THE USE OF ANTIBODIES TO STUDY STRUCTURAL CHANGES RELATED TO CATALYTIC AND REGULATORY PROPERTIES OF MUTANT PHOSPHOENOLPYRUVATE CARBOXYLASES

A DISSERTATION

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ABBREVIATIONS USED

1. PEPC'ase.....Phosphoenolpyruvate carboxylase
2. PEP.....Phosphoenolpyruvate
3. OAA.....Oxaloacetic acid
4. WT.....Wild-type PEPC'ase
5. CORD.....Co-regulatory/dissociation
6. CAD.....Catalytic activity deficient
7. NADH.....Nicotinamide adenine dinucleotide
8. FDP.....Fructose 1,6-bisphosphate
9. TCA.....Tricarboxylic acid cycle (Krebs)
10. EDTA.....ethylenediaminetetraacetic acid
11. MDH.....Malate dehydrogenase
12. IAC.....Iodoacetic acid
13. DTT.....Dithiothreotol
14. TPCK.....L-1-Tosyl-2-phenylethylchloro-methylketone
15. SDS.....Sodium dodecyl sulfate
16. TLC.....Thin layer chromatography
17. IgG.....Immunoglobulin G
18. BSA.....Bovine serum albumin
19. HTP-HA.....Hydrox apitite column

INTRODUCTION

The ability of any living organism to survive resides, to a large extent, in its ability to maintain an internal homeostatic environment, with respect to metabolite production and utilization. This homeostasis is controlled by the normal functioning of biochemical pathways within the organism. These pathways are in turn controlled by the action of specific protein molecules (enzymes). The enzymes, varying in size, shape and specificity, catalyze a particular reaction or group of closely related reactions.

One requirement for an enzyme to function properly is structural integrity of the protein molecule. Correlation between the structure, catalytic and regulatory relationships of these enzymes constitutes an important area of research. One approach to the study of these relationships is by alteration of the normal enzyme through genetic mutation, isolation of the defective enzyme and determination of the physical and chemical bases for the altered function. The enzyme studied in this research is PEPC'ase (phosphoenolpyruvate carboxylase) from E. coli K-12. The native protein has a molecular weight of approximately 400,000 as determined by sedimentation equilibrium analyses. Upon denaturation of the enzyme, analyses showed a single

species of approximately 100,000 molecular weight indicating that the carboxylase enzyme is composed of four subunits of identical size (Smith, 1971). PEPC'ase catalyses the essentially irreversible carboxylation of PEP by bicarbonate to oxaloacetate and inorganic phosphate (Maruyama, et.al., 1966). In some bacteria, it appears to fulfill an anaplerotic function in their metabolism (Kornberg, 1965). This bacterial enzyme is an allosteric protein whose activity is stimulated by acetyl CoA (Canovas, 1965), fructose 1,6-diphosphate (Samuel, et.al., 1966), and fatty acids (Izui, et.al., 1970), each of which might be expected to accumulate if the cycle were not operating efficiently. Activation of the enzyme by cytidine 5-diphosphate also compensates for aspartate inhibition under conditions where high concentrations of aspartate are required for pyrimidine biosynthesis (Samuel, et.al., 1966). Oxaloacetic acid produced through the action of this enzyme is used by the TCA cycle. This enzyme is also inhibited strongly by aspartate and by fumarate, malate and some other TCA cycle intermediates (Nishikido, et.al., 1965; Smith, 1970; Maeba, 1965). It appears that the enzyme is subjected to three types of regulatory processes: <u>contiguous</u> by acetyl coenzyme A and C-4 dicarboxylic acids, i.e. aspartate and malate; <u>precursor</u> activation by FDP, <u>compensatory feedback</u> by certain pyrimidine nucleotides.

Presently, two mutant E. coli organisms have been produced through treatment with N-methyl-N-nitro-N-nitroso-guanidine. One of the mutants designated CORD for co-regulatory/dissociation, has as much as 50-60 percent of its PEPC'ase activity, yet it will not sustain growth on media where glucose is the only carbon source. In the dimeric state, the CORD enzyme has essentially the same specific activity as the wild-type (WT); however, it is not activated by FDP or inhibited by aspartate. The second mutant enzyme in this study has less than 5 percent of the WT enzyme activity, and it can be grown on glucose media only if the media is supplied with TCA cycle intermediates. It does appear to produce more than 5 percent of the inactive enzyme protein. This mutant has been designated CAD for catalytic activity deficient. Neither of the mutant enzymes has been purified to homogeneity due to differences displayed by the mutants to normal extraction techniques.

It is the objective of this research to obtain the mutant enzymes in a homogeneous state through the use of an antibody prepared against the WT enzyme. Development of this technique allows examination of structural differences which may account for the catalytic and regulatory changes shown by these mutants. The results obtained may also shed some light on the structural requirements for catalysis, regulation and subunit interaction of this enzyme.

LITERATURE REVIEW

In 1836, years before Buchner's discovery of cell-free fermentation, J.J. Berzelius coined the term "catalysis". This term described the phenomenon by which some agents were capable, at lower temperatures, of accelerating chemical reactions which normally occurred at higher temperatures. By the middle of the nineteenth century, M. Traube proposed that this catalytic power of organic tissues resided in its proteins. The catalysts had been given the name enzymes by the end of that century. Since then and through the development of new biochemical techniques, research efforts have expanded in an attempt to elucidate the mechanism of enzyme actions as well as their structure and regulatory properties.

To study physical and chemical properties of proteins, it was first necessary to isolate and purify them in large quantities. This basic method was first accomplished in 1926 when Sumner crystallized urease from jackbean extracts. In 1930-36, Northrop and Kunitz crystallized pepsin, trypsin and chmotrypsin, these studies provided the first real proof that enzymes were indeed proteins. Another area of investigation which contributed to the correlation of structure-function relationships in enzymology was the sequencing of the protein insulin by Sanger and the resolution of the three dimensional structure of protein using X-ray

crystallographic techniques pioneered by Perutz and Kendrew.

The importance of the accurate functioning of an enzyme in many systems has long been recognized. Since the accuracy of function of an enzyme, including the allosteric enzyme is related to structure, a brief background is presented on the nature of the allosteric enzyme.

When enzymes act in a sequence so that the product of one enzyme becomes the substrate of the next, a multienzyme system or pathway is produced. The enzymes which are responsible for regulation of these multienzyme sequences are generally termed "allosteric" enzymes (other space; other site). An allosteric enzyme not only catalyzes a specific unique reaction in a sequence, but it has a second property not present in most enzymes; its catalytic activity is sensitive to the concentration of some crucial metabolites not necessarily related to its substrate. This metabolite is called a regulator and can have a positive or negative effect on the enzyme. Allosteric enzymes have been found to possess at least two types of binding sites: one for the substrate, termed catalytic site; and another for the regulator, termed effector site. Therefore, the activity of the allosteric enzyme maybe altered by molecules that are bound to sites other than the catalytic site (Theodore, et.al., 1964).

Several models have been proposed to explain allosteric

effects. The most common of these are the models of (Monod, et.al., 1965 and Koshland, et.al., 1966). The general properties of an allosteric system according to Monod are:

1. Most allosteric proteins are polymers which are associated in such a way that the subunits all occupy equivalent positions. This implies that the molecule possesses at least one axis of symmetry.
2. Allosteric interactions frequently appear to be correlated with alterations of the quaternary structure. In essence, alterations of the bonding between subunits.
3. While heterotropic effects (i.e. interaction between different lignads) maybe either positive or negative, homotropic effects (interaction between identical ligands) appear to be always co-operative.
4. Few, if any, allosteric systems exhibiting only heterotropic effects are known. In other words, co-operative homotropic effects are almost invariably observed with at least one of the two ligands of the system.
5. Conditions, or treatments, or mutations which alter the heterotropic interactions also simultaneously alter the homotropic interactions.

There is a co-operative binding effect in the allosteric system. The general premise of this model is that the enzyme can exist in two and only two conformations, the R and T states. In the absence of substrate, nearly all

terminal subunits and therefore the interior subunits each interact with two neighbors, whereas the two terminal units interact only with one neighbor. Thus in this model, the nature of subunit-subunit interactions are important. As illustrated in this representation, the structural integrity of the subunit-subunit interactions account to some extent for the enzyme's ability to bind the substrate. In essence, the Koshland sequential model makes three assumptions:

1. There are two conformational states accessible to any one subunit.
2. The binding of substrate changes the shape of the subunit to which it is bound. However, the conformation of the other subunits in this enzyme molecule is not appreciably altered.
3. The conformational change elicited by the binding of substrate in one subunit can increase or decrease the substrate binding affinity of the other subunits in the same enzyme molecule.

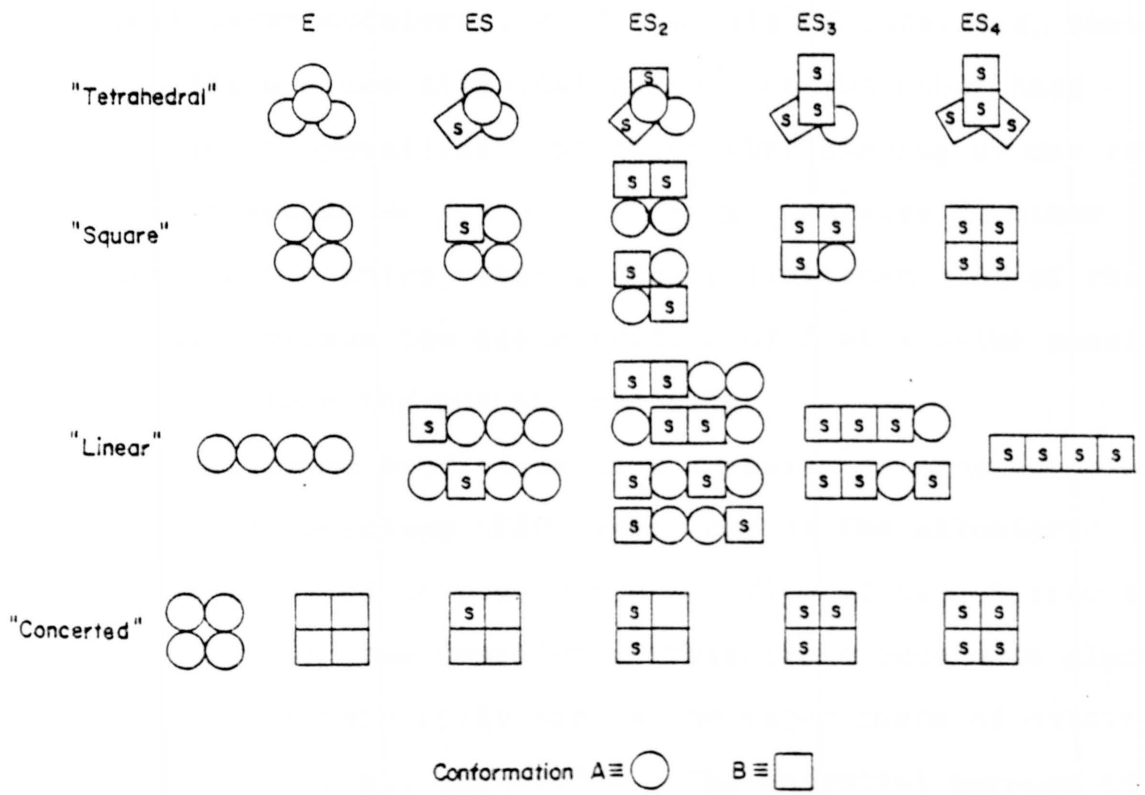
Another difference observed with the allosteric enzymes is the kinetic properties. Often these properties of the allosteric enzyme do not fit the hyperbolic curve associated with the Michaelis-Menten model of enzyme activity. Instead, when initial velocity is plotted against the substrate concentration, the curve is sigmoidal, particularly with homotropic allosteric enzymes. The sigmoid curve implies

molecules are in the T form if the enzyme shows sigmoidal binding with the substrate. Binding of the substrate to the T form will shift the conformation to the R form providing there is homotropic activation by the substrate. Allosteric activators (other than substrates) and inhibitors can also cause a shift in the enzyme forms. Activators appear to have a greater affinity for the R form, therefore shifting concentrations from T to R. On the other hand, inhibitors have a greater affinity for the T form, shifting concentrations from R to T. This considered to be the "concerted model" since the conformations of all subunits change simultaneously.

Koshland in 1966 diagrammed the various modes of binding of the ligand to a tetrameric enzyme for four major cases (Figure 1). Conformation A of the subunit is denoted by circles. Conformation B, the one capable of binding substrate S, is denoted by squares. In the models involving a progressive change, it is assumed that a subunit in Conformation B is present only when S is bound to it. In the inactive and/or tetrahedral case, each subunit presumably interacts with each of the other subunits. In the square case the subunits are arranged so that each subunit interacts with each of two neighbors, it being presumed that diagonal interactions are negligible. In the linear case, it is assumed that there is no interaction between

FIGURE 1: Schematic illustration of the various modes of binding the ligand S to tetrameric enzyme for the four major cases. Conformation A of the subunit is denoted by circles. Conformation B, the one capable of binding S, is denoted by squares. In the models involving a progressive change it is assumed that a subunit in Conformation B is present only when S is bound to it. In the concerted model all conformations change to B together.

BINDING OF LIGANDS TO A TETRAMERIC ENZYME



that binding the first substrate molecule to the enzyme enhances the binding of subsequent substrate molecules to other substrate sites (Lehninger, 1975). Allosteric enzymes are generally capable of showing positive or negative co-operativity or both. In the case of positive co-operativity, a rather small increase in substrate concentration can cause a very large acceleration of the rate of catalysis, thus producing a steep sigmoidal curve. On the other hand, negative co-operativity indicates that binding of one substrate molecule decreases the binding ability of other substrate molecules, thus giving a flattened plot of the initial V versus the concentration of S at a value considerably less than the maximum velocity.

The enzyme studied in this research is phosphoenolpyruvate carboxylase (PEPC'ase), one of the allosteric enzymes involved in regulating the flow of carbon into the TCA cycle for some organisms. This cycle occurs in almost all heterotrophic cells and is the major route of oxidative metabolisms of all foodstuffs. The essential purpose of this cycle is to produce energy and metabolites through oxidation of the acetyl CoA moiety to yield two molecules of CO_2. It is important to note that oxaloacetate, which is essential for operation of the cycle, is regenerated through the operation of the cycle, but the only source of this metabolite from outside the cycle is by the action of PEPC'ase.

This is true in many bacterial organisms such as E. coli. The following diagram shows the reaction catalyzed by this enzyme and its relationship to the TCA cycle (Figure 2).

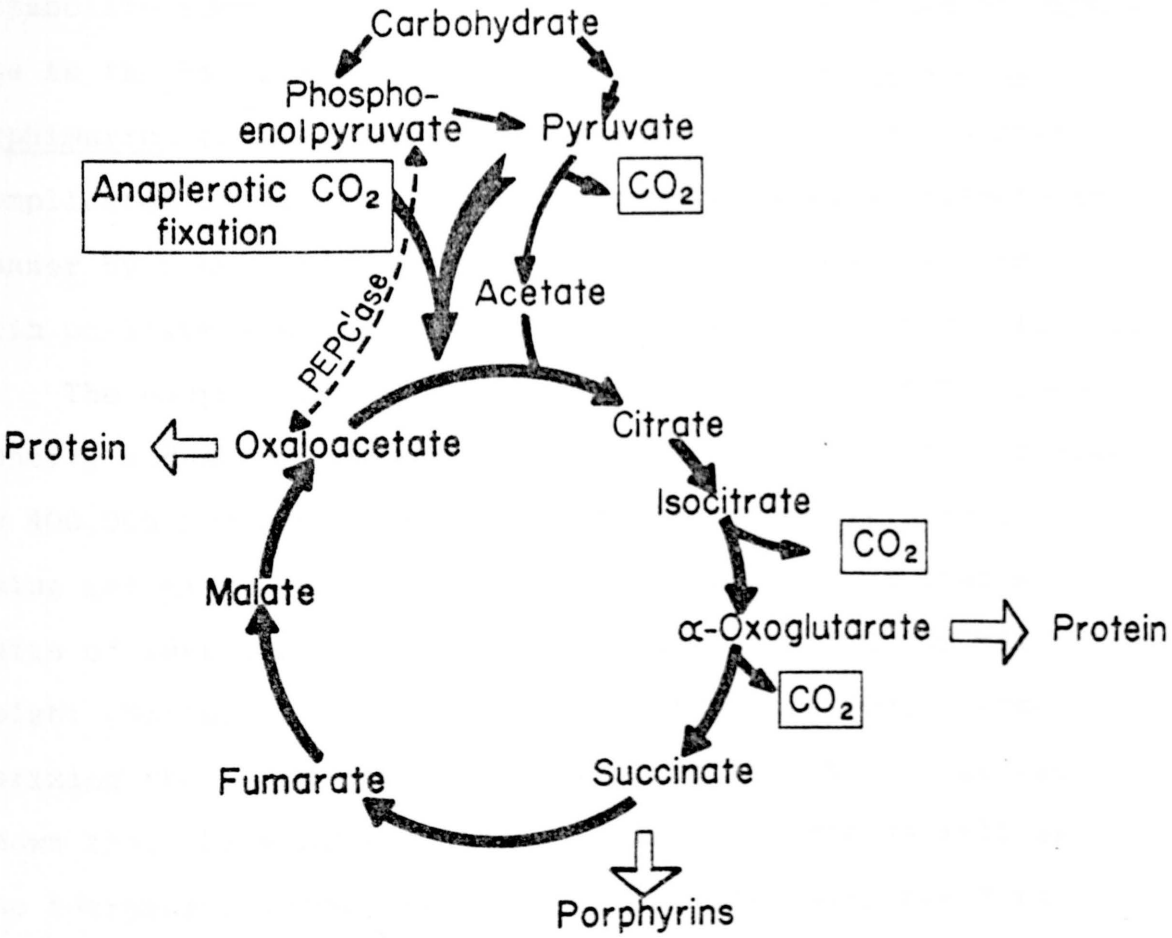
The function and hence the importance of this enzyme in metabolism was established when it was demonstrated that mutants devoid of its catalytic activity would not grow on medium where glucose was the sole carbon source. Instead, the organism required either some intermediate of the TCA cycle or a compound like glutamate (in the case of the enzyme under study in this paper, a compound like aspartate) that could easily produce a TCA cycle intermediate (Theodore, et.al., 1964; Ashworth, et.al., 1965). These observations clearly indicated that this reaction was the only viable one which could replace intermediates of the TCA cycle as carbon skeletons are pulled out of the cycle for growth. Thus, this enzyme is involved in an anaplerotic mechanism for replenishing oxaloacetate (OAA) for the cycle.

PEPC'ase was first discovered by Bandurski and Greiner (1953). The enzyme was extracted from plants and found to catalyze the carboxylation of PEP by bicarbonate to yield OAA and inorganic phosphate. The plant enzyme has a high affinity for bicarbonate and apparently functions primarily as a mechanism for trapping carbon dioxide as the four carbon dicarboxylic acid. The activity of the plant enzyme

FIGURE 2: Reaction sequence catalyzed by PEPC'ase and the relationship of the end product of the catalyzed reaction (oxaloacetate) to the TCA cycle.

In most bacterial systems the only source of OAA is by the action of PEPC'ase. Mutants devoid of PEPC'ase catalytic activity will not grow on medium where glucose is the sole carbon source.

RELATIONSHIP OF PEPC'ase TO THE TCA CYCLE



does not change in response to any intermediary metabolite and is therefore not subjected to any known allosteric control mechanism. Another class of PEPC'ase, represented by the Euglena enzyme shows allosteric inhibition by TCA cycle intermediates but is not activated by any known metabolite (Ohmann, et.al., 1969). The third class of PEPC'-ase is the bacterial enzyme of which the E. coli and S. typhimurium enzymes are representative. This is the most complicated of all in that it is regulated in an allosteric manner by a number of intermediary metabolites which have both positive and negative effects on its catalytic activity.

The enzyme from E. coli, B has been purified to homogeneity and shown to have a molecular weight of approximately 400,000 as determined by sedimentation equilibrium. Using gel electrophoresis, the enzyme consist of four subunits of identical size, each approximately 100,000 molecular weight (Smith, 1971). In a study by Smith (1968) characterizing the sedimentation behavior of the enzyme, it was shown that the enzyme could exist in a dimeric as well as the tetrameric form. Analyses of the partially purified enzyme in sucrose gradients indicated association-dissociation reactions involving the monomeric units. PEPC'ase has a S_{20w} of 8.4 (corresponding to a dimer) in the absence of substrates, metal ions, metabolic regulators or urea. This value of 8.4 decreases to 5.8 (considered a monomer) when

1M urea is included in the sucrose gradient mixture and increases to 12.2 with Mg^{++}. When aspartate is added, the $S_{20,w}$ of the enzyme reaches 13.2 (considered the tetramer). Neither PEP nor acetyl-CoA alone nor the two in combination can promote the association of the dimer to the tetramer in the absence of Mg^{++}. However, in the presence of urea, PEP, Mg^{++} and acetyl-CoA (which yield the dimer forms), activity assays indicate the dimer has 62-75 percent of maximum activity. Since Mg^{++} and other divalent metal ions exist in the cell at a consistent concentration, it is implied that the enzyme exists in the cell as a tetramer, and that association-dissociation normally is not involved in regulation activity.

As noted, this bacterial enzyme is activated by acetyl-CoA (Canovas, et.al., 1965), fructose-1,6-bisphosphate (Izui, et.al., 1970), GTP and by long chain fatty acids or their CoA derivatives (Taguchi, et.al., 1977); Izui, et.al., 1970). Furthermore, it is activated by various organic solvents such as dioxane and alcohols which are presumed to bind at the same site as the fatty acids (Katsuki, et.al., 1967). Studies on the activators or PEPC'ase and their binding sites are used for identification of the amino acid moieties forming the allosteric sites, along with the spatial arrangement of the allosteric sites and the active site on the surface of the enzyme. Studies on the interaction of

the effectors of PEPC'ase have also been done in order to determine binding sites (as related to synergism) and mechanisms of regulation. Smith (1977) demonstrated through kinetic analyses, that fructose-1,6 bisphosphate (FDP) can function very effectively as an augmenting ligand and synergistically with acetyl-CoA, can reverse the inhibitory effect of aspartate. However, the inhibition by aspartate is still competitive with acetyl-CoA. Two mechanisms for the interaction of the enzyme, PEP, FDP and acetyl-CoA have been proposed. In these schemes, binding of these moieties is random. Figures 3A and 3B show the proposed interactions, where E-PEPC'ase, A=acetyl-CoA, B=FDP and S=PEP. The activation of PEPC'ase by FDP is kinetically anomalous by comparison with that of acetyl-CoA (Smith, 1977). It appears that FDP is independent of the order of addition of the reaction components, and that binding of FDP makes the enzyme more accessible for interaction with PEP or acetyl-CoA. The enzyme complex resulting from the binding of each activator, including PEP, has increased affinity for the other activators. It would appear that the cellular concentration of FDP is not high enough for it to function alone as an effective activator for the enzyme. However, it may "prime" the enzyme for more effective activation by preventing the immediate binding of inhibitors, thereby causing an easier transition of the enzyme from the inactive

FIGURE 3A: Pathway for the interaction of PEPC'ase, PEP and FDP. In this scheme, binding of these metabolite is random.

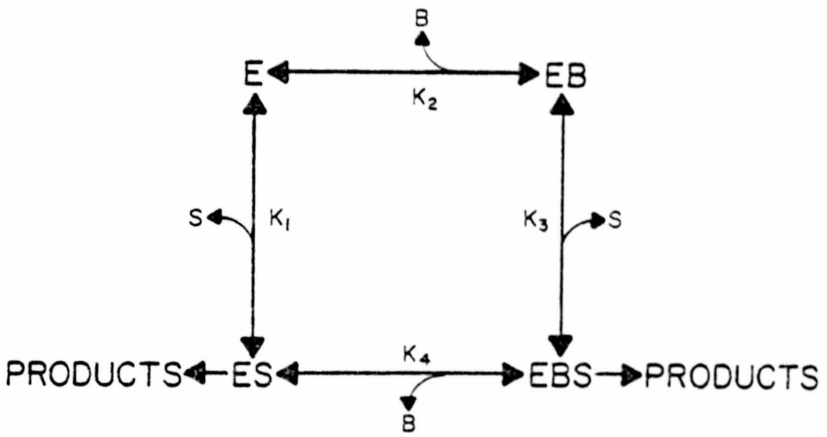
FIGURE 3B: Proposed interactions of PEPC'ase, PEP, acetyl-CoA and FDP. Again the scheme shows the binding of these metabolite is random. Binding of FDP is independent of the order of addition of the reaction components. It appears that binding of FDP makes the enzyme more accessible for interaction with PEP or acetyl-CoA. The enzyme complex resulting from the binding of each activator including PEP, has an increased affinity for the other activators.

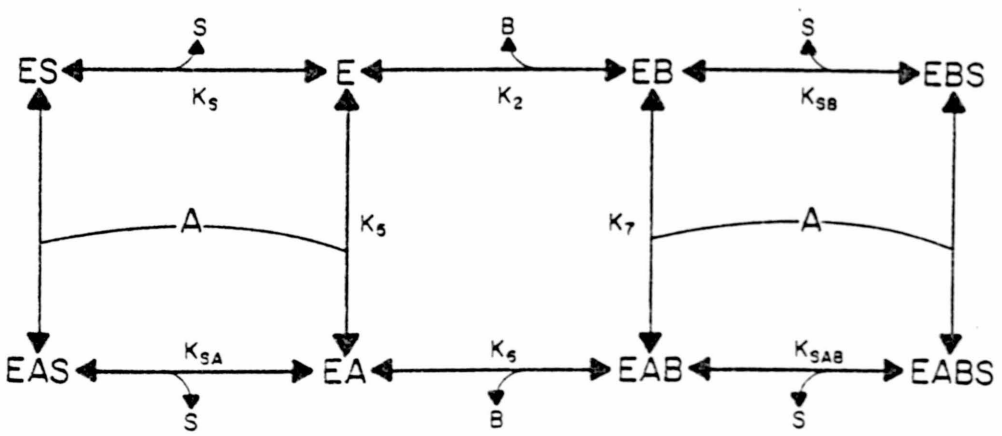
E = PEPC'ase

A = acetyl-CoA

B = FDP

S = PEP


B
E
EB
K2
S
K1
K3
S
PRODUCTS
ES
K4
EBS
PRODUCTS
B


S
B
S
ES
E
EB
EBS
K5
K2
K58
A
K5
K7
A
K5A
K6
K5AB
EAS
EA
EAB
EABS
S
B
S

to the active conformation.

Silverstein and Willis (1973) demonstrated that activation of this enzyme by acetyl-CoA and laurate was additive rather than synergistic, and that synergistic relief from aspartate inhibition did not occur with these two activators. This suggested that they may share the same binding site. Silverstein also indicated that fatty acids, fatty acyl CoA derivatives as well as acetyl-CoA may share the same binding site.

