

STUDIES IN PORPHYRIA

I. A DEFECT IN THE REDUCTIVE TRANSFORMATION OF NATURAL STEROID HORMONES IN THE HEREDITARY LIVER DISEASE, ACUTE INTERMITTENT PORPHYRIA*

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(Received for publication 7 June 1972)

The hereditary liver disease, acute intermittent porphyria (AIP),¹ is characterized clinically by a disabling, sometimes lethal, neurological-visceral symptom complex and biochemically by excessive activity of the porphyrin-heme biosynthetic pathway (1-3). The mitochondrial enzyme δ -aminolevulinic synthetase (ALAS), which is rate limiting for this pathway (4), is found at high levels of activity in the livers of AIP patients (5, 6), and this accounts for the excessive formation and subsequent excretion into the urine of porphyrin precursors which characterizes the disorder.

The high levels of hepatic ALAS in AIP have been postulated to reflect an operator gene defect (5, 7, 8) which ultimately finds expression in the "over-production" of this enzyme. ALAS is, however, readily inducible in the liver by a variety of drugs and foreign chemicals (9-11), and recent studies from these laboratories have demonstrated that many 5β steroid metabolites derived from hormones natural to man are also potent inducers of this enzyme (12-14). The occurrence of this endogenous class of potent inducers of ALAS raised the possibility that AIP patients might have abnormalities in steroid hormone biotransformation which lead to the disproportionate formation of 5β metabolites from precursor compounds. If so, such steroids could contribute by an induction mechanism to the high levels of hepatic ALAS activity which characterize AIP patients.

To explore this possibility the metabolic fate of tracer doses of testosterone (Δ^4 -androstene-17 β -ol, 3-one)-4-¹⁴C was examined in 15 patients with AIP and compared with that in a matched control group of 12 normal subjects. Testosterone was selected for initial study, since it is a structural prototype of endogenous steroids which are normally transformed in man to approximately equal quantities of 5α and 5β metabolites; thus a derangement of hormone metabolism in AIP in which one or the other pathway predominated could be readily ascertained.

* These studies were supported in part by U.S. Public Health Service Grants CA-07304 and HD-04313 and General Clinical Research Center Grants RR-53 and RR-102.

¹ *Abbreviations used in this paper:* AIP, acute intermittent porphyria; ALAS, δ -aminolevulinic synthetase; PCT, porphyria cutanea tarda.

The results of these studies indicate that individuals carrying the genetic lesion of AIP display a marked abnormality of testosterone metabolism manifest by the excessive reductive transformation of this compound along the 5β pathway, the pathway through which metabolites having the potential capacity for inducing ALAS in the liver are generated. Studies with a second steroid, dehydroisoandrosterone(Δ^5 -androstene- 3β -ol,17-one)- 4 - ^{14}C , confirmed the defect in hormone metabolism demonstrated with ^{14}C -labeled testosterone in the AIP patients. The enzymatic basis for this defect will be described in a subsequent report.² The findings in this study clearly establish the existence of a major abnormality of hormone biotransformation in AIP and support the view that there is a significant interplay of endocrine as well as genetic factors in the pathogenesis of this hereditary liver disease (15, 16).

Methods and Clinical Material

Tracer Hormones.—Testosterone- 4 - ^{14}C and dehydroisoandrosterone- 4 - ^{14}C were purchased from the New England Nuclear Corp., Boston, Mass. Paper chromatography of an aliquot of each steroid indicated that the materials were $>98\%$ pure and they were used without further recrystallization or other purification. The compounds were dissolved in sterile redistilled propylene glycol at a concentration of approximately 10^6 cpm/ml, and weighed aliquots of the solution were assayed to determine its exact specific activity. Using a Venopack and a 28-gauge butterfly needle, a slow 5% glucose-in-water intravenous infusion was started in each patient, and weighed portions of the propylene glycol solution were then injected slowly over a 10 min period into the tubing just above the needle. This procedure serves to sweep the steroid solution intravenously and prevent loss of steroid by absorption to the polyethylene tubing (17).

