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Biochemical Studies of a Patient with Hereditary Hepatorenal Tyrosinemia: Evidence of Glutathione Deficiency

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ABSTRACT. Metabolic and enzymatic studies in a patient with hereditary tyrosinemia demonstrated for the first time a deficiency of erythrocyte and hepatic glutathione. Markedly decreased hepatic fumarylacetoacetate hydrolase activity was demonstrated in this patient. The activities of hepatic enzymes not involved in tyrosine metabolism were also determined. Assay of mixed function oxidase activity demonstrated low levels of aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase, suggesting decreased hepatic detoxification capacity. 5-Aminolevulinic acid dehydratase activity was undetectable. Succinylacetone (4,6-dioxoheptanoic acid), an abnormal metabolic product secondary to fumarylacetoacetate hydrolase deficiency was found in serum and urine. Succinylacetone was demonstrated to inhibit 5-aminolevulinic acid dehydratase *in vitro*, as did the urine, plasma, and red cell lysates of the patient. (*Pediatr Res* 18:1332–1336, 1984)

Hereditary hepatorenal tyrosinemia is a rare metabolic disorder characterized by increased levels of tyrosine in the blood and urine. It presents during early childhood with hepatocellular disease, Fanconi's syndrome, and hypophosphatemic rickets. A pathognomonic feature of this disease is the presence of succinylacetone (4,6-dioxoheptanoic acid) in serum and urine (18). Malignant hepatoma, prevalent in this disorder, is not explained solely by cirrhosis (35).

The primary enzymatic defect in tyrosinemia is not known. Recently it has been postulated to be a deficiency of fumarylacetoacetate hydrolase (2, 18). Several additional enzymes have also been found to be deficient, as in this report and previous publications. Many clinical and biochemical findings remain unexplained in tyrosinemia. The aim of our study was to increase our understanding of this disease by examining the biochemical abnormalities and hepatotoxicity in such a patient. Our investigation revealed hepatic and erythrocyte glutathione deficiency, and decreased activity of several hepatic enzymes: fumarylacetoacetate hydrolase, 5-aminolevulinic acid dehydratase, and mixed function oxidases. The deficiencies of glutathione and mixed function oxidases, which have not been previously reported in tyrosinemia, may play a significant role in the carcinogenic potential associated with this disorder.

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CASE HISTORY

The patient was a 1½-year-old female admitted to the Pediatric Clinical Research Center at The New York Hospital-Cornell Medical Center. Pregnancy and neonatal course were uncomplicated. There was no jaundice. The baby was breastfed for the first 10½ months. Although she fed well, she was irritable, and vomited daily. At the time of the evaluation she was on a diet appropriate for her age. Developmental milestones occurred normally until 6 months of age, but she was not standing or making sounds at 13 months.

At 9 months of age, hemoglobin was 12.5 g/dl. By 1 year, it had decreased to 10.5 g/dl, and the patient had a palpable spleen.

Physical examination at 14 months of age revealed a weight of 9.34 kg (25th percentile), height of 79.3 cm (50th percentile), and head circumference of 44.5 cm (15th percentile). Abnormal physical findings included a grade II/VI systolic murmur at the apex without radiation, an enlarged liver (10.5-cm span at the midclavicular line), and the tip of the spleen was palpable 2 cm below the left costal margin. Neurologic examination revealed poor muscle strength proximally in the lower extremities, but was otherwise within normal limits.

Plasma tyrosine, methionine, bile acids, and ammonia were elevated (Table 1). Renal tubular dysfunction with acidosis was noted. α-Fetoprotein was markedly elevated. Serum cholesterol and triglycerides were also elevated, associated with fasting hypoglycemia and undetectable serum insulin. There was no response to glucagon in the fasting state or postprandially. Galactose-1-phosphate uridyltransferase was within normal limits. Serum calcium was normal, with a markedly decreased serum phosphorus. Alkaline phosphatase, primarily of bone origin, was elevated. Parathyroid hormone and vitamin D levels were normal. Bone age was appropriate for age. Early signs of rickets in the wrists and knees were demonstrated radiographically. An abdominal sonogram showed hepatosplenomegaly with no signs of increased portal pressure or tumor. An electroencephalogram

