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GENETIC HETEROGENEITY IN COMPLEMENTATION GROUPS OF PROPIONIC ACIDEMIA

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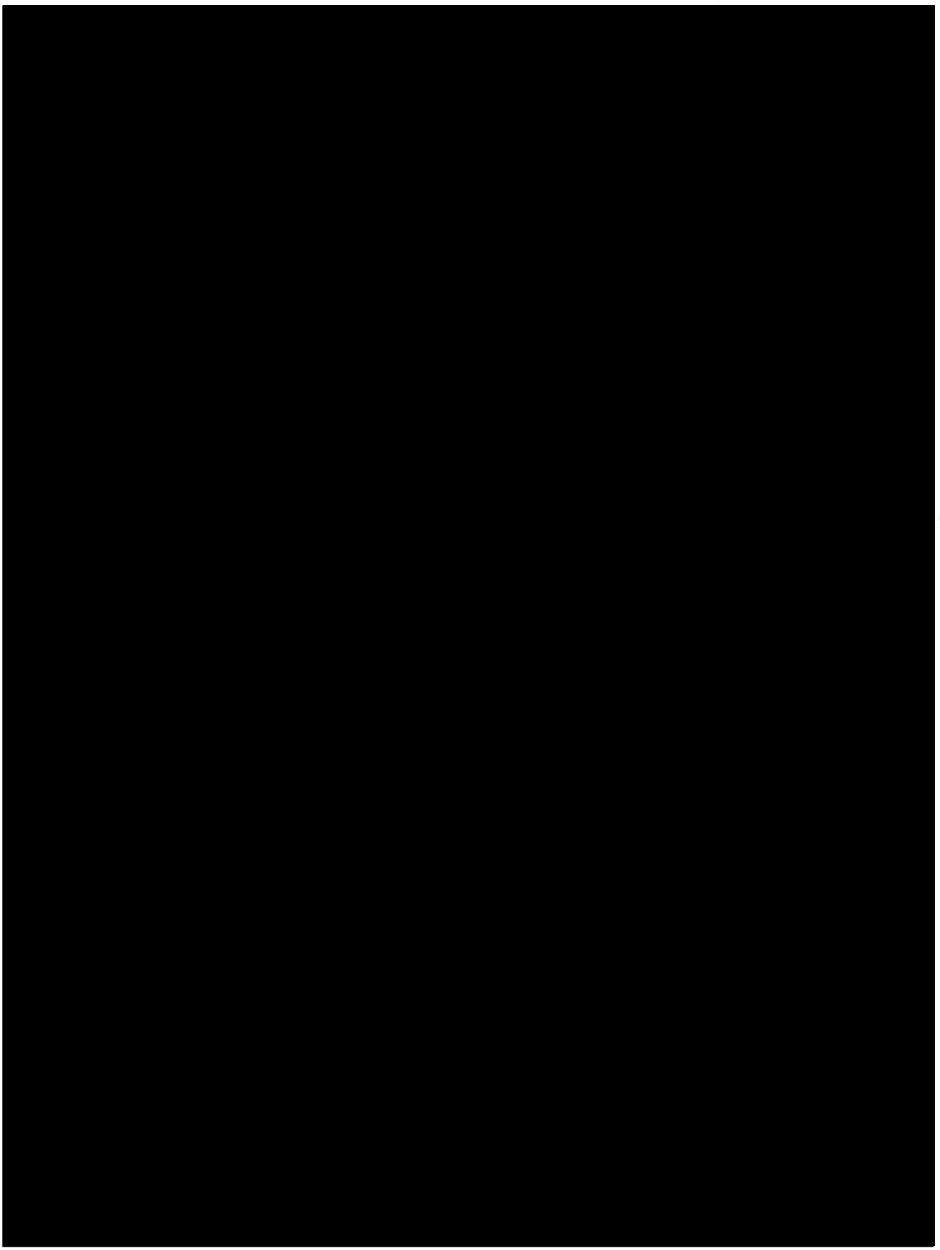

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INTRODUCTION

Propionic acidemia is an autosomal recessively inherited disorder of organic acid metabolism caused by deficient activity of propionyl CoA carboxylase. This enzyme is required for the catabolism of the odd-chain fatty acids, the side chain of cholesterol and the amino acids: isoleucine, methionine, and threonine. Although the clinical expression of this disorder is variable, affected children usually develop ketoacidosis and hyperammonemia which may progress to seizures, coma and possibly death. Some patients may exhibit vomiting, lethargy and hypotonia in the first few weeks of life whereas others may remain asymptomatic for months or even years.(1) The degree of residual enzyme activity in the tissues from these patients does not correlate to this apparent clinical heterogeneity. The relationship between the clinical variation observed among propionyl CoA carboxylase deficient patients and the corresponding genetic and biochemical make up, is not yet understood. The aim of this project is to study the biochemical and immunological properties of propionyl CoA carboxylase in liver homogenates and fibroblast samples from several patients with propionic acidemia, to gain a better understanding of the nature of the defect in this disorder.

Previous comparisons of propionyl CoA carboxylase from fibroblasts of patients with this enzyme deficiency and unaffected individuals have suggested that propionyl CoA carboxylase is structurally altered in the patients with propionic acidemia. The fibroblast lines from these patients can be categorized into two major genetic

complementation groups, pccA and pccBC, based on the increase in activity observed in heterokaryons formed by pairwise cell fusions.(2) In addition, the defective propionyl CoA carboxylase can be differentiated biochemically from the normal enzyme and from each complementation group.(3) These differences suggest that the alterations in propionyl CoA carboxylase structure in each complementation group represent mutations in different subunits. In addition, mutations within a single gene resulting slightly different enzyme structures, would explain the clinical variation within a complementation group. Therefore, biochemical differences among mutant propionyl CoA carboxylases from the same complementation group were investigated.

To provide further evidence that propionic acidemia is the result of structural alterations in propionyl CoA carboxylase, immunologic techniques were used to determine if equal quantities of cross-reacting material (CRM) were present in liver and fibroblast homogenates from propionyl CoA carboxylase deficient patients from the various genetic complementation groups. Antiserum prepared against purified pig heart propionyl CoA carboxylase which cross-reacts with human propionyl CoA carboxylase and another antiserum prepared against the human biotin-containing enzymes, were compared using immunotitration techniques. These tests demonstrated that there are equal quantities of cross-reacting material in the tissue homogenates of propionic acidemia patients.(4, 5)

Investigations were undertaken to biochemically characterize and evaluate the heterogeneity within the pccBC genetic complementation

group. This group was chosen because previous complementation and biochemical studies with fibroblasts from the pccBC subgroups have suggested the existence of interallelic complementation in this group. (6) Specific biochemical differences among propionyl CoA carboxylases from cells belonging to patients in this group could identify heterogeneity and characterize the complementation pattern. Normal and mutant propionyl CoA carboxylase from the pccBC complementation group were highly purified and their biochemical properties were compared using their isoelectric point, thermostability, and enzyme affinity for substrates. The properties of the purified enzymes were then compared with propionyl CoA carboxylase from other mutants in this group. These comparisons demonstrated biochemical heterogeneity within the pccBC complementation group.(7)

The results provide compelling evidence that the defect in propionic acidemia represents a structural alteration of propionyl CoA carboxylase in the pccA and pccBC complementation groups. In addition, the biochemical heterogeneity demonstrated within the pccBC complementation group suggests that several different structural mutations, possibly of the same subunit, are involved that result in slightly different biochemical parameters for each mutant enzyme. These strutural alterations may explain the complicated complementation map for the pccBC subgroups. Since interallelic complementation is based on the heteropolymer being slightly more active than the homopolymer, different structural alterations in the mutants may interfere with their ability to participate in interallelic complementation.

Furthermore, biochemical differences within the group may be reflected in the clinical phenotype of the disease and may be an indicator of the clinical variation which has been observed in these patients.

LITERATURE REVIEW - CLINICAL REVIEW

A. Identification of the Syndrome

Propionic acidemia was initially one of a group of metabolic disorders which was characterized by the clinical finding of hyperglycinemia.(8) In 1961, Childs et al. (9) described a patient with "idiopathic hyperglycenemia" who presented in infancy with vomiting, lethargy, and ketoacidosis. Amino acid analysis of his serum revealed elevated glycine concentrations as well as moderate elevations in the concentrations of leucine, isoleucine, and valine. The administration of loading doses of leucine, isoleucine, valine or threonine, precipitated attacks of ketosis and vomiting, whereas glycine failed to induce symptoms.

Subsequently, another patient was described with the clinical findings of hyperglycinemia and seizures but without ketosis.(10) This patient could also be differentiated from the first case by his response to the loading tests. Infusion of loading doses of isoleucine had no effect, however, the infusion of glycine did result in aggrevation of the clinical symptom in the absence of ketosis.(10) These two cases demonstrate the clinical variation seen among the hyperglycinemia syndromes, the presence and absence of ketosis and they are, therefore, designated as the ketotic and nonketotic forms, respectively.(11) *In vivo* studies of patients with the nonketotic form of hyperglycinemia demonstrate a defect in the conversion of glycine to serine. The catabolism of glycine-1-^{14}C was measured by $^{14}CO_2$ expired and glycine -2-^{14}C utilization was measured by its incorporation into

serine. Both functions were shown to be impaired in these patients. (12)

For several years, investigators searched futilely for a defect of glycine metabolism in the ketotic form of hyperglycinemia. In 1968, Hommes et al.(13) observed a patient with this disease who also had elevated propionate excretion and, therefore, this form of the syndrome became known as propionic acidemia. Autopsy examination of the liver from this patient showed increased concentrations of C_{15}- and C_{17}-odd-chain fatty acids. To explain these findings, Hommes proposed that these patients had a metabolic defect in propionyl CoA carboxylase which would prevent propionyl CoA from being catabolized to methylmalonyl CoA. This defect accounts for the elevated serum concentrations of isoleucine and the presence of increased odd-chain fatty acids since both are degraded via the propionate degradation pathway.

Concurrently, Rosenberg et al.(14) reported another patient with another form of hyperglycinemia who in addition to propionic aciduria excreted elevated concentrations of methylmalonic acid. The massive excretion of methylmalonic acid in this child led Rosenberg and his colleages to postulate that his patient had a defect in the propionate catabolic pathway which prevented the conversion of methylmalonyl CoA to succinyl CoA explaining the build up of methylmalonic acid. Restudying Childs' original patient failed to reveal increased methylmalonic excretion. The clinical similarity of these two patients convinced the authors that the original proband might have a defect in

the previous step of the same pathway, the carboxylation of propionyl CoA to methylmalonyl CoA. Again, the identification of a new ketotic hyperglycinemia syndrome, methylmalonic acidemia, indicated further heterogeneity among the hyperglycinemias and suggested the possible defect in a similar syndrome.

In 1970, Hsia et al.(15) demonstrated a block in propionate metabolism in the leukocytes from the affected sibling of Childs' original patient and in the proband with methylmalonic acidemia. Leukocytes from both patients and controls were incubated with either 3-^{14}C-propionate, ^{14}C-methylmalonate or ^{14}C-succinate. The quantity of ^{14}C incorporated into trichloroacetic acid precipitatable material was measured as an indication of metabolism through the propionate pathway. The propionic acidemic patient was not able to metabolize the labelled propionate but could utilize both methylmalonate and succinate normally. The methylmalonic acidemia patient's cells could neither utilize labelled propionate nor methylmalonate but could metabolize the labelled succinate normally. Since this is the major pathway by which propionate is metabolized, any substances entering this pathway prior to a block cannot be metabolized further. This experiment, therefore, localized the block in this pathway for each disorder. The block in the disorder, propionic acidemia, occurred at the enzyme propionyl CoA carboxylase as previously postulated. The block in methylmalonic acidemia is in methylmalonyl CoA mutase, methylmalonyl CoA racemase or cobalamin metabolism. The defect in propionic acidemia was confirmed by Gompertz et al.(16) who measured

propionyl CoA carboxylase activity directly in a liver mitochondrial preparation from a deceased patient with propionic acidemia and confirmed a deficiency of this enzyme. This finding was later confirmed in fibroblasts from the original proband and his affected sibling who were found to have less than 2% of normal carboxylase activity.(17) Propionyl CoA carboxylase activity in fibroblasts from each parent showed approximately 50% of normal activity. Because there was more than one affected sibling within this family and because the parents demonstrated propionyl CoA carboxylase activity intermediate between the deficient and control values, the disorder was considered to be inherited as an autosomal recessive condition.(17) Further evidence for autosomal recessive inheritance was provided by several families with multiple affected siblings and documented parental consanguinity.(16, 18)

B. Clinical Manifestations

The clinical features of propionic acidemia are extremely variable. The classical manifestations of this disorder include severe metabolic acidosis, vomiting, dehydration and lethargy which may progress to coma if untreated. Biochemical analysis may show high concentrations of propionate, ammonia, and glycine in the serum or urine but these latter findings are not constant. The incidence of compromised metabolism is usually episodic and must be treated aggressively with fluids, glucose, and bicarbonate replacement to minimize the effect on the patient.(19)

Many patients with propionic acidemia have presented with ketosis

early in the neonatal, others have not exhibited symptoms until several months of age. Some of the variability in the age of onset of symptoms may be explained by differences in dietary protein intake or the occurrence of infections. These two factors often interact to precipitate a crisis by causing an overload of endogenous protein catabolism not tolerated by the patient.(20)

The mode of presentation of this disorder is also variable. Ketoacidosis signals immediately that the problem may be metabolic; however, recently some patients have been reported who have not presented with acidosis. Non-specific symptoms such as vomiting, dehydration and lethargy can mimic commonly occurring problems such as sepsis, gastrointestinal obstruction, cardiopulmonary problems or birth trauma making diagnosis difficult.(21) The only consistent biochemical finding is elevated serum propionate which would not be detected by routine testing.

Rapid diagnosis of this disorder is essential for proper treatment because the acidosis in these children may be persistent and require agressive treatment.(21) The reduction in protein intake to a tolerable level is a major factor in restoring metabolic balance. Several patients have been misdiagnosed as non-ketotic hyperglycinemia (22, 23) because of the absence of ketosis or as a dysautonomia and subsequently, a urea cycle defect because of the presence of hyperammonemia in the absence of hyperglycinemia.(24) A further demonstration of the clinical heterogeneity is the report of a totally asymtomatic patient who has a comparable enzyme deficiency to other

propionic acidemia patients including her brother who clinically manifests the disorder. This patient has never been ketotic, even when challenged with an isoleucine loading test.(25) Clearly, the clinical variability seen in this disorder makes diagnosis difficult in many cases as well as results in affected symptomatic or asymptomatic individuals who are never diagnosed.

Hyperglycinemia, frequently observed during crises has been a major diagnostic indicator of propionic acidemia and suggests further definitive testing. Perturbation in propionate metabolism caused by the enzyme defect may also result in hyperammonemia especially during metabolic crises.(26) These two biochemical features are probably secondary to the primary defect because they are frequently found in patients with other metabolic disorders such as methylmalonic acidemia and isovaleric acidemia. Several studies have been performed to elucidate the etiology of hyperglycinemia. The cleavage of 1-^{14}C-glycine in vivo as measured by $^{14}CO_2$ expired, has been shown to be impaired. (27, 28, 29) However, the ability of 2-^{14}C-glycine to be converted to labelled serine seems to be normal in propionic acidemia patients in contrast to the findings in nonketotic hyperglycinemia.(30) A secondary inhibition of the glycine cleavage system by propionate or its metabolites may account for the elevated glycine concentrations observed in propionic acidemia.

There is growing evidence that the increase concentration of propionate or its metabolites which occurs in body tissues of these patients causes a secondary hyperammonemia. Serum concentrations of

ammonia have been shown to be directly proportional to the increased levels of serum propionate.(31) The observation of a decrease in carbamyl phosphate synthetase activity in two propionic acidemia patients suggests that propionate concentrations may influence this enzyme.(32, 24) Although, propionate does not inhibit carbamyl phosphate synthetase, the CoA derivatives tiglyl CoA, propionyl CoA, and methylmalonyl CoA as well as free Coenzyme A inhibit the activity of carbamyl phosphate synthetase from normal human liver up to 70%.(33) An alternate mechanism by which propionyl CoA may influence carbamyl phosphate synthetase activity is suggested by its ability to inhibit N-acetylglutamate synthetase. N-acetylglutamate is an activator of carbamyl phosphate synthetase; therefore, a decrease in its synthesis would result in a decrease in the activity of carbamyl phosphate synthetase.(34) A third mechanism implicates the depletion of ATP by high levels of propionate which in turn inhibits carbamyl phosphate synthetase. The inhibition of this enzyme is relieved by the addition of excess ATP to the system.(35)

Another mechanism for urea cycle inhibition has been proposed by Williamson et al. (36) which involves the tying up of free acetyl-CoA by the acyl intermediates which build up along the pathway. The decline in free cellular acetyl-CoA inhibits pyruvate carboxylase which has been shown to decrease both gluconeogenesis and urea synthesis by 50% in isolated rat liver cells. This mechanism may also explain the hypoglycemia often accompanying this disorder.

Other findings which have been described in association with

propionic acidemia include neutropenia and intermittent thrombocytopenia which may possibly account for the frequent infections in affected children. Chronic features such as seizures and mental retardation have been described frequently in this disorder but may be secondary to the acute early incidences of acidosis which are accompanied by hyperammonemia which is probably injurious to brain development and function.(21) There is hope that with earlier recognition and better metabolic control, many of these problems can be averted.

C. Abnormal Metabolites

The deficiency of propionyl CoA carboxylase activity in these patients causes an accumulation of propionate in body tissues resulting in the metabolism of propionate by alternate minor pathways. Many of these pathways have been identified by elucidating abnormal metabolites in the serum or urine of patients with this disorder. The use of isotopes to identify abnormal metabolites has also contributed to our knowledge of normal human metabolism by identifying reactions which had not been previously described in man.

The first observation of propionate being shunted through an alternate pathway was made by Hommes et al.(13) in one of the original proband with the finding of increased C_{15} and C_{17} odd-chain fatty acids. These fatty acids can be generated by substituting propionyl CoA for acetyl CoA in fatty acid synthesis causing the addition of three carbon units rather than two carbon units. This process would

occur even though the affinity of the enzyme for propionate may be low, due to the high concentrations of propionyl CoA in the cells.

Composition of the ketones found in propionic acidemia patients are different from those found in other children with ketosis. Menkes (37) compared the ketone found in the ketotic crisis of Childs' original patient to five other children with varying causes of ketosis. In the children with ketosis from other causes, acetone formed approximately 95% of their ketone. During ketotic crises, 60% of the ketone from patients with propionic acidemia were acetone whereas the remainder consisted of four or more carbon units. Three unusual ketones were found, 2-butanone, 2-pentanone and 2-hexanone. The 2-butanone which formed 25% of the patients' ketones had not previously been described in humans. This finding was confirmed by Sweetman et al. (38) using gas-liquid chromatography and mass spectroscopy (GC/MS) who postulated that this ketone was derived through isoleucine metabolism from the intermediate 2-methylacetoacetyl CoA as shown in step 4 of the pathway in figure 1.

The most abundant abnormal metabolite found in the urine of propionic acidemia patients is methylcitrate. This substance was found in the urine of a patient after being fed 1-^{14}C-propionate and identified by GC/MS. Methylcitrate is formed by the consolidation of propionyl CoA with oxaloacetate by citrate synthetase.(39) Studies of this enzyme from pig heart show that it can use propionyl CoA approximately 1/1000 as efficiently as acetly CoA.(40)

Another pathway metabolises propionate by β-oxidation to

3-hydroxypropionate.(41) The finding that a labeled ^{14}C-valine can be converted to hydroxypropionate indicated that valine must be normally metabolized through propionate.

Other metabolites of isoleucine catabolism are found in the urine of propionic acidemia patients. The most abundant is tiglic acid.(42) Two previously unrecognized urinary metabolites, 2-methylbutyric acid and 2-methyl-3-hydroxybutyric acid, are derived from their CoA intermediates in the isoleucine degradative pathway.(Steps 1, 2, and 3 in figure 1) (38) These metabolic intermediates accumulate because the block in propionate utilization causes a shift in the equilibrium along the pathway.

A general mechanism for detoxifying CoA derivatives is their conjugation with glycine. Propionylglycine (43), tiglylglycine (44), and 2-methyl butyrylglycine (38) have all been identified. It has been postulated that the high glycine levels in this syndrome stimulates detoxification. The high glycine concentrations should then result in a decrease in propionate concentrations and be inversely proportional to the propionate concentration instead of directly proportional, as has been observed.(43)

Two condensation products of propionyl CoA and acetyl CoA have been observed: 3-oxovaleric acid and 3-hydroxyvaleric acid. (Steps 6 and 7 in figure 1) The thiolase from pig heart which normally catalyzes the condensation of two acetyl CoA molecules has been shown to use propionyl CoA at about 2% of its maximal efficiency.(38) The occurrence of this abnormal condensation indicates that the

ABNORMAL METABOLITES

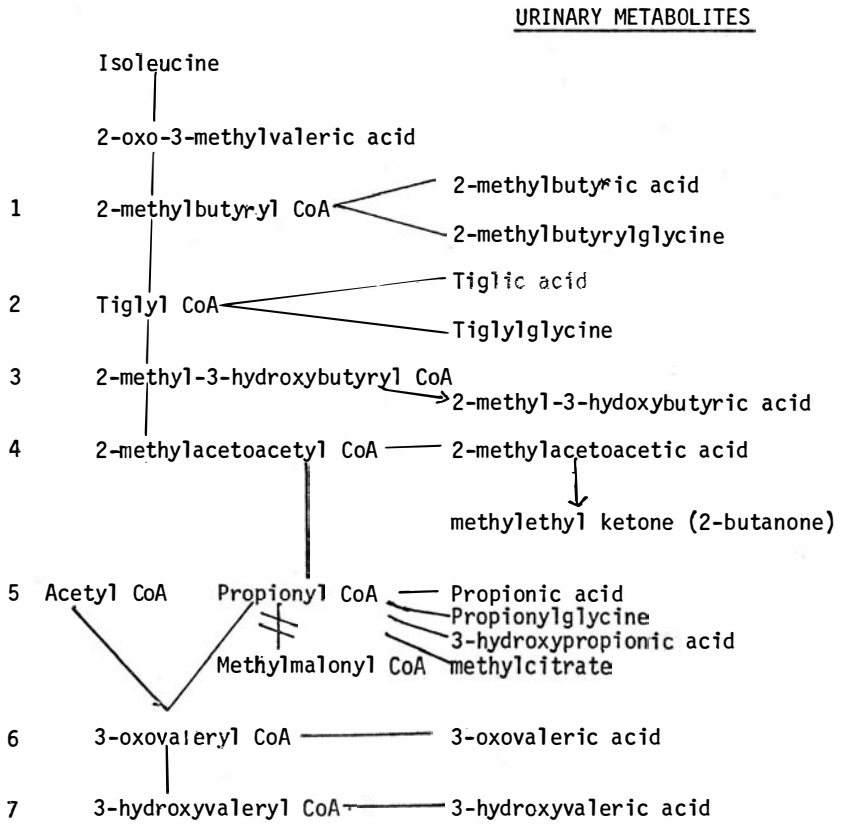


Figure 1 - Abnormal urinary metabolites. A proposed pathway for the production of abnormal urinary metabolites. (38)

condensation of acetyl CoA in man may occur in normal metabolism since the enzyme must be present.

The urinary metabolite excretion patterns in propionic acidemia are similar to those found in β-methylcrotonyl CoA carboxylase deficiency and methylmalonic acidemia, but they differ significantly from those seen in non-ketotic hyperglycinemia.(45, 46)

D. Treatment

The major method of treating this disorder has been to permit normal protein synthesis without causing endogenous protein catabolism. This is accomplished by restricting dietary protein thereby limiting potentially harmful amino acids isoleucine, valine, methionine and threonine. The restricted protein intake ranges from 1 to 1.5 grams per kilogram body weight per day while normal caloric requirements are made up largely from foods rich in carbohydrates. This diet requires a delicate balance between the amount of protein these children can tolerate without developing symptoms and the amount required for normal mental and physical growth. Even minor childhood infections can alter body protein catabolism and compromise these patients.(20)

The success of this form of therapy has been variable. Management of these patients seems to be most difficult between birth and two years, when the metabolic demands of the body are high and the amount of protein required exceeds the amount tolerated by the propionic acidemic patient.(24) During that critical period, many patients develop severe metabolic compromise, irreversable brain damage or die.

Patients surviving this period seem to be easier to control. The earlier literature indicates that most patients died in the first few months of life, even with early dietary treatment.(21) However, with earlier recognition, diagnosis and institution of appropriate therapy, the prognosis is somewhat improved.

E. Biotin Responsive-Propionic Acidemia

Another example of clinical heterogeneity within this disorder was the identification of a growing number of patients who demonstrated clinical improvement after the administration of pharmacologic concentrations of the vitamin, biotin. In the similar disorder methylmalonic acidemia, the vitamin B_{12} which is required for activity of methylmalonyl CoA mutase has been shown to ameliorate the symptoms in a proportion of the cases.(47) This finding led to the speculation that biotin, the coenzyme for propionyl CoA carboxylase could be useful in the treatment of propionic acidemia. The importance of biotin in the treatment of some of these children was confirmed when Barnes et al. (48) reported a child who exhibited marked clinical improvement after being treated with 5 mg. of biotin daily. Subsequently, two other patients have been reported who, in addition to the urinary metabolites previously found in propionic acidemia, had β-methylcrotonylglycine.(49, 50, 51) This abnormal urinary metabolite signalled a second defect in the enzyme β-methylcrotonyl CoA carboxylase. Studies of the fibroblasts from one of these patients demonstrated that both propionyl CoA carboxylase and β-methylcrotonyl

CoA carboxylase activities were deficient. Enzyme activities increased to normal when fibroblasts were incubated in high concentrations of biotin.(50) This finding lead to the hypothesis that the enzyme deficiencies were secondary to a defect in a common holocarboxylase synthetase which attaches biotin to both enzymes or, an alternate hypothesis, that there was a defect in intercellular biotin transport. Recently, fibroblasts from two of these patients have been restudied and have been shown to have a deficiency of a third mitochondrial carboxylase, pyuvate carboxylase.(52) Two additional patients have been reported in whom all three fibroblast mitochondrial carboxylase deficiencies were shown to be biotin responsive.(53, 54) In addition, eight patients have been reported in whom the biotin responsive multiple carboxylase deficiency was demonstrated in leukocytes.(55, 56, 57, 58) Alopecia and dermatitis seem to accompany the syndrome. In one family a defect in immune response has been reported which may account for a candida type skin rash which is frequently observed.(56) An unusual case has been reported where the patient had typical findings for a multiple carboxylase deficiency, including alopecia, dermatitis, hypotonia and large quantities of 3-hydroxyisovaleric acid, β-methylcrotonylglycine, and 3-hydroxypropionate in the urine. But the enzyme assay of the three carboxylases in fibroblasts from this patient showed normal activity. The patient responded clinically to high doses of biotin and therefore the transport of biotin was apparently defective in this patient.(59) A case of biotin responsive propionyl CoA carboxylase deficiency with normal β-methylcrotonyl CoA

carboxylase activity has also been reported.(60) This may represent still another form of the biotin responsive disorders.

