A Method for the Quantitative Fractionation of Urinary 17-oxo Steroids with some Observations on Steroid Excretion During Administration of ACTH and in the Adrenogenital Syndrome

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Many methods have been described for the estimation of the separate fractions of urinary 17-oxo steroids. However, the earlier methods used hot-acid hydrolysis to cleave the 17-oxo steroid conjugates and it is well known that this procedure causes considerable destruction of steroids and the formation of artifacts (Dorfman & Ungar, 1953). Most of the earlier methods also made no attempt to resolve the individual 17-oxo steroids possessing an oxygen function attached to $C_{(11)}$ of the steroid nucleus. More recently two methods have been described which do this (Rubin, Dorfman & Pincus, 1954; Kellie & Wade, 1956*a*). In the first of these the urine is hydrolysed with hot acid and the 3α-hydroxy ketonic fraction separated into its constituents by repeated paper chromatography in different systems. Artifacts formed during the hydrolysis and Girard separation are individually estimated and the quantities obtained added to the assays of their presumed precursors. Kellie & Wade hydrolyse an extract of urinary conjugates by mild methods and then separate the liberated 17-oxo steroids by gradient elution on a column of alumina.

The method to be described here was devised particularly to fractionate the individual 17-oxo steroids of urines containing low concentrations of these compounds.

NOMENCLATURE

The trivial names androsterone, aetiocholanolone, dehydro*epi*androsterone, 11-oxoandrosterone, 11-oxoaetiocholanolone, 11β-hydroxyandrosterone and 11β-hydroxyaetiocholanolone are used for 3α-hydroxy-5α-androstan-17-one, 3α-hydroxy-5β-androstan-17-one, 3β-hydroxyandrost-5-en-17-one, 3α-hydroxy-5α-androstane-11:17-dione, 3α-hydroxy-5β-androstane-11:17-dione, 3α:11β-dihydroxy-5α-androstan-17-one and 3α:11β-dihydroxy-5β-androstan-17-one respectively throughout this paper.

EXPERIMENTAL

Apparatus

The glass columns for alumina chromatography were 17 cm. long and had an internal diameter of 4 mm. They were tapered at the lower end and a bulb of 100 ml. capacity was fused to the upper end. For paper chromatography, Shandon all-glass tanks were used.

Methods

The flow sheet (Fig. 1) shows the overall plan of the procedure.

Hydrolysis and extraction. A volume of urine is taken which routine 17-oxo steroid analysis (Norymberski, Stubbs & West, 1953) has shown to contain 2 mg. of crude 17-oxo steroid. This specimen is extracted by the method of Edwards, Kellie & Wade (1953) and the ether–ethanol solution evaporated to give a residue containing the urinary 17-oxo steroid conjugates. This residue is dissolved in water (35 ml.), 3 ml. of M-acetate buffer, pH 4·3, added and the solution incubated with β-glucuronidase (20 000 units) at 37°. The batch of β-glucuronidase used in this laboratory was prepared from the visceral humps of limpets (*Patella vulgata*) by a slight modification of an early method (Mills, 1948) and had an activity of 20 000 units/ml. After incubation for 48 hr. the solution is extracted with ether (2 × 30 ml.) and the combined ether extracts are washed with water (1 × 10 ml.), 2N-NaOH (1 × 10 ml.) and water (2 × 10 ml.). The washed ether extract is evaporated to give a residue containing the 17-oxo steroids liberated from the glucuronides. The aqueous solution, together with the first water washing, is placed in the upper vessel of a liquid–liquid extractor and 10N-H_2SO_4 (2 ml.) added. Ether (100 ml.) is placed in the lower vessel of the extractor, which also contains a few chips of marble to neutralize any acid carried down, and the extraction is maintained by refluxing the ether for 3 days.

The ether extract is then washed with water (1 × 15 ml.), 2N-NaOH (1 × 15 ml.) and water (2 × 15 ml.), and evaporated to dryness to give a residue containing the 17-oxo steroids liberated from conjugation with H_2SO_4. The first water washing is added to the aqueous solution, which is then treated with 14 ml. of 11N-HCl and heated on a boiling-water bath for 30 min. After cooling, the solution is extracted with ether (2 × 30 ml.) and the combined ether extracts are washed with 2N-NaOH (2 × 10 ml.) and water (2 × 10 ml.) and then evaporated to dryness.

Portions of the dried residues from each hydrolytic procedure containing 5–50 μg. of 17-oxo steroid are taken for Zimmermann assay. The method employed for assaying here, and subsequently, is essentially that of Edwards *et al.* (1953). The dry fraction is treated in turn with 1% *m*-dinitrobenzene in ethanol (0·1 ml.) and ethanolic 2·5N-KOH (0·05 ml.). After 1 hr. at 25° the mixture is diluted with 2·5 ml. of ethanol and the optical density of the mixture is measured with Ilford spectrum green (no. 604) and violet (no. 601) filters in a Spekker absorptiometer with cells having an optical depth of 1 cm. and a volume of 2·5 ml.

