Evaluation of Deficiency of 21-hydroxylation in Patients with Congenital Adrenal Hyperplasia

O. M. GALAL, B. T. RUDD, and N. M. DRAYER

From the Institute of Child Health, University of Birmingham

Congenital adrenal hyperplasia can manifest itself in a variety of clinical and biochemical abnormalities (Bongiovanni and Root, 1963). The salt-losing tendency in some of these patients can be due to the absence of specific enzymes: dehydrogenases or hydroxylases (Bongiovanni and Root, 1963; Ulick *et al.*, 1964; Visser and Cost, 1964). In addition to the impaired production of certain steroids, antagonism by steroids or other compounds could, theoretically, account for the salt-losing tendency (Neher, Meystre, and Wettstein, 1959; Jacobs *et al.*, 1961).

The purpose of this study was to discover whether, despite the continuation of steroid therapy, stimulation of the adrenal cortex with ACTH would measure the capacity of the 21-hydroxylase enzyme system to convert steroid precursors to cortisol. This procedure would then support the diagnosis of this type of congenital adrenal hyperplasia, if withdrawal of therapy was considered to be undesirable.

This study was also intended to investigate whether the degree of 21-hydroxylase deficiency in the pathway leading to cortisol production could be related to the two clinical variants of 21-hydroxylase deficiency—salt-losers and non-salt-losers.

Children Studied

Eighteen normotensive patients were diagnosed as having congenital adrenal hyperplasia. 13 of these patients were girls, all of whom had signs of virilism. The abnormal quantities of total 17-oxogenic steroids, and pregnanetriol excreted in the urine by the 18 patients indicated a 21-hydroxylase deficiency. In 10 girls and 3 boys the salt-losing condition was shown in the first weeks of life by the occurrence of vomiting accompanied by a low serum sodium and a high serum potassium. All 18 patients received glucocorticoid therapy, either as dexamethasone or as prednisolone (1 patient). In addition, the 13 salt-losers received salt, 8 also received 9- α -fluorohydrocortisone, and 1 received DOCA. Steroid

therapy in these 13 children started within the first 3 weeks of life.

In addition to the 18 patients with congenital adrenal hyperplasia, 3 children with shortness of stature and 3 with early signs of puberty were given ACTH to investigate their adrenal function. None of these patients had received steroids and all had normal free cortisol and 17-hydroxycorticosteroid urinary excretion rates. The age and sex of the patients in the 3 groups and details of the steroid therapy are listed in Table I.

ACTH test. Twenty-four hour urine collections were obtained before and during ACTH stimulation. ACTH gel (Organon) was given intramuscularly at a dose of 20 I.U. twice daily for 3 consecutive days.

Methods

One-twentieth volume of a 24-hour urine collection was hydrolysed with a β -glucuronidase preparation from *Patella vulgata*. The liberated steroids were extracted with ethyl acetate-ether (1 : 2), washed with alkali and water, and chromatographed in the system light petroleum-toluene-85% acetic acid (1 : 1 : 2). Three widely separated fractions were obtained (Bush, 1961). The fraction of medium polarity, containing pregnanetriol, was oxidized with sodium bismuthate. The oxidized product was chromatographed in the system light petroleum-dekalin-85% methanol (1 : 1 : 2) (Bush, 1961). The Zimmermann chromogen, with a mobility similar to authentic aetiocholanolone, was eluted with methyl sulphoxide and measured spectrophotometrically (Epstein and Zak, 1963).

The most polar fraction containing the 17-hydroxycorticosteroids was eluted. The Porter-Silber reaction (Silber and Porter, 1954) was used for quantitative determination and the Allen formula (Allen, 1950) was applied.

Recoveries of added tetrahydrocortisol to urine. When 5–10 μ g. of tetrahydrocortisol (MRC reference steroid) were added to different urine samples, and the urines were taken through the described method, the recoveries ranged from 60 to 112%, with a mean of 84.5%. The levels of tetrahydrocortisol standard added to the urines corresponded to 0.1 mg. to 2 mg. of Porter-Silber chromogens excreted per 24 hours.

Received December 28, 1967.

