The Partial Characterization of 1-Oxygenated Steroids from Urine of a Hypertensive Newborn Child

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1. Four substances from the urine of a hypertensive newborn girl were partially characterized and shown to be 17 α -hydroxy-5 β -pregnane-1,3,20-trione, 3 α ,17 α -dihydroxy-5 β -pregnane-1,20-dione, 3 α ,17 α ,20 α -trihydroxy-5 β -pregnan-1-one and 5 β -pregnane-1 β ,3 α ,17 α ,20 α -tetrol. 2. The characterization rested mainly on R_M analysis of the substances and their derivatives by glycol fission, providing evidence for position and degree of substitution and for steroidal character. Supporting evidence was provided by chemically specific location reactions. 3. Certain problems in the manipulation of these β -disubstituted steroids are discussed.

The high 17-hydroxy corticosteroid excretion of patient S.C. when 1-3 weeks old and grossly hypertensive has been described elsewhere (Edwards, Harvey & Knight-Jones, 1968). Further examination of her urinary steroids by two-dimensional chromatography (Edwards, 1968*a*) after β -glucuronidase hydrolysis indicated two zones that were located by reduction of tetrazolium in a characteristically slow manner and were not superimposable on those of any reference steroids. Larger quantities isolated by paper-chromatographic purification provided 100-200 μ g. each of four substances designated H1, H2, H3 and H4. With these small quantities further characterization was limited to R_M analysis and the interpretation of the results of location reactions on paper chromatograms.

MATERIALS AND METHODS

Full details of the procedures used for the isolation of urinary steroids are described elsewhere (Edwards, 1968*a*) and in the present instance consisted of the following steps. Urine samples (6 days' collection, age between 8 and 20 days) from S.C. during her period of high 11-oxygenation index (ratio of metabolites of 17-hydroxylated precursors of cortisol to metabolites of cortisol; Edwards, Makin & Barratt, 1964) were combined (total volume 270 ml.), acidified (to pH 2) and near-saturated with (NH₄)₂SO₄ (50 g./100 ml.). Three ether-propan-2-ol (3:1, v/v) extracts (130 ml. each) were combined, evaporated (at 40°), dissolved in 0.1M-sodium acetate buffer, pH 4.5 (20 ml.), and treated with *Patella vulgata* β -glucuronidase [10⁵ units (Talalay, Fishman & Huggins, 1946); Baylove, Edinburgh]. After standing overnight (in the dark at 37°) the steroids were extracted into ether-ethyl acetate (1:1, v/v) (three 50 ml. portions).

Several stages of paper chromatography in light petroleum (b.p. 80-100°)-methanol-water (5:4:1, by vol.) mixtures with benzene substituted for different proportions of the

light petroleum (Bush, 1952) provided four substances located by a characteristic very slow reduction of styryl-tetrazolium reagent (Stevens, 1964). Each substance was homogeneous on the criterion that portions from the forward and rear portions of a chromatographed zone on rechromatography gave zones side by side of identical R_F values.

The chemical transformations used were (i) borohydride reduction and (ii) periodate oxidation, both carried out as described by Edwards *et al.* (1964), and (iii) oxidation with Jones reagent carried out as described by Djerassi, Engle & Bowers (1956), but with trebled proportion of acetone.

R_M analysis procedure. R_M values were calculated from R_F values and referred to the root system of the family (light petroleum-methanol-water, 5:4:1, by vol.) as R_{M0} values by correcting for the effects of benzene replacing light petroleum (Kabasakalian & Basch, 1960; Edwards, 1968*a*). Observed ΔR_M values were compared with reference values (Table 1) and the latter used to indicate likely structural changes. For clarity the ΔR_M value observed in changing from substance of structure A to structure B was signified $\Delta R_M [A \rightarrow B]$. It was presumed that substances with R_{M0} values differing by less than 0.07 (s.d. of repetition of a value on different occasions) were in fact one substance.