Certain nucleotides have been included as PEPC'ase activators. It was observed that synergistic activation of the enzyme occurs with acetyl-CoA and all of the co-activators, but there is no observed synergistic activation with FDP and other activators. Synergistic activation implies separate binding sites, and would be expected to occur only under conditions where separate binding sites exist for activators. The fact that acetyl-CoA could activate the enzyme in a synergistic manner with all other activators suggested that the binding site for acetyl-CoA was different from that of each of the activators. However, according to Smith (1980) synergism was not observed with any combination of FDP, CDP or GTP suggesting this group of activators all interact at the same site---totally or partially (Figure 4). This might also suggest that the interaction of either of them with the enzyme mutually

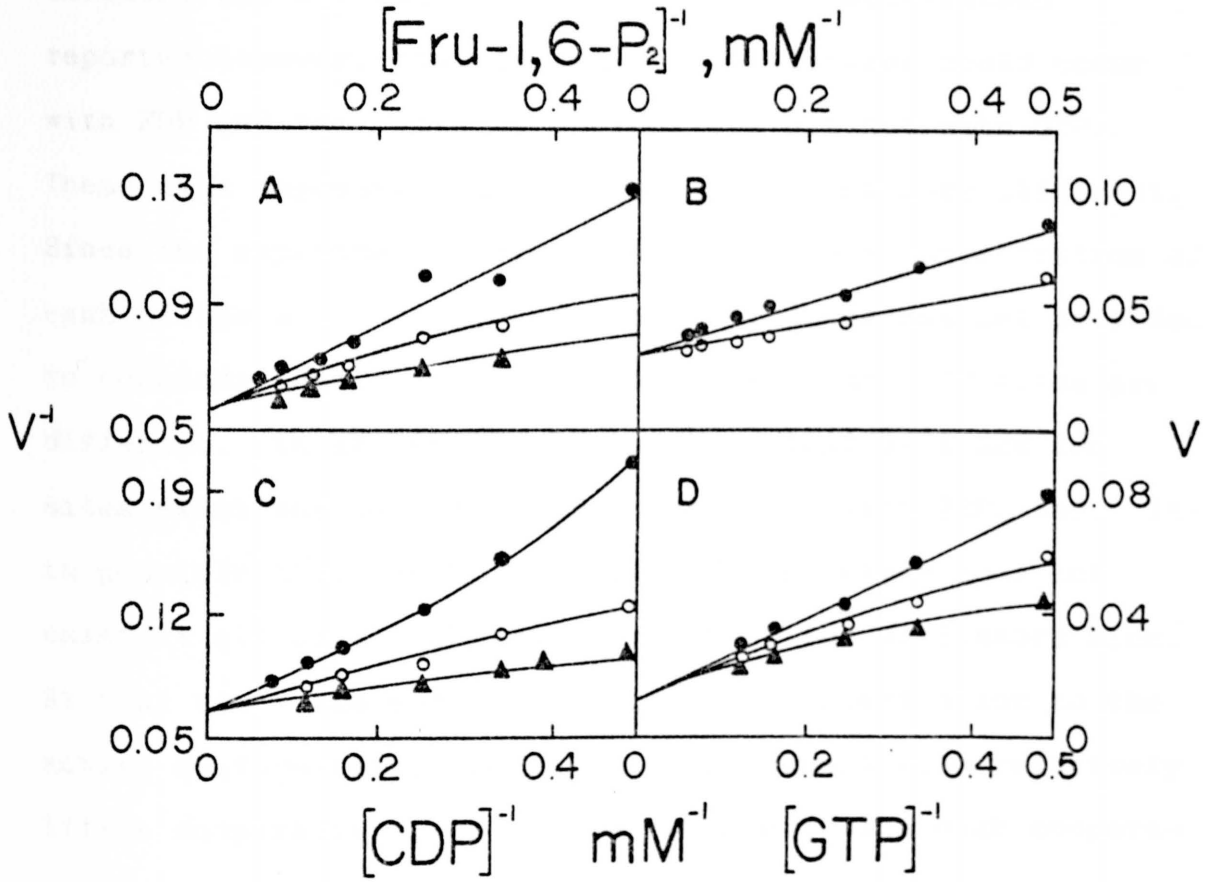
FIGURE 4: Graphical representation of the competitive activation of P-enolpyruvate carboxylase by fructose-1,6-bisphosphate (FDP), CDP, and GTP.

A. Double reciprocal plots of velocity versus FDP concentration at 0 mM (●), 1 mM (0), and 3 mM (▲) concentrations of CDP.

B. Double reciprocal plots of velocity versus FDP concentration at 0 mM (●) and 2 mM (0) concentrations of GTP.

C. Double reciprocal plots of velocity versus CDP concentration at 0 mM (●), 0.5 mM (0), and 2 mM (▲) concentration of FDP.

D. Double reciprocal plots of velocity versus GTP concentration at 0 mM (●), 1 mM (0), and 2 mM (▲) concentrations of FDP.


[Fru-1,6-P2]-1, mM-1
0
0.2
0.4
0
0.2
0.4
0.5
A
B
C
D
0.13
0.09
0.05
0.10
0.05
0
0.19
0.12
0.05
0.08
0.04
0
V-1
V
[CDP]-1 mM-1 [GTP]-1

excludes the interaction of the others. Silverstein reported however, that synergistic activation could occur with FDP and the cytidine nucleotides but not with GTP. These data suggested that the binding sites were different. Since the experiments were done at only one concentration of each of the activators, sufficient evidence was not provided to conclude that the cytidine nucleotide and FDP sites are different. In any event, it is clear that at least two sites exist for Co-activator interaction with PEPC'ase. It is possible that the active form of the enzyme does not exist at all until substrate or one of the activators bind. At that time, the enzyme undergoes an isomerization to the active conformation, yielding active dimers with relatively little cooperativity or active tetramers with high cooperativity.

The activators do fit into at least two classes and can cause different effects relative to subunit-subunit interaction (Smith, 1980). The nucleotides and FDP (Type I) presumably bind at one site and acetyl-CoA (Type II) at another site on the enzyme. FDP like aspartate, stabilizes the teterameric form of the enzyme in the active conformation. Not only do these two classes exhibit differences in the subunit-subunit interactions, they show a difference in cooperativity effects. Virtually no cooperativity is shown with acetyl-CoA whereas significant cooperativity exists for

the Type I interaction. The PEPC'ase dimer active species also require acetyl-CoA for activation. However, little cooperativity is observed by acetyl-CoA activation alone. This suggest that the association between dimers is a loose one.

The primary inhibitor of PEPC'ase appears to be aspartate which is produced in a single reaction sequence from OAA. In competitive binding studies using Salmonella enzyme, neither acetyl-CoA, FDP nor GTP alone could displace aspartate from its binding site. However, FDP with acetyl-CoA or GTP with acetyl-CoA could effectively prevent the binding of aspartate. The enzyme alone shows low cooperativity and appears to exist in an inactive state which binds aspartate more easily. Aspartate even in the presence of substrates can inhibit the enzyme completely. Wohl and Markus (1972) showed that the limit of aspartate inhibition approached 100 percent as the concentration of aspartate approached infinity. In the inactive state with bound aspartate, the enzyme is more stable to elevated temperatures (Izui, et.al., 1967). Under these conditions, the enzyme exists predominantly as the tetramer and shows increased cooperativity with respect to PEP binding. Other strong inhibitors appear to be malate and fumarate which are intermediates of the citrate cycle. Several other compounds have been reported to be mild inhibitors of the enzyme such

as citrate, succinate and isocitrate. Corwin and Fanning (1968) suggested that the best inhibitors of PEPC'ase were four-carbon dicarboxylic acids substituted with either an amino or a hydroxyl group.

Smith and Gold (1974) compared the effects of various allosteric inhibitors on the enzyme. The results of the study indicated that there was a relationship between the stereochemistry of the potential inhibitor and its ability to reduce activity or stabilize the tetrameric form of the enzyme. Most of the inhibitors were competitive with acetyl-CoA and shared with aspartate the ability to stabilize the enzyme. Malate also inhibited activity but it did not compete with acetyl-CoA or stabilize the enzyme. However, the report showed that each class of inhibitors is competitive with the other class of inhibitors and shared the same binding site. Pertinent information obtained from the study included:

1. Compounds that are four-carbons in length with carboxyl groups at both ends or three carbons in length with a carboxyl and a SH group at opposite ends, are able to bind at the inhibitor site of the enzyme and stabilize the tetrameric form of the enzyme.
2. At pH 8.5, the optimum for PEPC'ase activity, both of the end groups of the potential inhibitors must be at least partially ionized. This implies that the groups

on the enzyme to which they bind are electronegative.

3. The degree of inhibition caused by these compounds, is also related to the type of moiety substituted on the carboxyl groups. The strongly inhibitory compounds have electronegative substitutions in this position.

The tetrameric form of the enzyme is not only stabilized by allosteric inhibitors but also by high concentration of protein, Mg^{++} ions and ethanol. However, this does not prevent the enzyme fron undergoing a spontaneous inactivation that is apparently related to its dissociation. The rate of inactivation, as expected, is decreased by conditions that stabilize the tetramer form. Data presented by Smith and Perry (1973) showed the dimer and tetramer forms of the enzyme could undergo inactivation by dissociation to monomer units. The tetramer can dissociate directly to monomers and become inactivated or it can go to the dimer state and then to the monomer state before being inactivated. Monomer-to-dimer reassociation occurs to form a catalytically active species, but monomer-to-tetramer reassociation to an active species is not apparent. In order for reassociation to occur, Mg^{++} or aspartate must be present. If the enzyme is in 1M urea, the tetrameric and dimeric form of the enzyme can be maintained.

The regulation of PEPC'ase in E. coli is also

important, if the system is expected to function and the organism survive. At least three different mechanisms are known for the regulation of the enzyme. These mechanisms include:

1. Precursor or feed-forward activation which results from the interaction of FDP in anticipation of the need for the enzyme's activity. When E. coli is growing on glucose as the sole carbon source, the continuous metabolism of glucose is essential. The interaction of FDP with these enzymes would be very important in assuring that the steady-state intracellular concentrations of the allosteric inhibitors are not so effective that they inhibit these reactions altogether.
2. Contiguous regulation characterized by acetyl-CoA and by C-4 dicarboxylic acids as aspartate and malate. This is the classical allosteric regulation which results from the interaction of these metabolites that are closely associated with the reaction catalyzed.
3. Compensatory feedback activation results from the interaction of pyrimidine nucleotides to counteract the increased concentration of aspartate resulting from the inhibition of aspartate transcarbamylase by CTP.

A great degree of structural integrity must exist in order to accommodate so many specific binding sites on each subunit. Even if the binding sites are not close to each

other or overlapping, alterations in one might significantly influence the ability of the other to function. Figure 5 shows the mechanisms of PEPC'ase regulation.

Some structural studies have been done using PEPC'ase in regard to essential amino acids necessary for catalytic and regulatory interactions. Structural analyses of the enzyme from E. coli indicated the enzyme has thirty-six cystine residues per mole which appeared to exist as free sulfhydryl groups. At least six of the eight SH groups of the enzyme are essential for its catalytic activity, since it has been reported that modification of more than two of the groups by certain effectors affected the activity of the enzyme. Although conclusive evidence is lacking, it is most likely that the essential SH groups are located at or near the active site. Recent reports have also indicated the importance of arginyl residues (Kameshita, et.al., 1978). By chemically modifying the enzyme with 2,3 butanedione, it was observed that one residue was necessary for the catalytic activity of the enzyme. In addition, it was indicated that one to two arginyl residues were involved in the regulatory interaction of the enzyme with FDP. These observations that inactivation and desensitization occurred independently with butanedione modification, implies that the arginyl residue essential for catalytic activity is distinct from those required for the sensitivity to FDP.

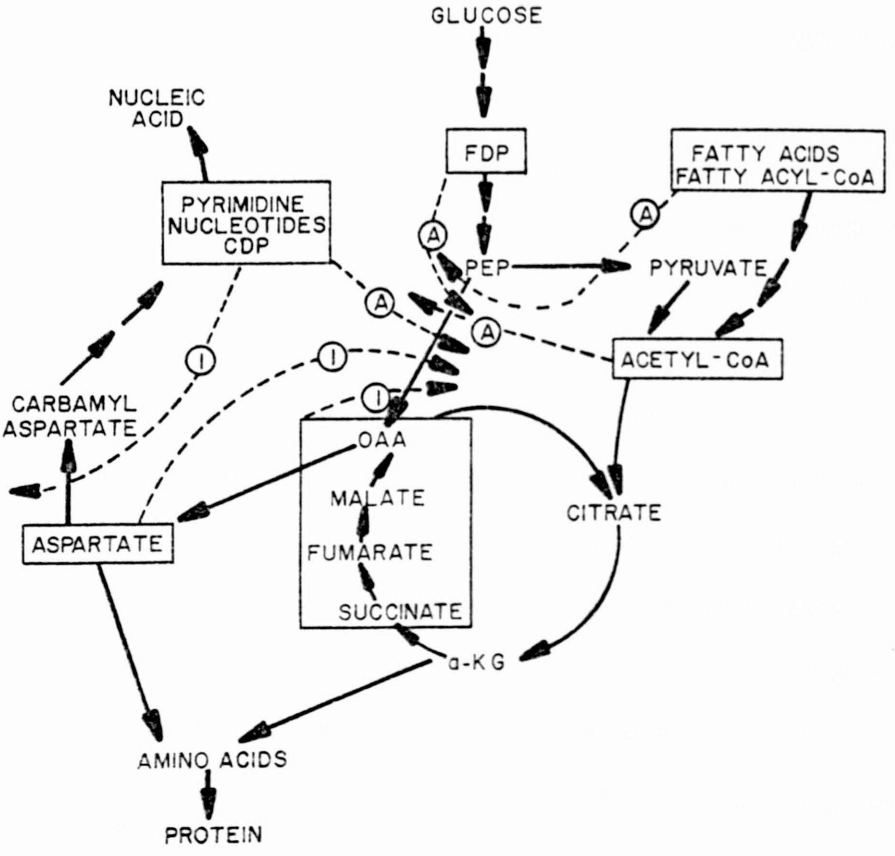
FIGURE 5: Mechanisms of PEPC'ase regulations in metabolism.

The three types or regulatory processes to which PEPC'ase is subjected are: 1) contiguous by acetyl-CoA and by C-4 dicarboxylic acids as aspartate and malate. 2) precursor activation by FDP and 3) compensatory feedback activation by pyrimidine nucleotides as CDP.

A = activators

I = inhibitors

MECHANISMS OF ENZYME REGULATION IN METABOLISM



Carboxylation of *P*-enolpyruvate

Studies done by Naide, et.al., (1979) also demonstrated the involvement of lysyl residues in the function of the bacterial enzyme. Here again the enzyme was modified with TNBS (2,4,6 trinitrobenzene sulfonate), a reagent known to attack amino acid groups. It was reported that the lysyl residues participated in the regulatory activity by FDP, GTP and aspartate. Suggestions were also made that the lysyl residue necessary for the regulation by aspartate or malate was different from the one or ones necessary for regulation by FDP and GTP. This implication based on these findings suggests that the sensitivity of the enzyme to aspartate was maintained whereas those to FDP and GTP were lost upon modification in the presence of malate. The fact that inactivation and desensitization did not occur concomitantly upon TNBS modification suggest that the lysyl residues necessary for the catalytic activity of the enzyme is not the same as those necessary for the regulation by the above effectors. The presence of malate during modification protected the enzyme not only against desensitization to aspartate but also against inactivation. This presumably due to a conformational change at the catalytic site caused by malate bound at the allosteric site.

Certain proteases have recently been used to study the catalytic and regulatory properties of this enzyme (Kameshita, et.al., 1979). The investigation centered

around the effect of protease treatment on the enzyme's activity and sensitivity to effectors, and the resulting conformational changes occurring. The study showed that three types of kinetics occur in the inactivation of PEPC'ase by the proteases. The first type of inactivation which is rapid initially and gradually tappering off, suggested the formation of an intermediate enzyme species which retained some enzyme activity and became resistant to further attack. This type of reaction was characteristic when trypsin and pepsin were used. The second type of reaction showed a constant rate of hydrolysis of a peptide bond which was essential for catalytic activity. The proteases which exhibited this type of reaction were papain, ficin and bromelain. The third type of inactivation showed increasing rate of proteolysis with time suggesting an intermediate species was produced which was susceptible to more proteolytic attack. Subtilisin and chymotrypsin demonstrated this type of inactivation.

The ability of the different effectors to influence inactivation during the protease treatment was also studied. GTP and acetyl-CoA gave strong protection against inactivation, whereas FDP offered moderate protection. In contrast, laurate stimulated the inactivation. Combined addition of FDP and acetyl-CoA protected the enzyme activity suggesting that these combined activators induced a different

conformational change in the enzyme then when the activators were used alone. It was reported that aspartate not only protected the enzyme from proteolytic attack but caused a temporary activation of the enzyme.

As demonstrated from this literature review, very few studies have been undertaken to determine comparative structural differences in a mutant versus the WT enzyme. This procedure may provide a more direct method of detecting structural areas involved in catalysis and regulation. In addition, no studies thus far have attempted to use an antibody to purify the enzyme, mutant or wild-type. From the literature review, most of the structural studies have used a partially purified enzyme thus limiting the type of information that can be obtained. These studies have also centered around determining specific amino acids necessary for catalytic and regulatory activities. This indicates that there exists a need for further structural elucidation of the mutational differences of the enzyme. It appears reasonable that immunological studies could also be useful in determining the differences that exist in the tertiary or quaternary structures of the enzyme, and surface receptors related to a specific antibody.

Two mutant enzymes from E. coli K-12 have been produced in our laboratory in order to study the relationship between the structure, catalytic and the regulatory properties of

this enzyme. These two organisms have been genetically altered using N-methyl-Nnitroso-N-nitroguanidine. The mutants either fail to grow or grow very slowly on medium where glucose is the only carbon source. The first mutant has been designated as CORD for co-regulatory/dissociation and the second mutant designated CAD for catalytic activity deficient. Both mutants are similar in that they will grow at near wild-type (WT) rate when the medium is supplied with an intermediate from the TCA cycle.

The CORD mutant has been partially characterized through kinetic studies and found to have as much as 50-60 percent of its PEPC'ase, it was found to have a diffuse fast moving activity band in 5 percent polyacrylamide gels when stained for activity using fast violet. This suggested that the enzyme had a greater negative charge or that it existed in a more highly dissociated form.

McAlister (personal communication) in characterizing the mutants found a difference in the sedimentation of the CORD and the WT enzymes. Sucrose gradient sedimentation analyses of the WT enzyme in the presence of a complete assay mixture, a 5mM $MgSO_4$ mixture, a 5mM $MgSO_4$ plus 10mM FDP mixture, FDP alone and 5mM aspartate gave S values of 13.4, 11.0, 12.7, 12.2 and 12.2 respectively. In similar experiments with the CORD enzyme, the S values were 8.1, 7.6, 8.1, 8.0 and 7.9 respectively. Thus the CORD mutant

is not stabilized in the tetrameric conformation in the presence of any of the reaction components. However, the S values were more similar if no additions were made to the sucrose gradients, S values being 7.2 and 6.6 for the WT and CORD, respectively.

A measure of the specific activity of the mutant and WT enzymes as a function of protein concentration also indicated that the CORD enzyme was more active in the dissociated state. This inverse relationship between protein concentration and specific activity suggest that higher aggregates can be formed but that they show considerably diminished catalytic activity. Figure 6 shows a decrease in specific activity upon dilution suggesting that the most active species of the CORD enzyme is the dimer.

Aspartate has been shown to increase cooperativity as demonstrated by changes in the PEP saturation curves. At 1mM aspartate, a sigmodial curve was obtained from the WT enzyme, however, with the CORD enzyme at even 10mM aspartate, the saturation curve was hyperbolic. Thus, aspartate is not capable of inducing cooperative interactions with the CORD enzyme. Figure 7 shows the result of these studies.

The mutant enzyme described has lost several very important properties. Among them are the ability to interact effectively with aspartate, FDP, CTP, GTP and the loss of cooperativity in its interaction with substrates and

FIGURE 6: Activity of CORD and WT enzyme showing an inverse relationship between the CORD protein concentration and specific activity. The graph suggest that higher aggregates of the CORD enzyme can be formed but they show considerably diminished catalytic activity.

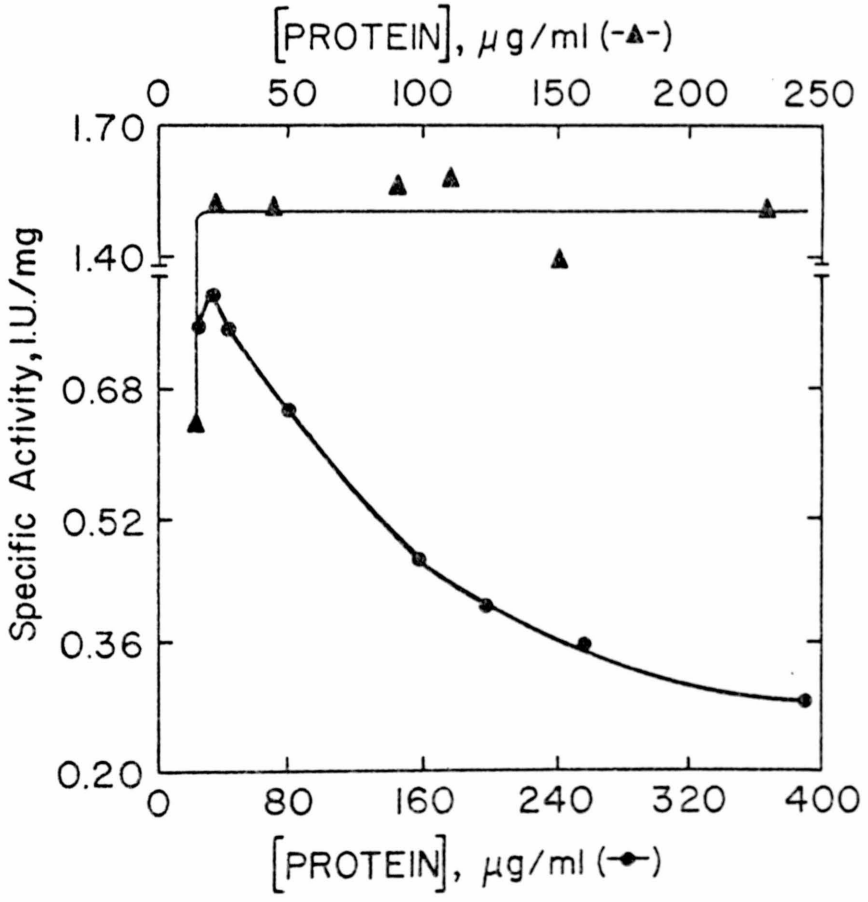
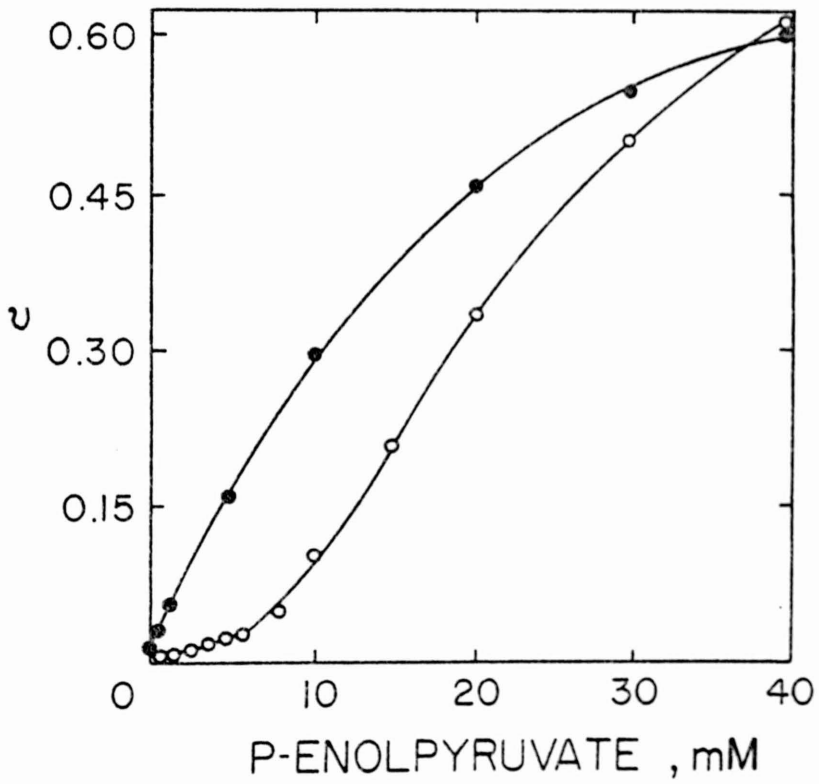

[PROTEIN], μg/ml (-▲-)
0
50
100
150
200
250
1.70
1.40
0.68
0.52
0.36
0.20
Specific Activity, I.U./mg
0
80
160
240
320
400
[PROTEIN], μg/ml (-●-)

FIGURE 7: The effect of aspartate on the WT and CORD enzymes.

I. The CORD mutant enzyme shows no co-operativity in the presence of aspartate upon substrate binding. This is illustrated by the hyperbolic curve.

II. The sigmoidal curve of the WT enzyme illustrates cooperativity in the presence of aspartate upon binding of the substrate to the enzyme.


0.60
0.45
0.30
0.15
v
0
10
20
30
40
P-ENOLPYRUVATE , mM

activators. Figures 8 and 9 show the interaction of the CORD and WT enzymes with FDP. Figure 8 demonstrates no activation of the CORD mutant with FDP but activation with acetyl-CoA. The insert in Figure 8 shows that activity exists in situ, but the activity is greater in the extract, where the enzyme exists in a more dilute form. Figure 9 compares the reaction of the CORD and WT enzymes with respect to FDP activation.

The CAD mutant enzyme has not been characterized kinetically or with regards to its ability to bind substrates and activators due to the fact it can not be assayed for activity by the classical assay mixture used for the WT or CORD enzymes. However, the CAD mutant shows about 5 percent as much activity as the WT cells. The mutant organism can be grown on media supplied with TCA cycle intermediates. The inability to extract and assay the CAD mutant indicates the importance of the development of the immunological studies undertaken in this research.

FIGURE 8: Interaction of the CORD enzyme with FDP. The graph demonstrates no activation of the CORD enzyme with FDP but activation with acetyl-CoA. The insert shows *in situ* activity. The *in situ* activity is greater in the extract where the enzyme exists in a more dilute form.

