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THE ADRENOGENITAL SYNDROME WITH DEFICIENCY OF 3β–HYDROXYSTEROID DEHYDROGENASE *

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A brief account of three cases of the adrenogenital syndrome due to a deficiency of 3β-hydroxysteroid dehydrogenase has been published (1, 2). It is the purpose of this report to extend the original observations, detail the methodological aspects, and present certain clinical correlations.[1]

The deficiency of 11β-hydroxylase or 21-hydroxylase as two possible causes of the adrenogenital syndrome with adrenocortical hyperplasia is now well recognized. These aspects have been reviewed in detail recently (2). A direct demonstration of an enzymatic defect in adrenocortical tissue has been possible with respect to 21-hydroxylase in only two instances (3). Such experiments are not generally possible because of the obvious difficulties in obtaining suitable tissue from human subjects with this disorder. The evidence is generally indirect and depends upon the demonstration of "abnormal metabolites" in relatively large amount in the urine or blood. The nature of these metabolites provides the clue to the site of the defect, hence the indirect evidence for the deficient enzymatic transaction. A deficiency of the enzyme 3β-hydroxysteroid dehydrogenase represents a third basis for the disorder which is characterized by an accumulation of steroidal compounds retaining the configuration (Δ^5-3β-ol) of the antecedents of this enzymatic step with an absolute or relative lack of the anticipated sequential compounds.

Six cases of this new form of the disorder are herein reported and compared with five cases due to a deficiency of 21-hydroxylase and five normal subjects.

MATERIALS AND METHODS

A summary of the clinical data on six cases of the adrenogenital syndrome due to a deficiency of 3β-hydroxysteroid dehydrogenase (I–VI), five due to a deficiency of 21-hydroxylase (VII–XI), and five normal individuals (XII–XVI) is presented in Table I. The post-mortem findings in four (I, III, IV, and V) were consistent with those of adrenocortical hyperplasia. The sex of the cases with the adrenal disorders was determined by examination of the sex chromatin in cells from the buccal mucosa, examination of the internal ducts by X ray, and histologic examination of the gonads in Case VI and in those who succumbed. Cases III and IV were known to have one prior affected sibling who died and showed bilateral adrenal hyperplasia at autopsy.

Urine specimens were frozen immediately upon the completion of the 24-hour collection and stored at −10° C until analysis. The urinary steroidal compounds were first hydrolyzed with beta glucuronidase (Ketodase, Warner Chilcott) 750 U per ml, pH 4.5, at 37° C for forty-eight hours. The urine was then extracted with methylene chloride and ethyl acetate. These extracts were pooled and are designated *G* in the results. Further hydrolysis was performed by the method of Burstein and Lieberman (4) for all cases except Case V, where the method of Fotherby (5) was employed. Comparison of the two methods, with dehydroepiandrosterone sulfate and pregnenediolone sulfate as models, revealed recoveries of 60 to 65 per cent with the technique of Fotherby and 85 to 90 per cent with that of Burstein and Lieberman (4). Hence the latter was preferred in most of these studies. With neither method were artefacts encountered with the model compounds. The residues of this second hydrolysis were analyzed separately and are designated *S* on the assumption that the cleaved conjugates were sulfates. No proof of the true nature of

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[1] The following abbreviations have been employed for the steroids under discussion: dehydroepiandrosterone = 3β-hydroxy-Δ^5-androstene-17-one; androsterone = 3α-hydroxyandrostane-17-one; etiocholanolone = 3α-hydroxyetiocholane-17-one; pregnanetriol = pregnane-3α,17α,20α-triol; 11-ketopregnanetriol = 3α,17α,20α-trihydroxypregnane-11-one; tetrahydro-E = 3α,17α,21-trihydroxypregnane-11,20-dione; pregnenetriol = Δ^5-pregnene-3β,17α,20α-triol; pregnenediolone = 3β,17α-dihydroxy-Δ^5-pregnene-20-one; pregnenediol = Δ^5-pregnene-3β,20α-diol; pregnenetetrol = Δ^5-pregnene-3β,17α,20β,21-tetrol; 16-OH-pregnenolone = 3β,16α-dihydroxy-Δ^5-pregnene-20-one; 16-OH-dehydro = 3β,16α-dihydroxy-Δ^5-androstene-17-one.