TABLE I
Results of ACTH Stimulation Test in 24 Children

Case No.	Sex	Treatment			Age at Time of Test	Test				21-hydroxylation Deficiency (%)
		Dexamethasone (mg./24 hr.)	9 α -fluoro-hydrocortisone (mg./24 hr.)	Salt (g./24 hr.)		17-OHCS (mg./24 hr.)		Pregnanetriol (mg./24 hr.)		
						BL*	ACTH*	BL*	ACTH*	
Salt-losing patients with congenital adrenal hyperplasia										
1	F	0.5	0.1	4	5 wk.	ND	0.1	ND	0.4	80
2	F	0.5	0.1	5	6 "	ND	0.1	ND	1.6	94
3	F	0.28	0.1	3	3 mth.	ND	ND	ND	1.3	100
4	M	0.375	0.1	3	3 "	0.1	0.1	ND	0.5	100
5	M	0.375	0.1	5	3 "	0.3	0.3	ND	2.4	100
6	F	0.3	0.1	2	3 "	0.1	0.3	ND	0.4	67
7	F	0.375	0.1	4	2½ yr.	0.2	0.4	0.9	2.2	87
8	F	0.5		'Ad lib'	3 "	0.2	0.3	1.1	4.0	96
9	F	0.25		6	4 "	0.1	0.2	ND	0.5	83
10	M	0.16	0.1	4	5½ "	0.4	0.5	5.5	8.5	97
11	F	0.5		'Ad lib'	6 "	0.2	0.7	0.4	9.9	95
12	F	0.5		'Ad lib'	8½ "	0.1	0.3	ND	17.8	98
		Prednisolone (mg./24 hr.)	DOCA Implant (mg.)	Salt (g./24 hr.)						
13	F	3	200	2	3 mth.	0.2	0.3	ND	5.4	98
		Dexamethasone (mg./24 hr.)	Age when Steroid Therapy Started							
Non salt-losing patients with congenital adrenal hyperplasia										
14	F	0.125	4 wk.	5 wk.	0.1	2.3	ND	0.3	12	
15	F	0.6	3 mth.	3 yr.	0.5	2.1	0.7	2.6	54	
16	M	0.5	2½ yr.	3 "	0.1	0.6	0.2	0.3	17	
17	M	0.75	2½ "	3½ "	0.1	1.2	0.2	1.1	45	
18	F	0.5	3 "	12 "	0.1	0.2	0.1	0.5	80	
Patients without congenital adrenal hyperplasia										
19	F			2½ yr.	0.9	6.5	0.1	0.3	3	
20	F			2½ "	0.6	16.0	0.2	0.5	2	
21	F			5 "	1.2	12.5	0.1	0.3	2	
22	M			6 "	1.1	15.0	0.1	0.3	1	
23	F			6½ "	0.7	7.5	0.1	0.4	4	
24	M			6½ "	1.1	11.2	0.2	0.4	2	

* 24-hour urine collections before (BL) and on the 3rd day of ACTH stimulation. ND = Not detected.

Recovery and oxidation efficiency of pregnanetriol. To normal urines after hydrolysis was completed, 5, 10, and 20 μ g. 11-oxy aetiocholanolone were added.

The recovery of this steroid after extraction and paper chromatography was 79% (range 69–89%). The fraction which contains 11-oxy aetiocholanolone also contains pregnanetriol, and it is assumed that the recovery of pregnanetriol after extraction and chromatography is similar to that of 11-oxy aetiocholanolone.

Pregnanetriol, 20 μ g., was used as a standard, undergoing the same subsequent oxidation and second paper chromatographic step as the biological sample. By this procedure, losses of pregnanetriol from the biological sample, occurring during the oxidation and second stage chromatography, are compensated for by similar losses from the standard (the recovery of pregnanetriol after this procedure was 76–80%).

Precision. The precision of the method for the 17-hydrocorticosteroids was established by the analysis of paired data. Seven pairs of urines were studied with

a Porter-Silber chromogen concentration range of 0 to 2.6 mg./24 hours. The standard deviation was ± 0.12 mg./24 hours. Thus, for a single analysis the expected variation from the true value would be ± 0.24 mg./24 hours (95% confidence limits). The precision of the method for the steroids present in the fraction of medium polarity, containing pregnanetriol, was similar (Rudd and Galal, 1967).

Results

Preliminary studies in 7 of the children with congenital adrenal hyperplasia have shown that, on average, the maximum Porter-Silber chromogen excretion was obtained on the third day of ACTH stimulation (Fig. 1). The increase in the urinary excretion of the Porter-Silber chromogens and pregnanetriol on the third day of ACTH is shown in Table I.