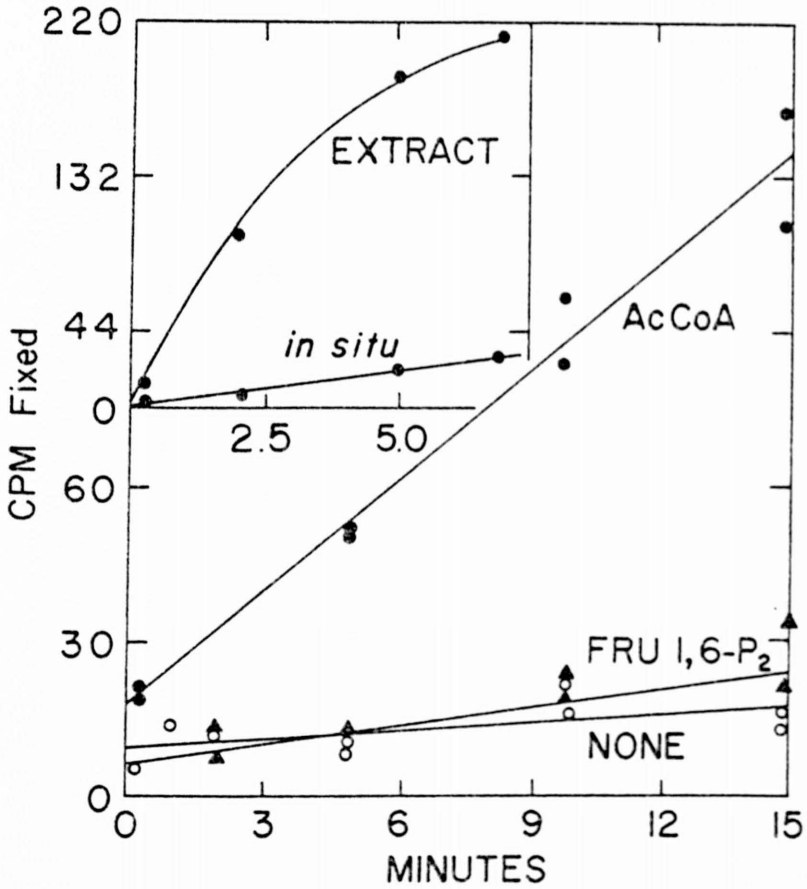
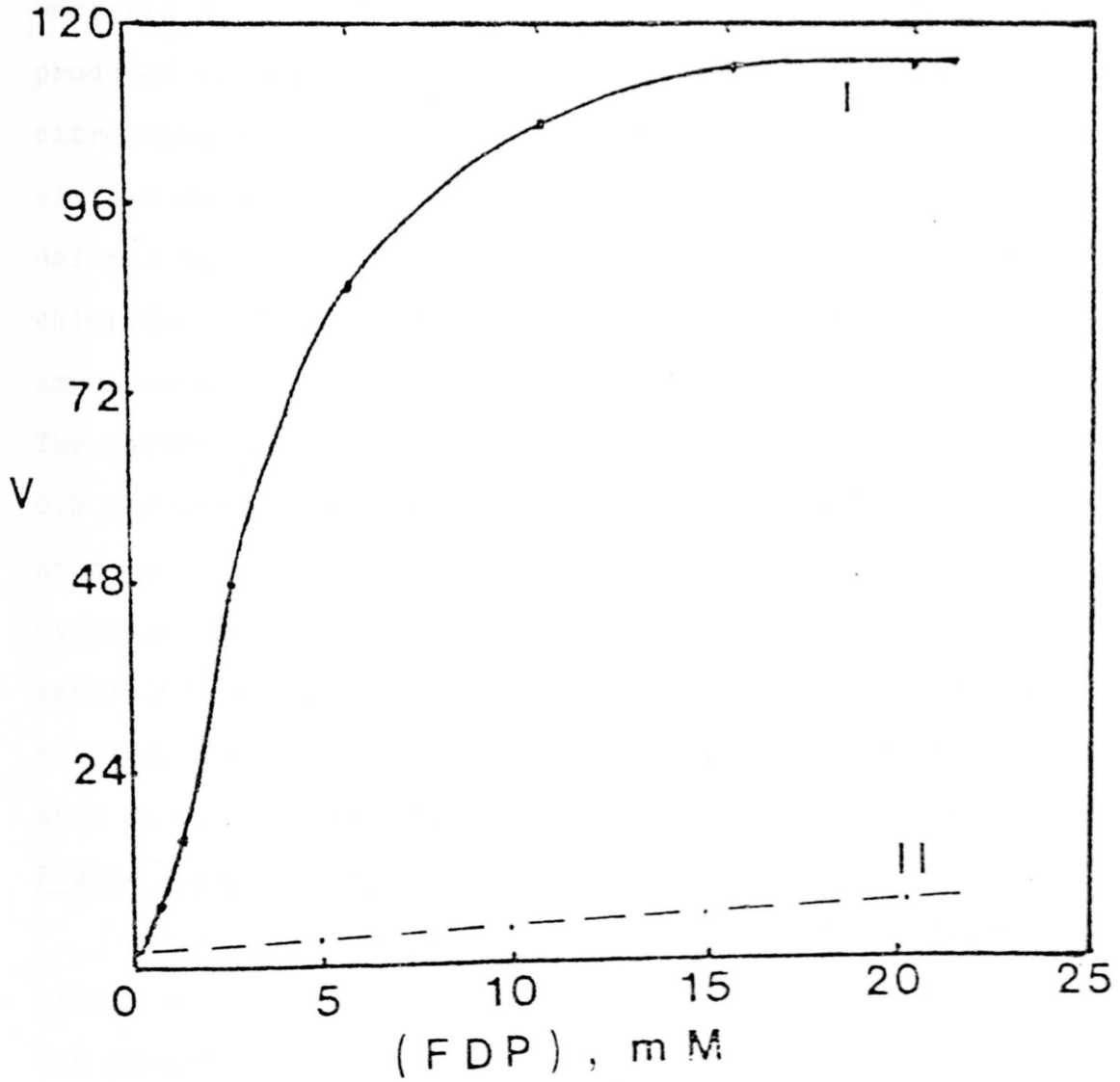

220
132
44
0
60
30
0
CPM Fixed
EXTRACT
in situ
2.5
5.0
AcCoA
FRU 1,6-P2
NONE
0
3
6
9
12
15
MINUTES

FIGURE 9: Reaction of the WT and CORD enzymes with FDP activation.

I. The WT enzyme shows activation by FDP.

II. The CORD enzyme is not activated by FDP.

F D P ACTIVATION



METHODS AND MATERIALS

The source of the enzyme used in these studies was the bacteria E. coli K-12. The mutant enzyme, which had been produced in our laboratory by treatment of the bacteria with nitrosoguanidine was extracted from this K-12 strain in a similar manner. The organisms were grown in a chemically defined medium of dihydrogen phosphate (.15%), calcium chloride (.001M), magnesium sulfate x 7 H_2O (0.02M), ammonium sulfate (0.2M) and dibasic sodium phosphate (1.64M). The carbon source was glucose at a final concentration of 0.2 percent. When the mutants were to be grown, aspartic acid was also added to the medium. The cells were grown overnight at 37°C, collected by centrifugation and washed several times with 0.15M KCl. Each batch of cells was weighed and stored frozen until enough material was available for enzyme purification (150-400g.).

Enzyme Purification

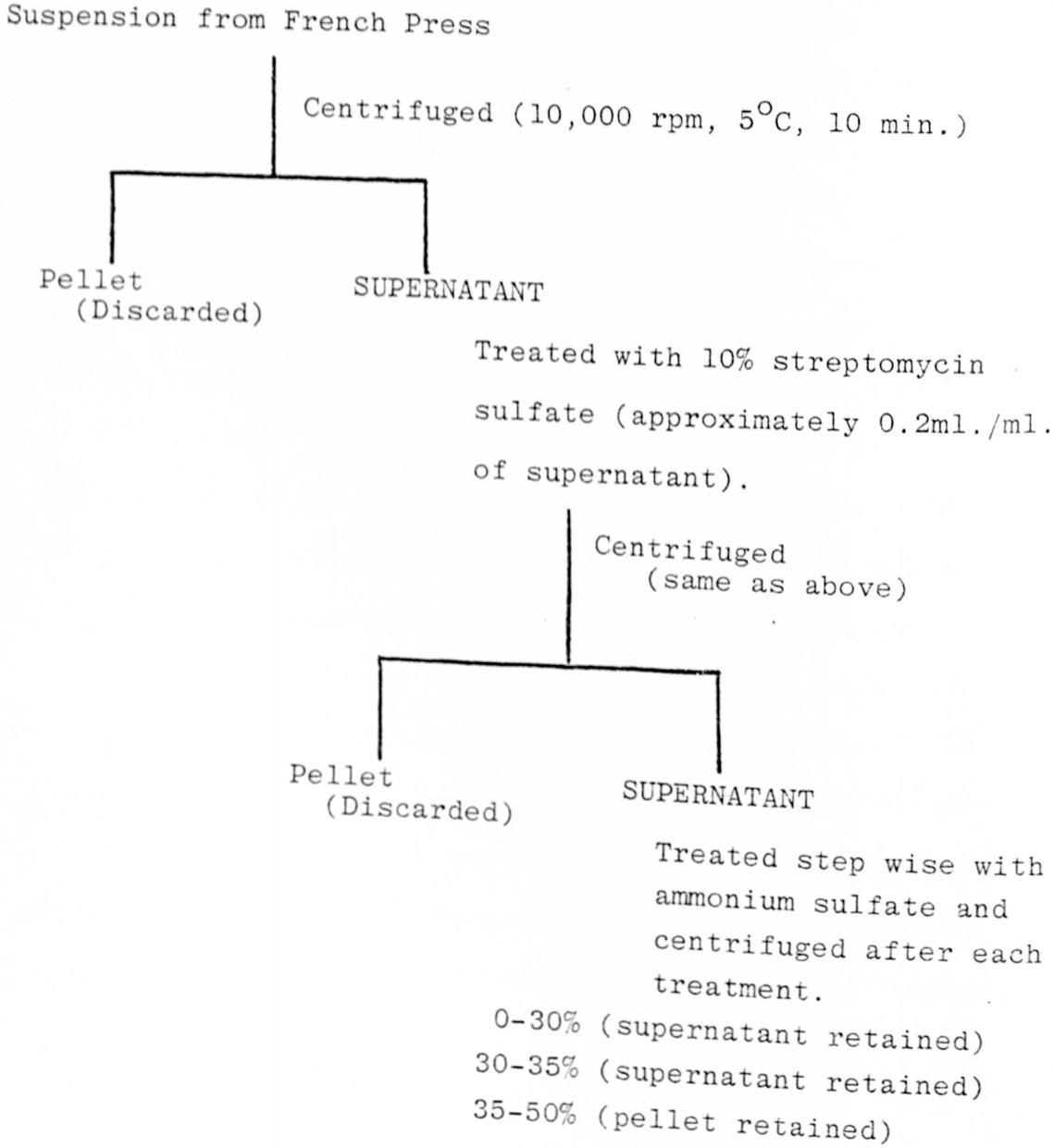
The enzyme was purified according to the procedures of Smith (1971). The following diagram is a flow sheet of the purification procedures (Figure 10).

1. The frozen cells were placed in an equal volume of PEA buffer pH 7.8 (phosphate 10^{-2}M; EDTA 10^{-4}; aspartate 5×10^{-3}M).

2. The cell suspension was mixed by homogenization in a Waring blender at medium setting. The cells were then broken in a French press. This extract was diluted 1:1 with the PEA buffer and centrifuged for 10 minutes at 10,000 rpm (all centrifugation was done at 10,000 rpm in the Beckman J21 rotor). The temperature during the purification procedure was 5°C.

Each supernatant retained from the ammonium sulfate precipitation was assayed for activity to monitor the effectiveness of the purification step. The pellet from the 35-50 percent precipitation was dissolved in the extraction buffer and assayed for activity and protein content. The enzyme activity was measured spectrophotometrically by following the rate of oxidation of NADH at 340nm in a coupled reaction with malate dehydrogenase. A unit of enzymatic activity is defined as the amount of enzyme necessary to catalyze the formation of one micromole of OAA per minute per ml. reaction mixture measured by the coupled reaction. The assay mixture for the wild-type (WT) enzyme was Tris (0.5M) HCl, pH 8.5, $KHCO_3$ (0.1M), MgSO4 (0.1M), PEP (0.1M), MDH (2mg./ml.) and FDP. The FDP was omitted when the CORD enzyme was assayed. Protein was determined by the standard Lowry technique and by the $A_{280/260}$ ratio when the enzyme solutions were essentially free of nucleotides.

FIGURE 10: FLOW SHEET FOR ENZYME PURIFICATION;



Heat Treatment

The resulting supernatant (in aliquots of approximately 40mls.) was made 50mM with aspartate. This solution was heated quickly to 50°C in a water bath and held for three minutes, then cooled rapidly to below 20°C in an ice water bath. The precipitated protein was pelleted by centrifugation and discarded. The supernatant generally contained better than 80 percent of the starting activity. This solution was then dialyzed overnight against 0.01M PEA containing 10^{-4}M mercaptoethanol to remove excess aspartate. This heat procedure was only used for the WT enzyme since the CORD and CAD mutant enzymes are not protected by increased concentrations of aspartic acid.

Column Purification

1. The next purification step involved the use of ion exchange chromatography on DEAE cellulose (DE-52, Whatman). The columns were maintained at 10°C with the use of circulating refrigerant. They were developed with a linear gradient or PEA ranging from 0.05M to 0.2M in phosphate at a flow rate of 64mls./hr. The flow rate was maintained constant and the gradient developed with the use of an ISCO gradient maker. The column was equilibrated overnight at the same flow rate with 0.01M PEA before loading the dialyzed enzyme.

2. Gel filtration on an AcA-34 column was used to resolve

the proteins by molecular size. The column was developed with 0.02M PEA buffer at a constant flow rate of 32mls./hr. Equilibration of the column was accomplished overnight with the same buffer prior to use. After elution of the protein (as determined by 280nm absorbence) the column was rinsed with 0.02M PEA containing 0.02M azide and stored in the cold for further use.

3. Hydroxylapatite (HTP) chromatography for abosrption purification was the last column used. The column was developed using a two step gradient varying the buffer concentration from 0.02M to 0.073M PEA in the first step and 0.073M to 0.25M PEA in the second step. Before applying the enzyme, which was extensively dialyzed, the column was equilibrated with 0.01M PEA for 5-6 hours.

Sucrose Gradient Centrifugation

This was the final method employed for purification. A linear sucrose gradient ranging in concentration from 5 percent to 20 percent (w/w) in 0.01M PEA buffer was used. The sucrose solutions were made 20mM with respect to aspartate. The gradient was developed at 25,000 rpm for 48 hours at 5°C. The enzyme, having an $S_{20/w}$ of 12-13, sedimented faster than the other proteins under these conditions. It was collected, assayed for activity and protein, concentrated and frozen for the remaining studies.

It should be noted that the enzyme from the above

columns was collected from tubes having the highest ratio of activity to protein. PEPC'ase was concentrated by precipitation with 55 percent ammonium sulfate. In each case, the pellet was dissolved in 0.01M PEA and dialyzed extensively against the same buffer except when the AcA-34 column was used. This method of purification and activity assay was done for both the WT and CORD enzymes. However, assays for the CAD enzyme were done using an antibody elicited against the purified WT enzyme. During the extraction procedures for the CAD enzyme, the sample fractions were reacted with the antibody using the Ouchterlony technique. Only the sample tubes giving a positive reaction were taken through the next purification step. The two mutant enzymes could not be purified to homogeneity by aforementioned protein extraction method. This was due to the ease by which the enzyme underwent dissociation resulting in multiple species that could not be stabilized by aspartate or the fact that one of them could not be assayed directly. Thus, for the assays using the antibody, each step in the purification was increased by one day. Complete purification of the mutant enzymes was obtained by a test tube precipitation of the two enzymes with the antibody followed by electrophoresis on a 9 percent polyacrylamide gel. The enzyme band was cut from the gel and the protein eluted. The complete method is described in a following section.

Electrophoretic Procedures

Disc tube gels of 5 percent acrylamide were run on native enzyme samples (WT enzyme only) at pH values of 8.5, 7.5 and 6.5. These gels were stained for activity as well as for protein. SDS gels of 9 percent acrylamide were also employed using the method of Laemmli (1970). These two gel techniques were used to verify the purity of the WT enzyme preparation, that is the presence of one band in both systems.

Immunological Procedures

Once the WT enzyme was shown to be homogeneous, aliquots were injected into a female goat in an attempt to elicit an antibody response. Approximately 500ug. of the enzyme (mixed with Freunds complete adjuvant) were initially injected into the animal subcutaneously. The animal was boosted every two weeks with 350ug. until serum from the goat gave a positive Ouchterlony test (when tested with the crude and purified WT enzyme). After the antibody response was accomplished, the animal was injected every three weeks and 150-225mls. of blood drawn before each booster injection. The injections following the initial one were given in the lymph glands under the neck at 0.1ml. per site on injection. The immunoglobulins from the serum were then partially purified by a modification of the method of Weir. This procedure was as follows:

1. The serum (from the clotted blood) was dialyzed overnight against 0.1M phosphate buffer pH 8 (if the amount of serum collected was below 100mls., the volume was adjusted to 100mls. with the phosphate buffer).
2. 18g/100ml. of sodium sulphate was added and the proteins allowed to precipitate. The suspension was centrifuged at 10K rpm for 15 minutes at 10^{o}C.
3. The supernatant was discarded and the pellet dissolved in 60 percent of the original volume with buffer. This solution was clarified by low speed centrifugation.
4. The supernatant proteins were precipitated with 12g/100 ml. sodium sulphate, centrifuged and again the pellet dissolved in buffer (approximately 30 percent of the original volume).
5. Final precipitation was effected using 12 percent sodium sulphate (w/v). This suspension was centrifuged, the pellet dissolved in an appropriate amount of buffer and clarified with low speed centrifugation.
6. The resulting supernatant was dialyzed overnight against several changes of 0.01M $KHPO_4$ pH 7.7. The precipitated protein was pelleted and discarded and the supernatant assayed for protein using the $A_{280/260}$ ratio.

This method produced a partically purified IgG fraction. For more complete purification (as done with the first IgG fraction), the suspension was placed on an equilibrated

DE-52 resin column and eluted with 0.01M $KHPO_4$. A control (pre-injection) IgG serum was also partially purified. All fractions were stored frozen for further use. The protein banding pattern of the IgG fraction was determined by electrophoresis on 9 percent SDS acrylamide tube gels.

The Ouchterlony immunodifussion slide test was the first test used to determine specificity of the enzyme for the antibody elicited against the WT enzyme. Four and one half mls. of the reaction medium were pipetted onto a standard bacterial slide. The medium consisted of 1.5 percent Noble agar dissolved in a buffered saline solution pH 7 (0.1M sodium chloride; 0.05M sodium phosphate; 0.01 percent sodium azide). In some cases, the antibody 20ul., was placed in the center well with varying dilutions of the enzyme placed in the surrounding wells. In the second case, the enzyme was placed in the center well and varying concentrations of the antibody placed in the surrounding wells. The antigen-antibody reaction was carried out in a closed humid chamber overnight at room temperature.

Rocket Immunoelectrophoresic

The rocket technique allowed quantification of the two mutant enzymes through the use of a standard protein curve, constructed from the rocket results of the WT enzyme. This procedure used a slide medium which consisted of a one percent agarose in a Tris-glycine-barbital buffer (Buffer 1;

sodium barbital 2.07g/l, barbital 13g/l: Buffer 2: glycine 56.3g/l, Tris 45.2g/l). These two buffers were mixed in a 1:1 ratio before use to give a final pH of 8.6. This was also the electrophoresis buffer used to run both experiments. The following procedures were employed for the rocket test:

1. Fifteen/mls. of the antibody-containing agarose was poured onto the slide. After congealing (10-20 min.) the wells were punched out using a template as a guide. Five wells (horizontal) were used per glass plate.
2. The gel plate was placed on the electrophoretic apparatus and the plate connected to the buffer system by means of paper wicks (Whatman #1). The system was cooled to 15-20^{o}C with a circulation water bath.
3. Samples of the enzyme were applied in the wells in increasing concentrations of 1:20, 1:15, 1:10 and 1:2. The samples were diluted with the running buffer.
4. The samples were electrophoresed at 280V for 3.5 hours. Upon completion of the run, the gel plates were pressed under pressure, washed with 0.1M NaCl (2X), rinsed in distilled water and dried at 70^{o}C. The plates were stained with Coomassie blue (5g/l in 450ml. ethanol, 100mls. acetic acid and 450mls. water) for 5 minutes. Destaining was with acetic acid, water and ethanol. The precipitant arcs were measured and a correlation made between the heights obtained with the enzyme and the mutant enzymes.

This method allowed for the quantification of enzyme present in the two mutants, relative to that in the WT strain.

Crossed-Immunoelectrophoresis

The combination of electrophoretic separation of a protein in agarose followed by electrophoresis in an antibody containing gel in a direction perpendicular to the first, makes this technique superior by providing a higher resolution. Furthermore, the area enclosed by an individual precipitate is proportional to the antigen-antibody ratio of the enzyme. Also the number of antigenically reactive units of the progein enzyme can be detected.

For this two-dimensional system, native (aliquots of the three enzymed diluted in the barbital buffer) and SDS treated samples were used. Sixty microliters of each enzyme were mixed with a sample buffer solution containing barbital buffer, SDS and bromophenol blue. The aliquots were boiled for two minutes and used in the first dimension. The two-dimensions were carried out as followed:

1. 15mls. of the heated one percent agarose in barbital buffer was poured onto the glass plates. After gelation, three wells were made using a template as a guide.
2. 20ul. of the sample enzyme were applied to each of the three wells and the gels connected to the buffer by

means of the paper wicks.

3. Electrophoresis in the first dimension was carried out at 280V for three hours at a constant temperature of 15°C which was maintained by a circulating water bath.
4. After the time allotted, a 5mm broad edge was cut around the gel plate. The gel was then divided into three slabs and transferred to the second plate.
5. For the second dimension plate, 15mls. of the antibody containing agarose were poured onto the first dimension slab and the antibody-agarose solution allowed to congeal.
6. After approximately ten minutes, the solidified gel was placed on the electrophoretic apparatus, connected to the buffer by means of paper wicks and electrophoresed overnight at 80V. Temperature was maintained at 15°C.
7. The completed plates were then treated as above with pressing, washing and rinsing. The plates, including a slab from the first dimension, were stained, destained and then photographed.

Inhibition Test (Binding ability of WT and Cord Enzymes):

Two types of classical precipitation test were used to determine the binding properties of the enzyme to the WT elicited antibody. Binding differences were determined by the ability of the IgG serum to inhibit the activity of the WT and CORD mutant. The first inhibition test could not be

carried out using the CAD mutant enzyme since the amount of inhibition exerted by the antibody was followed spectrophotometrically. The other two enzymes were diluted with 0.01M PEA such that the activity per ml. was equivalent.
The following is an example of the experimental procedures:

IgG#2 (50mG/ml.) Diluted 1:7
CORD (131.4U/ml.) Diluted 1:5
WT-Wild Type (450U/ml.) Diluted 1:20
Control Serum Diluted 1:7

Buffer	21	19	17	15	13	11	9	7	5	3	ul.
Serum	5	5	5	5	5	5	5	5	5	5	
Enzyme	4	6	8	10	12	14	16	18	20	22	

After incubating the reaction mixture overnight in the cold, the samples were centrigufed and the supernatant assayed for activity.

The second titration experiment used, employed reacting all three enzymes with the IgG in larger quantities. The precipitated enzyme-antibody complex obtained was sufficient to be used for purification of the mutant enzymes. In this reaction, the antibody was added in such quantities that the activity of the WT and CORD enzymes were almost totally absent. The aliquots of enzyme, buffer and IgG fraction were mixed and allowed to react overnight in the cold. This

solution was centrifuged and the supernatant assayed for activity. Fifteen ul. of each enzyme were removed and to this were added 10ul. of the WT enzyme having a known activity. The three enzymes were assayed along with a control WT for activity. This constituted the second assay. The supernatant fraction was assayed a third time using another aliquot of 10ul. of the enzyme and 15ul. of the WT control enzyme. Calculations were made to determine the inhibitory ability of the antibody on the three enzymes.

The pellet from the enzyme-antibody complex was fractionated into component parts by SDS tube gel electrophoresis on 9 percent acrylamide. The enzyme bands along with the heavy chain moiety of the IgG immunoglobulin were excised and the protein eluted from the gel (complete procedures will follow). The PEPC'ase protein and the heavy chain IgG protein were assayed and a ratio established. This procedure also allowed for a measurement of the binding ability of the three enzymes to the antibody.

Peptide Mapping

The pellet from the antibody-antigen reaction was washed with 0.1M. NaCl (2X), centrifuged and dissolved in an SDS sample buffer. Aliquots of each enzyme were electrophoresed on a 9 percent SDS acrylamide gel to separate the enzyme from the IgG fractions. Upon completion of the electrophoresis, one sample gel from each enzyme series was

stained for 25 minutes in a solution containing final concentrations of 0.1 percent Coomassie blue, 50 percent methanol and 10 percent acetic acid. The excess stain was removed from the gel by treatment in a solution of 5 percent methanol and 10 percent acetic acid for one hour. The stained gels were matched against their corresponding unstained gels and areas in the unstained gels corresponding to the position of the enzyme band in the stained gels were excised. Elution of the enzyme from the gel band was aided by homogenization of the gel in an SDS peptide sample buffer (0.125M Tris-HCl pH 6.8, 0.5 percent SDS, 10 percent glycerol and 0.001 percent bromophenol blue) Cleveland (1977). Protein was determined by the Lowry procedure on the eluted enzyme before treatment with the various proteases.

The purified enzyme fractions were then treated for three hours with the various proteases. The proteases were added in an amount equivalent to 1 percent (w/w) of the enzyme protein that was to be digested. The two proteases used were trypsin and chymotrypsin. The enzyme/protease solution was immersed in boiling water to stop the reaction. This solution was then placed on an exponential gradient slab gel for electrophoresis (Finkelstein, in press). The gradient gel (15-8 percent acrylamide) was run in a Tris-glycine buffer, pH 8.3 (0.05M Tris, 0.38M glycine, 0.1 percent SDS and 0.002M EDTA), at 35-40ma until the dye

reached approximately one half inch from the bottom of the slab plate. The gels were stained in 0.05 percent Commassie blue, 0.05 percent amido black dissolved in a solution of acetic acid, methanol and water. Destaining was in a 10 percent solution of acetic acid. The gels were photographed and evaluated for changes.

Two Dimensional Chromatography and Mapping

The enzymes were precipitated as before with the antibody. The rinsed pellet was suspended in an appropriate amount of freshly prepared 0.1M Tris-HCL buffer pH 8.5 containing 6M granidinium hydrochloride and 0.01M iodoacetic acid (IAC). The solution was allowed to incubate in the dark with shaking for 30 minutes. This solution was made 0.02M with dithiothreitol (DTT). Each enzyme solution was then dialyzed overnight against 0.2M ammonium bicarbonate pH 8. The enzymes were collected and lyophylized. The PEPC'ase was then dissolved in SDS sample buffer and electrophoresed using the Laemmli method (1971).

Enzyme bands containing the protein were eluted according to the previous procedures and dissolved using 0.2M ammonium bicarbonate pH 9.5. These protein aliquots were assayed for protein, dried, and stored in the dried state for further use.