TABLE I

Clinical cases

No.	Sex	Age	External genitalia	Salt loser	Outcome
I	F	2 wks	Clitoral hypertrophy Labial fusion	+	Died 6 wks
II	F	3 wks	Labial fusion	+	Living, 3 mos
III	F	7 wks	Labial fusion	+	Died 5 mos
IV	M	8 wks	Perineal hypospadias	+	Died 3 mos
V	M	3 mos	2° hypospadias	+	Died 6 mos
VI	M	6 yrs	Perineal hypospadias	0	Living, 10 yrs
VII	M	3 wks	Normal	+	Living, 2 yrs
VIII	M	8 wks	Normal	+	Living, 3 yrs
IX	F	18 mos	Clitoral hypertrophy Labial fusion	0	Living, 6 yrs
X	M	2½ yrs	Macrogenitosomia	0	Living, 3 yrs
XI	M	4 yrs	Macrogenitosomia	0	Living, 7 yrs
XII	M	1 wk	Pool of 8 normal infants		
XIII	M	1 wk	Normal		
XIV	M	4 wks	Normal, respiratory infection		
XV	M	30 yrs	Normal		
XVI	M	32 yrs	Normal		

either portion, *G* or *S*, can be adduced from these studies, however.

After preliminary crude analyses, *G* and *S* were separated into alpha and beta fractions by digitonin precipitation (6), and into ketonic and nonketonic moieties by treatment with Girard's T reagent (7). Preliminary chromatography was conducted on silica gel with apportionment of the residues into three broad eluates: 3 per cent ethanol: methylene chloride (A); 10 per cent ethanol: methylene chloride (B); 25 per cent ethanol: methylene chloride (C). For the most part each portion, in order, was further resolved by chromatography on paper as follows: (A) in ligroin (bp 110 to 115° C): methanol: water (500: 308: 42); (B) in isoöctane: tertiary butyl alchohol: methanol: water (500: 225: 225: 50); (C) in benzene: methanol: water (500: 250: 250). In some instances where chromatography in alternate systems was applied to some compounds, or overlapping occurred among the column fractions, the time was considerably prolonged for better resolution. A variety of stains was employed for the development of selected strips of the paper chromatograms, including phosphoric acid, the Zimmermann reagent, blue tetrazolium, antimony trichloride, phosphomolybdic acid, and phosphotungstic acid (8, 9). Regions of the chromatograms corresponding to discrete compounds were eluted with methanol and repurified by application to a small column of silica gel. In a single instance, Case V, all extracts were re-

TABLE II

Chromatography of steroids described *

Steroid	Column fraction	Paper system †	Chromatography distance	Suitable stains employed
			cms/hrs	
Androsterone	A	X	33.0/18	Zimmermann
Etiocholanolone	A	X	25.5/18	Zimmermann
Dehydroepiandrosterone	A	X	19.0/18	Zimmermann H_3PO_4, BT
Tetrahydro-E	C	Z	14.0/9	BT
Pregnanetriol	B	Z	35.0/6	$SbCl_3$
11-Ketopregnanetriol	B	Z	14.0/6	Phosphomolybdate
Δ^5-Pregnenetriol	B	Y	31.0/24	H_2PO_4, PT
Δ^5-Pregnenediolone	A-B	Z	40.0/10	H_3PO_4, PT
Δ^5-Pregnenediol	A	Y	39.0/24	H_3PO_4, PT
Δ^5-Pregnenetetrol	B-C	Y	8.0/24	H_3PO_4, PT
16-OH-Dehydro	B	Y	20.0/24	BT
16-OH-Pregnenolone	B-C	Y	23.0/24	H_3PO_4, Zimmermann

* A, B, C = 3%, 10%, 25% ethanol:methylene chloride; X = ligroin:methanol:water (500:308:42); Y = isooctane:tertiary butyl alcohol:methanol:water (500:225:225:50); Z = benzene:methanol:water (500:250:250) BT = blue tetrazolium; PT = phosphotungstic acid.