For patients with the vitamin responsive carboxylase deficiencies, the administration of biotin doses as high as 50 mg. per day is necessary to ameliorate the symptoms. In contrast, for most propionic acidemia patients, the administration of high doses of biotin does not increase the enzyme's activity even though β-methylcrotonyl CoA carboxylase activity in these patients is increased slightly above normal indicating that biotin is entering these cells.(61)

F. Prenatal Detection

Prenatal diagnosis of this disorder has been attempted using several different methodologies. Direct measurement of propionyl CoA carboxylase activity in cultured amnionic cells is a very sensitive, exact method although considerable time is required to culture enough cells for the enzyme assay. This technique was used by Gompertz et al. (62) to diagnose the first case of propionic acidemia *in utero* by comparing the activity of cultured amnionic cells obtained by amniocentesis with cells obtained from eight other pregnancies which were not at risk.

A method has been proposed by Hill et al.(63) that does not require culturing of the fibroblast cells. Instead, the fibroblasts obtained at amniocentesis are incubated with ^{14}C-propionate for 10 hours, then washed and examined by autoradiographic techniques. This method is rapid and can identify both propionic and methylmalonic

acidemia since neither can incorporate labelled propionate. A quantatative variation of this method was devised utilizing the trichloroacetic acid precipitable material which is counted in a scintillation counter to determine propionate incorporation.(64) Willard and colleagues (65) used this method in four at risk pregnancies for methylmalonic acidemia. They predicted the outcome correctly in all cases and identified one affected fetus.

Another method of identifying a fetus at risk for propionic acidemia is analysis of amnionic fluid for two unique metabolites of propionic acidemia methylcitrate and 3-hydroxypropionate by GC/MS.(66) This procedure is rapid permitting diagnosis within 48 hours.

The importance of rapidly identifying an affected fetus is especially crucial in the vitamin responsive disorders so that prenatal vitamin treatment can be initiated.

Biochemical Review

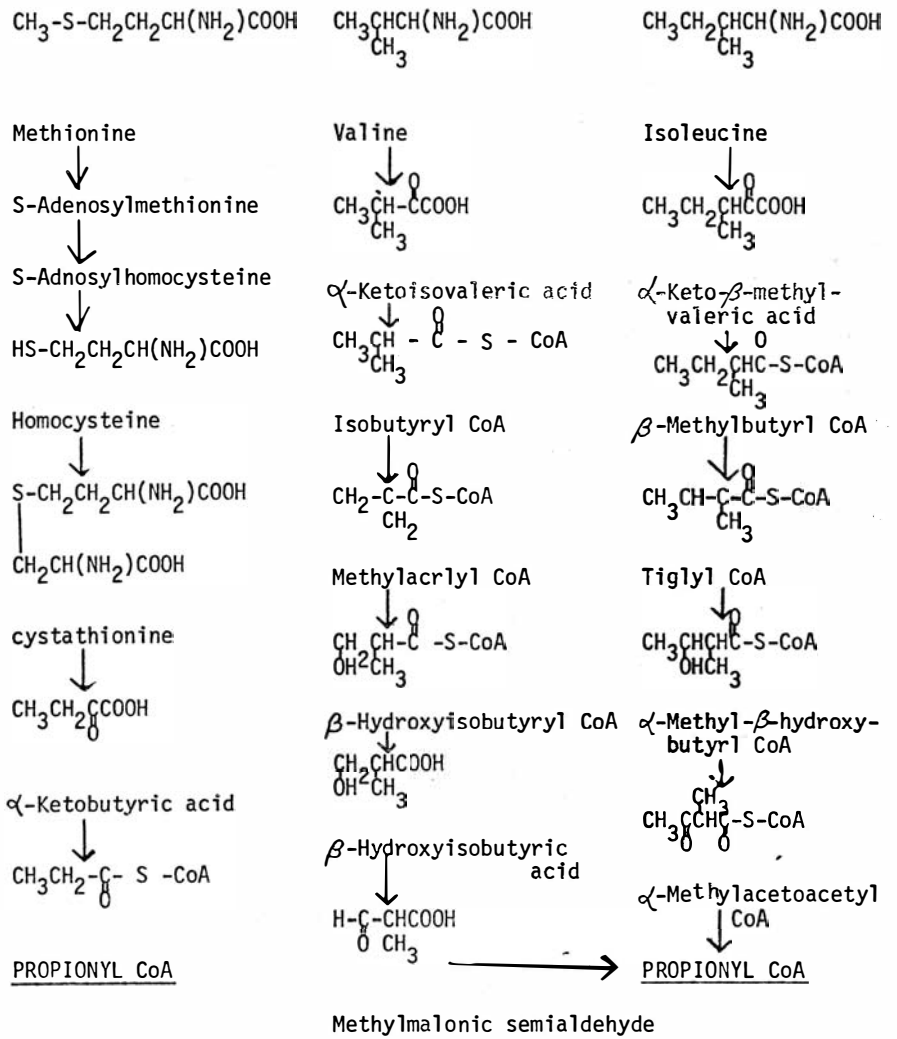
A. Propionate Metabolism

Propionate is produced by the oxidation of the branch-chain amino acids, valine , methionine, and isoleucine, (Figure 2), the β-oxidation of odd-chain fatty acids and the side chain of cholesterol. Propionate is further catabolized to methylmalonyl CoA and subsequently, succinate where it enters the tricarboxylic acid cycle.

Prior to 1950, the catabolism of propionate was thought to occur by direct conversion of another three carbon intermediate such as pyruvate.(47) Lorber et al. (48) first suggested an alternative metabolic pathway, after observing that when α or β-labelled propionate was infused into a rat, both the α and β carbons of the pyruvate isolated were equally labelled. In order to explain the randomization of the label, the investigators postulated that the conversion of propionate to pyruvate was occurring through a symetrical four-carbon intermediate such as succinate. Lardy (49) observed that the $^{14}CO_2$-fixation reaction required the presence of propionate and ATP in crude rat liver mitochondria. This product was extracted and identified as succinate by paper chromatography.(50, 51) Flavin et al. (52), using an enzyme preparation from pig heart, found that the C^{14}-labelled product of carboxylation co-migrated with succinate in their chromatographic system, but it was not converted to fumarate or malate when incubated with succinoxidase. Using several solvent systems, they demonstrated that the actual labelled product was methylmalonic acid.

FIGURE 2

DEGRADATION OF METHIONINE, VALINE, AND ISOLEUCINE TO PROPIONYL CoA
from-Leninger, A.L. in Biochemistry p 577, Worth Publishers Inc., New York, 1978.



This observation was confirmed in rat liver by Katz and Chaikoff (53), although in rat, the majority of the product was quickly converted to succinate. If propionyl CoA was used as the substrate, the activity increased substantially, suggesting that it was the actual substrate for the enzyme.(54, 55) The propionate carboxylation reaction therefore required propionyl CoA, ATP, and Mg^{2+}. The reaction was further enhanced by glutathione which stabilized the thiol-groups and thereby indicated their importance in the carboxylation reaction.(49)

Since other carboxylation reactions have been shown previously to be biotin-dependent, Lardy and Adler (51) studied propionyl CoA dependent $^{14}CO_2$-fixation in a series of rats made biotin deficient by adding avidin to their diets. Carboxylation of propionyl CoA was reduced to approximately 10% of normal which could be restored and even enhanced above normal levels after three days of oral biotin supplementation. These results suggested that biotin was an essential cofactor for propionyl CoA carboxylase activity.

By 1957, the propionate degradative pathway, including the factors necessary for optimal activity was known as follows:

$$\text{Propionyl CoA} + \text{ATP} + CO_2 \xleftarrow[\text{biotin}]{Mg^{2+}} \text{methylmalonyl CoA} \xleftarrow[B_{12}]{} \text{Succinyl CoA}$$

$$\text{ADP} + \text{Pi}$$

B. Enzyme Characterization

The compartmentalization of propionyl CoA carboxylase in rat liver revealed that 75% of the enzyme activity was localized in the mitochondria.(56) Propionyl CoA carboxylase has been purified from many

organisms and the enzymatic properties of the enzyme from a variety of sources shows remarkable similarity. For example, the Km values for the various substrates listed in Table I are very similar, if not identical. In addition to catabolizing propionyl CoA, the enzyme can also catalyze the carboxylation of several of acyl compounds including acetyl CoA, butyryl CoA and valeryl CoA but uses these 1.03, 4.71, and 0.49% respectively as efficiently as propionyl CoA.(57)

Propionyl CoA carboxylase activity is extremely sensitive to inhibition by avidin, the white protein which binds biotin and biotin-containing enzymes almost irreversibly.(64, 65) The addition of excess biotin to avidin prior to incubation with propionyl CoA carboxylase protects the enzyme from avidin inactivation.(65) Purified pig heart propionyl CoA contains four molecules of biotin for every 700,000 molecular weight molecule of the enzyme as determined by gel filtration.

The carboxylation reaction was also shown to be sensitive to sulfhydryl reagents such as para-chloromericuri benzoate, iodoacetamide and N-ethymaleimide and is protected from inactivation by glutatione. (66, 67) Pre-incubating with propionyl CoA prevents the inactivation by these sulfhydryl reagents. Moreover, addition of either of the sulfhydryl reagents causes a change in the Km of propionyl CoA but does not alter the Km's for ATP or bicarbonate indicating that a cystiene residue is involved in or near the binding site of propionyl CoA on the enzyme. (68)

Propionyl CoA carboxylase has also been shown to be activated by

TABLE I

COMPARISON OF Km VALUES FOR VARIOUS SUBSTRATES OF PROPIONYL CoA CARBOXYLASE FROM DIFFERENT SOURCES

SUBSTRATE	BACTERIA	BOVINE LIVER		PIG HEART	HUMAN FIBROBLAST		HUMAN LIVER	
	1	2	3	4	5	6	7	8
ATP	1.4×10^{-4}	5.5×10^{-5}		5.5×10^{-5}	9×10^{-4}	8×10^{-4}	1×10^{-4}	8×10^{-5}
Propionyl CoA	1.3×10^{-4}	2.6×10^{-4}	1.5×10^{-3}	2×10^{-4}	1.7×10^{-3}	5×10^{-4}	2×10^{-4}	2.9×10^{-4}
Bicarbonate	5.3×10^{-3}	1.9×10^{-3}	4×10^{-3}	1.9×10^{-3}	4.5×10^{-3}	2.1×10^{-3}	1.6×10^{-3}	3×10^{-3}
Mg^{2+}			5×10^{-3}			2.1×10^{-3}		
K^{+}						9×10^{-3}		

1. I. Olsen & J. M. Merrick. J. Bact. 95:1774-1778, 1968.(58)
2. D. R. Halenz, J. Feng, C. S. Hegre & M. D. Lane. J. Biol. Chem. 237:2140-2147, 1962.(57)
3. A. J. Giorgio & G. W. E. Plaut. Bioch. Bioph. Acta 139:487-501, 1967. (59)
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6. B. Wolf, Y. E. Hsia, & L. E. Rosenberg. Am. J. Hum. Genet. 30:455-464, 1978.(3)
7. P. Divry, M. O. Rolland, N. Dingeon, M. Mathieu, & J. Cotte. J. Inher. Metab. Dis. 1:3-7, 1978.(62)
8. F. Kalousek, M. Darigo, and L. E. Rosenberg. J. Biol. Chem. 255:60-65, 1980.(63)

monovalent cations such as potassium. Potassium has been reported to slightly lower the Km for bicarbonate.(69) Giorgio and Plaut did not find a lowered Km for bicarbonate but rather an increase in the velocity of the reaction.(59)

C. Mechanism

Kaziro et al. (70) and Lane et al. (71) independently proposed the following two step carboxylation reaction for propionyl CoA carboxylase:

Partial Reaction 1

$$(EnZ - Biotin) + ATP + HCO_3^- \xrightarrow{Mg^{++}} (EnZ\text{-}Biotin\ -CO_2) + ADP + Pi$$

Partial Reaction 2

$$(Enz - Biotin - CO_2) + Propionyl\ CoA \text{ ------ } Methylmalonyl\ CoA + (Enz - Biotin)$$

Net Reaction

$$ATP + HCO_3^- + Propionyl\ CoA \text{ ---------- } Methylmalonyl\ CoA + ADP + Pi$$

This concerted Ping-Pong mechanism was proposed, based on several observations which were consistent only with this mechanism.(72) The first partial reaction which required Mg++ was supported by the following two observations. First, Kaziro et al. (73) showed that the exchange of label between ADP^{32} and ATP or Pi^{32} and ATP required the presence of both ADP and Pi. Secondly, the exchange of labelled ADP^{32} with ATP or Pi^{32} with ATP was CO_2 dependent.(74) The second reversible

partial reaction was deomonstrated by the propionyl CoA carboxylase mediated catalysis of an exchange reaction between methylmalonyl CoA and ^{14}C- propionyl CoA which was independent of ATP, ADP or Mg++.(67) (70, 71, 75) The reverse reaction has also been demonstrated by the exchange of 3-^{14}C-methylmalonyl CoA with propionyl CoA.(67) Both partial reactions are avidin sensitive indicating that biotin is required. The rates for the two partial reactions have been determined by Kaziro et al.(76) and are shown in Table II.

Studies of these reactions using labelled oxygen showed that one oxygen from HCO_3^- was necessary for the ATP cleavage and was incorporated into orthophosphate, whereas, two labelled oxygens were found in the free carboxyl group of methylmalonyl CoA. Since both oxygens in the carboxyl group of methylmalonyl CoA were labelled, the reactive species must be HCO_3^- rather than CO_2 which would have only labelled a single oxygen of methylmalonyl CoA.(73)

The site of carboxylation on the enzyme has been shown to be the 1-N-position of the biotin miety.(72) Chromatographic studies of the hydrolysis of the purified beef liver mitochondrial enzyme has identified on ϵ-N-1-N-carboxy(+)biotinyl lysyl residue.(77) This indicates that the biotin binds to the enzyme via the ϵ-amino group of lysine.

D. Biotin as a Cofactor for Propionyl CoA Carboxylase

Biotin is the coenzyme for the four known human carboxylase enzymes: acetyl CoA carboxylase, β-methylcrotonyl CoA carboxylase, pyrvate carboxylase and propionyl CoA carboxylase. The biotin

TABLE II

RATE OF REACTIONS CATALYZED BY PROPIONYL CoA CARBOXYLASE

Reaction	Rate (25^{o}) moles/min/Unit forward
Overall, forward	1.000
Overall, backward	0.015
P_i^{32}-ATP exchange	0.002
Propionyl CoA -1-C^{14}- Methylmalonyl CoA exchange	0.600

From- Y. Kaziro and S. Ochoa. Adv. Enz. 26:283-378, 1964.(96)

molecule consists of a ureido ring fused to a tetrahydrothrophene ring with a five carbon aliphatic side chain as shown in Figure 3.(47) Biotin cannot be synthesised in man and is therefore supplied by diet and by rudimentary bacteria. A dietary deficiency of biotin causes a severe illness which is a phenocopy of the multiple carboxylase deficiency since biotin is required to transform the four carboxylases into their active forms. Biotin seems to be covalently attached to each of the carboxylases via its aliphatic side chain by the action of a common holocarboxylase synthetase. The reaction requires ATP and Mg++ and is specific for d-biotin.(78) The proposed mechanism for holocarboxylase synthesis is represented by the following two partial reactions:(79, 80)

Partial 1

$$\text{d-Biotin} + \text{ATP} \xrightarrow[\text{synthetase}]{\text{Mg++}} \underset{\text{d-biotinyl-5'-AMP}}{\text{R-C(=O)-O-P(=O)(-O-)-O-Adenosine}} + PP_i$$

Partial 2

$$\text{d-Biotinyl-5'AMP} + \underset{\text{apoenzyme}}{\text{Enz-lysyl-}\epsilon\text{-NH}_2} \xrightarrow[\text{synthetase}]{} \underset{\text{holoenzyme}}{\text{Enz-lysyl-}\epsilon\text{-NH-C(=O)-R}} + \text{5'AMP}$$

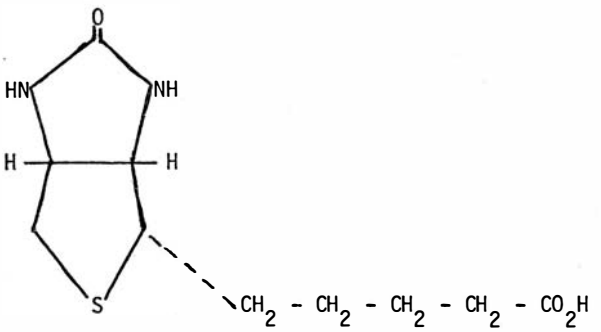
Net Reaction

$$\text{d-Biotin} + \text{ATP} + \text{apoenzyme} \xrightarrow{\text{Mg++}} \text{Holocarboxylase} + \text{AMP} + PP_i$$

The reaction involves the formation of an amide linkage between the aliphatic side chain of biotin and the ϵ-amino group of a lysine residue in the active site of the enzyme. This reaction was established

FIGURE 3

STRUCTURE OF d-BIOTIN



by demonstrating the formation of biocytin (biotin-lysine) from enzyme digests of purified carboxylases and seems to be a common feature of all four carboxylases.(80)

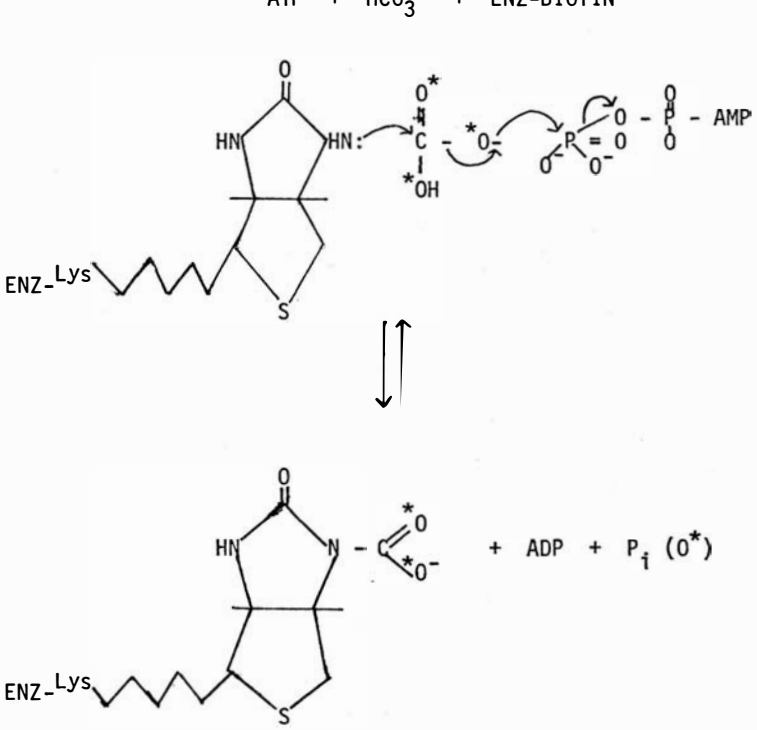
Once attached to the enzyme, the ureido ring of biotin accepts a carboxyl group by nuecleophylic attack in the first partial reaction. (Figure 4) In the second partial carboxylation reaction, biotin translocates to the acceptor site and releases the carboxyl to the receptor substrate. Biotin acts then, to transfer the carboxyl from one site on the molecule to another.(81)

E. Avidin

Avidin is an egg white protein which has a very high affinity and specificity for biotin of 10^{15}.(82) It has been a useful tool, therefore, in identifying biotin-containing enzymes. Avidin is a glycoprotein that has a molecular weight of 67,000 with four identical subunits, each with a molecular weight of 16,000. Each subunit binds one molecule of biotin and consists of 129 amino acid residues, four residues of mannose and three residues of glucosamine.(83, 84) Chemical modification studies using bromosuccinimide, demonstrated that biotin protects three to four tryptophan molecules in each subunit from oxidation implicating these residues in the biotin-avidin interaction. (85) These tryptophans probably form a non-covalent attachment to the ureido group of biotin. The identification of tryptophan in biotin binding explains the red shift in the avidin absorption spectrum which is observed after biotin binding. Although, there are four binding

FIGURE 4

MECHANISM FOR BIOTIN CARBOXYLATION



From- Y. Kaziro, L. F. Hass, P. D. Boyer, and S. Ochoa. J. Biol. Chem. 237:1460-1468, 1962.(73)

sites for biotin on the avidin molecule , there is no evidence of co-operativity between the sites.(86)

Avidin has been used by several investigators to bind and isolate biotin-containing proteins. Swack et al. (87) used an affinity column of avidin-coupled to Sepharose to remove carboxylases from avian and rat liver homogenates. Elution from the column required 6M quanidine hydrochloride which unfortunately denatured the enzymes. This method was modified by Green and Toms (88) using avidin-monomers coupled to Sepharose which bound biotin-containing proteins less tightly and allowed their elution without denaturation. This latter column was used by Gravel et al. (89) for the purification of propionyl CoA carboxylase. Avidin has also been coupled to fluorescent dyes such as fluorescamine and used for the localization of biotin-containing proteins or subunits.(87)

F. Structure of the Normal Enzyme

The molecular weight of purified pig heart propionyl CoA carboxylase was initially estimated by gel filtration to be approximately 700,000 daltons.(60) The large size of the molecule and the demonstration that it contained four biotins per molecule, suggested that the enzyme was probably a tetramer. Lau et al. (90) described the physical properties of this enzyme after purification from bovine kidney and demonstrated that it was composed of two non-identical subunits with molecular weights 58,000 and 74,000 as determined by SDS-polyacrylamide gel electrophoresis.(Table III) The larger subunit was

TABLE III

MOLECULAR WEIGHT OF PROPIONYL CoA SUBUNITS FROM DIFFERENT SPECIES

	AVIAN	BOVINE	HUMAN	
	1	2	3	4
α	76,000	74,000	72,000	75,000
β	42,000	58,000	56,000	60,000

1. J. A. Swack, G. L. Zander, & M. F. Utter. Anal. Biochem. 87:114-126, 1978.(87)
2. E. P. Lau, B. C. Cochran, L. Munson, & R. R. Fall. Proc. Nat. Acad. Sci. 76:214-218, 1972.(90)
3. F. Kalousek, M.D. Darigo, & L. E. Rosenberg. J. Biol. Chem. 255:60-65, 1980.(63)
4. R. A. Gavel, K. F. Lam, D. Mahuran, & A. Kronis. *Arch. Biochem.* Biphy. 201:669-673, 1980.(89)

considered to contain biotin because of its ability to bind avidin. Since the native molecule contains four biotin molecules, the structure $(\alpha\beta)_4$ was proposed to explain the native molecular weight of 540,000. Similar results have been reported for the enzyme from avian liver.(87)

The human enzyme was purified 20-fold from normal human liver and showed similar properties and Km's to avian and other mammalian propionyl CoA carboxylase.(91) As predicted, the enzyme was activated by monovalent cations and inactivated by avidin and parachloromercuribenzoate.

Recently, Kalousek et al.(63) purified the enzyme up to 3000-fold from human liver by using column separations and a Blue Sepharose affinity resin which binds the ATP fold of the enzyme. The homogeneous enzyme shows very similar characteristics to the bovine kidney enzyme. The native enzyme has a molecular weight of 540,000 with two non-identical subunits with molecular weights of 72,000 and 56,000. The larger subunit appears to contain one biotin molecule. The apparent Km's for propionyl CoA, ATP and bicarbonate are similar to those reported for other mammalian propionyl CoA carboxylases and those reported for the human enzyme in crude fibroblast homogenates. (Table I)

Using the property of avidin to bind biotin-containing carboxylases, Gravel et al. (89) have devised a two step procedure to isolate propionyl CoA carboxylase from human tissue. The procedure involves a carbon tetrachloride tissue extraction and an avidin-monomer-Sepharose affinity column. Since the avidin-monomer binds the biotin-containing

carboxylase less tightly than the avidin tetramer, the enzyme can be eluted from the column by the addition of excess free biotin. The enzyme prepared in this manner was purified to homogeneity and demonstrated similar structure to the other purified enzymes. Estimates of the subunits' molecular weights were 75,000 and 60,000. (Table III)

Over-all, the structure of propionyl CoA carboxylase seems to be similar in the enzyme from bovine, avian, rodent and human sources.(92) The basic structure of the enzyme seems to have been conserved throughout species evolution.

Mutant Enzyme

The mutant enzyme was purified 200-fold from normal fibroblasts and those of several mutant lines. The mutant cell lines had between 1-5% of normal propionyl CoA carboxylase activity. The size of the normal and the mutant enzyme, as measured by sucrose density gradient centrifugation, was 700,000 similar to that previously reported. The Km values of the normal and mutant enzyme were also similar to those reported as compared in Table I. These studies were confirmed by Divry et al. (62) who also failed to demonstrate a difference in the Km values for substrates in the enzyme from mutant fibroblast lines. However, the mutant enzyme was extremely unstable even when frozen at -80°C. The enzyme from the mutant fibroblasts showed greater thermolability with a $t_{\frac{1}{2}}$ of 3 minutes at 45°C where the normal enzyme had a $t_{1/2}$ of 15 minutes.(61) The instability of the mutant enzyme has made its study more difficult.