Digestion of the enzymes with trypsin-TPCK and chymotrypsin-TLCK was carried out in 0.2M ammonium bicarbonate for the two-dimensional chromatrography mapping and SDS peptide mapping sample buffer for the gradient slab gel mapping. The enzyme-protease solutions were incubated at 37^{o}C for six hours with the protease being added in two steps; one-half at zero time and the other half at three hours. After digestion, the SDS-enzymes were loaded and

electrophoresed on the gradient gel.

The enzymes in the ammonium bicarbonate buffer were spotted on TLC cellulose plates, approximately .2 to 1 nanomole of protein per plate. The sample spots were placed 3cm. from each edge in one corner of the plate. The chromatogram was electrophoresed for one and one half hours at 350V with the spot placed initially closest to the anode. The plate was then dried and chromatographed in the second direction. Upon completion, the chromatogram was dried and sprayed with triethylamine followed by a flourescamine spray (Felix, et.al., 1974; Tilly, et.al., 1974; Kempe, et. al., 1974).

Special Solutions

1. Electrophoresis Buffer: pyridine; acetic acid; water (1:10:89). The pH of this solution was approximately 3.7.
2. Chromatography solvent: 1-butanol:pyridine:acetic acid: water (50:33:1:40).
3. Fluorescamine Spray: 0.1% fluorescamine (w/v) in acetone.
4. Triethylamine: 10% triethylamine in methylene chloride.

RESULTS

Production of a Homogeneous WT Enzyme

All purification procedures were standard according to Smith (1971). However, modification on one of the columns was necessary. This modification allowed for a cleaner separation of the protein fractions on the ion exchange HTP column. The largest portion of the enzyme was eluted in the 0.073M to 0.15M range of the PEA buffer, with activity showing a peak at approximately 0.087-0.097M range PEA.

A typical graph showing a sucrose gradient separation is shown in Figure 11. The specific activity of fractions obtained indicated that the PEPC'ase enzyme was probably pure. Other tests substantiated this. Upon concentration of the enzyme from the sucrose gradient, the following data were obtained:

UNITS/ML.	18.46
PROT. (MG./ML.)	0.20
UNITS/MG.PROT.	92.30

Tube gel electrophoresis using the 9 percent SDS acrylamide and a 5 percent native acrylamide gel were run on the purified WT enzyme. The 9 percent SDS gel showed one band, even with other loading, (Figure 12C), indicating the presence of a homogeneous enzyme. The native gels, without

FIGURE 11: Graph showing the sedimentation pattern of the WT enzyme in a sucrose gradient. The sucrose gradient was linear varying in concentration from 5 percent to 20 percent (w/w) sucrose in 0.01M PEA buffer, made 20mM with aspartate. The sedimentation was developed at 25,000 rpm for 48 hours, 5°C. The enzyme, having an $S_{20/w}$ of 12-13 sedimented near the bottom of the tube under these conditions.

A = Enzyme associated with peak A.

Sucrose Gradient for WT Enzyme

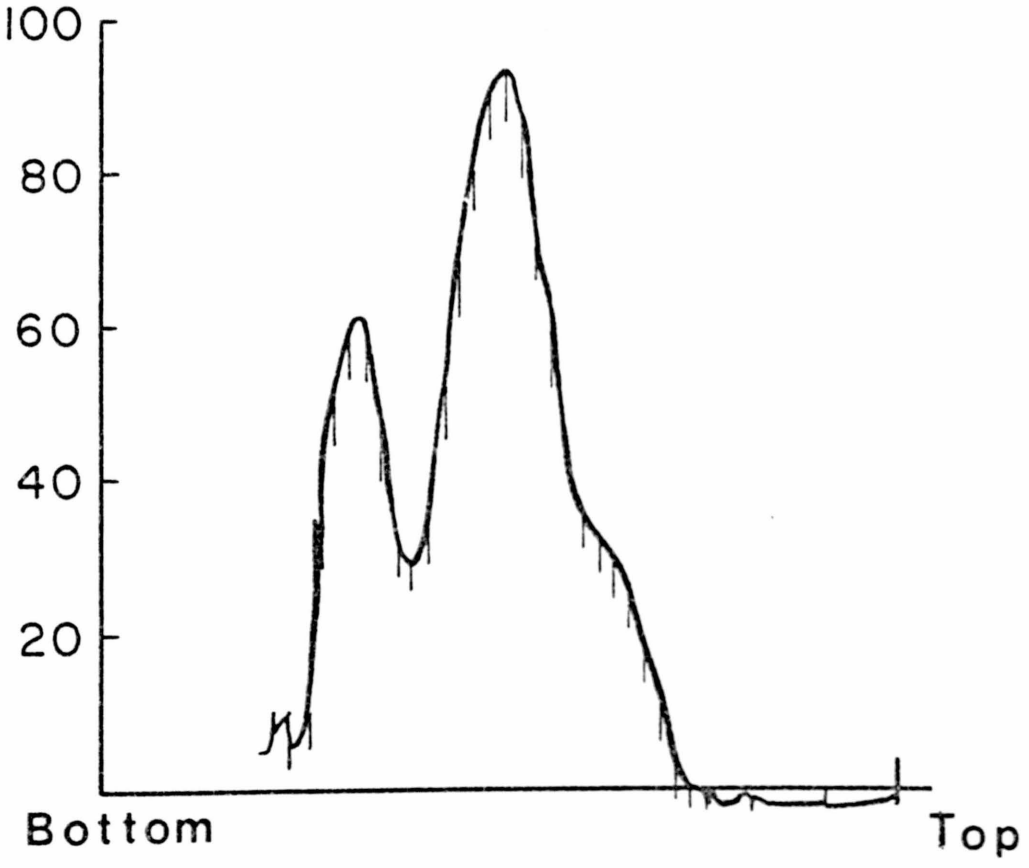


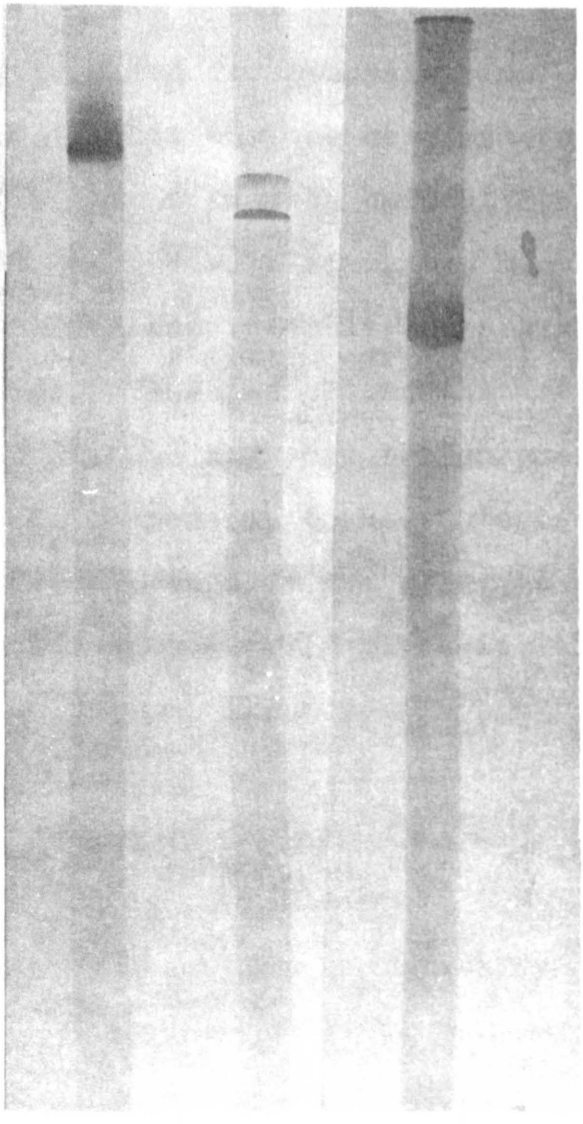
FIGURE 12: Results of WT enzyme electrophoresed on 5 percent native and 9 percent SDS treated tube gels.

A. Native gel of 5 percent acrylamide with a 50 percent increase in aspartic acid (pH 6.5).

B. Native 5 percent acrylamide gel using the standard concentration of aspartic acid (pH 6.5).

C. Purified WT enzyme electrophoresed on 9 percent SDS acrylamide gel. The presence of one band indicates a homogeneous enzyme species.

The second band shown in B was a product of dissociation of the purified WT enzyme due to a change from the optimum pH of 7.8. An increase in aspartic acid stabilized the tetrameric form of the enzyme thus reducing dissociation (A).


A
B
C

SDS, run at pH values of 6.5 (Figure 12B) and 7.5 (not shown) showed a second band. Because of the results with SDS, this second band was believed to be due to dissociation of the enzyme which may occur when the pH differed from the optimum normally required for stabilization of this enzyme. To determine if this was the case, the gels were rerun in the same buffer but with a 50 percent increase in aspartic acid. At pH 6.5 where dissociation was most noticeable, aspartic acid decreased the intensity of staining of the second band (Figure 12). Thus indicating that it most likely resulted from dissociation and that the enzyme was indeed pure. It was proper, therefore, to use this preparation to elicit an antibody response in the goat. A summary of the complete purification procedure is given in Table 1.

TABLE 1: SUMMARY OF PURIFICATION PROCEDURES.

PROCEDURES	VOL	U/ML	PROT	SP.ACT.	YIELD	PURIF.
1. Crude	600	14.0	17.47	0.8	100	1
2. Strep	650	9.0	17.19	0.52	69.6	0.65
3. A.S.	99	81.9	42.64	1.92	96.6	2.4
4. Heat	98	65.75	28.16	2.33	76.6	2.9
5. DE-52	16	281.36	43.38	6.48	---	8.1
6. ACA-34	3.4	546.62	29.75	18.37	---	23.0
7. HA-HTP	1	627.0	17.35	36.14	---	45.2
8. SUC/GR	11.9	18.46	0.2	92.3	---	115.4

Notes on steps in table: (2) Supernatant after streptomycin sulfate precipitation of nucleic acids. (3) Ammonium sulfate precipitate between 35 & 50 % saturation. (4) Heated in the presence of 50 mM aspartate at 50 degrees for 3 min. (6) Tow ACA-34 columns were run. The first column, the cut was to steep. From the #2 column, two fractions were collected. Fraction 2, which contained 6 ml: 165.27 U/ml : 35.08 mg/ml (protein) was frozen. (8) After the sucrose gradient, the enzyme was concentrated to a total volume of 2 ml using the Amicon pressure filtration system.

Immunodiffusion Results

The first indication of an antibody production was four weeks after the initial injection of 500ug. of the enzyme. Immunodiffusion tests (standard Ouchterlony slide test), using a partially purified IgG fraction showed a positive reaction to the purified PEPC'ase. Another WT enzyme purified up to the hydroxylapatite step was also used to test the specificity of the antibody (Figure 13). The specificity of the antibody was important since the CORD and CAD enzymes could not be purified past the HA column by standard techniques. However, since the two mutant enzymes were needed in the homogeneous state for studies of structural differences, specific antibodies would be needed to effect that purification. The antibody (IgG #6 fraction) reacted with the crude CORD enzyme also, using the standard Ouchterlony test. Figure 14 shows the precipitant lines formed from this reaction. The diffusion test also determined if there was identity between the WT and CORD enzymes.

The CAD enzyme was partially purified using the standard procedures for the WT enzyme. However, location of the enzyme during purification was carried out by reacting aliquots of the tubes from each extraction step with the IgG fraction. Using this method, it was shown that the CAD enzyme would produce a positive Ouchterlony reaction with the WT specific antibody (Figure 15). In each slide

reaction, the CAD enzyme showed two precipitant bands, also the precipitant bands formed were very light. However, this method still indicated that the CAD enzyme could precipitated.

FIGURE 13: Ouchterlony immunodiffusion test showing specificity of the antibody for the purified WT enzyme and the partially purified enzyme.

A. Purified enzyme showing precipitin band formation. 20ul. of the IgG fraction was placed in the center well with 15ul. of the enzyme in each of the outer wells.

B. Specificity of partially purified WT enzyme to the IgG fraction. The IgG fraction was placed in the center well with the partially purified enzyme in each of the outer wells.

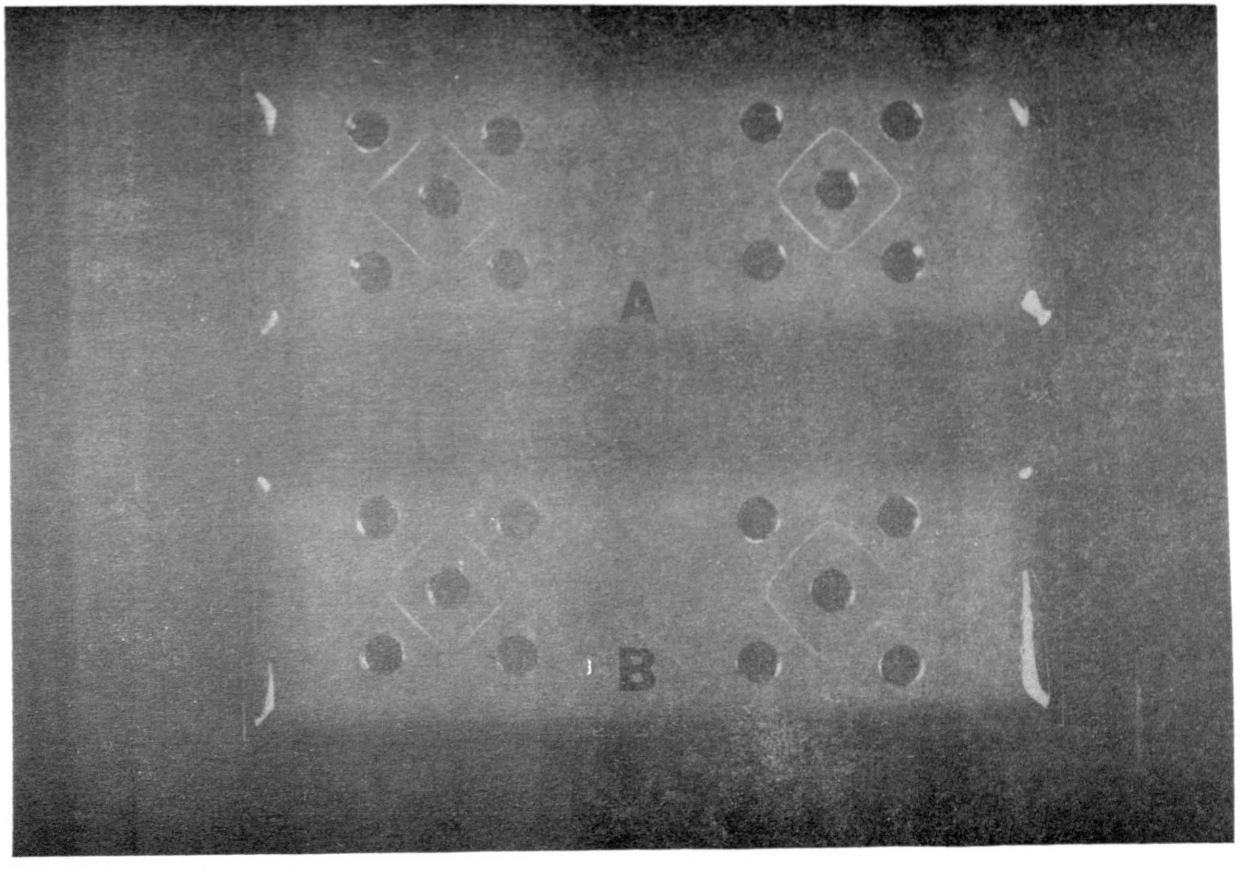

A
B

FIGURE 14: Immunodiffusion test showing specificity of the IgG fraction for WT and CORD enzymes. The enzymes used were partially purified as was the IgG fraction. In all plates shown, the IgG fraction was placed in the center well with the enzyme in the outer wells.

a. WT enzyme

b. CORD enzyme

c. WT and CORD enzyme placed in alternating wells (clockwise starting in the upper left corner).

A single precipitin band was formed with both enzymes.

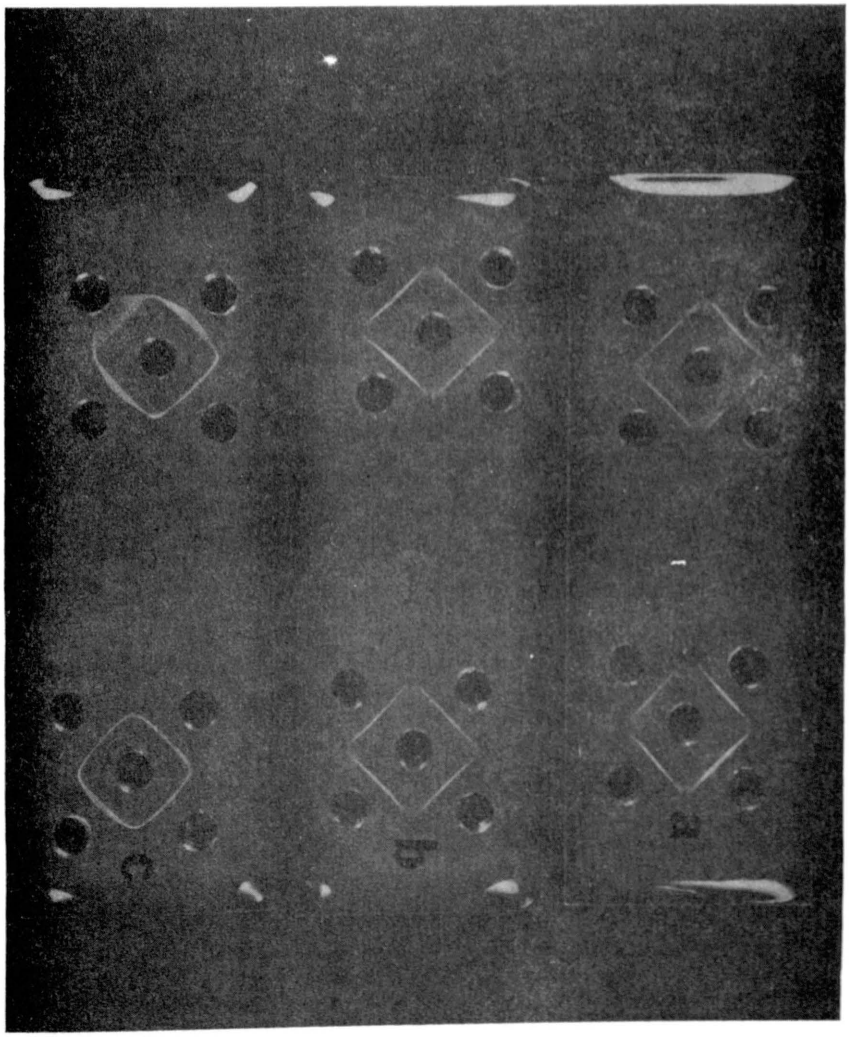
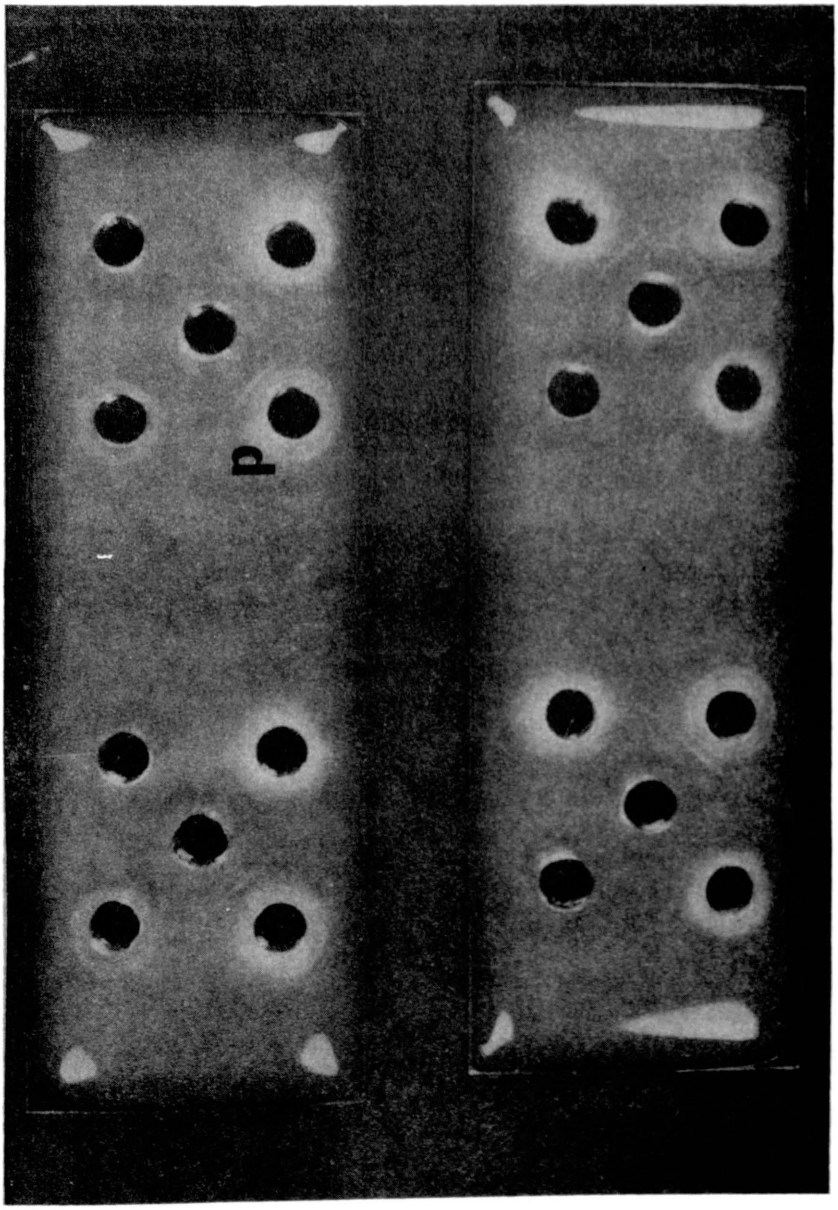


FIGURE 15: Immunodiffusion reaction of the CAD mutant enzyme. The IgG fraction was placed in the center well. In all cases, double precipitin bands were formed indicating two immunological reactive species (d).


P

Large Scale Precipitation

Although seven fractions of the serum containing antibody were collected and partially purified, fraction #2 has been used in most of the experiments. This fraction was shown to contain 51mg/ml. of protein and was stored in 50ml. of buffer. In order to distinguish between the IgG heavy and light protein chains and the bands occurring from the enzyme preparations, the native IgG fraction was electrophoresed on 9 percent acrylamide SDS gel. Using this method, the nature of the IgG contaminating proteins was checked. Figure 16 shows the results of the electrophoresis.

Even though the antibody produced was specific for the WT enzyme and would also produce a positive Ouchterlony reaction with CORD and CAD mutant enzymes, experiments were done to determine if the antibody could precipitate the enzymes from a crude preparation, and to see if the enzymes could be separated from the precipitated enzyme-antibody complex. The key was to react the antibody with a crude WT and a purified WT enzyme preparation, electrophorese the complex on a 9 percent SDS acrylamide gel. If the separation was complete, three bands could be observed corresponding to the two chains of the IgG immunoglobulin and the enzyme subunit. This technique could then be used to purify the mutant enzymes through elution of the enzyme from the gel. Figure 17 shows that all IgG fractions gave

similar results with respect to precipitation of the WT enzyme. Figure 18 allows unequivocal identification of the enzyme and the IgG staining bands. In this figure, the protein pattern of a purified IgG #1 is identified as well as the bands obtained when the purified IgG fraction was reacted with the purified WT enzyme.

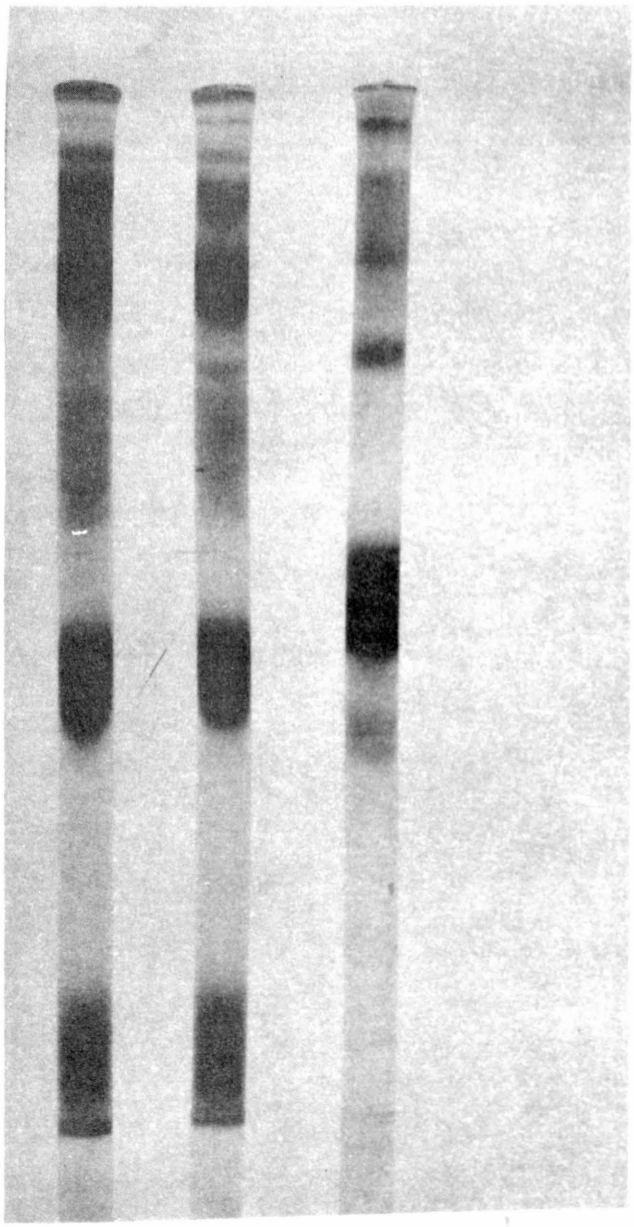
FIGURE 16: Protein band pattern of IgG fraction after separation on 9 percent SDS acrylamide tube gels.

A-B. IgG band pattern after partial purification of the antibody serum.

C. Control BSA, molecular weight 60,000 (III).

I = Heavy chain of IgG with molecular weight of approximately 55,000.

II = Light chain of IgG, molecular weight approximately 22,000.



A B C

FIGURE 17: Resolution of WT-IgG complex after electrophoresis on 9 percent SDS tube gels. The results show that the partially purified IgG fractions gave similar protein patterns with respect to precipitation of the WT enzyme. In all, seven antibody fractions were collected and partially purified.

