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ACUTE INTERMITTENT PORPHYRIA: THE FIRST "OVERPRODUCTION DISEASE" LOCALIZED TO A SPECIFIC ENZYME

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Acute intermittent porphyria is an inborn error of metabolism characterized chemically by the excretion in the urine of excessive amounts of porphyrin precursors [δ-aminolevulinic acid (ALA) and porphobilinogen (PBG)][1-6] and clinically by neurologic dysfunction. It is transmitted as an autosomal dominant disorder with an incidence of about 1.5 per 100,000 in most areas.[7] Chemical manifestations which resemble those of acute intermittent porphyria can be produced in animals by administration of any one of several compounds.[8, 9] The increased excretion of porphyrin precursors seen in experimental porphyria produced by at least one of these compounds (allylisopropylacetamide) is now thought to result from the induction in liver of δ-aminolevulinic acid synthetase (ALA synthetase), the rate-controlling enzyme of porphyrin biosynthesis.[10-12] The enzyme occurs exclusively in mitochondria.[10, 12] The induction of this enzyme can be inhibited by carbohydrate administration,[12] a phenomenon previously demonstrated with certain other inducible enzymes and termed the "glucose effect." The inhibition of induction of hepatic ALA synthetase by carbohydrate explains the observation that the ability to produce experimental porphyria is reciprocally related to the intake of carbohydrate in the diet.[13] The fact that changes in diet in the human genetically determined disease produced similar effects on porphyrin precursor excretion to those seen in experimental porphyria suggested that ALA synthetase was induced in the livers of patients with the disease and that the induction in man was subject to the "glucose effect."[14] Using a new method for the measurement of ALA syn-

thetase,[15] the present study demonstrates the high level of this enzyme in a patient with the disease. This is thought to be the first example in which the biochemical manifestations of a disease are directly attributed to an increased level of a specific enzyme.

Materials and Methods.—The patient with acute intermittent porphyria was a 19-year-old white female who had a well-documented family history of the disease. A fulminating attack of the disease produced abdominal and back pain, quadriplegia, aphonia, and respiratory paralysis. She was maintained on a positive pressure respirator and survived many serious complications for nearly six months before she expired.

PBG and ALA excretion were high during her entire hospital course (PBG 100–200 mg/day, ALA 70–200 mg/day). Liver was obtained within 30 min after death for the enzyme determinations described below. The control values of nonporphyric patients were determined on liver biopsy samples obtained in the operating room during various surgical procedures described in Table 1. In two control patients (C. G. and R. V.) liver was obtained at autopsy 3–4 hr after death.

δ-Aminolevulinic acid synthetase (ALA synthetase) activity was measured in homogenates under conditions where ALA utilization was almost completely inhibited and production of ALA was optimal. Liver obtained from autopsy or from the operating room was immediately placed in ice-cold 0.05 *M* phosphate buffer pH 7.0 containing 0.14 *M* KCl. It was then homogenized in 2 vol 0.15 *M* tris buffer pH 7.2 containing 0.02 *M* sodium Versenate. The homogenate was incubated in 0.08 *M* tris buffer pH 7.2 containing 0.01 *M* sodium Versenate and 0.1 *M* glycine. ALA production was measured at multiple time intervals and values are expressed on the basis of the early portion (the first 30 min) of the curve of ALA production. After addition of trichloracetic acid to end the incubations, the tissue supernatant was adjusted to pH 5.0 with NaOH and sodium acetate buffer and then passed over Dowex-1 acetate to remove PBG. The effluent was reacted with acetylacetone at 100°C for 15 min. This solution was then passed over Dowex-1 acetate again, which quantitatively retains both the aminoacetone (AA)pyrrole (2,4-dimethyl-3-acetylpyrrole) and ALA pyrrole (2-methyl-3-acetyl-4-propionic acid pyrrole). The AA pyrrole was eluted with n-butanol containing 0.01 *M* NH_4OH and the ALA pyrrole with a mixture of 2 vol methanol and 1 vol glacial acetic acid.[15] The pyrroles were quantitatively determined after reaction with modified Ehrlich's reagent.[16]

ALA dehydrase was measured by the method of Gibson *et al.*[17] For determination of catalase activity, liver was homogenized for 2 min in a Waring Blendor with 49 vol of ice-cold distilled water and then assayed spectrophotometrically on a continuous recording spectrophotometer with a log absorbance attachment by the method described by Beers and Sizer[18] as modified by Rechcigl *et al.*[19] Lactic dehydrogenase in liver was measured by the oxidation of DPNH to DPN using the Cary recording spectrophotometer under the conditions described by Meister.[20]

Results and Discussion.—The levels of ALA synthetase, ALA dehydrase, catalase, and lactic dehydrogenase in the liver of a patient with acute intermittent porphyria and those of nonporphyric controls are presented in Table 1. The values of AA in Table 1 represent the net amount of AA produced in liver *in vitro* under conditions which are optimal for ALA production. These do not represent the maximal ability of liver to produce AA from glycine under certain other conditions which are optimal for AA synthetase.