† Whatman no. 1 paper, 45 × 45 cms was used and equilibrated at 24° C for 3 hours.

solved by gradient elution on silica gel with ethanol: methylene chloride; the early eluates (up to 2 per cent ethanol) were rechromatographed on paper as with Fraction A, above. The chromatographic treatment pertinent to the steroids discussed herein is outlined in Table II.

The following methods were employed for the analysis of the preliminary crude fractions and for the quantification of the eluted pure steroids. Oxidation with periodate and measurement of the acetaldehyde or formaldehyde produced thereby was according to published methods (10). The Allen reaction (11) was employed only for preliminary analysis. The Oertel reaction was used as a tentative index of the Δ^5-3β-ol grouping, since steroids with this configuration generally exhibit a peak of absorption at 405 mμ (12, 13). With the aid of the Allen correction factor, this last device was also applied as a means of quantification, with dehydroepiandrosterone as a standard for crude fractions or other steroids closely allied to the isolated pure compounds. The quantity of pregnane-3α,17α,21-triol-11,20-dione was measured with the Porter-Silber reagent (14) on appropriate eluates from the paper chromatograms. Blue tetrazolium (15) was utilized primarily for the measurement of steroids with the 16α-ol-17-one grouping, since these do not react with the Zimmermann reagent. The Zimmermann reaction and the measurement of total urinary 17-ketosteroids were conducted as reported elsewhere (16).

Tentative identification of the steroids reported herein was based on the absorption characteristics in concentrated sulfuric acid and the Oertel reagent through the visible and ultraviolet region. The latter method was proved to be especially useful and reliable (12, 13). Other criteria of identification included the mobilities in the chromatographic systems, the staining reactions, melting points, and in several instances, the infrared spectrum. In those experiments where compounds were not examined by infrared spectroscopy, they were identical in all other stated respects to those which were so analyzed.

RESULTS

Preliminary analyses of the crude fractions of urinary extracts are described in Table III. In Cases I to VI, the subjects of this report, the preponderant quantity of steroid was present in the extract after solvolysis rather than after hydrolysis with beta-glucuronidase. On occasion, however, respectable amounts of steroids yielding acetaldehyde on periodate oxidation or reacting with the Allen or Oertel reagents were also present in the latter, but over 90 per cent of this material was precipitable with digitonin and was pooled with the beta fraction of the residue extracted after solvolysis. Further chromatography, as described, revealed that steroids identical with those in the solvolytic portion were indeed present in the enzymatically released material in smaller amounts. It is not possible, however, to

TABLE III

*Preliminary analyses of crude fractions * (milligrams per 24 hours)*

	Total 17-ketosteroids	Allen		Oertel		Acetaldehydogenic steroids		Formaldehydogenic steroids	
		G	S †	G	S †	G	S †	G	S †
I	4.5	0	4.5			1.4	6.7	0	3.1
II	4.0			5.9†	5.6	3.0	3.9	0	0
III	10.0	1.0	10.0	1.0	5.9	3.7†	13.0	2.0†	3.5
IV	1.8	0	1.8	0	5.9				
V	6.0	2.0†	5.9			1.0	6.2		
VI	11.5	0	4.3	1.2	4.1	10.0†	14.0	2.7	7.0
VII	11.7	0	1.2	0	0.9	1.8	0.5	0	0
VIII	3.2	0	0	0	0.5	4.4	0.4		
IX	3.2	0	0	0	0	19.2	3.6	1.6	0
X	10.8	0	0			4.4	1.2	2.0	0
XI	10.4	0	0			8.0	1.6		
XII	0.8	0	0.8	0	0.8	0.1	0.3	0.4	0.1
XIII	0.5	0	0	0	0.5	0.1	0.3	0.3	0.2
XIV	1.2	0	0.8	0	0.7	0.2	0.5	0.4	0
XV	16.0	0	2.0			0.9	0.8	4.9	1.9
XVI	14.1	0	2.9			1.6	0.9	6.1	1.0

* G = fraction hydrolyzed with beta glucuronidase; S = fraction treated by solvolysis after enzyme. Dehydroepiandrosterone was employed as standard for Allen and Oertel reaction (Note: pregnenetriol yields a relatively weak Allen reaction.) Pregnenetriol (20-β) was employed as standard in acetaldehyde reactions, and cortisol in formaldehyde reactions.