Table 1. *Biochemical results in patient with hereditary hepatorenal tyrosinemia*

Metabolic	Hepatic
Plasma amino acids*	α-Fetoprotein, 182,000 ng/ml (<49 ng/ml)
Tyrosine, 751 μM (21–87 μM)	CEA, 15 ng/ml (1–5 ng/ml)
Methionine, 989 μM (6–39 μM)	Serum NH_3, 94 mM (<40 mM)
Urinary amino acids: increased threonine, serine, glycine, alanine, methionine, isoleucine, tyrosine	Alkaline phosphatase, 1518 units/liter (182 heat stable) (30–110 units/liters)
Cholesterol, 206 mg/dl, Triglycerides, 122 mg/dl (>97th percentile for age)	Bilirubin, 0.8 mg/dl (0.1–1.0 mg/dl)
Ferric chloride test, within normal limits	SGOT, 99 units/liter (0–45) units/liter
Berry spot test, negative	SGPT, 51 units/liter (0–45) units/liter
Urinary catechols, 52 μg (8–160 μg)	Total protein, 6.3 g/dl (5.5–8.0 g/dl)
Urinary uroporphyrins, nondetectable (WNL)†	Total albumin, 4.1 g/dl (3.0–5.0 g/dl)
Urinary porphobilinogen, nondetectable (WNL)	γ-GTP, 126 units/liter (7–50 units/liter)
Thyroid function tests, within normal limits	72-h stool fat, 12.6 g/72 h (25% intake) (normal, <5%)

Renal	Hematologic
Creatinine clearance, 112 ml/min/m^2 (90–150 ml/min/m^2)	Prothrombin time, 16/12.2
HCO_3, 12–15 mM (22–32 mM)	Partial thromboplastin time, 61.5/<40
Serum pH 7.29 (7.34–7.47)	Fibrinogen, 219 mg/dl (180–400 mg/dl)
Urine analysis, significant for 1+ glucose, 2+ protein, and intermittent ketonuria	Specific clotting assays ↓ factor II, V, VII, VIII, and X
Urinary quantitative glucose, 4.5 g/12 h	Platelet functions within normal limits
Urinary β_2-microglobulin, 4050 (4.0–370)	Serum immunoglobulins within normal limits
Urinary quantitative protein, 0.04 g/24 h	Bone marrow within normal limits; 15% T cells; 14% B cells

* Normal values are in parentheses.

† WNL, within normal limits; CEA, carcinoembryonic antigen; SGOT, serum glutamate oxaloacetate transaminase; SGPT, serum glutamate pyruvate transaminase, γ-GTP, γ-glutamyl transpeptidase.

showed mild and nonspecific changes characterized by rare sharp wave activity. Computerized transaxial tomography of the brain demonstrated mild cerebral atrophy.

Liver biopsy showed cirrhosis with nodular areas and fat deposition on gross examination. Light microscopy revealed focal regenerative nodules with a variable degree of fibrous tissue. Individual nodules showed variable amounts of steatosis, cholestasis, and numerous acidophilic bodies. All evaluations were performed under a protocol approved by this institution's review boards. Informed consent was obtained from the parents.

MATERIALS AND METHODS

Hepatic enzymes of tyrosine metabolism. Tyrosine aminotransferase and 4-hydroxyphenylpyruvate dioxygenase activities were measured according to the method of Whelan and Zannoni (36), and maleylacetoacetate isomerase and fumarylacetoacetate hydrolase activities were measured by the methods of Edwards and Knox (4).

Pyrrole metabolism. Porphyrins, 5-aminolevulinic acid dehydratase, and uroporphyrinogen synthetase were measured using methods previously reported (29, 30). A portion of the liver obtained at biopsy was homogenized in 0.1 M sodium phosphate buffer, pH 7.4, and the homogenate was then centrifuged at 9000 $\times$ *g* for 10 min. The porphyrin content of the liver (9) and the activity of 5-aminolevulinic acid dehydratase (31) was measured in the homogenate. The activity of 5-aminolevulinic acid synthase was measured in the 9000 $\times$ *g* pellet (31).

