Metabolism of 11-Oxygenated Steroids

2. 2-METHYL STEROIDS

By I. E. BUSH and V. B. MAHESH
Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford

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In previous papers we have explained our reasons for studying the metabolism of 11-oxygenated steroids, and especially the interconversion of steroid 11β-alcohols and 11-ketones (Bush, 1956; Bush & Mahesh, 1958*a*, 1959*a*). The 2α-methyl steroids are particularly interesting because of a peculiar feature of their biological activity. The natural adrenocortical steroids with biological activity show a fairly constant relationship between the activity of 11-ketones such as cortisone (17α:21-dihydroxypregn-4-ene-3:11:20-trione) and the related 11β-alcohols, in this case cortisol (11β:17α:21-trihydroxypregn-4-ene-3:20-dione). In most biological assays, and particularly those related to so-called glucocorticoid activity in mammals, the 11-ketones have 0·3–0·5 time the activity of the 11β-alcohols. In these cases the 11-ketones and 11β-alcohols are readily interconvertible *in vivo* and in the steady state the latter have by far the greater concentration in blood (Bush, 1956; Peterson, Pierce, Wyngaarden, Bunim & Brodie, 1957). On oral administration of cortisone, and of other steroid 11-ketones having a relatively flat α-surface (Bush & Mahesh, 1957, 1959*a*), reduction is so efficient that little or no unchanged 11-ketone reaches the blood, and, with the exception of 5β-(H)-3α-hydroxy metabolites, all the urinary metabolites are largely in the 11β-hydroxy form.

With 2α-methylcortisol and 2α-methylcortisone, however, this relation broke down and the former was found to be from 10 to 50 times as active as the latter in assays for both glucocorticoid and mineralocorticoid activity (Liddle, Richard & Tomkins, 1956; Dulin, Bowman & Stafford, 1957). Peterson *et al.* (1957) suggested that this might be due to a failure of the liver to carry out the usual reduction of the 11-oxo group and it was shown that this was so both *in vivo* in man (Bush & Mahesh, 1957) and *in vitro* with rat-liver microsomes (Glenn, Stafford, Lyster & Bowman, 1957). This exceptional behaviour seemed to offer an excellent opportunity of deciding whether the pharmacological action of the 11-oxygen function in the 11-oxygenated adrenocortical steroids was confined to the 11β-hydroxyl group or depended upon the oxidation–reduction reaction undergone by this oxygen function (Bush, 1956; Bush & Mahesh, 1959*a*). In order to have sure grounds for adopting the former hypothesis, however, it was necessary to examine all the metabolites of 2α-methylcortisone and to compare its absorption with that of 2α-methylcortisol. To obtain further information on the factors determining oxidation–reduction at C-11, the metabolism of 2α-methyladrenosterone (2α-methylandrost-4-ene-3:11:17-trione) was also investigated. These experiments are described in this paper; experiments on cortisone and adrenosterone were carried out by the same methods to enable a direct comparison to be made between the methyl-substituted and unsubstituted precursors.

METHODS

Materials and reagents

These were prepared by the earlier methods (Bush & Mahesh, 1959*a*; Bush & Willoughby, 1957), except for the following.

2α-Methylcortisone (17α:21-*dihydroxy-2α-methylpregn-4-ene-3:11:20-trione*) *and 2α-methylcortisol* (11β:17α:21-*trihydroxy-2α-methylpregn-4-ene-3:20-dione*). These were gifts from the Upjohn Co., Kalamazoo, Mich., U.S.A. Purity was high judged by chromatography of the esters and the derivatives prepared from them (see below). Less than 2% of any single steroid impurity giving fluorescence with NaOH, reduction of alkaline blue tetrazolium, or a 17-oxo steroid from oxidation with sodium bismuthate was present.

2α-Methyladrenosterone (*2α-methylandrost-4-ene-3:11:17-trione*). 2α-Methylcortisone (60 mg.) was dissolved in 50 ml. of 50% (v/v) acetic acid and 1 g. of sodium bismuthate added. The flask was screened with black paper and shaken for 1·25 hr., after which 10 ml. of 10% sodium metabisulphate was added and the flask shaken a further 0·5 hr. The mixture was brought to pH 5·0 with 4N-NaOH, cooled in ice, and extracted thrice with 3 vol. of ether–chloroform (1:1, v/v). The combined extract was washed twice with 0·1 vol. of N-NaOH and then twice with 0·1 vol. of water. The extract was dried with about 5 g. of anhydrous Na_2SO_4, filtered, and evaporated under reduced pressure at 40–45°. The residue was recrystallized from ethanol to give 41 mg. of a crystalline product which was chromatographically pure to 2% of any single impurity (m.p. 212–214°; uncorr.).

Cortisone. A dry, powdered preparation of the free alcohol was used (gift from Dr L. H. Sarett, Merck and Co., Rahway, N.J., U.S.A.). This was chromatographically pure to 1% (or less) of any single impurity as judged by the

Table 1. *Excretion of normal steroid metabolites in Experiments A–E*

Prednisone (15 mg.) or prednisolone (10 mg.) was given on both days of the urine collections. C, control day; T_1 first estimation; T_2, second estimation approximately 1 month after T_1. The results are expressed as mg./24 hr.