ALA synthetase activity in the porphyric liver exceeded that in the biopsy control livers approximately 7-fold and in the post-mortem controls by at least 14-fold. The porphyric liver was obtained about 30 min after death, whereas the liver biopsy samples from controls were utilized immediately after removal from live patients. The values of the biopsy control specimens which were obtained under general anesthesia are about the same as those of normal unanesthetized animals.[15] Hence it is unlikely that the differences between porphyric and control livers are related to administration of anesthetics.

TABLE 1

ENZYME LEVELS IN NORMAL AND PORPHYRIC HUMAN LIVERS

Patient (age, sex)	Diagnosis	ALA Synthetase (mμM ALA/60 min/) /gm liver	ALA Synthetase (mμM ALA/60 min/) /mg N	ALA Dehydrase (mμM PBG/60 min/) /gm liver	ALA Dehydrase (mμM PBG/60 min/) /mg N	AA Synthetase (mμM AA/60 min/) /gm liver	AA Synthetase (mμM AA/60 min/) /mg N	Catalase (units/) /gm liver	Catalase (units/) /mg N	LDH† (units/) /gm liver	LDH† (units/) /mg N
B. T. (19, F)	Acute intermittent porphyria	171	6.0	1140	40	94	3.3	20	0.7	4	0.1
	Biopsy Controls										
I. I. (76, F)	Bullous pemphigus, cholelithiasis, and recurrent cholecystitis	29	1.4	*	*	135	6.7	38	1.9	10	0.5
O. S. (37, F)	Rheumatic heart disease, peptic ulcer	24	1.2	964	48	121	6.1	38	1.9	11	0.6
A. H. (23, M)	Peptic ulcer	15	0.9	*	*	90	6.0	*	*	*	*
A. B. (58, M)	Hypertension, peptic ulcer	21	1.0	890	41	91	4.1	51	2.3	*	*
I. C. (52, F)	Carcinoma of the vagina without evidence of hepatic metastases	31	*	1010	*	117	*	*	*	*	*
P. C. (45, F)	Possible "Cushing's syndrome" with recurrent cholecystitis and cholelithiasis	27	1.3	983	45	110	5.0	50	2.3	21	1.0
L. L. (48, F)	Essential hypertension, cholelithiasis	19	0.9	830	37	96	4.2	58	2.6	16	0.7
	Mean	*24*	*1.1*	*935*	*43*	*109*	*5.4*	*47*	*2.2*	*14*	*0.7*
	Autopsy Controls										
C. G. (36, F)	Interstitial pneumonia; specimen obtained 3 hr after death	12	0.6	768	35	25	1.2	20	0.9	38	1.7
R. V. (30, F)	Congenital heart disease, acute blood loss; specimen obtained 4 hr after death	<10	<0.5	420	20	15	0.7	22	1.0	35	1.6

* The tissue available was insufficient for this determination.
† Lactic dehydrogenase.

The values of ALA synthetase obtained by the present technique do not represent the measurement of the activity of the isolated enzyme, but rather the ALA produced from added glycine and endogenously generated succinyl coenzyme A. A number of previous observations[15] using liver homogenates from other species suggest that the ALA obtained by this method is a good measure of ALA synthetase activity: (1) Under the conditions of homogenate incubation, ALA utilization does not occur to a significant extent at the concentrations at which it is produced. (2) That the generation of succinyl coenzyme A is not the rate-limiting process in the formation of ALA in the homogenate incubations described above is supported by several lines of evidence. (*a*) Addition of various tricarboxylic acid cycle (TCA cycle) intermediates at different concentrations does not increase ALA production above that of endogenous TCA cycle substrates. (*b*) The enzyme is probably readily saturated by endogenously generated succinyl coenzyme A, since the K_m for this substrate is probably very much lower than for glycine in both liver and nucleated erythrocytes.[10, 12, 21, 22] (*c*) The concentration of succinyl coenzyme A which is the optimal range for ALA synthetase activity in rat liver mitochondria[12] is approximately that concentration actually found for TCA cycle intermediates in fasting rat liver.[23] (*d*) Estimates of TCA cycle capacities in rat liver by several techniques[24, 25] show that only a small fraction (probably $< 1\%$) of the succinyl coenzyme A generated is required for maximal ALA production.[12] These findings indicate that the level of ALA synthetase is the rate-limiting step in the formation of ALA by liver homogenates under the conditions described above. (3) Earlier methods for measuring ALA synthetase have used isolated mitochondria with added glycine and TCA cycle intermediates.[10, 12, 21, 22] The present homogenate method produces somewhat greater amounts of ALA per unit liver than the previous methods.