Glutathione determination. A portion of the liver biopsy specimen was sonicated for 30 s in 5 volumes of 1% picric acid, centrifuged, and glutathione concentration was measured using the method of Tietze (33). Erythrocyte glutathione was measured by the same method immediately after hemolyzing the cells by dilution with 100 volumes of water.

Mixed function oxidases. The activities of aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase were measured in the 9000 $\times$ *g* supernatant (26).

RESULTS

Activity of enzymes of tyrosine metabolism measured in liver tissue are presented in Table 2. These studies indicated the absence of 4-hydroxyphenylpyruvate dioxygenase activity, markedly decreased fumarylacetoacetate hydrolase activity, decreased tyrosine aminotransferase activity as well as normal maleylacetoacetate isomerase and increased homogentisate dioxygenase activity.

Liver and erythrocyte glutathione concentrations were significantly decreased (Table 3).

Serum and urinary determination of organic acids by mass spectrometry showed the presence of succinylacetone and 4-hydroxybenzaldehyde, as well as markedly elevated levels of lactate and 4-hydroxyphenyllactate (39).

The activities of enzymes and intermediates involved in heme biosynthesis and microsomal hemoprotein function are presented in Table 4. In erythrocytes, 5-aminolevulinic acid dehydratase was undetectable, whereas uroporphyrinogen-I synthetase was at the upper limits of normal. Erythrocyte protoporphyrin content was slightly elevated, a finding consistent with new erythrocyte production in response to anemia. In liver, 5-aminolevulinic acid dehydratase activity was also undetectable. Consistent with the marked inhibition of 5-aminolevulinic acid dehydratase activity, hepatic porphyrins were below normal (3). In contrast, the activity of 5-aminolevulinic acid synthase, the rate-limiting enzyme for heme synthesis in liver, was more than three times higher than values reported for normal adults, and was in the range observed in patients with porphyria (12, 22, 34).

Activities of the mixed function oxidases, aryl hydrocarbon hydroxylase and 7-ethoxycoumarin deethylase (Table 4), were at the lower end of the range observed in human fetal liver (25, 27), and only 1–10% of the mean values reported for autopsy specimens of adult liver (11).

DISCUSSION

In this study, we have documented the extensive hepatic, renal, and skeletal abnormalities previously reported in tyrosinemia,

and we report several additional biochemical derangements. The primary defect in this disease has been postulated to be a decreased activity of fumarylacetoacetate hydrolase (18). This enzyme, first reported in mammalian liver, kidney, and other tissues as having acylpyruvic acid-hydrolyzing activity (19), catalyzes the final step in the main phenylalanine and tyrosine degradative pathway (Fig. 1, *Reaction 6*). We found a low hepatic activity of this enzyme in our patient, confirming similar findings by others (2, 5, 14, 15). A deficiency of this enzyme is postulated to account for the presence of succinylacetone and succinylacetoacetate in the urine of patients with tyrosinemia (18). Succinylacetoacetate presumably arises by reduction of either maleylacetoacetate or fumarylaceotacetate or both. The mechanism of its formation is unknown. Its absence in the urine of normal individuals may be attributable to the fact that it is a substrate of the hydrolase (13). Neither fumarylacetoacetate nor maleylacetoacetate has been found in the urine of patients with tyrosinemia.

Hypoglycemia and acidosis were prominent findings in this patient (Table 1). The failure of lactate to increase with concomitant hypoglycemia after glucagon stimulation differentiates this disorder from glycogen storage diseases. The lack of response to glucagon may be secondary to cirrhosis and a resultant decrease in total liver glycogen. Alternatively, the enzymes necessary for glycolysis may be inhibited by abnormal metabolic products. Impaired gluconeogenesis and glycogenolysis induces lipolysis, causing the increase in serum cholesterol and triglycerides noted. The delay in developmental milestones and cerebral cortical atrophy may be the result of prolonged episodes of hypoglycemia or may be secondary to the toxic effects of the metabolic derangement of amino acid metabolism.

