Heme Biosynthesis in Intermittent Acute Porphyria: Decreased Hepatic Conversion of Porphobilinogen to Porphyrins and Increased Delta Aminolevulinic Acid Synthetase Activity*

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Abstract. Hepatic conversion of porphobilinogen to porphyrins was less than 50% of control levels in human subjects with the genetic disease, intermittent acute porphyria. This relative block in heme biosynthesis may be relevant to a concomitant 6- to 10-fold elevation in δ -aminolevulinic acid synthetase activity, since this first and rate-controlling enzyme in the biosynthetic pathway is subject to negative feedback regulation by the end product, heme. A micro-radiochemical assay of δ -aminolevulinic acid synthetase, and some of its applications, are described.

Intermittent acute porphyria (IAP) is a rare genetic disease characterized by an acute neurological syndrome often precipitated by therapeutic dosages of drugs of diverse structure. There is an increased urinary excretion of porphyrin precursors. In the two other types of genetically transmitted hepatic porphyrias, variegate porphyria and hereditary coproporphyria, the neurological syndrome is identical but each type has a distinct pattern of porphyrin and precursor excretion.¹ Another form of hepatic porphyria, porphyria cutanea tarda, often appears to be acquired. Patients with this disorder do not display neurological abnormalities. They primarily excrete excessive quantities of uroporphyrin but normal amounts of porphyrin precursors. Because increased activity of hepatic δ -aminolevulinic acid synthetase (ALA-S) has been observed in IAP,^{2,3} and IAP is transmitted as an autosomal dominant, a defect in genetic regulation, possibly caused by an operator constitutive mutation, has been considered.^{2,4,5} Alternatively, the increased ALA-S activity may be due to a genetic defect resulting in diminished activity of an enzyme beyond ALA-S in heme or hemoprotein^{1,2,6,7} biosynthesis that results in derepression of ALA-S. The marked increase in the porphyrin precursors, δ -aminolevulinic acid (ALA) and porphobilinogen (PBG), in relation to the normal porphyrins in the urine of patients with IAP has suggested that the conversion of PBG to uroporphyrinogen is defective. Heilmeyer and Clotten observed a decreased hepatic production of porphyrins from ALA in a patient with IAP.⁶ However, Nakao *et al.* reported that PBG disappearance catalyzed by liver homogenate from a patient

with IAP was similar to that in controls.³ We have determined the conversion of PBG to porphyrins in liver from patients with different types of porphyria and have correlated it with ALA-S activity as measured by a new, specific, and sensitive radiochemical assay.

Materials and Methods. Porphyrins⁸ and precursors⁹ were quantitated in excretory products and/or liver of all subjects studied. Both enzyme assays employed are sufficiently sensitive to permit their use on only a portion of needle biopsy specimens of liver obtained for diagnostic purposes. Informed consent was obtained in all cases. The liver from one patient with IAP (Case no. 3) was obtained shortly after her death. The biopsy specimens were washed with saline and homogenized with 19 vol of Tris·HCl, 0.05 M, pH 7.7 (assay of PBG conversion to porphyrins) and in 9 vol of 0.9% NaCl containing 0.5 mM EDTA and 10 mM Tris·HCl (pH 7.4) for the ALA-S assays.

Conversion of PBG to porphyrins was quantitated by porphyrin fluorescence after incubation of homogenate containing about 2 mg of liver with 0.05 M Tris·HCl, and PBG, 60 μ M, in a total volume of 0.15 ml, at pH 7.7, in the dark for 3 hr at 37°C. The reaction was stopped with an equal volume of 2 N perchloric acid-ethanol (1:1) in the cold; supernatant fluorescence was compared to a standard coproporphyrin solution at excitation and emission wavelengths of 405 and 595 nm respectively, with an Aminco-Bowman spectrophotofluorometer, using a high intensity light source. Nonenzymatic porphyrin formation was determined with liver tissue that had been boiled for 30 min. Under the conditions of assay, the ratio of enzymatic to nonenzymatic porphyrin formation was greater than 10:1. The product was formed linearly with respect to time and enzyme concentration. With higher protein concentrations and a longer time period, it was shown that the rate of PBG disappearance paralleled porphyrin appearance. Differential porphyrin extraction⁸ indicated that the ratio of 8-carboxyl/4-carboxyl/2-carboxyl porphyrins was 1:2:1. The fluorescence excitation spectra of the porphyrins formed enzymatically was routinely scanned and found to be characteristic of the extracted porphyrins. The constancy of these findings permitted us to equate the increase in fluorescence (with coproporphyrin as standard) to porphyrin formation from PBG in the small specimens of liver tissue available. This activity is presumably proportional to uroporphyrinogen I synthetase activity since the next enzyme in the pathway is not limiting.¹⁰ Under the conditions of incubation there was no significant utilization of porphyrins, as judged by the fact that the high zero-time porphyrin values from patients with porphyria cutanea tarda, and from rats made porphyric with 2-allyl-2-isopropylacetamide (AIA) failed to disappear. PBG was synthesized¹¹ by Dr. G. Kohan and purchased from Protex Research and Development, Registered, Montreal, and was recrystallized from hydrochloric acid just before use. Purity was verified by UV absorption, reaction with Ehrlich's reagent, and melting point.¹²