G. Other Human Carboxylases

In addition to propionyl CoA carboxylase, there are three other human carboxylases: pyruvate carboxylase, β -methylcrotonyl CoA carboxylase and acetyl CoA carboxylase. All of the carboxylases have the same basic carboxylation mechanism with two biotin dependent. However, each enzyme recognizes a different acceptor substrate for the carboxyl group.(47)

Partial 1

$$\text{Enz- Biotin} + CO_2^- + \text{ATP} \xrightarrow{Mg^{++}} \text{Enz- biotin - } CO_2^- + \text{ADP} + P_i$$

Partial 2

$$\text{ENZ- biotin - } CO_2^- + \text{Acceptor} \text{ ------ Enz-Biotin} + \text{Product}$$

Net Reaction

$$CO_2^- + \text{ATP} + \text{Acceptor} \text{ ---------- Product} + \text{ADP} + P_i$$

Pyruvate carboxylase is a mitochondrial enzyme which is important in the regulation of gluconeogenesis. The enzyme recognizes pyruvate and forms oxaloacetate. Acetyl CoA is an allosteric cofactor for the enzyme and manganese is an activator cation.(47) This enzyme has not been isolated from man but the rat mitochondrial enzyme has been shown to be a tetramer consisting of four identical biotin-containing subunits with a molecular weight of 125,000.(87)

Another mitochondrial carboxylase, β -methylcrotonyl CoA carboxylase is necessary for the catabolism of leucine. The enzyme converts

β-methylcrotonyl CoA to β-methylglutaconyl CoA. When this enzyme is defective as in multiple carboxylase deficiency, β-methylcrononyl-glycine and β-hydroxyisovaleric acid accumulate and are excreted in the urine of these patients. The structure of human β-methylcrotonyl CoA carboxylase has not been determined but the enzyme from bovine kidney consists of a tetramer composed of two non-identical subunits of 62,000 and 80,000 molecular weight with the larger subunit containing the biotin moiety.(90) The structure is similar to that of propionyl CoA carboxylase however the evidence suggests that the subunits are not common to both enzymes.(90)

Acetyl CoA carboxylase is the only cytosolic carboxylase enzyme. It converts acetyl CoA to malonyl CoA which constitutes the first committed step of fatty acid synthesis.(93) Citrate is a powerful activator of this enzyme and may regulate fatty acid synthesis. The structure of the human enzyme has not been reported; however, in rat liver the enzyme seems to be composed of a protomer of 215,000 molecular weight which consists of two non-identical subunits with molecular weight of 118,000 and 125,000, one of which contains the biotin moiety.(94) The protomers form linear polymers with molecular weights of several million which have a fiber-like appearance under the electron microscope. The fiber structures may be important in cytoplasm ultrastructure.(47)

H. Complementation

Genetic complementation is a somatic cell technique which has

recently been used to identify and investigate the genetic heterogeneity of human metabolic disorders. This technique was initially developed in bacterial systems in order to determine whether a defect was the result of mutations involving separate genes or alleles. Homozygous mutant bacteria were crossed and if their defects were the result of two different genes or gene units, the genomes would complement one another and give a wild type phenotype.(95)

An extension of this technique to human fibroblasts was devised by fusing two populations of fibroblasts carrying independently derived mutations using inactivated sendai virus and testing for correction of the defect.(96) If two different gene products are involved in the enzyme deficiency, then the genome of one cell should complement that of the other cell and the enzyme activity would be increased. This process is called intergenic complementation. Even when different defects occur within the same gene locus, there is occasionally some degree of complementation. This occurs sometimes in multimeric proteins because heteropolymers of the two different defective subunits are sometimes more active than the respective homopolymers, probably due to steric interaction.(97) This complementation is usually of a lesser magnitude and is called interallelic complementation. This technique has been used to elucidate genetic heterogeneity in several human metabolic disorders such as methylmalonic acidemia and xeroderma pigmentosum.(98, 99)

In 1977, Gravel et al. (2) identified two major complementation groups by consecutive pairwise fusions of seven mutant fibroblast

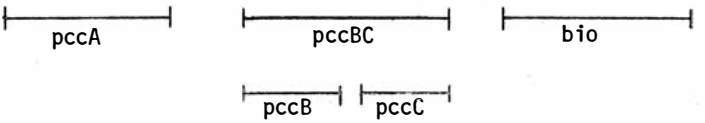
lines from patients with clinically indistinguishable propionic acidemia. These groups were designated pccA and pccBC. Thus, when cells from pccA were fused to cells from the pccBC group, propionyl CoA carboxylase activity increased markedly over unfused cells as measured by the ^{14}C- propionate incorporation demonstrated by autoradiography. When the cells from different complementation groups were mixed and not fused or when cells were fused to themselves, no increase in labelled propionate incorporation was observed. The complementation picture became more complicated when fibroblasts lines from the pccBC group were fused to each other. There were some cells which did not complement within the group which comprised subgroup, pccBC. There were some cells which complemented each other by showing a slight increase in grain count. These fell into two subgroups designated pccB and pccC. Neither pccB nor pccC complemented pccBC. A complementation map, indicating the different complementation groups, is shown in Figure 5. The demonstration of more than one complementation group was the first indication that more than one gene was probably involved in propionyl CoA carboxylase deficiency.

A third major complementation group has been established with cells from patients with biotin-responsive multiple carboxylase deficiency. This group designated, bio, complements all other propionyl CoA carboxylase mutants which supports the theory that this defect involves a different gene locus, possibly responsible for holocarboxylase synthetase or a biotin transport protein.(52)

Evidence that two genes are responsible for propionyl CoA

FIGURE 5

COMPLEMENTATION MAP



From- M. Saunders, L. Sweetman, B. Robinson, K. Roth, R. Cohn and R. A. Gavel. J. Clin. Invest. 64:1695-1702, 1979.(52)

carboxylase deficiency depends on whether the complementation between these groups was intergenic or interallelic. Studies on the kinetics of complementation were undertaken on polyethyleneglycol-fused cells using a quantitive propionate fixation assay in order to choose between these alternatives. The results of these studies revealed that complementation between pccA or pccC with bio had the same kinetics as fusions of two unrelated gene defects. The complementation of pccA and pccC was much slower, although it achieved the same magnitude of enhanced activity as did the bio X pccA crosses. This suggested that pccA X pccC complementation may be due to subunit exchange or de novo subunit synthesis. The magnitude of this complementation also suggested an intergenic complementation consistent with a two non-identical subunit model. Complementation of the pccB and pccC subgroups revealed only a small increase in propionyl CoA carboxylase activity compared to the other crosses and possibly represents interallelic complementation.(6)

Further evidence for the mechanism of complementation was obtained by determining whether de novo protein synthesis was necessary for complementation. Cyclohexamide added after fusion of bio cells with either pccA or pccC cells did not alter complementation.(100)

Studies were initiated to try and determine the significance of the two complementation groups by identifying biochemical differences between them. The enzymes from several fibroblast lines in each group were compared for thermostability, for substrates, Km's and for avidin interactions. In most parameters the mutant enzymes were similar to

the enzyme in normal fibroblast extracts. However, propionyl CoA carboxylase from the dialyzed pccA group fibroblast extracts demonstrated an increased Km for potassium ions, whereas, that in pccC extracts was similar to normal cell extracts. In addition, the activity from pccA fibroblast extracts was less stable at 45°C than was either the normal or pccC fibroblast activity. The pccC activity was intermediate to that of normal and pccA.(6) These studies demonstrated a few differences between the complementation groups although only a few cell lines in each group were studied. The demonstration of two identifiable complementation groups also raises the possibility of two different subunits in these enzymes.

Still another difference between the pccA and pccC groups was disclosed in studies of obligate carriers of propionic acidemia. The propionyl CoA carboxylase activity in fibroblast extracts from pccA heterozygotes was 52% of that of controls as expected in a recessive disorder. However, pccC heterozygotes had propionyl CoA carboxylase activity which was in the normal range. There are several possible explanations for this phenomenon. One explanation is that a heteropolymer of normal subunits and mutant pccC retain normal activity. Another explanation is that there is a differential rate of synthesis of one subunit, so that enough normal enzyme is formed to compensate for the defective enzyme.(101)

Further studies on the minor complementation groups pccB and pccBC showed them to be indistinguishable from the pccC group when compared for heat stability and Km values.(102) The data supports the

hypothesis that pccB, pccBC and pccC represent at least three inter-allelic mutations and part of a single complementation group now designated pccBC. This would be expected if the addition of a missing enzyme such as holocarboxylase synthetase is needed to activate propionyl CoA carboxylase and therefore only the mixing of cell contents would be necessary for enhanced propionyl CoA carboxylase activity. However, when cyclohexamide was added after fusion of pccA and pccC, very little increase in activity was observed, indicating that de novo protein synthesis was required. It is possible that protein synthesis is needed in order for subunits from both complementation groups to be incorporated into the enzyme to increase propionyl CoA carboxylase activity.

MATERIALS AND METHODS

A. Tissue Procurement and Preparation

Liver and kidney tissue was obtained one to eight hours postmortem from individuals ranging in age from newborn to 55 years old, who died suddenly from various non-metabolic causes. Permission for all tissue procurement was obtained using consent statements approved by the Medical College of Virginia human research committee and signed by legal responsible parties. (Appendix I) The patient's history and cause of death was obtained from medical records and for the purpose of confidentiality the liver sample was assigned a specific identification number. At the time of procurement, livers were sliced into one centimeter sections and immediately stored at -80^{o}C. No deterioration in propionyl CoA carboxylase activity was noted with storage up to three years. Normal human placentas were obtained immediately after delivery, placed on ice and frozen.

Four liver samples were obtained from patients with propionic acidemia who died because of complications of the disease. Genetic complementation studies using fibroblasts from the same patients assigned these cell lines to the pccA, pccBC, and pccC (2) complementation groups. One placenta was obtained from the pregnancy of a second affected offspring with propionic acidemia. The fibroblasts of this patient were assigned to the pccBC complementation group. These tissues were frozen and sent to the Medical College of Virginia in dry ice and stored subsequently at -80^{o}C.

Various tissues were homogenized in 100mM Tris-HCl, 0.5%, pH 8.0 (10% w/v) at 4^{o}C using a Potter-Elvehjem homogenizer. Following

homogenization, cell debris was removed by centrifugation at 10,000xg at 10°C for 20 minutes. Homogenates of tissues from affected individuals were prepared similarly except that in several instances 2M sucrose was added to the homogenizing solution to stabilize the mutant enzyme. Mitochondria were isolated for several experiments by homogenizing liver (10%w/v) in 0.25M sucrose-10mM Hepes, pH 7.4 and centrifuging at 7200xg for 15 minutes at 2°C. The mitochondrial pellet was resuspended and recentrifuged before lysis by sonication.

Control fibroblast cultures were derived from skin biopsies obtained from volunteers or from individuals tested for other abnormalities and determined to be normal. Fibroblast cultures were grown and incubated in Eagles' minimum essential medium with glutamine containing 10% fetal calf serum and 1% Kanamycin. Mutant cell lines of the pccA (2), pccBC, pccB, pccC (2), and bio genetic complementation groups were grown to confluency and harvested using 1% trysin -0.2% EDTA in Hank's phosphate balanced salt buffer. The cells were washed three times in Hank's buffer and centrifuged at 1000xg at 27°C for 10 minutes. Cell pellets were disrupted by a 15 minute incubation in 100mM Tris-HCl, 0.5% Triton (pH 8.0) using a pasteur pipet. Cell extracts were then centrifuged at 10,000xg at 15°C to remove cell debris.

B. Enzyme Assays

Propionyl CoA carboxylase activity was measured by determining the incorporation of ^{14}C-bicarbonate into methylmalonyl CoA using the procedure of Wolf et al. (3) wherein 50 ul. of enzyme-containing

sample was mixed with 50ul. of buffer-substate mixture and incubated in a 37°C shaking water bath for up to 30 minutes. The final assay mixture contains: 50mM Tris-Hcl, pH 8.0, 10mM $MgCl_2$, 2mM sodium adenosine triphosphate (ATP) (Sigma), 100mM KCl, 1mM propionyl CoA (Sigma), 5mM reduced glutatione (Sig ma) and 10mM sodium ^{14}C- bicarbonate (New England Nuclear, Boston, Mass.; final specific activity 12.2 mC_i/mmol). The blank included all reactants except propionyl CoA in order to determine nonspecific labelled bicarbonate incorporation. In all cases the reaction was initiated by the addition of labelled bicarbonate to the reaction mixture. The reaction was terminated by the addition of 50ul of 10% trichloroacetic acid and the denatured protein was removed by centrifugation at 2000xg at room temperature for 10 minutes. A 100ul alliquot of the supernatant was placed in a scintillation vial and dried under heat lamp liberating $^{14}CO_2$ which was not incorporated into methylmalonic acid. The residue was solublized in 0.2ml of deionized water; four ml of Aqua-sol (New England Nuclear) was added and samples were counted in a Beckman LS 7500 liquid scintillation counter. In earlier experiments, liberated $^{14}CO_2$ was trapped by methylbenzethonium hydroxide in an enclosed vessel and an alliquot of the assay mixture was counted directly in Aqua-sol. Identification of the final reaction product, by high voltage paper chromotography, demonstrated that the ^{14}C-labelled product ran identically with methylmalonic acid in 0.5M Acetic acid- Formic acid, pH 2.9 solvent system. In all cases $^{14}CO_2$ incorporation into nonvolatile material was linear for periods up to 30 minutes with enzyme

preparations containing up to 20mg of protein per milliliter.

This assay system was modified to measure the activities of two other carboxylases, β-methylcrotonyl CoA carboxylase and pyruvate carboxylase. In the β-methylcrotonyl CoA carboxylase assay 3mM (final concentration) β-methylcrotonyl CoA was substituted for propionyl CoA. In the pyruvate carboxylase assay final concentrations of 0.75mM acetyl CoA, an activator of this enzyme, and 3mM pyrvate were substituted for propionyl CoA. The blank for the pyruvate carboxylase assay contained acetyl CoA in the absence of pyruvate.

Glutamic dehydrogenase activity was used as a reference mitochondrial matrix-enzyme in several experiments. The reaction was monitored spectrophotometerically (Beckman D.U. with Gilford Components) at 340^{o}nm to detect the oxidation of nicotinamide adenine dinucleotide, reduced form (NADH). The final reaction mixture of one milliliter contained: 33.5mM triethanolamine-2.6mM EDTA, pH 8.0, 0.1M ammonium acetate, 0.15mM NADH (Sigma), 6mM ADP (Sigma), 8mM sodium α-ketoglutarate, and 50ul of enzyme containing-sample. Liver homogenates were diluted one to one hundred for this assay that was initiated by the addition of α-ketoglutarate. The velocity was measured as the change in optical density over time at 23^{o}C.

All enzyme activities are reported in pmoles or umoles product formed per minute per milligram of protein. The protein concentrations were determined by the method of Lowry et al.(123) Detailed assay procedures are included in Appendix II.

C. Antibody Preparation

The unique property of biotin and biotin-containing enzymes to bond specifically and essentially irreversibly to avidin was used to isolate antibodies to the human carboxylases. An avidin affinity column synthesized as described by Landman and Landman (124) was used to selectively remove carboxylases from a human liver preparation. This Sepharose-avidin-carboxylase complex was in turn used as an antigen to produce anti-carboxylase antibody in rabbits.

a) Avidin-Affinity Column Synthesis

One gram of Sepharose 4B (Pharmacia) was swollen, washed and activated with cyanogen bromide (CNBr) by the method of March et al.(125) All procedures were conducted at 4°C unless otherwise noted. The Sepharose was suspended in an equal volume of 2M sodium carbonate. (This procedure is based on the volume of the Sepharose which is approximately 10ml in this preparation.) A stock solution of five milligrams of cyanogenbromide (Aldrich) was mixed with 2.5 ml of acetonitrile and stored at 4°C for further use. A volume of the CNBr-acetonitrile solution, equal to approximately 0.1 volume of the Sepharose solution (1ml), was added to the Sepharose and stirred vigorously under the hood for 2 minutes at 23°C. The mixture was filtered on a sintered glass filter and washed with 10 volumes of 0.1-M sodium bicarbonate pH 4.5, 20 volumes deionized water, and 10 volumes of a 0.1M sodium bicarbonate-0.5M sodium chloride solution at pH 9.0. The resin was then stirred overnight in 0.5 volumes of 0.1M sodium bicarbonate-0.5M sodium chloride solution (pH 9.0) containing

10mg avidin (Sigma). The preparation was filtered and the filtrate was saved for analysis. The resin was washed and resuspended in one volume of the bicarbonate-NaCl buffer and placed on ice. Excess ethanolamine was added to the resin suspension to give a final concentration of 1M. This was stirred overnight to bind any remaining unreacted CNBr-active sites.

b) Characterization of the Avidin Affinity Column

An estimation of the amount of avidin bound to the column and its ability to bind biotin were assessed by using two procedures. First, the concentration of avidin removed in the filtrate was measured by a sensitive method developed by Lin and Kirsh (126) in which the unknown concentration of avidin is titrated by a known concentration of biotin. The titration, performed at 23°C, was followed at 350nm on an Amino-Bowman Spectrophotofluorometer with an excitation wave length of 290nm so as the biotin binds to avidin, a red shift occurs due to the interactions of biotin with the tryptophans in the active site. The quantity of biotin required to complete this shift is determined by the concentration of avidin in the unknown solution. (Method in Appendix III) The amount of avidin bound to the column equals the initial amount of avidin minus the amount unreacted in the filtrate.

The second method determined the ability of the avidin-coupled sepharose to bind biotin. As well as binding biotin, avidin also reversably binds 2-(4-hydroxyazobenzene) benzoic acid (HABA, Sigma) which is a yellow dye that turns magenta when bound to avidin.(124)

The addition of the dye through the column and its altered color is a quick way to demonstrate the ability of avidin to bind liginds. The binding capacity of a 0.8 X 2 cm column can be determined by using 0.5ml of a 20 mg/ml solution of HABA standardized spectrophotometrically at 340^{o}nm visable light prior to passing over the column. After collecting the eluate from the column, the concentration of HABA can be determined by its absorption and the amount of HABA through the column can be calculated after the sample is corrected for volume. The amount of HABA bound is then determined by the amount of HABA put on the column. The amount of HABA bound determines the binding capacity of the column. The normal binding capacity of avidin for biotin is reduced due to steric hinderence from the coupled sepharose.

c) Preparation of liver - Avidin-Sepharose Complex

Twenty volumes of cold distilled acetone (0^{o}C) was added, drop by drop, to one volume of liver homogenate and stirred for 15 minutes at 4^{o}C. The precipitate was vacuum filtered in a buchner funnel and washed with excess cold distilled acetone to remove any remaining acetone soluble material. The acetone was removed by washing the preparation with several volumes of peroxide-free ether and the sample was air dried.(85) This procedure removed lipid material from the preparation which could have interfered with column binding as well as denaturing large quantities of protein while leaving propionyl CoA carboxylase β-methyl crotonyl CoA and pyruvate carboxylase active after the procedure. The liver acetone powder was dissolved in 100mM Tris-HCl buffer (pH 8.0) by vortexing for 10 minutes and centrifuged

at 10,000xg for 20 minutes at 15°C to remove undissolved acetone powder. This solution was applied to a 0.8 X 2.0 cm avidin affinity column until the binding sites on the column were determined to be saturated as determined by the appearance of increased propionyl CoA carboxylase activity in the eluate. A solution of 1mM biotin (Sigma) was applied next to the column to bind to any unreacted avidin sites. Propionyl CoA carboxylase retained activity while bound to the column matrix as determined by assaying a suspension of column packing. A second column was prepared in an identical manner; however, this column was saturated first with biotin and was used as a control.

d) Preparation of Sepharose-avidin-carboxylase complex for immunization

The column material was suspended in an equal volume of 5mM potassium phosphate buffer, pH 7.0. One half milliliter was mixed with 0.5 ml of Freund's adjuv ant and emulsified by vigorous agitation in a syringe. The emulsion was subsequently injected in two separate abdominal subcutaneous sites in two New Zealand white rabbits.(127) An emulsion of the biotin-saturated column with no measurable carboxylase activity was injected into a third rabbit as a control. The rabbits were given boosters of the same antigen 14 days after the primary immunization. Blood was obtained by cardiac puncture at each antigen injection. The serum was prepared by centrifugating the coagulated blood sample at 1200xg for 20 minutes at 15°C and was examined for antibody production by the Ouchterlony double diffusion test. (Method in appendix IV) A 1% agarose gel in 0.05M sodium barbitol

buffer, pH 8.6 was poured into a small petri dish. Wells were cut into the agar using a template and 100ul of serum; samples were tested against an antigen which was placed in the center well.

D. Immunotitration Experiment

Sequential dilutions of anti-carboxylase antiserum were used to titrate the amount of cross reacting material present in liver and fibroblast homogenates with equivalent total protein concentrations. Fibroblasts and liver homogenates were sonicated in 50mM Tris-HCl buffer, pH 8.0 to prevent interference from triton on antibody-antigen complex formation. Antiserum was diluted sequentially with equal parts of a phophate balanced salt solution, pH 7.5 to a dilution of 1 to 4096. The protein concentration of the homogenates were standardized to 10mg/ml and that of the fibroblasts to 5 mg/ml as determined by the method of Lowry et al.(123) Equal volumes of the diluted antiserum and the enzyme-containing liver or fibroblast extract were incubated for 30 minutes at 37°C and centrifuged at 10,000xg for 10 minutes to remove immunoprecipitates. The supernatant were then assayed for propionyl CoA carboxylase activity in order to determine the residual activity. Serum obtained from the same rabbit prior to immunization was used as a control. Identical experiments were performed using an antibody prepared against homogeneous pig heart propionyl CoA carboxylase. This antiserum (donated by Dr. R. R. Fall from the University of Colorado) was prepared against pig heart propionyl CoA carboxylase which was isolated from pig heart by a procedure

similar to that used for the isolation of the bovine kidney enzyme. (110) A rabbit antiserum, obtained after subcutaneous injections of the purified enzyme in incomplete Freund's adjuvant, was partially purified by an ammonium sulfate fractionation procedure.(128) The immunotitration experiments with antibovine propionyl CoA carboxylase were used to confirm our findings with heterologous antiserum.

E. Purification of Propionyl CoA carboxylase from Normal Liver

The enzyme was purified, using a modification of the procedure of Kalousek et al. (83) which involves three basic column procedures. All procedures were conducted at 4°C unless otherwise noted.

a) Ion Exchange Column Chromatography

Four hundred grams of liver from a 28 year old male who died of congestive heart failure was homogenized in 300ml of 40mMKCl - 10mM potassium phosphate buffer, pH 7.5 and centrifuged for 60 minutes at 25,400xg at 15°C. The supernatant was placed on a 2.5 X 44 cm column of DEAE Sephacel cellulose (Pharmacia) which had been previously equilibrated with the same KCl-KPO_4 buffer. After addition of the liver supernant, non-bound protein was removed by washing with 10 volumes of the same buffer until less than 0.1 OD was recorded spectrophotometerically at 280°nm. Following removal of non-bound protein, the enzyme was eluated using a potassium chloride gradient between 40mM to 190mM in 10mM potassium phosphate buffer, pH 7.5 (800 ml of each). Fractions of approximately 24 ml were collected and assayed for propionyl CoA carboxylase activity. The peak containing activity was located in the final volumes of the gradient which was followed by

excess 190mM KCl- 10mM potassium phosphate buffer to remove any residual propionyl CoA carboxylase activity. The fractions containing a high ratio of propionyl CoA carboxylase activity relative to protein concentration were pooled and concentrated in an Amicon ultrafiltration cell with a PM 30 Diaflomembrane, then equilibrated against 10mM potassium phosphate buffer, pH7.5, and stored at -80°C.

b) Affinity Resin

The concentrated enzyme-containing solution from the DEAE column was passed over a 2.5 X 7.0 cm Blue Sepharose column (Pharmacia) which had been equilibrated with 5mM potassium phosphate buffer, pH 7.5. Blue Sepharose is a commercially prepared affinity resin in which blue dextran is coupled to Sepharose 4B. After protein addition, the column was washed with 15mM potassium phosphate buffer (pH 7.5) until no protein was detected spectrophotometrically at 280nm in the eluate. The enzyme was selectively removed from the column by 3mM ATP in 5mM potassium phosphate buffer, pH 7.5. Enzyme activity was determined in the eluate immediately after ATP addition. The fractions with activity were pooled, concentrated and equilibrated against 50mM potassium phosphate buffer- 200mM KCl, 0.5M sucrose and 1mM EDTA using the Amicon system. This step resulted in a major purification of approximately a 1000-fold.

c) Gel Filtration

Further purification to remove low molecular weight proteins was obtained by running the sample on 1.1 X 115 cm column of Sephacryl-200 superfine (Pharmacia) which was equilibrated with 50mM

potassium phosphate buffer- 200mM KCl -0.5M sucrose-1mM EDTA. Propionyl CoA carboxylase activity was detected in the elution volume. These samples were pooled, concentrated and stored in 2M sucrose- 100mM Tris-HCl-1mM EDTA-1mM glutathione at -80°C. No loss of activity was detected during storage of the purified enzyme over several months.

F. Purification of the Mutant Enzyme from Liver

A 200-fold purification of the mutant enzyme from fibroblasts has previously been reported but the process was hampered by the instability of the enzyme and the lack of material.(81) Twenty-seven grams of liver from a propionic acidemia patient in the pcc-BC complementation group was homogenized in 50ml of 40mM KCl-10mM potassium phosphate buffer, pH 7.5 in a similar manner to that used to purify the normal enzyme. The supernant solution was passed over a 2 X 25 cm DEAE column and the fractions were collected, following the initiation of the 40mM to 190mM KCl gradient in 10mM potassium phosphate. The fractions were assayed for activity, pooled, concentrated and equilibrated in a solution of 5mM potassium phosphate- 0.5M sucrose, pH 7.5. Half molar sucrose was used through the remainder of the purification in order to stabilize the enzyme. It could not be used in the DEAE column procedure because the presence of the sucrose inhibited the association of the mutant enzyme with the DEAE column.