Reference 1-hydroxy steroids. 1 α -Hydroxy-5 α -androstane-3,17-dione samples from Dr Fukushima and Dr Foell were found to be identical with the exception of a small proportion of contaminant in the latter. This was assumed to be the 1 β -isomer, since other isomerization was unlikely from the method of preparation. A similar contaminant was present in the 1 α -hydroxyandrost-4-ene-3,17-dione sample. The 1 α ,3 β -dihydroxy-5 α -androstan-17-one sample was uncontaminated.

Other reference steroids were prepared by unambiguous chemical transformations and were purified by chromatography, but there was insufficient material available for isolation and characterization. Attempted oxidation of 1 α -hydroxy-5 α -androstane-3,17-dione with the Jones reagent yielded only highly polar material of the nature of a 17-oxo-seco acid. Since such extensive oxidation was probably due to the lability of the enolized 3-oxo group, use

Table 1. *Reference ΔR_M values*

Values were determined in this Laboratory during the present study or, marked *, are the mean of the values obtained by other workers [see Edwards (1968a), where many other values not pertinent to the present work are given]. A, Androstane; β P, pregnane with β -side-chain orientation.

Substitution at position	ΔR_M		
	Hydroxyl group		Oxo group
	Axial	Equatorial	
1†	α 1.50	β 1.19	0.94
2	β 0.33	α 0.41	
3	α 1.45	β 1.6	0.83
6	β 1.23	α 1.69	0.51
7	α 1.13	β 1.40	0.82
9	α 1.0*		
11	β 0.86	α 1.50	0.73
12	α 1.06	β 1.24*	0.84*
Quasi			
14	α 1.31*		
15	α 1.64*	β 1.5	0.5*
16	α 1.65	β 1.81	0.16*
17A	α 1.50	β 1.67	0.83
17 β P	α 0.81		
18		1.75	
19		1.80*	
20 β P	α 1.65	β 1.53	0.88
21		0.80	
Interconversion			
	From	To	ΔR_M
5 α		5 β	0.14
1-One		1 α -Ol	0.55
1-One		1 β -Ol	0.25
3-One		3 α -Ol	0.35
17 α -Ol-20-one side chain		17-One	-0.66
17 α ,20 β -Diol side chain		17-One	-0.89
17 α ,20 α -Diol side chain		17-One	-1.06
17 α ,21-Diol-20-one side chain		17-One	-1.49
17 α ,20 β ,21-Triol side chain		17-One	-1.86
17 α ,20 α ,21-Triol side chain		17-One	-2.05

† 5 β -Series not available for study

was made of the ease of oxidation of 1- rather than 3-hydroxyl groups (Fieser & Fieser, 1959). When borohydride reduction preceded oxidation with Jones reagent a good yield of the trione was obtained together with a little seco acid and presumed 3 β -hydroxy 1-ketone (location was effected with the Zimmermann reagent).

RESULTS

Observed R_F and R_{M0} values for the substances isolated from the urine samples, for glycol fission products, for products of oxidation with Jones reagent and for reference steroids are presented in Table 2. Indications of chemical character as shown by specific location reactions (Edwards, 1968b) are also given. It should be particularly noted that substances H1-H4 did not react with

the Zimmermann reagent whereas the various glycol fission products (designated with an extra Z) did do so.

DISCUSSION

The basis and reliability of structural characterization by comparison of R_F values (ideally by attainment of identical R_F values from supposedly identical substances) have been discussed by Bush & Mahesh (1959) and Bush (1961). The present extension of the procedure by the use of R_M values suggested by Bush & Mahesh (1964) and Monder (1964) provides consistency comparable with the use of molar rotation values. Bush (1961) studied the metabolism of steroids of well-established

Table 2. R_{M0} values of isolated substances, derivatives and reference steroids

R_F values are quoted in percentage units. The ΔR_M values in column 3 correct for the effect of benzene incorporated in the solvent mixture (Edwards, 1968a). The R_{M0} value in column 4 is a mean when more than one R_F value is quoted in column 2.