ALA-S was determined by the rate of incorporation of radioactive succinate into ALA,¹³ and subsequent isolation of labeled ALA by sequential column chromatography (manuscript in preparation). Assay was at 37°C for 20 min, at a pH of 7.4 and total volume of 0.1 ml. The incubation mixture contained Tris·HCl, 0.05 M; glycine, 0.1 M; sodium succinate, 0.01 M; MgCl₂, 0.02 M; glutathione, 2 mM; pyridoxal-5-phosphate, 1 mM; ATP, 0.025 M; CoA, 0.425 M; antimycin A, 2.5 mg/ml; sodium DL-malate, 5 mM; sodium malonate, 10 mM; sodium arsenite, 5 mM; purified bacterial CoA synthetase 0.25 unit, and tissue homogenate. The specific activity of the sodium [1,4-¹⁴C]succinate was 21.1×10^6 dpm/ μ mol in the human liver assays. The optimum conditions of assay were obtained for embryonic avian liver in cell culture by altering the concentrations of tricarboxylic acid cycle inhibitors. The succinyl CoA synthetase (EC 6.2.1.5) was purified from *Rhodopseudomonas spheroides*¹⁴ and generously supplied to us by Dr. Bruce F. Burnham. In some of the studies, crude succinyl CoA synthetase from an ALA-requiring mutant¹⁵ of *Rhodopseudomonas spheroides* H-5 (culture kindly supplied by Dr. June Lascelles) was found to be satisfactory. The ALA-S reaction was stopped

with 25% trichloroacetic acid (TCA); sodium succinate and ALA were added as carriers. The pH of the supernatant was adjusted to pH 7.0 with NaOH, and the ALA isolated by three-step column chromatography employing tandem Biorad columns of Dowex-1-acetate and Dowex-50-H⁺. The eluate was reacted with 1,4-pentanedione to form 2-methyl-3-acetyl-4-(3-propionic acid) pyrrole (ALA pyrrole)¹⁶ and further isolated and concentrated on Dowex-1-acetate by elution with methanol-acetic acid 2:1. Recovery was determined colorimetrically after reaction of an aliquot of the eluate with modified Ehrlich's reagent¹⁶ or by double-label counting with [3,5-³H]ALA. With 10 μ Ci of sodium [1,4-¹⁴C]succinate, about 40 dpm above background were found in the succinyl coenzyme A synthetase blank, less than 20% of the counts obtained with liver of minimum enzymatic activity. Under the conditions of the assays, ALA formation was linear with time and protein concentration. Recovery of ALA was between 65 and 80%. Authenticity of product was verified by paper (butanol-acetic acid and butanol-ammonia)¹⁶ and thin-layer chromatography.¹³ ALA was not detectably utilized by liver during the reaction.

Protein was determined by the method of Lowry *et al.*¹⁷ with crystalline human albumin as standard. All assays were done in duplicate or triplicate and agreed within 5%.

Avian embryonic hepatocytes were cultured by a modification (manuscript in preparation) of the method of Granick.⁴ Human fibroblasts were cultured by minor modifications of standard methods.¹⁸ Methods of preparation of these cells and their enzymatic assay, as well as of erythrocytes and Harderian gland studies (done in collaboration with Dr. Lennart Wetterberg), will not be detailed here.