The pooled DEAE peak was placed on a 2 X 8cm Blue Sepharose column which was equilibrated with 5mM potassium phosphate-0.5M sucrose and non-associating proteins were removed as before. The enzyme was dislodged by 3mM ATP- 5mM potassium phosphate - 0.5M sucrose, pH7.5, and

concentrated ten-fold in the 50mM potassium phosphate -200mMKCl- 0.5M sucrose-1mM EDTA buffer. The concentrate was passed through a 1.1 X 115 cm Sephacryl-200 superfine column and the mutant enzyme was collected in the elution volume. The enzyme was concentrated and stored in 2M sucrose-100mM Tris-HCl-1mM EDTA -1mM glutathione, pH7.5 at -80^{o}C.

G. Sodium Dodecyl Sulfate -Polyacrylamide Gel Electrophoresis

Purification of these enzymes was determined by SDS-polyacrylamide gel electrophoresis. Since the molecular weight of the native protein has been shown to be approximately 550,000, the native enzyme would be excluded from most polyacrylamide gels. Therefore, SDS-polyacrylamide gel electrophoresis was chosen for identification of the enzyme by its subunits. The enzymes were denatured to subunits with sodium dodecyl sulfate and mercaptoethanol. The molecular weight of the subunits of propionyl CoA carboxylase could be compared with that which has been reported by other investigators and with that of purified bovine kidney propionyl CoA carboxylase.

A sample from each of the purification steps was run on a 7.5% polyacrylamide horizontal slab gel (25cm X 11cm X 1mm) with 0.1% SDS using an LKB-Multiphor unit.(129) The gel contained a final concentration of 0.125M glycine which was adjusted to pH 8.3 with Tris. The electrode buffer was 0.5 M glycine-Tris buffer, pH 8.3. (Appendix V)

The protein samples were diluted in the same buffer with 0.2% SDS and were boiled for 3 minutes to insure denaturation of the molecule and maximal association with SDS. In addition, 5 ul of mercaptoethanol and 5 ul of a 5% solution of bromthymol blue tracking dye was

added to the sample. Fifteen ul of sample which gave between 0.1 - 0.003 mg of protein could be run on each lane of the gel. Each gel was run at a constant current of 80 mA for approximately 3 hours until the tracking dye reached the end of the gel.

The gel was immediately fixed in 10% trichloroacetic acid and stained for protein in 50% methanol- 5% acetic acid- 5% Coomassie blue overnight and destained in 50% methanol- 5% Acetic acid. (see Appendix V)

H. Isoelectric Focusing

The isoelectric point for propionyl CoA carboxylase was determined in several normal tissues including 8 livers, 1 kidney, 1 placenta and 2 fibroblast lines, and from 3 livers and 1 placenta from patients with propionyl CoA carboxylase deficiency. Tissues were homogenized in 10mM Tris-HCl buffer and sonicated. These isoelectric points were confirmed by focusing the purified normal and pccBC$_1$ enzyme. This procedure was performed on a 110ml capacity LKB-preparatory isoelectric focusing column, using a sucrose density gradient ranging from 0 to 1M with 2% ampholines (LKB) ranging in pH from either 3.5- 10 or 4 -6, depending on the experiment. Temperature of -10^{o}C was maintained throughout the run.(130) (Appendix VI) For isoelectric focusing of the purified enzymes, 0.2% ampholines was used since the higher concentration caused a decrease in enzyme activity and the increased buffering capacity was not necessary to maintain the pH gradient. In addition, 1 mg of bovine hemoglobin was focused as a visible standard to indicate that focusing was completed. The column was run at a constant output

of 600 volts for 48 hours or until the current became zero. Two milliliter fractions were collected and assayed for propionyl CoA carboxylase activity and the pH gradient was determined from these fractions. The isoelectric points of pyruvate carboxylase and β-methyl crotonyl CoA carboxylase from normal fibroblasts and liver, were similarly determined.

I. Heat Denaturation Experiments

Homogenates from 5 normal livers, 1 normal placenta, 3 PCC deficient livers and 1 PCC deficient placenta and the two purified enzymes were studied for thermostability. The enzyme-containing homogenates, prepared as described previously, were dialyzed against 50mM Tris-HCl buffer (pH 8.0) overnight to remove any small molecules. These homogenates were incubated at either 45°C or 52°C and the residual activity was determined over time. The protein concentration was determined and standardized to 10mg per ml. The samples were incubated with either 10mM Tris-HCl buffer, pH 8.0 alone or with 5mM ATP or 0.5M sucrose in a 10mM Tris-HCl buffer, pH 8.0, for 15 minutes at 23°C. The samples were then incubated in either a 45°C or 52°C waterbath for lengths of time varying between 0 and 30 minutes and assayed for remaining propionyl CoA carboxylase activity. The results are expressed as the percent of the initial activity and plotted versus time.

J. Determination of Km's for Various Substrates

The apparent Km's for ATP, propionyl CoA, potassium and bicarbonate were determined for the purified normal enzyme, the purified

$\underline{pccBC}_1$ enzyme and the homogenates from the $\underline{pccC}_1$ liver and the $\underline{pccBC}_2$ placenta. The homogenates were dialyzed against 10mM Tris-HCl buffer, pH 8.0, prior to assay and the purified enzymes were dialyzed for the potassium Km determination. During these experiments, all assay conditions were kept constant while the studied substrates concentration was varied. All Km's were determined at pH 8.0 which is the reported pH optimum for the enzyme. The assay contained 50mM Tris-Hcl, 10mM $MgCl_2$, 2mM sodium adenosine triphosphate, 100mM KCl, 1mM propionyl CoA, 5mM reduced glutathione and 10mM sodium bicarbonate. For the determination of potassium, ATP, and propionyl CoA, the concentrations of these substances were varied as follows: potassium between 5mM and 200mM, ATP between 0.25mM and 5mM and propionyl CoA between 0.5mM and 5mM. Each assay was run for 10 minutes in order to maintain the concentrations of the substrates near the initial concentrations except in the purified mutant which was run for 30 minutes but forms so little product that it probably did not appreciably alter the initial concentrations. In the ATP Km determination, magnesium is known to interact with ATP and was maintained at a two-fold excess even at the higher concentrations of ATP. When the Km for bicarbonate was determined, the total uCi of ^{14}C-bicarbonate per assay was kept constant while the concentration of cold sodium bicarbonate was varied. This resulted in a different specific activity correction for each assay condition. The Km values were calculated by linear regressions of a double reciprocal Lineweaver-Burk plot.(131)

CASE REPORTS

Patient 1

A white female presented at 3 days of age with metabolic acidosis, hepatomegly and hypoglycemia. She was the full term product of an uncomplicated pregnancy and was breast fed for the first days of life. Metabolic testing revealed the presence of ketones in the urine, an anion gap of 39mmol/L indicating severe metabolic acidosis, and all amino acids within normal limits.

On day 4, the baby became increasingly hypotonic without evidence of infection. Blood ammonia was shown to be elevated at 150ug/dl (nl= 50ug/100ml). This level increased on 5 to 350ug/dl producing coma. Peritoneal dialysis was begun to reduce the hyperammonemia. During the course of the dialysis, her ammonia levels reached 1350ug/dl. She experienced a respiratory arrest and responded to resuscitation. She was maintained on a respirator for approximately 27 hours until her blood ammonia levels returned to 150ug/dl and spontaneous breathing resumed.

On day 8, she was begun on a low protein diet. Propionic acidemia was diagnosed, following the demonstration of 3-hydroxypropionic acid, methylcitrate, tiglyglycine and propionylglycine in the urine as determined by GC/MS. She gained weight and survived several bouts of viral illness which precipitated ketotic crises. At the age of 20 months, the patient developed severe metabolic acidosis as a result of a viral gastroenteritis and died.

The patient's fibroblasts were complemented and determined to

belong to the pccA group and a liver sample was obtained at autopsy. (This patient was reported by M. F. Roberts, D. J. Schultz, B. Wolf, W. D. Cochran and A. L. Schwartz. Arch. of Diseases in Childhood, 54:962-964, 1979.(132)

Patient 2

A black male presented at 11 days of age with vomiting, hypotonia and apparent sepsis. The patient responded to antibotics and intervenous fluids; however, he developed metabolic acidosis when fed milk. The patient was released on a low protein diet after a repeat hospitalization. Severe neutropenia was noted at this time.

At 5 months of age the patient was admitted with symptoms of Kwashiorkor. The patient was released on a revised diet, on which he began to gain weight. At the age of 10 months, hyperglycinemia and hyperammonemia were noted and propionic acidemia was suspected. The diagnosis was confirmed by assay of the patient's fibroblasts. The patient died at 4 years, 7 months after a bout of acute pancreatitis and pneumonia which resulted in ketosis and coma. The patient's fibroblasts were complemented and determined to be in the pccC group. A liver sample was obtained and frozen at autopsy which is designated $pccC_1$ in this study.
(This patient was previously studied by Landes, R. D., G. B. Avery, F. A. Walker and Y. E. Hsia. Pediat. Res. 6:394, 1972.(32))

Patient 3

A white male presented at 2 weeks of age with vomiting, lethargy

and hypotonia which progressed to coma. The patient showed no evidence of ketosis and responded to IV fluids. At this time, the diagnosis of dysautonomia was considered. The patient was readmitted within 3 days with vomiting and lethargy. A plasma ammonia level of 1,100ug/100ml was obtained and glycine was also elevated. A liver biopsy was performed to determine the activity of urea cycle enzymes and carbamyl phosphate synthetase activity was found to be 20% of normal. The patient was maintained on 1 g of protein/kg body weight/day and the hyperammonemia abated. Neutropenia was also noted at this time. Decreases in plasma valine, phenylalanine and tyrosine levels, suggested amino acid depletion and a ketoacid-amino acid nutritional supplementation diet was begun. This load of protein initiated a ketoacidotic crisis with lethargy, neutopenia and hypoglycemia which suggested the diagnosis of an organic acidemia. The activity of propionyl CoA carboxylase in leukocytes was found to be approximately 2% of normal, confirming the diagnosis. The patient was maintained on 1.4 g of protein/Kg body weight/day. The patient died at 2½ years old as a result of the disease. The studies of patient's fibroblasts, resulted in their assignment to the pccC complementation group. A liver sample was obtained and frozen at autopsy which is designated $pccC_2$ in this study.

(This patient was previously reported by D. J. Harris, B. I. Y. Yang, B. Wolf, P. J. Snodgrass. Pediatrics 65:107-110, 1980.(22))

Patient 4

A black female presented at 2 days of age with tonic-clonic

seizures and progressive lethargy which was present from birth. She was worked up for sepsis and started on antibiotics. Marked hyperammonemia and ketosis were observed on day 7 with elevated serum propionate reaching 6800uM. The patient was treated with eleven 2-volume exchange transfusions over a 48 hour period with the addition of IV glucose and assisted ventilation. The diagnosis of propionic acidemia was confirmed by enzyme assay of cultured fibroblasts. The patient was hospitalized four times for refusing formula and for dehydration. She died at 4 1/2 months of meningitis. The patient's fibroblasts were complemented and were determined to be in the pccBC group. A sample of liver, obtained at autopsy, was frozen and subsequently, purification of the mutant enzyme in this study was designated pccBC.

Patient 5

A white female was diagnosed prenatally to have propionic acidemia as a result of abnormal metabolites in the amnionic fluid. The mother had a previous child with propionic acidemia who died at the age of 8 days. This patient was started on a low protein diet from birth. She had hypotonia from birth and had intermittent episodes of ketosis and hyperammonia. The patient is alive at 14 months and shows some delay in gross motor skills as a result of severe hypotonia. Studies of the patient's fibroblasts resulted in their assignment to the pccBC complementation group. The placenta from this pregnancy was frozen after delivery and is designated $pccBC_2$ in this study.

(This patient was reported by L. Sweetman, W. Weyler, T. Shafai, P. E.

Young and W. L. Nyhan. J. Am. Med. Assoc. 242:1048-1052, 1978.(66)

RESULTS

A. Propionyl CoA Carboxylase Activity in Normal Tissue and Tissue from Propionic Acidemis Patients

Biochemical characterization of normal and mutant propionyl CoA carboxylase from various human tissues was undertaken to gain insight into the biochemical defect causing the deficiency in these patients. It was necessary to biochemically characterize normal propionyl CoA carboxylase in various tissues in order to determine which were appropriate for study. The specific activity of samples of this enzyme, from several human tissue homogenates, are shown in Table IV. Liver and kidney tissue demonstrated comparable and high specific activities. Since normal and mutant liver tissue was readily available, it was chosen as a source of enzyme for most of this study. The donors of the fifteen control livers used in this study, ranged in age from newborns to a 55 year old, but most of the livers were from infants. There was no significant correlation between the age of the donor and the specific activity of propionyl CoA carboxylase. However, the time the liver was obtained after death, did influence the specific activity of the enzyme and also its subcellular distribution. In general, the longer time before obtaining and freezing the tissue resulted in a lower specific activity. A scatter plot of the specific activity of 8 livers showed a correlation of -0.83 between specific activity and time before freezing of the liver. Early attempts to localize propionyl CoA in isolated mitochondria from both liver and placenta, demonstrated only 3% of the propionyl CoA carboxylase activity in this

TABLE IV

PROPIONYL CoA CARBOXYLASE ACTIVITY IN NORMAL TISSUE
AND TISSUE FROM PROPIONIC ACIDEMIA PATIENTS

Tissue	Mean Specific Activity nmoles/min/mg	Range
Normal Liver (n =9)	6.97	0.8-16.1
Mutant Liver (n= 3)	0.26	0.23-0.36
Fibroblast Normal (n=5)	0.49	0.3-0.7
Fibroblast Mutant (n=8)	0.0045	0.0022-0.056
Placenta Normal (n=3)	0.49	0.41-0.56
Placenta Mutant (n=1)	0.17	
Kidney Normal (n=2)	8.67	7.6, 9.7

fraction as well as only 3% of another mitochondrial enzyme activity, glutamic dehydrogenase, suggesting the breakdown of mitochondria during liver autolysis. The percentage of propionyl CoA and glutamic dehydrogenase activities was found to be particularly low in the mitochondrial fraction of livers which were obtained a long time after death. This finding was confirmed by a control study in freshly obtained pig liver which monitored the leakage of propionyl CoA and glutamic dehydrogenase. In this experiment, pieces of liver, maintained on ice, were processed 1, 4 and 7 hours after death and demonstrated 15% decrease in mitochondrial propionyl CoA carboxylase activity. Because of the loss of propionyl CoA carboxylase activity in isolated mitochondria, whole cell homogenates were used as the source of human enzyme in most experiments.

The three livers and one placenta from patients in the pccC and pccBC complementation groups all retained residual propionyl CoA carboxylase activity of less than 4% of normal activity. The complementation class was determined in fibroblast cultures obtained from these patients prior to death. Two of these livers, designated $pccC_1$ and $pccC_2$ belong to the subgroup pccC, whereas the other liver and placenta belonged to the subgroup pccBC, designated $pccBC_1$ and $pccBC_2$ respectively.

The activity of glutamic dehydrogenase was used as an indicator of total mitochondrial enzyme activity in the mutant livers because it correlated with propionyl CoA carboxylase activity in normal livers. As shown in Table V, the mutant livers have glutamic dehydrogenase

TABLE V

THE SPECIFIC ACTIVITY OF PROPIONYL CoA CARBOXYLASE AND GLUTAMIC DEHYDROGENASE IN LIVERS FROM NORMALS AND PROPIONIC ACIDEMIA PATIENTS

	Propionyl CoA Carboxylase Activity nmoles/min/mg			Glutamic Dehydrogenase Activity umoles/min/mg	
	Mean	Range		Mean	Range
Normal Liver (n=9)	6.97	0.8 -16.1	Normal Liver (n=7)	1.06	0.4-1.98
Mutant Liver (n=3)	0.255	0.23-0.36	Mutant Liver (n=2)	1.7	1.38, 2.02

activities within the range obtained in normal livers indicating that the differences in propionyl CoA carboxylase activity are not likely to be caused by variation in time before these tissues were frozen. The residual activity in both the mutant liver and placental tissues parallels that observed in mutant fibroblast cell lines exhibiting 0.5- 10% of the specific activity found in normal tissue. Although the problem of autolysis is circumvented using fibroblast cultures, the disadvantage in working with fibroblasts is that they have only 10% of propionyl CoA carboxylase activity found in liver, therefore, requiring more material to obtain adequate activity for experimentation.

The other two mitochondrial carboxylases, pyruvate carboxylase and β-methylcrotonyl CoA carboxylase are less stable than propionyl CoA carboxylase. So their activity is found in reduced amounts in most liver samples. Both enzymes can be readily assayed in fibroblast homogenates, even though they are approximately 1/2 and 1/4 the activity of propionyl CoA carboxylases, respectively. Pyruvate and β-methylcrotonyl CoA carboxylase activities have been shown to be equivalent in both normal and propionyl CoA carboxylase deficient lines except for the bio complementation group. The bio group represents cell lines with deficiencies in all three carboxylase enzyme activities when incubated in medium not supplemented with biotin. The addition of 5 ug/ml of biotin to the cell culture medium restores the activities of these carboxylases to near normal levels. Deficient activity of all three carboxylases in the bio mutant and ability to correct this deficiency with excess biotin suggests that the defect is the result of

either abnormal intracellular biotin transport of a defect in a common holocarboxylase synthetase, the enzyme which covalently binds biotin to the apocarboxylase.

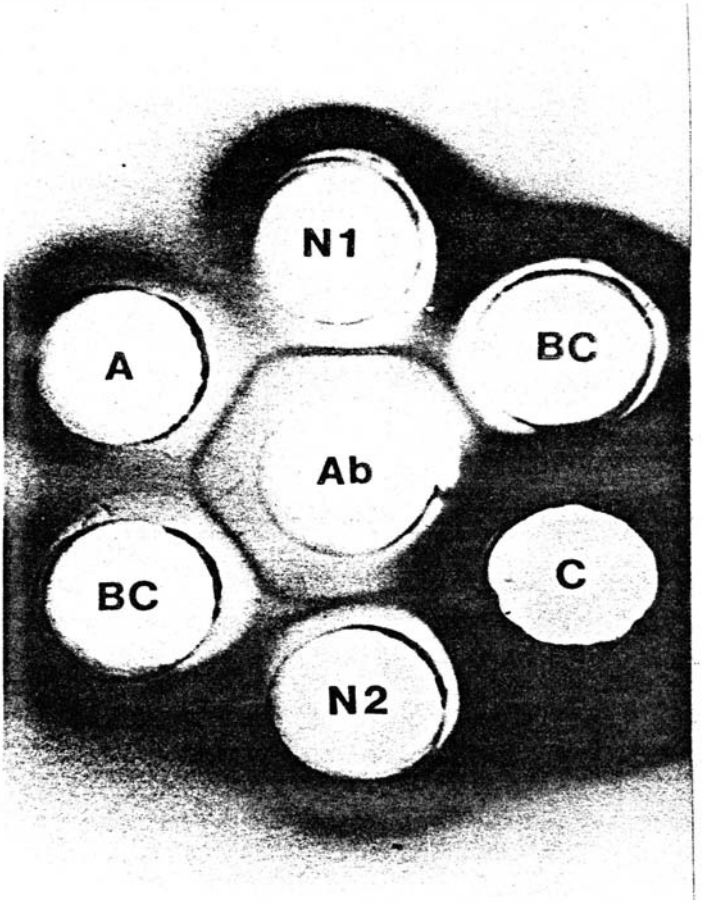
B. Immunotitration of Propionyl CoA Carboxylase in Normal and Mutant Tissues

To determine if propionyl CoA carboxylase deficiency was the result of a normal quantity of less active defective enzyme or a decreased synthesis of a structurally normal enzyme, immunologic methods were employed. Antiserum against purified pig heart propionyl CoA carboxylase, when added to fibroblast extracts, inhibited propionyl CoA carboxylase activity, but did not inhibit the activity of pyruvate carboxylase, nor glutamic dehydrogenase. The activity of human β-methylcrotonyl CoA carboxylase was slightly inhibited by incubation with the antiserum.

A precipitated complex could readily be removed from solution by centrifugation at 5000 X g for 5 min. Non-centrifuged antibody-PCC complexes retained activity comparable to that of extracts to which antibody was not added. In addition, the antiserum only complexed to PCC, since the activities of the other three carboxylases were unchanged in the supernatant following centrifugation.

Ouchterlony double-diffusion studies of extracts of normal and PCC-deficient livers against anti-pig heart PCC yielded single precipitin arcs for each preparation (Fig. 6). In addition, these studies revealed patterns of identity between the mutant PCC and normal PCC, with no evidence of spurring. Since the precipitin arcs were about an

Figure 6. Double diffusion on Ouchterlony plates of crude extracts of normal liver (N) and liver from the PCC-deficient patients (10 mg/ml) belonging to the pccA (A), pccBC (BC) and pccC (C) genetic complementation groups against undiluted antiserum to pig heart PCC in the center well (Ab). Variable darkly staining regions around extract wells appear to be due to lipid material.

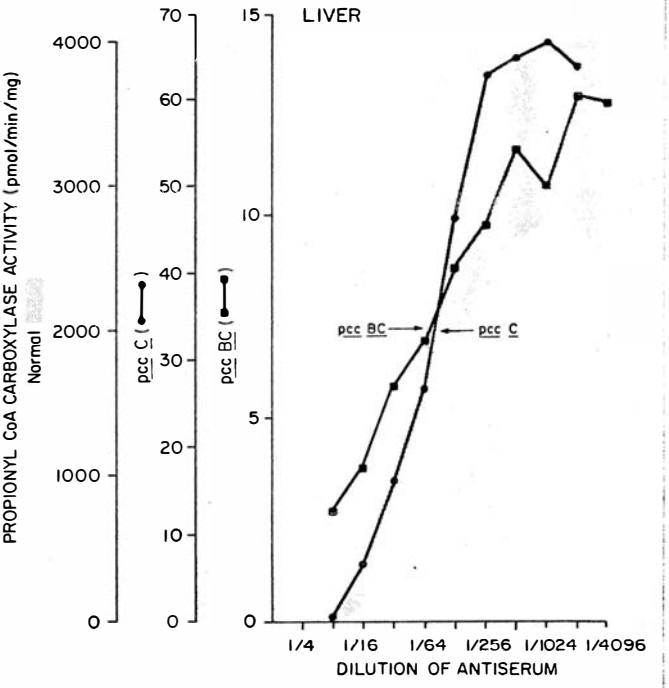

N1
A
BC
Ab
BC
C
N2

equal distance from each of the liver extract wells containing equivalent protein concentrations, the cross-reacting material (CRM) in each liver sample appears to be about the same concentration.

To measure the amount of the CRM in these liver extracts more accurately, we titrated PCC activity in extracts of normal and PCC-deficient livers by determining the residual PCC activities in the supernatant solutions of extracts following incubation with decreasing titers of antiserum (Fig.7). The results strikingly revealed the difference in the activities of normal (2600-4100 pmol/min/mg protein) and mutant PCC; activity of the pccC$_1$ liver was 67 pmol/min/mg (2.1% of normal) and that of the pccBC$_1$ liver was 13.5 pmol/min/mg (0.4% of normal). PCC activity in extracts incubated without antiserum was similar to that of extracts incubated with 1/2048 to 1/4096 dilutions of antiserum. The shapes of the hepatic immunotitration curves for the two mutant extracts corresponded closely to each other and to those for normal liver extracts with 50% of the maximum PCC activity inactivated at antiserum titers of about 1/64-1/128. Extracts of the liver of the pccA group could not be titrated because the residual activity was so low, because of its reduced thermo stability, that it would not be linear with respect to the lengthy time of incubation necessary under the assay condition.

To compare the CRM in fresh tissues derived from individuals assigned to the major complementation groups, we titrated extracts of fibroblasts from normal and PCC-deficient individuals with anti-pig heart PCC in the same manner as we titrated the liver extracts. As

Figure 7. Inhibition of PCC activity by increasing dilutions of antiserum to pig heart PCC in normal liver extracts and in extracts from PCC-deficient patients belonging to the pccBC and pccC complementation groups. The extract protein concentrations were adjusted to 10 mg/ml. Extracts were incubated with the several dilutions of antiserum at 37°C for 30 min. The samples were then centrifuged at 5000 X g for 5 min. and the supernatant solutions assayed for PCC activity. Shaded areas indicate the range of five normal extracts; pccC and pccBC extracts are indicated by (●) and (■), respectively. PCC-specific activity is plotted against the titer of antiserum. The arrows indicate titers of antiserum at which 50% of maximum PCC activity is inactivated. Note the different ordinate scales of PCC activity for the various liver samples.


LIVER
PROPIONYL CoA CARBOXYLASE ACTIVITY (pmol/min/mg)
Normal
4000
3000
2000
1000
0
pcc C ( )
70
60
50
40
30
20
10
0
pcc BC ( )
15
10
5
0
pcc BC
pcc C
1/4
1/16
1/64
1/256
1/1024
1/4096
DILUTION OF ANTISERUM

shown in Fig. 8A, the residual PCC activity in the supernatant solutions of mutant fibroblast extracts varied from 2.2 to 56 pmol/min/mg protein (normal = 500-700 pmol/min/mg) or from 0.4% to 9.3% of normal. As in the hepatic immunotitration studies, the titration curves of mutant fibroblasts from each of the complementation groups were comparable to those of normal fibroblasts, with 50% inhibition of PCC activity at an antiserum titer of 1/256 to 1/512. The fibroblast line (Fig. 8A) and the liver (Fig. 7) of the pccC complementation group were from the same patient. Immunotitration of another pccC fibroblast line (data not given) gave similar results. Two other pccA lines and a pccBC line were also titrated, but the PCC activity was heat labile under the conditions of the titration assay.

PCC activity in fibroblast cultures of the bio mutant incubated in medium without supplemental biotin (biotin in unsupplemented medium 2 ng/ml) was 36 pmol/mn/mg. Activity in the same cells incubated in medium supplemented with biotin (1 ug/ml) was sixteenfold higher (610 pmol/min/mg) and within the normal range of activity. Immunotitration of PCC activity in fibroblast extracts of the bio mutant incubated in medium without supplemental biotin gave curves similar to those of normal extracts (50% of maximum activity inactivated at 1/256 to 1/512 dilution of antiserum). Thus, not only do PCC-deficient liver and fibroblasts evidently contain CRM to the antiserum, but, based on unit protein concentration, each mutant liver or fibroblast extract has a concentration of CRM similar to that of normal extracts.

