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A Microassay for Uroporphyrinogen I Synthase, One of Three Abnormal Enzyme Activities in Acute Intermittent Porphyria, and its Application to the Study of the Genetics of this Disease*

(spectrofluorimetry/erythrocytes)

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ABSTRACT **A new spectrofluorometric assay is described for quantitating uroporphyrinogen I synthase (EC 4.3.1.8) activity in volumes of human blood as small as 2 μl. By this sensitive assay the inheritance of the enzyme's activity has been studied and the genetic defect for acute intermittent porphyria has been confirmed to be autosomal dominant in nature. There is a 3-fold range of uroporphyrinogen I synthase activity in erythrocytes in the normal population, with a mean V_{max} ± SD of 35.7 ± 8.4 nmol of uroporphyrinogen I formed per ml of erythrocytes per hr, at 37°. One-half this level of enzyme activity (18.0 ± 5.0) is found in erythrocytes from patients with clinically manifest acute intermittent porphyria; and in erythrocytes from those of their relatives, including prepubertal children, who have the latent gene defect for the disease. The K_m of erythrocyte enzyme of normal people is 12.3 ± 3.9 μM, whereas the K_m of the erythrocyte enzyme of patients with acute intermittent porphyria is 6.2 ± 3.9 μM, as determined on whole blood lysates. Three enzymic changes have now been identified in patients with acute intermittent porphyria; a high level of δ-aminolevulinate synthase activity; a low level of uroporphyrinogen I synthase activity; and a deficiency of steroid Δ^4-5α reductase activity.**

Acute intermittent porphyria (AIP) is a genetically determined liver disease that is characterized clinically by a disabling neurological–visceral symptom complex and biochemically by the over-production, and excretion into urine, of the porphyrin precursors, δ-aminolevulinic acid (ALA) and porphobilinogen (PBG). Granick and Urata (1) demonstrated that δ-aminolevulinate synthase (EC 2.3.1.37; ALA synthase) is the rate-limiting step in hepatic porphyrin synthesis; and subsequently Tschudy *et al.* (2) and Nakao *et al.* (3) showed that AIP patients had substantially higher than normal levels of this mitochondrial enzyme activity in their livers.

The fact that excessive amounts of porphyrin precursors rather than porphyrins are produced in AIP suggested the idea (4) that, in addition to elevated levels of hepatic ALA-synthase activity, there is in this disease a biochemical defect of the porphyrin pathway at an enzymatic site beyond ALA synthase (4–6). This suggestion was confirmed when Strand *et al.* (7) demonstrated low levels of uroporphyrinogen I synthase (EC 4.3.1.8; URO synthase activity) in the livers of patients with AIP. Meyer *et al.* (8) later reported that URO synthase activity was also diminished in the erythrocytes of such individuals. This enzymatic deficiency results in impaired conversion of PBG to porphyrins and explains the excessive excretion of ALA and PBG in AIP patients.

The recent studies of Kappas and associates (9, 10) have defined a third enzymatic lesion in AIP, involving a deficiency of steroid Δ^4-5α-reductase activity which leads to the disproportionate increase in the ratio of 5β to 5α steroid metabolites from natural steroid hormones. 5β steroid metabolites are potent inducers of hepatic ALA synthase experimentally (11–13). The 5α-reductase deficiency found in patients with AIP, coupled with the dramatic increase in steroid hormone production which occurs at puberty, may thus be critical in determining clinical expression of this genetic disease postpubertally.

Studies of the inter-relationships of the three enzymatic abnormalities so far identified in patients with AIP are of central importance to an understanding of the pathogenetic mechanisms of this hereditary disorder. To facilitate such studies, we have developed a highly sensitive micromethod for assaying the level of URO synthase activity in circulating erythrocytes. This method, which can be performed on 2 μl of blood, is suitable for the mass screening of individuals, including infants, suspected of carrying the potential for AIP. In this report, we have utilized this microassay to establish the range of erythrocyte URO synthase activities in normal subjects and in clinically manifest AIP patients, to identify individuals, including one as young as 5 months of age, with latent AIP, and to confirm the autosomal dominant transmission of the URO synthase gene defect for this disease. In addition, in studies of steroid Δ^4-5α-reductase and URO synthase activities in one propositus-parent set, evidence has been obtained that indicates that the level of steroid Δ^4-5α-reductase activity in humans is, in part, determined by genetic influences.

