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Chronic Tyrosinemia Associated with 4-Hydroxyphenylpyruvate Dioxygenase Deficiency with Acute Intermittent Ataxia and without Visceral and Bone Involvement

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Summary

A 17-month-old girl, with acute intermittent ataxia and drowsiness, had hypertyrosinemia (serum tyrosine, 62 μmole/dl) and phenolic aciduria in the absence of hepatic, renal, eye or skin lesions. Serum methionine and urinary δ-aminolevulinic acid concentrations were normal. Her psychomotor development was also normal. Protein restriction and vitamin C therapy failed to correct the biochemical abnormality. Liver biopsy was histologically normal.

Analysis of the enzymes in the liver biopsy, taken at 25 months of age, showed no detectable activity of 4-hydroxyphenylpyruvate dioxygenase (4HPPD), either in whole homogenate or cytosol fraction. Mixing experiments revealed no inhibitor of either 4HPPD or tyrosine aminotransferase (TAT).

TAT in unfractionated liver was 0.23 μmole/mg protein/h (control, 0.10–0.30 μmole/mg protein/h; $n = 5$). In mitochondria, TAT was 0.24 μmole/mg protein/h (control, 0.09–0.12 μmole/mg protein/h; $n = 3$) whereas in cytosol fraction it was 0.23 μmole/mg protein/h (control, 0.27–0.44 μmole/mg protein/h; $n = 3$). Glutamate dehydrogenase activity appeared in the cytosol fraction suggesting some rupture of mitochondria during fractionation of the patient's liver and indicating that true cytosol TAT might be somewhat lower than indicated; however, the kinetics of the patient's cytosol TAT were normal: Km for tyrosine, 4.5×10^{-3} M (control, 4.0×10^{-3} M); Km for α-ketoglutarate, 98×10^{-6} M (control, 75×10^{-6} M); approximate Km for pyridoxal phosphate, 2.1×10^{-6} M (control, 4.0×10^{-6} M). Vmax in patient liver was 0.37 μmole/mg protein/h (control, 0.88 μmole/mg protein/h). These data argue against a primary abnormality of TAT but are consistent with a defect of 4HPPD; thus, this patient appears to represent a previously undescribed form of tyrosinemia.

Speculation

Demonstration of deficient activity of 4-hydroxyphenylpyruvate dioxygenase in a patient with a unique form of tyrosinemia raises the possibility that a similar defect may be present in several other patients with atypical forms of tyrosinemia.

There are two well recognized forms of hereditary tyrosinemia. In the first (type I), associated with failure to thrive, rickets, Fanconi syndrome, and progressive liver failure, the levels of blood tyrosine and frequently methionine are elevated and there is increased urinary excretion of tyrosine and its metabolites (tyrosyluria) as well as increased δ-aminolevulinic acid. Deficient activity of a number of enzymes in the liver, including cytosol tyrosine aminotransferase (TAT; EC 2.6.1.5.) and 4-hydroxyphenylpyruvate dioxygenase (4HPPD; EC 1.13.11.27) has been reported. Recently, a deficiency of hepatic fumarylacetoacetate hydrolase (EC 3.7.1.2) has been demonstrated in several patients (18) and is now felt to be the primary enzyme defect in this disease.

Tyrosinemia type II has been described in over twenty patients, most of whom have manifest the Richner-Hanhart syndrome with severe persistent keratitis and more variable hyperkeratosis on the fingers and palms of the hands and soles of the feet. These lesions respond completely to dietary tyrosine restriction. Blood tyrosine is greatly elevated and there is massive tyrosyluria. Absent activity of hepatic cytosol TAT has been demonstrated in two patients (9, 16) and a milder deficiency has been reported in two other patients, in liver (11) and fibroblasts (4) respectively.

Another form of tyrosinemia has been reported in two children with severe metabolic acidosis who excreted the unusual tyrosine metabolites hawkinsin and cis- and trans-4-hydroxycyclohexylacetic acids in the urine (3, 22, 26). The primary abnormality is thought to be in the rearrangement of an intermediate formed in the 4HPPD reaction.

We now report a case of persistent tyrosinemia and tyrosyluria associated with deficient activity of 4HPPD in liver. The clinical picture was characterized by sporadic ataxia and drowsiness in a child who was otherwise normal.