In order to obtain an antibody to human propionyl CoA carboxylase,

Figure 8. Inhibition of PCC activity by increasing dilutions of antiserum to pig heart PCC in normal fibroblast extracts and in extracts from PCC-deficient patients belonging to the various complementation groups. Titration conditions are as described in Fig. 7, except that the extract protein concentrations were adjusted to 6 mg/ml. Shaded areas indicate the range of three normal extracts. PCC-specific activity is plotted versus the titer of antiserum. The arrows indicate titer of antiserum at which 50% of maximum PCC activity is inactivated. Note the different ordinate scales of PCC activity for the various fibroblast lines. (A) Immunotitration of PCC activity in representative fibroblast lines of the pccA (■) and pccC (●) complementation groups. The pccC fibroblast line shown in this figure and the liver of the pccC complementation group shown in Fig. 7 were from the same individual. (B) Immunotitration of PCC activity in the biotin-responsive fibroblast line of the bio complementation group with supplemental biotin (● :1 ug/ml) and without added biotin (■ ; biotin in unsupplemented medium 2 ng/ml).

A. FIBROBLAST
PROPIONYL CoA CARBOXYLASE ACTIVITY (pmol/min/mg)
Normal
pcc C
pcc A
pcc A
pcc C
DILUTION OF ANTISERUM
1/4
1/16
1/64
1/256
1/1024
1/4096

B. FIBROBLAST
PROPIONYL CoA CARBOXYLASE ACTIVITY (pmol/min/mg)
Biotin Responsive Line with Supplemental Biotin
Biotin - Responsive Line Without Supplemental Biotin
Normal
WITHOUT BIOTIN
WITH BIOTIN
DILUTION OF ANTISERUM
1/4
1/16
1/64
1/256
1/1024
1/4096

we developed a simple method to prepare antibodies to the human carboxylases. Exploiting the biotin-binding property of avidin, an avidin affinity column was synthesized to retain carboxylase from human liver homogenates. Evidence that the biotin-containing enzymes did in fact bind to the column is provided by the following observations: 1) less than one percent of the liver supernant protein which was passed through the column adhered to the column matrix; 2) the loss of this small quantity of protein was associated with a complete loss of propionyl CoA carboxylase activity in the eluant while glutamic dehydrogenase activity remained; 3) saturability of the column for propionyl CoA carboxylase could be demonstrated, indicating specific binding sites for the enzyme, and 4) propionyl CoA carboxylase activity could be demonstrated on the column following extensive washing. Since enzyme activity could be detected in the enzyme-avidin-Sepharose complex, one or more unbound sites on the enzyme molecule must retain their activity following binding to the column matrix.

When serum, obtained from rabbits on days 0 and 14 after inoculation with this enzyme-avidin-Sepharose complex, was diffused against total liver homogenate, no precipitant arcs which indicate the presence of antibody were visible. However, precipitant arcs were observed when the serum obtained seven days after the secondary immunization was tested in the same manner. The control rabbits did not demonstrate any precipitant arcs. When serum from rabbits treated with carboxylase-saturated column was incubated with liver homogenates and centrifuged, the activity of propionyl CoA carboxylase, pyruvate carboxylase, and

β-methylcrotonyl CoA carboxylase was inhibited. Inhibition of these enzyme activities, following the addition of antiserum, appears to be related to a specific antigen-antibody reaction since the activity of glutamic dehydrogenase was not inhibited by the antiserum. A precipitable complex could readily be removed from solution by centrifugation at 5000xg for 5 minutes. Non-precipitated complexes retained enzyme activity. The precipitation reaction was temperature dependent and was substantially inhibited by incubation at 4°C. In addition, Ouchterlony double diffusion studies in agarose showed a broad precipitin region between antiserum and the supernatant from normal liver extracts which retained fluorescamine-tagged avidin, indicating the presence of biotin-containing protein. When sera from inoculated rabbits were passed over an avidin-carboxylase saturated column, both the ability to inhibit propionyl CoA carboxylase activity and to form precipitant arcs on Ouchterlony double diffusion studies, was removed.

The precipitation of propionyl CoA carboxylase by anti-carboxylase antiserum was compared with the antiserum prepared against purified pig heart propionyl CoA carboxylase. Both antisera similarly inhibited propionyl CoA carboxylase activity almost totally in human fibroblast and liver homogenates. (Table VI) The antibody against pig heart propionyl CoA carboxylase was shown to cross react slightly with human β-methylcrotonyl CoA carboxylase but not with human pyruvate carboxylase. Since the structures of β-methylcrotonyl CoA carboxylase and propionyl CoA carboxylase are probably similar, some degree of cross reactivity at high antibody titers is not surprising. The antiserum

TABLE VI

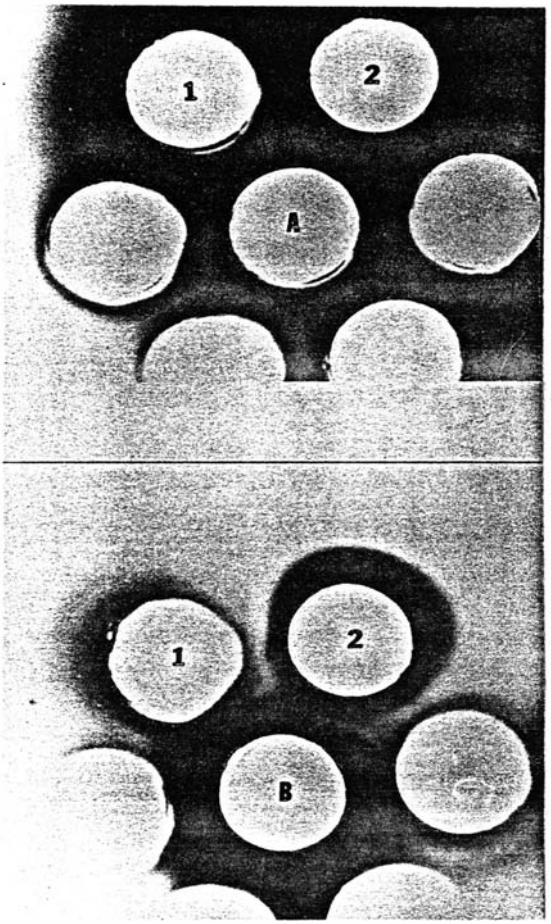
Comparison of Percent Inhibition of Anti-porcine Propionyl CoA and Anti-human Carboxylase Antiserum

Fibroblast	Anti-human carboxylase antiserum	Anti-porcine propionyl CoA carboxylase antiserum
Propionyl CoA carboxylase	95%	83%
β -Methylcrotonyl CoA carboxylase	62%	28%
Pyruvate carboxylase	71%	0%
Liver Homogenate		
Propionyl CoA carboxylase	80%	60%
β -Methylcrotonyl CoA carboxylase	59%	43%
Pyruvate carboxylase	73%	2%

prepared against the human carboxylases, as expected, inhibited both pyruvate carboxylase and β-methylcrotonyl CoA carboxylase, but not as dramatically as it inhibited propionyl CoA carboxylase.

Further comparisons of the antisera by Ouchterlony double diffusion, demonstrated that both antisera precipitated purified pig heart propionyl CoA carboxylase. (Figure 9A) The spur observed, indicates that the anti-pig heart propionyl CoA carboxylase recognizes different antigenic determinants in this preparation than those recognized by the human anti-carboxylase. There are several possible explanations for the observed spur. There is a possibility that there is, in the purified pig heart preparation, a contaminant not observed by gel electrophoresis to which an antibody has been made, or that the human anticarboxylase serum may be missing some antigenic determinants which are covered by the avidin binding. However, the most likely possibility is that this spur results from different determinant in the pig heart enzyme as opposed to the human enzyme. Whatever the correct explanation, the anticarboxylase antiserum precipitated the purified pig heart propionyl CoA carboxylase as shown by the precipitant arc. Figure 9B shows the precipitant arcs formed when the anti-carboxylase antiserum and the anti-pig heart propionyl CoA carboxylase antiserum were diffused against normal liver homogenate which contained measurable activity of propionyl CoA, pyruvate and β-methylcrotonyl CoA carboxylase activity. The figure shows the same spurring pattern observed in the previous plate and, in addition, a spur by the anti-carboxylase antiserum. This spur is probably caused by precipitation

Figure 9. Double diffusion on Ouchterlony plates of anti-human carboxylase antiserum (1) and anti-pig heart propionyl CoA carboxylase antiserum (2) against purified pig heart propionyl CoA carboxylase (A) and human liver homogenate (10mg/ml protein (B)).


1
2
A
1
2
B

of the other carboxylases in the liver homogenate which are not recognized by the anti-pig heart propionyl CoA carboxylase antiserum.

Immunodiffusion studies revealed a pattern of identity when the anti-human carboxylase antiserum was tested against crude liver homogenates from both propionyl CoA carboxylase deficient and non-deficient subjects. (Figure 10) The absence of precipitin spurs indicate no differences in antigenic sites between mutant and normal tissue extracts. These are identical to those observed with anti-pig heart propionyl CoA carboxylase antiserum. Propionyl CoA carboxylase activity was titrated in fibroblast extracts from a normal line, a pccA line and a pccC line. Each line has equivalent total protein concentrations (5mg/ml) and comparable pyruvate and β-methylcrotonyl CoA carboxylase activity. The titration curves were similar in each of the lines tested, indicating equivalent propionyl CoA carboxylase cross-reacting material. (Figure 11)

Finally, propionyl CoA carboxylase activity was measured in supernant solutions of normal, $pccC_1$ and $pccBC_1$ liver extracts containing identical total protein concentrations. These were titrated with antiserum and subsequently centrifuged. (Figure 12) The results indicated that a 1:2 dilution of antiserum inhibits 59% to 63% of the maximum propionyl CoA carboxylase activity in both normal and mutant extracts. The immunotitration curves for the mutant extracts are similar to each other and to those for normal liver extracts. These curves are also similar to those obtained using the anti-propionyl CoA carboxylase antiserum. Thus, not only do the mutant livers evidently contain

Figure 10. Double diffusion on Ouchterlony plates of extracts of normal liver and liver from the PCC-deficient patients against antiserum to human carboxylases. 1 and 4, normal liver; 2 and 5, liver of patient $\underline{pccC}_1$ and 6, liver of patient $\underline{pccBC}_1$. The protein concentrations of extracts in wells 1, 2, and 3 were 10 mg/ml and 5mg/ml in wells 4, 5, and 6.

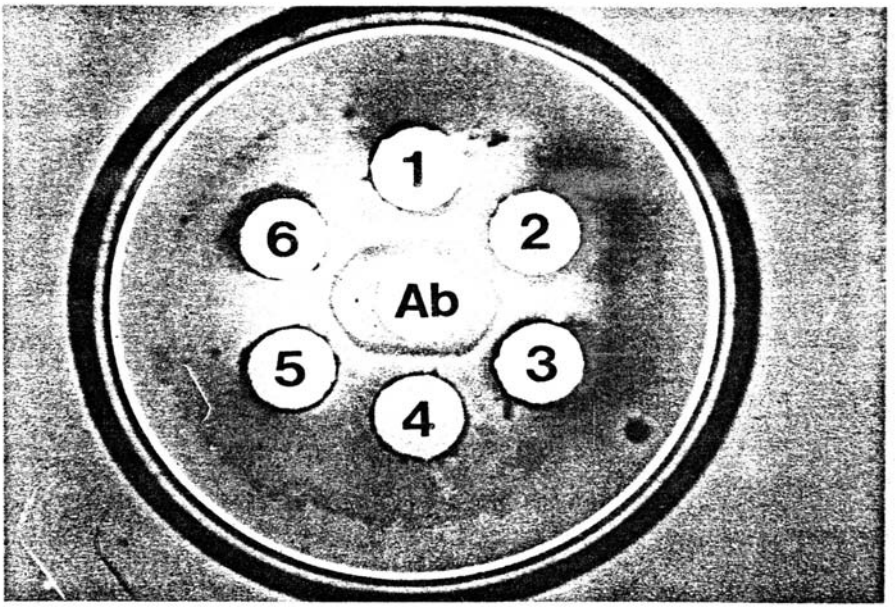

1
2
3
4
5
6
Ab

Figure 11. Titration curve of PCC activity in normal fibroblast extracts (●) and in extracts from PCC-deficient patients belonging to the pccA (△) and pccC (○) complementation groups (5mg protein/ml) inhibited by increasing dilutions of antisera to human carboxylases. Extracts were incubated with each dilution of antiserum at 37°C for 30 min. and centrifuged at 5000xg for 5 min. PCC activity was then determined in the supernatant solutions.

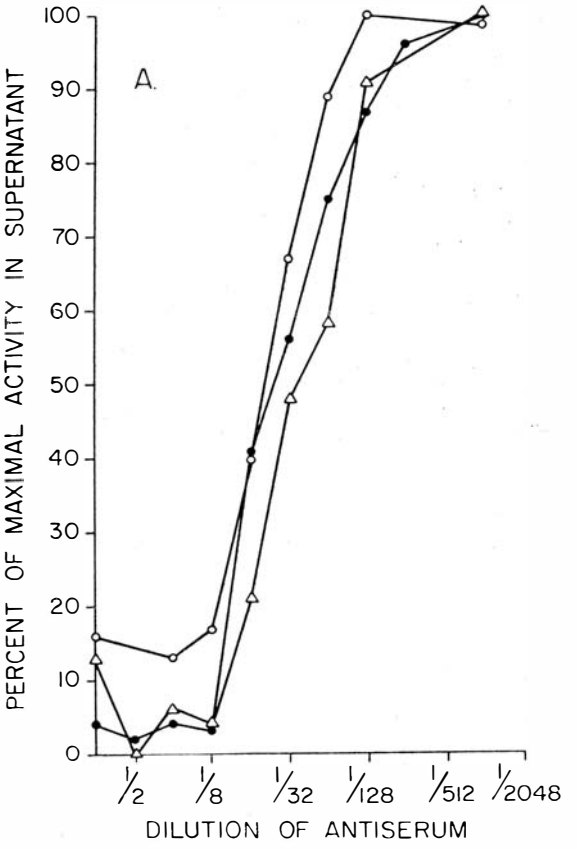
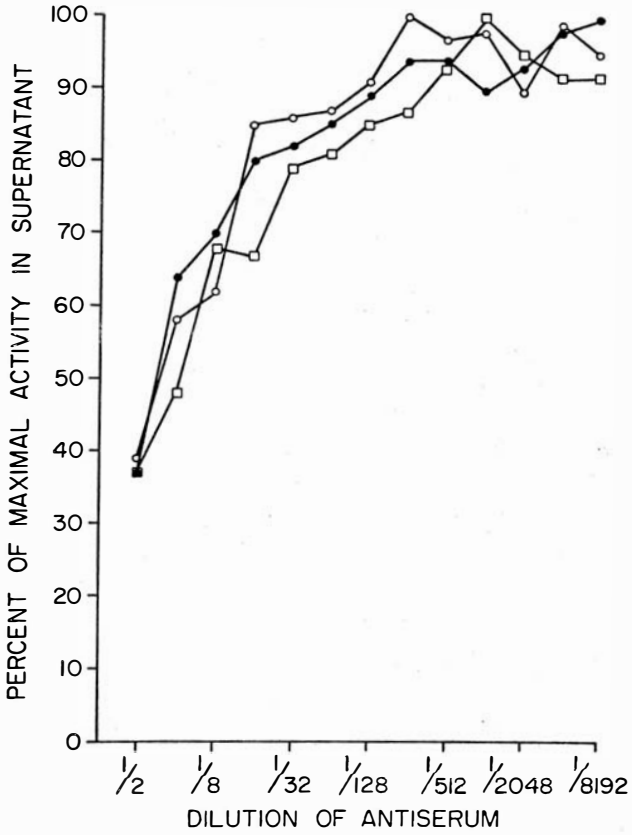

A.
PERCENT OF MAXIMAL ACTIVITY IN SUPERNATANT
100
90
80
70
60
50
40
30
20
10
0
1/2
1/8
1/32
1/128
1/512
1/2048
DILUTION OF ANTISERUM

Figure 12. Inhibition of PCC activity in a normal liver extract (●) and in extracts from PCC-deficient patient $\underline{pccC}_1$ (o) and $\underline{pccBC}_1$ (□) by increasing dilutions of antiserum to PCC and other acyl-carboxylases. The extract protein concentrations were adjusted to 14mg/ml. Extracts were incubated with the several dilutions of antiserum at 37°C for 30 min. The samples were centrifuged at 5000xg for 5 min. and the supernatant solutions assayed for PCC activity.


PERCENT OF MAXIMAL ACTIVITY IN SUPERNATANT
100
90
80
70
60
50
40
30
20
10
0
1/2
1/8
1/32
1/128
1/512
1/2048
1/8192
DILUTION OF ANTISERUM

cross-reacting material to the antiserum, but based on unit protein concentration, each mutant liver extract has a concentration of cross-reacting material comparable to that in the normal liver extracts. Furthermore, the results demonstrate that the heterologous antiserum gave similar results to those derived from purified pig heart antiserum.

C. Isoelectric Focusing of Propionyl CoA Carboxylase from Normal Tissue

Since the isoelectric point is a valuable indication of minor differences between enzymes from different sources, and since it had not been previously used in the investigation of propionyl CoA carboxylase, the isoelectric point of propionyl CoA carboxylase from four different human tissue homogenates, liver, kidney, placenta and fibroblast, was determined and compared in order to look for different isozymes of the enzyme. Studies of seven normal livers, showed that the enzyme had an isoelectric point of 4.66 ± 0.2. Similar profiles were obtained using homogenates of kidney, placenta and fibroblasts and a representative profile of each tissue is shown in Figure 13.

In addition, cell mitochondrial fractions from several liver samples were compared by isoelectric focusing to determine if there are multiple isozymes of propionyl CoA carboxylase. These comparisons in three different livers failed to show any difference between mitochondrial propionyl CoA carboxylase and that found in whole tissue homogenates. All mitochondrial fractions demonstrated a pI for the enzyme of about 4.7 as shown in the representative profiles in Figure 14. From these data, no isoelectric focusing differences in propionyl

Figure 13. Isoelectric focusing profiles of PCC in liver, kidney, placenta and fibroblast extracts on a 3.5 - 10.0 pH gradient for 48 hours. PCC activity (o) and pH (•) were determined in each 2ml fraction collected.

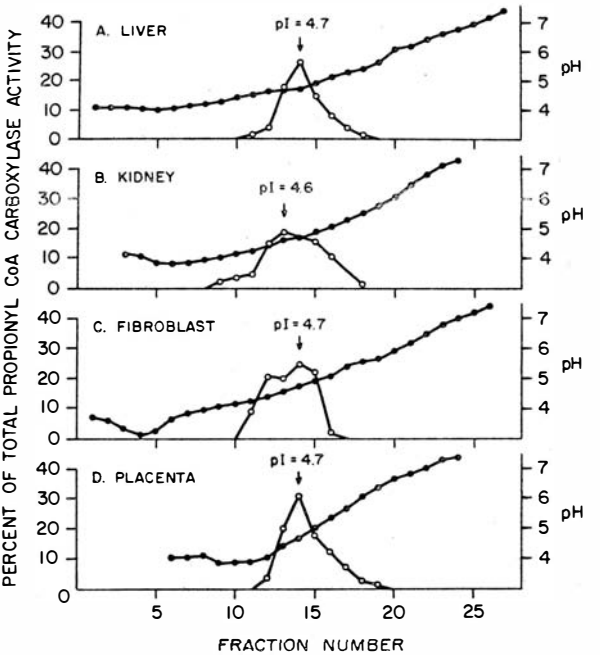
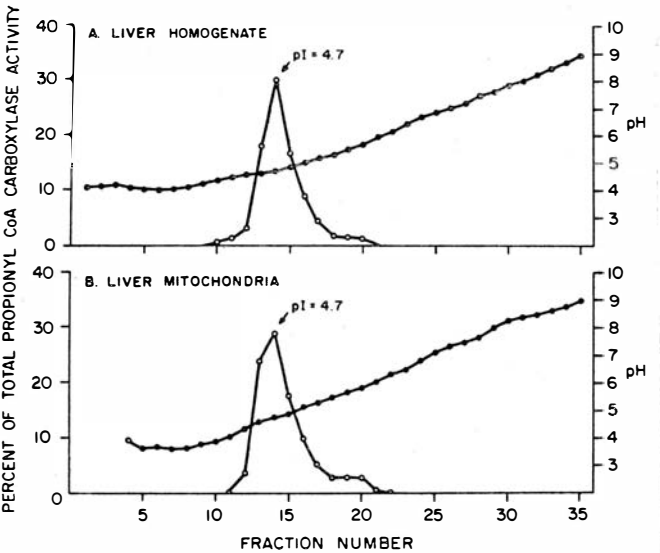

A. LIVER
pI = 4.7
B. KIDNEY
pI = 4.6
C. FIBROBLAST
pI = 4.7
D. PLACENTA
pI = 4.7
pH
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
FRACTION NUMBER

Figure 14. Comparison of isoelectric focusing profiles of PCC from liver homogenate and from the mitochondrial fraction isolated by centrifugation at 7200xg for 20 min. PCC activity (o) and pH (•) were determined in each 2 ml fraction.


A. LIVER HOMOGENATE
pI = 4.7
B. LIVER MITOCHONDRIA
pI = 4.7
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
pH
FRACTION NUMBER

CoA carboxylases from different tissues or different subcellular fractions were observed, suggesting the absence of different isozymes.

The isoelectric point of propionyl CoA carboxylase was also compared to the other two mitochondrial carboxylases, pyruvate carboxylase and β-methylcrotonyl CoA carboxylase in a freshly obtained human liver which had been stored in sucrose to preserve both pyruvate carboxylase and β-methylcrotonyl CoA carboxylase activity. Isoelectric points for these enzymes have not been previously reported in human tissue. Although all three enzymes gave different peaks, the peaks for propionyl CoA carboxylase and pyruvate carboxylase were overlapping with pI values of 4.9 and 5.3, respectively. β-Methylcrotonyl CoA carboxylase had a pI of about 6.4. (Figure 15) Similar profiles were obtained when the isoelectric points of these enzymes were determined in normal fibroblasts.

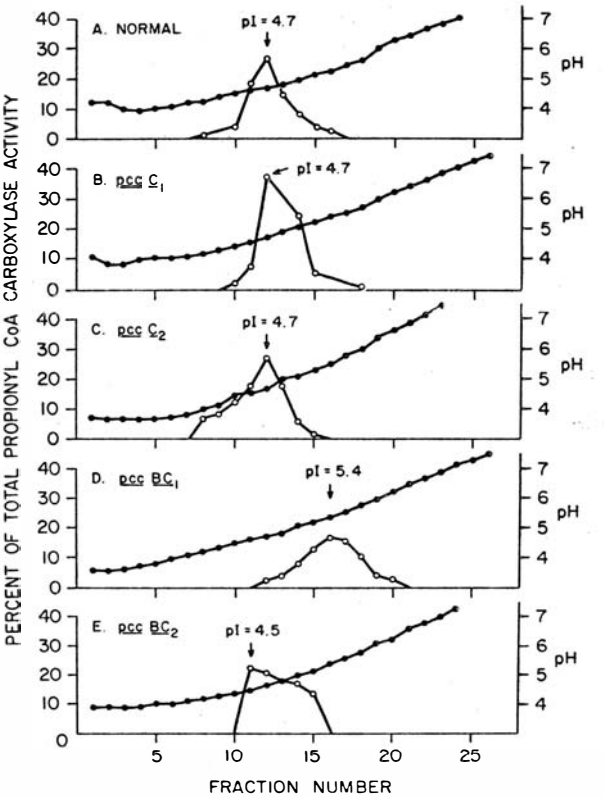
D. Characterizing the Mutant Enzyme

Propionyl CoA carboxylase from three livers ($\underline{pccC}_1$, $\underline{pccC}_2$ and $\underline{pccBC}_1$) and from one placenta ($\underline{pccBC}_2$) of patients with propionic acidemia in the pccBC complementation group were characterized by studying three basic biochemical properties: isoelectric point, thermostability, and binding affinity for the various enzyme substrates. The isoelectric points of these enzymes in tissue homogenates from the $\underline{pccC}_1$, $\underline{pccC}_2$ and $\underline{pccBC}_2$ mutants demonstrated a pI within the normal range, whereas the enzyme from the $\underline{pccBC}_1$ liver homogenate had a pI of 5.3. (Figure 16) This altered pI was observed even when equal amounts of liver homogenates, as determined by protein concentration of $\underline{pccC}_1$

Figure 15. Isoelectric focusing profile of the three mitochondrial carboxylases was determined by focusing a normal liver homogenate on a 3.5 - 10.0 pH gradient and analysing the 2 ml fractions for percent total PCC activity (o). βMCC activity (▲), PC activity (■) and pH (•).

PERCENT OF TOTAL SPECIFIC
ENZYME ACTIVITY
0
10
20
30
40
5
10
15
20
25
30
35
40
FRACTION NUMBER
pH
4
5
6
7
8
9
10
PCC (pI=4.9)
PC (pI=5.3)
βMCC (pI=6.4)

Figure 16. The isoelectric focusing pattern of PCC from liver (3) and placental (1) homogenates from PCC-deficient patients was determined by PCC activity (o) and pH (•) in each 2ml fraction.


A. NORMAL
pI = 4.7
B. pcc C1
pI = 4.7
C. pcc C2
pI = 4.7
D. pcc BC1
pI = 5.4
E. pcc BC2
pI = 4.5
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
pH
FRACTION NUMBER

and $\underline{pccBC}_1$ (altered pI), were preincubated for 30 minutes at 23°C and then focused. The profile demonstrates the presence of different enzymes by the biphasic peak at pH 4.9 and 5.4. (Figure 17) A similar profile was obtained when a ten-fold excess of this mutant liver homogenate was incubated with a normal liver homogenate.