METHODS AND CLINICAL MATERIAL

Microassay for URO Synthase Activity in Erythrocytes. Blood was collected by finger-stick into a heparinized glass capillary (Micro-Natelson Blood Collecting Tube, 280 μl volume, Sherwood Med. Ind. Inc.) and ejected into a 0.3-ml plastic test tube with a captive plug. Blood (200–300 μl) can be readily collected in this manner. The hematocrit was deter-

Abbreviations: AIP, acute intermittent porphyria; PBG, porphobilinogen; ALA, δ-aminolevulinic acid; URO, uroporphyrinogen I; F.U., fluorescence unit.

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mined with a pre-cal microhematocrit capillary tube (no. 1025 Clay Adams). Blood was usually stored in liquid nitrogen, although this is not essential since URO synthase activity in whole blood is stable for many weeks even when stored at −20°.

The activity of URO synthase was determined by a modification of the micromethod of Granick *et al.* (14). In a 1-ml disposable glass test tube (6 × 50 mm, Kimble Products) used both for the reaction and as a cuvette, 2 μl of frozen–thawed whole blood lysate was mixed with 25 μl of PBG solution in 0.1 M phosphate buffer (pH 7.4). To determine the V_{max} and K_m of the enzyme, concentrations of PBG were used in the range from 10 to 500 μM. The V_{max} and K_m were determined for all the blood samples reported here. Incubations were carried out in a water bath in a tightly sealed chamber at 37° for 1 hr in the dark. The reaction was stopped by freezing the tube. Porphyrins were extracted by the method of Sassa *et al.* (15). Briefly, 300 μl of ethylacetate–acetic acid (2:1, v/v) mixture was added to the tube and the contents were quickly mixed on a vibrator. 300 Microliters of 0.5 N HCl were then added and the tubes were again mixed. After 5 min to permit the two phases to separate, the tubes were inserted into a semi-microcell holder of a Hitachi–Perkin Elmer MPF2A spectrofluorometer so that the exciting beam traversed the lower aqueous phase, and the fluorescence was determined as described (15). Under these conditions, 86% of uroporphyrin, 83% coproporphyrin, and 80% of protoporphyrin were extracted into the aqueous phase.

Products of the reaction were mainly uroporphyrin with some coproporphyrin and negligible amounts of protoporphyrin. Since the fluorescence intensity and recovery of uroporphyrin and coproporphyrin in the aqueous phase under these conditions were quite close, the activity of the enzyme was expressed as the rate of formation of uroporphyrin in nmol/ml of erythrocytes per hr, at 37°. Blank fluorescence readings were less than 2 fluorescence units (F.U.) from 0 to 50 μM PBG; 10 F.U. at 100 μM PBG; and 60 F.U. at 500 μM PBG. An F.U. is defined as 0.01 division of the full-scale recording on a chart 23 cm in height at maximal sensitivity. Sample readings were in the range of 1000 to about 3000 F.U. This assay has the advantage over previously published methods such as that of Strand *et al.* (16) because it requires only 2 μl of blood. Its sensitivity is also greatly increased by the use of the two-step extraction which removes heme and protein from the aqueous phase; these would otherwise interfere with the assay. The V_{max} and K_m were calculated on a Hewlett Packard Calculator model 9810A by using an equation of $1/V = M(1/S) + B$, where V_{max} is $1/B$ and K_m is M/B. The correlation coefficient of the regression line was 0.97 ± 0.02.

The reaction was linear for at least 2 hr at 37°. In a 2-hr. incubation, about 10% of the PBG was consumed at the lowest PBG concentration used; of this amount 95% was converted to porphyrin. The reaction was also linear with the amount of blood added to the tube up to at least 5 μl of whole blood per tube. URO synthase activity is entirely localized in the erythrocytes and not in the plasma. The presence of normal or porphyric plasma did not interfere with the assay. Porphyrinogens were sufficiently autooxidized by room light during the extraction procedure so that further exposure of the sample to ultraviolet light, or addition of I_2, did not increase the fluorescence. Variations in the duplicate assays were less than ±5% at all concentrations of PBG. PBG used in this assay was kindly supplied by Dr. Jürgen Fuhrhop of the Institut für Molek. Biol. Bioch. u. Bioph., Stockheim, W. Germany, and its purity was more than 95%, as confirmed by chromatographic and spectroscopic criteria.

