

## LACTIC ACIDEMIA

### I. Background

Several inborn errors of metabolism are characterized by a metabolic acidosis in which the major anionic species is lactate. The lactic acid that circulates in the human body is a product of the anaerobic metabolism of glucose that occurs primarily in the red cells, in kidney medulla and white skeletal muscle. Some of the lactate is oxidized by red muscle and the kidney cortex, but the bulk of it is taken up by the liver and converted to glucose or oxidized.

The only metabolic reaction which produces lactate is the NADH<sub>2</sub>-dependent reduction of pyruvate which is catalyzed by lactate dehydrogenase, and lactate is always removed by a reversal of this reaction. Therefore, any condition which results in the accumulation of pyruvate may lead to inadequate removal of both pyruvate and lactate from the circulation with consequent lactic acidemia. The metabolic fates of pyruvate are shown in the Figure below.

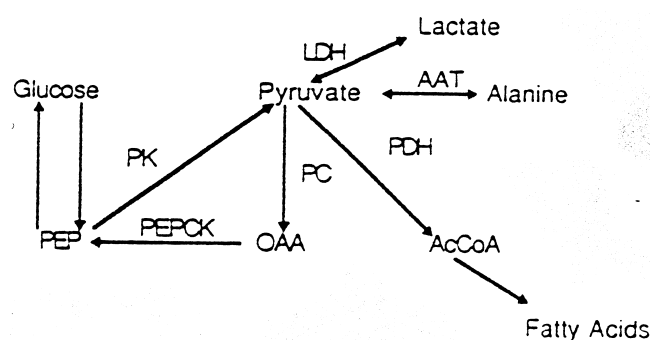


Fig. 1. The metabolic fates of pyruvate. Pyruvate can be converted to phosphoenolpyruvate (PEP), oxaloacetate (OAA), acetyl coenzyme A (AcCoA), alanine or lactate. The enzymes involved are pyruvate kinase (PK) phosphoenolpyruvate carboxykinase (PEPCK), pyruvate dehydrogenase (PDH), pyruvate carboxylase (PC) alanine aminotransferase (AAT), and lactate dehydrogenase (LDH).

The oxidative metabolism of pyruvate proceeds through pyruvate dehydrogenase, the Krebs cycle and the respiratory chain, whereas the anabolic utilization of pyruvate proceeds primarily through the pyruvate carboxylase reaction. Normally, only a small amount of the total pyruvate is transaminated to alanine. A defect in any of the pathways of pyruvate utilization may lead to the accumulation of pyruvate and lactate. A number of vitamin deficiencies have also been shown to result in lactic acidemia.

The most common of the disorders leading to lactic acidemia is a deficiency of the pyruvate dehydrogenase complex. Genetic defects in the pyruvate dehydrogenase complex (PDC) are associated with a range of clinical abnormalities. The most striking disorders occur in the nervous system, although impaired development and poor function of other tissues, including muscle and liver, do occur. The brain, unlike other organs, normally produces ATP almost exclusively from glucose oxidation via glycolysis and tricarboxylic acid cycle. Blockage of this process at the step of pyruvate oxidation catalyzed by PDC results in ATP deprivation and damage to the brain and other

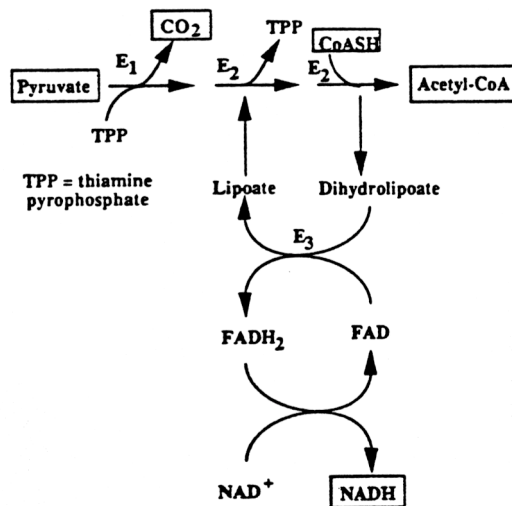
tissues in patients with moderate and severe deficiencies of PDC activity. Currently; there is no effective treatment, and infants with severe PDC deficiency survive for only two to three years at most. Less severe deficiencies in PDC activity can result in decreased production of acetyl CoA in brain. This, in turn, leads to decreased synthesis of the neurotransmitter acetylcholine which may be a factor contributing to the poorly coordinated movements seen in patients with mild PDC deficiency.