In order to show that the pI shift in the $\underline{pccBC}_1$ mutant was not the result of a non-specific interaction with some extraneous substance within the homogenate as suggested by Kalousek et al.(83), the enzymes from a normal liver and from the $\underline{pccBC}_1$ liver were purified. The purification of the mutant enzyme was made possible by the addition of 0.5M sucrose which stabilized the enzyme during most purification procedures. The extent of purification and yields are shown in Table VII. The apparent low purification of the $\underline{pccBC}_1$ enzyme is the result of its instability throughout the procedures, especially DEAE column chromatography where sucrose had to be omitted because it interfered with enzyme-column interactions. A loss of 98% of the enzyme's activity occurred following this step. The purity of the normal enzyme was determined by SDS-polyacrylamide gel electrophoresis. The separation on the gel showed the presence of three bands, two of which ran identically to a purified pig heart propionyl CoA carboxylase standard which showed molecular weights of 76,000 and 62,500 was determined by comparing them to known standards. (Figure 19) The third band may be a degradation product of one of the subunits or a contaminant in the preparation. The mutant enzyme could not be visualized on electrophoresis because of the low yield after purification and the limited

Figure 17. Isoelectric focusing profile of the $\underline{pccC_1}$ and $\underline{pccBC_1}$ liver PCC. The percent total PCC activity (o) and pH (•) was determined on an isoelectric focusing gradient where equal volumes of (10mg protein/ml) liver homogenates derived from patients from the $\underline{pccC_1}$ and $\underline{pccBC_1}$ complementation group were incubated at 23°C for 30 min. prior to focusing on a 3.5 - 10.0 gradient.

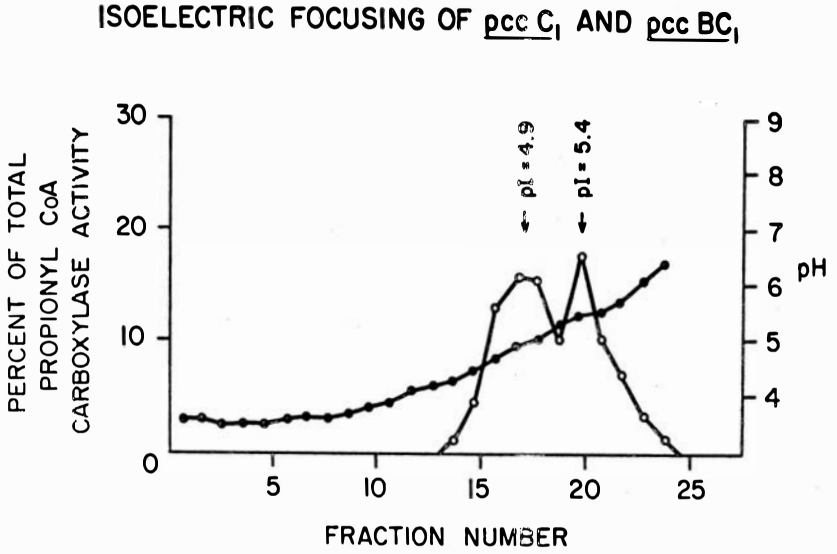

ISOELECTRIC FOCUSING OF pcc C1 AND pcc BC1
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
30
20
10
0
5
10
15
20
25
FRACTION NUMBER
← pI = 4.9
↓ pI = 5.4
9
8
7
6
5
4
pH

TABLE VII

PURIFICATION OF THE NORMAL AND MUTANT ENZYME

Step	Specific Activity nmol/mg/ml	Purification -fold	Yield %
Normal			
Homogenate	3.64×10^1	1.0	100
DEAE	3.91×10^2	11	18
Blue Sepharose	5.27×10^4	1447	3
Sephacryl	6.02×10^4	1654	4
Mutant			
Homogenate	0.1037	1	100
DEAE	0.037	0.36	2.2
Blue Sepharose	1.53	11	2.6
Sephacryl	3.56	34	0.6

Figure 18. The electrophoretic profile of four steps of the purification compared on a 7.5% polyacrylamide -2% SDS gel under reducing conditions. Lane 1 - Purified PCC After Sephacryl, Lane 2 - After Blue Sepharose, Lane 3 - After DEAE-cellulose, Lane 4 - Whole liver homogenate, Lane 5 - Standards (from top to bottom): bovine serum albumin, glutamic dehydrogenase, alcohol dehydrogenase, and avidin.

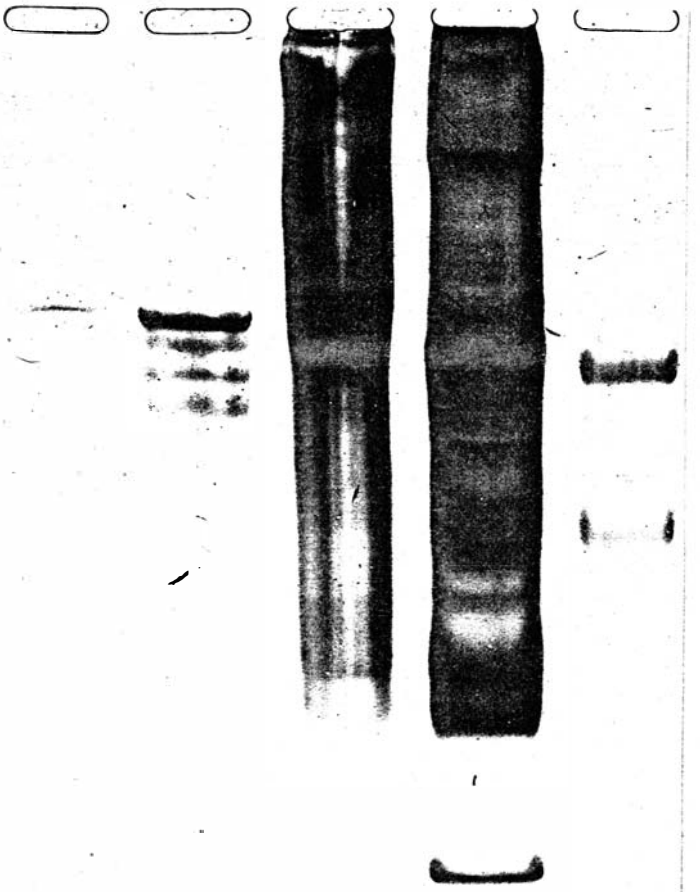
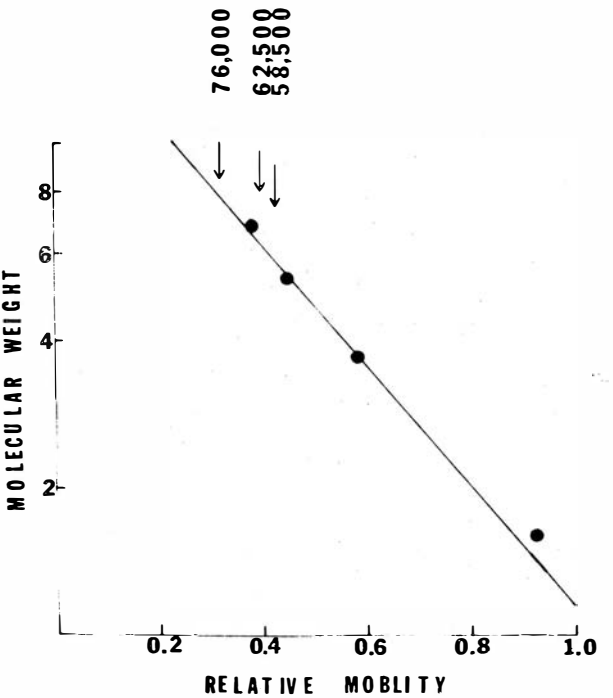


Figure 19. Determination of molecular weight of propionyl CoA carboxylase subunits by SDS-polyacrylamide gel electrophoresis. The following standards were used: bovine serum albumin 68,000, glutamic dehydrogenase 53,000, alcohol dehydrogenase 37,000 and avidin 16,000.


76,000
62,500
58,500
MOLECULAR WEIGHT
8
6
4
2
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBLITY

amount of liver available for purification.

Isoelectric focusing of the purified enzymes (Figures 20 & 21) demonstrates a similar profile to those observed in crude liver preparations with a pI of 4.7 for the normal enzyme and 5.3 for the mutant enzyme. An explanation was sought for the pI shift reported by Kalousek et al. (83) during purification of the normal enzyme from 4.7 to 5.2. Similar shifts were observed in both the crude and purified normal enzyme upon incubation with 5mM ATP prior to focusing. Since Kalousek et al. (83) observed their pI shift after the elution of the enzyme from the Blue Sepharose column with 3mM ATP, the addition of this nucleotide could easily account for the apparent shift, especially if the ATP is not completely removed from the system. A shift to a slightly higher pI was observed when the $\underline{pccBC}_1$ liver homogenate was pre-incubated with ATP. The demonstration of an altered pI in one $\underline{pccBC}$ mutant demonstrates heterogeneity within the mutant complementation class as well as providing the first definitive evidence of a structural alteration in a propionyl CoA carboxylase deficient mutant.

Previous studies have demonstrated differences between normal and propionyl CoA carboxylase deficient fibroblasts with respect to their thermostability.(3) In order to further investigate the thermostability in the mutant $\underline{pccBC}$ complementation group, the study was extended to include the interactions of sucrose or ATP with the enzyme and their influence on thermostability. Thermostability of propionyl CoA carboxylase activity was determined by incubation of dialyzed tissue homogenates containing 10mg of protein per ml at $45^{o}C$ for various

Figure 20. The isoelectric focusing profile of the normal enzyme was determined in a normal liver homogenate in a 3.5 - 10.0 (top) and a 4-6 pH gradient (middle) and compared to the highly purified sample focused on a 4-6 pH gradient (bottom) with pH (•) and percent total PCC activity (o) being determined in each fraction.

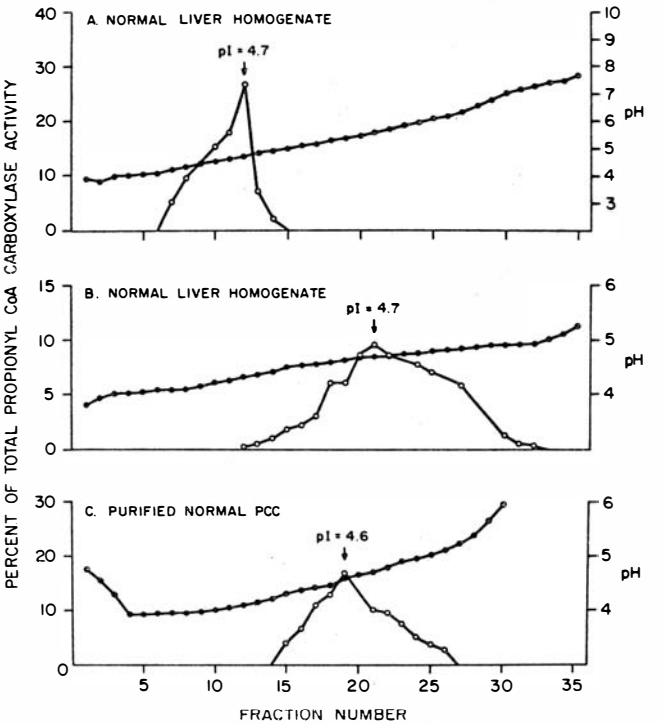
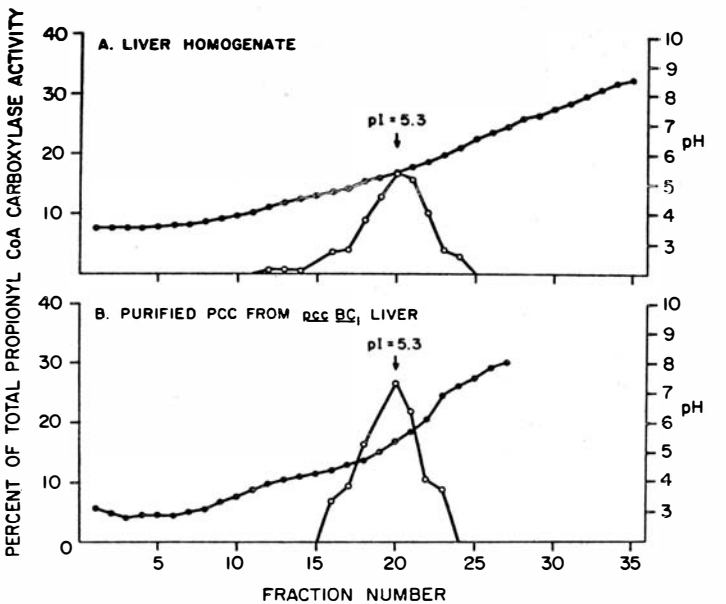

A. NORMAL LIVER HOMOGENATE
pI = 4.7
B. NORMAL LIVER HOMOGENATE
pI = 4.7
C. PURIFIED NORMAL PCC
pI = 4.6
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
pH
FRACTION NUMBER

Figure 21. The isoelectric focusing profile of the highly purified mutant (below) was compared with the pccBC$_1$ liver homogenate (above) on a 3.5 - 10.0 pH gradient with the pH (•) and percent Total PCC activity (○) being determined in every fraction.


A. LIVER HOMOGENATE
pI = 5.3
B. PURIFIED PCC FROM Pcc BC1 LIVER
pI = 5.3
PERCENT OF TOTAL PROPIONYL CoA CARBOXYLASE ACTIVITY
pH
FRACTION NUMBER

lengths of time between 0 and 30 minutes. The shaded area in Figure 22 represents the normal range determined from the data on five normal livers. Under these conditions, the enzymes from the $\underline{pccC_2}$ and $\underline{pccBC_1}$ were considerably more labile than the enzyme from homogenates of either normal liver or mutant $\underline{pccC_1}$ or $\underline{pccBC_2}$ tissue. Both the $\underline{pccC_2}$ and $\underline{pccBC_1}$ mutant had a half-life of approximately 2-3 minutes at 45°C, whereas the enzyme in the $\underline{pccC_1}$ and $\underline{pccBC_2}$ tissue homogenates had a half-life of greater than 15 minutes.

Further differences were observed by studying thermostability in homogenates with 5mM ATP or 0.5M sucrose added during experiments. Tissue homogenates were incubated for 30 minutes at 23°C prior to heat denaturation experiments. Sucrose was tried because it was demonstrated to stabilize the enzyme by Hsia et al. (81) and ATP was used as a measure of substrate stabilization. Both of these reagents stabilized propionyl CoA carboxylase in a representative normal liver homogenate; however, the mutant enzymes demonstrated a heterogeneous response. (Figure 23) Propionyl CoA carboxylase in the $\underline{pccC_1}$ liver homogenate gave a similar heat denaturation profile to the normal enzyme being stabilized by ATP or sucrose at 45°C. (Figure 23B) The $\underline{pccBC_1}$ mutant homogenate was stabilized dramatically by the addition of sucrose; however, the addition of ATP had little or no effect. (Figure 23C) The $\underline{pccBC_2}$ mutant homogenate was stabile up to 30 minutes under all conditions as was the normal placental control. (Figure 23A & D)

In a further attempt to distinguish between the $\underline{pccC_1}$ mutant and the normal liver enzyme by thermostability, the experiment was repeated

Figure 22. Heat denaturation curves for normal and PCC-deficient livers. Residual PCC activity was determined in dialyzed homogenates from normal livers (5) (range in shaded area) and livers from PCC-deficient patients (3) after incubation at 45°C for various times between 0 - 15 minutes.

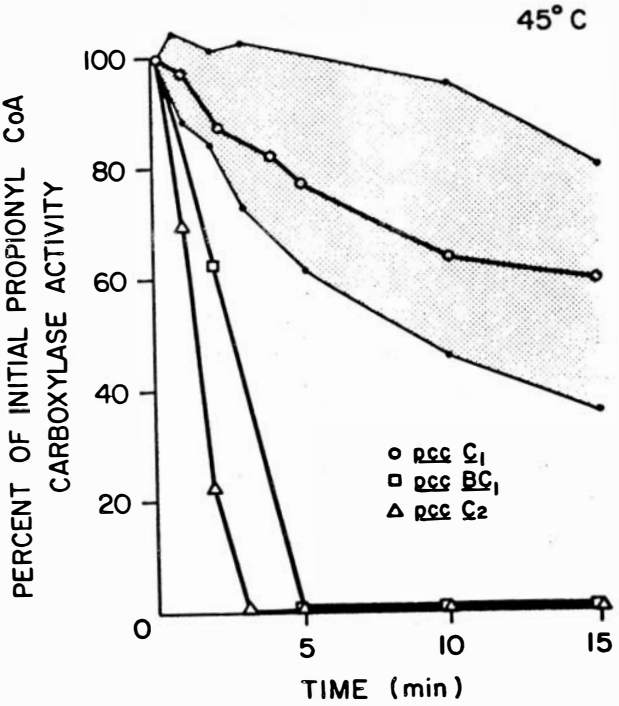
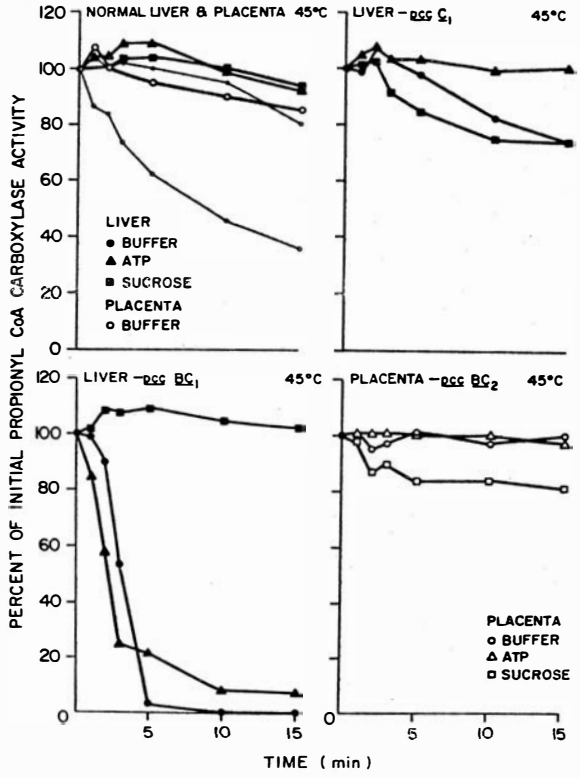

45° C
PERCENT OF INITIAL PROPIONYL CoA CARBOXYLASE ACTIVITY
100
80
60
40
20
0
5
10
15
TIME (min)
o pcc C1
□ pcc BC1
△ pcc C2

Figure 23. Comparison of heat denaturation profiles of dialyzed liver homogenates (closed symbols) and placental homogenates (open symbols) (10 mg protein/ml) after a 30 min. incubation at 45°C in either 10mM Tris-HCl buffer, pH 8.0 (●). 5mM ATP- 10mM Tris- HCl buffer, pH 8.0 (▲), or 0.5M sucrose- 10mM Tris-Hcl buffer, pH 8.0 (■). Normal range of liver homogenate heat denaturation is shown by shaded area.


NORMAL LIVER & PLACENTA 45°C
LIVER – pcc C1
45°C
LIVER
● BUFFER
▲ ATP
■ SUCROSE
PLACENTA
○ BUFFER
LIVER – pcc BC1
45°C
PLACENTA – pcc BC2
45°C
PLACENTA
○ BUFFER
△ ATP
□ SUCROSE
PERCENT OF INITIAL PROPIONYL CoA CARBOXYLASE ACTIVITY
TIME (min)
0
20
40
60
80
100
120
5
10
15

at 52°C. (Figure 24) Even at the higher temperature, the $\underline{pccC}_1$ mutant behaved similarly to the normal enzyme. Both the $\underline{pccC}_1$ mutant and the normal liver homogenate were stabilized by sucrose and slightly by ATP.

The purification of the normal enzyme did not alter its stability as determined at 45°C in 10mM Tris-HCl buffer, pH 8.0, from that in normal liver homogenates. However, the enzyme was no longer stabilized by the addition of either 5mM ATP or 0.5M sucrose. (Figure 25A) The purified $\underline{pccBC}_1$ mutant enzyme showed increased thermostability of the enzyme over that in the $\underline{pccBC}_1$ liver homogenate demonstrating a half-life of 17 minutes but the enzyme remained less stable than the normal enzyme. (Figure 25B) In contrast to the normal enzyme, the purified $\underline{pccBC}_1$ enzyme continued to be stabilized following the addition of sucrose. Sucrose was also noted to stabilize both enzymes when stored at -80°C. The addition of ATP failed to stabilize either enzyme.

In summary, $\underline{pccC}_2$ and $\underline{pccBC}_1$ mutants demonstrated differences in the thermostability. The $\underline{pccBC}_1$ homogenates also showed a dramatic stabilization of enzyme activity by the addition of sucrose to both crude and purified preparations. Mutant $\underline{pccC}_1$ amd $\underline{pccBC}_2$ gave similar profiles to the normal controls. In general, thermostability has been a useful parameter for demonstrating heterogeneity between the mutants in the $\underline{pccBC}$ group; however, thermostability seems to be a function of the molecular environment of the enzyme as well as the enzymes' structure.

A third parameter used to compare the properties of the mutant and normal enzymes in tissue preparations was to determine the apparent

Figure 24. Heat denaturation profile. Residual PCC activity was determined for dialyzed normal and pccC$_1$ liver homogenates (10 mg protein/ml) after incubation in a 52°C waterbath for various periods of time between 0 - 15 min. Prior to heat denaturations samples were incubated at 23°C for 30 min. in either 10mM Tris-HCl buffer, pH 8.0 (●), 5mM ATP- 10mM Tris-HCl buffer, pH 8.0 (▲), or 0.5M sucrose-10mM Tris-HCl buffer, pH 8.0 (■).

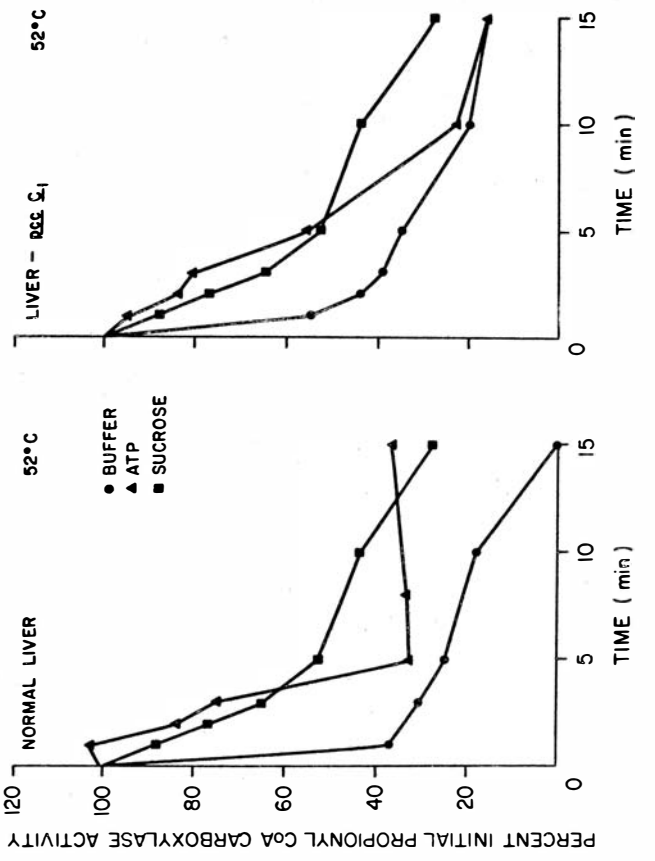

PERCENT INITIAL PROPIONYL CoA CARBOXYLASE ACTIVITY
120
100
80
60
40
20
0
NORMAL LIVER
52°C
● BUFFER
▲ ATP
■ SUCROSE
0
5
10
15
TIME (min)
LIVER – RAT
52°C
0
5
10
15
TIME (min)

Figure 25. Heat denaturation profile of residual PCC activity of purified normal enzyme and highly purified pccBC$_1$ enzyme after incubation in a 45^{o}C waterbath for various periods of time. Prior to heat denaturation purified normal enzyme (diluted 1:100 with 10MM Tris-HCl buffer, pH 8.0) and dialyzed highly purified pccBC$_1$ enzyme were incubated at 23^{o}C for 30 min. in either 10mM Tris-HCl buffer pH 8.0 (●), 5mM ATP- 10mM Tris-HCl buffer, pH 8.0 (▲) or 0.5 M sucrose- 10mM Tris-HCl buffer, pH 8.0 (■).

PERCENT INITIAL PROPIONYL CoA CARBOXYLASE ACTIVITY
120
100
80
60
40
20
0
PURIFIED NORMAL PCC 45°C
● BUFFER
▲ ATP
■ SUCROSE
0 5 10 15 20 25 30
TIME (min)
PURIFIED MUTANT PCC 45°C
pcc BC1
0 5 10 15 20 25 30
TIME (min)

Km's of the enzyme for its substrates. An increased Km for any substrate could affect the ability of the enzyme to function and explain the defect in these cases. The Km values for the substrates were determined with both of the purified enzymes and with homogenates of $\underline{pccC}_1$ and $\underline{pccBC}_2$ tissues. The apparent Km's were determined for ATP, propionyl CoA, bicarbonate and potassium ions. All other substrates were held constant at standard assay conditions while the substrate of interest was varied. The Km's for the four reactants are reported in Table VIII and the individual graphs follow in figures 26 to 29. There is good agreement between the values obtained for the purified normal and mutant enzymes. The values obtained in tissue homogenates showed approximately a two-fold increase over the values obtained in the purified enzyme; however, this variation should be expected in crude preparations. The Km values obtained in the dialyzed homogenates were in good agreement with values obtained in both the purified enzymes and those reported by other investigators. The largest deviation from the normal enzyme Km value was observed in the Km for propionyl CoA in the $\underline{pccBC}_2$ tissue homogenate. This value was not dramatically different from the Km observed in the $\underline{pccC}_1$ homogenate or the value obtained by Hsi et al. (81) in crude fibroblast extracts. In general, no heterogeneity was found in the study of Km's especially among the values obtained for the purified enzyme. The Km values seemed to be comparable between homogenates as well as between normal enzymes.