Results. The radioisotopic method for ALA-S is sufficiently sensitive and reproducible to permit determination of ALA-S in small amounts of tissue with vastly different amounts of activity (Table 1). For example, this method

TABLE 1. *Some representative δ -aminolevulinic acid synthetase (ALA-S) activities in various tissues.*

Tissue	ALA-S activity (pmol ALA/hr/mg protein)
Cultured human fibroblasts	8-19
Rat harderian gland	14,005-22,250
Murine spleen	184-301
Human erythrocytes (1.8-8.8% reticulocytes)	750-4,115*
Cultured avian embryonic liver†	349
After 300 μ g/ml AIA	27,672
After 100 μ g/ml heme	385
After 300 μ g/ml AIA and 100 μ g/ml heme	3,814

* Expressed in picomoles of ALA per hr per ml erythrocytes.

† In embryonic avian liver in cell culture, 2-allyl-2-isopropylacetamide (AIA) and/or heme were added and ALA-S activity was assayed at 20 hr.

allows direct assay of ALA-S activity in cultured avian hepatocytes, thereby permitting quantitative study of its regulation in an *in vitro* system. Ebert *et al.*¹⁹ have recently described a microchemical method for ALA-S that omits an exogenous generating system for succinyl CoA and employs single-column chromatography. In our studies, omission of succinyl CoA synthetase frequently resulted in lower and more variable ALA-S activity in some tissues; also, when 5-10 μ Ci of ¹⁴C precursor per assay was employed, chromatography of the reaction product through a single column gave high blanks.

Patients 1 and 2 with IAP (Table 2) had markedly elevated urinary levels of ALA (14-22 mg/liter) and PBG (69-79 mg/liter) with no increase in fecal porphyrins. The third patient with IAP had an increased concentration of PBG (10⁻⁴ M) and low porphyrin concentration (3 $\times$ 10⁻⁶ M) in her liver, of com-

TABLE 2. *Hepatic δ -aminolevulinic acid synthetase (ALA-S) and porphobilinogen (PBG) conversion to porphyrins.**

Subjects	ALA-S activity ($\mu\text{mol ALA/mg protein/hr}$)	PBG conversion to porphyrins ($\mu\text{mol porphyrin/mg protein/3 hr}$)
Intermittent acute porphyria		
1	1760	22
2	1760	16, 23
3 (acute attack)	3035	6
Variegate porphyria		
1 (acute attack)	2807	73
2	499	47
Porphyria cutanea tarda		
1	282	—
2	290	65
3	248	66
4	220	65
Nonporphyric controls	260 ± 35 (12 subjects)	55 ± 7.6 (9 subjects)
Control rats	216	78
AIA-treated rats	5024	92

* Mean value for nonporphyric subjects followed by SEM. Rats treated with 2-allyl-2-isopropylacetamide (AIA) 300 mg/kg per day for 4 days. The rat experiments represent results from pooled liver homogenate of four animals. The two values listed for PBG conversion to porphyrins for Patient 2 with IAP are from biopsy specimens obtained on two different occasions (the first value corresponds to the ALA-S value shown).

position consistent with the diagnosis. Both variegate porphyria patients had increased fecal protoporphyrin (500–1300 mg/g stool) and coproporphyrin (500–1200 mg/g stool), and patient 1 had a large increase in urinary ALA (15 mg/liter) and PBG (62 mg/liter). Porphyria cutanea tarda patients 1, 2, and 3 had markedly increased urinary uroporphyrin (475–2063 mg/liter) excretion. Patient 4 had been treated with phlebotomy and chloroquine with a decrease in urinary uroporphyrin concentration to normal at the time of biopsy. All of the control subjects had normal urinary and fecal porphyrins and precursors, as determined in our laboratory.^{7,9}

Hepatic ALA-S activity was increased in both patients with IAP and variegate porphyria, but only in IAP was it associated with a decreased enzymatic conversion of PBG to porphyrins (Table 2). That the decrease in PBG conversion to porphyrins is not a secondary effect of increased ALA-S activity (e.g. substrate inhibition) is suggested by the following observations: (1) Patient 1 with variegate porphyria had an extremely high ALA-S activity, while PBG conversion to porphyrin was not decreased (Table 2). (2) This was also observed in rats made porphyric with AIA (Table 2); PBG concentration of these livers was extremely elevated (0.5 mM). (3) Up to 10-fold increases in PBG concentration did not produce substrate inhibition of enzymatic porphyrin formation from PBG in rat liver or in several of the human porphyric and nonporphyric livers tested. We were not able to demonstrate an inhibitor of PBG conversion to porphyrins in the liver of patients with IAP. Mixing an equal concentration of liver homogenate from nonporphyric and IAP liver in the assay gave an additive porphyrin yield. Furthermore, heating the homogenate of normal rat liver or liver from a patient with IAP at 56°C for 30–60 min had no effect on the formation of porphyrins from porphobilinogen, suggesting the lack of extrinsic factors

affecting the stability of this enzymatic step. Moreover, these data confirm that the differences in PBG conversion to porphyrins are not a consequence of uroporphyrinogen III cosynthetase, since this enzyme, unlike the synthetase, is heat labile.¹⁰