A. WT enzyme plus IgG #5

B. WT enzyme plus IgG #6

C. WT enzyme plus IgG #7

I. PEPC'ase band (M.W. 100,000)

II. Heavy IgG chain

III. Light IgG chain

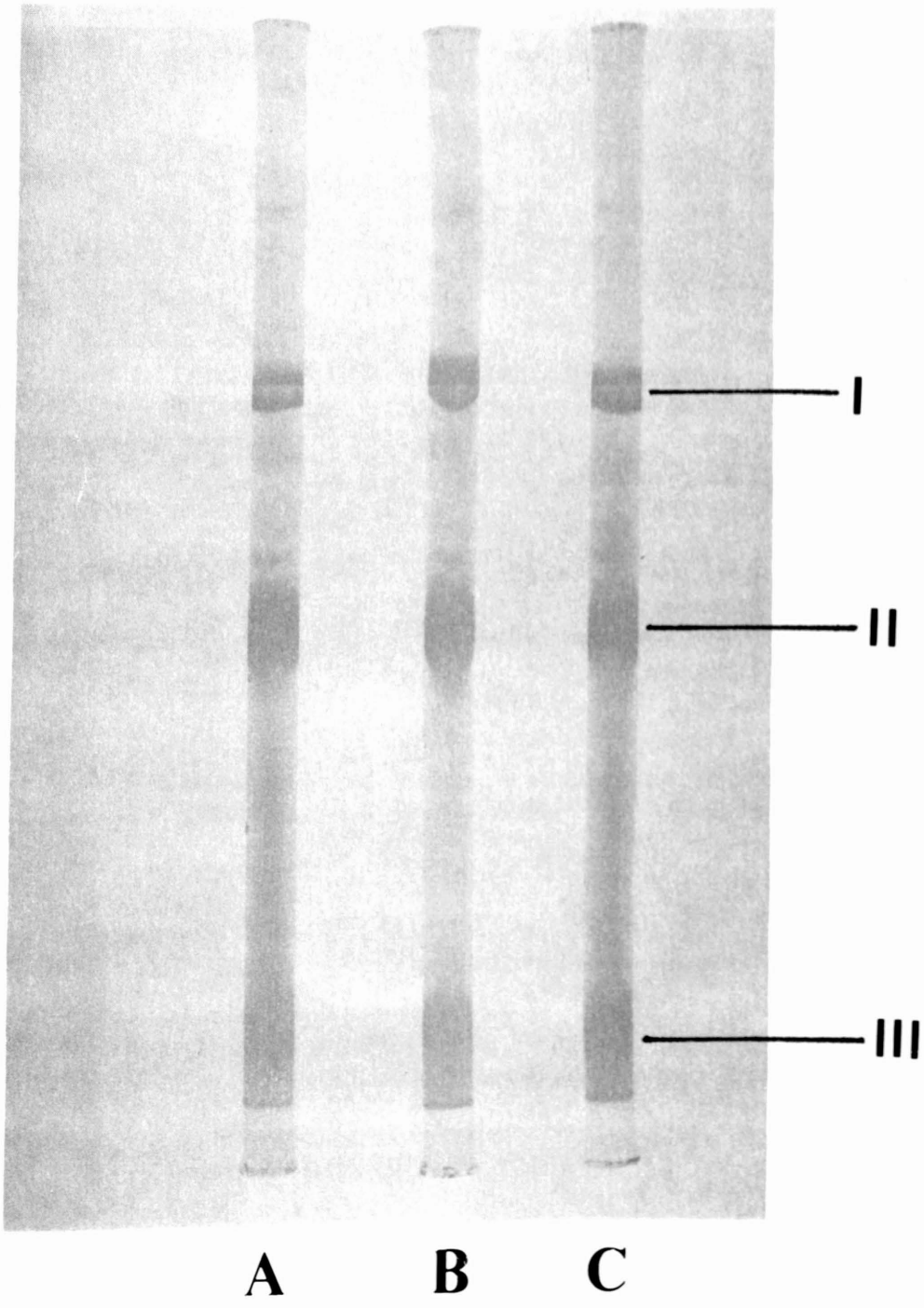

I
II
III
A
B
C

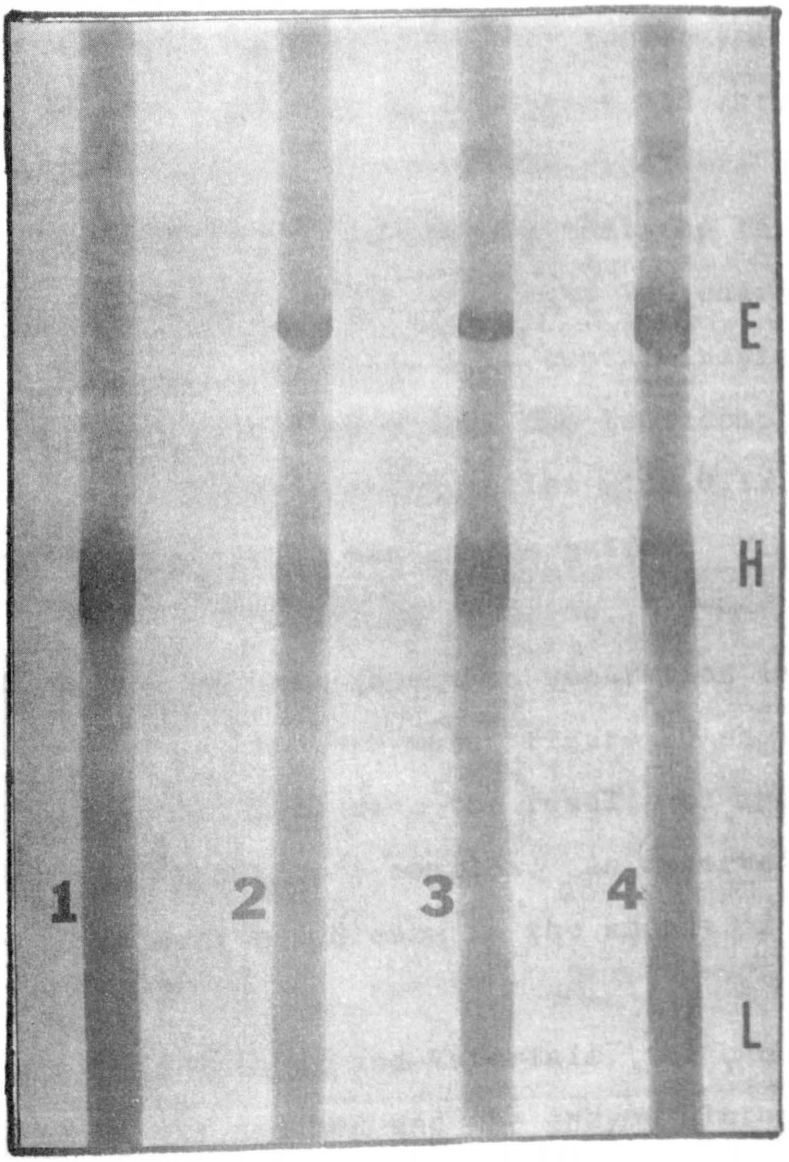
FIGURE 18: Comparison of protein patterns of IgG and WT enzyme obtained after large scale precipitation and separation on 9 percent SDS polyacrylamide gels. The following materials were used to obtain these patterns.

1. Purified IgG alone showing the positions of the light (L) and heavy (H) chains.

2. Precipitate obtained from the purified IgG and the purified WT enzyme, showing, in addition, the position of the enzyme band (E).

3. Precipitate obtained from the partially purified WT enzyme and the purified IgG fraction.

4. Precipitate obtained from the partially purified WT enzyme and the partially purified IgG fraction.


E
H
1
2
3
4
L

By reacting the CORD enzyme with the IgG fractions on a preparative scale, it was found that the enzyme could be precipitated and separated on 9 percent SDS acrylamide tube gels. The protein band patterns observed were similar to those produced by the WT enzyme, in that the three major bands (WT enzyme band, heavy and light IgG chain bands) were very distinct. However, more contaiminating proteins were noted. This could have been due to incomplete washing of the enzyme-antibody complex pellet with 0.1M NaCl before dissolving the pellet in the sample buffer. Figure 19 shows the result of the CORD enzyme reaction.

The CAD enzyme was subject to separation in the same manner as the other two enzymes. Figure 20 shows the results of this separation, including the results of increased washing of the enzyme-antibody complex. As observed, an increase in washing produced a decrease in the amount of enzyme present.

As given in Methods and Materials, the bands from each type of enzyme was excised and the enzyme eluted using SDS sample peptide buffer. Before the enzymes were used for further studies, they were re-run on the 9 percent SDS acrylamide gels to test purify after their excision from the tube gels. Figure 21 shows all three enzymes obtained from the preparative enzyme-antibody complex. The appearance of one band for the enzymes indicated the presence of a

homogeneous PEPC'ase species. These purified enzymes were then used for further studies.

FIGURE 19: Results obtained from large scale precipitation of crude CORD enzyme extracts using the WT specific antibody. The precipitation was followed by separation of the IgG-CORD complex on 9 percent SDS polyacrylamide gels. All three gels show:

I. CORD enzyme band

II. IgG heavy chain

III. IgG light chain

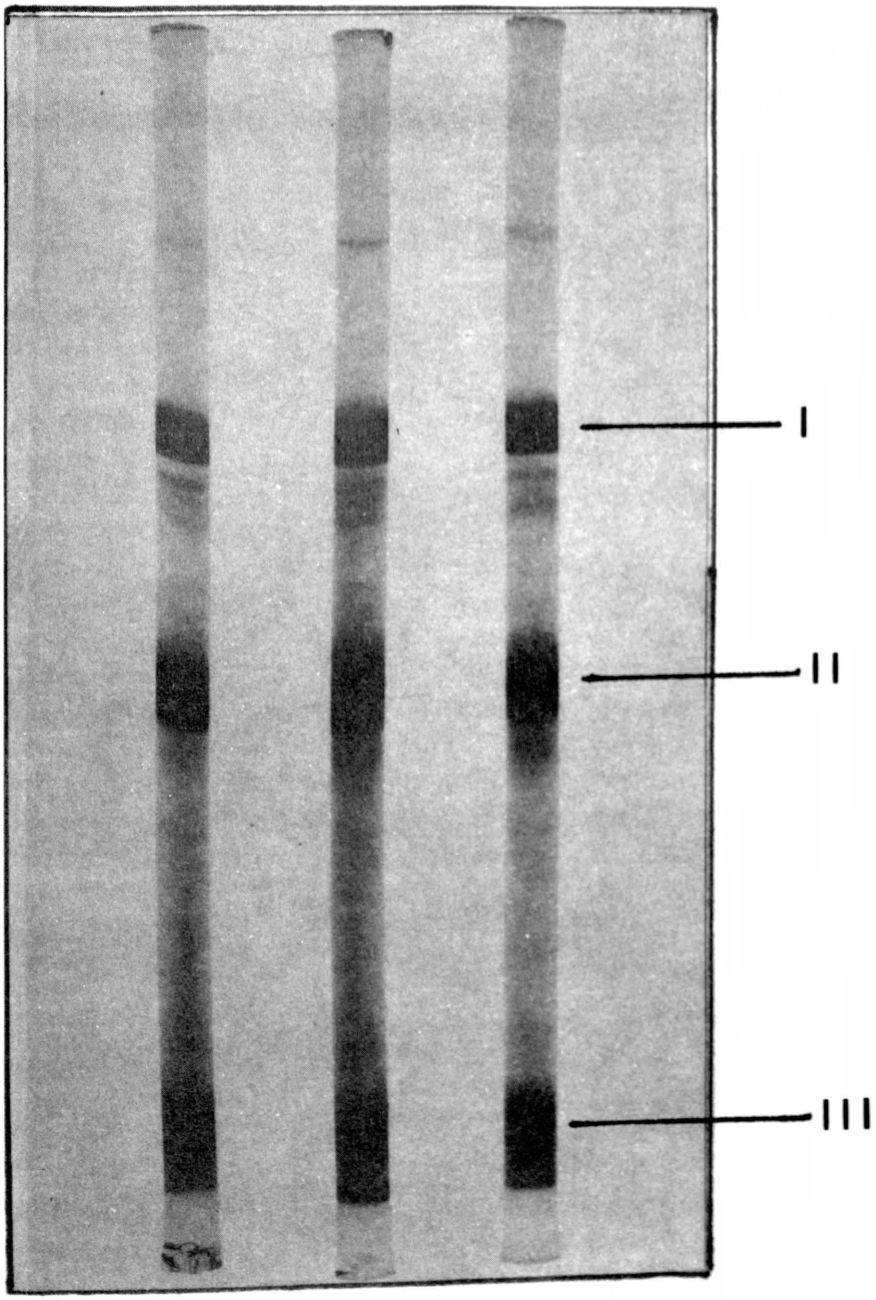

I
II
III

FIGURE 20: Results obtained from large scale precipitation of the CAD enzyme from crude extracts using the WT specific antibody. The precipitation was followed by separation and purification of the enzyme on 9 percent SDS polyacrylamide gels. From left to right the results show the effect of increased washing of the enzyme-IgG complex before electrophoresis.

I. CAD enzyme band

II. IgG heavy chain

III. IgG light chain

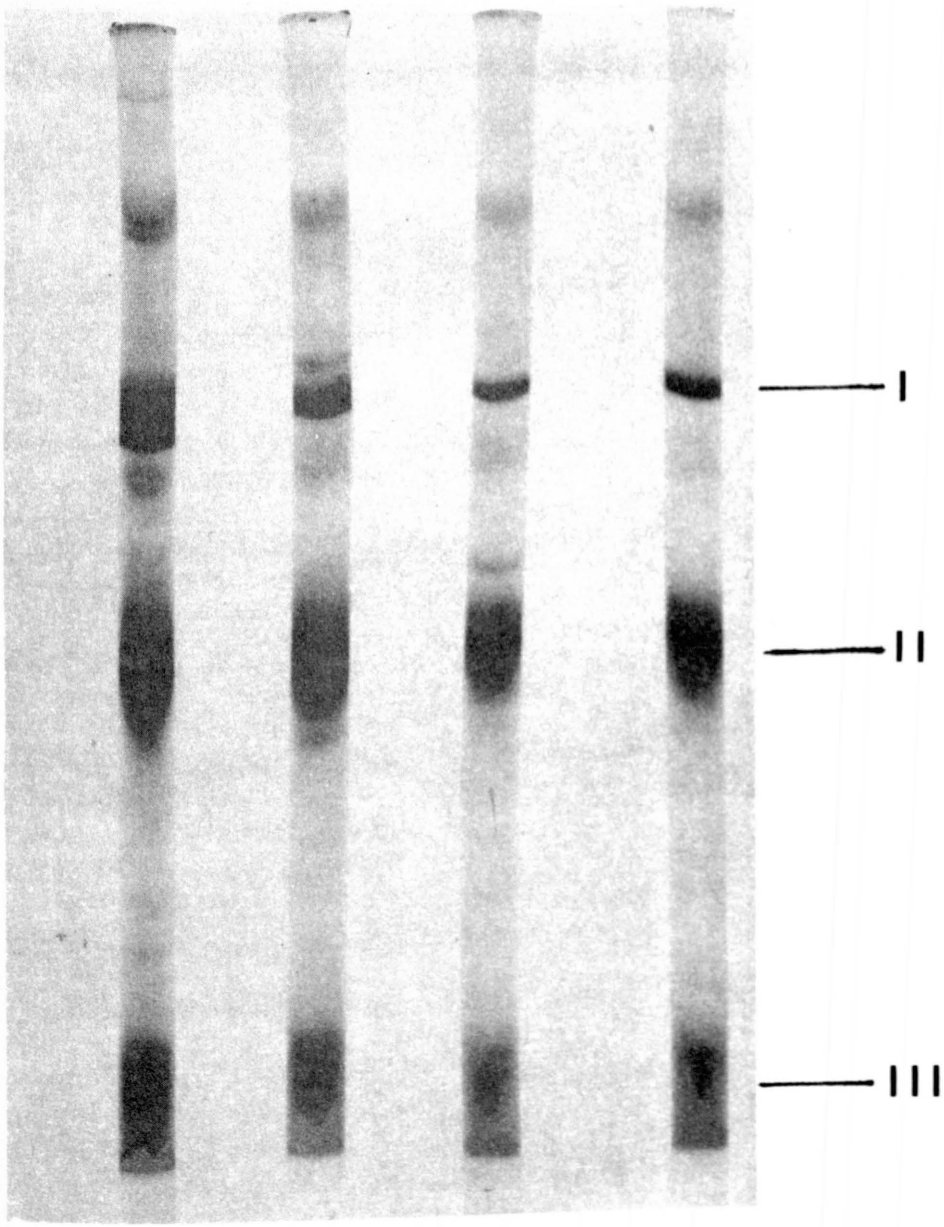

I
II
III

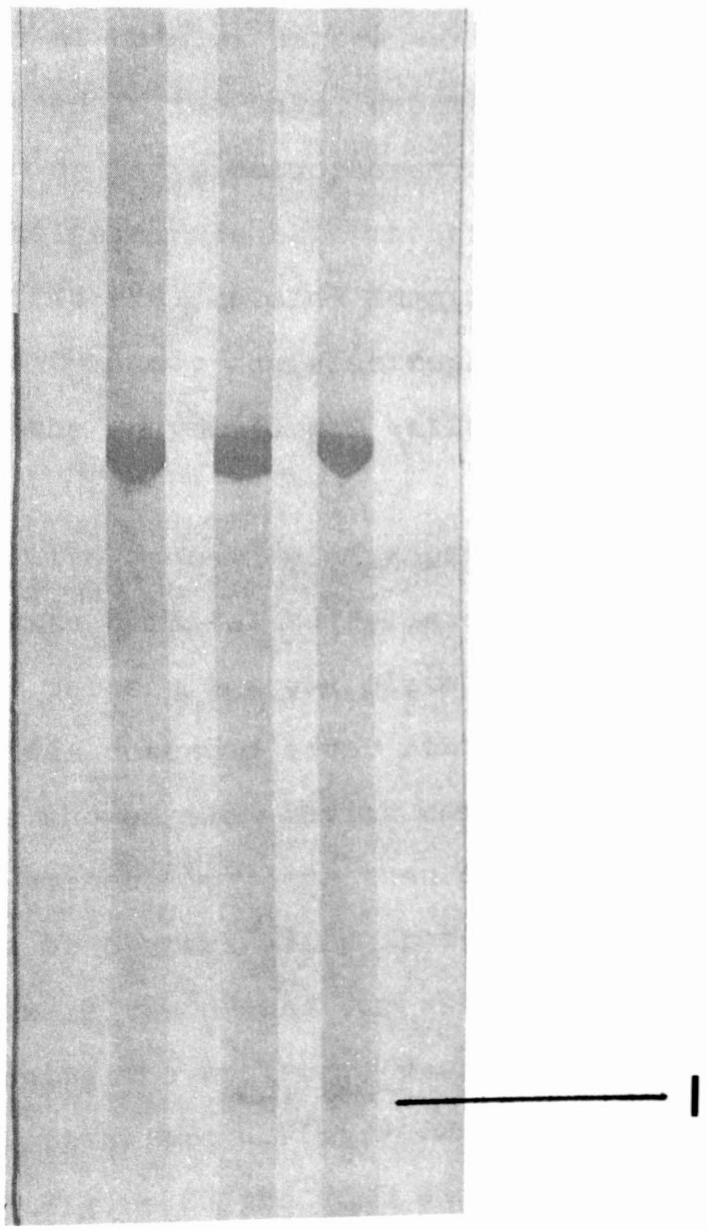
FIGURE 21: Homogeneous enzymes obtained from large scale precipitation of the enzymes from crude extracts using the WT specific antibody. The enzymes eluted from the gels were subjected to electrophoresis on 9 percent SDS polyacrylamide gels. The presence of one protein band for each enzyme indicates a homogeneous PEPC'ase species.

A. CORD enzyme

B. WT enzyme

C. CAD enzyme

I. Tracer dye


1
A B C

Crossed-Immunoelectrophoresis

Crossed-immunoelectrophoresis is an effective tool even in the studies of complex antigen-antibody systems. The method maybe used in two ways, depending on whether the quantification or the electrophoretic resolution is the emphasis of analysis. In this manner, a number of antigens can be quantified and proteins identified. This method was basically used to study the electrophoretic patterns (active components of the enzyme to the antibody) of the three enzymes.

In the native crossed-electrophoresis, enzyme samples were diluted with barbital buffer and run in the first dimension of 1 percent agarose plates. For the WT enzyme, only one spot was detected after staining. However, both mutant enzymes showed slow moving components. This possibly indicated the presence of more than one immunologically active species or contamination from other proteins. After electrophoresis in the second dimension, using 1 percent agarose containing the antibody, each enzyme showed a distinct precipitant arc. Figure 22 shows the results with the WT enzyme, Figure 23 the CORD enzyme and Figure 24 the CAD enzyme. Although the protein concentrations of the partially purified mutant enzymes were higher than the partially purified WT enzyme (CORD 56.55mg./ml; CAD 50.6mg./ml.; WT 38.8mg./ml.), the precipitant arcs were smaller for

FIGURE 22: Crossed-immunoelectrophoresis of the WT native enzyme. The results show the precipitin arc (A) pattern of the WT enzyme electrophoresed in the second dimension. The 1 percent agarose gel of the second dimension contained the IgG fraction. 15ul of the protein extract was used in the experiment. The protein concentration of the three enzymes were: CORD 56.55mg/ml, WT 38.8mg/ml and CAD 50.6mg/ml.

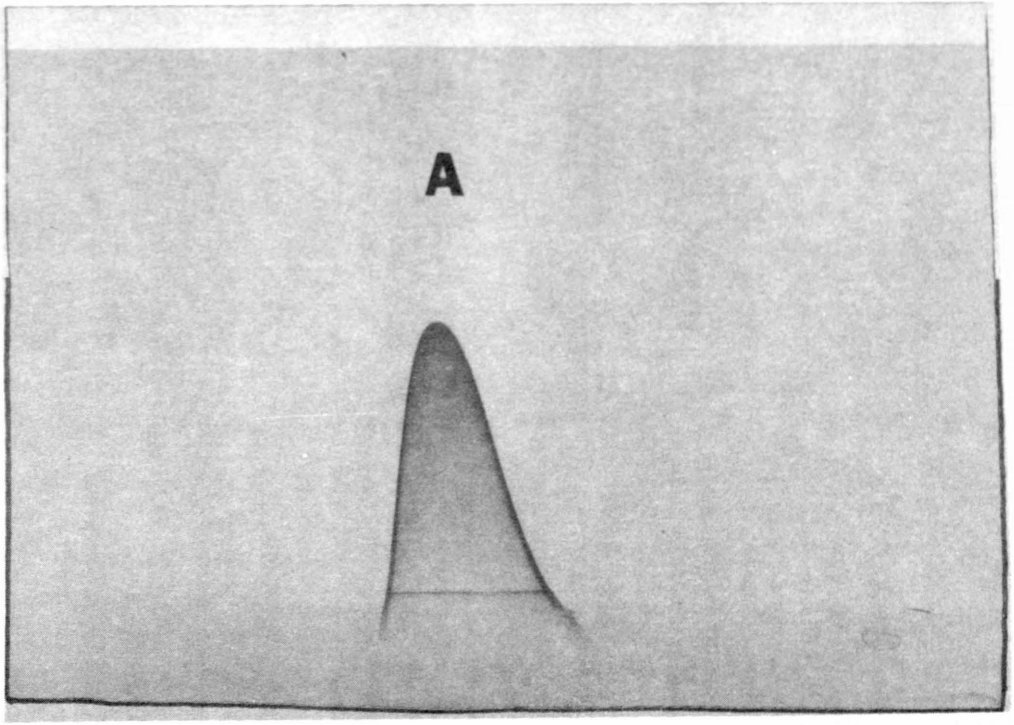

A

FIGURE 23: Crossed-immunoelectrophoresis of the CORD native enzyme (B). The results show the precipitin arc pattern of the enzyme after electrophoresis in the second dimension gel containing 1 percent agarose and IgG. The height of the precipitin arc is less than the arc of the WT enzyme (Figure 22) even though the amount of crude protein used was larger (CORD 56.55mg/ml.: WT 38.8mg/ml.

15ul. of each enzyme extract was electrophoresed.

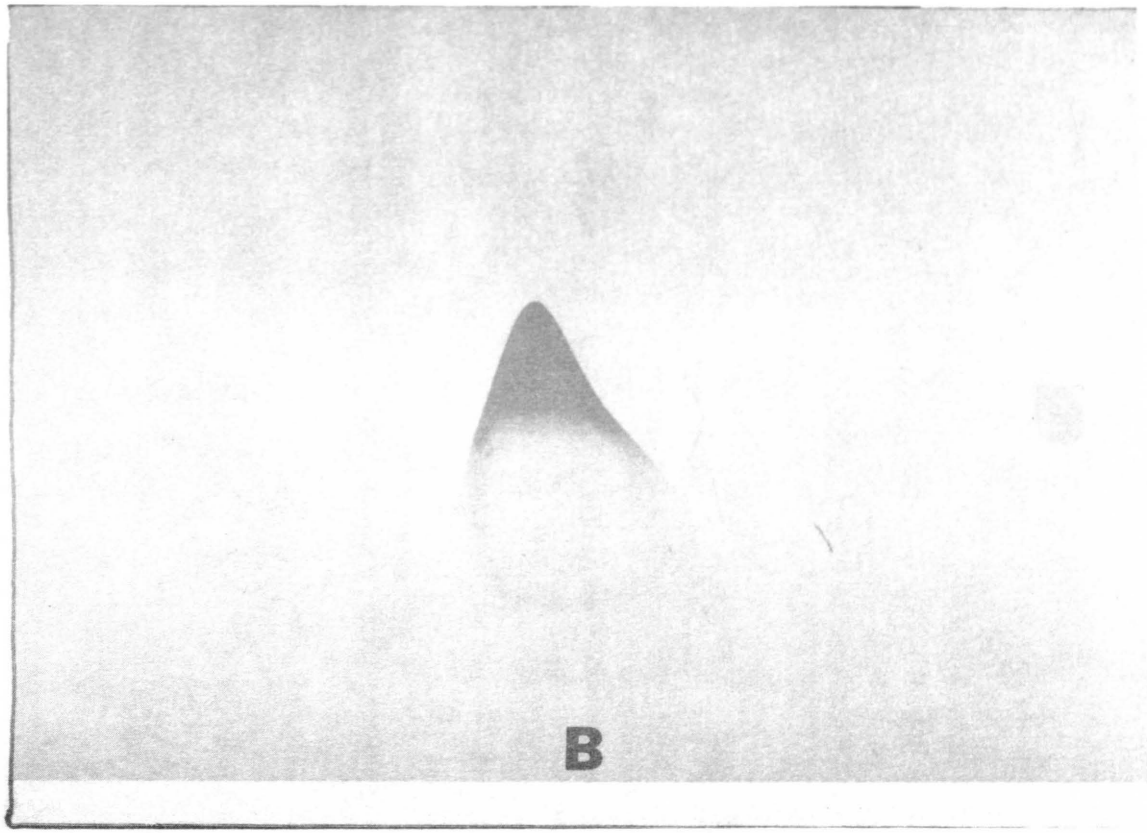

B

FIGURE 24: Crossed-immunoelectrophoresis of the CAD native enzyme. The results show the protein pattern of the enzyme in the first dimension (b) and the precipitin arc protein pattern of the enzyme in the second dimension (a). The second dimension gel contained the IgG fraction 15ul. of the WT and CAD protein extract were used. The height of the CAD precipitin arc is less than the WT (Figure 22) indicating the amount of immunological reactive protein is less.

b
a

the mutant enzymes. This indicated that the total amount of actual enzyme available to react with the antibody was less with the mutants or that the antibody binding sites for the mutants were distorted (resulting from conformational changes in the mutant enzymes) for binding.