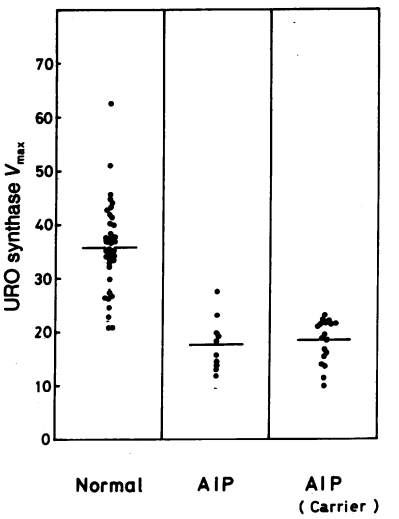

FIG. 1. Human erythrocyte URO synthase activities from 37 normal subjects, 10 patients with AIP, and 18 individuals with low URO synthase but without overt AIP. The enzyme activity (V_{max}) is expressed as nmol of URO per ml of erythrocytes per hr at 37°. The *line* represents the mean of each group.

Clinical Material. The total number of subjects tested was 114; these consisted of 37 normal subjects, 10 patients with clinically manifest AIP, and 67 siblings and other relatives of these patients. Among these relatives of patients were 18 individuals who were totally asymptomatic but who had abnormally low levels of URO synthase activity in their erythrocytes (Fig. 1, AIP carriers). Nine of these individuals with low URO synthase activity were prepubertal children, including one child 5 months of age and one child 7 years of age who was ingesting phenobarbital and dilantin for treatment of epilepsy. The data on 49 members of the patients' relatives who had normal values for URO synthase activity (mean ± SD, 39.7 ± 10.4) are not charted in the normal group in Fig. 1 or in Table 1. The data shown for the normal group are thus derived from individuals totally unrelated to the AIP subjects.

RESULTS AND DISCUSSION

Erythrocyte URO Synthase. Range of Activities in Normal and AIP Subjects. The levels of URO synthase activity, expressed as the V_{max} of the enzyme in 37 normal subjects, had a 3-fold spread from 20.7 to 62.5 nmol of URO formed per ml of erythrocytes per hr, at 37°, with the mean normal value ± SD being 35.7 ± 8.4 (Fig. 1). In 10 patients with clinically manifest AIP the enzyme activity ranged from 11.7 to 27.3 nmol of URO formed per ml of erythrocytes per hr, at 37° with a mean value ± SD of 18.0 ± 5.04. A paired *t*-test between the normal and clinically expressed AIP groups was highly significant ($P < 0.001$), confirming the findings of Meyer *et al.* (8) and Strand *et al.* (16).

TABLE 1. *Erythrocyte uroporphyrinogen I synthase activity (nmol of uroporphyrinogen per ml of erythrocytes per hr, at 37°) in families with acute intermittent porphyria*

Family		Erythrocyte URO synthase activity, V_{max}	Family	Erythrocyte URO synthase activity, V_{max}
Subject, sex, age			Case no., sex, age	
Family A			Family F	
Propositus	F 31	20.9*	1 M 65	13.1†
Husband	32	37.9	3 F 63	16.0†
Son 1	10	46.4	4 Father 64	33.3
2	2	22.2†	5 Mother 61	22.0†
			6 M 59	31.9
Family B			15 F 33	21.5†
Propositus	F 46	27.3*	17 F 33	16.4†
Sister	45	47.9	18 F 28	14.9†
			20 Propositus F	15.3*
			21 F 25	35.1
			22 F 32	21.2†
Family C			23 M 34	45.5
Propositus	F 31	11.7*	26 F 32	43.5
Husband	36	20.7	27 F 28	38.8
Son	6	9.5†	28 M 22	29.9
			29 F 17	55.9
			30 F 11	25.9
			31 F 9	33.3
Family D			32 F 6	31.0
Propositus	F 25	14.0*	33 M 4	26.4
Father	55	20.0	37 F 7	19.1†
Mother	57	10.9†	38 M 5	38.7
			39 F 4	21.8†
			40 M 9	18.8†
			41 M 7	22.8†
Family E			42 M 2	18.3†
Propositus	F 28	19.2*	43 M 12	35.0
Father	60	22.1†	44 F 10	21.3†
Mother	58	43.0	45 M 9	13.4†
			48 M 16	34.8
			49 F 12	44.6
			50 F 3	41.7

* Active AIP.
† Carrier AIP.