## II. Deficiencies of the pyruvate dehydrogenase complex (PDC)

PDC is a multienzyme complex consisting of three different enzymes (E1, E2 and E3) and requiring five different cofactors and coenzymes. The overall reaction catalyzed by the PDC is

$$\text{pyruvate} + \text{NAD} + \text{CoASH} \rightarrow \text{Acetyl CoA} + \text{NADH}_2 + \text{CO}_2$$

The activities of the individual components of the complex are illustrated in the figure below.



Deficiencies of the pyruvate dehydrogenase complex have been shown to result from inherited defects in the E1, pyruvate decarboxylase and the E2, lipoyl transacetylase components or from an acquired defect in one of the vitamins which serves as a precursor to the cofactors and coenzymes required by PDC. The E3 (dihydrolipoyl dehydrogenase) is also a component of the alpha-ketoglutarate dehydrogenase complex, the branched chain alpha-ketoacid dehydrogenase complex and the glycine cleavage system. Infants with deficiencies in dihydrolipoyl dehydrogenase have very serious medical problems and rarely survive more than a few months. Deficiencies in pyruvate dehydrogenase phosphatase, the enzyme catalyzing the dephosphorylation and consequent activation of pyruvate dehydrogenase, have also been reported. Fibroblasts obtained from skin biopsies and grown in tissue culture for several generations have been widely used to assay PDC activity in patients suspected of having PDC defects.

Case report 1.

A full term infant male weighing 2.9 kg was born without complications, In the neonatal period he failed to gain weight and had episodes of vomiting and metabolic acidosis. At eight months of age he was diagnosed with renal tubular acidosis. Physical examination at that time showed marked failure to thrive (4.1 kg), hypotonia, small muscle mass and poor coordination movement. A computer tomography scan of the head revealed moderate atrophy of the cerebral cortex. Severe auditory and optic atrophy were present.

Laboratory studies revealed persistent acidosis (pH 7.0 to 7.2), with blood lactic acid concentration of 9.0 mM (normal, <2.2 mM), pyruvic acid of 2.4 mM (normal, <0.7 mM), and alanine of 1.4 mM (normal = 0.09 - 0.31 mM). Treatment with thiamine (600 mg/day) and biotin (10 mg/day) for four weeks had no effect on either the clinical state or the blood lactate and pyruvate values. Serum bicarbonate levels were below 20 mM (normal, 25-26 mM), and remained low despite daily therapy with 4.9 g of oral sodium bicarbonate. Analysis of urine samples for organic acids by gas chromatography - mass spectrometry revealed high concentrations of lactate, 2-hydroxybutyrate, 3-hydroxyisovalerate and either 2-hydroxyglutarate or 2-ketoglutarate. These compounds are not detected in normal urine.

Extracts from fibroblasts were assayed for PDC activity, alpha-KGDH complex activity, and lipoamide dehydrogenase (LAD) activity. The results of these assays are shown in the table below.

