

Similar to PEP carboxylase, the rate-concentration curve with PEP as variable substrate shows sigmoidity, which is unaltered in the presence of FDP, but becomes hyperbolic or normalized in the presence of AMP. For regulation of glycolytic and terminal oxidation sequences AMP is a sensitive indicator of the state of ATP supply. From the calculation of Krebs (1964) when the level of ATP falls 43%, the level of AMP rises 720%. Since pyruvate kinase is one of the key enzymes in glycolysis, any decrease in the energy supply will be reflected in the stimulation of pyruvate kinase with a consequent generation not only directly of more ATP but also indirectly by furnishing a substrate which may be used for energy generation through the terminal oxidative supply.

The FDP activation of pyruvate kinase is more complex. Not only is the catabolic channel for PEP (via pyruvate kinase) as well as the anaplerotic channel (via PEP carboxylase) controlled by FDP but it also controls the pathway for fatty acid biosynthesis through FDP activation of acetyl CoA carboxylase (Wakil, Goldman and Williamson, 1966). The activation of three interlocking pathways by the same metabolite would tend to create certain problems in regulation, especially in the partitioning of PEP into the anaplerotic and catabolic channels--unless the effect of FDP is itself modulated by other effectors. That this may be so is indicated by the multiplicity of effectors that have been found for enzymes which are activated by FDP (see Figure 43).

FIGURE 43. Schematic representation of the feedback systems affecting the various control point enzymes in the glycolytic and citric acid cycle in E. coli. (+) and (-) represent activation or inhibition, respectively. The numbers in brackets are references to publications given below:

(1) Maeba, P., and Sanwal, B. D. (This thesis).
(2) Sanwal, B. D., and Maeba, P. (1966a).
(3) Maeba, P., and Sanwal, B. D. (1965b).
(4) Cánovas, J. L., and Kornberg, H. L. (1965).
(7) Wakil, S. J., Goldman, J. K., Williamson, I. P., and Toomey, R. E. (1966).
(8) Blangy, D. (1967).
(9) Wright, J. A., Maeba, P., and Sanwal, B. D. (1967).
(10) Weitzmann, P. D. J. (1967).


F-6-P
(+)
ADP (8)
(8)
(-)
FDP
(2), (+)
PEP
(1)
(+)
(7)
(+)
AMP
(1)
(+)
PROTEINS
OAA
PYRUVATE
(3,4)
(+)
ACETYL CoA
FATS
(-)
(9)
CITRATE
(+)
AMP (10)
α-KG

As shown in Figure 40, the presence of both AMP and FDP causes a cumulative effect on pyruvate kinase velocity, i.e., at both unsaturating and nearly saturating concentrations of AMP the percentage activation by FDP is constant and the K_a (AMP) does not change in the presence of FDP. This is in marked contrast with the effect of mixtures of FDP and acetyl CoA on PEP carboxylase (Fig. 25). At concentrations of 10.0 mM bicarbonate and 1.0 mM PEP (0.1 K_m), FDP causes half-maximal activation at 2.5 mM, but in the presence of only 0.2 mM, acetyl CoA this figure is reduced to about 0.5 mM. Also the velocity of the reaction in the presence separately of 0.04 mM acetyl CoA and 1.5 mM FDP is 0.024 but changes to 0.15 in the presence of activators together. Instead of cumulative activation effect of FDP and AMP on pyruvate kinase, the effect of FDP and acetyl CoA together on PEP carboxylase is a strong cooperative effect.

As mentioned previously when ATP levels are low, the AMP and ADP levels are high (Krebs, 1964; Atkinson, 1965) leading to high levels of FDP through activation of phosphofructokinase by ADP (Blangy, 1967). Cumulative activation of pyruvate kinase by AMP and FDP will lead to ATP formation directly and indirectly through the increased availability of acetyl CoA for combustion through the tricarboxylic acid cycle. However, were PEP carboxylase not activated simultaneously by FDP it would lead to a virtual cessation of the production of oxalacetate and indirectly the production of amino acids. This statement is based on the experimental values of K_m of PEP carboxylase (10.0 mM) and pyruvate kinase (0.1 mM). The fact that acetyl CoA activates PEP carboxylase may not have much significance under conditions where AMP

concentrations are high and the catabolic channel is optimally active because acetyl CoA concentration is bound to be low due to its oxidation through the tricarboxylic acid cycle (Weitzmann, 1967; Atkinson, 1965).