				Location reactions (Edwards, 1968 <i>b</i>)		
	R_F	ΔR_M [observed $R_M \rightarrow R_{M0}$]	R_{M0}	Tetrazolium reduction	Absorption at 254m μ	Zimmermann reaction
<i>Substances isolated from urine</i>						
H1	59	1.84	1.68	+	+	
	65	1.95				
H2	38	1.84	2.01	+		
	48	1.95				
H3	45.5	2.47	2.55	+		
	49	2.54				
H4	32.4	2.47	2.77	+		
	38.0	2.54				
<i>Derivatives</i>						
H1ZA	12.3	0.96	1.82			+
H1ZB	19.2	0.96	1.59			+
H2Z	34	1.56	1.84			+
	12.3	0.96				
H3ZA	54	1.56	1.49			+
H3ZB	36	1.56	1.81			+
H3ZC	46.5	1.56	1.62			+
H4Z	46	1.56	1.63			+
H1T	42	0.49	0.63	+	+	+
H2T	39.0	0.49	0.68	+	+	+
H3T	42.5	0.49	0.62	+	+	+
H4T	40.5	0.49	0.66	+	+	+
<i>Reference steroids</i>						
5 β -Pregnane-3 α ,17 α ,20 α -triol (Pt*)	10.8	0.49	1.40			
3 α ,17 α -Dihydroxy-5 β -pregnan-20-one (Pk*)	25	0.49	0.97			
3 α -Hydroxy-5 β -androstan-17-one (Ae*)	35.5	0.00	0.26			+
5 α -Androstane-3,17-dione	69.5	0.00	-0.36			+
1 α -Hydroxy-5 α -androstane-3,17-dione	22.5	0.49	1.03	+		
1 β -Hydroxy-5 α -androstane-3,17-dione	59.5	0.96	0.79			
3 β -Hydroxy-5 α -androstane-1,17-dione	17.6	0.49	1.15	+		+
	18.6	0.49				
1 α ,3 β -Dihydroxy-5 α -androstan-17-one	4.2	0.49	1.85			+
5 α -Androstane-1,3,17-trione (Tk*)	42.5	0.49	0.62			+
	43.1	0.49				
5 β -Androstane-3,6,17-trione	32.5	0.49	0.81			+
5 β -Androstane-3,7,17-trione			0.43†			
5 β -Androstane-3,11,17-trione	58.5	0.49	0.34			+
5 β -Androstane-3,12,17-trione			0.45†			
Androst-4-ene-3,17-dione	45.6	0.00	0.08		+	+
1 α -Hydroxyandrost-4-ene-3,17-dione	8.3	0.49	1.54	+	+	+
1 β -Hydroxyandrost-4-ene-3,17-dione	12.8	0.49	1.32	+	+	+
Androst-4-ene-1,3,17-trione	24.3	0.49	0.99		+	+

* Abbreviation used in Table 3.

† Calculated from ΔR_M values (Table 1).

structure and could be sure that the metabolites were also steroids. The present study of substances excreted by S.C. were, on first recognition, tentatively assumed to be steroids since they chromatographed among the known urinary steroids and were located by reagents generally used for steroids.

The interrelationship of R_M values now discussed shows both the character of the substances under investigation and the efficiency and value of R_M analysis.

The initial steps of characterization of the substances excreted by S.C. were based on the

following: (1) it was assumed that the substances were steroids; (2) it was assumed that oxidation in the clinical 17-hydroxy corticosteroid procedure (Wilson & Fairbanks, 1955; Few, 1961a) implied 17-hydroxylation and 20-oxygenation of a 17 β -pregnane derivative; (3) it was assumed that the periodate oxidation products were 17-oxo steroids, though the characteristic colour production in the Zimmermann reaction was the only evidence; (4) it was assumed that in common with all other biosynthesized steroids the substances were 3-oxygenated; (5) it was assumed that these urinary excretion products were likely to have followed the usual pattern of metabolism and be 'tetrahydro' products of inactivation of another substance, with the 3 α -hydroxy-5 β -epimer being the most likely product; (6) as a preliminary hypothesis it was presumed that the four substances differed from one another in mode of substitution at one centre, being epimeric alcohols or the corresponding ketone; (7) it could be presumed that the only neighbouring substituents were on the side chain since the 17-oxo steroid product was neutral and stable to periodate oxidation; (8) substitution of positions 15, 16 and 18 was not considered, since any of these would block the Zimmermann reaction of the 17-ketone (Neher, 1960).