The percentage of 21-hydroxylase deficiency

FIG. 1.—Porter-Silber chromogen excretion before and during 3 days of ACTH stimulation in 7 children with congenital adrenal hyperplasia.

present at the time of the test was calculated using the following formula.

$$\frac{\text{Increase in pregnanetriol}}{\text{Increase in pregnanetriol} + \text{increase in 17-OHCS}} \times 100.$$

The distribution of the calculated degree of 21-hydroxylase deficiency found for the three groups of children studied is shown in Fig. 2. Table II illustrates that the difference in the calculated degree of 21-hydroxylase deficiency between days 2 and 3 of ACTH stimulation was minimal (0–5%) in 4 patients, and 9–17% in 2 other patients. There is no linear correlation between the duration of therapy and the calculated degree of 21-hydroxylase deficiency in the patients with the salt-losing condition (Fig. 3). However, in the non-salt-losing patients there appears to be a progressive rise in the degree of 21-hydroxylase deficiency as the duration of therapy increases (Fig. 3).

Discussion

Non-salt-losing patients with congenital adrenal hyperplasia who are not receiving steroid treatment excrete much larger amounts of pregnanetriol than of tetrahydrocortisone. In contrast, normal sub-

FIG. 2.—Calculated degree of 21-hydroxylation deficiency in 24 children.

jects excrete more tetrahydrocortisone than pregnanetriol (Bongiovanni and Eberlein, 1958). On the basis of this finding, the adrenals of these non-salt-losing patients with congenital adrenal hyperplasia were estimated to work 10–100 times as hard as the adrenals of normal subjects in order to make the same amount of cortisol from the precursor 17-hydroxy progesterone. The amount of tetrahydrocortisone excreted in the urine by the salt-losing patients with congenital adrenal hyperplasia was minimal. It was concluded that the basic difference between the salt-losers and the non-salt-losers was the amount of cortisol being produced.

In the present study, when ACTH was administered to the 6 children without a deficiency of 21-hydroxylation, the increase in urinary pregnanetriol excretion was minimal. In contrast, the Porter-Silber chromogen excretion increased. The patients with congenital adrenal hyperplasia were receiving steroid treatment and had suppressed adrenocortical activity, as shown by the urinary steroid excretion (Table I). These patients reacted quite differently to ACTH; there was only a marginal increase in Porter-Silber chromogens but the urinary pregnanetriol excretion was much increased (Table I).

If the adrenal cortex is stimulated, and a block in the steroid biosynthetic pathway exists, the excretion of metabolites of precursors is expected

FIG. 3.—Duration of steroid therapy versus the degree of 21-hydroxylation deficiency.

to increase while those of the end product, in this instance cortisol, will not increase. In this study this concept has been used to define the degree of 21-hydroxylase deficiency present, by measuring the urinary pregnanetriol and urinary Porter-Silber chromogens before and after ACTH. The choice of the metabolites measured could influence the evaluation of the 21-hydroxylation deficiency, because on theoretical grounds the biosynthetic pathways in adrenocortical steroid production could be characteristically altered by analogues of cortisol. Steroid therapy, as such, might also affect the method, described above, for calculating 21-hydroxylase deficiency.

The 6 children without congenital adrenal hyperplasia showed a minimal degree of 21-hydroxylation deficiency. However, these children had not received any steroids before or during the test. It would be of some interest to see what the degree of 21-hydroxylation deficiency is in children without congenital adrenal hyperplasia who are receiving prolonged steroid therapy.

In the present study even those patients who received steroid therapy for several years (Table I) still responded to ACTH by demonstrating an increase in urinary steroid excretion. The calculated degree of 21-hydroxylase deficiency is not related to the duration of therapy in the salt-losing patients (Fig. 3), or to stimulation with ACTH for 2 or 3 days (Table II).

The relatively small quantities of dexamethasone administered do not significantly contribute to the amount of Porter-Silber chromogens excreted in

the urine. If patients are receiving cortisone, then the steroid therapy can conveniently be changed to dexamethasone before the ACTH test. One salt-losing patient (Case 8), who was on cortisone therapy, was changed to dexamethasone 5 days before the test. The calculated degree of 21-hydroxylase deficiency in this patient was 96%, comparing well with the result (98%) obtained in her older sister (Case 12) who had been on dexamethasone for over 6 months.

In one salt-losing patient (Case 13) a febrile illness with vomiting occurred during the second day of ACTH stimulation. A low serum sodium and a high serum potassium were subsequently found. This patient had received a DOCA implant of 200 mg. before the ACTH test and was receiving her usual daily dosage of 3 mg. prednisolone and 2 g. salt.

TABLE II

Comparison of Degree of 21-hydroxylation Deficiency Present on 2nd and 3rd Day of ACTH Stimulation

Case No.	Degree of 21-hydroxylation Deficiency (%)		
	ACTH Stimulation		Difference Between 2nd and 3rd day
	2nd day	3rd day	
1	75	80	5
4	100	100	0
7	87	87	0
9	100	83	17
10	93	97	4
15	45	54	9

No adverse effects were noted in any of the other patients who were all receiving dexamethasone during the ACTH test and who, if losing salt, were given salt and usually 9- α -fluorohydrocortisone in addition.

