Type II Hyperprolinemia

Δ^1-PYRROLINE-5-CARBOXYLIC ACID DEHYDROGENASE DEFICIENCY IN CULTURED SKIN FIBROBLASTS AND CIRCULATING LYMPHOCYTES

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ABSTRACT Type II hyperprolinemia is an inherited abnormality in amino acid metabolism characterized by elevated plasma proline concentrations, iminoglycinuria, and the urinary excretion of Δ^1-pyrroline compounds. To define the enzymologic defect of this biochemical disorder, we developed a specific, sensitive radioisotopic assay for the proline degradative enzyme Δ^1-pyrroline-5-carboxylic acid dehydrogenase. Using this assay, we have shown an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity in the cultured fibroblasts from three patients with type II hyperprolinemia. We confirmed this result on cultured cells by demonstrating a similar absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity in extracts prepared from the peripheral leukocytes of these patients. Additionally, we found significantly decreased levels of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity in the leukocyte extracts from five obligate heterozygotes for type II hyperprolinemia. We also demonstrated a reduction in leukocyte Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity in three successive generations of a family.

These results prove that an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase is the enzymologic defect in type II hyperprolinemia and that this defect is inherited in an autosomal recessive fashion.

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INTRODUCTION

The hyperprolinemias are rare metabolic disorders caused by inherited biochemical abnormalities in the pathway of proline degradation (Fig. 1). Both type I hyperprolinemia (HP1)[1] and type II hyperprolinemia (HP2) are characterized by elevated plasma proline concentrations. The increase in renal filtered load of proline results in iminoglycinuria with increased urinary excretion of proline, hydroxyproline, and glycine (1). The distinguishing feature of HP2, however, is the urinary excretion of large amounts of Δ^1-pyrroline compounds. The presence of these compounds results in a characteristic orange color when the urine of HP2 patients is reacted with *o*-aminobenzaldehyde (OAB) (1).

The cause of HP1 has been shown to be a deficiency of proline oxidase, the first enzyme in the proline degradative pathway (2) (Fig. 1). In a preliminary paper we

[1] *Abbreviations used in this paper:* HP1, type I hyperprolinemia; HP2, type II hyperprolinemia; OAB, *o*-aminobenzaldehyde; PC, Δ^1-pyrroline-5-carboxylic acid; 30HPC, Δ^1-pyrroline-3-hydroxy-5-carboxylic acid.

TABLE I

Clinical and Laboratory Features of Type II Hyperprolinemia Patients

	Age	Sex	Clinical findings	Plasma proline	Urine amino acids			Urine OAB reaction	Other
					Proline	Hydroxy-proline	Glycine		
	yr			*μmol/ml*	*μmol/mg creatinine*				
E. D.	12	F	Normal	1.66–2.53	26.9	1.7	9.5	+	—
M. B.	5	M	Normal	2.76	41.7	2.3	23.1	+	Abnormal EEG
K. K.	3	F	Normal	3.16	72.8	—	21.0	+	—
G. F.	8	M	Normal	1.9	9.0	—	—	+	Abnormal EEG
Normals				0.07–0.15	0	0	<1.9	−	—

reported an absence of Δ^1-pyrroline-5-carboxylic acid dehydrogenase (PC dehydrogenase) in the cultured skin fibroblasts of a single individual with HP2 (3). In the present report we extend this observation to include the fibroblasts from three patients with HP2. Furthermore, we verify the findings in cultured cells by demonstrating the absence of PC dehydrogenase in leukocyte extracts from these patients. Additionally, we show intermediate levels of enzyme activity in the leukocyte extracts of asymptomatic obligate heterozygotes and thus verify the autosomal recessive inheritance of this biochemical abnormality.