The increased level of tyrosine in the blood has been attributed

Table 2. *Hepatic enzymes of tyrosine metabolism**

Enzyme	Patient	Range of normal controls ($n = 10$)
Tyrosine aminotransferase	0.024	0.027–0.091
4-Hydroxyphenylpyruvate dioxygenase	<0.004	0.085–0.144
Homogentisate dioxygenase	1.50	0.65–1.24
Maleylacetoacetate isomerase	0.77	0.31–2.21
Fumarylacetoacetate hydrolase	0.15	0.60–1.17

* Values are expressed as units/mg protein (1 unit = 1 μmol of substrate metabolized/60 min at 22° C, except for homogentisate dioxygenase which was measured at 37° C).

Table 3. *Hepatic and erythrocyte glutathione*

Hepatic glutathione (μmol/g tissue)		
Patient		3.4
Normal control liver biopsy		5.9
Erythrocyte glutathione	μmol/ml of blood	nmol/mg Hb
Patient	0.35*	2.9*
Father	0.84	4.7
Mother	1.32	6.8
Controls	0.92†	6.1†

* Mean of four determinations over 4 months (range, 0.26–0.46 μmol/ml of blood; 2.3–3.6 nmol/mg Hb).

† Mean of five samples (range, 0.74–1.12 μmol/ml of blood; 4.0–8.7 nmol/mg Hb).

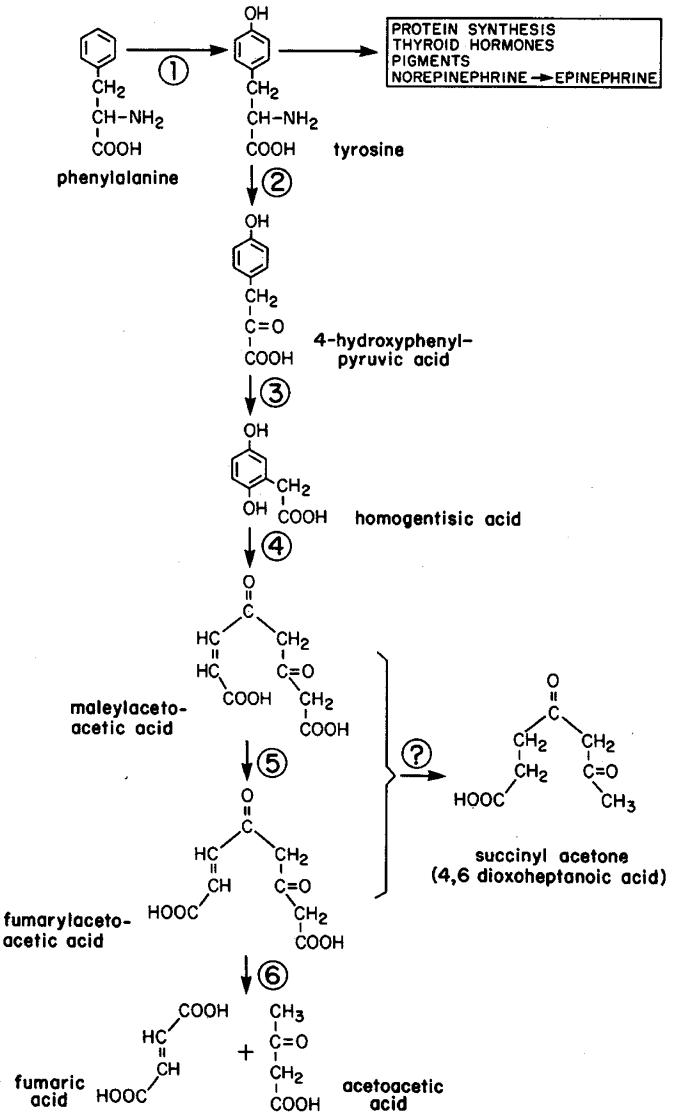

Fig. 1. Tyrosine degradative pathway: intermediary metabolism. *1*, phenylalanine hydroxylase; *2*, tyrosine aminotransferase; *3*, 4-hydroxyphenylpyruvate dioxygenase; *4*, homogentisate dioxygenase; *5*, maleylacetoacetate isomerase; *6*, fumarylacetoacetate hydrolase.