† Over 80 per cent precipitable with digitonin.

TABLE IV

Urinary steroids (milligrams per 24 hours)

Cases	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI
Androsterone		0.1			1.0*	0.9					5.1*				5.2	
Etiocholanolone		0.1			0	0.4					1.9				3.9	
Dehydroepiandrosterone		1.4			4.5*	4.0*					1.6				2.1	
Tetrahydro-E	0	0	0	0	0	1.8	0	0	1.3	0.7	2.1	1.0		4.6	5.1	5.0
Pregnanetriol	0	2.0*	0	0	0	0.7	1.0	2.9*	8.3*	4.3	7.6*	0	0	0	0.9	1.7
11-Ketopregnanetriol	0		0	0	0		3.9	5.2*	4.5*						0.2	
Δ⁵-Pregnenetriol	4.0	5.0*	6.8*	6.5*	3.2	8.2*	0.5	0.4	2.3	1.7	1.2	0.3	0.2	0.9	0.6	0.8
Δ⁵-Pregnenediolone		3.5	4.0*	4.6*	3.9*	2.2		1.5	1.4	0.9		0.5	0.6	0.5	1.2	0
Δ⁵-Pregnenediol		1.8*		2.5*	2.2											
Δ⁵-Pregnenetetrol	†	0	†	4.0*	3.1	5.0*		0	0			0.2				
16-OH-Dehydro		1.9	3.5*		2.0*	1.7		0.3	1.4	1.0	1.0	0.2	0.3		0	
16-OH-Pregnenolone			3.0	4.5*								0	0			
		3β-ol-Dehydrogenase						21-Hydroxylase						Normal		

* Infrared identification.
† Present, not quantified.

state whether these steroids were truly conjugated as glucuronosides or as other conjugates released nonspecifically during the enzymatic hydrolysis.

Detailed and specific results are presented in Table IV. Insufficient urine was available in Cases I and III for better definition of other steroids detected. In those cases attributed to a deficiency of 3β-hydroxysteroid dehydrogenase (I–VI), the largest part of the steroids isolated and identified was comprised of compounds with Δ⁵-3β-ol structure, almost to the exclusion of those "normal" pregnane derivatives found in the usual form of the disorder (VII–XI, 21-hydroxylase deficiency) or in healthy subjects (XII–XVI). There are two partial exceptions in the first group. Case VI revealed not only readily detectable amounts of pregnanetriol but of tetrahydro-E as well. This individual probably represents the single instance of "compensation" in the group and has fared well clinically, as would be expected from the analogous situation in those with the 21-hydroxylase deficiency (17). In Case II, although the generalization is correct as presented, abnormal amounts of pregnanetriol (although relatively smaller in quantity) were clearly present. This remains unexplained unless a deficiency of two enzymes is postulated. This possibility is supported by the lack of Δ⁵-pregnenetetrol (see below). In addition to those compounds designated in Table IV, there was regularly present a highly polar steroid that reacted with blue tetrazolium and is believed to be 3β,16α-dihydroxy-Δ⁵-androstene-7,17-dione, but it has not been satisfactorily identified. The steroid Δ⁵-pregnene-3α,17α,20β,21-tetrol, recently reported (18), was also present, but was accurately measured only in the three cases indicated and was absent from Case II.

The clinical observations (Table I) merit inclusion with the results in this newly recognized disorder. The three males (IV–VI), contrary to expectation from the observations in other forms, failed to completely realize masculine organogenesis, and all had hypospadias. In two, (IV and VI), this was so extreme as to suggest the diagnosis of "male pseudohermaphrodism" of unknown cause early in their course before the nature of the disorder was clarified. The females, although exhibiting fusion of the labia majora and enlargement of the clitoris, did not have the usual displacement of the urethral orifice as in the urogenital sinus of the other types of the disease.