Glycol fission characterization. Periodate selectively oxidizes 17 α ,20 α - and 17 α ,20 β -glycols of the steroid series to 17-ketones (Few, 1961a), but does

not oxidize α -ketols such as the 17 α -hydroxy-20-oxopregnane derivatives (Brooks & Norymberski, 1953). Preliminary borohydride reduction makes the latter susceptible to glycol fission oxidation (Appleby, Gibson, Norymberski & Stubbs, 1955), though there is then the possibility of simultaneous reduction of nuclear oxo groups.

Substances H1, H2, H3 and H4. Borohydride reduction and periodate oxidation of substance H4 gave one product, H4Z. This was also given as minor product by substances H1 and H3 but was not noticed from substance H2, perhaps because less of this substance was available. Substance H2 gave the same product as the major product from substances H1 and H3. These observations were taken to indicate that substances H1-H4 were closely related structurally.

Substance H4 gave substance H4Z with or without reduction before periodate oxidation, indicating absence of ketonic substituent. The value of ΔR_M [H4 $\rightarrow$ H4Z] (Table 3) thus arose only as a result of side-chain fission, and comparison with reference ΔR_M values (Table 1) indicated that substance H4 had 17 α ,20 α -dihydroxy substitution if it had a pregnane side chain.

Substance H3 gave substance H3ZA when borohydride reduction was omitted, and the value of ΔR_M [H3 $\rightarrow$ H3ZA] also indicated presence of the 17 α ,20 α -dihydroxy side chain. With borohydride reduction substance H3 gave two products differing

Table 3. *Errors of match of observed and tabulated R_M values*

Items are set out in the order of consideration in the text. Pt, Pk, Ae and Tk are defined in Table 2. For variance analysis, errors not listed were neglected for reasons discussed in the text; those listed were summed as variances and pooled with 10 degrees of freedom, giving s.d. ± 0.054 .

Transformation	ΔR_M		Structural change(s)	Error (R_M unit)
	Observed	Tabulated		
H4 $\rightarrow$ H4Z	-1.14	-1.06	17 α ,20 α -Diol $\rightarrow$ 17-one	-0.08
H3 $\rightarrow$ H3ZA	-1.06	-1.06	17 α ,20 α -Diol $\rightarrow$ 17-one	0.00
H2 $\rightarrow$ H3	0.54	0.61	17 α -Ol-20-one $\rightarrow$ 17 α ,20 α -diol	-0.07
H1 $\rightarrow$ H2	0.33	0.35	3-One $\rightarrow$ 3 α -ol	-0.02
Pt $\rightarrow$ H4	1.37	1.20	1 β -Hydroxylation	
Ae $\rightarrow$ H4Z	1.37	1.20	1 β -Hydroxylation	
Ae $\rightarrow$ H3ZC	1.36	1.20	1 β -Hydroxylation	
Ae $\rightarrow$ H1ZB	1.33	1.20	1 β -Hydroxylation	
Ae $\rightarrow$ H3ZB	1.55	1.50	1 α -Hydroxylation	+0.05
Ae $\rightarrow$ H2Z	1.58	1.50	1 α -Hydroxylation	+0.08
Ae $\rightarrow$ H1ZA	1.56	1.50	1 α -Hydroxylation	+0.06
Pt $\rightarrow$ H3	1.15	0.94	1-Ketonation	
Ae $\rightarrow$ H3ZA	1.23	0.94	1-Ketonation	
Pk $\rightarrow$ H2	1.04	0.94	1-Ketonation	
Pk $\rightarrow$ H1	0.71	0.59	1-Ketonation and 3 α -ol $\rightarrow$ 3-one	
Tk $\rightarrow$ H1T	0.01		Presumed identity except for 5 α -5 β -isomerism	+0.01
Tk $\rightarrow$ H2T	0.06		Presumed identity except for 5 α -5 β -isomerism	+0.06
Tk $\rightarrow$ H3T	0.00		Presumed identity except for 5 α -5 β -isomerism	0.00
Tk $\rightarrow$ H4T	0.04		Presumed identity except for 5 α -5 β -isomerism	+0.04

from substance H3ZA and these, substances H3ZB and H3ZC (minor product), were evidently epimeric alcohols from reduction of a nuclear ketone.