Girard separation and adsorption chromatography. The extracts obtained by the three methods of hydrolysis were combined, dried and separated into ketonic and non-ketonic fractions by the Pincus & Pearlman (1941) modification of the Girard procedure. Since some acetylation of the free hydroxide groups takes place under these conditions, it is necessary to hydrolyse the acetates which have been formed so that subsequent fractionation is simplified. The evaporated ketonic fraction is therefore dissolved in methanol (1 ml.), 0·2 ml. of an aqueous solution of $KHCO_3$ containing 50 mg./ml. is added and the mixture allowed to stand overnight at 37°. The solution is then partitioned between ether (30 ml.) and water (7 ml.) and the aqueous phase extracted with a further 10 ml. of ether. The combined ether extracts are washed with water (2 × 5 ml.) and evaporated to dryness.

The dry ketonic fraction is separated into less polar and more polar fractions by a simple chromatogram on alumina. The column is prepared in the following way. A small plug of cotton wool is inserted into the lower (tapered) end of the glass column. A slurry of alumina (1·5 g., Savory and Moore, Brockmann activity II–III) in 4–5 ml. of benzene is poured into the top of the bulb and the column gently tapped while the alumina settles down. The ketonic fraction is dissolved in benzene (5 ml.) and 4 ml. of this solution carefully run on to the top of the column; a further 0·1 ml. portion of the benzene solution is taken for Zimmermann assay. The steroids are eluted successively with 50 ml. of benzene containing 0·3% ethanol (fraction 1) and 20 ml. of benzene containing 10% ethanol (fraction 2). Development of the column may be considerably hastened by the application of pressure (100 mm. Hg). A 2·5 l. bottle (e.g. a Winchester) fitted with a manometer and a hand bellows forms a convenient air reservoir and can provide a stable source of pressure for up to six columns.

Fraction 1 contains those 17-oxo steroids which do not possess an oxygen function in the $C_{(11)}$ position and fraction 2 contains those which do. Portions of the two fractions may be taken for Zimmermann assay: one-thirtieth of the less polar fraction and one-tenth of the more polar fraction.

Paper chromatography. Whatman no. 2 filter paper is cut as shown in Fig. 2. The less polar fraction (1) is run in a Bush (1952) *A* system (light petroleum–methanol–water, 5:4:1, by vol.) at room temperature with standard

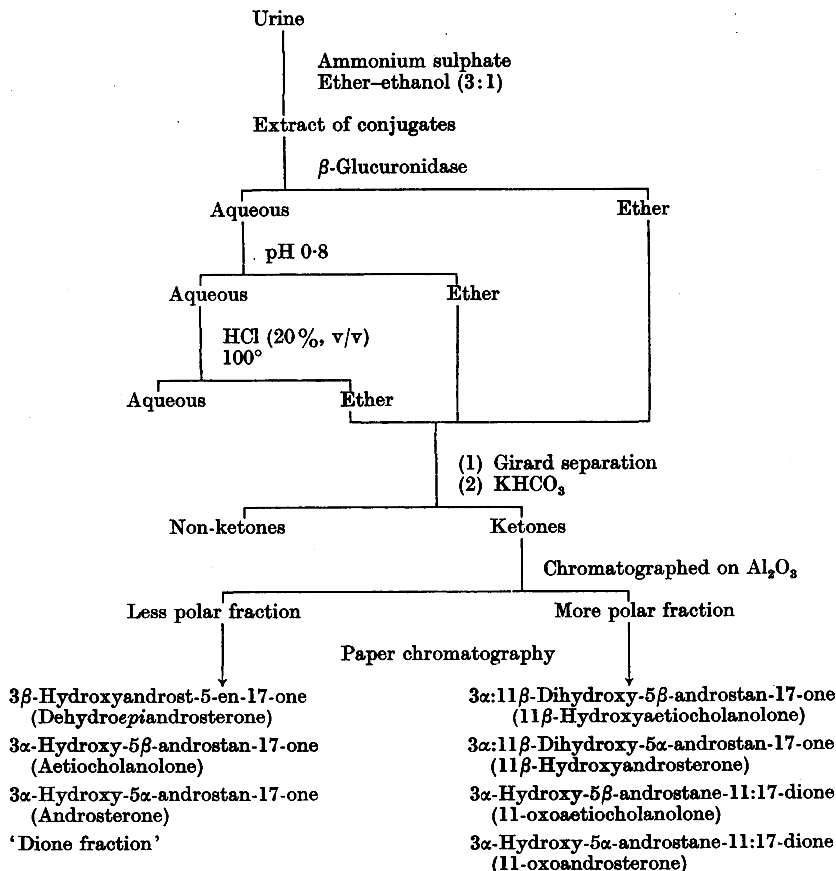

Fig. 1. Scheme for fractionating urinary 17-oxo steroids. The individual steroids isolated in the less polar and more polar fractions are listed in the order in which they appear on their respective paper chromatograms, compounds with the greatest mobility being at the bottom of the list.

reference compounds on lanes 1, 3 and 5. Lanes 1 and 5 have only aetiocholanolone, but lane 3 has, in addition, dehydro*epi*androsterone, androsterone and 5α-androstane-3:17-dione. A mixture containing 20 μg. of each of these four standards in 20 μl. of ethyl acetate is used.