METHODS

Patient material. The pertinent data describing our four patients is presented in Table I. Three of these patients

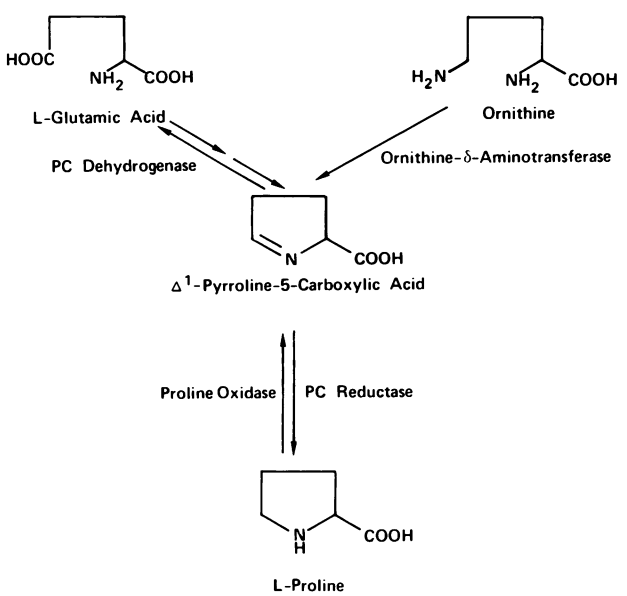

FIGURE 1 The biosynthetic and degradative pathways of proline metabolism. The enzyme(s) converting glutamic acid to PC are not defined in higher organisms. Although ornithine-δ-aminotransferase is theoretically reversible, the equilibrium constant is 70-fold in favor of PC; therefore, for clarity the reaction is shown as irreversible.

(G. F., M. B., and E. D.) have been previously reported in detail (4–6). The fourth patient (K. K.) was discovered as a neonate by the prospective Massachusetts State Newborn Screening Program. All of our patients are presently functioning as normal children.

Fibroblast cultures. Fibroblasts were cultured from punch skin biopsies by standard techniques. All cells were grown in Eagles' minimal essential media (Gibco Diagnostics, The Mogul Corp., Chagrin Falls, Ohio) with added nonessential amino acids and 10% fetal calf serum (Gibco Diagnostics). Cell cultures were periodically checked for mycoplasmal contamination by the method of Hayflick (7) and were negative throughout the period of study. Antibiotics were not used.

For biochemical studies late log-phase cells were harvested by scraping with a soft rubber policeman and collected by centrifugation. The cell pellet was washed three times with phosphate-buffered saline and sonicated with the micro tip of a Branson model W180 sonicator (Branson Sonic Power, Co., Danbury, Conn.) at a setting of 2–3 for 40 s in 1 ml buffer appropriate for the enzyme assay. The enzyme activity of these extracts was usually assayed on the day of preparation although activity was stable for at least 4 wk at − 20°C.

Leukocyte extract preparation. Purified leukocytes were prepared from 10 ml fresh heparinized (10 U/ml) blood by dextran sedimentation and osmotic lysis of erythrocytes (8). The leukocytes were washed three times with phosphate-buffered saline and sonicated for 40 s in 1 ml of 50 mM Tris buffer pH 8.2.

Enzyme assays. We devised a specific radioisotopic assay for PC dehydrogenase similar to our previously reported assay for PC reductase (9). The standard reaction mixture, as modified from that of Strecker (10), was pH 8.2 and included 50 mM Tris, 1 mM Na_2EDTA, 360 μM NAD^+, 72 μM PC, 0.12 μCi of [U-^{14}C]PC, and 10–50 μg protein in a final volume of 0.25 ml. The [U-^{14}C]PC was prepared enzymatically from [U-^{14}C]ornithine (New England Nuclear, Boston, Mass.) using partially purified ornithine-δ-amino transferase according to our previously published method (11). After incubation at 30°C for 30 min in a Dubnoff shaker, the reaction was stopped by the addition of 0.05 ml 6 N HCl. 0.2 ml of this acidified reaction mixture was then mixed with an equal volume of a solution of OAB (10 mg/ml in 1 N HCl and 10% ethanol). The OAB quantitatively combines with unreacted PC forming a dihydroquinazolinium compound which remains at the top of a Dowex-50W column (150 × 5 mm) while the reaction product, [U-^{14}C]glutamate, is eluted with 1 N HCl (9). The glutamate elutes as a peak between 7 and 12 ml eluent. A 3-ml aliquot of this 5-ml fraction is mixed with 12 ml