A summary of the data comparing the normal and mutant enzymes from the $\underline{pccBC}$ tissue studied is given in Table IX. All of the

TABLE VIII

COMPARISON OF APPARENT Km's OF PROPIONYL CoA CARBOXYLASE FROM VARIOUS SOURCES

SOURCE	PROPIONYL CoA (mM)	ATP	Bicarbonate	Potassium
Purified Normal	0.424	0.117	1.49	4.87
Purified pccBC$_1$	0.420	0.103	2.98	8.33
pccC$_1$ Liver	0.90	0.440	9.09	10.65
pccBC$_2$ Placenta	1.67	0.4 8	6.30	8.5
Previous investigators normal mammalian enzyme	0.2 - 1.7	0.06 - 0.9	1.6 - 4.5	7.0

Figure 26. A double reciprocal Line weaver- Burk plot of velocity against varying concentrations of propionyl CoA between 0.5 and 5mM was used to determine apparent Km in homogenates of liver $\underline{pccC}_1$ (■) and placenta $\underline{pccBC}_2$ (▲) (above) and highly purified normal (o) and $\underline{pccBC}_1$ (●) propionyl CoA carboxylase (below).

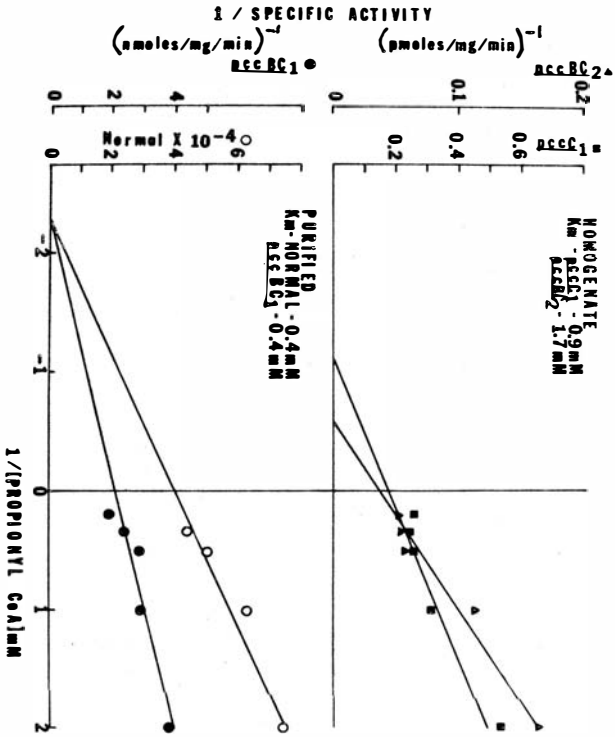

1 / SPECIFIC ACTIVITY
(nmoles/mg/min)$^{-1}$
(pmoles/mg/min)$^{-1}$
acc BC$_1$ ●
acc BC$_2$ ▲
acc$_1$ ■
Normal X 10^{-4} ○
PURIFIED
Km-NORMAL - 0.4mM
acc BC$_1$ - 0.4mM
HOMOGENATE
Km - acc$_1$ - 0.9mM
acc BC$_2$ - 1.7mM
1/(PROPIONYL CoA)mM

Figure 27. A double reciprocal Line weaver- Burk plot of velocity against varying concentrations of ATP between 0.25 and 5mM was used to determine apparent Km in homogenates of liver $\underline{pccC}_1$ (■) and placenta $\underline{pccBC}_2$ (▲) (above) and highly purified normal (o) and $\underline{pccBC}_1$ (●) propionyl CoA carboxylase (below).

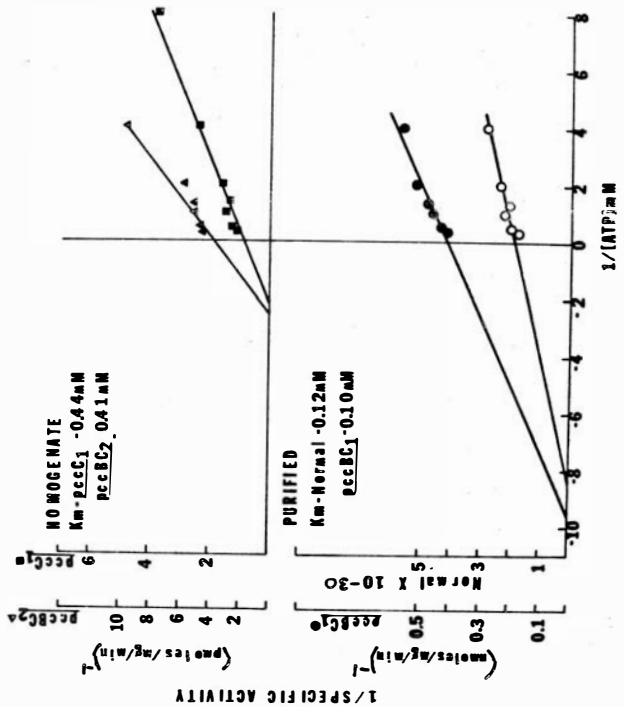

1/SPECIFIC ACTIVITY
(pmoles/mg/min)⁻¹
(nmoles/mg/min)⁻¹
HOMOGENATE
Km-pccC1 -0.44mM
pccBC2 0.41mM
PURIFIED
Km-Normal-0.12mM
pccBC1-0.10mM
Normal X 10-30
1/[ATP]mM

Figure 28. A double reciprocal Line weaver- Burk plot of velocity against varying concentrations of total bicarbonate between 0.5 and 20mM by varying cold sodium bicarbonate and keeping total cpms per assay constant was used to determine apparent Km in homogenates of liver $\underline{pccC}_1$ (■) and placenta $\underline{pccBC}_2$ (▲) (above) and highly purified normal (o) and $\underline{pccBC}_1$ (●) propionyl CoA carboxylase (below).

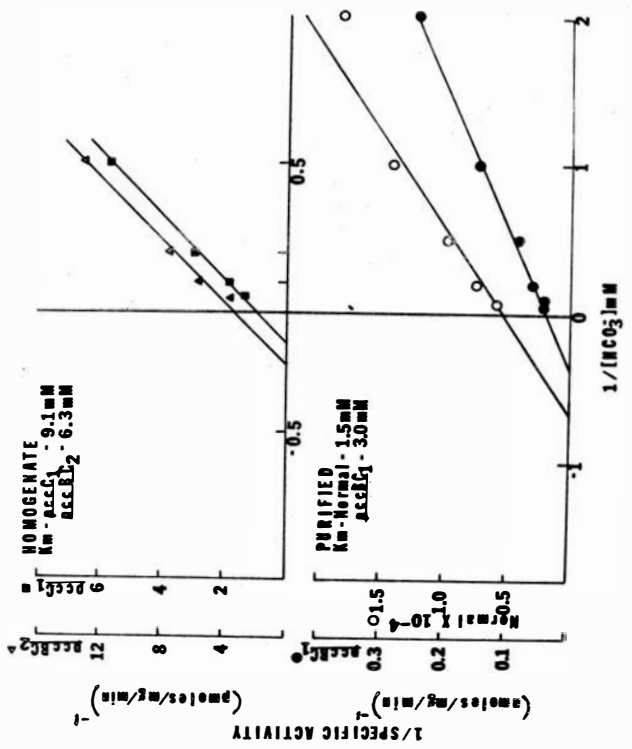

HOMOGENATE
Km - acetate
acetate1 - 9.1mM
acetate2 - 6.3mM
PURIFIED
Km - Normal - 1.5mM
acetate1 - 3.0mM
1/[HCO3-]mM
1/SPECIFIC ACTIVITY
(pmoles/mg/min)-1

Figure 29. A double reciprocal Line weaver- Burk plot of velocity against varying concentrations of the activator, potassium between 5 and 100mM was used to determine apparent Km in homogenates of liver $\underline{pccC_1}$ (■) and placenta $\underline{pccBC_2}$ (▲) (above) and highly purified normal (o) and $\underline{pccBC_1}$ (●) propionyl CoA carboxylase (below).

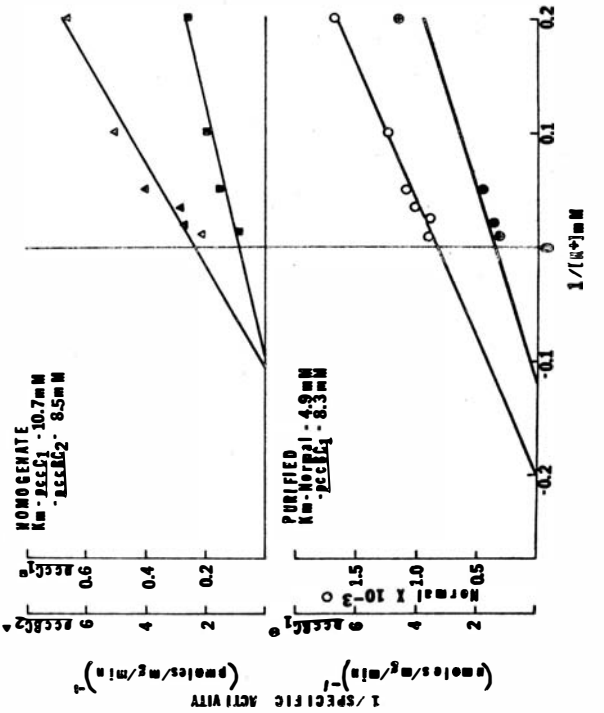

HOMOGENATE
Km - AcCh1 - 10.7mM
- AcCh2 - 8.5mM
PURIFIED
Km - Normal - 4.9mM
- AcCh1 - 8.3mM
AcCh1
AcCh2
1/[S] mM
1/SPECIFIC ACTIVITY
(pmoles/mg/min)-1
(pmoles/mg/min)

TABLE IX

COMPARISON OF PROPERTIES OF PROPIONYL CoA CARBOXYLASE FROM NORMAL AND MUTANT TISSUE

Source	CRM	pI	Thermostability at 45°C ($t_{1/2}$) min		
			Buffer	w/ATP	w/Sucrose
Normal Liver	+	4.7	9	>30	>30
pccC$_1$ Liver	+	4.7	> 30	>30	>30
pccC$_2$ Liver	+	4.7	3	NA	NA
pccBC$_1$ Liver	+	5.4	2	2	>15
Normal Placenta	+	4.6	>30	>30	>30
pccBC$_2$ Placenta	+	4.6	>30	>30	>30

variants that were studied represent CRM-positive mutants, suggesting that the mutation causes a structural alteration. The data demonstrate heterogeneity within the pccBC group in two of the parameters tested with mutants $pccC_1$ and $pccBC_2$ being indistinguishable from the enzyme in the normal tissue. Mutant $pccBC_1$ enzyme demonstrated differences in thermostability and isoelectric point. Mutant $pccC_2$ showed a difference in thermostability but not in isoelectric point. Unfortunately, there was not enough sample for further study. The differences observed are suggestive of further heterogeneity in the structure of the enzyme within the pccBC complementation group.

DISCUSSION

The propionic acidemias are a group of genetic defects that demonstrate a similar clinical phenotype. This disorder has been divided into three groups: pccA, pccBC and bio, on the basis of genetic complementation studies of fibroblasts derived from patients with propionic acidemia. Previous evidence has suggested that the defects in each complementation group are caused by a structurally abnormal enzyme. In order to provide additional evidence for delineating these defects in tissues from propionyl CoA carboxylase deficient patients, biochemical characterization of propionyl CoA carboxylase in normal tissues was required. Fibroblast lines and tissues from four patients with propionyl CoA carboxylase deficiency, representing each of the complementation group, were studied using immunologic techniques to demonstrate the presence of equal amounts of cross-reacting material in normal and mutant tissue homogenates. These experiments provided evidence that propionic acidemia is caused by a structural alteration which resulted in a less active enzyme.

In order to further characterize the structural alteration, tissues from patients belonging to one complementation group, pccBC, were studied. One such mutant enzyme from this group was purified and compared to normal enzyme. The findings suggested the presence of multiple mutations within the pccBC group.

Initially, the activity of propionyl CoA carboxylase was determined in four normal human tissues: fibroblasts, liver, kidney and placenta. These determinations provided information about the

distribution of propionyl CoA carboxylase in the various body tissues. As expected, kidney and liver demonstrated the highest specific activity, with liver being the tissue of choice for further study because of the availability of both normal and propionyl CoA carboxylase deficient samples. The distribution of propionyl CoA carboxylase between mitochondria and supernatant fractions was also investigated in several normal livers. The greatest percentage of propionyl CoA carboxylase, as well as glutamate dehydrogenase, a mitochondrial marker enzyme, was found in the supernatant solution; a finding that contradicts information from animal studies in our laboratory, as well as in others, showing that these enzymes are located in mitochondria. It seems likely that the human liver samples had undergone autolysis with time, which caused mitochondrial matrix enzymes to be released into the supernatant fraction. The localization of propionyl CoA carboxylase was studied in porcine liver which was obtained immediately after death. The same phenonenon of release of GDH and PCC into the supernatant fraction was observed over a six hour period. That confirmed the conclusion that the phenomenon was not an artifact of the mitochondrial isolation procedure. The breakdown of mitochondria could also explain the findings of Giorgio and Whitaker (111) who reported that the enzyme isolated from human liver mitochondria showed greatly reduced activity after one hour postmortem. Although propionyl CoA activity was shown to decrease with time postmortem, the enzyme activity was detectable up to eight hours postmortem, in conflict with the observations of Giorgio and Whitaker (111). The release of both GDH and PCC from the

mitochondria was, however, found to occur during this time period. The decrease in PCC activity reported by Giorgio and Whitaker probably represents the release of PCC from the mitochondria which occurs rapidly after death and results in an apparent decrease in the enzyme in isolated mitochondria. Because of the rapid decrease in PCC in liver mitochondria over time, and the time elapsed during the course of obtaining tissue, whole liver homogenates were used for most experiments in this study.

Propionyl CoA carboxylase activity was compared in normal tissue and tissue from patients with propionyl CoA carboxylase deficiency. In liver, placenta and fibroblast tissue obtained from propionic acidemia patients, less than 5% of normal propionyl CoA carboxylase activity was found in homogenates of each tissue type. In agreement with similar findings for fibroblasts by Hsia et al. (81) and Wolf et al. (3) the mutant enzyme from these tissues was generally less stable than the enzyme from normal tissue. The observation that the glutamic dehydrogenase activity in the mutant livers is comparable to that of the controls also indicates that there was no significant autolysis in these livers and, therefore, the dramatic decrease in PCC activity results from the mutant enzyme. The residual PCC activity found in the mutant livers corresponds to the deficiency observed in fibroblasts in which the time required to obtain and process tissue is constant. The activities of the other two mitochondrial carboxylases, pyruvate carboxylase and β-methylcrotonyl CoA carboxylase were more labile in liver than was PCC, and were greatly reduced in most liver samples

obtained one hour postmortem. Both of these enzymes could be stabilized by 2M sucrose and could be assayed in fibroblasts. PCC deficient fibroblast lines demonstrated normal βMCC and PC activity except fibroblasts lines belonging to the bio complementation group had reduced activity of all three mitochondrial carboxylases. The activity of these carboxylases returned to normal when cell culture media was supplemented with 10mg/L biotin.

Further proof was obtained that propionic acidemia is the result of a structural alteration of the enzyme as studied in livers and fibroblast lines from patients with propionic acidemia belonging to the various genetic complementation groups when studied by immunodiffusion and immunotitration. Comparisons between the anti-human carboxylase antiserum and the anti-porcine PCC antiserum demonstrated that the anti-human carboxylase antiserum precipitates the activity of the three carboxylases, whereas the anti-PCC serum inhibits the activity of PCC and, to a minor degree, that of βMCC. This cross-reaction with MCC is not surprising since βMCC is very similar in structure to PCC as demonstrated when both enzymes were purified from bovine kidney (110).

Differences between bovine and human propionyl CoA carboxylase were demonstrated by the Ouchterlony double diffusion technique. Both antisera formed precipitant arcs when diffused against purified pig heart PCC but a spur was observed between the two antisera. The presence of the spur suggests some differences in antigenic determinants between human and porcine PCC. When both antisera were diffused

against a liver homogenate containing activity for the three carboxylases, a second spur signaled the difference between the two antiserums. The non-identity spurs indicate that different antigens were recognized by the anti-human carboxylase antiserum which were not recognized by the anti-porcine PCC antiserum. This spur probably results from the presence of βMCC and PC, which were precipitated by the anti-human carboxylase antiserum and not by the anit-PCC antiserum.

The anti-human carboxylase antiserum demonstrated the presence of CRM in liver extracts of PCC-deficient patients in two ways. First, precipitin arcs were observed signifying identity between the antiserum and normal extracts as well as between the antiserum and mutant extracts. Fluorescense in these arcs, when treated with avidin-fluorascamine, confirmed the presence of biotin-containing protein. The absence of spur formation on double diffusion plates suggested that normal tissue extract has no antigenic sites that are lacking in mutant tissue extract. Second, we demonstrated that the binding of cross-reacting material in mutant extracts to antibodies in the antiserum prevented the inhibition of PCC activity in normal liver extracts. These findings are similar to those observed with the anti-pig heart PCC.

The immunotitration results further confirmed the presence of CRM in mutant liver extracts, but revealed that the same dilution of antiserum inhibits PCC activity in both normal and PCC-deficient liver extracts at equivalent total protein concentrations to the same percentage of initial activity. The immunodiffusion curves obtained for

both mutant extracts closely resembled the curve of normal extract. Thus, the quantities of CRM in each extract, when extract protein concentration is held constant, are approximately equal. The finding presented here, therefore, confirms the findings with pig heart PCC that the two PCCs studied from liver, including one from a patient assigned to the pccC complementation subgroup ($pccC_2$), and one from the pccBC subgroup ($pccBC_1$), represent CRM positive mutants, synthesizing approximately normal quantities of structurally altered PCC.

In addition, this technique demonstrates that heterologous anti-human antiserum produced against various carboxylases, by a simple technique, can also be used to determine CRM in immunotitration techniques where the concentration of other carboxylases are known to be constant. The activity of MCC and PC in fibroblast lines were assayed and were shown to be similar in the cell lines used. Since the protein levels in the liver homogenates are kept constant, the activity of the other carboxylases should constitute a similar percentage of the total protein resulting in similar concentrations in these homogenates. Therefore, even if the antiserum cross-reacts with the other carboxylases, any difference in the amount of cross-reacting protein would correspond to the difference in the concentration of PCC and would shift the equivalence point of the curve. The immunotitration profiles of PCC activity show similar amounts of PCC protein using either antiserum. The fact that these curves correspond to those obtained with the anti-pig heart PCC, demonstrates their value in immunotitration experiments. The results of the immunotitration

experiments using both antisera are consistent with near normal quantities of an inactive enzyme in all three complementation groups, bio, pccBC and pccA. In addition, CRM was demonstrated in all livers by Ouchterlony double diffusion. In the bio group, the inactive enzyme is probably the result of the inability to bind biotin, whereas, in pccA and pccBC, it suggests a structural mutation.

This study has provided further evidence for a structural mutation by biochemically characterizing the normal enzymes and the enzyme from a single mutant complementation group, pccBC. The pccBC group is especially interesting since there exists three subgroups which are designated pccBC, pccC and pccB because of their complementation properties. When pccB cells are fused to pccC cells, there is a slight but reproducible increase in PCC activity which is approximately 10% of that seen in complementation between either group and pccA cells. Previous investigation of the kinetic properties for the small increase in activity during complementation and the similarity in the biochemical properties between pccB and pccC have suggested that this is the result of interallelic complementation (6, 122). The mutants in this study comprised two of these subgroups, pccC and pccBC. The identification of biochemical heterogeneity in the isoelectric point and the thermostability of the enzyme among these mutants in this study suggests that the complementation within this group is interallelic.

Differences were observed in the pI of one mutant enzyme, $pccBC_1$, as compared to normal and other mutant PCCs. The isoelectric point of human PCC was determined to be 4.7 for the normal enzyme from seven

different liver homogenates (133). This was the first determination of the pI for the human enzyme although the pI of 6.1 was reported previously for bovine liver.(80) The investigation of normal enzyme from different subcellular fractions and different tissue showed no normal variant or isoenzyme of PCC. The pI of the enzyme derived from liver mitochondria preparations was compared with the total cell homogenates and these profiles were almost identical providing further evidence that there exists only a single subcellular enzyme which is localized in both fractions due to mitochondrial leakage during liver autolysis. The pI of PCC from liver, kidney, placenta and fibroblast homogenates are in the same range as the normal enzyme from liver.

In addition, the isoelectric points for the other two mitochondrial carboxylases had not been previously reported and they were determined for the purpose of purification of this enzyme. The pI of 6.2 for βMCC, which is structurally similar to PCC, is quite different from that of PCC and this was useful for separation of the two enzymes during the purification procedure by ion exchange chromatography and eliminated the possibility of residual βMCC or PC activity contributing to the determination of mutant PCC activity in the 4.7 peak.

Although other investigators have provided evidence suggesting that the defect in propionic acidemia is due to structural mutations, the first direct evidence was the identification of a mutant enzyme with an altered pI of 5.3 in the $\underline{pccBC}_1$ mutant. From the studies in the normal tissue, it is unlikely that this altered pI represents a

normal variant of PCC. The other three mutants had pI's in the normal range, demonstrating the heterogeneity within the pccBC group. The $pccBC_1$ mutant enzyme and the normal enzyme were purified from liver in order to confirm the finding of an altered pI.

The purification of the normal enzyme was accomplished by a modification of the procedure of Kalousek et al.(83) The extent of purification was comparable to that reported, although the yield was lower. This was probably because only the central regions of the peak were pooled during the column procedures. The SDS-polyacrylamide gel showed three bands with molecular weights of 58,500, 62,500 and 76,000. The location of the 76,000 and 62,500 molecular weight bands corresponded to the bands obtained with a sample of purified pig heart PCC (61,500 and 76,000). These two bands are of equal intensity which would be expected for two subunits which are in equal proportions in the enzyme molecule. The smaller band may be either a contaminant or a degradation product of one of the PCC subunits similar to that observed in purified bovine kidney PCC, since it became more prominent as the preparation aged. These molecular weight values are similar to those obtained by Swack et al. (167) for chicken PCC and Kalousek et al. (83) for human PCC.

The strongest evidence in favor of a structurally altered mutant enzyme was provided by the purified mutant enzyme. The enzyme from a PCC-deficient human liver has not been previously purified because of the difficulty in obtaining enough material and the instability of the enzyme. The purification of the mutant was only accomplished by

conducting most procedures in 0.5M sucrose which had been shown previously to stabilize the partially purified mutant enzyme from fibroblasts.(81) The dramatic stabilization effect of sucrose on this mutant was demonstrated in the heat denaturation experiments where the stability of the mutant becomes comparable to normal with the addition of 0.5M sucrose. The presence of 5mM ATP slightly decreased the stability of the mutant in the thermostability experiments and decreased its stability during the purification procedure when 3mM ATP was used to elute the enzyme from the Blue Sepharose column. However, the addition of sucrose to the 3mM ATP solution stabilized the enzyme, even in the presence of ATP. The specific activity of the mutant is approximately 1000 times less than that of the purified normal enzyme, indicating the mutant enzyme is structurally altered, since a regulatory mutant would result in low yields but a similar specific activity to the normal enzyme. The low specific activity is due to the instability of the enzyme during the isolation procedures, especially the DEAE column step where sucrose had to be omitted. The purifications of the mutant and the normal enzyme were comparable beyond this step with the mutant being purified 90-fold over the next two steps and the normal 150-fold. These data suggested that the mutant enzyme actually was purified around 1500-fold like the normal enzyme, but the fold increase was obscured by loss of activity.

The isoelectric points in the purified enzymes supported the data obtained with the crude homogenates. The pI shift, during the purification reported by Kalousek et al. (83), was not observed in either

purified enzyme. A shift approximating that reported could be produced in both purified and crude enzyme preparations by preincubating with 5mM ATP. Since the shift occurs after the elution from the Blue Sepharose column with 3mM ATP and a proportion of their purified enzyme still focuses at the lower pI, this observation is consistent with the binding of ATP to the majority of the enzyme, causing a pI shift. The reason why a shift is not observed in our preparation is probably due to the higher salt concentrations employed during the Sephacryl column procedure which would minimize the enzyme-ATP interaction.

Several possible explanations for the altered pI in the $\underline{pccBC}_1$ enzyme can be invoked. The pI of the $\underline{pccBC}_1$ mutant enzyme is shifted approximately 0.6 of pH units which probably cannot be explained by one charge difference. Since PCC is a tetramer, any charge difference would occur in all four subunits causing a four-fold effect on the pI of the enzyme. One obvious explanation for a charge difference would be the substitution of an amino acid in the polypeptide chain of the subunit. Such a substitution could either alter the pI directly by changing the charge of the molocule or indirectly by altering the conformation of the enzyme. A substitution in the amino acid sequence could alter the post-translational modification of the enzyme, possibly causing the addition or deletion of charged moiety. Also, a substitution could cause local conformational changes causing exposure of previously internalized groups. The data on sucrose stabilization has already suggested that such a change may occur in this mutant.

Several or all of these phenomenon may interact to alter the pI of this enzyme.

Another explanation for the altered pI is the tight binding of some ligand such as ATP to the mutant enzyme. The presence of 5mM ATP has been shown to shift the pI of the mutant enzyme indicating that it is not responsible for the pI alteration originally observed in this mutant. In addition, the Km of the purified $\underline{pccBC}_1$ mutant enzyme for ATP is not different from that obtained for the normal enzyme which suggests similar affinity for ATP. The presence of some other tightly bound ligand remains a possibility.

Comparisons of the property of thermostability also demonstrated differences between the mutant enzymes. Two of the four mutant enzymes, $\underline{pccC}_2$ and $\underline{pccBC}_1$, had a half-life of approximately 3 minutes at 45°C, whereas, the other two mutants, $\underline{pccC}_1$ and $\underline{pccBC}_2$, demonstrated half-lifes of over 30 minutes, which is within the normal range. The dramatic stabilization of the $\underline{pccBC}_1$ mutant by sucrose may give a clue to the nature of the defect in this mutant and suggests a defect in the outer structure of the molecule which is involved in hydrophobic interactions. Sucrose, at the concentrations required to stabilize this enzyme, has been shown to strengthen hydrophobic interactions between groups and reduces the driving force for the transfer of a hydrophobic group to a non-polar environment.(134) From the data, a substitution of a hydrophobic group on the outside of the molecule might be suggested.