The more polar fraction (2) is run in a system consisting of light petroleum–toluene–methanol–water (667:333:800:200, by vol.) at 37°. Reference standards 11β-hydroxyaetiocholanolone, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone are run in lane 3 and 11β-hydroxyandrosterone only in lanes 1 and 5. It is desirable to apply 150–200 μg. of the unknown mixture of 17-oxo steroids to the 3 cm. wide lanes. In practice this usually means taking one-quarter of the less polar fraction or all the more polar fraction. Standards and unknowns are applied to the strips with 200 μl. graduated pipettes and simultaneously dried by a jet of nitrogen directed at the under side of the strip. The papers are then hung in the tanks overnight to equilibrate and run the following day for 6 hr. When the papers have been removed from the tanks and dried, the 3 mm. wide strips marked on the middle of lanes 2 and 4 are cut out and, together with lanes 1, 3 and 5, dipped in a mixture of equal volumes of 2% *m*-dinitrobenzene in ethanol and ethanolic 2·5 N-KOH. The strips are allowed to drain for 30 sec. before being laid on a horizontal sheet of glass. After 5–10 min. at room temperature they are transferred to an oven at 90° for 3 min.: this procedure gives minimal background colour.

Regions of the remainder of the 3 cm. lanes corresponding to the spots obtained on the test portion are then cut out and placed in stoppered test tubes overnight with 2·5 ml. of ethanol. Two 1 ml. portions from each of these solutions are evaporated to dryness and assayed in the usual way. If there is reason to doubt the identity of any Zimmermann-reacting spot on the test strip, the two coloured solutions obtained after assaying the corresponding eluted zone are combined and acidified and the 17-oxo steroid–*m*-dinitrobenzene complex extracted as described by Kellie & Smith (1956). The extract may then be rechromatographed on paper together with the appropriate 17-oxo steroid–*m*-dinitrobenzene standard in the same system as was used for the corresponding free 17-oxo steroid.

11-Oxoaetiocholanolone and its 5α-epimer do not separate well in the system used for resolving the more polar fraction. If the amount of 5β-epimer is large compared with the 5α-epimer the two zones may be confluent, making it impossible to distinguish the separate components. In such cases the complete section of the 3 cm. lane corresponding to the two 3α-hydroxyandrostane-11:17-diones is cut out and eluted in the usual way. One 2 ml. portion is removed from the solution, evaporated and the residue so obtained applied to the start line of a 3 cm. lane. The two standard substances are applied to neighbouring 1 cm. wide lanes and the chromatogram is developed in a Bush *A* system at room temperature for 24 hr.; this procedure gives very good separation.

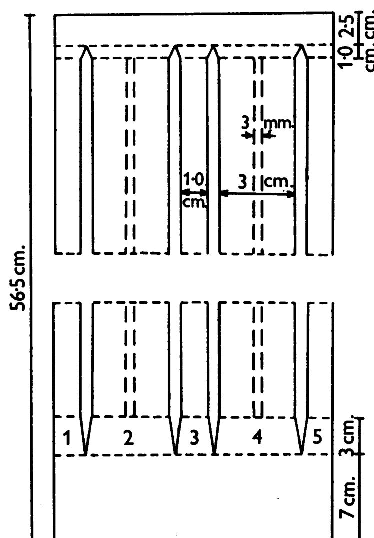

Fig. 2. Plan of paper for quantitative chromatography of 17-oxo steroids. The full lines indicate the shape in which the paper is cut before chromatography and the interrupted lines are for reassessment. The 3 mm. strips (indicated by interrupted lines) in the middle of the 3 cm. lanes are cut out after chromatography and, together with the standard strips 1, 3 and 5, treated with Zimmermann reagents.

RESULTS

Recovery of crystalline steroids in the Girard separation

In the fractionation of the urinary 17-oxo steroids from the cases reported in this paper (Tables 2 and 3), the hydrolysis of any acetates formed during the Girard separation was effected by dissolving the ketonic fraction in ethanolic 0·5 N-KOH and allowing the mixture to stand at room temperature overnight. However, in two experiments in which 11-oxoaetiocholanolone (98 μg.) was added to the specimens of urine from two patients with hypopituitarism, recoveries of less than 40% were obtained when the complete fractionation procedure was followed. The loss was traced to the hydrolysis with ethanolic KOH after the Girard separation. Table 1 shows the results of an experiment in which 200 μg. quantities of six crystalline 17-oxo steroids were individually put through the Girard separation, after which the ketonic fractions were divided into three equal parts. Part (*A*) was chromatographed on paper and in three cases this gave rise to two Zimmermann-reacting spots, the more mobile of which was probably 17-oxo steroid acetate. Part (*B*) was hydrolysed overnight at room temperature with ethanolic 0·5 N-KOH before being chromatographed; part (*C*) was hydrolysed overnight with $KHCO_3$ in aq. methanol at 37° and then chromatographed on paper. In every case parts (*B*) and (*C*) gave only one Zimmermann-reacting spot, although part (*B*) of the 11-oxoaetiocholanolone and 11-oxoandrosterone series gave a non-specific grey–blue chromogen 8 cm. in front of the 17-oxo steroid zone.