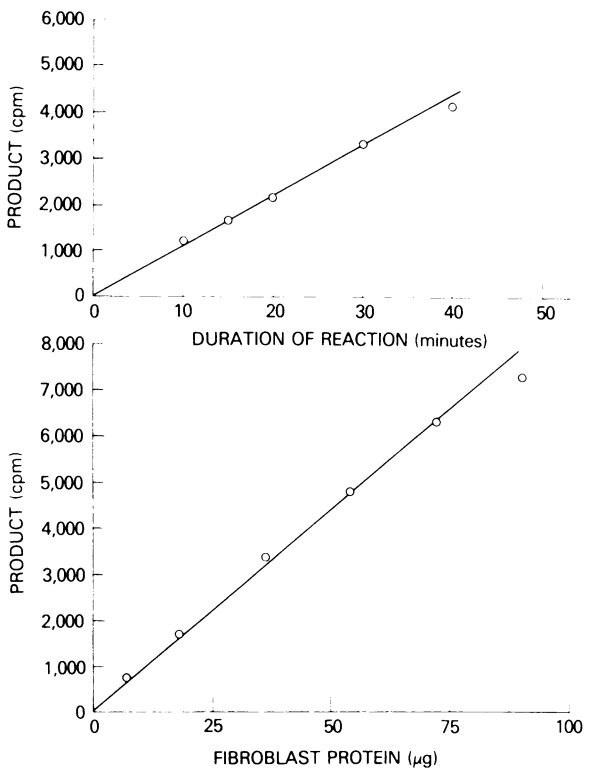

FIGURE 2 Formation of product glutamate vs. duration of reaction and quantity of fibroblast protein. In the top panel linearity of recovered product with increasing duration of reaction is shown in a standard reaction mixture containing 52 μg fibroblast protein. In the bottom panel linearity of recovered product with increasing amounts of fibroblast protein in a standard reaction mixture incubated for 30 min at 30°C is shown. In both panels, each point represents the mean of three separate determinations.

Aquasol (New England Nuclear) and the radioactivity is quantitated in a liquid scintillation counter. Collection of product glutamate by this method recovers 89±3% of the glutamate added to the column. Reaction blanks lacking either enzyme or NAD^+ were identical and yielded 275–300 cpm. At usual protein concentrations, a complete reaction mixture results in a 10-fold increase in counts per minute recovered over blank. Using this assay we are able to detect as little as 0.2 nmol of product.

PC reductase, ornithine-δ-aminotransferase, and proline oxidase were assayed as previously described (9, 11, 12). Protein was determined by the method of Lowry et al. (13). DNA was determined by the method of Burton (14).

RESULTS

Fibroblast PC dehydrogenase activity. PC dehydrogenase was readily detectable in extracts of normal human fibroblasts. This activity was stable for 1 mo at −20°C and was not affected by freezing and thawing.

At standard reaction conditions of pH 8.2 and 30°C, enzyme-dependent production of product [^{14}C]glutamate increased linearly with increasing amounts of added fibroblast protein from 5 to 70 mg (Fig. 2). Also with standard amounts of fibroblast protein, product formation increases linearly for up to 30 min (Fig. 2). The sensitivity of this method allows several assays to be performed on an extract of as few as 10^7 fibroblasts.

PC dehydrogenase in HP2 fibroblasts. PC dehydrogenase activity was completely absent in extracts of HP2 fibroblasts with an assay able to detect as little as 0.2 nmol product (Fig. 3). Addition of as much as 180 μg HP2 fibroblast protein to a standard assay mixture did not increase the [^{14}C]glutamate radioactivity recovered above either NAD^+ or enzyme blanks. Comparable amounts of control fibroblast protein produced a greater than 25-fold over blank increase in recovered radioactivity. Furthermore, 100-fold increases in PC concentration to levels well above the K_m or substitution of $NADP^+$ for NAD^+ as cofactor also were ineffective in increasing HP2 fibroblast PC dehydrogenase activity above blank.

The possibility of an inhibitor causing the absence of PC dehydrogenase activity in HP2 cells was eliminated by an experiment in which the addition of 93 μg HP2 protein to an assay mixture containing 16 μg normal fibroblast protein did not alter the activity of the normal PC dehydrogenase enzyme (3).