Three complete 24-hr urine collections were then obtained from each subject. In all studies the subject voided before the administration of the tracer and the collection was started from that time. Completeness of collections was judged by creatinine determinations; studies in our laboratory have conclusively established that creatinine measurements are a reliable index of completeness of collection when adequate precautions are observed.³

The first 2-day collections, which invariably contained the bulk of the excreted radioactivity in all subjects, were combined and hydrolyzed by methods which we have described in details previously (18). In the case of the dehydroisoandrosterone- 4 - ^{14}C studies, 3 days of urine collections were combined before hydrolysis and further chemical analysis since this steroid is excreted more slowly than testosterone in man. The hydrolytic methods included adjustment of the urine to pH 5 after the addition of 10% v/v of an 0.3 M pH 5 acetate buffer solution and 300 Fishman units/ml of the enzyme β -glucuronidase (Ketodase). After incubation of the treated urine for 5 days at 37°C the urine was extracted continuously with ethyl ether for 48 hr. The ether extract was washed with 10% NaOH and saturated brine until neutral and then dried and concentrated in vacuo. The residual urine and the aqueous washings of the ether extract were combined, acidified to 1 N in sulfuric acid, and again continuously extracted with ethyl ether for an additional 48 hr. The residual urine after this solvolysis and the aqueous washings of the latter extract were combined, made 5% v/v in sulfuric acid, refluxed for 30 min, and then extracted continuously with ethyl ether for a further 48 hr. This

² Bradlow, H. L., P. N. Gillette, T. F. Gallagher, and A. Kappas. Steroid studies in porphyria. II. Evidence for a deficiency of steroid Δ^4 - 5α -reductase activity in acute intermittent porphyria. Manuscript in preparation.

³ Zumoff, B. Personal communication.

ether extract was washed, dried, and concentrated again as above. The neutral extracts were identified as glucuronide, sulfate, and hot acid extracts, respectively, according to the above procedures, and each of the three extracts was separated into ketonic and nonketonic fractions with Girard's "T" reagent.

Paper chromatography of each of the ketonic fractions was carried out on 118×18 cm strips of Whatman No. 1 paper slit to give two 2.5-cm side strips for standards and dye indicators and two 5-cm strips for samples. After sample application, the strips were inserted into the tanks and run in appropriate systems without preequilibration until the dye marker was in the lower portion of the strip. The side strips were sprayed to detect the concurrently run

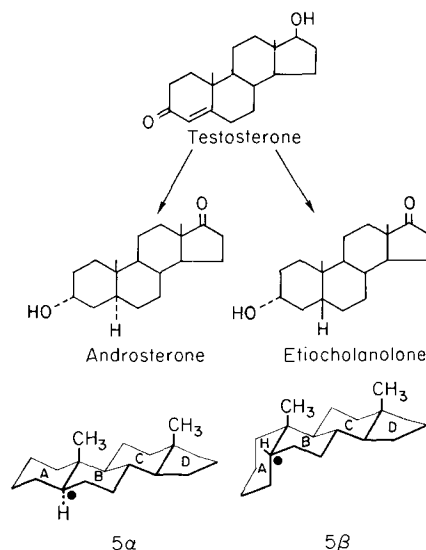


FIG. 1. Structures of testosterone and the two principal metabolites, androsterone and etiocholanolone, into which it is transformed in man. Stereochemical representations of these 5 α and 5 β metabolites are shown at the bottom. The 5 α derivative androsterone is planar like the parent hormone; the 5 β derivative etiocholanolone is highly angulated at the A:B ring junction. The black dots indicate the differing orientations of the C5 hydrogen atom in each type of metabolite.

standards and the sample strips were scanned in a Vanguard 880 scanner to locate the radioactive peaks, which were identified by comparison with the standards. The radioactive peaks were then eluted with ethyl alcohol and counted to determine the conversion of the administered hormone to the various metabolites. For determination of the endogenous output of 17 ketosteroids, separate aliquots of the ketonic extracts were chromatographed in the same manner. Etiocholanolone (5 β -androstane-3 α -ol, 17-one) and androsterone (5 α -androstane-3 α -ol, 17-one) were located as described above, eluted, and quantitated by the micro-Zimmerman reaction. All radioactive samples were counted in the Diotol scintillant of Herberg (19) using Butyl PBD as the phosphor in a Packard 3320 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Correction for quenching was made by using the internal standard procedure.