Table 1. *Recovery of crystalline 17-oxo steroids*

Recoveries recorded after Girard separation followed by (*A*) paper chromatography, (*B*) hydrolysis with ethanolic 0·5N-KOH and paper chromatography, (*C*) hydrolysis with $KHCO_3$ in aq. methanol and paper chromatography.

	Recovery (%)			
	(*A*) Girard separation		(*B*)	(*C*)
Compound	Main spot	Mobile spot	Girard separation + KOH hydrolysis	Girard separation + $KHCO_3$ hydrolysis
Androsterone	64	7	70	85
Aetiocholanolone	61	—	79	86
Dehydro*epi*androsterone	71	5	77	82
11-Oxoandrosterone	68	—	13	79
11-Oxoaetiocholanolone	81	5	11	87
11β-Hydroxyandrosterone	87	—	96	90

The recovery of these two compounds was very low after hydrolysis with KOH, but not when $KHCO_3$ was used. There was no significant difference in the recoveries of the other compounds by the three procedures described above and it therefore seems justifiable to use hydrolysis with $KHCO_3$ as the standard method.

Recovery from alumina chromatograms

In chromatography on alumina the most polar of the major urinary 17-oxo steroids without an oxygen attached to $C_{(11)}$ is aetiocholanolone. Recovery experiments were therefore carried out with an extract of hydrolysed urine divided into three equal parts, to one of which was added aetiocholanolone and to another 11-oxoaetiocholanolone. The mean recovery for the two compounds in the appropriate fractions was essentially quantitative, being 97 % for aetiocholanolone and 103 % for the 11-oxo analogue. Any considerable departure of the alumina from this performance would, of course, be revealed on the subsequent paper chromatography, i.e. by Zimmermann-reacting material moving more slowly than dehydro*epi*androsterone in the paper chromatography of the less polar fraction or by a spot near the solvent front in the more polar system.

Recovery from paper chromatograms

The recovery of crystalline androsterone (80 µg.) and dehydro*epi*androsterone (52 µg.) chromatographed together on 3 cm. wide lanes in the Bush *A* system and eluted in the way described in the method was tested in a series of six experiments. After development of the chromatogram the zones were eluted on the basis of the distances run by the standards androsterone, aetiocholanolone and dehydro*epi*androsterone and assayed in duplicate. For androsterone the mean recovery was 87 ± 2·8 % (S.D.) and for dehydro*epi*androsterone 92 ± 3·6 % (S.D.). Aetiocholanolone, which travels between these two compounds in this system, was not applied to the recovery lanes, so that any material found in this zone must have been derived from the neighbouring androsterone and dehydro*epi*androsterone zone and is therefore a measure of the inefficiency of separation. The percentage of the total 17-oxo steroids applied which was found in this region was 3·7 ± 1·2 % (S.D.).

Recovery of added steroid through the complete method

In addition to the two 11-oxoaetiocholanolone recovery experiments already reported, three other overall recovery experiments were done. In the first 11β-hydroxyaetiocholanolone (91 µg.) and sodium dehydro*epi*androsterone sulphate (110 µg.) were added to one-sixth of a 24 hr. urine collection from a patient with Addison's disease. This specimen was put through the complete fractionation procedure in parallel with a similar portion to which no steroid had been added. The recoveries of these two compounds in their appropriate fractions were 73 and 72 % respectively. In the second and third recovery experiments, androsterone (250 µg.) was added to specimens of urine from two patients with hypopituitarism. The recoveries of the added steroid in the androsterone regions of the paper chromatograms were 75 and 82 % when compared with their respective control specimens.

Normal values

Table 2 shows the results of fractionating the urinary 17-oxo steroids in two normal men and six normal women. The proportion of the crude total 17-oxo steroids hydrolysed by β-glucuronidase varied from 58 to 86 %, and continuous extraction at pH 0·8 released 12–34 % and hot acid 2–13 % of the total. These figures agree well with those reported by Kellie & Wade (1956*b*). However, the colours obtained when performing the Zimmermann reaction with the fractions liberated by the final hot-acid hydrolysis were very impure, usually showing a greater absorption with the violet filter than with green. The apparent 17-oxo steroid content of the ketonic fractions represent from 57 to 71 % of the crude total fractions. This range is narrower than

Table 2. *Fractionation of the urinary* 17-*oxo steroids in normal human individuals*

All amounts are in mg./24 hr. and for individual compounds they are corrected for the colour equivalent of the compound concerned in the Zimmermann reaction. F, female; M, male.