The subjects with 21-hydroxylase deficiency (VII–XI) show the anticipated predominance of pregnanetriol (and 11-ketopregnanetriol). In addition, those steroids that were detected and measured as predominant with a deficiency of 3β-hydroxysteroid dehydrogenase were present as well in these subjects, and are probably to be regarded as elevated in relation to healthy subjects of approximately similar age. Nonetheless, they are relatively small in quantity as compared to the major urinary metabolites that have been measured.

The high mortality early in the course of the life of the individuals with 3β-hydroxysteroid dehydrogenase deficiency, despite adequate treatment with steroids, is notable. All were "salt losers"

with the exception of VI. Post-mortem examination of the four who succumbed revealed the classical findings of bilateral adrenocortical hyperplasia. Unfortunately, the events did not permit suitable chemical study of the tissues. In three instances (II, III, IV), examination of urine collected two or more weeks after the institution of treatment with cortisol revealed the virtual disappearance of the dominant compounds described.

DISCUSSION

The data in six cases of the adrenogenital syndrome due to congenital adrenocortical hyperplasia represent the basis for a third biochemical defect in this disorder, namely a lack of 3β-hydroxysteroid dehydrogenase. As anticipated, large amounts of the steroidal precursors of this enzymatic transaction dominate the urinary steroidal pattern in all instances. Previous studies, reviewed elsewhere (2), have indicated a deficiency of 21-hydroxylase as the cause of the commonest form of the disorder, under which circumstances a group of C-21 methyl steroids account for the major portion of the urinary metabolites (19, 20). In the hypertensive form, 11β-hydroxylase is lacking, as evidenced by the abundant 11-desoxy steroidal compounds in both blood and urine.

Five of the six cases of this newly described disorder were associated with the complication termed "salt losing" in the other forms. The results indicate that the five salt losers suffered from a complete block in the biosynthesis of the major biological adrenocortical secretory products, cortisol, as reflected by the absence of urinary tetrahydro-E. This relationship between the severity of the block and the salt-losing state has been established earlier in the case of 21-hydroxylase deficiency (17). More recently, it has been shown that the secretion of aldosterone is significantly diminished in subjects with the adrenogenital syndrome who have a "complete block" (21). Case VI represents a partial deficiency of 3β-hydroxysteroid dehydrogenase, since significant quantities of tetrahydro-E were present in the urine. In addition, in this last subject, pregnanetriol was present in quantities which might be regarded as normal for the age (22). Nonetheless, the dominant pattern of urinary steroids is identical with that of the other cases. It is difficult at this time to assess the frequency of the complete block with salt losing in the latest variety of the disease. Often, in the evolution of the understanding of metabolic disorders, the more severe types are first recognized. Case VI will be described in detail elsewhere, by a group of workers who recognized the compensation in this patient (23).

Case II is curious in that the over-all pattern coincides with the proposed locus of the biochemical disorder. The amount of pregnanetriol measured, however, is abnormally great for the age of this subject. The absence of tetrahydro-E suggests a complete block in the biosynthetic goal, and the facts may be best explained by a major but partial defect of 3β-hydroxysteroid dehydrogenase and a second deficiency of 21-hydroxylase. This hypothesis is supported by the absence of Δ^5-pregnenetetrol (having a C-21 OH), which was present in the other five cases.