The findings which are the basis for the chemical diagnosis of the disease, i.e., increased excretion of ALA and PBG, are shown by the present data to result from an increased ability to synthesize ALA in the livers of patients with acute intermittent porphyria. The evidence cited above indicates that this overproduction is caused by a high level of ALA synthetase, previously shown to be an inducible enzyme. The relationship of the increased ALA synthetase in the liver to the neurologic aspects of the disease remains unknown, since some patients who are asymptomatic produce large amounts of porphyrin precursors.

The fact that acute intermittent porphyria is a genetic disease with a high hepatic level of ALA synthetase raises the question of whether it could be caused by a defect of a regulator gene, i.e., a constitutive regulator mutation. Evidence against this hypothesis is the fact that such genetic defects are recessive,[26] whereas acute intermittent porphyria is a dominant disorder.[27] A constitutive operator mutation[26] as discussed by Watson *et al.*[28] could explain the genetic and biochemical findings. This requires that the neurologic symptoms result from overproduction of porphyrin precursors. However, if this etiologic relationship does not exist and one postulates a single genetic defect as the explanation of both chemical and clinical aspects of the disease, then the defect may not be in a gene involved in the primary regulation of ALA synthetase production.

Another possibility is that a defect in a structural gene in the heme biosynthetic pathway beyond PBG might result in defective end-product repression. For ex-

ample, if the end product of the porphyrin biosynthetic pathway (heme) were involved in the repression of ALA synthetase, as has been suggested in microorganisms,[29] then a partial block in this pathway could explain the induction of ALA synthetase. Evidence against this hypothesis is the fact that hepatic ALA synthetase can be induced in high levels in one type of experimental porphyria[12] where ability for heme synthesis appears to be increased.[30] Therefore, it is possible that the genetic defect lies outside the heme synthetic pathway and secondarily leads to induction of ALA synthetase.

Assuming ALA synthetase to be the rate-controlling enzyme in human hepatic porphyrin synthesis, then the maximal ability for heme synthesis in a normal adult liver is approximately $^1/_4$ to $^1/_3$ the normal unstressed marrow production per day. (Liver = 24 mμM ALA/gm/hr $\times$ 1500 g $\times$ 24 hr $\approx$ 0.9 mM ALA/day. Marrow, assuming a 120-day red cell life span in a 70-kg male with a total red cell hemoglobin of 11 mM = 0.37 mM heme/day or $\approx$3 mM ALA/day.)

A slight increase in ALA dehydrase activity in acute intermittent porphyria is suggested by the data in Table 1. As measured by the present techniques, the maximal capacity to utilize ALA exceeds the ability to generate it by a factor of about 80 in normal liver. The reason for the normally high ratio of ALA dehydrase to ALA synthetase probably lies in the fact that ALA is formed in mitochondria[10, 12] and diffuses out into the soluble portion of the cell where it is acted on by ALA dehydrase[17] to form PBG. This means that ALA is distributed over a relatively large intracellular volume at a low concentration before it is acted on by ALA dehydrase. Because of the low concentration of ALA normally presented to ALA dehydrase, a high level of the latter enzyme is required to ensure maximal utilization of the ALA which is formed and to minimize the escape of ALA from the cell. As the ratio of ALA synthetase to ALA dehydrase rises, as in acute intermittent porphyria, the increasing amount of ALA which is formed is partitioned between escape from the cell and conversion to PBG. Both of these processes are increased above those of normal liver cells. This explains the pattern of porphyrin precursor excretion seen in this disease. The large excess of ALA dehydrase capacity above ALA synthetase capacity demonstrated *in vitro* may be greater than actually exists *in vivo*, since the ALA dehydrase may be functioning under conditions which do not permit maximal activity.

Examination of three other enzymes outside the porphyrin synthetic pathway (AA synthetase, catalase, and lactic dehydrogenase), reveals a variable degree of decrease in activity in the porphyric liver as compared with biopsy samples from normal liver. Some or all of the decrease may be artifactual in that the porphyric liver was obtained shortly after death, whereas the control liver biopsy samples were studied immediately.