Case no.	1	2	3	4	5	6	7	8
Sex	F.	F.	F.	F.	F.	F.	M.	M.
Age (years)	28	21	23	30	26	22	25	40
Hydrolysed by β-glucuronidase	10·5	7·4	10·4	9·0	8·0	10·2	14·7	10·5
Hydrolysed at pH 0·8	3·9	1·0	1·7	3·2	2·4	2·4	8·8	5·2
Hydrolysed by HCl at 100°	0·6	0·2	0·6	0·9	1·5	0·9	2·1	1·0
Total	15·0	8·6	12·7	13·1	11·9	13·5	25·6	16·7
Ketonic fraction	8·6	5·3	8·2	7·6	7·5	8·6	15·0	11·9
Less polar fraction	7·8	5·1	8·4	6·5	6·5	6·4	14·0	10·2
More polar fraction	1·6	1·3	1·3	1·2	1·4	1·6	1·6	1·3
Unidentified mobile fraction	—	—	—	0·1	0·2	0·2	0·3	—
Dione fraction	—	—	0·1	—	—	—	—	—
Androsterone	1·9	2·2	3·4	2·8	2·9	2·3	4·6	4·0
Aetiocholanolone	2·3	1·4	2·5	2·0	1·5	3·0	3·0	2·7
Dehydro*epi*androsterone	1·1	0·1	0·2	0·3	0·4	0·3	2·7	2·4
11-Oxoaetiocholanolone	0·13	0·10	0·04	0·16	0·17	0·16	0·22	0·12
11β-Hydroxyandrosterone	0·85	0·75	0·82	0·61	0·85	0·54	1·40	0·46
11β-Hydroxyaetiocholanolone	0·47	0·21	0·21	0·23	0·36	0·51	0·47	0·41

that reported by Dobriner, Kappas, Rhoads & Gallagher, 1953 and by Kappas & Gallagher, 1955.

Of the individual 17-oxo steroids the relatively low excretion of dehydro*epi*androsterone in five of the six women should be noted. The ratio of androsterone to its 5β isomer covers the range 2:1 to 0·8:1. The two isomeric androstane-3:17-diones (5α- and 5β-) are not separated in this method but only in case 3 was any material found in this zone. However, four other individuals (cases 4–7) had very small amounts of unknown material which had greater mobility than the diones in this system.

The 17-oxo steroid found in greatest quantity in the more polar group is, in every case, 11β-hydroxyandrosterone, the daily amounts excreted varying from 0·46 to 1·4 mg. The amounts of 11-oxoaetiocholanolone found in this normal series are low compared with the quantities reported by some workers (Rubin *et al.* 1954; Kappas & Gallagher, 1955; Kellie & Wade, 1956*b*). This is presumably due to some destruction of this compound taking place during hydrolysis with ethanolic KOH which was used at this time in place of hydrolysis with $KHCO_3$ in aq. methanol, now the standard procedure. In the reports by other workers referred to above this loss did not arise either because the Girard separation was not used (Kellie & Wade, 1956*b*) or because no attempt was made to hydrolyse acetates formed during the Girard reaction (Rubin *et al.* 1954; Kappas & Gallagher, 1955). It would appear from the two experiments referred to earlier, in which 11-oxoaetiocholanolone was recovered from urine by the fractionation method incorporating hydrolysis with ethanolic KOH, that the actual values for the excretion of this 17-oxo steroid should be several times those reported here.

Effect of stimulation with adrenocorticotrophic hormone

Table 3, columns 1 and 2, shows the pattern obtained by fractionating the 17-oxo steroids in a 14-year-old boy before and during administration of adrenocorticotrophic hormone (ACTH) gel injected intramuscularly. Treatment causes the greatest changes in the excretion of dehydro*epi*androsterone and 11β-hydroxyaetiocholanolone, both of which show 15-fold increases.

Separate fractionation of the 17-*oxo steroids liberated by the different hydrolytic procedures*

The remaining columns of Table 3 show the results obtained on fractionating the 17-oxo steroids released in two patients after applying the successive methods of hydrolysis. Patient 10 was a girl (13 years old) with the adrenogenital syndrome due to congenital adrenal hyperplasia; she was at this time receiving no hormone treatment. Patient 11 had Klinefelter's syndrome.

The extract containing the urinary-steroid conjugates from case 10 was hydrolysed in the usual way, first with β-glucuronidase, then at pH 0·8 during continuous extraction with ether and finally by boiling with hot mineral acid. However, the three extracts obtained from these procedures were not combined before the Girard separation but were individually put through the entire fractionation procedure, the results being shown in columns 3, 4 and 5. Similarly, column 6 shows the composition of the fraction liberated by the action of β-glucuronidase on the extract from case 11, and column 7 shows the additional amounts released by the continuous extraction with ether at pH 0·8. Column 8 shows the residual amounts obtained by hot acid.