It is evident from Fig. 1 that the mean value of URO synthase activity in the AIP group is about 50% of that of normal subjects and that both groups have an approximately 3-fold spread in the enzyme activity values. There is a small but clear overlap in values for URO synthase activity between AIP subjects with the highest URO synthase levels, and normal subjects with the lowest URO synthase activities. We have also obtained evidence that a 3-fold spread of this enzyme activity in normal individuals reflects genetic variation (17, 18). Evidence that individuals with AIP (with overt or latent disease) having URO synthase activities that extend upwards into the lower limits of the normal range, do in fact, have a gene defect relating to this enzyme is indicated by the data in Table 1. In the AIP carriers or patients shown, levels of erythrocyte URO synthase activity were approximately one-half those of a normal parent, or normal sibling, thus confirming that AIP subjects carry a defective gene for URO synthase. AIP subjects can thus still have an enzyme value falling into the lowest normal range if the subject's family lineage is characterized by high URO synthase levels (families A, B, and E in Table 1). Conversely, eight normal subjects with low URO synthase values falling into the upper range of the AIP group, can be confirmed to have no gene defect for the enzyme, by family data like those shown for families C and D in Table 1. For example, the husband in family C has a low URO synthase value for normal subjects; however his son, as well as the propositus wife with manifest AIP, have a considerably lower URO synthase activity. The son is apparently getting one dose of the defective gene from his symptomatic mother. A comparable situation prevails in family D in which the father is normal although he has an unusually low level of URO synthase activity, while the propositus daughter with active AIP appears to have inherited the URO synthase gene defect from her mother, who is an asymptomatic AIP carrier.

Gene Carriers of AIP. Sixty-six relatives of the 10 patients with clinically overt AIP were studied; of these relatives, 18 were found to have low URO synthase levels (9.4–22.8 nmol of URO per ml of erythrocyte per hr, at 37°) similar to those of clinically active AIP patients and a total of 48 had URO synthase levels within the normal range (25.9–71 nmol of URO per ml of erythrocytes per hr, at 37°). The former 18 subjects thus represent carriers of the latent gene defect for AIP; they included one each of three sets of living parents of the 10 active AIP patients studied and 15 other blood relatives including children, siblings, aunts, and uncles. Nine children, ranging in age from 5 months to 12 years were included in this group. Thus, low erythrocyte URO synthase activity in AIP families extends to prepubertal children, a group known not to have the clinically manifest disease. Low URO synthase activity, therefore, is a valuable genetic marker for this inherited disorder, but does not of itself distinguish individuals having clinically manifest AIP from those in whom the disease is completely latent.

K_m of URO Synthase. It was found that the apparent K_m values for the erythrocyte enzyme in 10 AIP patients (6.2 ± 3.9 μM) were only one-half those of the normal values (12.3 ± 3.9 μM) when whole blood lysates were used for enzyme determinations. Strand *et al.* (16) reported, however, that the K_m for the enzyme in the washed erythrocyte lysate as well as in the partially purified erythrocyte enzyme from both sources was 6 μM. These differences suggest that there may be a factor in the plasma that affects the affinity of the enzyme for the substrate. However, we found that the porphyric plasma had no effect on the URO synthase activity in normal erythrocytes. We partially purified the URO synthase from normal and AIP subjects, but found no structural differences between them in electrophoretic mobility or resistance to heat. URO synthase activity from both sources migrated to 0.26 times the distance that the tracking dye, bromophenol blue, migrated when they were subjected to electrophoresis in 7.5% polyacrylamide gel in Tris-glycine buffer (pH 8.4). A half-denaturation time for URO synthase activity at 70° was 45 and 50 min for two normal subjects; and 45 min for enzymes from three AIP patients. Work with purified enzymes may provide more definitive data.