Fig. 1 depicts the structures of testosterone and the two principal 17-ketosteroids into which it is transformed in man; stereochemical representations of these 5 β and 5 α metabolites are also shown.

AIP Patients.—A total of 15 patients with well defined hepatic porphyria of the AIP type were studied. The diagnosis in each case was established on the basis of characteristic clinical and biochemical findings (1-3); typical values for the urinary excretion of porphyrins and precursors in the majority of these patients have been presented elsewhere (20). The age range of this patient group was 21-56 yr; the group comprised 12 women and 3 men. This is a somewhat higher than the average female:male sex incidence of AIP (21) but comprises our full referral experience for this disease at The Rockefeller University Hospital. The majority of the steroid metabolism studies were carried out in the Clinical Research Center of this hospital with the subjects consuming an ordinary hospital diet. A control group which comprised 12 normal individuals of the same sex incidence and approximate age range as the AIP patients was also studied. The majority of these subjects were also hospitalized and the tracer experiments were conducted under the same conditions as for the porphyric individuals.

It is not possible to give a quantitative assessment to the clinical severity of the porphyric process in AIP patients since it is well known that the urinary output of intermediates in the

TABLE I
Excretion and Metabolism of Testosterone-4-¹⁴C in Normal Subjects and in AIP Patients

Subjects	Urine	Glucuro- nide fraction	Sulfate fraction	Hot acid fraction	Androste- rone (5 α)	Etiocol- anolone (5 β)	Metabolite ratio, 5 β /5 α
Normal subjects	81 (69-93)	50 (41-57)	15 (10-22)	3 (0-5)	26 (22-35)	23 (17-36)	0.9:1
Group A AIP patients	76 (64-85)	41 (36-46)	12 (7-18)	5 (2-11)	10 (4-20)	28 (22-35)	3.3:1
Group B AIP patients	65 (44-89)	29 (23-37)	7 (4-10)	6 (2-10)	6 (3-11)	23 (10-30)	

Data are expressed as per cent of administered dose of the ¹⁴C-labeled hormone; figures in parentheses represent the range of values. The average 5 β /5 α metabolite ratio for the entire group of AIP patients is indicated.

porphyrin-heme pathway does not necessarily correlate with the symptomatic state of the disease. Therefore, to facilitate interpretation of the data obtained in this study, the AIP patients were categorized into two principal groups based on qualitative assessment of the clinical status of each subject at the time of and in the immediate months or years preceding the tracer experiment. Group A comprises 10 AIP patients who were either asymptomatic during this period or only occasionally ill with mild episodic abdominal cramps, etc. All subjects in this group were ambulatory and active, including school attendance or full-time work. Group B comprises five patients who suffered from chronic recurrent, frequently severe abdominal colic which was sufficiently disabling to prevent work, or had residual neurologic symptoms or muscular wasting from previous exacerbations of the disorder. The group B patients were clearly distinguishable from the group A patients on the basis of history and clinical symptoms or findings alone.

RESULTS

Normal Subjects.—The results of the testosterone-4-¹⁴C studies in the control group are recorded in Table I and are entirely similar to the data obtained in earlier studies of normal individuals in these laboratories (22). The ratio of

$5\beta/5\alpha$ metabolites derived from the exogenous ^{14}C -labeled hormone ranged from 0.6:1 to 1.8:1 with a mean of 0.9:1. The ratio of endogenously derived etiocholanolone (5β) and androsterone (5α) was similar (Table II).