Table 4. *Enzymes and intermediates involved in heme biosynthesis and function**

Enzyme or intermediate	Level or activity	Normal values	Reference
Liver			
5-Aminolevulinic acid synthase	114 nmol ALA/g liver/h	24–34	22, 34
5-Aminolevulinic acid dehydratase	<10 nmol porphobilinogen/g liver/h	2000–3700	22
Porphyrins	0.30 nmol porphyrin/g liver	0.80–0.87	3
7-Ethoxycoumarin deethylase	17.6 nmol umbelliferone/g liver/h	409.9 ± 292.5	11
Aryl hydrocarbon hydroxylase	6.9 nmol phenols/g liver/h	66.4 ± 58.6	11
Erythrocytes			
5-Aminolevulinic acid dehydratase	<10 nmol porphobilinogen/ml RBC/h	633 ± 234	8
Uroporphyrinogen-I synthetase	43.7 nmol uroporphyrinogen/ml RBC/h	30.8 ± 5.6	29
Porphyrins	208 μg protoporphyrin/100 ml RBC	40–140	30

* ALA, 5-aminolevulinic acid; RBC, red blood cells.

to low hepatic 4-hydroxyphenylpyruvate dioxygenase activity (7, 16, 17), (Fig. 1, *Reaction 3*). The activity of this enzyme may also be decreased by the toxic metabolites produced in this condition. The increased methionine level, a variable finding in this disorder (20), has been explained by secondary inhibition of methionine-activating enzyme and cystathionine synthase (6).

Several abnormalities of heme metabolism have been reported in tyrosinemia. These include increased urinary excretion of 5-aminolevulinic acid (6, 7, 10), decreased activity of liver and erythrocyte 5-aminolevulinic acid dehydratase (18, 32), increased activity of 5-aminolevulinic acid synthetase (7, 10), and porphyria-like symptoms.

In our patient, 5-aminolevulinic acid dehydratase activity was undetectable in the liver, less than 1% of normal in erythrocytes, and was not restored, even after transfusion and red cell washing. Both parents of the child had normal erythrocyte levels of 5-aminolevulinic acid dehydratase activity. The activity of 5-aminolevulinic acid synthase was elevated in the liver of the patient, and there was high urinary excretion of 5-aminolevulinic acid. Lindblad *et al.* (18) showed that succinylacetone directly inhibits 5-aminolevulinic acid dehydratase, and postulated that the increase in 5-aminolevulinic acid excretion observed in tyrosinemia is secondary to inhibition by succinylacetone.

The low levels of mixed function oxidase activity observed in this patient have not been described previously in tyrosinemia. Levels could be low as a consequence of impaired heme percursor and cytochrome P-450 synthesis, structural liver damage accompanying cirrhosis, direct inhibition of mixed function oxidase activity by succinylacetone, or other organic acids, or a combination of these factors. The low levels of mixed function oxidase activity could result in a decreased detoxification capacity of the liver and may contribute to the development of hepatic cirrhosis and hepatocellular carcinoma.

This is the first report of a depression of glutathione in erythrocytes and liver of a patient with tyrosinemia. Fumarylacetoacetate has been shown to react with glutathione to form an adduct (4). If fumarylacetoacetate accumulates in tyrosinemia as suggested, this reaction may explain the glutathione depletion we observe. Glutathione forms an adduct with maleylacetoacetate much more slowly or not at all. However, glutathione both catalyzes the isomerization of maleylacetoacetate to fumarylacetoacetate, with which it can then react, and also serves as a coenzyme for the isomerase (4). Thus, it is possible that the isomerase activity may be deficient in tyrosinemia because of the glutathione depletion. Another possible reason for the low glutathione concentrations is that its synthesis may be inhibited by some of the organic acids which accumulate in tyrosinemia.