If the function of FDP activation is to maintain a supply of oxalacetate through the anaplerotic channel when momentary adjustments of energy supply are occurring in the cell a cooperative activation of PEP carboxylase will be advantageous--if not necessary. The maintenance of a steady supply of oxalacetate for biosynthesis and for combustion of acetyl CoA cannot depend solely on the activation of PEP carboxylase by acetyl CoA. Firstly the mechanism of the activation ensures the oxidation of acetyl CoA (Kornberg, 1965) and secondly, acetyl CoA concentrations must be highly variable being drained into the tricarboxylic acid cycle when AMP levels are high and to fatty acid synthesis when AMP levels are low. Thus, when the energy level of the cell is high, a transient state is likely to occur during which the acetyl CoA concentration increases and that of FDP decreases and vice versa. By precisely modulating the effect of each other on PEP carboxylase, production of oxalacetate would become independent of the fluctuating concentration of the effectors which are necessitated by the need for energy regulation in the cell. Cooperative interaction in the regulation of the anaplerotic channel appears to be a device which ensures that adjustments needed for keeping the ATP/AMP ratio constant will not interfere with the withdrawal of PEP for biosynthetic purposes.

The levels of the two activators are also determined by controls independent of the energy state in the cell. The inhibition in E. coli of phosphofructokinase by PEP (Blangy, 1967) and citrate synthetase by α-ketoglutarate (Wright, Maeba and Sanwal, 1967) will augment the concentrations of FDP and acetyl CoA and be complementary to the control mechanisms on PEP carboxylase.