MATERIALS AND METHODS

Case report. K.W. was the second child of healthy, unrelated, Egyptian parents. Pregnancy and delivery were uncomplicated. Birthweight was 3200 g and the neonatal period was normal. No problems occurred until 17 months of age, when the child was admitted to the Pediatric Clinic of Rome University because of acute ataxia followed by drowsiness. On examination she demonstrated confusion, motor incoordination, muscular hypotonia and absent tendon reflexes. The pupils were unreactive to light, there was no response to painful stimuli, breathing was noisy and the liver edge was 2 cm below the costal margin. The examination was otherwise normal. Blood glucose was normal. Routine toxicologic analysis of serum and urine by gas-liquid chromatography revealed no poisonous agents.

The child was treated intravenously with fluids, glucose, and electrolytes. After 8 h, response to stimuli and reflexes reappeared and in the following days, voluntary movements improved although her gait was ataxic until day 10.

During this time, routine laboratory tests, including SGOT (20–38 IU/liter), SGPT (11–20 IU/liter), total serum protein (8 g/liter), alkaline phosphatase (110 IU/liter), total serum cholesterol (125 mg/dl), and triglycerides (48 mg/dl) were normal. Glomerular filtration rate (GFR), creatinine, creatinine clearance, and phosphate reabsorption were also normal. Plasma amino acids (Table 1) established the diagnosis of tyrosinemia. Fundus examination, E.E.G., E.C.G., skull x-rays and psychomotor devel-

Table 1. *Serum aminoacid levels (μmole/dl)*

	Patient				Mother	Normals[1]
Age	17 months	19 months	27 months	42 months		9–24 months
Dietary protein (g/kg/day)	normal	1.0	1.0	normal	normal	normal
Tyrosine	62.4	80.5	49.6	69.6	6.7	1.1–12.2
Phenylalanine	7.6	7.9	6.2	10.2	5.3	2.3–6.9
Methionine	2.6	5.4	3.3	2.8	3.4	0.3–2.9

[1] From Soupart (24).

opment were also normal (I.Q. 123). Skeletal x-rays showed mild osteoporosis but no rickets.

After the patient had recovered, an oral loading test with L-tyrosine (100 mg/kg in fruit juice acidified with ascorbic acid) was performed. The serum tyrosine increased from 80 μmole/dl at zero time to 101 μmole/dl at 1 h, 104 μmole/dl at 2 h, and 107 μmole/dl at 3 h. and the child became drowsy with mild ataxia which lasted 4 h. An open liver biopsy, performed at 25 months (after written informed consent was obtained) was histologically normal.

Further clinical course. On day 24 the infant was dismissed in good condition, on a low-protein diet (1 g/kg/day). There was no recurrence of symptoms except for a painful erythema of the limbs that disappeared after a few days. Serum tyrosine remained high despite protein restriction.

The girl has been on a regular diet since the age of 30 months and has continued excellent growth and psychomotor development. When last seen, at 3½ years of age, her protein intake was normal and she was doing well. Her height was 96.5 cm (25th percentile) and her weight was 15.4 kg (50th percentile); physical examination was normal. The serum tyrosine level was 69.6 μmole/dl (Table 1).

Gas chromatography of tyrosine metabolites. Urine was acidified to pH 1, extracted 3 times with 2 volumes of ethyl acetate, dried over sodium sulfate, filtered, evaporated *in vacuo* and derivatized with a 1:1 mixture of Sil Prep (Applied Science) and BSTFA (Pierce) at room temperature, overnight. Phenolic acids were quantitated on a Hewlett-Packard model 5830 A gas chromatograph equipped with a hydrogen flame ionization detector and an 8 ft x 2 mm column of 3% OV101 on 80/100 mesh Supelcoport (Supelco Inc.), using 4-phenylbutyric acid as internal standard. Identity of compounds was confirmed by gas chromatography/mass spectrometry on a Finnegan model 4021 mass spectrometer equipped with an all glass jet separator. Selective ion monitoring at 302, 287, 212, 204, 195, 147, 123, 95, and 81 m/e was used in an attempt to detect cis- or trans-4-hydroxycyclohexylacetic acids (22).