It has been proposed that the increased production of ALA in acute intermittent porphyria might result from inability to convert glycine to AA.[31] The production of AA from glycine in the porphyric patient is only slightly lower than in controls. The conditions used for the incubation were those for optimal ALA production and are not optimal for AA production. Hence, the AA produced cannot be considered a measure of maximum ability to produce AA from glycine. The present study clearly demonstrates, however, that significant amounts of AA can be produced from glycine and endogenously generated acetyl coenzyme A by the liver in acute

intermittent porphyria. As previously demonstrated in guinea pig liver,[32] normal human liver appears to produce much more AA than ALA from glycine.

The hepatic catalase of the porphyric patient is less than that of biopsied controls; it is not certain to what extent this decrease results from rapid post-mortem breakdown of the enzyme.

Summary.—A new method has been developed for the measurement of hepatic δ-aminolevulinic acid (ALA) synthetase. An increased level of this enzyme has been demonstrated in the liver of a patient with acute intermittent porphyria. This provides an explanation of the increased porphyrin precursor production and excretion seen in this disease. This is thought to represent the first example in which the biochemical manifestations of a disease are directly attributed to an increased level of a specific enzyme. The mechanism responsible for the induction of ALA synthetase remains unknown as does the relationship of the high hepatic ALA synthetase to the neurologic manifestations of the disease.

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*PEPTIDES CONTAINING THE FUNCTIONAL TYROSYL RESIDUES OF THE ACTIVE CENTER OF BOVINE PANCREATIC CARBOXYPEPTIDASE A**

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Tyrosine has been implicated by Simpson *et al.*[1] as being in the active site of bovine pancreatic carboxypeptidase A. Vallee *et al.*[2] showed that iodination and acetylation both cause a complete loss of peptidase activity, but an increase in esterase activity. However, the presence of the inhibitor β-phenylpropionate during either of these treatments prevents these alterations in the enzymatic properties.[1] These authors found that the alterations in the enzymatic activities correlate exactly with the acetylation and deacetylation of two tyrosyl residues and that the accompanying spectral shifts are characteristic of O-acetyltyrosine.

We have now isolated tyrosyl peptides derived from sequences which appear to involve tyrosines in the active center of this enzyme. This was done by means of a paired label iodination procedure.[3, 4] One portion of carboxypeptidase was iodinated with I^{125}-labeled hypoiodite. A second portion was iodinated with I^{131}-labeled hypoiodite in the presence of β-phenylpropionate to protect the site from iodination. The preparations were then mixed, digested by pepsin, and the peptides separated by paper chromatography followed by high-voltage paper electrophoresis in the second dimension. The ratio of I^{125}-labeled iodine to that of I^{131}-labeled iodine in each iodinated peptide was then determined, and a value larger or smaller than the average value was interpreted to mean that the associated peptide was derived from a sequence in the active site. Elution of these peptides followed by pancreatin digestion and high-voltage paper electrophoresis of the digest showed that moniodotyrosine was present both in peptides with high ratios and in peptides with low ratios. These peptides would appear to involve two different tyrosines in the site.

Materials and Methods.—Bovine pancreatic carboxypeptidase A (diisopropyl-fluorophosphate-treated) was obtained from the Worthington Biochemical Corp., Freehold, N. J. Pancreatin was from Merck and Co., and Pronase (enzyme P from *Streptomyces griseus*) from Biddle Sawyer Co., New York, N. Y. Other reagents were reagent grade. I^{131} was obtained from the Oak Ridge National Laboratories, and I^{125} from the Nuclear Science Corp., Pittsburgh, Pa.

Protein concentrations were based on Nesslerization following digestion.

The iodinations of carboxypeptidase were carried out with hypoiodite labeled with I^{125} or with I^{131} in glycine buffer at pH 9.[3] To 0.03 ml of I^{131} solution (360 μc) were added 1 ml of water, 0.20 ml of conc. HCl, and 0.2 ml of 4×10^{-4} *M* ICl in 2 *N* HCl. The solutions were mixed and 0.25 ml of KI solution (40 mg/l) added and mixed. The I_2 was next extracted into 2 ml of CCl_4 and then into 1.2 ml of pH 9, 1 *M* glycine buffer. This solution is referred to as the I^{131}-glycine buffer.

In a similar manner, 0.018 ml of I^{125} solution (360 μc) *plus* 0.015 ml of I^{131} solution (180 μc)[5]