The second phase of the crossed-immunoelectrophoresis required treatment of the three enzymes with an SDS sample buffer before electrophoresis. After treatment, the two mutant enzymes showed differences in the first and second dimensions. The WT enzyme (first and second dimension shown in Figure 25) showed the expected one protein formation pattern (precipitant arc) after staining. The CORD enzyme (Figure 26) showed a slow migrating component in the first dimension, but only one precipitant arc when the enzyme was electrophoresed in the second dimension. The slow component in the first dimension was therefore probably due to the presence of contaminating proteins. On the other hand, the CAD mutant enzyme, shown in Figure 27, exhibited a slower migrating component in the first dimension and the formation of at least two precipitant arcs in the IgG gel of the second dimension. These results indicated that the CAD enzyme contained two antibody reactive species.

FIGURE 25: Results of two-dimensional immunoelectrophoresis of the WT enzyme. The first dimension was run using 1 percent agarose gels. The WT enzyme was treated with SDS before electrophoresis in the first dimension. The completed gels were then run at a 90 degree angle into 1 percent agarose plates containing the IgG fraction.

A. First dimension WT enzyme gel.

a. Second dimension gel, showing the precipitin arc.

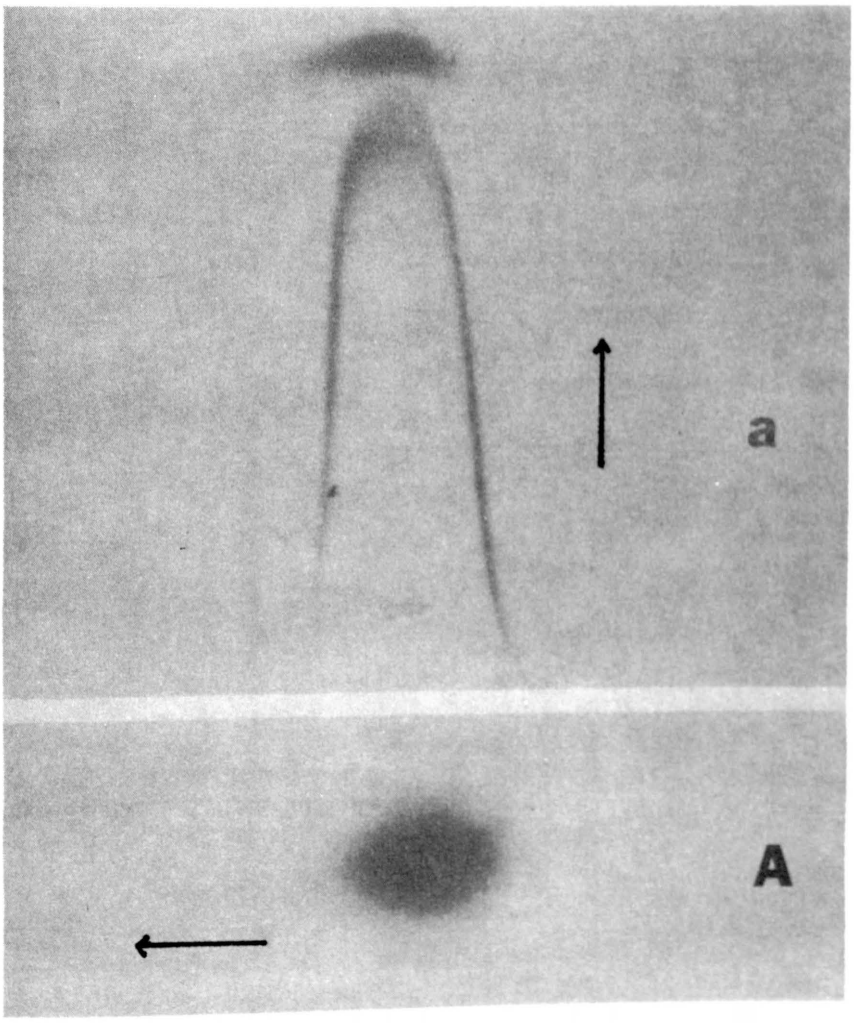

a
A

FIGURE 26: Results of two-dimensional immunoelectrophoresis of the CORD enzyme. The first dimension (B) was run in 1 percent agarose using SDS treated CORD enzyme. The slow migrating component noted in the first dimesnion was due to the presence of contaminating proteins, since only one precipitin arc was formed in the second dimension (b). The second dimension gel contained the IgG fraction.

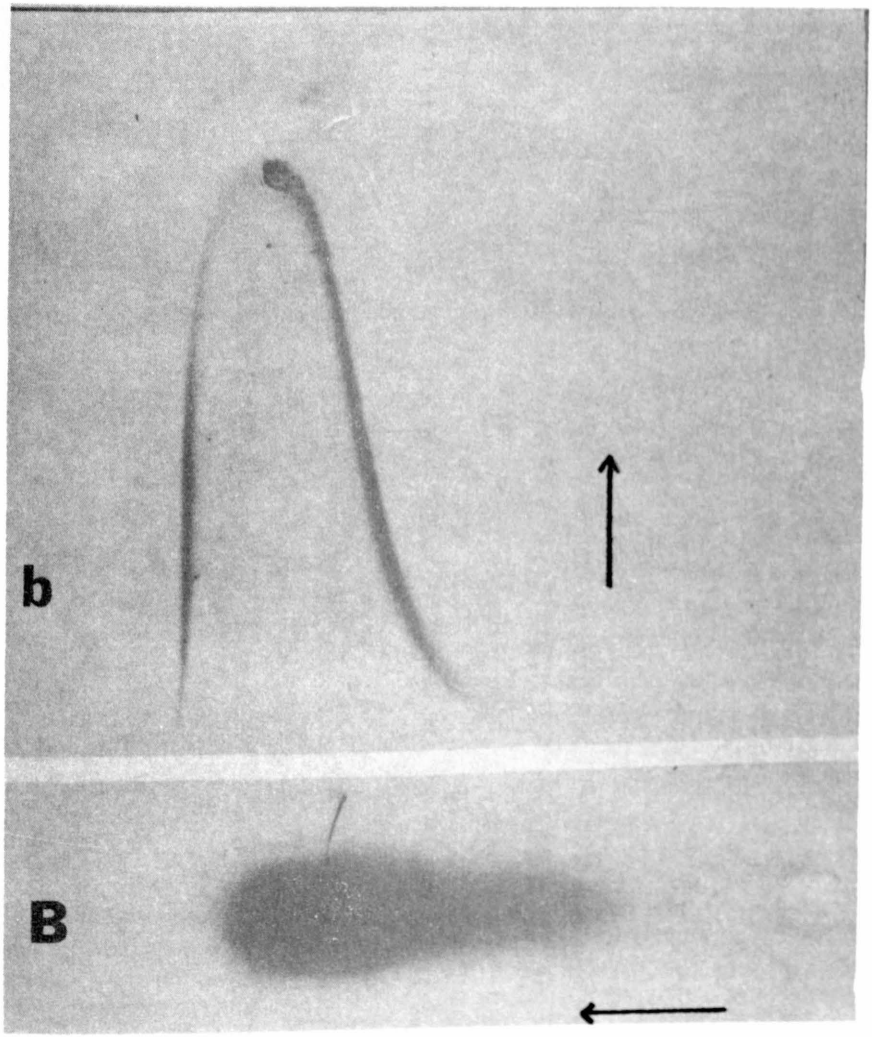
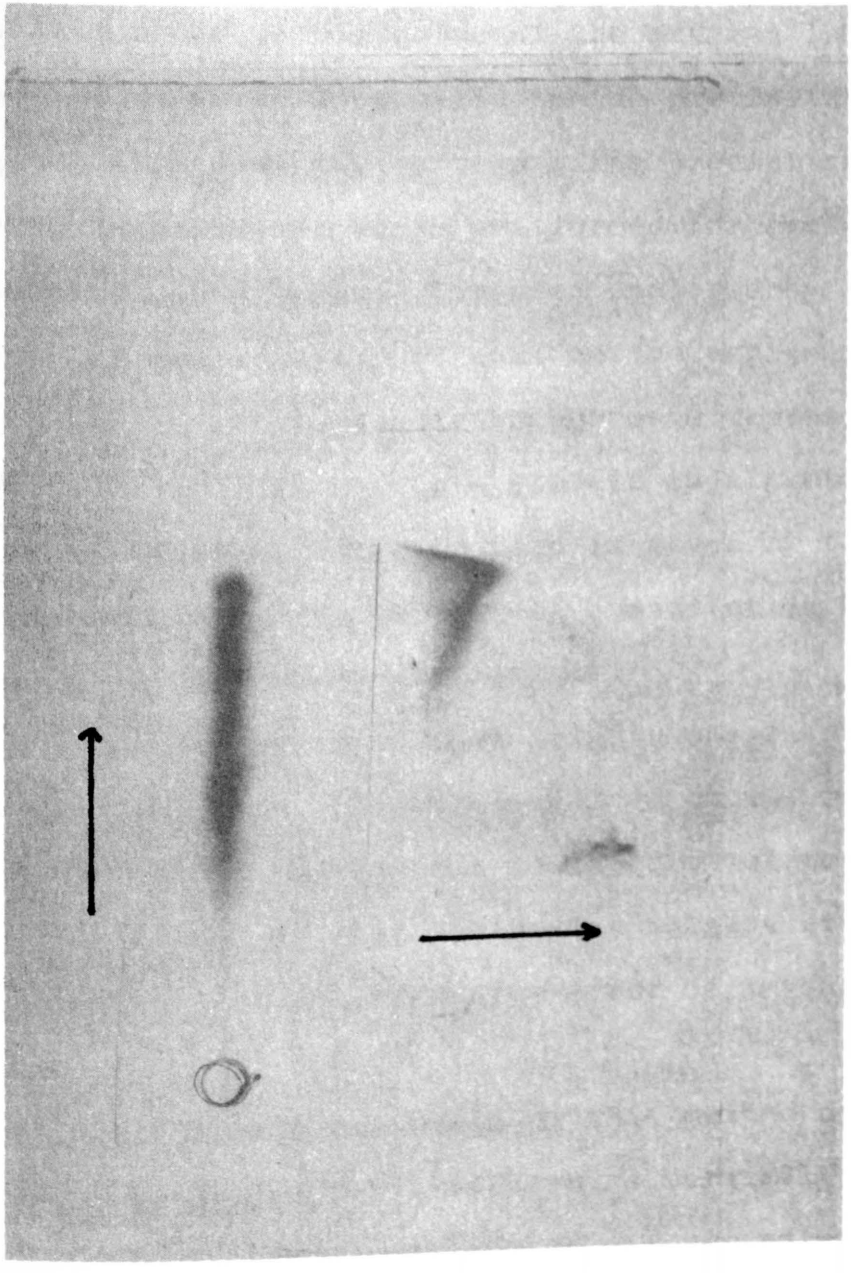

b
B

FIGURE 27. Results of two-dimensional immunoelectrophoresis of the CAD enzyme. A slower migrating protein component was noted in the first dimension when run on 1 percent agarose plates using SDS treated enzyme. At least two precipitin arcs were formed in the IgG gel of the second dimension (c). These results indicated the presence of at least two immunologically active protein species.



Rocket Immunoelectrophoresis Results

Quantitative immunoelectrophoretic methods are based on the electrophoretic migration of the antigen in antibody containing gels and a specific immuno-precipitation of the antigen by means of the corresponding precipitant antibodies. Individual precipitin bands are formed for each antigen/antibody system present. The area enclosed by these precipitant arcs is proportional to the antigen/antibody ratio. The rocket immunoelectrophoresis represents a method for determination of a single protein in a protein mixture. The identification of the protein is given by the rocket shaped precipitate formed and the quantitation based on measuring the height of the rocket.

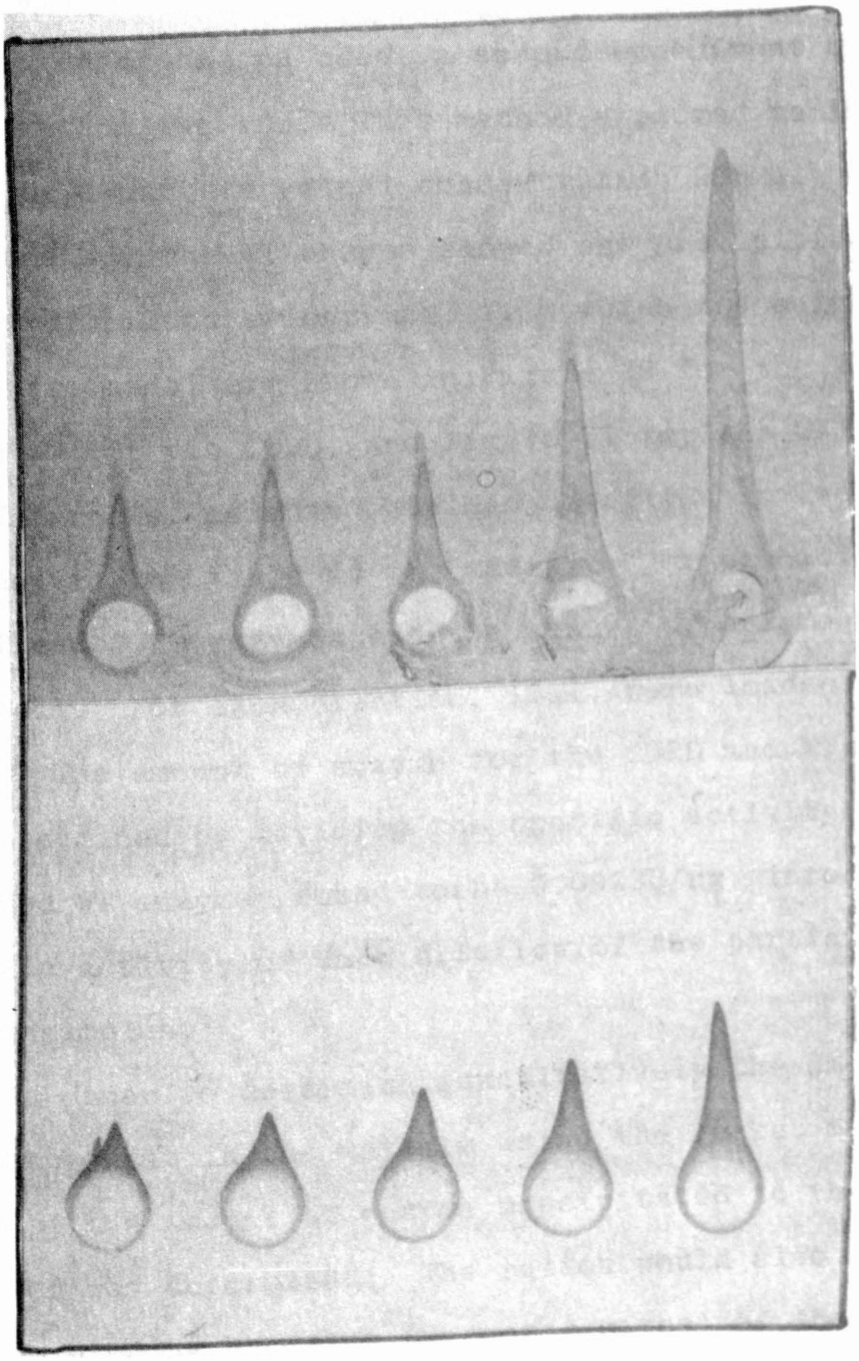
Measurement of the rocket heights was taken directly from the slide gels, in which readings on the rockets of 10-50mm in height corresponds to an error of measurement of about .2-0.4 percent. If the rocket heights are measured from an enlarged photograph, the error of measurement is reduced.

To test the effectiveness of this method using the PEPC'ase and the antibody elicited, a partially purified sample of the WT enzyme was run in a 1 percent agarose gel containing a 1:60 final concentration of IgG #2 (Figure 28). As noted in this figure, the enzyme showed more than one precipitin arc. This was believed to be due to dissociation

FIGURE 28: Preliminary results of WT enzyme using rocket immunoelectrophoresis.

A. Rockets of the WT enzyme run in a 1 percent agarose gel containing a 1:60 final concentration of IgG. 15ul of enzyme using 1:5, 1:10, 1:15, 1:20 and 1:25 dilutions (left to right) were placed in each well. The double rocket arcs were due to dissociation of the enzyme.

B. Rockets showing the effect of increased aspartate to stabilize the tetrameric form of the enzyme and prevent dissociation.



A

B

of the WT enzyme. To determine if the enzyme had indeed dissociated at the pH used, a second experiment using the rocket method was run. This method employed an increased amount of aspartate (final concentration 20mM). As observed in Figure 28, the WT enzyme showed one precipitin arc. Results of all three enzymes, from which the height measurements were made, are shown in Figure 29 for the WT enzyme; Figure 30 for the CORD; and Figure 31 for the CAD mutant.

The rocket heights obtained are given in Table 2. Included in the table is the activity for each dilution of the WT and CORD enzymes and the amount of enzyme placed in each well. For each dilution, 15ul. were loaded into the wells. The amount of enzyme for the CORD and WT enzyme was determined by dividing the specific activity of the purified WT enzyme, found to be 0.0923U/ug. into the specific activity of each dilution of the partially purified enzyme samples.

In order to determine quantitatively the amount of enzyme present in the mutants using the rocket method, a ratio of the amount of enzyme precipitated to the amount of IgG used was determined. The ratios would also indicate the surface binding capacity (receptor sites) of the mutant enzymes. The ratios were based on the average amount of enzyme eluted from the tube gels compared to the amount of protein content of the heavy chain of the IgG also eluted

TABLE 2: SUMMARY OF ROCKET IMMUNOELECTROPHORESIS HEIGHTS OF ALL THREE ENZYMES.

WT ENZYME (ACT. 450U/ml.; PROT. 38.8mg/ml. partially purified)

DILUTIONS	HEIGHT	ACT.	ug. PROT.
1:2	46	3.375	36.6
1:5	39.5	1.35	14.6
1:10	31	.675	7.3
1:15	23	.450	4.9
1:20	16.5	.3375	3.7

CORD ENZYME (ACT. 131.39U/ml.; PROT. 56.5mg/ml.)

1.2	35	.983	10.7
1:5	30	.393	4.26
1:10	25	.197	2.13
1:15	20	.131	1.42
1:20	14	.0983	1.07

CAD ENZYME (PROT. 42.6mg/ml.)

1:2	25.9
1:5	22
1:10	15.5
1:15	10.6
1:20	8.9

** Since the activity of this enzyme could not be measured, the amount of protein could not be determined.

FIGURE 29: Results of the rocket immunoelectrophoresis using WT enzyme. The height of each rocket (point a to point b) was used to construct a standard curve of height to enzyme concentration. The protein curve was then used to quantify the CORD and CAD enzyme concentration. The dilutions of the enzyme placed in each well were 1:20, 1:15, 1:10, 1:5 and 1:2 respectively from left to right.

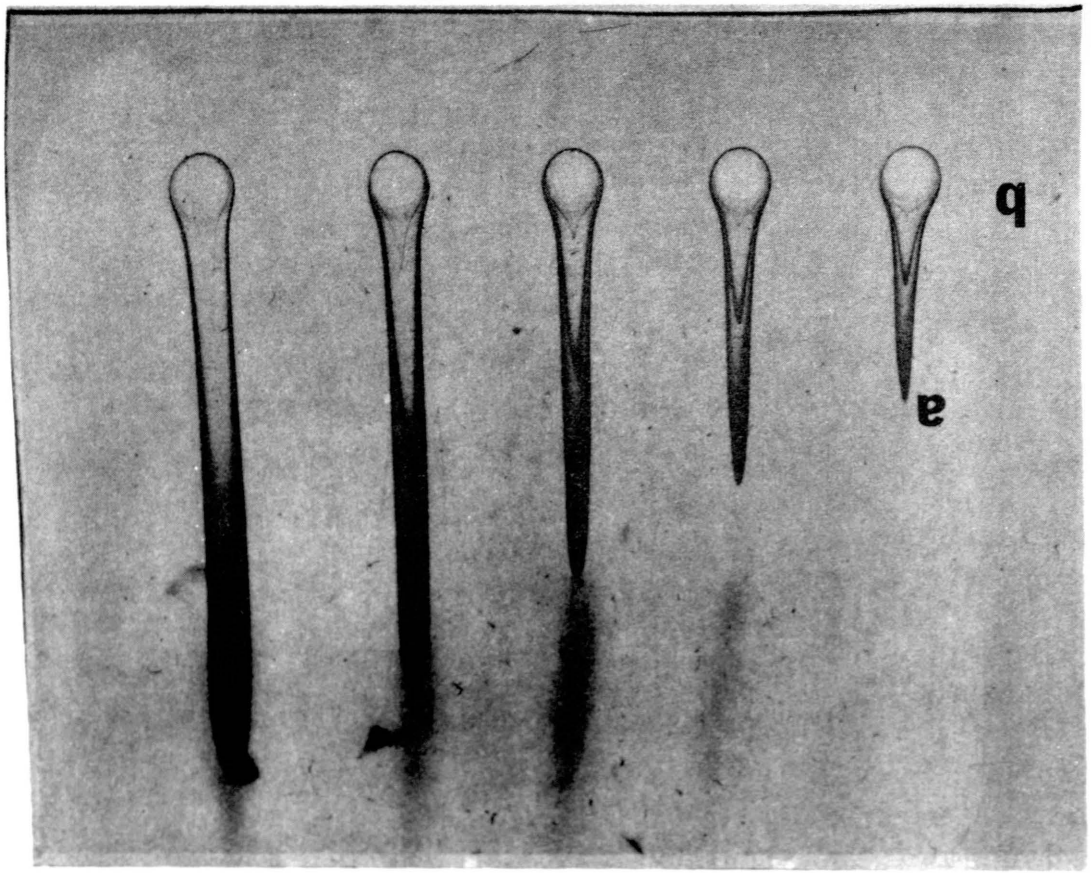

q
a

FIGURE 30: Rocket immunoelectrophoresis using CORD enzyme. 15uls of each dilution (1:20, 1:15, 1:10, 1:5 and 1:2 respectively from left to right) were placed in separate wells. Quantification of the CORD rocket heights indicated the amount of cross-reacting material produced by the CORD was 70-80 percent as much as that produced by the WT enzyme. The height of the rockets were measured from the same relative positions as shown in Figure 29.

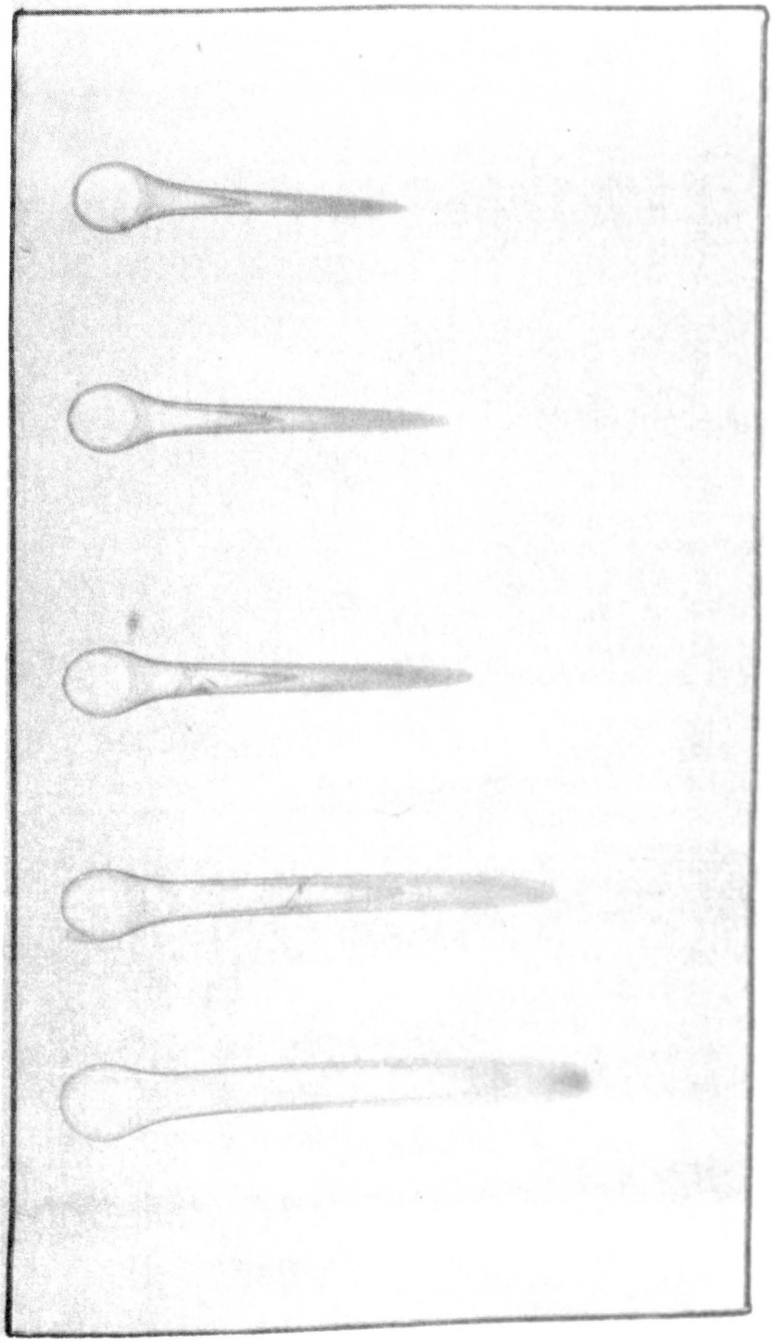
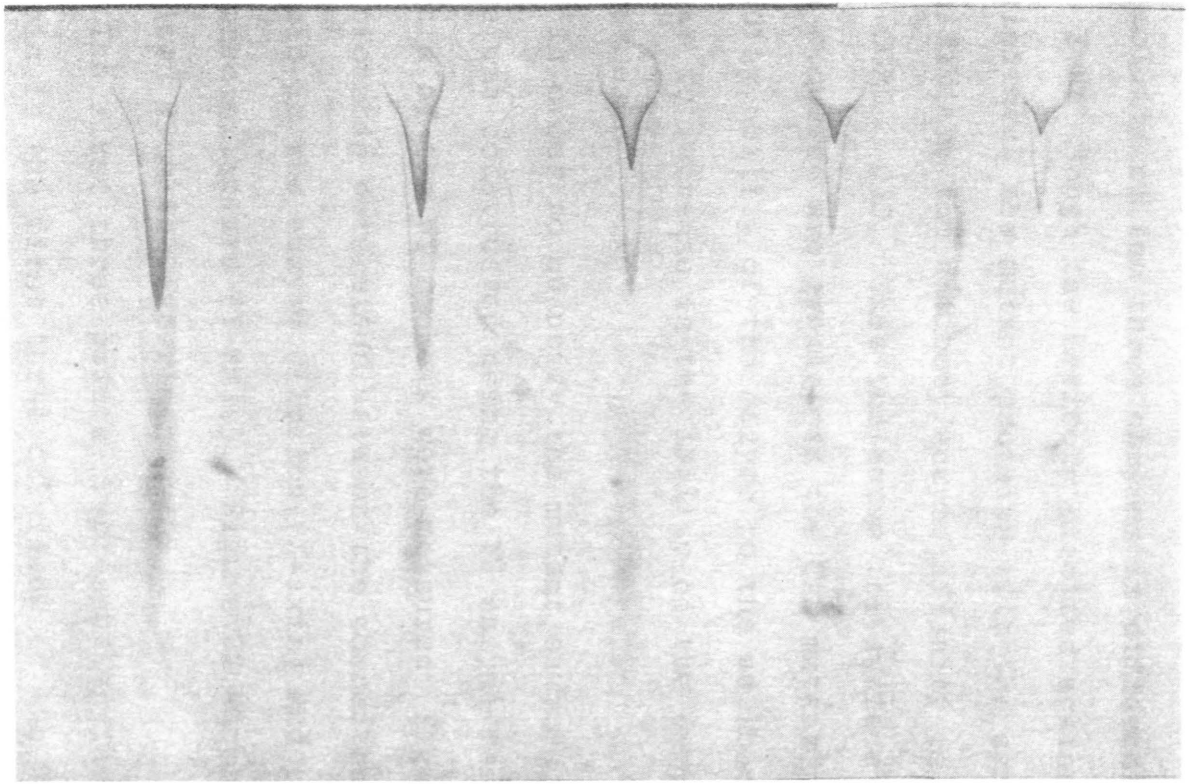


FIGURE 31: Rocket immunoelectrophoresis using the CAD enzyme. 15uls of each dilution (1:20, 1:15, 1:10, 1:5 and 1:2 respectively from left to right) were placed in separate wells. Quantification of the CAD rocket heights indicated the amount of cross-reacting material produced by the CAD was 30 percent as much as that produced by the WT enzyme. The height of the rockets were measured from the same relative positions as shown in Figure 29.



from the same gels. The ratios found are shown in Table 3, along with the amount of protein eluted for each entity. As indicated in Table 3 (ratio of enzyme/antibody) the binding ability of the three enzymes is different.