**Table I. Enzyme activities and kinetic data in cultured skin fibroblasts**

	<i>PDHC</i> ( $\mu\text{mol}/\text{min}/\text{mg protein}$ )	$\alpha$ - <i>KGDHC</i> ( $\mu\text{mol}/\text{min}/\text{mg protein}$ )	<i>LAD</i> ( $\text{nmol}/\text{min}/\text{mg protein}$ )	<i>LAD Km</i> ( $\text{mM lipoamide}$ )
Normal ( $n = 20$ )	204 $\pm$ 25 (80 to 504)	109 $\pm$ 11 (45 to 207)	62 $\pm$ 7 ( $n = 20$ ) (32 to 91)	1.41 $\pm$ 0.10 ( $n = 4$ ) (1.2 to 1.7)
Abnormal controls* ( $n = 26$ )	156 $\pm$ 17 (49 to 383)	107 $\pm$ 9 (4 to 266)	59 $\pm$ 4 ( $n = 12$ ) (35 to 80)	1.64 $\pm$ 0.37 ( $n = 4$ ) (0.7 to 2.5)
Ataxia† ( $n = 17$ )	139 $\pm$ 21 (35 to 329)	104 $\pm$ 10 (28 to 207)	52 $\pm$ 4 ( $n = 12$ ) (36 to 76)	1.51 $\pm$ 0.15 ( $n = 9$ ) (0.9 to 2.4)
Lactic‡ acidosis ( $n = 39$ )	134 $\pm$ 13 (25 to 387)	81 $\pm$ 6 (13 to 181)	50 $\pm$ 6 ( $n = 7$ ) (34 to 73)	2.3; 1.6; 1.3 ( $n = 3$ )
All controls ( $n = 102$ )	154 $\pm$ 9.0 (25 to 504)	97 $\pm$ 5 (14 to 266)	56 $\pm$ 2 ( $n = 39$ ) (32 to 91)	1.55 $\pm$ 0.11 ( $n = 20$ ) (0.7 to 2.5)
Patient (assayed $\times$ 4)	58.9 $\pm$ 27.6	19.8 $\pm$ 2.0	12.4 $\pm$ 1.9	1.29 $\pm$ 0.29

Values mean  $\pm$  SD; range in parentheses.

\*Patients with diseases without ataxia or lactic acidemia.

†Includes patients with Friedreich ataxia.

‡Patients with lactic acidosis other than LAD deficiency.

The  $K_M$  and  $V_{max}$  values for lipoamide dehydrogenase, measured in extracts of cultured skin fibroblasts, are shown below.

**Table II.  $K_M$  (apparent) and  $V_{max}$  of lipoamide dehydrogenase in extracts of cultured skin fibroblasts**

	<i>Lipoamide</i> (mM)	<i>NAD</i> <sup>+</sup> ( $\mu$ M)	<i>Lipoamide-H<sub>2</sub></i> (mM)	<i>NADH</i> ( $\mu$ M)
Control $K_M$	1.75	201.0	1.0	56.0
Patient $K_M$	1.30	382.0	1.17	83.0
Control $V_{max}$	44.5	34.5	36.0	52.0
Patient $V_{max}$	12.5	14.0	16.0	20.0

Experiments performed on fibroblast extracts of control cell line and patient fibroblasts as described.<sup>7</sup>

$V_{max}$ , Maximum velocity.

Treatment by moderate protein restriction (2.0 g/kg body weight/day) failed to alleviate the lactic acidosis. Restriction of dietary carbohydrate to 40% of the total caloric intake for two weeks carried a worsening of the acidosis. After informed consent, oral administration of lipoic acid (25 to 50 mg/kg body wt/day) greatly improved the lactic acid and pyruvic acidemia. This therapy also produced a dramatic cleaning of the abnormal organic aciduria. Maintenance therapy of 50 mg/kg/day lipoic acid and a diet containing 2.0 to 2.5 g protein/kg/day has been continued for two years without side effects. Episodes of acidosis have occurred when treatment was interrupted because of infection. The patient has made slow but consistent gains in development and at 30 months weighted 9.0 kg and showed improvement in both gross and fine motor development.

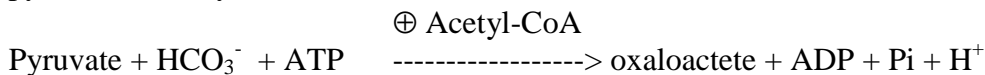
Questions on Case I.