AIP Subjects.—

Group A: The results of the testosterone- ^{14}C studies in this group of 10 patients are recorded in Table I. Excretion and hydrolysis of radioactive metabolites of the administered hormone in these subjects were within the normal range. However the relative proportions of etiocholanolone and androsterone differed significantly with the average ratio of these 5β to 5α metabolites in the AIP subjects being substantially higher than that found in the control subjects (Table I).

Group B: This group of five AIP patients was characterized by a normal

TABLE II

Excretion of Dehydroisoandrosterone, Androsterone, and Etiocholanolone from all Endogenous Sources in 18 Normal Subjects and in 12 AIP Patients

Subjects	Dehydroisoandrosterone	Androsterone (5α)	Etiocholanolone (5β)	Metabolite ratio, $5\beta/5\alpha$
Normal subjects	0.6 (0.1-2.9)	1.8 (0.3-3.4)	2.0 (0.5-6.5)	1.1:1
AIP patients	0.3 (0.1-0.7)	0.5 (0.1-1.1)	1.8 (0.3-4.0)	3.6:1

Data are expressed as mean values, with range in parentheses, of output in milligrams per day per gram creatinine. The ratio of endogenously formed 5β and 5α metabolites in the AIP group was comparable to the ratio produced from the exogenously administered radio-labeled hormone (Table I).

to low urinary excretion of administered radioactivity, significantly diminished glucuronide fraction (AIP mean 29% vs. normal mean 50%), and slightly decreased sulfate fraction (Table I). The recovery of "androsterone plus etiocholanolone" was low (AIP mean 29% vs. normal mean 49%), with a marked decrease in the output of the 5α compound. The mean $5\beta/5\alpha$ ratio of the group B AIP patients was somewhat higher than in the group A patients, the average $5\beta/5\alpha$ ratio of the entire group of 15 AIP subjects exceeding the normal average by approximately 350%. The ratio of endogenously derived 5β and 5α derivatives in the AIP patients (Table II) was comparable to that produced from the administered ^{14}C -labeled hormone (Table I).

In Fig. 2 are plotted the individual $5\beta/5\alpha$ metabolite ratios for all of the normal AIP subjects studied; in addition, the $5\beta/5\alpha$ ratios in five patients with the acquired form of hepatic porphyria, porphyria cutanea tarda (PCT), are shown for comparative purposes. Detailed studies of steroid hormone metabolism in these PCT patients will be reported elsewhere.

DISCUSSION

The data reported here indicate that AIP patients as a group have a significant abnormality of steroid hormone metabolism manifest by the disproportionate metabolism of the prototype hormone testosterone along the 5β pathway. The mean ratio of $5\beta/5\alpha$ metabolites derived from the tracer dose of the hormone in the AIP subjects exceeded the average in normals by about 350%,

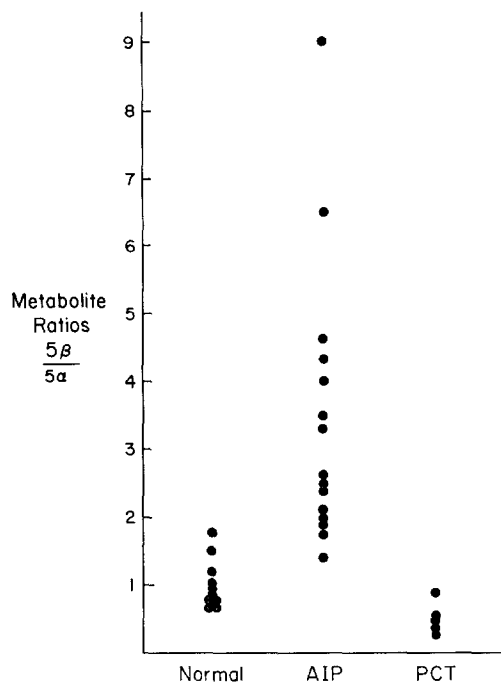


FIG. 2. The ratios of 5β and 5α metabolites formed from testosterone- $4\text{-}^{14}\text{C}$ in each of 12 normal subjects, 15 AIP patients, and 5 individuals with the acquired form of hepatic porphyria, PCT.