We also considered the possibility that the apparent lack of activity in HP2 extracts resulted from the con-

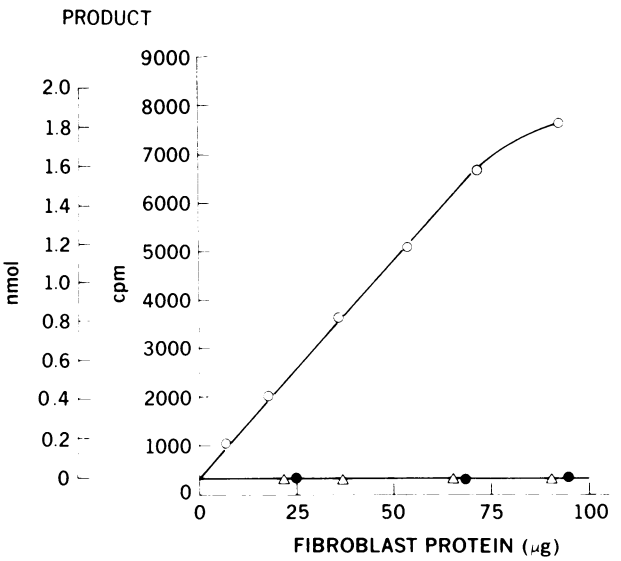

FIGURE 3 The formation of product [^{14}C]glutamate by extracts of control and HP2 fibroblasts. Increasing amounts of control (○) or of HP2 (●) fibroblast protein were incubated in a standard reaction mixture for 30 min. The amount of product recovered is shown on the ordinate as both counts per minute and as nanomoles. The counts per minute recovered in the reaction blanks lacking NAD^+ are also indicated (Δ). Each point represents the mean of three separate determinations.

TABLE II

Enzyme Activity in Fibroblast Extracts

Extract	PC dehydrogenase	Ornithine-δ-amino-transferase	PC reductase
	nmol/mg protein/h		
Type II hyperprolinemia patients			
E. D.	0	163	544
M. B.	0	140	472
K. K.	0	168	427
Normals	38.0±4.7	156±19	550±55

Late log-phase fibroblasts were sonicated in buffer appropriate for the enzyme to be assayed. All assays involved radioisotopic methods using 5–50 μg fibroblast protein in a final reaction volume of 0.25 ml. Reaction product was separated from precursor on Dowex-50 columns (150 × 5 mm) eluted with 1 N HCl. For assay of ornithine-δ-aminotransferase activity, the conversion of [^{14}C]ornithine to [^{14}C]PC in 0.1 M KPO_4, pH 8.0, at 37°C was measured. For assay of PC dehydrogenase activity, the conversion of enzymatically prepared [^{14}C]PC to [^{14}C]glutamate in 0.05 M Tris, pH 8.2, at 30°C was measured using NAD^+ as cofactor. For assay of PC reductase activity, the conversion of enzymatically prepared [^{14}C]PC to [^{14}C]proline in 0.1 M KPO_4, pH 6.8, at 37°C was measured using NADH as cofactor. Each value of enzyme activity represents the mean of at least three separate determinations. The normal values are the means ±1 SD of multiple determinations on six control skin fibroblast lines.

sumption of product glutamate by HP2 extracts but not by control extracts. We examined this possibility by adding [^{14}C]glutamate and NADH (0.01 mM) to standard reaction mixtures containing either HP2 or control fibroblast extract. In both, 90% of the glutamate added at the beginning of the reaction was recoverable at the end of the reaction. This result rules out excessive consumption of product glutamate in HP2.

Specificity of the enzyme defect in HP2 fibroblasts. To ensure that the absence of PC dehydrogenase was a specific defect in HP2 fibroblasts, we compared the activity of two proline biosynthetic enzymes, ornithine-δ-aminotransferase and PC reductase, in HP2 and control fibroblasts (Table II). Activities of these two enzymes did not differ significantly in HP2 and control cells. We also assayed control and HP2 extracts for proline oxidase, the enzyme deficient in HP1. Unfortunately, we were unable to detect proline oxidase activity in control cells despite the use of an assay capable of detecting as little as 100 pmol of product (12). Additional attempts at measuring proline oxidase using concentrated mitochondrial preparations from control cells yielded similar negative results.

Leukocyte PC dehydrogenase. To verify and extend our findings in cultured fibroblasts, we assayed PC dehydrogenase activity in extracts of fresh peripheral leukocytes obtained from individuals with HP2 and members of their families (Fig. 4). As with fibroblasts, leukocytes from three individuals with HP2 lacked PC dehydrogenase activity. This result verifies our observations on HP2 cells cultured in vitro. Furthermore, even though their plasma proline concentrations were normal (4–6), all five obligate heterozygotes for HP2 and one of two maternal grandparents in the E. D. family had leukocyte PC dehydrogenase activity at least 2 SD below the normal leukocyte mean (Fig. 5). These results prove the autosomal recessive inheritance of HP2.