Discussion. The present data indicate that of all the subjects studied, only those with IAP had concomitant elevations of ALA-S and diminished capacity to convert PBG to porphyrins enzymatically [which we presume is a function of uroporphyrinogen I synthetase (Table 2)]. This difference is entirely consistent with the fact that patients with IAP excrete excessive levels of the porphyrin precursors (ALA and PBG) and normal amounts of porphyrins. Of particular interest is whether decreased uroporphyrinogen I synthetase activity reflects familial or ethnic differences which modulate inappropriate overproduction of ALA, or whether this decrease is the result of a primary mutation unique to patients with IAP. At this time, the primary mutation appears more plausible for the following reasons: (1) IAP is reported to be characterized by a unique pattern of porphyrin and precursor excretion which generally runs "true to form" in heterogeneous populations;^{1,20} (2) On the basis of the present study, PBG conversion to porphyrins in the liver of patients with IAP is markedly below that of nonporphyric controls (Table 2), a point which should be further validated by sampling a larger population.

If the data supporting these arguments are correct, then the increased activity of hepatic ALA-S observed in patients with IAP should be due to diminished enzymatic conversion of PBG to porphyrins. These phenomena can be reconciled since a partial defect in the conversion of PBG to uroporphyrinogen would be expected to interfere with the formation of heme, which has been shown both to inhibit²¹ and to exercise feedback regulation²² of ALA-S in a number of systems. Table 1 indicates the ability of heme to prevent AIA induction of ALA-S in cultured avian hepatocytes. Dowdle *et al.* have suggested,²³ on the basis of indirect data obtained from the study of one patient with IAP, that the rate of hepatic heme synthesis is unimpaired. However, this suggestion is consistent with a partial defect in PBG conversion to porphyrins since the K_m of uroporphyrinogen I synthetase is probably higher than the normal concentration of PBG in the liver, as suggested by the fact that PBG is not detectable in normal liver using a method that can detect less than 10^{-6} M PBG. Although the hepatic enzyme has not been purified, the K_m of uroporphyrinogen I synthetase for PBG in our studies was approximately 10^{-6} M, and for enzyme partially purified from mouse spleen, 3.7×10^{-5} M.²⁴ Thus, induction of ALA-S may sufficiently increase the PBG concentration to compensate for an enzymatic defect in heme synthesis.

The increased sensitivity of the hepatocyte to drug-mediated induction of ALA-S could be readily explained by defective uroporphyrinogen I synthetase activity. The circumscribed induction of ALA-S by drugs⁴ would be accentuated since this defect would either diminish the capacity for heme synthesis, or greater precursor levels would be required for the same levels of heme.

Each of the three dominantly-transmitted hepatic porphyrias (IAP, variegate porphyria, and hereditary coproporphyria) is associated with an increase in hepatic ALA-S as shown in this paper for IAP and variegate porphyria (Table 2)

and in other studies.^{2,3,7} However, on the basis of the present studies, IAP and variegate porphyria appear to be enzymatically different (Table 2) and each appears to differ from hereditary coproporphyria on the basis of porphyrin and porphyrin precursor excretion. Therefore, if IAP is a consequence of a gene defect affecting enzymatic conversion of PBG, it would appear likely that the enzymatic differences characterizing each of the other genetically transmitted hepatic porphyrias result from independent mutations.

The relationship of these data to the multitude of clinical abnormalities which have been observed in patients with IAP and the other hepatic porphyrias^{1,2,5} awaits further investigation.

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Abbreviations: IAP, intermittent acute porphyria; ALA, δ -aminolevulinic acid; ALA-S, δ -aminolevulinic acid synthetase; PBG, porphobilinogen; AIA, 2-allyl-2-isopropylacetamide.

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