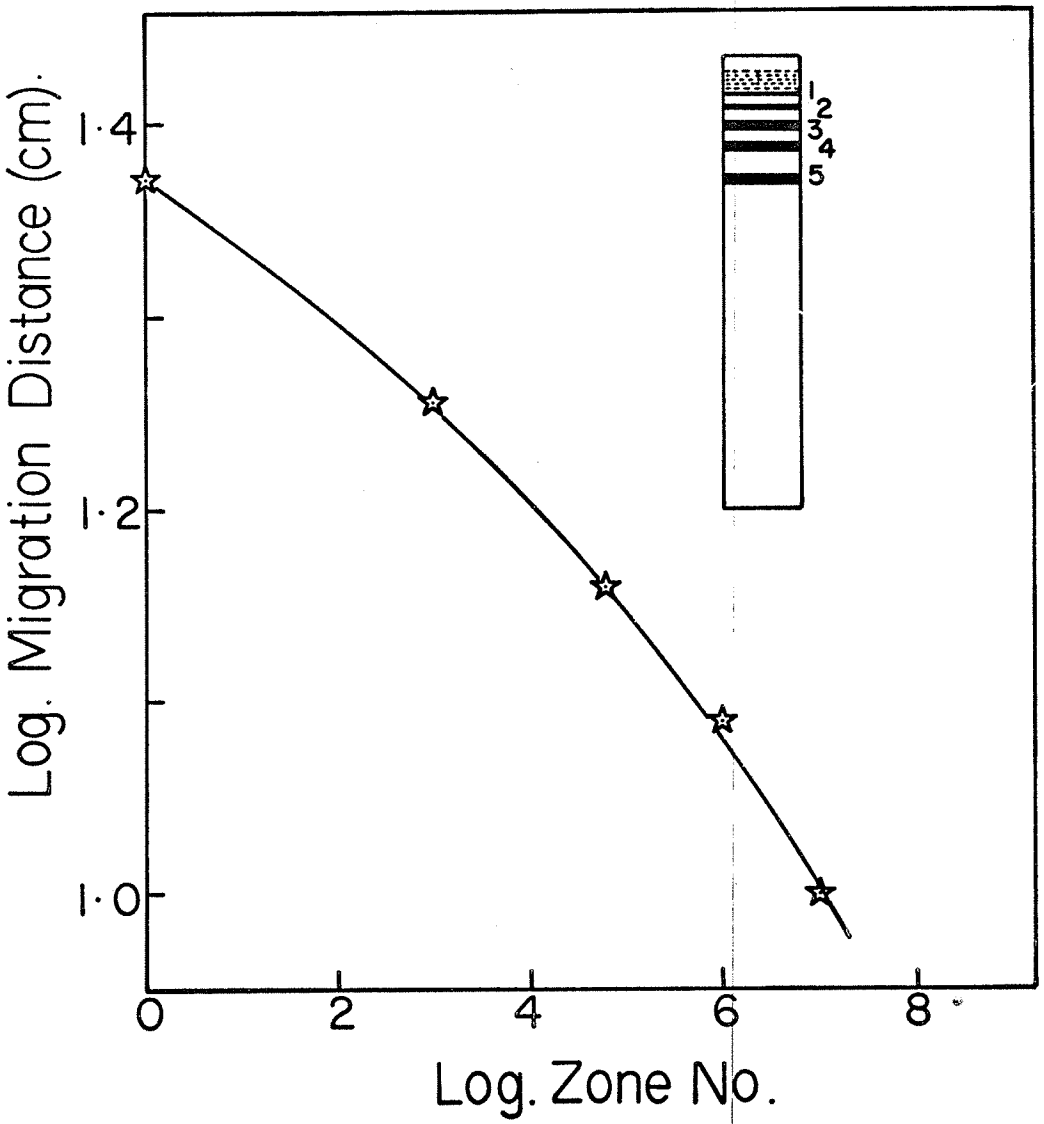
On the basis of the effects of macroions and dioxane (Sanwal, Maeba and Cook, 1967) an idea of the tertiary structure of PEP carboxylase can be formed. The results in Table 4 and Figure 29 show that non-specific electrostatic interactions occur between negatively charged groups on the enzyme surface and positively charged amino groups of the homo- and hetero-polymers leading to activation. That activation is due to electrostatic interaction is supported by the finding that the degree of activation is dependent on the lysine content of the polymer (Fig. 29). Secondly, the activation is dependent on pH (Fig. 30) which may be due to protonation of an increasing number of amino groups of lysine (Daniel, Katchalski and Stahmann, 1962, Applequist, Doty and Stahmann, 1962). Thirdly, the activation decreases with increasing ionic strength of the assay system (Fig. 31) and is likely due to the reduction of electrostatic interactions as a result of the swamping of charged groups by stabilizing counterions.

The effect of modification of enzyme activity by polyamino acids is a well known phenomenon (see Katchalski, Sela, Silman and Burger, 1964), but the mechanism is little understood. With PEP carboxylase it appears that a number of negatively charged groups are accessible to the amino group of lysine, binding of which possibly leads to conformational alteration and alteration of the catalytic site.

That there are hydrophobic regions involved can be inferred by the effects of non-polar solvents on enzyme activity (Fig. 33). Non-polar solvents are known to weaken hydrophobic bonds (Kauzmann, 1959). The possibility that alteration of pK values of ionizable groups, brought about by changes in the dielectric constant on addition of solvent, has been discounted on the grounds that solvents with different dielectric constants activate the enzyme to the same degree. The effect of activation must be due to a subtle change in the conformational state of the protein, rather than dissociation into subunits (Churchich and Wold, 1963; Kauzman, 1959), since there was no evidence for this in sedimentation velocity studies both at low and high enzyme concentrations.

PMB treatment of PEP carboxylase results in partial inactivation of the enzyme and in the formation of protein species with different mobilities as judged by polyacrylamide gel electrophoresis (Plate 4). At least five bands are visible in the gels. On plotting the logarithm of migration distance of the various bands against the logarithm of zone numbers, according to Smithies and Connell (1962), a smooth curve is obtained (Fig. 44) which suggests that these bands probably represent successive oligomers of a basic monomeric unit. Since mercaptoethanol partially restores activity of PMB-treated enzyme and results in the formation of two major bands in polyacrylamide gels, thiol groups are implicated in the quaternary structure. Many enzymes are known to be dissociated by PMB treatment, notably muscle phosphorylase b (Madsen and Cori, 1956), phosphorylase a (Chignell, Gratzer and Valentine, 1968),yeast alcohol dehydrogenase (Snodgrass, Vallee and

FIGURE 44. Plot of the distance migrated by protein species produced by PMB treatment of PEP carboxylase as detected by polyacrylamide gels after electrophoresis. Inset shows a diagrammatic representation of the appearance of the gel after staining with Coomasie blue ("Methods"). The numbers beside the bands represent zone numbers which have been assigned to them.