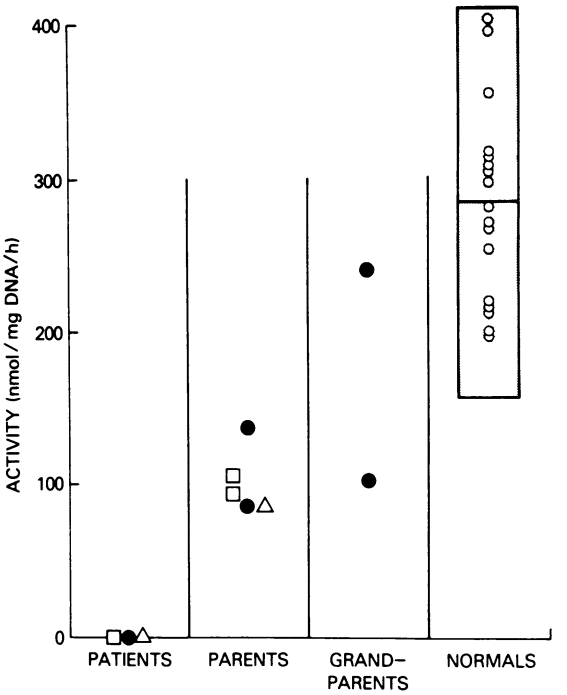

FIGURE 4 Leukocyte PC dehydrogenase activity in normals and in members of three families with HP2. The mean of the normal values (284 nmol/mg DNA per h) ±2 SD is indicated by the shaded rectangle overlying the normal values. Each point represents the mean of three separate determinations. The symbols refer to normals (○), E. D. kindred (●), G. F. kindred (□), and M. B. kindred (△).

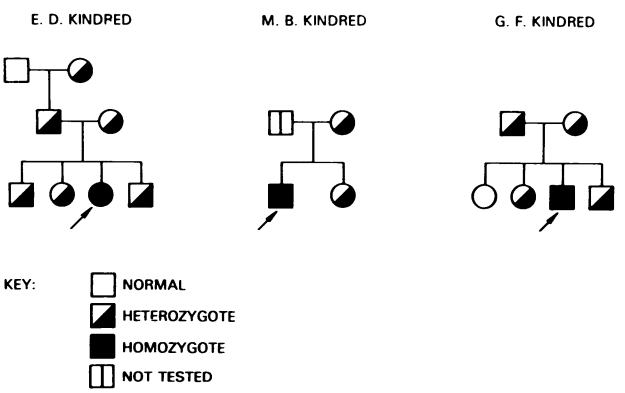

FIGURE 5 Pedigrees of three families with HP2. The genotypic designations are based on assays of leukocyte PC dehydrogenase activity. The affected individuals had no demonstrable activity, whereas the activity of the heterozygotes ranged from 85 to 130 nmol/mg DNA per h.

DISCUSSION

HP2 is a rare, inherited, metabolic disorder of proline degradation. The biochemical abnormalities of this condition include elevated plasma proline concentrations, iminoglycinuria, and the urinary excretion of OAB-reactive material. Our results clearly show an absence of PC dehydrogenase activity in the cultured skin fibroblasts and fresh peripheral leukocytes of HP2 patients. The metabolic block resulting from this enzymatic defect explains the biochemical abnormalities described in HP2. PC derived from either proline or ornithine accumulates because of the block in conversion of PC to glutamate (Fig. 1). A portion of the accumulated PC is excreted in the urine and results in the positive urine OAB reaction diagnostic of HP2. The bulk of the accumulated PC, however, is converted to proline by PC reductase resulting in markedly elevated plasma proline concentrations.

The inheritance of HP2 is clearly autosomal recessive. The leukocyte PC dehydrogenase activity of the heterozygotes is approximately 40% that of normals and thus allows heterozygote determination. This level of activity is apparently sufficient under usual conditions to maintain the plasma proline concentrations in the normal range. When stressed with a proline load, however, the heterozygotes, as shown by Similä (15), do demonstrate decreased proline tolerance in comparison with controls.