Substance H2 gave no product if borohydride reduction was omitted, indicating that the 17 α -hydroxy-20-ketonic side chain was present. After borohydride reduction the product, H2Z, was the same as substance H3ZB, and consequently substances H2 and H3 have the same nuclear structure. The side-chain structure is characterized by ΔR_M [H2 $\rightarrow$ H3] and this is consistent with the 17 α -hydroxy-20-ketonic side chain for substance H2.

Substance H1 also required borohydride reduction before oxidation and gave the same major (H1ZA) and minor (H1ZB) products as did substance H3. It followed that an extra oxo group in substance H1 was reduced to the same hydroxyl group as was present in substances H2, H3 and H4. Reduction of this ketone was characterized by ΔR_M [H1 $\rightarrow$ H2], a value consistent with 3-oxo and 3 α -hydroxy substitution. This was likely if substances H1–H4 were tetrasubstituted, since three other positions of substitution were accounted for by the above arguments.

The relationship of substances H2 and H3 as 20-ketone and 20 α -alcohol was likely, since biological reduction takes this form in the human (Dorfman & Ungar, 1965). Similarly the relationship of 3-ketone and 3 α -alcohol was also likely, and these have been related for the 5 β -series by borohydride reduction (Barton, 1953).

It was likely then that substance H4 was 3 α ,17 α ,20 α -trihydroxylated, and it was reasonable to take the 5 β -epimer (pregnanetriol) as reference substance for the characterization of the extra hydroxylic substituent and likewise, with substance H3, for the corresponding ketone. 3 α ,17 α -Dihydroxy-5 β -pregnan-20-one provided the reference substance for substances H2 and H1, giving in each case a ΔR_M value for the ketone but with ΔR_M [3-ketone $\rightarrow$ 3 α -alcohol] in addition for substance H1. The corresponding Z derivatives were related similarly to 3 α -hydroxy-5 β -androstan-17-one (aetiocolanolone). The observed values, listed in Table 3, gave the mean ΔR_M values of 1.12 for the ketone and 1.36 and 1.56 for the epimeric alcohols.

Tabulated ΔR_M values at the time of this work did not include any trio of values approximating to these, and 1-oxygenated reference steroids were sought on the basis of the evidence for β -disubstitution discussed below. These provided the best but by no means adequately accurate values (Table 1), and it was presumed that the evident errors were due to matching natural 5 β -isomers with the synthetic reference 5 α -steroids. The 1 β -hydroxy substituent would be expected to be most sensitive to the 5 α –5 β -epimeric change, since Stuart-type

models show markedly different steric hindrances by the angular methyl group. The 1 α -hydroxyl group would not be so sensitive, but the 1-oxo group may be so. The latter would also show marked differences in degrees of hydrogen-bonding with 3-hydroxyl groups in the two series. In continuing the discussion greatest reliance was put on match with data relating to 1 α -hydroxy substitution.

It was evident that substances H3ZB, H2Z and H1ZA were the 1 α -hydroxy derivatives, and this was to be expected since they were produced by borohydride reduction of the ketone reported by Schlegel & Tamm (1957) to produce this epimer in preponderance. Substances H4Z, H3ZC and H1ZB were thus 1 β -hydroxylated, as was the excreted substance H4. This orientation was the same as observed by Schneider & Bhacca (1966) for the 1-hydroxy derivatives in the urine of an adult male after ingestion of large doses of 3 α ,17 α ,20 β ,21-tetrahydroxy-5 β -pregnan-11-one, 3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione and 5 β -pregnane-3 α ,17 α -20 α -triol (pregnanetriol). These authors reported ΔR_M 1.10 between pregnanetriol and 5 β -pregnane-1 β ,3 α ,17 α ,20 α -tetrol and quoted R_F 0.25 for 1 β ,3 α -dihydroxy-5 β -androstan-17-one in their paper-chromatographic system 18 (toluene–iso-octane–methanol–water, 40:10:3:20, by vol., run at 25°). This gave R_{M0} 1.73 on using the conversion values obtained in this Laboratory (25° to 35°, –0.25; for toluene content, +1.45), a value comparing satisfactorily with ours (Table 2), which lay between 1.59 and 1.63. We did not find it possible to calculate R_{M0} values for the other substances isolated by Schneider & Bhacca (1966) or for their derivatives.