with the individual $5\beta/5\alpha$ ratios in each of the 15 patients ranging from approximately 50% to as much as 1000% above the normal mean ratio. The highest ratio (9.0:1) was found in an asymptomatic AIP patient and is a $5\beta/5\alpha$ ratio which we have never observed previously in any except severely myxedematous individuals (23). The pattern of biotransformation of the tracer-labeled hormone was also clearly representative of the manner in which structurally related endogenous hormones were metabolized, since the $5\beta/5\alpha$ ratios of metabolites from endogenous (Table II) and exogenous hormones (Table I) were similar in the AIP patients. Comparable studies with the second prototype hormone, dehydroisoandrosterone- $4\text{-}^{14}\text{C}$, confirmed this point as well. This hormone differs from testosterone in that the C-17 substituent is a ketone

rather than a hydroxyl group; the C-3 substituent is a 3β -hydroxyl rather than a ketone; and the double bond at the A:B ring junction is between C5 and C6 rather than C4 and C5. Despite these structural differences, its reductive transformation at the A:B ring junction markedly favored the *cis* or 5β configuration, as was the case with testosterone, in the AIP patients.

It should be noted that no excess in production of dehydroisoandrosterone itself was observed in this study (Table II), as was earlier reported by Goldberg et al. (24); rather, the principal abnormality defined in the isotope experiments with this hormone and with testosterone was characterized by the marked preferential metabolism of these compounds along the 5β pathway.

An attempt to relate $5\beta/5\alpha$ ratios in patients to specific effects of a given disease or therapeutic regimen are only meaningful if careful consideration is given to those factors which can alter the ratio in such a way as to obscure any specific disease-related effects. As has been recently reported by Zumoff et al. (25), the $5\beta/5\alpha$ ratio can be altered by age and illness. With increasing age the $5\beta/5\alpha$ ratio becomes elevated in both men and women; the apparent effect of nonspecific chronic illness on this ratio is even more pronounced. In both sexes the ratio becomes elevated primarily because of a decrease in the excretion of androsterone and a lower total recovery of administered isotope as the sum of the two compounds, androsterone plus etiocholanolone. Studies with labeled androsterone and with androsterone sulfate have demonstrated a low urinary recovery of the administered compounds in the form of androsterone in chronically ill patients (26), suggesting that the elevated $5\beta/5\alpha$ ratios in such individuals represent a diversion of androsterone to other as yet unidentified metabolites rather than a decreased formation of androsterone per se. This nonspecific effect of chronic illness no doubt includes a substantial contribution arising from the effects of the numerous drugs which such patients receive (25). In order to demonstrate that there is, in a specific disease state, an alteration in the relative reduction of a precursor hormone to 5β and 5α metabolites, it is necessary to establish that its over-all conversion to androsterone plus etiocholanolone is in the normal range and to show that a chance age effect in the given patient population is not a factor. These requirements have been met in the present study. The over-all excretion of isotopically labeled compounds, the extent of glucuronide formation, and the recovery as androsterone plus etiocholanolone were in the normal range for the great majority of the AIP patients studied (i.e., in all the group A patients) though not for the five chronically ill patients in group B (Table I). Further, these patients were restricted, of course, in their drug usage because of the provocative effects which many drugs have on their disease; drug effects on the $5\beta/5\alpha$ ratio can therefore be excluded. The findings in the asymptomatic or only mildly ill AIP patients (group A) particularly emphasize these points since these patients displayed a substantial increase in the $5\beta/5\alpha$ metabolite ratio (Table I) with normal recoveries of isotope as androsterone plus etiochol-