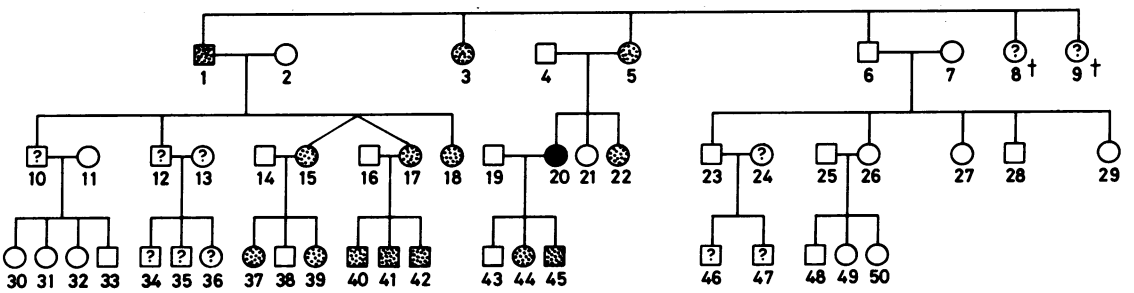

FIG. 2. Study of erythrocyte URO synthase activity in a family with AIP. This family is Family F in Table 1; cases 15 and 17 are fraternal twins. (□) Males; (○) females; (□, ○) individuals with low URO synthase activities; (●) female propositus.

Autosomal Dominant Inheritance of Low URO Synthase Activity. Fig. 2 shows a family study in which 63 members of an AIP lineage were examined. The lineage included the propositus (case 20), her three children (cases 43, 44, and 45), her parents (cases 4 and 5), her siblings (cases 21 and 22), and 31 other relatives examined out of 49 individuals on the maternal side; and 13 relatives examined on the paternal side. Fig. 2 shows the 49 members of the maternal lineage and the father. URO synthase levels in the father and all paternally related individuals were normal, and these individuals (except for the father) are not included in Fig. 2. The propositus and her mother both had low URO synthase levels, as did two of her prepubertal children and one of her two sisters. Among the siblings of her mother, two of four had low URO synthase levels; and of a total of 26 of their children and grandchildren studied, 11 had low URO synthase levels in circulating erythrocytes. In Fig. 2, a total of 14 individuals with low URO synthase activities are shown. These individuals were all entirely asymptomatic; and out of six children and three adults studied, only two (adults) had elevated ALA and PBG in urine. No prepubertal children displayed any biochemical evidences of AIP, regardless of the fact that some of them had low URO synthase activities in the range characterizing clinically active AIP patients. Case 37, the 7-year-old child, is of special interest since she had at the time of study been treated with dilantin and phenobarbital for $2^1/_2$ years for epileptic seizures. Nevertheless, she was completely asymptomatic and her urinary excretion of ALA and PBG was within normal limits.

URO synthase activity in the propositus did not vary beyond ± 7% on three different occasions over a $1^1/_2$ years, including periods of both relapse and remission of her disease. Thus URO synthase activity is not correlated with clinical severity of AIP, as is evident also by the fact that prepubertal as well as adult gene carriers of AIP have URO synthase levels as low as those found in patients with the clinically manifest disease (Fig. 1). The 14 individuals with low URO synthase activity in the family depicted in Fig. 2 showed a pattern of inheritance of the URO synthase deficiency compatible with an autosomal dominant mode. This pattern of inheritance may explain the early findings of Waldenström (19) that AIP is inherited as an autosomal dominant mode.

Hormone Metabolism Studies: Steroid Δ^4-5α Reductase Activity. The metabolism of [4-^{14}C] testosterone and 11β-hydroxy[4-^{14}C]androstenedione was studied in a 28-year-old woman with biochemically and clinically manifest AIP, and in each of her parents, by methods already described in detail (9, 10). The propositus had a level of URO synthase in her erythrocytes within the abnormally low range characterizing AIP patients (Table 2); studies of URO synthase activity in her parents indicated that her father, who was totally asymptomatic and had normal levels of ALA and PBG in his urine, carried the gene trait for this disease. Her mother had normal values for ALA and PBG output in her urine, and normal levels of URO synthase activity in her erythrocytes.