The general character of substances H1–H4 was thus revealed as a series of tetraoxygenated steroids, differing from one another by stepwise reduction of ketones. Metabolic reduction was known to occur preferentially with the 3-ketone rather than the 20-ketone (Dorfman & Ungar, 1965), and the present indications are that reduction of each of these is preferred to reduction of the 1-ketone.

A complete scheme of interrelationship of substances H1–H4 and their derivatives was constructed (Scheme 1) on the basis of 1-substitution. The errors of fit were calculated from experimental and tabulated ΔR_M values (Table 3) and variance analysis was used to show that the s.d. was of the same order as that of simple repetition of R_M determination ($\pm 0.07 R_M$ unit). It was concluded that the substances under investigation were correctly represented by the formulae shown in Scheme 1.

Though the structures of Scheme 1 adequately fit the available data it would be unwise to consider

Scheme 1. Chart of chemical transformations. The isolated substances and derivatives are here accounted for as 1-oxygenated pregnane derivatives as described in the text. Heavy arrows indicate the formation of major products. Tk, 5 α -Androstane-1,3,17-trione.

the scheme to be unique. Consideration of the 5α - rather than 5β -series produced large errors, principally because the 5α -3-ketone would be reduced to the 3β -alcohol (Barton, 1953). Likewise consideration of the effect of an isolated unsaturation centre or of 20β - rather than 20α -hydroxylation threw the errors out of balance about zero. Similar errors intruded on consideration of substances H1ZA and H1ZB as 3α - 3β -epimers rather than 1α - 1β -epimers or on consideration of substances H1 and H2 or substances H3 and H4 (or both pairs) as 5α - 5β -epimers.

The occurrence of 1-oxo groups in substances H1, H2 and H3 would not be expected to provide reactivity towards the Zimmermann reagent, since Schlegel & Tamm (1957) demonstrated that 1-oxocholestane, 1-oxo- 5α -etianic acid methyl ester and 1-oxo- 5β -etianic acid methyl ester were without reaction.

Correlation with 1-oxygenated steroids. The 1,3,17-trione was produced by oxidation with Jones reagent of substances H1ZA, H2Z, H3ZA and H4Z, the products being designated H1T-H4T, and these were chromatographed alongside reference 5α -androstane-1,3,17-trione. The R_{M0} values (Table 2) were not significantly different and support the contention of 1-oxygenation. It is not possible to decide between the 5α - and 5β -epimers on this basis, since these are unlikely to separate in the presence of the 3-ketone in the Bush (1952) systems.

Attempts to use the presumed seco acid oxidation products in cross comparisons failed, owing to difficulty in obtaining discrete spots on chromatography.

Number of functional groups and steroidal character. The number of functional groups was determined by the method of Trafford & Edwards (1968). It has been shown that the ΔR_M value arising from incorporation of a high concentration of formaldehyde in the aqueous methanol-light petroleum systems is proportional to the number of hydroxylic and ketonic substituents, irrespective of position of substitution. Substance H1ZA gave the value 0.12, indicating trioxxygenation, for which the expected value was 0.16 ± 0.07 (s.d., nine androstane derivatives). The expected values for dioxygenation and tetraoxygenation were -0.03 ± 0.07 (six derivatives) and $+0.33 \pm 0.05$ (two derivatives).

On the basis that substance H1Z was a dihydroxy ketone, mean ΔR_M values (Edwards, 1968a) indicated that these functional groups contribute +3.05 R_M units. It followed that the skeletal R_M was -1.56, which was very nearly -1.65, that tabulated for the androstane skeleton.