The identity of the Δ^5-pregnenetetrol has been established by comparison with this steroid prepared chemically and by biosynthetic means (24). The presence of this novel compound was initially revealed in the quantity of formaldehydogenic steroids present in the crude urinary extracts after solvolysis and digitonin precipitation. In addition to the chemical behavior noted, elemental analysis and identity with the product of sodium borohydride reduction of 3β,17α,21-trihydroxy-Δ^5-pregnene-20-one by several physical characteristics (including infrared) established its nature (24). The orientation at C-20, as indicated in our original report (18), was incorrect. However, minute quantities of a slightly more polar compound suggested the presence of the 20α epimer in urine, but the amounts were too small for precise identification. Pregnenetetrol was not detected in normal urine when volumes corresponding to a 24-hour output were examined, with the exception of the pool of urine of infants representing several days' collection when the total quantity was insufficient for definitive identification (XII, Table IV). Its occurrence indicates steroidal 21-hydroxylase activity, although the matter is somewhat obscured by our failure to induce 21-hydroxylation of suitable precursors (3β,17α-dihydroxy-Δ^5-pregnene-20-one) *in vitro* with bovine, porcine, and rabbit adrenal glands. Whatever the precise course of events, the presence of this compound reveals the

ability of the organism to hydroxylate the C-21 of these early precursors.

The occurrence of hypospadias in all three of the males with 3β-hydroxysteroid dehydrogenase is interesting. The deficient enzyme is known to play a role in the gonadal biosynthesis of testosterone and perhaps other androgens (25). The clinical picture suggests inadequate fetal testicular function necessary for the completion of the embryonic masculine development. The females reveal the presence of masculinization, as in other forms of the disease, although the anatomy is somewhat mitigated by the normal position of the urethral orifice, suggesting a lesser degree of virilization in this type. It must be assumed, however, that some androgens are produced that allow respectable, although not complete, development of the male and a degree of virilization of the female. Whether these androgens are lesser in biologic activity or smaller in amount is not clear. Further evidence of a biologic difference in the androgenic influences between the disease due to 3β-hydroxysteroid dehydrogenase and 21-hydroxylase deficiency became apparent when Subject VI, the oldest of the individuals with the former type, was compared with a patient of a similar age with the latter type. The somatic maturation, although advanced in both, is more striking in the instance of 21-hydroxylation deficiency. There was significantly greater advancement of bone age, considerably more genital development, and pubic hair. As of this writing, the question of the most active androgen responsible for the clinical manifestations in any of the forms is unresolved.

In the group with a deficiency of 21-hydroxylase, the predominant urinary steroidal pattern is as expected. A modest but perhaps significant increase of those species of compounds with the Δ^5-3β-ol grouping is evident. This circumstance is to be anticipated as a result of a degree of "backing up" in the biosynthetic pathway, and also has previously been proposed to explain the large quantities of pregnanediol regularly found in this particular group (26, 27).

Further details concerning the characteristics of certain of the 3β-hydroxy-Δ^5-steroids isolated in these studies are to be found in the recent publications of the Sloan Kettering Institute, wherein current information and a review is to be found (9, 28). To date, these series of compounds have generally been found in the urine of individuals with adrenal carcinoma. In this report, their association is with a congenital metabolic disorder. The steroid Δ^5-pregnene-3β,17α,20β,21-tetrol described in this series has not previously been isolated from urine. It was specifically sought in specimens from three children with adrenocortical tumors and was not found. Nor was it reported in the studies cited previously (9, 28). The limited information to date would suggest that it abounds in the congenital metabolic disorder owing to the absence of one critical enzyme (3β-hydroxysteroid dehydrogenase) but not another (21-hydroxylase). Perhaps this precise combination of circumstances does not obtain in tumor tissue; both enzymes may be relatively deficient.

SUMMARY

Six cases of the adrenogenital syndrome due to congenital adrenal hyperplasia have been described, and the etiology attributed to a deficiency of 3β-hydroxysteroid dehydrogenase. The urinary steroids, for the most part, were composed of a group of compounds with the Δ^5-3β-ol configuration in rings A and B. Five of the six cases revealed a complete block as indicated by the virtual absence of the metabolites of cortisol, and these cases were salt losers. The sixth was compensated, showing respectable quantities of 3α,17α,21-trihydroxypregnane-11,20-dione in the urine, and maintained salt and water equilibrium satisfactorily. Three of the subjects were male, all showing hypospadias. The three females were virilized with respect to labial fusion and clitoral hypertrophy. In this series, however, it appears that the degree of virilization noted in other forms of the disorder was less extreme.

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