Hepatic enzyme studies. Control liver was obtained within 4 h of death from children who died of trauma (#177 age 15 yr), lactic acidosis (#186, age 4 yr), nonketotic hyperglycinemia (#240 age 17 months), and Menkes disease (#262 age 28 months). Control 247 was a biopsy from a patient of 14 months with hypermethioninemia but no tyrosinemia or evidence of hepatic disease and control 178 was a biopsy of normal hepatic tissue from a 3-year-old patient with a hepatic hamartoma. Fresh tissue was homogenised in 19 volumes of 0.25 M sucrose containing 10 mM Tris or phosphate buffer pH 7.5, and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged at 20,000 × *g* for 30 min and the resulting supernatant carefully separated from the mitochondrial pellet. All procedures were carried out at 0–4°C. Fractionation of the patient's biopsy material was performed immediately as described above. The fractions were maintained at −70°C for 3 wk, shipped on dry ice from Italy to the USA where they arrived still frozen, and maintained at −70°C until assay. The mitochondrial pellet was suspended in a small volume of 10 mM Tris, pH 7.5 and sonicated three times for 15 sec each at 30–40 watts on a Braunsonic model 1510 sonicator with a micro probe. For measurement of enzyme activity in unfractionated homogenate, tissue was homogenized in 10 mM Tris pH 7.5 as above, centrifuged at 1000 × *g* for 10 min and the supernatant sonicated as described. All fractions were stored at −70°C.

TAT was measured by the radiochemical procedure of Fellman *et al.* (8, 9) except that the final concentration of α-ketoglutarate was 10 mM, the buffer was 0.2 M Tris, pH 7.5 and N, N'-diethyldithiocarbamate was omitted. This reaction is based on the release of [^3H_2O] from L-tyrosine (side-chain-2, 3, $-^3H$; Amersham) the [^3H_2O] being distilled in Thunberg tubes and measured by liquid scintillation counting. 4HPPD was measured by method II of Lindblad *et al.* (17). The incubation mixture contained bovine liver catalase (Sigma, 0.8 g/liter), glutathione (10 mM), 2, 6-dichloroindophenol (0.15 mM), Tris buffer, pH 7.5 (0.2 M) and 4-hydroxyphenyl-[1-^{14}C] pyruvate [0.2 mM; 5.2 μCi/mmole, generously provided by Dr. J. H. Fellman (7)] in a final volume of 2.0 ml. The sample was pre-incubated for 20 min at 4°C with all the above components except 4-hydroxyphenylpyruvate. This was then added and the mixtures incubated at 37°C for 15 min in stoppered vials fitted with a filter paper disc containing 0.04 ml of hyamine hydroxide (New England Nuclear) suspended from the stopper. The reaction was stopped with 0.2 ml of 1 N sulfuric acid and incubation continued for a further 30 min to allow absorption of the evolved [$^{14}CO_2$] on the filter paper. Radioactivity was determined by liquid scintillation counting in 10 ml of 0.4% omnifluor (New England Nuclear) in toluene:ethanol 4:1. Lactate dehydrogenase (E.C. 1.1.1.27) and 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44) were determined as described previously (15) and glutamate dehydrogenase (E.C. 1.4.1.3) by the method of Beaufay *et al.* (1). Protein was determined by the method of Lowry *et al.* (20).

Km values of tyrosine and α-ketoglutarate for TAT were determined on undialysed cytosol fractions and calculated from Lineweaver-Burke double reciprocal plots. The Km for pyridoxal phosphate was obtained on samples which had been dialysed twice against 25 volumes of 0.2 M potassium phosphate, pH 7.4, containing 5 mM mercaptoethanol and 1 mM EDTA, for 2 h each (4). Because dialysis did not result in complete removal of pyridoxal phosphate, as evidenced by the appreciable enzyme activity in samples to which no pyridoxal phosphate was added, a correction was made for the residual amount of pyridoxal phosphate in the sample after dialysis by extrapolating the curve of velocity *versus* substrate to zero velocity. The approximate Km for pyridoxal phosphate was then obtained from the double reciprocal plots.