anolone. The ages of these patients also eliminate any important age-related effect on this ratio. While the $5\beta/5\alpha$ ratios observed in the chronically ill AIP patients were comparable to those found in other (i.e., non-AIP) chronically ill individuals (25), the ratios between the group A and group B AIP patients were in fact not substantially different despite the poor glucuronidase hydrolysis and low recovery of isotope in the latter individuals. Finally, the low $5\beta/5\alpha$ ratios observed in patients with the acquired form of hepatic porphyria, PCT, (Fig. 2) make it clear that the high ratios found in the genetic form of the disease are distinctive of this disorder and not secondary to the porphyric process itself. The extremely high ratio (9:0/1) found in one asymptomatic AIP subject (Fig. 2) also suggests that the preferential formation of 5β metabolites is not a consequence of the active disease state, per se, even in AIP.

The possible pathogenetic significance of these findings merits comment. Individuals carrying the genetic lesion of AIP are well known to be highly susceptible to the provocative effects of many exogenous chemicals, i.e. drugs, etc., which induce ALAS in liver cells. Our previous studies have shown that 5β steroids, which are natural substances, are also potent inducers of this enzyme experimentally. The excessive generation of 5β steroids from precursor hormones in AIP patients may thus be viewed as constituting an endogenous source of chemicals which have the potential for contributing, by an induction mechanism, to the high levels of ALAS activity found in the livers of such patients. It is emphasized that the derangement in hormone metabolism described here is not unique to AIP, but may also be found in profound hypothyroidism (23). The postulated inducing action of 5β steroids in AIP must therefore be contingent upon the associated genetic defect(s) in control of the heme pathway which underlies the extreme susceptibility of hepatic ALAS in this disorder to induction by various endogenous as well as exogenous chemicals (27-31). This steroid action is probably also dependent upon other factors, such as the activities of steroid conjugation or deconjugation mechanisms in the liver, the efficiency of excretory processes, etc., which can influence the availability or the access of free 5β steroids to inducing sites for ALAS in hepatic cells (32). It is not evident at present whether the abnormal hormone metabolism demonstrated in AIP is acquired in some fashion or has partial genetic determinants itself; nor is the specific relationship between the degradative pathway for steroid hormones and the biosynthetic pathway for porphyrins and heme clear. The extent and consistency with which significant derangements of both of these pathways are demonstrable in AIP patients, however, strongly suggests that there is an important interplay of endocrine as well as genetic factors in the pathogenesis of this hereditary liver disease.

SUMMARY

A variety of 5β steroid metabolites derived from hormones natural to man are potent inducers experimentally of δ -aminolevulinic synthetase, the rate-

limiting enzyme in porphyrin-heme formation. This mitochondrial enzyme is found at high levels of activity in the livers of patients with the genetic disease, acute intermittent porphyria (AIP). In this study the metabolism of ^{14}C -labeled testosterone was examined in AIP patients to determine whether there was a disproportionate conversion of the hormone to its 5β , compared to its 5α metabolite. The results indicate that AIP subjects do generate a substantially greater than normal fraction of 5β metabolite from this steroid; the excessive degree of ring A reduction of testosterone taking place via the 5β pathway in the porphyric patients averages 350% greater than in the non-porphyrin subjects. In one asymptomatic AIP patient the disproportionate generation of 5β metabolite from the hormone reached a level 10 times the normal mean. Studies with a second ^{14}C -labeled hormone, dehydroisoandrosterone, whose metabolism in man resembles that of testosterone, confirmed the derangement in reductive transformation of steroids found in the individuals carrying the genetic lesion of AIP.

These findings define a new endocrine abnormality in AIP patients and raise the possibility that endogenously derived 5β steroids may contribute by an induction mechanism to the increased levels of hepatic δ -aminolevulinic synthetase activity found in AIP patients.

The authors are indebted to the Misses G. Gilman, N. Hellinger, and R. Jandorek for excellent technical assistance and to Miss Ann Marie Quatela for the preparation of this manuscript.

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