Even though the antibody elicited against the WT enzyme precipitated the mutant proteins with the same degree of specificity, the antibody/enzyme affinity is different; WT CORD CAD. This indicates the surface changes which have occurred are greater in the CAD mutant. The changes (binding ability of the enzyme to the antibody) observed appear to increase as the ability of the enzyme to function decreases.

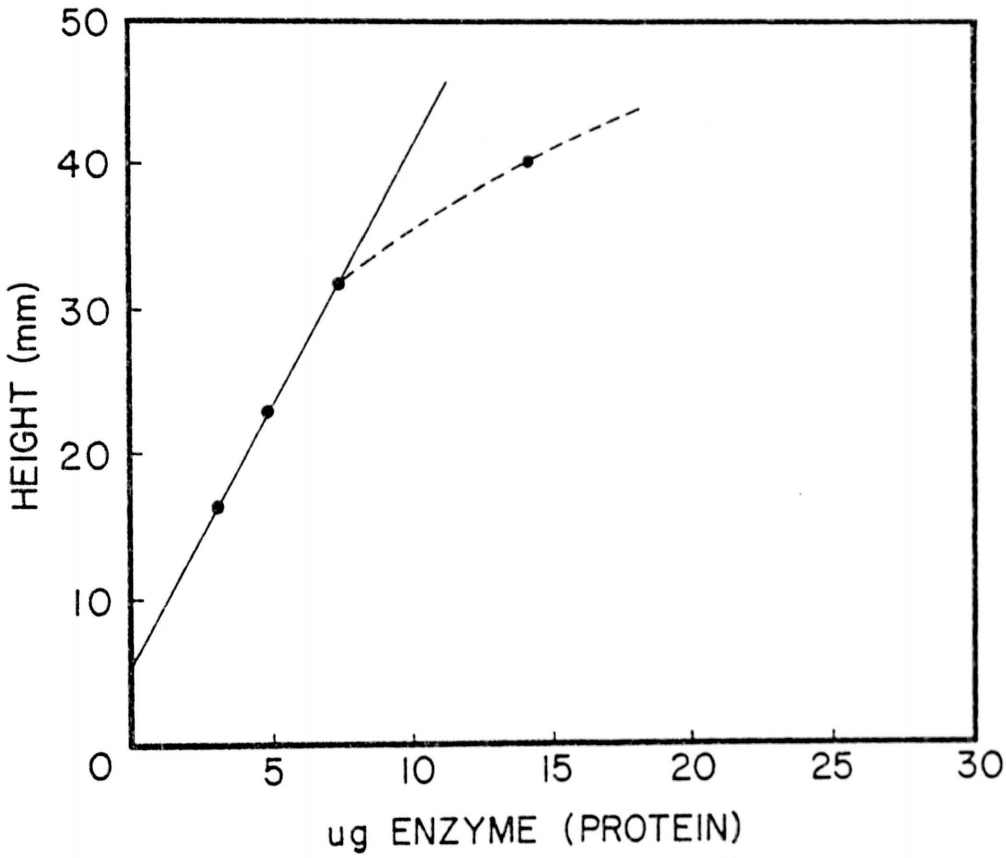
In order to quantify the mutant enzymes, a standard curve was constructed using the WT enzyme results. The peak heights of the WT rockets were measured (Figure 29) and the relationship determined between the concentration of the protein in the enzyme dilution (X-axis) and the height of the precipitin arc (Y-axis). Figure 32 shows the WT curve obtained from the experiment.

The first three points, representing the amount of protein in the first three dilutions of the WT enzyme (i.e. 1:10, 1:15, and 1:20) were used, since these points constituted a straight line. The remaining points from the 1:5 and 1:2 dilutions were omitted, since at these dilutions the protein was too concentrated to give a valid reading.

TABLE 3: RATIO OF ENZYME/ANTIBODY (Eluted from tube gel separation).

	ASSAY	VOLUME	PROT.	TOT. PROT.	RATIO (Enz./IgG)
1.	WT enzyme	0.57	1.33mg./ml.	0.76mg.	1.04
2.	IgG from WT	0.48	1.53	0.73	
3.	CORD enzyme	0.55	1.18	0.65	0.83
4.	IgG from CORD	0.53	1.48	0.78	
5.	CAD enzyme	0.51	1.07	0.545	0.78
6.	CAD IgG	0.51	1.37	0.70	

FIGURE 32: Standard protein curve (cross-reacting protein) of WT enzyme rocket immunoelectrophoresis. As indicated the heights of the first three concentrations were used to construct the curve (i.e. heights from 1:20, 1:15 and 1:10 dilutions of the enzyme). This WT enzyme curve was used to estimate the amount of CAD and CORD cross-reacting protein present.


50
40
30
20
10
0
HEIGHT (mm)
5
10
15
20
25
30
ug ENZYME (PROTEIN)

An example of the calculation method used to determine the equivalent amount of enzyme like protein present in the mutants, is given below.

1. The heights of two of the CAD rockets were measured and the corresponding amount of protein from the standard curve extropolated.

   Example: 22mm = 4.7mg of WT protein

   9mm = 1.0mg of WT protein

2. 42.6mg/5ml. (concentration of CAD protein)=8.52mg/ml.

3. A total of 25.6ug of CAD protein was placed in each well (15ul of a 1:15 dilution of the CAD enzyme equals 25.6ug protein).

4. Equivalent amount of enzyme like protein in CAD is

$$\frac{4.7 + 1}{25.6} = 0.22 \text{ or } 22\%$$

5. Correcting for the ratio of CAD/IgG taken from Table 3 gave a total of 7.7mg or $\frac{7.7}{25.6} = .30$ or 30%.

This figure of 30 percent indicates that the CAD mutant produces approximately 30 percent as much cross reacting material as the WT. The same calculation method was used for the CORD rocket. The results indicated that the CORD mutant had 75-80 percent as much cross reacting material as the WT mutant enxyme.

Quantitation of Inhibition Reactions

The previous section demonstrated that the antibody could cross react with each of the three enzymes, and that the WT as well as the mutant enzymes could be precipitated in preparative quantities. The following results are from studies used to determine to what extent the antibody could inhibit the WT and CORD enzymes. This procedure would establish the binding affinity of the CORD mutant when compared to the WT enzyme. Again, only the WT and CORD enzymes were used since the amount of inhibition was followed spectrophotometrically at 340nm.

Table 4A shows the results of the WT and CORD enzymes using a pre-injection IgG fraction as the control. This procedure demonstrated that there was no component in the pre-injection serum which would react to inhibit enzyme activity. Table 4B shows the differences observed in the ability of the antibody to inhibit the activity of the WT and CORD enzymes. From the activities represented in the tables, the CORD enzyme was observed to be less inhibited by the antibody when compared to the activities found in the WT enzyme. This indicated that the CORD mutant had less affinity for the antibody, thus indicating surface changes on the mutant enzyme.

As noted in Methods and Materials, the IgG fraction (51mg./ml.) was diluted 1:3 along with the control serum.

The activities of the two enzymes were diluted to a point such that the enzymes exhibited almost equal activity when assayed in 0.01M PEA buffer. All reactions were run overnight, centrifuged and the supernatant assayed.

Figure 33 shows the results of the experiment when the control activities given in Table 4A were plotted against the enzyme concentrations used (CORD and WT enzymes). Figure 34 illustrates the graph obtained when the activities from Table 4B (inhibition reaction of the enzymes) were plotted against the enzyme concentrations. These preliminary results indicated that the binding ability of the CORD mutant to the antibody was less than that of the WT enzyme. Since the ability of an antigen (enzyme) to bind successfully with its antibody resides in the integrity of the antigen surface and the availability of specific receptor sites, it is indicated from this study, that the aforementioned factors have been changed to a degree in the CORD mutant.

TABLE 4A: CONTROL REACTION

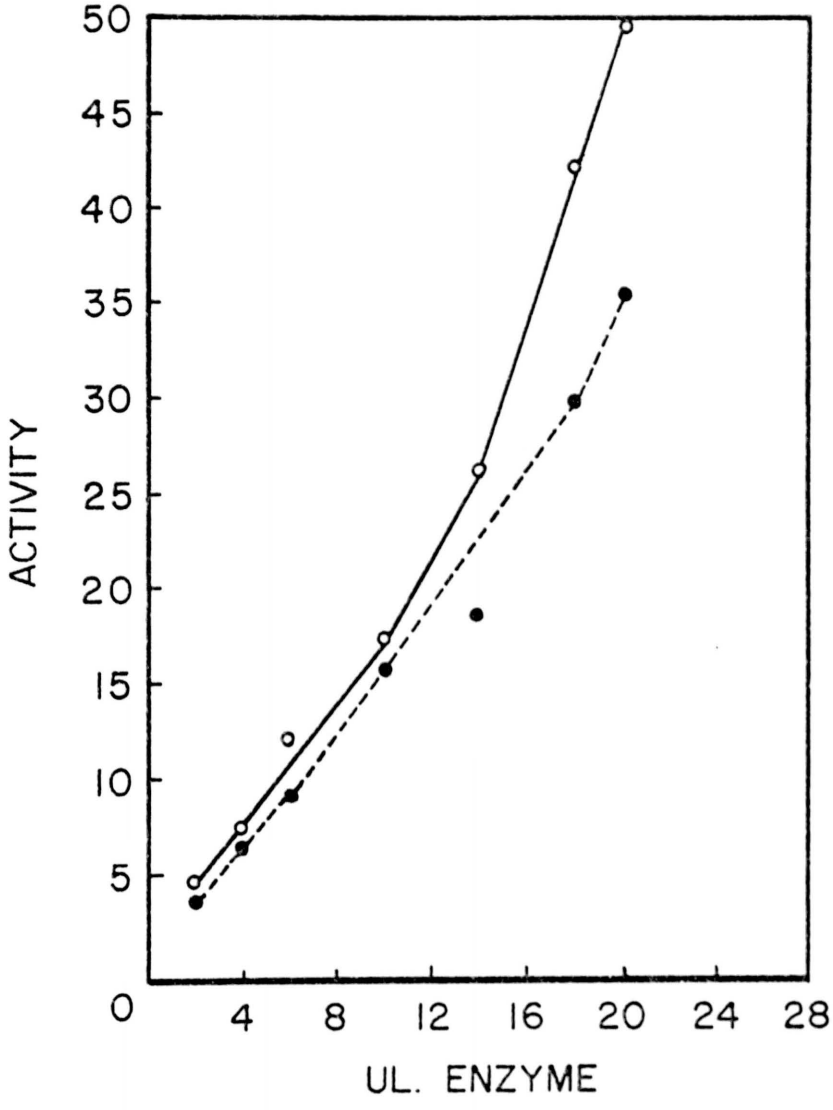
CONTROL SERUM + WT		CONTROL SERUM + CORD	
Enz. Vol.	Act.	Enz. Vol.	Act.
2ul.	4.35	2ul.	4.70
4	6.47	4	7.23
6	9.49	6	11.23
10	15.22	10	16.55
14	18.01	14	26.23
18	28.71	18	41.11
20	34.85	20	50.01

TABLE 4B: INHIBITION REACTION (IgG):

IgG + WT		IgG + CORD	
Enz. Vol.	Act.	Enz. Vol.	Act.
2ul.	---	2ul.	1.25
4	---	4	4.12
8	.16	8	7.38
12	.27	12	11.58
16	.51	16	15.69
20	1.51	20	21.93
22	2.56	--	---
24	3.49	24	29.5

FIGURE 33: Effect of pre-immunization serum on WT and CORD enzymes. The concentrations of the enzymes are plotted against the activity. The initial activity of the two enzymes were diluted such that the enzymes exhibited almost equal activity. The enzymes were diluted in 0.01M PEA buffer in a ratio of 28uls buffer to 2uls of enzyme. An aliquot of this solution was assayed spectrophotometrically at 340nm.

CONTROL REACTIONS

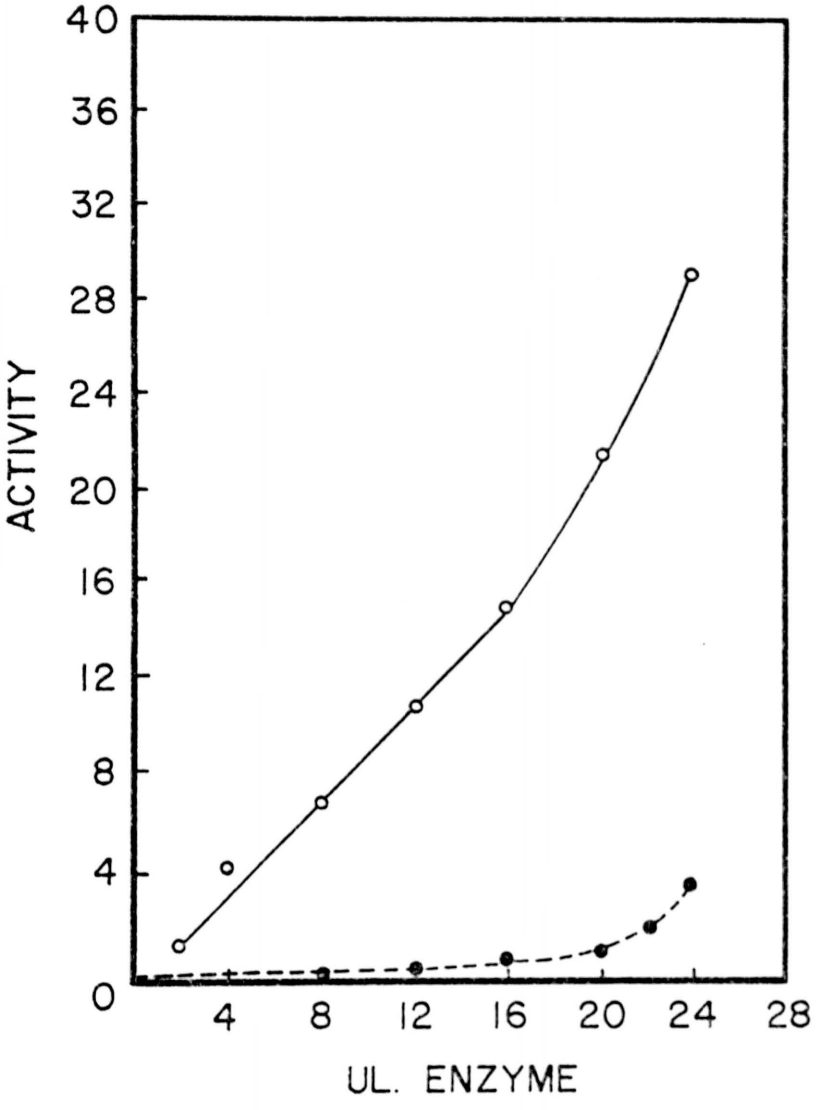


—— Cord Enzyme (Control)

----- WT Enzyme (Control)

FIGURE 34: Inhibition effect of IgG on WT and CORD enzymes. The results show a greater inhibitory effect, as indicated by a decrease in activity, on the WT enzyme than on the CORD enzyme. This indicates a greater binding potential of the WT enzyme to the IgG fraction. In this figure, the enzyme concentration is plotted against the enzyme activity which was followed spectrophotometrically at 340nm.

INHIBITION REACTION (IgG)



Titration studies were also undertaken to further establish the binding properties of the WT and CORD enzymes as well as to quantify the amount of antibody required by each enzyme to inhibit its activity. Again, the two enzymes were diluted such that the activity of both was approximately the same. The reactions were run according to the procedures given in Methods and Materials. Results of the activities obtained from the titration experiment are given in Tables 5A and 5B. Graphic representation of the data from Tables 5A and 5B is shown in Figure 35 (WT enzyme titration) and Figure 36 (CORD mutant titration).

Using these data, it was calculated that 151.7ug. of IgG/U WT enzyme was required for complete inhibition. The mutant enzyme required 293.4ug. of IgG/U CORD for complete inhibition. An example of the quantification of the data and the factors used is given below.

1. Experimental dilutions:

   IgG 1:7 = 7.1mg./ml.

   5ul. of IgG used = 35.5ug./ul.

   WT 1:20 (450U/ml. = 22.5U/ml.)

   CORD 1:5 (131.4u/ml. = 26.2U/ml.)

2. From a linear portion on the titration curve, a line was extropolated back to the X-axis to determine the amount of enzyme required for complete inhibition. In the case of the WT enzyme, 10.38ul. of enzyme was

22.5U/ml. X 0.01038ml. = 0.234U/ml.

$$\text{Ratio of IgG/U} = \frac{35.5\text{ug.}}{.234\text{U}} = 151.7\text{ug. IgG/U WT.}$$

The same calculation procedure was used for the CORD enzyme which showed that 4.6ul. of enzyme was needed for complete inhibition.

This quantification method reinforced the previous observation which demonstrated a difference in the binding affinities of the two enzymes. All of these results indicated a structural surface change had occured in the CORD mutant.

TABLE 5A: CONTROL TITRATION RESULTS FOR WT AND CORD.

CONTROL SERUM + WT ENZYME		CONTROL SERUM + CORD	
Vol. Enz. Sol.	Activity	Vol. Enz. Sol.	Activity
4ul.	2.74U/ml.	4ul.	3.51U/ml.
6	4.34	6	4.81
8	5.66	8	6.47
10	6.89	10	7.91
12	8.61	12	----
14	9.74	14	9.73
16	10.98	16	11.29
18	13.31	18	12.61
20	13.86	20	14.07
22	15.81	22	14.98

TABLE 5B: IgG TITRATION RESULTS FOR WT AND CORD ENZYMES:

IgG FRACTION + WT ENZYME		IgG FRACTION + CORD ENZYME	
Vol. Enz. Sol.	Activity	Vol. Enz. Sol.	Activity
4ul.	----	4ul.	.11
6	.092	6	1.09
8	.640	8	2.29
10	.89	10	3.54
12	1.54	12	4.82
14	3.39	14	5.5
16	4.63	16	7.43
18	6.31	18	8.63
20	8.58	20	----
22	9.97	22	11.29

FIGURE 35: Titration curve for the formation of the WT enzyme antigen-antibody complex. The concentration of enzyme in a total of 30ul varied as indicated on the abscissa of the figure. The top line of the figure is a control in which pre-immunization serum was used. The experimental format for the controls was the same as that for the IgG fractions (See Methods and Materials). From these data, it can be shown that approximately 152ug of this IgG fraction would be required to inhibit one unit of activity (See Table 5A-B).

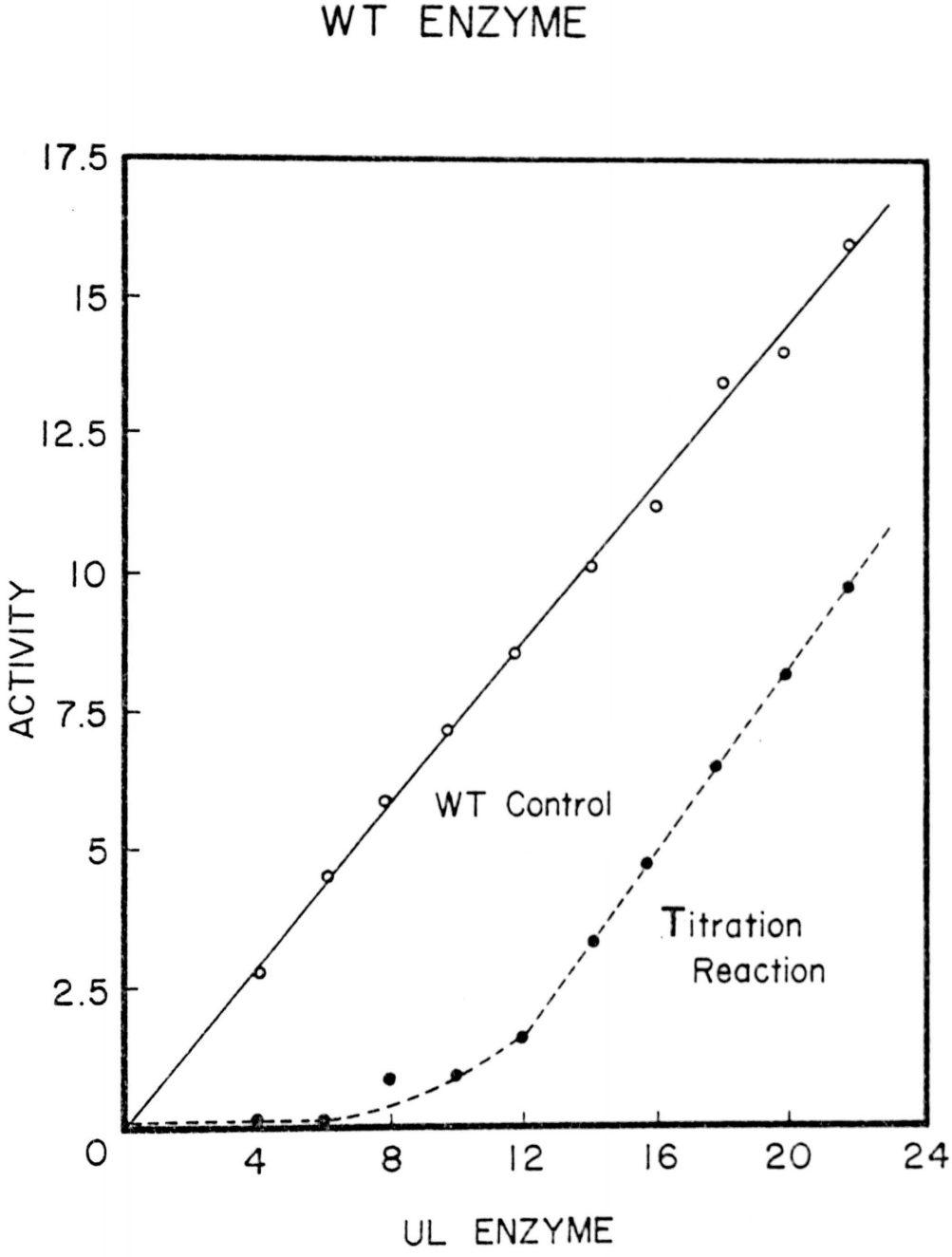
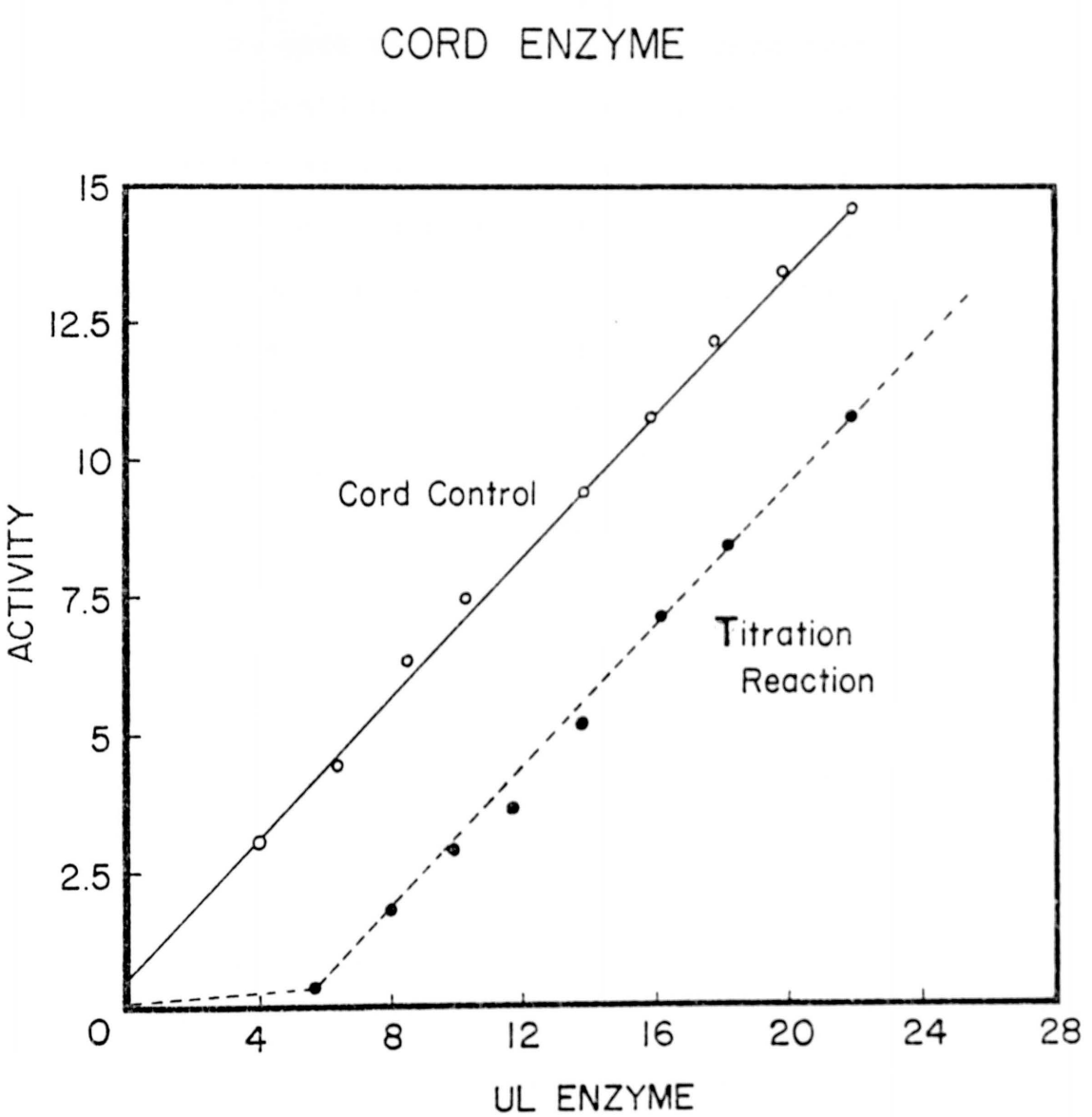

WT ENZYME
17.5
15
12.5
10
7.5
5
2.5
0
ACTIVITY
WT Control
Titration
Reaction
4
8
12
16
20
24
UL ENZYME

FIGURE 36: Titration curve for the formation of the CORD enzyme antigen-antibody complex. The top line of the figure is a control in which pre-immuinzation serum was used. The experimental format for the controls was the same as that for the IgG fractions (See Methods and Materials). From these data, it can be shown that approximately 293ug of IgG fraction would be required to inhibit one unit of CORD activity as compared to 152ug of IgG to inhibit the WT enzyme activity (See Tables 5A-B).