Table 3. *Urinary 17-oxo steroid excretion during treatment with adrenocorticotrophic hormone (ACTH) and in the adrenogenital syndrome*

Columns 3–8 show the amounts released by the successive application of the three hydrolytic procedures in cases 10 and 11. All amounts are in mg./24 hr. and for individual compounds they are corrected for the colour equivalent of the compound concerned in the Zimmermann reaction.

Column no.	1	2	3	4	5	6	7	8
Case no.	9	9	10	10	10	11	11	11
Treatment	Nil	ACTH	Nil	Nil	Nil	ACTH	ACTH	ACTH
Hydrolysed by β-glucuronidase	4·7	31·0	80·8	—	—	60·6	—	—
Hydrolysed at pH 0·8	1·8	24·0	—	15·7	—	—	11·7	—
Hydrolysed by HCl at 100°	0·7	8·0	—	—	7·0	—	—	4·0
Total	7·2	63·0	—	—	—	—	—	—
Ketonic fraction	5·0	40·4	—	—	—	41·7	2·9	—
Less polar fraction	3·2	26·2	37·8	7·4	0·6	27·3	1·5	0·48
More polar fraction	1·0	15·4	20·7	1·7	0·4	11·3	1·3	0·05
Dione fraction	0·02	0·3	—	0·7	—	—	—	—
Androsterone	1·3	6·2	21·1	1·0	0·06	10·3	0·2	—
Aetiocholanolone	0·9	5·7	12·1	0·7	0·05	16·5	0·4	—
Dehydro*epi*androsterone	0·4	6·8	0·3	2·8	0·06	0·4	0·6	—
11-Oxoaetiocholanolone	0·13	0·8	2·2	0·1	0·1	0·5	0·2	—
11β-Hydroxyandrosterone	0·71	7·1	22·4	1·0	0·1	2·6	0·6	—
11β-Hydroxyaetiocholanolone	0·43	7·1	4·4	0·2	0·1	7·7	0·7	—

The most notable feature of these results from the standpoint of methodology is that whereas in patient 10 the crude 17-oxo steroids obtained by hot-acid hydrolysis (column 5) form 7 % of the total obtained by the successive application of all three hydrolytic procedures (column 3 + 4 + 5), after the removal of the non-ketones by Girard separation this proportion is only 1·5 %. In patient 11 the corresponding proportions are 6 and 1·3 %. β-Glucuronidase released 78 % in patient 10 and 80 % in patient 11 of the total crude 17-oxo steroids. However, the results show that the extracts obtained by β-glucuronidase hydrolysis are relatively pure so that the predominance of the glucuronide mode of conjugation is greater than these percentages indicate.

In these two patients only one compound is found in greater amount in the fraction hydrolysed at pH 0·8 than in the β-glucuronidase fraction, namely dehydro*epi*androsterone. The small amount of this compound found in the fraction hydrolysed by β-glucuronidase (cf. Kellie & Wade, 1956*b*) may have been excreted as the glucuronide or they may have been present in the urine in the free state. Control experiments with sodium dehydro*epi*androsterone sulphate, prepared synthetically, showed that the enzyme preparation used did not liberate the free steroid under the standard conditions.

In case 10 the large amounts of more polar 17-oxo steroids excreted should be noted; especially significant is the excretion of 11β-hydroxyandrosterone which, together with its 11-deoxy analogue, forms two-thirds of the total. In spite of the level of 23·4 mg. of 11β-hydroxyandrosterone/24 hr. released by β-glucuronidase and hydrolysis at pH 0·8 only a further 0·1 mg. is liberated by hot acid. This contrasts with the evidence of Lieberman, Mond & Smyles (1954) for the existence of a conjugate of this compound which is not hydrolysed by enzyme in 60–65 hr. nor by mild acid treatment.

DISCUSSION

The method described is lengthy in that almost 2 weeks is required to complete the fractionation of one specimen. However, this is largely because of the mild methods of hydrolysis used: the manipulation time required is not inordinately great. One person can begin the fractionation of four specimens each week, so that during the hydrolysis of any one batch of four the subsequent purification of the preceding batch is being carried forward simultaneously. It is apparent from the results of the two experiments (cases 10 and 11), in which the residues obtained by the final hot-acid hydrolysis were further fractionated on their own, that this hydrolytic stage is unnecessary and may be omitted.

It was recommended earlier that a volume of urine containing 2 mg. of crude 17-oxo steroids should be taken for analysis. In fact the sensitivity of the method is greater than might be inferred from this figure, which was selected for normal cases in which the amount in the more polar fraction is less than one-quarter of that in the less polar fraction. In many cases in which the excretion of 17-oxo steroids is low (e.g. in children, old people, patients with hypopituitarism), the two fractions are more nearly equal and a sample containing as little as 0·5 mg. may be sufficient for analysis. The fairly extensive purification involved in this method

makes it possible to fractionate the 17-oxo steroids in such specimens without difficulty, since the final extracts for paper chromatography are relatively free from pigments and non-ketonic chromogens. In view of the comparatively large number of steps in the method with the inevitable losses associated with each stage in the purification, the overall recoveries of added crystalline 17-oxo steroids would appear to be reasonable and justified by the increased specificity which is ensured.