1. Why were the plasma concentrations of pyruvate, lactate and alanine abnormally high?
2. The enzyme activity of the PDC complex, the  $\alpha$ -KGDH complex and dihydrolipoyl dehydrogenase from sonicated fibroblasts were all low when compared with the enzymes from normal fibroblasts. Explain how a defect in the gene of a single enzyme would lead to these findings.
3. Lipoic acid, when added to fibroblast homogenates, did not stimulate dihydrolipoyl dehydrogenase activity, yet lipoic acid has a dramatic effect in vivo. Explain these findings.
4. What was the rationale for attempting therapy with each of the following: thiamine, biotin, bicarbonate, protein restriction?
5. Restriction of dietary carbohydrate to 40% of the caloric intake has been effective in alleviating the symptoms of lactic acidosis in patients whose genetic defect in PDC is in the E1 subunit. Provide an explanation for the observation that in this patient a dietary carbohydrate restriction resulted in a worsening of the acidosis.
6. Provide a biochemical explanation, which would account for the low content of  $\text{HCO}_3^-$  in the blood of the patient.
7. Dichloroacetate, an inhibitor of the pyruvate dehydrogenase kinase, has been useful in treating some patients with lactic acidemia. What enzyme defects would you expect to respond to this treatment and why?

8. Describe the mechanism(s) by which PDC activity is regulated (a) allosterically and (b) by covalent modification.
9. How would decreasing the concentrations of each of the following metabolites affect the rate of the TCA cycle? (a) ATP, (b) Acetyl CoA, (c) NADH<sub>2</sub>? How would these changes effect glycolysis? Gluconeogenesis?

### III. Deficiencies in Pyruvate Carboxylase (PC)

Pyruvate carboxylase is a mitochondrial enzyme consisting of four identical subunits. Each subunit has binding sites for the substrates, pyruvate, ATP, HCO<sub>3</sub><sup>-</sup> and the allosteric effector, acetyl-CoA. Each subunit also contains one molecule of covalently bound biotin. The reaction catalyzed by pyruvate carboxylase is shown below.



The activity is almost totally dependent on the presence of acetyl CoA as an allosteric activator. The pyruvate carboxylase reaction is the first enzyme reaction in the gluconeogenic pathway, and is activated under conditions where fatty acids are mobilized and are generating acetyl CoA. Although this enzyme has its highest activity in tissues that carry out gluconeogenesis (liver and kidney), it is present in all tissues containing mitochondria. Both brain and adipose tissue have significant concentrations of pyruvate carboxylase, where it plays an anaplerotic role which is important in fatty acid synthesis. PC is also important in muscle where, together with PEPCK, it regulates the pool size of the citric acid cycle intermediates.

Deficiencies of pyruvate carboxylase can be divided into two distinct groups in terms of the clinical and biochemical manifestations of the disease. In the simple form of the disease (form A), the patients present in the first few months a life with a mild to moderate lactic acidemia and delayed development. In the more complex form of the disease (form B), the patients present soon after birth with severe lactic acidemia accompanied by hepatomegaly, elevated blood ammonia, citrulline and lysine. Patients with the B form of the disease rarely survive to three months of age. A summary of the presentation of these two groups of patients with PC deficiency is provided in the following Table.

**The Presentation of Human Pyruvate Carboxylase (PC) Deficiency**

	<i>Group A (13 cases)</i>	<i>Group B (11 cases)</i>
Origin	8 Amerindian, 1 Canadian Italian parents, 3 U.S. Caucasian, 1 Japanese	2 Canadian (1 with Egyptian parents), 4 French, 2 U.K., 1 West German, 1 Saudi Arabian, 1 Swede
Presentation	Metabolic acidosis Delayed neurologic development	Metabolic acidosis Hepatomegaly
Age of presentation	Birth to 5 months	Neonatal
Survival	2 survive to 5 years; severe mental retardation	All dead within 3 months
Biochemical	$\alpha$ -Ketoglutarate in urine Lactic acidosis mild with severe attacks on infection	$\alpha$ -Ketoglutarate in urine Lactic acidosis chronically severe
Pyruvate carboxylase activity	<5% control	<5% control
Lactate:pyruvate ratio	Normal	Elevated (X5)
Acetoacetate:3HOB ratio	Normal?	Elevated (X5)
Blood ammonia	Normal	Elevated (X5)
Alanine	Elevated	Elevated
Citrulline	Normal	Elevated (X5)
Lysine	Normal	Elevated
Proline	Elevated	Elevated
PC [ <sup>3</sup> H]biotin protein (125 kDa)	Present	Absent in 6/10 cases
PC immunoreactive protein	Present	Absent in 6/10 cases
PC mRNA	Present	Absent in 4/10 cases
	Refs. 85 to 92 + 3 unpublished cases*	Refs. 93 to 98 + 3 unpublished cases*

Patients with the A form of the disease have some residual pyruvate carboxylase activity while those with the B form have no activity at all; some of the patients in this latter group have an absence of both mRNA and pyruvate carboxylase protein.