If substance H1ZA was trifunctional and the side-chain character was correct then substance H1 was tetrafunctional. The monohydroxy triketone requires the ΔR_M contribution of +3.2 for the

functional groups, which, subtracted from the molecular R_M (Table 2), gave the skeletal R_M value, 1.52. This on correction for the vicinal interaction of the 17-hydroxyl and 20-oxo groups (Edwards, 1968a) gave a skeletal R_M value of 1.9, which compared well with the tabulated R_M value of 1.87 for the pregnane skeleton. The similar calculation with substance H4, for which no correction for vicinal effects was necessary, gave 1.85 for the skeletal R_M value.

These skeletal R_M values for both androstane and pregnane derivatives strongly indicate the steroidal character of the substances under investigation.

Further evidence of β -disubstitution. The weak absorption of $254m\mu$ light described for 5α -androstane-1,3,17-trione by Noguchi & Fukushima (1965) was shown by substance H1, indicating enolization.

Reference β -ketols, 1α -hydroxy- 5α -androstane-3,17-dione and the presumed 3β -hydroxy-1-oxo isomer slowly reduced styryltetrazolium on warming. Substances H2 and H3 did likewise. So also did substances H1 and H4, though the reference 5α -1,3-dione and 5α -1 β ,3 β -diol did not and it will be necessary to account for this anomaly when 5β -reference substances are available. It is likely that there are marked differences of reactivity of the 5α - and 5β -epimers because of the proximity of alternating groups. It will also be necessary to consider the effects of elimination or rearrangement of the 19-methyl group in strong base, as discussed for 1α -hydroxy steroids by Smith, Foell & Teller (1965).

The hydroxamate- Fe^{3+} ion reaction for esters, lactones and amides (Robertson & Butler, 1964) was positive for substances H1 and H2, but was not tried on substances H3 and H4. None of these three functional groups was present, however, as acid and alkaline hydrolysis procedures did not alter the R_F values. It was considered possible that Fe^{3+} ions were reacting characteristically with the enolized form of substance H1, but the reaction was not reproduced in the absence of the treatment with hydroxylamine, even after pretreatment with alkali. Alternatively, reaction with Fe^{3+} ions occurred readily after treatment with the tetrazolium reagent. It is possible that a nitrogenous substance must be present to facilitate the enol- Fe^{3+} ion reaction.

As discussed above, the Zimmermann reactivity of the 17-ketones of oxidation precludes further substitution of the D-ring, and thus the only position available for β -disubstitution is 1,3, assuming that 2,4-disubstitution is highly unlikely.

Side-chain characterization. It has been presumed that substances H1-H4 have the pregnane side chain in using the ΔR_M values of Table 2. It is unlikely that a 17,20-dihydroxy side chain of more

than two carbon atoms need be considered, since each extra carbon atom would contribute $-0.14 R_M$ unit. The R_M relationship of substances H1-H4 relative to the pregnane reference derivatives would then show a consistent error when compared with the Z series of derivatives and the androstane reference steroids.

Further evidence of side-chain character was given by the lead tetra-acetate 17-oxogenic reaction (Bush, 1955), which on paper behaved similarly to periodate in solution; substances H3 and H4 were positive and substances H1 and H2 negative unless reduced by borohydride before oxidation. The vanillin-perchloric acid reaction (Few, 1961b) for 21-deoxy steroids was positive with substances H3 and H4, but was not tried with substances H1 and H2 owing to lack of material.

Naturally occurring 1-oxygenated steroids. The investigations of Schneider & Bhacca (1966) already discussed above were carried out under unphysiological conditions, but nevertheless indicate that 1β -hydroxylation occurs in man and that in the guinea pig it occurs in the liver. In an adult male, pregnanetriol was metabolized in part to 5β -pregnane- $1\beta,3\alpha,17\alpha,20\alpha$ -tetrol, of the constitution we propose for our substance H4.

Lieberman, Fukushima & Dobriner (1950) isolated 5α -androst-1-ene-3,17-dione from pooled human urine. It is likely that this substance was an artifact of boiling a 1-hydroxyandrostane-3,17-dione with strong acid in the hydrolytic process applied to the urine. The hypothesis that 1-hydroxylated androgens were intermediates of oestrogen biosynthesis was disproved by Gual, Morato, Hayano, Gut & Dorfman (1962). Idler & Truscott (1967) showed that 1α -hydroxycorticosterone was the circulating corticosteroid of the cartilaginous fish, *Raja ocellata*, and a potent mineralocorticoid when administered to the rat (Idler, Freeman & Truscott, 1967).