Ethanolic potassium hydroxide was the method initially chosen to hydrolyse any 17-oxo steroid acetates formed in the Girard separation, for two reasons: first, because this method has been used by other workers for splitting 17-oxo steroid acetates (Edwards *et al.* 1953), and secondly, because preliminary experiments with dehydro*epi*androsterone acetate indicated complete hydrolysis at room temperature overnight without loss of steroid or formation of artifacts. However, poor recoveries of 11-oxoaetiocholanolone from urine led to the performance of the comparative experiment, the results of which were shown in Table 1, and resulted in the adoption of potassium bicarbonate in aqueous methanol for hydrolysis; in this way therefore it should be possible to get a quantitative estimate of all the major urinary 17-oxo steroids.

The presence of large amounts of 17-oxo steroids in the more polar group of case 10 is of interest because of the light it sheds on the nature of the biochemical abnormality in the adrenogenital syndrome. The 17-oxogenic steroids (Gibson & Norymberski, 1954) excreted by this patient were 43 mg./day, a figure which could be accounted for by the level of pregnanetriol (5β-pregnane-3α:17α:20α-triol), which by the method of Bongiovanni & Clayton (1954) was 45 mg./day. Urinary 17-hydroxycorticosteroids determined by the method of Reddy, Jenkins & Thorn (1952) were normal (5·1 mg./day). This picture of the excretion of $C_{(21)}$ metabolites of the adrenal steroids by this patient was confirmed by the chromatography of the 17-oxogenic steroids on alumina. Only 26 % of the 17-oxogenic steroids were found to have an oxygen atom attached to $C_{(11)}$, whereas in the normal individual 11-oxygenated 17-oxogenic steroids predominate. There is therefore a contrast between the high proportion of 11-oxygenation among the C_{19} steroids and the low proportion among the C_{21} steroids. Jailer (1953) has suggested that in virilizing adrenal hyperplasia there is a metabolic block in the conversion of 17α-hydroxyprogesterone (17α-hydroxypregn-4-ene-3:20-dione) into cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione) and that this intermediate is metabolized by alternative pathways, e.g. to pregnanetriol and 17-oxo steroids. Dorfman (1954) has collected evidence which shows that whereas 11-oxygenated C_{21} compounds with the Δ^4-3 keto grouping are metabolized to C_{19} compounds, chiefly of the 5β-series, adrenosterone (androst-4-ene-3:11:17-trione) yields mainly 5α-urinary metabolites. The patterns of C_{21} and C_{19} steroid excretion in this patient conform to this concept well. Thus the very large amounts of 11-hydroxylated 17-oxo steroids are mainly the 5α-compound and would appear to be derived from a C_{19} precursor with a Δ^4-3 keto grouping. The high ratio of androsterone to its 5β-analogue found here has been reported also by Wilkins, Bongiovanni, Clayton, Grumbach & van Wyk (1955), in one of their cases with this syndrome and also by Pond (1954). This high 5α:5β ratio indicates that if any considerable proportion of the urinary 17-oxo steroids are derived from 17-hydroxyprogesterone then fission of the side chain at $C_{(17)}$ occurs before reduction of the A ring of the steroid nucleus.

SUMMARY

1. A method is described for the analysis of the major urinary 17-oxo steroids.

2. An extract containing the urinary 17-oxo steroid conjugates is hydrolysed first with β-glucuronidase, then by continuous extraction with ether at pH 0·8. The liberated 17-oxo steroids are purified by application of the Girard treatment and the ketonic fraction is treated with potassium bicarbonate in aqueous methanol to hydrolyse any acetates formed in the Girard separation. The product is then separated into two fractions by chromatography on a column of alumina. The first fraction eluted contains those 17-oxo steroids without an oxygen attached to $C_{(11)}$ and the second fraction contains the 17-oxo steroids with a $C_{(11)}$ oxygen function. These two fractions are resolved into their component 17-oxo steroids by paper chromatography, the final estimation being made by applying the Zimmermann reaction to residues obtained after eluting the individual steroids from the paper.

3. Recovery of added 17-oxo steroid through the complete procedure is 70–80 %.

4. Evidence is presented to show the effectiveness of the hydrolytic procedures.

5. Normal values for six females and two males are given, and also values after stimulation with adrenocorticotrophic hormone, and for one case of the adrenogenital syndrome.

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REFERENCES

Bongiovanni, A. M. & Clayton, G. W. (1954). *Johns Hopk. Hosp. Bull.* **94**, 180.

Bush, I. E. (1952). *Biochem. J.* **50**, 370.