Glutathione is known to act as a scavenger for a large number of toxic metabolites (1). It also serves to protect the integrity of cell membranes and to maintain protein sulfhydryl groups in the reduced state. Thus, low levels of glutathione may contribute to liver toxicity and malignant potential in tyrosinemia by impairing the detoxification of toxic metabolites.

Treatment with glutathione or other thiols may thus offer a means of decreasing the level of toxic and carcinogenic compounds in patients with tyrosinemia. Glutathione and cysteine have been shown to protect against chemically induced toxicity (21) and mutagenesis (28). Administration of glutathione has been reported to induce regression of liver tumors in animals (23). Treatment of acetaminophen toxicity with cysteamine or *N*-acetylcysteine, a glutathione precursor, has proven successful (24). L-2-Oxothiazolidine-4-carboxylate has been shown to be an effective glutathione precursor in experimental animals (37, 38) and may be a valuable potential therapeutic agent in tyrosinemia.

In summary, metabolic studies have demonstrated extensive enzymatic and biochemical abnormalities in a patient with tyrosinemia. We have demonstrated several enzyme abnormalities not previously described. The decreased glutathione in liver and erythrocytes which we report for the first time may play an important role in the pathophysiology of this disease. The decreased levels of glutathione demonstrated in our patient suggest the possible benefit of a new mode of therapy, aimed at increasing the concentration or availability of glutathione in tissues, particularly in liver.

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Cl^- Permeabilities in Red Blood Cells and Peripheral Blood Lymphocytes from Cystic Fibrosis and Control Subjects

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ABSTRACT. Recent studies have identified abnormalities in Cl^- permeation across two target cystic fibrosis (CF) epithelia (sweat duct and respiratory epithelium). In the present study, anion conductances of red blood cells (RBCs) and peripheral blood lymphocytes (PBLs) from CF and normal subjects were estimated and compared. For RBCs, the valinomycin-induced rate constant for K^+ loss (P_{K^+}) was taken as an index of P_{Cl^-}. For PBLs, the secondary volume increase after gramicidin pretreatment and hypotonic (0.67× isotonic) stress was used to estimate P_{Cl^-}. The Cl^- permeabilities of RBCs and PBLs from CF and control subjects were comparable. These findings suggest that the abnormality in P_{Cl^-} reported for CF sweat ductal and respiratory epithelia is not expressed in circulating blood elements. (*Pediatr Res* 18:1336–1339, 1984)

Abbreviations

CF, cystic fibrosis
PBL, peripheral blood lymphocyte
RBC, red blood cell
P_{Cl^-}, Cl^- permeability coefficient
P_{K^+}, K^+ permeability coefficient
HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
DIDS, 4-acetamide-4′-isothiocyanostilbene-2,2′-disulfonic acid
SITS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid

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Cystic fibrosis is a disease that is characterized by multiple abnormalities in the volume and composition of body "secretions" (5). This feature has led many investigators to suggest that the disease may reflect a generalized dysfunction in ion permeation (5). Evidence has accrued that the CF sweat ductal epithelium may be relatively impermeable to Cl^- ions (23, 26). A reduction in cell Cl^- permeability also appears to be a characteristic of CF respiratory epithelia (17, 18). The defect at each site appears to involve a path for Cl^- that is electrically conductive. It is not yet known whether this dysfunction reflects a structural or functional (control) abnormality.

Techniques have recently been developed to identify and partition Cl^- movements across membranes of circulating blood elements to either conductive or nonconductive paths. In the RBC, ion and water movement across valinomycin-treated cells reflect conductive Cl^- flows (13, 14). In the PBL, volume regulatory changes in response to an isosmotic medium have been used to study conductive paths for K^+ and Cl^- (9, 10). Because an abnormality in Cl^- channels might be generalized to blood elements in CF subjects, we compared the magnitude and regulation of conductive Cl^- paths in RBCs and PBLs from CF subjects with those from control (normal) subjects.

MATERIALS AND METHODS

Subjects. Blood was obtained in heparinized tubes and used for study within 4 h of venipuncture. Blood was obtained from