CORD ENZYME
15
12.5
10
7.5
5
2.5
0
ACTIVITY
Cord Control
Titration
Reaction
4
8
12
16
20
24
28
UL ENZYME

Peptide Mapping

As indicated in Methods and Materials, two types of peptide maps were run. One technique used trypsin and chymotrypsin digestion of the three enzymes in SDS peptide sample buffer without prior treatment with IAC. These enzyme samples used were those eluted from the separation of the enzyme-IgG complex on 9% SDS acrylamide tube gels. The results of this digestion procedure are shown in Figure 37 A and B. Figure 37 A shows the peptide band pattern obtained when the three enzymes were digested with trypsin. These patterns were similar with the exception that some of the WT enzyme bands were more intense than the corresponding bands of the mutant enzymes (*). However, the amount of enzyme protein placed on the gel was the same for each of the enzymes. Figure 37 B illustrates the band patterns obtained from chymotrypsin digestion of the enzymes using the same method. These pattern formations also showed differences in the band intensities of the mutant enzymes when compared to the WT enzyme pattern. It was also noted that some of the intermediate bands present in the WT enzyme pattern were missing in the CAD and CORD mutant patterns.

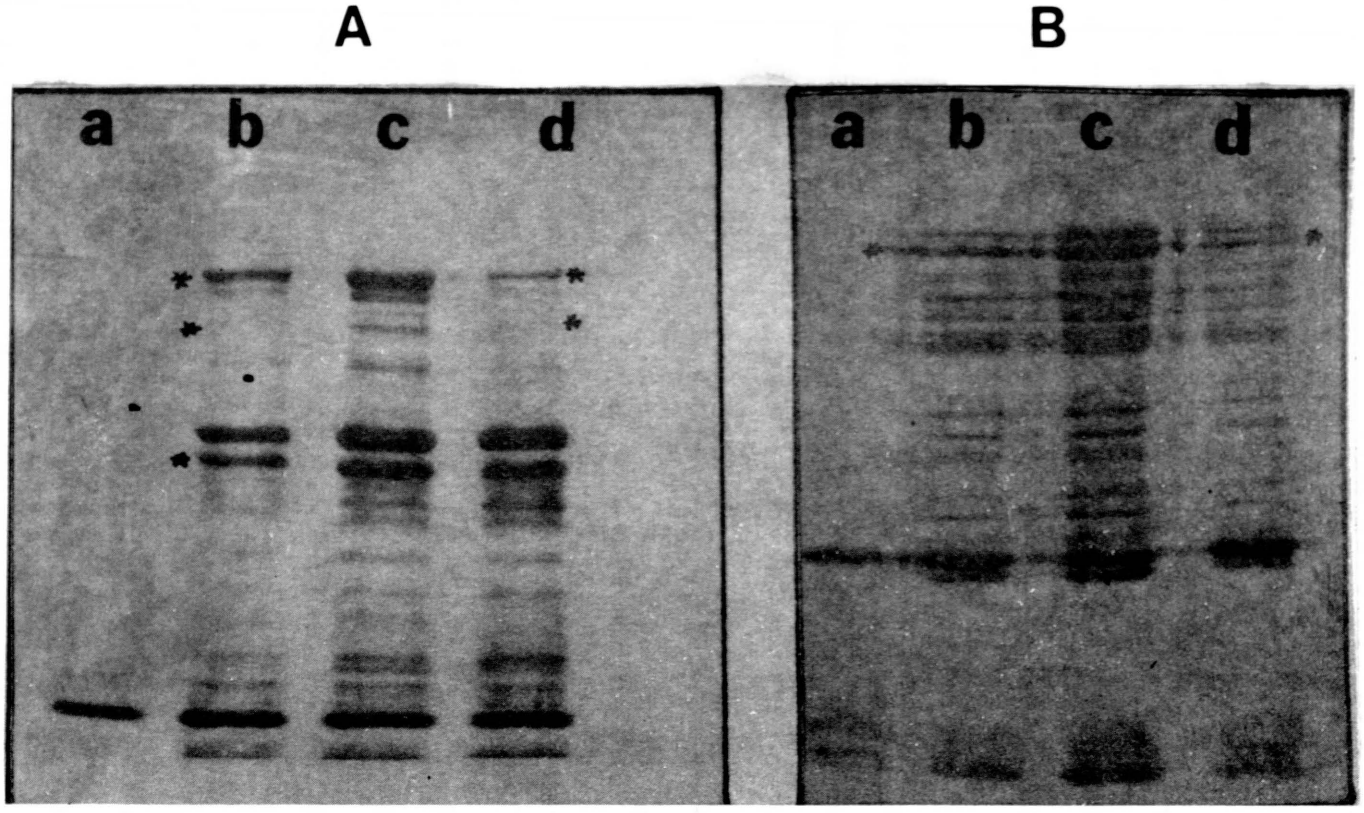
The procedure used appeared to produce only partially digested enzyme fragments, since the number of peptides actually generated by this method was less than the number calculated.

FIGURE 37A: Peptide map of enzymes partially digested with trypsin, without prior treatment with IAC. Approximately 150ug of each of the digested enzymes were run on an exponential gradient polyacrylamide slab gel. The results obtained showed similar band patterns between the WT enzyme and the two mutant enzymes. However, a number of the WT peptide bands were more intense than the corresponding bands of the mutant enzymes (I).

a. Trypsin control
b. CAD enzyme band pattern
c. WT enzyme band pattern
d. CORD enzyme band pattern

FIGURE 37B: Peptide map of enzymes partially digested with chymotrypsin, without prior treatment with IAC. The experimental format was the same as given in Figure 37A. These pattern formations also show differences in the mutant enzyme band intensities when compared to the WT enzyme pattern. It was also noted that some of the peptide bands present in the WT enzyme pattern were missing in the CAD and CORD mutants (I).

a. Chymotrypsin control
b. CAD enzyme pattern
c. WT enzyme pattern
d. CORD enzyme pattern


A
B
a
b
c
d
a
b
c
d

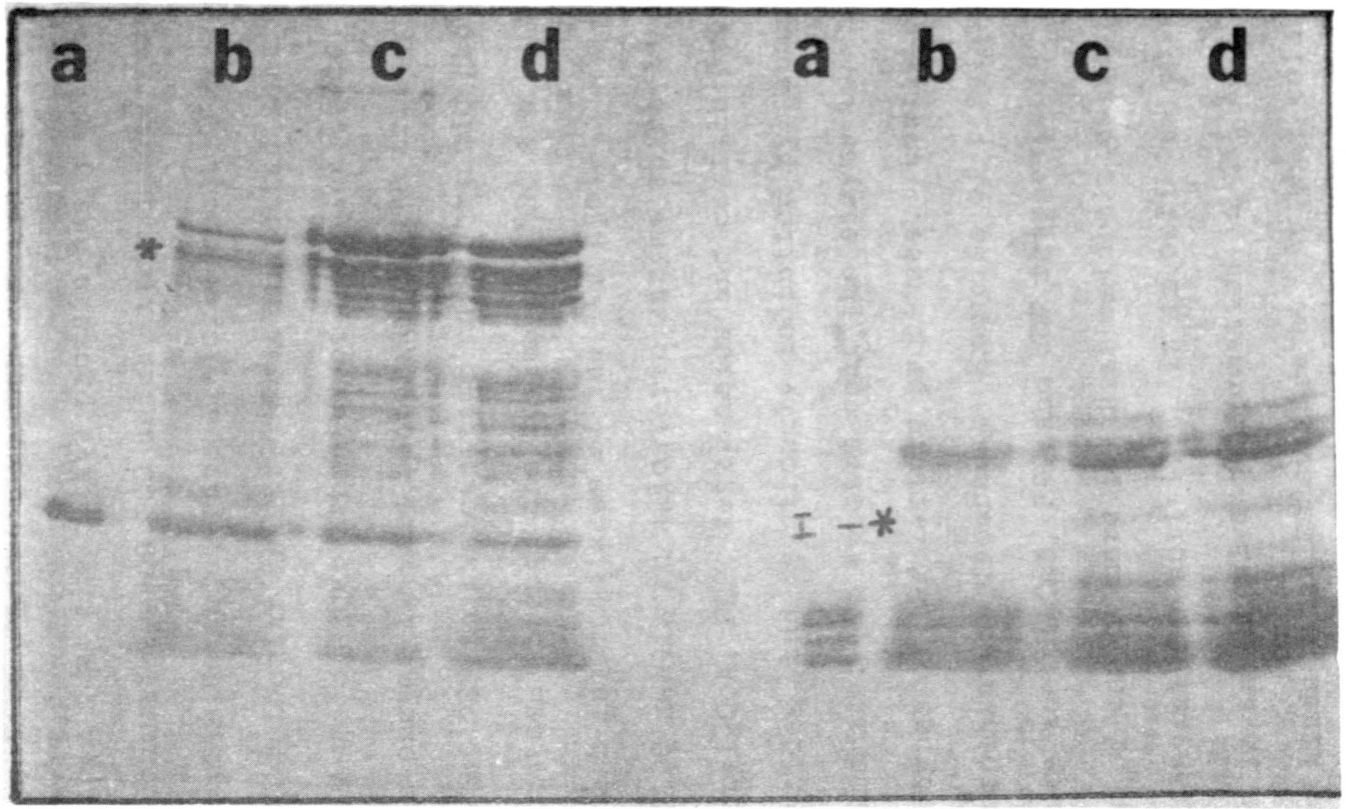
The second method used for peptide mapping employed IAC treatment of the enzymes before digestion of the three enzymes with trypsin and chymotrypsin. As noted in Methods and Materials, the IAC treated enzymes were dried to remove excess IAC and formic acid. Figure 38A shows the peptide pattern generated from IAC pre-treatment followed by trypsin digestion. Again, the amounts of protein placed on the gels were the same. The trypsin patterns showed differences in band intensities of the mutant enzymes when compared to the WT peptide pattern. In the CAD mutant, there appeared to be a difference in the number of intermediate bands present when compared to the WT and CORD. The greatest difference in the peptide patterns of the three enzymes was observed in the IAC-chymotrypsin digested enzymes (Figure 38B). A section of the intermediate peptides was missing in the CAD mutant pattern.

The reserved second portion of the IAC treated enzymes was dissolved in 0.2M ammonium-bicarbonate and used for two-dimensional peptide mapping on TLC plates. The results obtained from several experiments were not consistent, even-though there were changes noted in the CAD and CORD peptide patterns when observed under UV lighting. The consistently large peptide fragment which was located near the origin of application of the enzymes, indicated that the enzymes were not completely digested using this method.

FIGURE 38A: Peptide map of trypsin digested enzymes with prior IAC treatment. All three enzymes were treated with 0.01M IAC in the presence of guanidinium hydrochloride (See Methods and Materials). The trypsin digest patterns again show differences in the band intensities of the mutant enzymes when compared to the WT enzyme pattern. The CAD mutant pattern also appears to exhibit a difference in the number of bands when compared to the WT and CORD patterns.

a. Trypsin control
b. CAD enzyme pattern
c. WT enzyme pattern
d. CORD enzyme pattern

FIGURE 38B: Peptide map of chymotrypsin digested enzymes with prior IAC treatment. Although the protein concentrations of the purified enzymes placed on the gel were equal, a section of peptide bands in the CAD enzyme pattern were missing (I). Using this technique, the enzymes were only partially digested.



A

B

DISCUSSION

Previous studies using the enzyme PEPC'ase from E. coli have shown that the native protein has a molecular weight of approximately 400,000 which, upon denaturation in SDS polyacrylamide gels, gave a single species of approximately 100,000 molecular weight. This indicated that the enzyme is composed of four subunits of identical size. The function of this enzyme in this bacterium is the irreversible carboxylation of PEP to oxaloacetate and inorganic phosphate. The OAA produced through the action of PEPC'ase is used to replenish intermediates of the TCA cycle.

PEPC'ase is an allosteric enzyme which is activated by acetyl-CoA, FDP, fatty acids and certain pyrimidine nucleotides, and is strongly inhibited by aspartate, malate and fumarate. It appears that the enzyme is subjected to three types of regulartory processes: contiguous, precursor and compensatory feedback. Examples of regulators of each type are acetyl-CoA, FDP and CDP respectively. The interaction of one or more of these metabolites with the enzyme is essential for activity. It is believed that the enzyme exists in an inactive conformation in the cell until the enzyme binds with its substrate. At that point, all subunits change from the inactive (T conformation) to the

active conformation (R). Activators have a greater affinity for the R form and PEPC'ase inhibitors have a greater affinity for the T form. There are at least two types of activator binding sites for the enzyme; one for acetyl-CoA and another for FDP and CDP. In addition, there are binding sites for the substrate and for inhibitors.

The two mutant E. coli organisms, whose enzymes were studied in conjunction with the WT enzyme, were produced in the lab by treatment of the organisms with nitroso-guanidine. This chemical is an alkylating agent which would be expected to affect the N7 position of guanine in DNA. It is known that a change in the guanine moiety could result in mispairing and lead to an insertion of a different (wrong) amino acid. This insertion could result in termination of the protein chain or in an end product protein which shows defective catalytic and regulatory properties including changes in antigenic binding sites for the anti-WT antibody. As shown by the disc gel electrophoresis studies, however, chain termination did not occur for either of these mutant enzymes since both had subunits of aproximately 100,000 M.W.

The enzymes from the mutant organisms have shown changes in catalytic and regulatory activity. The CORD mutant, which has as much as 50-60 percent of its PEPC'ase activity, is not activated by FDP or inhibited by aspartate,

indicating that some structural anomalies exist in the binding sites. The second mutant, CAD, has less than 5 percent of the WT activity and is produced at about 30 percent (equivalence) of the WT enzyme. Without the ability to replenish TCA cycle intermediates, these mutants can not sustain growth on medium where glucose is the sole carbon source.

In order to determine the nature of the structural changes in the two mutant enzymes by comparison to the WT enzyme, it was necessary to obtain them in a pure form. A purification procedure was developed, since it had been demonstrated that the two mutants could not be purified by the WT extraction method. The enzyme from E. coli strain B has been purified to homogeneity, however, the enzyme from the K-12 strain which was used in this study had not previously been purified. This WT enzyme was purified to homogeneity, in this study, as indicated by the presence of one band upon electrophoresis in SDS polyacrylamide gels (9 percent) and by having a specific activity of 92.3 Units/mg. of protein. Antibodies were raised in a goat against the WT enzyme. This antibody was subsequently used to purify the two mutant enzymes. Specific antibodies have been used for the purification of other enzymes but not PEPC'ase.

The enzyme-antibody complexes of the two mutants were separated on 9 percent SDS acrylamide tube gels. As shown

in the Results, the enzymes were eventually obtained in a homogeneous state.

The results of the titration-inhibition studies reinforced the idea of structural changes in the CORD enzyme, i.e., it showed a different binding affinity from the WT enzyme indicating fewer antigenic sites on the protein. The CAD enzyme could not be tested by this method since it had no catalytic activity.

As indicated in Figures 30-31 (also Tables 5A and B), the amount of antibody required to inhibit one unit of CORD enzyme activity was almost two times that required to inhibit one unit of activity of the WT enzyme (151.7ug.IgG/U of WT compared to 293.4ug.IgG/U of CORD). These differences are indicative of either; (1) changes in the amino acids at the antigenic binding sites or (2) a change in the number of antigenic binding sites due to changes in the tertiary and/or quaternary structure in the mutant enzymes. Either one or both of the above structural changes could be expressed in the mutants.

Immunological differences in the CAD mutant were observed with the Ouchterlony immunodiffusion method. Although all three enzymes showed a positive reaction (noted by the precipitant lines formed), the CAD mutant showed two precipitant bands indicating two immuno-reactive species. There are several possible explanations for these bands which are

discussed below.

Crossed-immunoelectrophoresis is an effective tool even in the studies of complex antigen-antibody systems. This method maybe used in two ways depending on whether quantitation or the electrophoretic resolution is the intent of the analysis. This technique enabled us to study and correlate observed immunological changes exhibited by the three enzymes.

Results using the crossed-immunoelectrophoretic technique, demonstrated the presence of two immuno-reactive components in the CAD mutant enzyme, as compared to the WT and CORD enzymes (Figure 18-23). In the second dimension, using SDS treated enzyme, two precipitin arcs were formed in the antibody gel for the CAD mutant. This result maybe indicative of tertiary and/or quaternary changes in the CAD enzyme when compared to the CORD and WT enzymes. There definitely appears to be more than one immuno-reactive protein which does not result from breakage of or formation of interchain disulfide bridges. The second band maybe the result of partial degradation of the altered enzyme.

The rocket-immunoelectrophoresis technique was used to determine the amount of mutant enzyme present. This method represents a reproducible technique for determination of a single protein in a complex mixture of protein. Since the cellular enzyme concentration of the mutants could not be

determined through purification procedures, this method represented an important technique for quantification of the mutant enzymes using the WT protein concentration as a standard.

The identification of the proteins is given by the rocket-shaped precipitate formed in the antibody gel, and quantitation of protein content is based on a correlation of the height of the precipitant rocket arc to the amount of WT protein used. To quantitate more accurately the amount of enzyme precipitated by the antibody, the percentage (ratio) of enzyme to antibody (amount of IgG heavy chain) on the gel was determined and incorporated (Results Table 3 and Figure 32), into the calculations.

As given in the results, the CAD mutant produces about 30 percent as much WT-equivalent cross-reacting material. The CORD mutant, on the other hand, produces approximately 75-85 percent as much cross-reacting material as the WT. This indicates that although the antibodies elicited against the WT enzyme react with each of the enzymes with the same degree of specificity, the antibody binds to the enzymes with different affinities; WT CORD CAD. From these results, it can be concluded that certain surface changes on the CORD and CAD enzymes have occurred. It is probable that these surface changes observed, to a greater degree in the CAD mutant, are linked to the inability of this enzyme to

function catalytically.

Peptide mapping studies (without prior IAC treatment) were done to identify differences in the amino acid composition of the mutant enzymes when compared to the WT enzyme, more specifically to identify peptides that behaved differently on peptide maps. The peptide pattern of each enzyme was identified on gradient slab gels after undergoing partial proteolytic digestion with trypsin or chymotrypsin. In the first study, the purified enzymes were not treated with IAC prior to digestion (See Methods and Materials).

Since the WT enzyme consist of approximately 101 lysine plus arginine residues (according to studies using E. coli B), it is estimated that 102 peptides would be generated from trypsin digestion. However, approximately one-third that number of peptides were actually observed for the WT enzyme under these conditions. It was noted that the peptide patterns of the three enzymes (using trypsin digest) were similar, but the intensities of some of the bands were different in the CORD and CAD enzyme patterns. Specific conditions were used to eliminate artifacts and insure valid results. These conditions included: running the three enzymes on the same gradient, using equal amounts of the enzyme protein and maintaining constant external conditions.

Changes noted in the mutant enzymes (band intensity) upon digestion can be interpretated as changes in the number

of peptides produced having the same molecular weight. An increased number of the same molecular weight peptides produced would cause increased band intensity and the reverse could occur for a decrease in intensity. In either case, production of greater or lesser number of peptides would result from the susceptibility or availability of the lysine and/or arginine residues to proteolytic attack.

The chymotrypsin digested peptides of the CORD and WT enzymes also gave similar band patterns. However, there were differences again in band intensities. The CAD enzyme did show fewer intermediate bands indicating a change in either the susceptibility or the availability of tyrosine phenylalanine and/or tryptophan residues to proteolytic attack.

Similar results were obtained with trypsin digested peptides that had been reduced and treated with IAC, except the CAD enzyme pattern showed a slight difference in band intensity and the number of intermediate bands. In the case of chymotrypsin digested peptides, where the proteins were pretreated with IAC, considerable differences were observed between the CAD enzyme (fewer intermediate size peptides) and the WT and CORD enzymes. The latter two still gave similar patterns.

Since it has been demonstrated that specific arginine and lysine residues are necessary for catalytic activity and

regulatory function of the enzyme, a loss in the availability of these residues or conformation around them (as indicated by the observed results) could account for the deficient catalytic and regulatory properties exhibited by the mutant enzymes.

Attempts to determine peptide patterns by complete digestion with trypsin and the use of two-dimensional finger printing techniques, i.e. chromatographic/electrophoretic separation on thin layer cellulose plates, were unsuccessful due to the presence of an apparently large undigestible core. Preliminary results using this technique did show some differences in the peptides generated by the CAD mutant when compared to the WT enzyme pattern. However, the results were inconclusive. It is suggested that attempts be made to completely digest this large core. Reasons for the inability to digest the core of the three enzymes are not known. However, this could be due to the presence of bound SDS which had been used throughout the prior procedures.

SUMMARY AND CONCLUSION

The enzyme studied in this research phosphoenolpyruvate carboxylase (PEPC'ase), is one of the allosteric enzymes involved in regulating the flow of carbon into the TCA cycle for some organisms. This enzyme catalyzes the formation of oxaloacetate from PEP. The function and hence the importance of this enzyme in metabolism was established when it was demonstrated that mutants devoid of its catalytic activity would not grow on medium where glucose was the sole carbon source.

To gain some insight on the relationship between the structure and the catalytic and regulatory properties of PEPC'ase, two mutationally altered PEPC'ase enzymes were purified. The aforementioned properties plus immunological properties and peptide maps of the mutants were compared to the WT enzyme. The two mutant enzymes were designated CORD for co-regulatory/dissociation and CAD for catalytic activity deficient. In the dimeric state, the CORD enzyme has essentially the same specific activity as the WT enzyme, the CAD, on the other hand, has less than 5 percent of the activity of the WT enzyme.

The two mutant enzymes and the WT enzyme have been purified to homogeneity by the use of specific interactions with antibodies prepared against the WT enzyme.

Antibodies elicited against the WT enzyme precipitated each of the proteins but with different affinities, i.e., WT CORD CAD. It is concluded that these binding differences indicate surface changes in the two mutant enzymes, changes which are linked to changes in the structure of the surface antigenic binding sites. Thus, the immunological differences observed probably reflect changes in the tertiary or quaternary structures of the mutant enzymes.

Some changes in the mutant enzymes, when compared to the WT enzyme, have been detected in the primary structure as judged by peptide maps run in polyacrylamide gels. Partial digestion with trypsin and chymotrypsin without prior treatment with IAC gave similar pattern. However, some of the WT enzyme bands were more intense than the CAD or CORD mutant bands, even though the amount of homogeneous enzyme run on the gels was the same.

In cases where the proteins were pretreated with trypsin and chymotrypsin, considerable differences were observed between the CAD enzyme (fewer intermediate size peptides) and the WT and CORD enzymes. The latter two still gave similar patterns, with the exception that the CORD peptide band intensity was less than the WT peptide bands.

Attempts to determine peptide patterns by complete digestion with trypsin and the use of two-dimensional finger

printing techniques, i.e., chromatographic/electrophoretic separation on thin layer cellulose plates, was successful due to the presence of an apparently large undigestible enzyme core. However, the preliminary results using this technique indicated a change in the finger printing pattern of the CAD enzyme when compared to the WT or CORD enzymes. The differences observed indicate that whatever the structural change is that resulted in the loss of activation by FDP or complete loss of activity of the CAD mutant enzyme, may have also caused a change in the susceptibility of specific amino acids to proteolytic attack.

The structural alteration in the CORD mutant affects both subunit-subunit interaction and co-regulator activity suggesting a causal relationship between these two phoenomena. The structural alteration in the CAD mutant affects catalytic activity but it is not yet clear whether activation binding or substrate binding is affected most.

It is apparent from these studies that a primary structural change has occurred. The extent to which it also affects tertiary or quaternary structure is not known. It is also concluded that this research has set the foundation for further studies to determine the exact structural change which has occurred. Our intent is to develop a technique whereby the three enzymes can be completely digested with the protease used and a two-dimensional fingerprint pattern

elucidated. Peptides with different properties will be characterized further with respect to their amino acid content.

This premise is based on the conclusion that changes in the band intensities of the mutant enzymes (as demonstrated in this research) reflect a change in the susceptibility and/or availability of the lysine and arginine residues in the case of trypsin digest and tyrosine, phenylalanine and tryptophan in the case of chymotrypsin digest.

Since it has been demonstrated in previous studies, that specific lysine and arginine residues were necessary for catalytic and regulatory function of the enzyme, loss of these amino acids or changes in their environment by mutation could result in the deficiencies noted in the two mutant enzymes studied in this research. In essence, a change in the availability of these amino acids, necessary for binding the activators and regulators, would be reflected in lost or diminished activity as well as lost or diminished regulatory properties.

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VITAE

Beverly Ann Kerl Mitchell was born in Dallas, Texas, the youngest child of Mr. and Mrs. J.M. Kerl. She graduated from James Madison High School in 1964. Upon graduation, she was awarded a four-year scholarship to the college of her choice from the Alpha Kappa Alpha Sorority. She attended Dillard University in New Orleans, where she also received a university scholarship. She received her B.S. degree in biology in 1968. She married Don M. Mitchell in August of that year and also entered Texas Woman's University Graduate School. She worked during that time as a graduate research assistant under Dr. R.A. Gerdes. In October of 1969 her first daughter, Teri Dawn Mitchell was born. In May of 1970, she became the first black to receive an M.S. degree in pure science from Texas Woman's University.

She entered TWU's doctoral program in Molecular Biology the following year. That same year, October, 1971 her second daughter, Stacy Ann Mitchell was born. From 1971-1975 she held a graduate teaching assistantship, a University fellowship, and an NDEA fellowship.

In 1975 she left to work at Bishop College as a faculty researcher, in the biology department. In 1978 she returned to the University to complete the research aspect of the doctoral program. She was accepted into

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