Patients in the A group who survive are severely mentally retarded. This seems to be due to loss of cerebral neurons, despite the fact that in normal individuals there is no pyruvate carboxylase activity in neurons, although there is significant activity in astrocytes. These observations suggest that pyruvate carboxylase has an essential anaplerotic role in astrocytes and that its absence deprives the neuron of one or more obligatory nutrients which are normally supplied by astrocyte metabolism. Current evidence suggests that the neurotransmitter pools are replenished, in part, by de novo synthesis within the presynaptic terminals and that glutamine derived metabolically from astrocytes appears to be a major precursor for both glutamate and  $\gamma$ -aminobutyric acid (GABA).

A deficiency in PC may be suspected on the basis of metabolic acidosis associated with lactic acidemia. However, a definitive diagnosis of pyruvate carboxylase deficiency routinely depends on the direct measurements of PC activity in cultured skin fibroblasts. In fibroblasts that are found to be deficient in PC activity, it is often desirable to determine whether or not PC protein is present. In order to examine directly the pyruvate carboxylase protein in cultured skin fibroblasts, the techniques of [<sup>3</sup>H]-biotin labeling and immunoprecipitation of [<sup>35</sup>S]-methionine labeled protein have been used. In the first technique, cells are starved of biotin, and then incubated with high specific activity [<sup>3</sup>H]-biotin. The proteins are extracted and separated by SDS-PAGE electrophoresis. After fluorography of the dried gels, three bands are evident. The first band of M = 125,000 corresponds to the polypeptide of pyruvate carboxylase, the second to  $\beta$ -methylcrotonyl-CoA carboxylase (M = 75,000) and the third to propionyl CoA carboxylase (M = 73,000). Typical results from the biotin-labeling assay are shown in the the following figure.

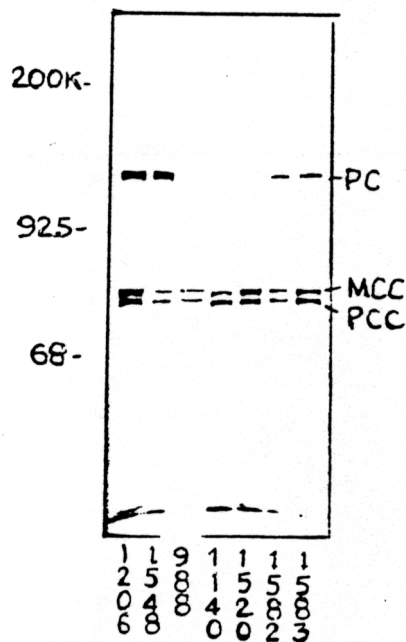


FIGURE [<sup>3</sup>H]-biotin labeling of carboxylase proteins in cultured skin fibroblasts. SDS/PAGE gelectrophoresis was carried out on amniocyte or skin fibroblast extracts after labeling with [<sup>3</sup>H]-biotin followed by fluorography of the dried gel. Cell strains used were lane 1, control cell strain 1206; lane 2, control cell strain 1548; lane 3, PC-deficiency cell strain 988 (presentation I); lane 4, PC-deficient cell strain 1 1140 (presenntation II); lane 5, PC-deficient cell strain 1520 (presentation II) lane 6 cell strain 1582 (mother of 1520); lane 7, cell strain 1583 (father of 1520).