The ratio of the intracellular concentrations of PC and proline in patients with HP2 probably reflects the activities of PC reductase and proline oxidase. PC reductase in liver and kidney has a two to three times higher maximal velocity and a 10-fold lower K_m for PC (0.2 mM) than proline oxidase has for proline (2 mM), thus favoring the accumulation of proline[2] (16). Furthermore, PC reductase in liver and kidney is relatively insensitive to inhibition by proline (16–18) so the high proline concentration in HP2 should not prevent conversion of PC to proline. Additionally, preliminary data suggests that PC is a potent inhibitor of proline oxidase.[3] Thus, elevation of intracellular PC concentration decreases the conversion of proline to PC. The net result of these enzymologic and regulatory features is to favor the accumulation of proline over PC.

Interestingly, patients with HP2 have higher plasma proline concentrations than those with HP1 (1). This difference in proline accumulation may be explained by a partial rather than a complete deficiency of proline oxidase in HP1. Enzyme assays, in a single patient with HP1, however, indicated that the defect was virtually complete (2). A more likely explanation for the higher plasma proline concentrations in HP2 involves the contribution from ornithine to the proline pool in HP2. Normally, a significant amount of ornithine may be converted to PC by ornithine-δ-aminotransferase and thence to glutamate by PC dehydrogenase (19). This pathway is not interrupted in HP1. In HP2, however, with a block in the conversion of PC to glutamate, PC derived from ornithine will accumulate and, in turn, add to the proline pool. In support of the significance of this contribution by ornithine to PC, Similä and co-workers (20) have shown a 10 to 20-fold increase in urine OAB-reactive material after an ornithine load in patients with HP2. Thus, in HP2, the proline pool derives not only from undegraded proline but also from ornithine, and therefore it is reasonable to expect that proline concentrations will be higher in HP2 than in HP1.

The urinary excretion of OAB-reactive material is characteristic of HP2. This OAB-reactive material was initially thought to be PC, but recent evidence shows that the majority of the OAB-reactive material is Δ^1-pyrroline-3-hydroxy-5-carboxylic acid (30HPC), the oxidized degradative product of hydroxyproline (5, 6). This observation suggests an abnormality of hydroxyproline degradation in HP2. Furthermore, on the basis of co-purification of the dehydrogenase activities for PC and 30HPC, Adams and Goldstein have previously suggested (21) that a single enzyme catalyzes the oxidation of both PC and 30HPC. We have taken advantage of HP2 as a naturally occurring mutant and have recently obtained evidence proving a complete block in oxidation of 30HPC to γ-hydroxyglutamate in HP2 (22). This result provides strong genetic evidence for a single dehydrogenase catalyzing the oxidation of both PC and 30HPC.

Although significant amounts of PC and 30HPC are excreted by patients with HP2, these substances are undetectable in their plasma (6). Thus, the source of the urinary PC and 30HPC may be from renal metabolism of proline and hydroxyproline. Holtzapple and coworkers (23) have shown in human kidney cortex slices that 90% of proline transported by the tubule cells is rapidly metabolized to glutamate. Presumably, intracellular metabolism is equally important for hydroxyproline transported by the kidney. In HP2 the kidney tubule cells are exposed to saturating concentrations of proline and hydroxyproline. In HP2, however, the degradation of these amino acids is blocked. Thus, the tubule cells must accumulate large amounts of 30HPC and PC which could diffuse back into the tubule lumen and appear in the urine.

The clinical implications of HP2 are still unclear (1, 24). Possible bias resulting from the method of patient ascertainment prevents a clear delineation of the clinical phenotype. Two of our four patients (E. D. and M. B.) were diagnosed during evaluation for transient behavior problems. Another (G. F.) was diagnosed dur-

[2] Phang, J. M., S. J. Downing, D. Valle, and E. Kowaloff; unpublished results.

[3] Phang, J. M., and S. J. Downing; unpublished results.

ing a work-up for focal seizures associated with fever. Our fourth patient (K. K.) was ascertained by the mass newborn screening program in Massachusetts. Despite moderate dietary protein restriction, her plasma proline concentrations have remained markedly elevated. At age 3 yr, she has normal developmental milestones and has not had seizures. All of our patients have had normal physical growth and have I.Q.'s in the normal to low-normal range (4–6). Thus, HP2 certainly is not a cause of severe illness. Long-term prospective study of patients found by mass screening will be necessary to delineate completely the clinical significance of HP2.

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