The Km values for propionyl CoA, ATP, bicarbonate, and potassium

were compared in the normal and the mutant enzymes and no major differences were found. The lack of large differences in the Km's for the enzyme substrates similar to that observed in studies of fibroblast, suggests the defect in these enzymes does not interfere with substrate binding.(3, 82) The Km values calculated for the purified normal enzyme is within the range reported for mammalian PCC by other investigators. Surprisingly, none of the mutants showed a deviation from these values of more than 6-fold. The Km for propionyl CoA in the pccBC$_2$ mutant which is approximately four times normal is still less than the Km calculated for normal human fibroblast extracts by Hsia et al. (81) and is probably not significant. The crude cell homogenates gave a good approximation of the values determined in the purified normal enzyme although more variation was observed among the Km values from the homogenates. In the study by Wolf et al. (3), fibroblasts were separated into different complementation groups; the pccA group demonstrated an increased Km for potassium but the pccBC group was the same as the normal. Their finding in the pccBC group was confirmed in the mutants studied.

This study has demonstrated the heterogeneity within one mutant complementation group, pccBC. All of the enzymes demonstrated normal quantities of CRM and Km values for substrates. Mutant pccBC$_1$ had an altered pI on abnormal heat denaturation profile, and was not stabilized by ATP. Mutant pccC$_2$ had a similar heat denaturation profile but a normal pI. The demonstration of three different patterns in these mutants suggests that each represents a different structural mutation

within a single subunit. Since mutants $\underline{pccC}_1$ and $\underline{pccC}_2$ comprise one subgroup and $\underline{pccBC}_1$ and $\underline{pccBC}_2$ another, there is no pattern of behavior common to any one subgroup. If there exists many possible structural mutations within a complementation group, some mutations may interfere with the ability to participate in interallelic complementation by interfering with stable tetrameric form. The varied ability of cells within the pccBC complementation group to participate in complementing one another may be just another example of heterogeneity within the pccBC group.

The heterogeneity observed within this complementation group parallels the clinical heterogeneity observed in this disorder. No difference in severity of the disorder has ever been observed between the two major complementation groups and great heterogeneity has been observed within the groups.( ) Of course, no conclusions can be reached with such a small sample; however, it is interesting to note that the $\underline{pccBC}_2$ placenta is the only one of the samples from a patient still living with PCC deficiency and is one of the two samples which did not show any differences from normal in the parameters tested. The patient whose liver enzyme ($pccBC_1$) was deviant in these tests, died at the youngest age of those in this study. It is possible that in the future, parameters such as the ones used in this study, could be used to predict the clinical course for these patients.

Alteration of enzyme stability, if it occurs in vivo, would represent a severe problem to these patients. However, the discovery that other molecules influence the stability of this enzyme suggests

another possible approach to treatment of this disorder. Investigations are needed to elucidate substances which would increase the stability of the enzyme. It is possible that a substance could be found which could be used to increase the stability of mutant PCC leading to a treatment for this disorder.

In summary, the data presented demonstrated that the defect in mutant pccBC$_2$ is the result of a structural mutation which is stabilized by the addition of 0.5M sucrose. Additionally, the immunotitration experiments support the hypothesis that the defect in the other mutants studied from the other two complementation groups are also the results of a structural mutation. The finding that none of the binding constants for the pccBC$_2$ mutant were altered and that sucrose stabilizes the enzyme, hints that the defect involves the hydrophobic outer area of the molecule rather than the substrate binding sites.

The demonstration of biochemical heterogeneity in the pccBC group suggests that this group represents a collection of different structural mutations. If different mutations result in slightly different properties, the interallelic complementation observed in some of these mutants may be interfered with in other mutants such as those comprising the pccBC subgroup.

Clearly, this study is only a beginning in the understanding of the molecular defect in propionic acidemia. Many questions on the relationship between the biochemical and clinical heterogeneity in this disorder remain to be answered. Hopefully, a better understanding of the nature of the defect will initiate innovations in the

treatment of propionic acidemia which, at present, is so devastating to the affected children and their families.

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APPENDIX I

CONSENT FORM AND LIVER INFORMATION SHEET

MEDICAL COLLEGE OF VIRGINIA
VIRGINIA COMMONWEALTH UNIVERSITY
Richmond, Virginia

RELEASE OF ORGANS AT POSTMORTEM EXAMINATION

I, _____, as the parent and/or guardian of _____, give permission to Dr. _____ to remove any organs necessary for medical investigation at the postmortem examination of my child. This permission and authority is granted subject to the following restrictions:_____

_____

_____

Physician:_____ Signed:_____
(Signature of physician obtaining permission)

Signed:_____

Date:_____

House and attending staff to be called:

Dr._____ Phone:_____

Dr._____ Phone:_____

Human Autopsy Liver: Identification No.:_____

Date of Birth: / / Gestational Age:___wks. Sex: M F

Mode of Delivery: V C-S Birthweight:_____gms. Race: C N M

Maternal Complications:

Birth Complications:

Date of Death: / / Time of Death:_____a.m. p.m.

Body Weight at Death:_____gms

Postmortem handling of body:

Time at which liver removed
and iced down:_____a.m./p.m.: Date: / /

Cause of Death:

Therapy preceding death:

Assisted Ventilation: Yes No O_2 Therapy:_____%

Hyperalimentation: Yes No

Hyperalimentation fluid:

I.V. Fluids:

Medical Therapy:

Anticonvulsants:

Antimicrobial Agents:

Diuretics:

Cardiovascular Agents:

Hormones:

Other:

Abnormal Findings at Autopsy:

Abnormal Findings at Autopsy (Cont'd.)

Liver Studies:

Weight at Autopsy: _____ g.
Appearance:
Histology:
Light Microscopy:

Electron Microscopy:

Biochemical Findings:
Protein Content: _____ mg./g. liver

Protein Distribution:
Nuclear Fraction: _____ mg./g. liver
Mitochondrial Fraction: _____ mg./g. liver
Microsomal Fraction: _____ mg./g. liver
Soluble Fraction: _____ mg./g. liver

Bile Acids:

Enzyme Content:	Total	Mitochondrial	Microsomal	Cytosol	Nucleus
Branched-Chain α-Ketoacid Dehyd.					
Propionyl CoA Carboxylase					
Glutamic Dehydrogenase					
Acid Phosphatase					
Glucose-6-Phosphatase					

APPENDIX II

ENZYME ASSAYS

a) Propionyl CoA Carboxylase

b) β-Methylcrotonyl CoA carboxylase

c) Pyruvate Carboxylase

d) Glutamic Dehydrogenase

e) Lowry Protein Determination

The Assay for Propionyl CoA carboxylase, β-Methylcrontonyl CoA carboxylase, and Pyruvate Carboxylase

A. Reagents

1. Magnesium Chloride, Analytical Reagent
2. Sodium Bicarbonate, Analytical Reagent
3. Potassium Chloride, Analytical Reagent
4. Glutathione, Reduced form, MW = 307.3; Sigma Chemical Co., Grade III, cat. # G4501.
5. Adenosine-5'-triphospnate, disodium salt, MW = 551.2; Sigma Chemical Co., cat. # A3127.
6. Tris base, MW - 121.1; Calbiochem, grade A, metal ion free, cat. # 648311.
7. Ethylenediaminetetraacetic acid, disodium salt, MW = 336.2 Sigma Chemical Co. cat. # ED2SS.
8. Propionyl CoA, MW = 823.6; Sigma Chemical Co., Grade II, lithium salt, cat. # P4386.
9. β-Methylcrotonyl CoA, lithium salt, MW = 849.7(free acid); Sigma Chemical Co., cat. # M9507.
10. Pyruvic acid, sodium salt, MW = 110.1; Sigma Chemical Co., cat. # P2256.
11. Acetyl CoA.
12. Trichloroacetic acid, Analytical Reagent.
13. $NaH^{14}CO_3$, 40-60 mCi/mmole, MW = 84.0; New England Nuclear, cat. # NEC-086H.

B. Solutions

1. 40mM ATP

0.119 gm ATP-Na_2 was dissolved in 3.0 ml of deionized water, titrated to pH 8.0 with NaOH and made to a final volume of 5.0 ml. 0.1 ml of this solution was diluted to 100 ml in deionized water and the absorbance of the final solution was determined through a 1.0 cm light path at 259nm. The concentration of the initial solution was determined using a millimolar absorption coefficient for ATP of 15.4. The concentration was adjusted to 0.040M.

2. 10mM Propionyl CoA

0.01 gm of propionyl CoA was dissolved in 0.5 ml of deionized water. The final concentration was determined by using a millimolar absorption coefficient of 16 at a wavelength of 260nm through a 1.0 cm lightpath.

or 30MM β-Methylcrotonyl CoA

0.003 gm of -methylcrotonyl CoA was dissolved in 0.1ml deionized water and standardized as above.

or a) 60mM Pyruvate

0.33gm pyruvate was dissolved in 5 ml of deionized water.

b) 15mM Acetyl CoA

3. 200mM $MgCl_2$

4.066 gm of $MgCl_2$-$6H_2O$ was dissolved and brought to a final volume of 100ml.

4. 2.5M KCl

18.6 gm of KCl was dissolved and brought up to a final volume of 100 ml.

5. 1M Tris-Hcl, pH 8.0, 100mM glutathione, 0.1mM EDTA.

a) 1M Tris-HCl
12.1 gm of Tris was brought up to a volume of 50 ml and pH with HCl to 8.0.
b) 3.07 gm of glutathione were added to the solution.
c) 0.0037 gm EDTA were added and the final volume was brought up to 100ml.

6. 50mM Sodium Bicarbonate - ^{14}C- labelled

   a) 100mM Sodium Bicarbonate
   0.084 gm of Sodium bicarbonate was dissolved and made up to a final volume of 10ml.

   b) One ml of ^{14}C labelled bicarbonate containing 1 mCi was diluted with 0.64 ml of the 100mM sodium bicarbonate solution.

7. 10% Trichloroacetic acid

   10 gm of Trichloroacetic acid were made up to a final volume of 100ml with deionized water.

8. Reaction Mixture
   0.5 ml of solutions 1, 3, 4 and 5 are mixed together the day of the assay.

C. Assay System

Blank

10ul of deionized water or 5ul of deionized water and 5 ul of acetyl CoA (for pyruvate carboxylase)

20ul of reaction mixture
50 ul of sample
20 ul of bicarbonate is added to begin the assay

Complete system

10 ul of either propionyl CoA or -methylcrotonyl CoA or 5 ul of acetyl CoA and 5 ul of pyruvate for pyruvate carboxylase
20 ul of reaction mixture
50 ul of sample
20 ul of bicarbonate to begin assay

The first three reagents are mixed together and the samples are incubated at 37°C for 2 minutes in a water bath. The reaction is begun by the addition of the labelled bicarbonate and is allowed to proceed for up to 30 minutes. To stop assay, 50 ul of 10% TCA is added. 100ul alliquot is removed from each and evaporated to dryness under a heat lamp to volitalize any unreacted $^{14}CO2$. The residue was solublized in 100 ul of deionized water and dissolved in 4.0 ml of Aqua-sol and counted in a Beckman LS7500 scintillation counter.

Analysis of Supernatants of Mitochondrial Sonicates for Glutamic Dehydrogenase:

1. Reagents

   A. Triethanolamine: Sigma, cat. no. 7-1377, lot no. 120-2290, mol. wt. = 149.2

   B. EDTA: Sigma, cat. no. ED, lot no. 36B 0950, mol. wt. (free acid) = 292.25

   C. Ammonium Acetate: Fisher, A.C.S., cat. no. 1-637, mol. wt. = 77.08

   D. -NADH: Sigma, cat. no. N8129, lot no. , disodium salt with 1.5 moles water/mole, mol. wt. = 736.02

   E. ADP, monosodium salt: Sigma, cat. no. A-0127, 19t no. mol. wt. =

   F. ∝-Ketoglutaric acid, monosodium salt: Sigma, cat. no. K1875, lot no. , mol. wt. = 168.1.

2. Solutions

   A. 0.067 M Triethanolamine-5.2mM EDTA, pH 8.0:*

      0.99964g. triethanolamine-0.15197 g. EDTA (free acid) dissolved in 85 ml. water, then titrated to pH 8.0 at 25°C and made to a final volume of 100 ml. Store frozen when not in use.

   B. 2.1 M Ammonium Acetate

      1.6187 g. Ammonium acetate dissolved in deionized water and made to a final volume of 10.0 ml. Store frozen when not in use.

   C. 1 mM NADH:

      Approximately 0.00585 g. NADH-Na_2-1.5H_2O dissolved in 5.0 ml. water. 0.1 ml. of this diluted with 1.9 ml of deionized water. The absorbance of the resulting dilution determined at 340 nm through a 1.0 cm. light path. O.D. = .365. Calculated conc'n. of the undiluted solution = mM. The concentration of the undiluted original solution adjusted to 1.0 mM. Prepare fresh daily.

D. 12 mM ADP:

0.02736 g. monosodium ADP dissolved in 2.0 ml of water, titrated to pH 6.8, and made to a final volume of 5.0 ml.

E. 0.16M ∝-Ketoglutarate:

0.13448 g. of the monosodium salt of -ketoglutarate is dissolved in 4.0 ml of deionized water. The solution is titrated to pH 7.0 with NaOH.

Adjust the final volume to 5.0 ml. Store at -20°C.

3. Glutamic Dehydrogenase Assay:

0.5 ml of 0.067M triethanolamine-5.2 mM EDTA, pH 8.0
0.05 ml of 2.1M ammonium acetate
0.10 ml deionized water
0.15 ml of 1.0 mM NADH
0.05 ml of 12 mM ADP

0.05 ml of enzyme-containing solution (the mitochondrial supernatant from a mitochondrial suspension containing approximately 10 mg of protein/ml is diluted 1:4 with ice-cold deionized water immediately before assay). Following addition of enzyme, the change of optical density at 340 nm is followed for 1-2 minutes at 25°C., then 0.05 ml of 0.16M sodium ∝-ketoglutarate is added and the recording continued. Results are recorded as initial ratios of absorbance change at 340 nm through a 1.0 cm light path at 25°C. Full scale absorbance for the instrument is set for 0.200.

In order to insure complete rupture of the mitochondria in order to release glutamic dehydrogenase, the triethanolamine-EDTA reagent is probably best made containing approximately 0.1% Triton X-100. This is best made with Packard purified Triton X-100 on the day of use. Dilute 0.01 ml of Triton X-100 with triethanolamine-EDTA reagent to a final volume of 10.0 ml. Without triton X-100, dilutions of mitochondrial preparations when added to the assay system show non-linear rates of reaction with a steady declining rate of reaction as a function of time.

When triton X-100 is present, the rate of reaction remains linear.

Measurement of Protein Concentrations in Tissue Homogenates and Tissue Extracts:

A. Reagents

1. $CuSo_4$-$5H_2O$, mol. wt. = 249.68; Fisher Certified, A.C.S.
2. Sodium Potassium Tartrate, $NaKC_4H_4O_6$. mol. wt. = 282.23; Fisher Certified, A.C.S.
3. Sodium Hydroxide, mol. wt. = 40.00; Fisher Certified A.C.S.
4. Na_2CO_3, anhydrous, mol. wt. = 105.99; Fisher Certified, A.C.S.
5. Folin-Ciocalteau Reagent; Fisher Scientific Co.
6. Bovine Serum Albumin, crystallized and lyophilized; Sigma Chemical Co.
7. Phosphorus Pentoxide, mol. wt. = 141.95; Fisher Scientific

B. Solutions

1. Lowry Method for Protein Determination

Reagent A:

20g. of anhydrous Na_2CO_3 dissolved and made to 1000 ml in 0.1N NaOH.

Reagent B:

This is prepared by mixing equal volumes of 1 $CuSO_4$ $5H_2O$ (1g. of $CuSO_4.5H_2O$ dissolved and made to 100 ml in water) with 2 sodium potassium tartrate. (2.0g. of $NaKC_4H_4O_6.$ $4H_2O$ dissolved and made to 100 ml with water)

Reagent C:

Mix 50 ml of reagent A with 1 ml of reagent B. Use freshly made only.

Reagent D:

Folin-Ciocalteu Reagent (Phenol Reagent)
The normality of the commercial preparation is determined by titration using standardized HCl. Prior to use, the Folin-Ciocalteu Reagent is diluted to 1N.

2. Preparation of Standard Protein

A stoppered weighing bottle is dried to constant weight over P_2O_5 in an evacuated desiccator at room temperature.

Then add 0.8g. of purified, lyophilized bovine serum albumin and dry it to constant weight in the desiccator. Final stable weight is dissolved in 10 ml water and 1.0 ml samples stored in vials at -20°. Each 1.0 ml diluted to 10.0 ml with water and the resultant used for a standard in the biuret assay.

C. Analytical Methods

0.2 ml of tissue homogenate or tissue extract, containing 2-40 ug protein, is mixed with 1 ml of Lowry Protein Reagent C and allowed to interact for 10 min. at room temperature. Add 0.1 ml of Lowry Protein Reagent D to the above mixture, shake immediately and wait for 30 min. before reading the final color. The absorbance of the final mixture is determined either at 750 nm or at 500 nm. Color development is compared against that yielded by bovine albumin standard.

When it is desirable to determine the protein content on protein precipitates, these are first dissolved in 0.1N NaOH before a sample is taken for analysis. In some cases it may be necessary to heat the protein in 0.1 N NaOH to effect solution.

The presence of uric acid, guonine, xonthine, sucrose, monosaccharides, hexosamine in significant quantities may interfere with the accuracy of the method. The presence of glycine (0.5) can cause a decrease in color by 50 ; hydrazine at greater than 0.5mg/10ml interferes. Phenols (except nitrophenols) reduce the reagent and thereby cause interference. Ammonium sulfate (at a concentration greater than 0.15), sulfosalicylic acid and thymol may interfere. The presence of inorganic phosphate in the tissue extract or homogenate causes precipitation of the molybdate reagent.

D. References

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APPENDIX III

AVIDIN ASSAY

Appendix III - Avidin Assay

Standard Solutions

1. Biotin standard - 5mg. d-biotin (Sigma) in 5ml of 0.15M sodium phosphate buffer, pH7.0. This stock solution is diluted 1:50 (0.1 ml in 5ml) to a concentration of 20ug/ml for the assay.

2. Avidin standard - 10ug of avidin (Sigma) is dissolved in 0.15m sodium phosphate buffer, pH 7.0. This solution is standardized spectrophotometrically at 280 nm.

Procedure

With the exicitation monochromator set at 290nm, using a bandwidth of 4nm, the fluorescence is monitored at 350nm with a bandwidth of 16nm. For standard, 2ml of avidin solution is pipetted into the cuvette and fluorescence decrease is monitored after the addition of 20ul aliquots of the biotin standard solution. The point where no further decrease in fluorescence is observed is the point of equivalence between avidin-biotin bind. Unknown avidin solutions can be titrated in the same manner and compared to the standard.

Lin, H. J. and J. F. Kirsch. Anal. Biochem. 81:442-446, 1977. (126)

APPENDIX IV

OUCHTERLONY DOUBLE DIFFUSION

Ouchterlony Double Diffusion

Reagents	Grams
1. Barbitol MW 184.2; Fisher Co.	3.44
2. Sodium Barbitol MW 206.2; Fisher	7.57
3. Noble Agar; Difco	10
4. Sodium Azide MW 65.0; Fisher	0.1

Procedure

Dissolve barbitol and sodium barbitol in 900 ml deionized water. Add agar and heat mixture in an earlmyer flask while swirling constantly over a bunsen burner until boiling. Heat gently until all of the agar has dissolved. Add the sodium azide and bring up to 1000 ml. Let cool until luke warm and pour 10 ml in 5cm in diameter petri dishes. After these cool, refrigerate overnight. Cut wells for antibody and antigen with template and fill wells with 100ul of sample and let diffuse for 24 hours.

APPENDIX V

SDS-polyacrylamide Gel Electrophoresis

SDS-Arcylamide Gel Electrophoresis

I. Reagents

A. Glacial Acetic Acid, mol. wt. = 60.05, Reagent Grade
B. Methanol, Reagent Grade
C. Acrylamide, mol. wt. 71.08, electrophoretic grade, Bio-Rad Laboratories, Cat. No. 161-0100
D. N,N'-methylene bisacrylamide, mol. wt. 154.2, electrophoretic grade, Bio-Rad Laboratories, Cat. No. 161-0200.
E. N,N,N',N'-tetramethylethylene diamine, mol. wt. 116.21, Eastman Organic Chemicals, Cat. No. 8178
F. Ammonium Persulfate, mol. wt. 228.2 electrophoretic grade, Bio-Rad Laboratories, Cat. No. 161-0700
G. Sodium dodecyl sulfate, mol. wt. 288.4, Sigma Chemical Co., Cat. No. L-5750
H. Coomassie Brilliant Blue R, Sigma Chemical Co., Cat. No. B-0630
I. Bromthymol Blue, Allied Chemical Co., Cat. No. 241
J.
K.
L. β-Mercaptoethanol mol. wt. 78.13, Sigma Chemical Co., Cat. No. M6250
M. Tris base, mol. wt. 121.1 calbiochem grade A metal ion free, Cat. No. 648311
N. Glycine MW75.07, Reagent grade

II. Solutions:

A. Acrylamide - N,N'-Methylene Bisacrylamide:

20.0g. Acrylamide + 0.667g. N,N'-Methylene bisacrylamide dissolved and made to a final volume of 50 ml in deionized water. Store this solution in a brown bottle at 0-2°. Note: This mixture is unstable and tends to polymerize - prevent exposure to light as much as possible. Do not use this solution after one month of storage. Warm up the bottles to room temperature prior to opening them. These compounds are neurotoxins - avoid excessive exposure to them.

B. Ammonium Persulfate Solution:

0.56g. of $(NH_4)_2S_2O_8$ is dissolved and made to a final volume of 10ml in deionized water. Make fresh just before use.

C. N,N,N',N'-Tetramethylethylene diamine Solution:

0.092ml of n,n,n',n',-tetramethylethylene diamine is dissolved and made to a final volume of 10.0ml. in deionized water. Make fresh before use.

D. Tris-Glycine Buffer: For Gel Preparation (0.25M Glycine). 3.7535g. Glycine dissolved in 180ml of deionized water and the pH of the solution is adjusted to 8.3 with tris 0.4 gm of SDS is added and final volume is made to 200ml with deionized water.

E. Tris-Glycine Buffer: For Electrode Compartment 37.535g. Glycine is dissolved in 970ml of deionized water and the pH is adjusted to 8.3 with tris. Following the pH adjustment, the volume is made to 1000ml. Store at 0-2°C. For use as the electrode solution it is diluted 1:5 with deionized water so 300 mls is made up to 1400ml per compartment and 15gm SDS is added.

F. Protein Carrier Solution:

502 of Mercaptoethanol mixed with 1.0ml of Solution E.

G. Dissolution of Protein for Electrophoresis:

Up to 100mg of protein dissolved in 0.10ml of Solution F.

H. Marker Solution.

2.0mg of Bromthymol blue (Bromphenol blue may also be used) dissolved in solution E. This solution can be used in place of Solution E to make Solution F.

I. Methanol-Acetic Acid Staining Solution:

0.125g coomassie blue dissolved in 113.5ml of 50% methanol and the final volume adjusted to 125ml by addition of glacial acetic acid.

J. Destaining Solution:

10ml of Glacial Acetic Acid diluted to 250ml with deionized water and 250ml Methanol.

III. Gel Preparation:

Gel%	ml. Sol'n. D	ml. sol'n. A	ml. H_2O	ml. sol'n. C	ml. sol'n. B
7.5	40	15	15	5	5

The solutions (D through C) are mixed in a vacuum flask and degassed 10 min. Then Solution B added, immediately mixed and the resulting solution used immediately to fill gel mold.

IV. Electrophoresis of the Protein Mixture

The protein sample was dissolved in the Tris-HCl, Mercaptoethanol, SDS mixture and boiled for 3 minutes. Fifteen ul of sample is placed in each of the preformed slots. Up to 100 ug of protein can be run in each lane; however, for the most accurate determination only 10ug should be run. The gel is run at constant current of 80mA while being cooled by circulating water at 15°C, for approximately three hours until the tracking dye reaches the end of the gel.

V. Staining the Gel

The gel is placed in 10% TCA for 1 hour and is then stained in the solution I overnight. The staining solution is then poured off and the destaining solution is added. It requires several changes of destaining solution to remove all of the stain from the gel.

APPENDIX VI

ISOELECTRIC FOCUSING

Required Reagents:

Solution D: Dense Solution: 1.5ml of 40% Ampholine (pH 3-10)+ 28c. sucrose + glass distilled water to a final volume of 60ml.

Solution L: Light Solution: 1.5ml of 40% Ampholine (pH 3-10)+ water to a final volume of 60ml.

Anode Solution: 1% H_2SO_4 in dense sucrose.
0.2mT of concentrated sulfuric acid (98%) added to 14.0ml of water. 14.0g of sucrose dissolved in the acidic solution.

Cathode Solution: 0.1g. NaOH + 10.0ml distilled water - to give a 1% solution.

Preparation of the Sucrose Ampholyte Solutions Needed to Prepare the Sucrose Gradient:

Tube #	ml. Solution D	ml. Solution L	Tube #	ml. Solution D	ml. Solution L
1	4.6	-	13	2.2	2.4
2	4.4	0.2	14	2.0	2.6
3	4.2	0.4	15	1.8	2.8
4	4.0	0.6	16	1.6	3.0
5	3.8	0.8	17	1.4	3.2
6	3.6	1.0	18	1.2	3.4
7	3.4	1.2	19	1.0	3.6
8	3.2	1.4	20	0.8	3.8
9	3.0	1.6	21	0.6	4.0
10	2.8	1.8	22	0.4	4.2
11	2.6	2.0	23	0.2	4.4
12	2.4	2.2	24	-	4.6

Preparation of Collection Tubes:

60 small glass tubes (12 x 100mm.) are calibrated (with 2.0ml of water) so that they can be used to collect 2.0ml fractions after the isoelectric focusing run.

When desirable, mercaptoethanol may be added to Solutions D and L to provide stability for certain enzymes. To make these solutions approximately 1mM with respect to mercaptoethanol, add 4.2 of mercaptoethanol /60ml.