RESULTS

The patient's urine gave a positive reaction to 2,4-dinitrophenylhydrazine, suggesting the presence of an α-ketoacid, and to nitrosonaphthol, indicating the presence of tyrosine metabolites. Serum and urine levels of tyrosine were much increased: 62 μmole/dl and 9.39 μmole/kg/24 h, respectively. Other aminoacids showed no consistent abnormalities. Serum methionine was normal except on one occasion when several other amino acids were also mildly elevated (Table 1). Urinary δ-aminolevulinic acid was normal. The urine contained high levels of *p*-hydroxyphenyllactic acid (pHPLA, 3.80 mg/mg creatinine), *p*-hydroxyphenylpyruvic acid (pHPPA, 0.80 mg/mg creatinine) and *p*-hydroxyphenylacetic acid(pHPAA, 0.27 mg/mg creatinine). Only pHPAA (0.005–0.042 mg/mg creatinine) is normally detected in control urines (2). The

identity of these metabolites was confirmed by mass spectrometry; selective ion monitoring gave no evidence for the presence of cis- or trans-4-hydroxycyclohexylacetic acids.

The stability of the hepatic enzymes from control tissue to storage at −70°C was investigated (Fig. 1). All showed slow decline but retained at least 50% of their original activity even after a 12-month period.

The results of the enzyme analysis of the patient's liver biopsy are shown in Table 2. 4HPPD activity was undetectable in both whole homogenate and cytosol. Mixing experiments indicated that no inhibitor, either of TAT or of 4HPPD, was present in the cytosol. TAT activity was normal in whole tissue, slightly lower than normal in cytosol and approximately twice normal in mitochondria. Mitochondrial TAT in tissues from three controls represented 11–17% of the total TAT activity whereas in the patient it was 19% of the total; however, the activity of glutamate dehydrogenase, a mitochondrial enzyme, was much higher in the patient's cytosol than in the controls, suggesting considerable rupture of mitochondria during the fractionation procedure. In order to investigate whether the kinetics of TAT were normal, Km values for both substrates and coenzyme were determined (Table 3).

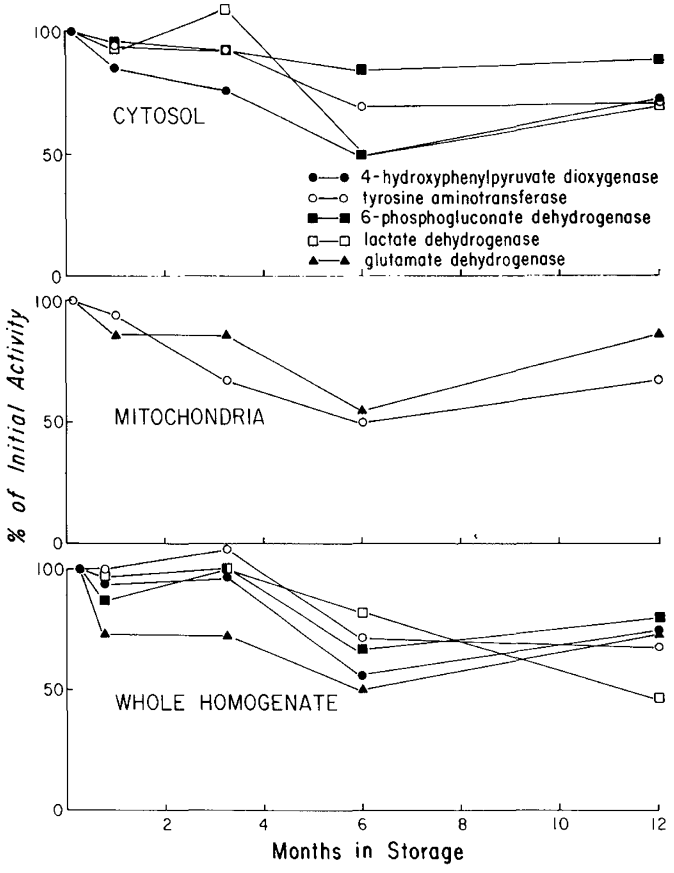

Fig. 1. Stability of hepatic enzymes to storage at −70°C.