Identical results to those shown above were obtained on extracts of fibroblasts grown in the presence of [<sup>35</sup>S] methionine. In this case, the radiolabeled pyruvate carboxylase was isolated by immunoprecipitation with anti-PC antiserum and examined by SDS-PAGE. This experiment showed that the absence of a detectable PC band after [<sup>3</sup>H]-biotin labeling in cell lines 1140 and 1520 (Fig. above) was due to the absence of cross-reactive protein (CRM) and not due to defective biotinylation of the carboxylase. Labeling experiments like those described above have been used to correlate the clinical presentations with the biochemical lesion. Groups of patients with the simple (Type A) disease are CRM-positive, i.e., have cross-reacting material corresponding to pyruvate carboxylase, while the group of patients with the more complex presentation are found to be CRM-negative; these individuals may synthesize a protein that is neither biotinylated nor immunoprecipitated by anti-PC antibody, although a more likely explanation is that the CRM-negative individuals do not synthesize PC protein at all.

The metabolic consequences of a CRM-negative pyruvate carboxylase deficiency appear to be profound. The evidence suggests that these individuals suffer from a lack of intracellular aspartate. If no pyruvate carboxylase is present, affected individuals must derive any required oxaloacetate from dietary aspartate, glutamate or glutamine, making these a trio of "essential amino acids". CRM-negative patients manifest their lack of intracellular oxaloacetate and aspartate in

disturbances of (1) the urea cycle, (2) the equilibration of mitochondrial and cytosolic redox states, and (3) lysine metabolism.

### Case report II.

A three-month old female seemed normal until she developed seizures. The infant became progressively worse, showing hypotonia, psychomotor retardation and poor head control. Plasma levels of both lactate and pyruvate were more than seven-times the normal concentrations. Plasma alanine concentration was above normal, and administering an alanine load failed to restore a normal gluconeogenic response.

Extracts of cultured fibroblasts were measured for pyruvate carboxylase activity and was found to be less than 1% the normal level. Both the mother and father had intermediate levels of fibroblast pyruvate carboxylase. Fibroblasts from the patient accumulated five-times greater than the normal amounts of lipid.

### Questions on Case II.

1. What is the metabolic function of pyruvate carboxylase?
2. How would you assay a fibroblast extract for PC activity?
- (3.) Patients with type B deficiency in PC also have a redox disturbance in which the cytosolic compartment is more oxidized as judged by a higher than normal ratio of blood acetoacetate to  $\beta$ -hydroxybutyrate. Give a biochemical explanation for this observation.
4. Explain the failure of an alanine load to induce gluconeogenesis in the patient.
5. Why did lipids accumulate excessively in the patients fibroblasts?
6. Explain why glutamine, when added to the cell culture medium, greatly stimulated the growth of fibroblasts from the patient.
7. What treatment would you suggest for a patient with this disease? Explain.
8. Most of the patients with PC deficiency die at a very early age, and those who survive are mentally retarded. What biochemical findings would you use to do genetic counseling?
9. Why would the urea cycle be affected by a deficiency in PC?
- (10.) What is biotinidase? What effect would a defect in biotinidase have on PC activity?
11. What is meant by the statement "PC plays an anaplerotic role in fatty acid synthesis"? or "PC plays an anaplerotic role in brain metabolism?"

### IV. Prenatal diagnosis of Pyruvate Carboxylase Deficiency

In their paper, Marsac et al. describe the first prenatal diagnosis of pyruvate carboxylase deficiency. The activity of pyruvate carboxylase was measured in amniotic fluid cells obtained from a pregnancy at risk for PC deficiency. The couple had two children who had previously died of PC deficiencies. The diagnosis was corroborated by four independent laboratories, and the diagnosis was confirmed by the demonstration that pyruvate carboxylase activity was undetectable in all the tissues examined from the aborted fetus.

#### Biochemical questions

1. Why were the following enzyme activities measured in Marsac's paper; propionyl CoA carboxylase, methylcrotonyl CoA carboxylase, phosphoenolpyruvate carboxylase?
2. Which are the chemical reactions catalyzed by the enzymes listed above? (equations, formulas)
3. How would you assay pyruvate carboxylase activity?



4. Why was the enzyme activity measured in liver, kidney and brain? What is the function of pyruvate carboxylase in these tissues?

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