Log. Migration Distance (cm).
1·4
1·2
1·0
0
2
4
6
8
Log. Zone No.
1
2
3
4
5

Hoch, 1960) and aspartate transcarbamylase (Gerhart and Schachman, 1965). The effects of this reagent are quite complex since it has great affinity for sulfhydryl groups and may also act as a "hydrophobic wedge" by virtue of the benzene ring structure. It has not been determined whether inactivation of PEP carboxylase is primarily due to oxidation of sulfhydryl groups involved in catalysis or to disruption of the quaternary structure or both.

That there are thiol groups present has been tested with DTNB which reveals that 0.4 moles of sulfhydryls per mole of enzyme are readily accessible to this reagent. It is only after complete denaturation with sodium dodecyl sulfate that four moles of thiol groups could be titrated. The fact that most of the thiol groups are "buried" within the molecule may explain why PMB can only partially inactivate the enzyme.

It is known that more extensive denaturation is obtained with sodium dodecyl sulfate compared to the partial denaturation experienced with PMB (Neurath, Greenstein, Putnam and Erickson, 1944). The effectiveness of sodium dodecyl sulfate stems in part from the repulsive forces generated as a result of binding of the anionic detergent and also from the rupture of hydrophobic bonds in the protein through interaction with the long hydrocarbon chain of the detergent. This is true with sodium dodecyl sulfate-treated PEP carboxylase which shows complete dissociation into a protein species sedimenting with an $S_{20,w}$ of 4.1. In polyacrylamide gel electrophoretic analysis, a single band with increased mobility over that of the untreated enzyme is visible. The fact that this band migrates faster than the "monomer" band of PMB-dissociated enzyme cannot be taken as proof of different

subunits since migration depends both on charge and size of the particle, the latter being dependent on the binding of detergent to the molecule.

The subunits formed by sodium dodecyl sulfate treatment are totally devoid of activity, yet the sulfhydryl groups are intact and can be titrated with DTNB, indicating possibly that the quaternary structure is more important for activity than the thiol groups. This is unlike β -galactosidase (Craven, personal communication) and aspartate transcarbamylase (Gerhart and Schachman, 1965) which retain activity after dissociation by SDS and PMB, respectively.

Dioxane and other non-polar reagents are generally good denaturants, but they were ineffective in dissociating PEP carboxylase. Organic solvents are effective in this role because of their affinity for non-polar side chains in the protein, but on the other hand, ionic bonds are strengthened by virtue of the decrease in the dielectric constant of the medium induced by addition of these reagents. Such is not the case with PMB or SDS because the charged groups on these molecules form repulsive forces on binding the protein (Schachman, 1963). Although as stated earlier, non-polar solvents alter the conformation of the enzyme through weakening of hydrophobic bonds, the increased strengthening of ionic bonds is probably sufficient to maintain the integrity of the molecule.

The subunits formed by SDS treatment sediment at 4.1 $S_{20,w}$ (or a rough molecular weight of 48,000). This is in good agreement with the minimal molecular weight determined by amino acid analysis, i.e., 48,900. Since the molecular weight of the untreated enzyme is

193,000 ± 7,000, this would indicate that at least four subunits are involved in the oligomeric protein.

A qualitative picture of PEP carboxylase that arises from the data is that of a tetrameric molecule; the subunits of which are held together by hydrophobic and ionic bonds. Buried within the oligomer are four sulfhydryl groups, while on the surface are regions composed of acidic groups. Although the nature of the catalytic site is unknown, conformational changes brought about by modifying hydrophobic bonds within the molecule or the surface charges greatly alters activity of the catalytic site. The capacity for profoundly altering catalytic activity as a result of modification of the structural features of the enzyme makes PEP carboxylase an enzyme ideally suited for modulation by effectors.

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