DISCUSSION

Cytosolic TAT, the first and rate limiting enzyme of tyrosine metabolism, catalyses the transamination of tyrosine and α-ketoglutarate to pHPPA and glutamate and requires pyridoxal phosphate as coenzyme. In the mitochondria, tyrosine can be transaminated by aspartate aminotransferase (EC 2.6.1.1), which utilizes a wide range of substrates (21). It is not thought to be involved in normal tyrosine catabolism. The second enzyme in the pathway, 4HPPD, converts pHPPA to homogentisic acid. Absent activity of hepatic cytosol TAT (tyrosinemia type II) is associated with the Richner-Hanhart syndrome, tyrosinemia, and tyrosyluria. The eye and skin lesions respond rapidly to dietary tyrosine restriction. The enzyme defect was first described by Fellman *et al.* (9) in the patient subsequently reported by Kennaway and Buist (13); mitochondrial TAT and 4HPPD were normal. In this condition pHPPA, the product of the missing enzyme is thought to be formed by mitchondrial transamination of tyrosine in many tissues which lack the 4HPPD. The pHPPA thus formed would enter the blood stream and utimately be cleared by the kidney (6, 14).

Table 3. *Kinetic characteristics of cytosol tyrosine aminotransferase*

	Control	Patient
V max[1]	0.88	0.37
Km for tyrosine	4.0×10^{-3} M	4.5×10^{-3} M
Km for α-ketoglutarate	75×10^{-6} M	98×10^{-6} M
Km for pyridoxal phosphate[2]	4.0×10^{-6} M	2.1×10^{-6} M

[1] (μmole/mg protein/h).

[2] These values are approximations (see "Materials and Methods" section).

Table 2. *Enzyme activities in liver fractions*[1]

	Time in storage	Tyrosine amino-transferase	4-Hydroxyphenylpyruvate dioxygenase	LDH[2]	6PGD[2]	GDH[2]	Protein (mg/g wet wt)
Whole homogenate							
Control 177	18 months	0.13	0.43	24	1.1	2.2	172
Control 178	13 months	0.14	0.67	44	1.3	2.5	129
Control 186	11 months	0.10	0.61	44	2.3	1.7	126
Control 240	13 wk	0.27	0.31	39	1.5	1.6	137
Control 262	12 days	0.30	0.36	28	1.6	1.6	152
Patient K.W.	14 wk	0.23	undetectable	31	1.6	1.7	102
Cytosol							
Control 247	7 days	0.36	0.38	37	2.0	0.25	155
Control 240	13 wk	0.44	0.55	74	2.3	0.49	88
Control 262	12 days	0.27	0.64	55	1.7	0.34	97
Patient K.W.	12 wk	0.23	undetectable	38	2.2	2.5	68
Mitochondria							
Control 247	7 days	0.09				1.5	78
Control 240	13 wk	0.12				1.9	37
Control 262	12 days	0.12				2.7	42
Patient K.W.	12 wk	0.24				3.1	15

[1] Units of measurement for tyrosine aminotransferase, 4-hydroxyphenylpyruvate dioxygenase, LDH, 6PGD, and GDH: μmoles/mg protein/h.

[2] LDH, lactate dehydrogenase; 6PGD, 6-phosphogluconate dehydrogenase; and GDH, glutamate dehydrogenase.

Although many cases of tyrosinemia type II have been recognized, absent cytosol TAT activity has only been reported in one other patient (16); however, Goldsmith *et al.* (11) described a mildly retarded 55-year-old male with the Richner-Hanhart syndrome and tyrosinemia. Hepatic cytoplasmic TAT was half the mean control value and although within the control range, this was assumed to be responsible for the tyrosinemia. It seems to us unlikely that tyrosinemia would result from a reduction of only 50% of normal enzyme activity. These findings could however be explained by a mutant TAT with decreased affinity for substrate or cofactor, because the enzyme assays were presumably performed under saturating concentrations. Alternatively, this patient might represent a case of 4HPPD deficiency. Unfortunately, this enzyme activity was not measured.

Tyrosinemia and tyrosyluria were reported in another patient, aged 3 years, with developmental delay and seizures but no other manifestations of the Richner-Hanhart syndrome (4). Enzyme assays in cultured skin fibroblasts revealed normal cytosol TAT under saturating conditions, normal Vmax and Km for tyrosine, but markedly decreased Km for pyridoxal phosphate. 4HPPD was normal. This report is interesting, not only because of the unique nature of the enzyme defect but because enzymes were detectable for the first time in cultured skin fibroblasts. This conflicts with our failure to detect these enzymes in fibroblasts but this could be due to differences in the culture conditions or some other unknown factor. These reports illustrate the importance of studying enzyme kinetics in patients with apparently normal levels of enzyme activity.