Dobriner, K., Kappas, A., Rhoads, C. P. & Gallagher, T. F. (1953). *J. clin. Invest.* **32**, 940.
Dorfman, R. I. (1954). *Recent Progr. Hormone Res.* **9**, 5.
Dorfman, R. I. & Ungar, F. (1953). *Metabolism of the Steroid Hormones*, p. 22. Minneapolis: Burgess Publishing Company.
Edwards, R. W. H., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.* No. 2, 53.
Gibson, G. & Norymberski, J. K. (1954). *Ann. Rheum. Dis.* **12**, 59.
Jailer, J. W. (1953). *Bull. N.Y. Acad. Med.* **29**, 377.
Kappas, A. & Gallagher, T. F. (1955). *J. clin. Invest.* **34**, 1566.
Kellie, A. E. & Smith, E. R. (1956). *Nature, Lond.*, **178**, 323.
Kellie, A. E. & Wade, A. P. (1956*a*). *Biochem. J.* **62**, 1 P.
Kellie, A. E. & Wade, A. P. (1956*b*). *Acta endocr. Copenhagen*, **23**, 357.
Lieberman, S., Mond, B. & Smyles, E. (1954). *Recent Progr. Hormone Res.* **9**, 113.
Mills, G. T. (1948). *Biochem. J.* **43**, 125.
Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **1**, 1276.
Pincus, G. & Pearlman, W. H. (1941). *Endocrinology*, **29**, 413.
Pond, H. M. (1954). *J. Endocrin.* **10**, 202.
Reddy, W. J., Jenkins, D. & Thorn, G. W. (1952). *Metabolism*, **1**, 511.
Rubin, B. L., Dorfman, R. I. & Pincus, G. (1954). *Recent Progr. Hormone Res.* **9**, 213.
Wilkins, L., Bongiovanni, A. M., Clayton, G. W., Grumbach, M. M. & van Wyk, J. (1955). *Ciba Foundation Colloq. Endocrin.* **8**, 460.

The Synthesis of *N*-Acetylneuraminic Acid

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The term neuraminic acid is now reserved for a hypothetical amino sugar whose derivatives are found combined in many animal tissues, secretions and excretions. These derivatives of natural occurrence, which are known as sialic acids (for nomenclature see Blix, Gottschalk & Klenk, 1957), have acyl groups bound to the amino group and sometimes also to a hydroxyl group of the parent neuraminic acid; the commonest substituent is acetyl. The first sialic acid to be crystallized was isolated from bovine-submaxillary-gland mucin by Blix (1936); it is now known to be an *ON*-diacetylneuraminic acid (Blix, Lindberg, Odin & Werner, 1956). Crystalline *N*-acetylneuraminic acid was first prepared from the same mucin by Klenk & Faillard (1954), the *O*-acetyl group having been lost when the mucin's acidity was used to catalyse its own hydrolysis. Later, the influenza-virus enzyme neuraminidase (Gottschalk, 1951, 1957*a*) was used to release *N*-acetylneuraminic acid from human urinary mucoprotein (Klenk, Faillard & Lempfrid, 1955). Since then, the acid has been isolated from pseudomyxomatous cysts (Odin, 1955*a*), human-serum proteins (Odin, 1955*b*; Svennerholm, 1956; Böhm, Ross & Baumeister, 1957), human-cervix-uteri mucus (Odin, 1955*c*), human meconium (Odin, 1955*b*), human gangliosides (mucolipids) (Blix & Odin, 1955; Svennerholm, 1956), human lipid-free brain tissue (Svennerholm, 1956), human liver (Martinsson, Raal & Svennerholm, 1957), stroma of human erythrocytes (Klenk & Lempfrid, 1957), human milk (Svennerholm, 1956), fetuin (Klenk & Faillard, 1957), boar-seminal mucin (Odin, 1955*c*), ovine-submaxillary-gland mucin (Blix *et al.* 1956), and from ovomucin (Odin, 1955*c*).

In mucoproteins and mucolipids, the sialic acid forms part of a very large molecule, but Kuhn & Brossmer (1956*a*) have isolated from cow colostrum a trisaccharide in which *ON*-diacetylneuraminic acid is bound glycosidically to lactose. Gynaminic acid from the non-dialysable fraction of human milk is identical with *N*-acetylneuraminic acid (Zilliken, Braun & György, 1955). Sialic acid of porcine-submaxillary-gland mucin and of the stroma of erythrocytes from horse, ox and swine was shown to be *N*-glycolloylneuraminic acid (Blix *et al.* 1956; Klenk & Uhlenbruck, 1957). Horse-serum lactaminic acid as prepared by Yamakawa & Suzuki (1955) is almost certainly the methyl ester of *N*-acetylneuraminic acid.

When Klenk's (1941) original method of heating at 100° with 5% methanolic hydrogen chloride is used to split sialic acid from mucoids, the methylglycoside of neuraminic acid is obtained, all acyl groups being lost by alcoholysis. This glycoside has been prepared from gangliosides (Klenk, 1941),