The propositus (Table 2) had a 5β/5α steroid metabolite ratio, derived from testosterone, of 1.9:1, a ratio that is twice the normal mean (9); her mother also metabolized testosterone preferentially along the 5β pathway (5β/5α ratio, 1.8:1). Her father, who had low URO synthase activity but was a completely latent AIP carrier, had a normal 5β/5α ratio (0.7:1). Moreover, both the propositus and her mother displayed deficient steroid Δ^4-5α-reductase activity (43% and 59% below the normal mean, respectively), as determined by metabolism of the adrenal hormone 11β-

TABLE 2. *Erythrocyte URO synthase activity and steroid metabolism in an AIP family*

Subject	Erythrocyte URO synthase activity (V_{max})*	Testosterone metabolism (5β/5α ratio)†	5α-metabolism of 11β-hydroxyandrostenedione (%)‡	Urinary excretion of ALA and PBG	Clinical symptoms
Propositus 28F	19.2	1.9:1	47	Increased	Typical for AIP
Father (AIP carrier)	22.1	0.7:1	72	Normal	None
Mother	43.0	1.8:1	34	Normal	None

* The propositus and her father have abnormally low levels of URO synthase activity; the mother's URO synthase activity is normal.

† The 5β/5α ratios of the propositus and her mother are twice the normal mean ratio (9).

‡ The percent 5α-metabolism of this hormone reflects the level of steroid Δ^4-5α-reductase activity. The propositus and her mother have levels of 5α-reductase activity substantially below those of normal subjects (10).

hydroxy-androstenedione (10), while the father metabolized this hormone in essentially normal fashion (Table 2). Thus in this patient–parent set, the daughter with clinically expressed AIP appears to have inherited from her mother, who had a normal URO synthase level, a tendency to metabolize steroid hormones in the abnormal pattern characteristic of active AIP subjects; while she inherited from her father, who metabolized hormones normally, the URO synthase gene lesion of this disease.

CONCLUDING COMMENTS

Three hepatic enzyme abnormalities have now been identified in AIP individuals in whom the disorder has become clinically expressed; elevated ALA synthase activity; decreased URO synthase activity; and low steroid Δ^4-5α-reductase activity. A low level of URO synthase activity characterizes all individuals carrying the gene defect for AIP, but this enzymic defect is clearly not of itself sufficient to determine clinical expression of the AIP trait. At least one other nonlinked biochemical defect, decreased steroid 5α-reductase activity, appears necessary to bring the latent disorder to the state where excessive porphobilinogen is excreted in urine. There may also be as yet unidentified metabolic factors associated with clinical and biochemical expression of this genetic disorder, and these could, for example, be particularly related to the pathogenesis of the neurological-visceral symptoms of the disease.

It should be emphasized that while the assay we describe in this report can identify low URO synthase activity in children, this finding is not adequate in itself to support the diagnosis of AIP. As noted, there is a 3-fold genetically determined range of activities for this enzyme, resulting in an overlap of URO synthase values of AIP individuals into the low normal range. However, it is clear that prepubertal children with URO synthase values in the range characterizing the average normal level of enzyme activity can be excluded as carriers of the gene defect for this inherited disorder.

It has been proposed (7) that the low levels of URO synthase activity in the livers of AIP patients may block hepatic heme synthesis and that, as a consequence, heme repression of ALA synthase synthesis is lessened, thus resulting in enhanced production of this enzyme. Our recent studies on avian embryo hepatic cells in culture indicate that it is necessary to decrease concentrations of liver heme considerably below normal before the enhanced synthesis of ALA synthase will occur (20). It seems unlikely, therefore, that the low URO synthase activity in the liver cells of AIP patients can alone depress hepatic heme synthesis sufficiently to lead to secondary stimulation of ALA synthase production. To elicit the overproduction of this enzyme in individuals carrying the low URO synthase trait, other metabolic factors appear to be required; of these factors, the endogenous hormonal milieu of the AIP subject clearly is of great significance. There is little doubt, however, of the central importance of the URO synthase gene lesion in the pathogenesis of AIP and of the fact that this biochemical lesion sensitizes affected individuals to the ALA synthase inducing action of endogenous chemicals such as steroids and of exogenous substances such as drugs and other foreign chemicals.

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