Several other patients with tyrosinemia and tyrosyluria have been described who do not clearly fit the picture of tyrosinemia type I or II. The patient described by Louis *et al.* (19) was a 35-year-old male with only moderate mental retardation and marked tremor of the hands, head, and feet. Enzymatic studies of this patient were inconclusive but suggested a possible deficiency of 4HPPD activity (5). Wadman *et al.* (25) described a patient with severe mental retardation and cataracts; enzyme studies were not performed. Finally, Danks *et al.* (3, 22) reported a patient with mild prolonged, transient tyrosinemia who presented with severe metabolic acidosis in whom the unusual tyrosine metabolites, hawkinsin and cis- and trans-4-hydroxycyclohexylacetic acid were found in the urine. It was postulated that these metabolites were derived from an intermediate of the 4HPPD reaction and that the child and her mother were heterozygous for a defect of this enzyme which was able to oxidize and decarboxylate pHPPA but was unable to rearrange the intermediate to homogentisic acid. A second family, with five affected members in three successive generations, has recently been described (26).

The patient described here differs from all other reported patients with prolonged tyrosinemia because of the absence of any persistent clinical symptom. In particular, her development is normal, she does not have liver or kidney dysfunction, nor does she have the eye or skin lesions characteristic of tyrosine toxicity in the Richner-Hanhart syndrome. The cause of her transient ataxia and drowsiness is unknown although, on at least one occasion, it was associated with an increase in the concentration of serum tyrosine. The child's normal psychomotor development (she learned very quickly to speak Italian) indicated that the hypertyrosinemia *per se* cannot account for mental impairment. This has previously been suggested by Pelet *et al.* (23) with reference to four other patients (5, 10, 12).

Our patient has persistent hypertyrosinemia and tyrosyluria. The serum tyrosine was not modified by high doses of vitamin C nor, surprisingly, by a low protein diet. Strict adherence to this diet could not be verified and might explain this finding; in view of the mild clinical course, more severe tyrosine restriction was not felt warranted. The absence of activity of 4HPPD in liver is unlikely to be due to instability of this enzyme to freezing for 12 wk because enzymes in the control tissue showed little decline in activity over this period, whether stored as intact tissue or supernatant fraction. In all controls studied, 4HPPD activity was readily detectable even in tissues stored up to 18 months. The patient's liver showed no evidence of an inhibitor of this enzyme, suggesting that 4HPPD deficiency represents the primary enzyme defect in this form of tyrosinemia. 4HPPD catalyses a complex reaction which involves hydroxylation of the aromatic ring, decarboxylation, and rearrangement of the side chain. Because our assay method is based on the release of CO_2 from pHPPA, the defect in our patient must be at an earlier step of this enzyme activity than in Danks' patient who excreted hawkinsin and the hydroxycyclohexylacetic acids, metabolites presumed to be formed after hydroxylation and decarboxylation of pHPPA. This is consistent with our failure to demonstrate these metabolites in the urine from our patient. Absence of metabolic acidosis in our patient and most other patients with tyrosinemia suggests that the more commonly observed tyrosine metabolites, pHPPA, pHPLA and pHPAA, are cleared more rapidly by the kidneys than the acids which accumulate in Danks' patient.

The specific activity of TAT in mitochondria from the patient was twice normal, a finding previously observed in two patients with cytosol TAT deficiency (10, 15). Although TAT activity in cytosol was close to the lower limit of normal, the apparent rupture of a major proportion of mitochondria suggests that a considerable amount of the cytosol TAT activity could have derived from this source; thus, it is not possible to rule out at least a partial deficiency of cytosol TAT in this patient. It does however seem unlikely that this represents the primary enzyme defect, first because it is certainly not totally deficient and second because the kinetics of the enzyme in the patient were entirely normal.

In conclusion, we have described a patient with transient ataxia and drowsiness and no persistent clinical abnormality, who has tyrosinemia and tyrosyluria associated with deficient activity of hepatic 4HPPD. It is possible that several other patients, particularly those described by Wadman *et al.* (25), Faull *et al.* (5), and Goldsmith *et al.* (11) may have had a similar enzyme defect.

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