The conclusion from this study is that a low degree of 21-hydroxylation deficiency for cortisol production is found in patients with the non-salt-losing state, while a high degree of 21-hydroxylation deficiency is found with the salt-losing condition (Fig. 2), with the exception of one patient (Case 18). There appears to be a relation between the duration of therapy and the degree of 21-hydroxylation deficiency in the patients without the salt-losing tendency (Fig. 3), though the number of patients studied in this group is small. It could be, therefore, that in Case 18 the deficiency of 21-hydroxylation in the cortisol pathway has been impaired by the 9 years of dexamethasone therapy.

It has been shown that aldosterone production is deficient in the salt-losing variant of congenital adrenal hyperplasia, but not in the non-salt-losing variant (Kowarski *et al.*, 1965). It would, therefore, be reasonable to suggest that in the non-salt-losing patient (Case 18) with an 80% deficiency of 21-hydroxylation in the cortisol pathway, there is an adequate capacity for 21-hydroxylation in the biosynthetic pathway leading to aldosterone production.

In all other patients, however, the observed correlation between the inefficiency of cortisol production and the presence or absence of the salt-losing condition suggests a close interrelationship of the genetic factors ultimately responsible for cortisol and aldosterone production.

Summary

The measurement of urinary steroid metabolites before and after ACTH stimulation was used to determine the extent of the 21-hydroxylase defect present in the cortisol pathway of 18 patients with

congenital adrenal hyperplasia, who were receiving steroid therapy. The results indicate that the degree of 21-hydroxylase deficiency in the cortisol pathway is directly related to the two clinical variants of the disease, salt-losing and non-salt-losing.

We wish to express our gratitude to Professor D. V. Hubble for his helpful criticisms and permission to study his patients.

Our grateful thanks are due to Dr. W. H. P. Cant, Consultant Paediatrician at the Birmingham Children's Hospital, and to Dr. G. M. Komrower, visiting physician, Royal Manchester Children's Hospital, for permission to study their patients.

REFERENCES

- Allen, W. M. (1950). A simple method for analysing complicated absorption curves, of use in the colorimetric determination of urinary steroids. *J. clin. Endocr.*, **10**, 71.
- Bongiovanni, A. M., and Eberlein, W. R. (1958). Defective steroidal biogenesis in congenital adrenal hyperplasia. *Pediatrics*, **21**, 661.
- , and Root, A. W. (1963). The adrenogenital syndrome. *New Engl. J. Med.*, **268**, 1283, 1342, and 1391.
- Bush, I. E. (1961). *The Chromatography of Steroids*, pp. 336–346. Pergamon Press, Oxford.
- Epstein, E., and Zak, B. (1963). A new staining technique for ketosteroid chromatograms. *J. clin. Endocr.*, **23**, 355.
- Jacobs, D. R., Van der Poll, J., Gabrilove, J. L., and Soffer, L. J. (1961). 17- α -hydroxyprogesterone—a salt-losing steroid: relation to congenital adrenal hyperplasia. *ibid.*, **21**, 909.
- Kowarski, A., Finkelstein, J. W., Spaulding, J. S., Holman, G. H., and Migeon, C. L. (1965). Aldosterone secretion rate in congenital adrenal hyperplasia. A discussion of the theories on the pathogenesis of the salt-losing form of the syndrome. *J. clin. Invest.*, **44**, 1505.
- Neher, R., Meystre, C., and Wettstein, A. (1959). Neue 16 α hydroxysteroides aus menschlichen urin und aus Schweine-nebennieren. Isolierung, Konstitution, Synthesen. *Helv. chim. Acta*, **42**, 132.
- Rudd, B. T., and Galal, O. M. (1967). The Zimmermann reaction—past and present. *Proc. Ass. clin. Biochem.*, **4**, 175.
- Silber, R. H., and Porter, C. C. (1954). The determination of 17, 21-dihydroxy-20-ketosteroids in urine and plasma. *J. biol. Chem.*, **210**, 923.
- Ulick, S., Gautier, E., Vetter, K. K., Markello, J. R., Yaffe, S., and Lowe, C. U. (1964). An aldosterone biosynthetic defect in a salt-losing disorder. *J. clin. Endocr.*, **24**, 669.
- Visser, H. K. A., and Cost, W. S. (1964). C₂₁-corticosteroid excretion pattern in a familiar salt-losing syndrome. A new enzyme defect in the biosynthesis of aldosterone. *Acta endocr. (Kbh.)*, Suppl. 89, 31.