The many 1β -hydroxy steroids of the plant kingdom are exemplified by ouabagenin (Tamm, 1953), acovenosigenin (Schlegel, Tamm & Reichstein, 1955) and the sapogenin, ruscogenin (Benn, Colton & Pappo, 1957), which is 1β -hydroxydiosgenin. It is not appropriate to review here the extensive range of 1-oxygenated products isolated after incubation of steroids with fungi or moulds, though it is necessary to carry in mind the possibility that substances found in urine may be products of the activity of gut flora.

Conjugation. The four urinary substances became solvent-extractable after the action of a β -glucuronidase preparation, and it may be presumed that all were excreted as β -glucosiduronosides as is common with steroids. Such conjugation may readily be envisaged with substances H2, H3 and H4, since they are probably 3α -alcohols. Substance

H1 is unlikely to conjugate at the strongly hindered 17-hydroxyl group, so it is suggested that conjugation occurs in the enolized form, H1G.

Conclusions. It is tentatively proposed that the four substances excreted by S.C. were 17α -hydroxy- 5β -pregnane-1,3,20-trione, $3\alpha,17\alpha$ -dihydroxy- 5β -pregnane-1,20-dione, $3\alpha,17\alpha,20\alpha$ -trihydroxy- 5β -pregnan-1-one and 5β -pregnane- $1\beta,3\alpha,17\alpha,20\alpha$ -tetrol, as shown in Scheme 1. 17-Oxo steroids of the polarity of 1-hydroxy- and 1-oxo-aetiocholanolone were not expected at the outset and would not have been detected by the procedures used.

Three hypotheses for the origin of the 1-oxygenated pregnane derivatives may be considered. First, following Schneider & Bhacca (1966), they may be considered as metabolites of pregnanetriol and thus of 17α -hydroxypregn-4-ene-3,20-dione. This would be so if S.C. were producing the latter as the result of a 21-hydroxylase defect in cortisol biosynthesis. Though this could have been true in the first week of life there has been no indication of it from the eighth week to the time of writing, because S.C. is now, at 12 months old, healthy and thriving. Perhaps the early defect was transitory and the result of a late maturation of the 21-hydroxylating system.

A second hypothesis concerns the metabolism of a presumably secreted substance such as 17α -hydroxypregn-4-ene-1,3,20-trione. This is unlikely, since substitution in or near the A-ring largely prevents reduction of the 4-en-3-one system as shown by 1,2-unsaturation (Gray, Green, Holness & Lunnon, 1956; Bush & Mahesh, 1964), 2α -methyl substitution (Bush & Mahesh, 1959) and 9α -fluoro substitution (Bush & Mahesh, 1964).

The secreted substance, as third hypothesis, may be one of the substances found in the urine. Since metabolism occurs by reduction, the triketone H1 is the most likely to be secreted. There is, of course, no indication of the gland of origin of these substances, but the occurrence of 17-hydroxylated pregnane derivatives draws attention to the adrenal cortex, as does the occurrence of 1α -hydroxycorticosterone in the fish (Idler & Truscott, 1967).

It may also be considered that the excretion of the 1-oxygenated steroids in urine was related to the

hypertension shown by S.C. The evidence from one case is only of such weight as to indicate the need to investigate the physiological properties of the 1-oxygenated steroids and the correlation of excretion with clinical states. We hope that it may be possible to carry out such investigations and, if the opportunity arises, to obtain larger quantities of the substances of the present study for full characterization.

Very generous samples of 1 α -hydroxy-5 α -androstane-3,17-dione, 1 α ,3 β -dihydroxy-5 α -androstane-17-one and 1 α -hydroxyandrost-4-ene-3,17-dione were supplied by Dr D. K. Fukushima of the Sloan-Kettering Institute and of the first steroid by Dr T. J. Foell of Wyeth Laboratories. Professor Tizard and his colleagues of the Institute of Child Health, The Post-graduate Medical School, Hammer-smith, kindly sought our aid in this steroid study and we are pleased to acknowledge their full co-operation.

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