Experiment	*A*		*B*		*C*		*D*		*E*		
Precursor given	Cortisone		2α-Methyl-cortisone		2α-Methyl-cortisol		Adrenosterone		2α-Methyl-adrenosterone		
Dose (mg.)	100		50		50		50		40		
Urine sample	*C*	T_1	T_1	T_2	T_1	T_2	*C*	*T*	T_1	Recovery value (%)	Method of estimation
Steroids excreted											
Tetrahydrocortisol	0·79	13	1·7	1·4	1·4	1·1	1·4	0·86	0·86	70	Scanning; blue tetrazolium
Tetrahydrocortisone	1·0	21	2·4	2·3	2·0	1·9	1·0	0·87	0·53	75	
*allo*Tetrahydrocortisol	0·57	7·7	0·86	0·72	0·86	0·86	0·57	0·57	0·57	70	
20-Dihydrocortisol	<0·01	0·43	0·014	—	0·010	—	—	—	—	70	Visual; NaOH fluorescence
20-Dihydrocortisone	<0·01	0·05	<0·01	—	—	—	—	—	—	70	
Cortisol	<0·01	0·29	<0·01	—	—	—	0·014	0·01	0·029	70	
Cortisone	<0·02	0·021	<0·02	—	—	—	—	—	—	70	
11β-Hydroxyaetiocholanolone	0·21	1·6	0·63	0·55	0·44	0·33	0·20	1·6	<0·25	80	Scanning; alkaline *m*-dinitrobenzene
11-Oxoaetiocholanolone	0·36	3·3	0·94	0·75	0·48	0·34	0·26	4·1	<0·15	80	
11β-Hydroxyandrosterone	0·24	0·88	0·50	0·36	0·36	0·30	0·23	12	<0·25	80	
Dehydroepiandrosterone	<0·06	<0·06	2·0	1·4	1·3	1·0	<0·06	<0·06	<0·06	80	
Aetiocholanolone	2·4	1·7	3·4	2·8	2·9	2·0	2·3	3·1	1·8	85	
Androsterone	2·8	2·5	3·3	2·4	3·3	2·8	3·2	3·1	2·0	85	

alcohol, the 21-acetate, and oxidation with sodium bismuthate to adrenosterone.

Adrenosterone (*androst-4-ene-3:11:17-trione*). This was a gift from Dr L. H. Sarett and was chromatographically pure to 1% (or less) of any single 17-oxo steroid or impurities giving a fluorescence with NaOH.

11β-Hydroxy-2α-methylandrostenedione (*11β-hydroxy-2α-methylandrost-4-ene-3:17-dione*). This was prepared in amounts of 25–50 μg. as required for chromatographic correlations by oxidizing 2α-methylcortisol with sodium bismuthate [1·0 ml. of 50% (v/v) acetic acid with 25 mg. of $NaBiO_3$].

3β:11β-Dihydroxy-2α-methyl-5α-androstan-17-one (Chart 3). 2α-Methylcortisol (3 mg.) was hydrogenated in acetic acid (2 ml.) in the presence of activated platinum oxide (1 mg.) for 8 hr. and the product extracted with ethyl acetate. The extract was evaporated at 40–45° under reduced pressure and oxidized with sodium bismuthate as above to 3β:11β-dihydroxy-2α-methyl-5α-androstan-17-one (XXV). Owing to a faulty sample of bismuthate, 50% of the product was the related 11-ketone (XXVI). This was proved by acetylation of the two products of the reaction and oxidation with chromic acid to obtain identical products. The oxidation product of the ester of the 11β-hydroxyl group gave the rapid pink coloration with alkaline *m*-dinitrobenzene characteristic of 11:17-dioxo steroids. Both products of the original oxidation with bismuthate were oxidized with chromic acid to the same substance which had the properties expected of 2α-methyl-5α-androstane-3:11:17-trione (XXIII).

Methods

The administration of steroids by mouth, collection of urine, extraction, estimation of steroids, chromatography and identification were carried out by the earlier methods. The solvent systems used (Bush & Mahesh, 1959*a*) were: (1) benzene–methanol–water (2:1:1, by vol.); (2) toluene–methanol–water (4:3:1, by vol.); (3*a*) light petroleum (b.p. 100–120°)–benzene–methanol–water (67:33:80:20, by vol.); (3*b*) light petroleum–toluene–methanol–water (67:33:85:15, by vol.); (4*a*) light petroleum–methanol–water (25:24:1, by vol.); (4*b*) light petroleum–methanol–water (20:17:3, by vol.).

EXPERIMENTAL AND RESULTS

Metabolism of cortisone (*Experiment A*, *Table* 1)

A male subject aged 21 years took 5 mg. of prednisone (1:2-dehydrocortisone) by mouth on rising (7.0 a.m.), at noon and at 7.0 p.m. for 2 days. He collected his urine from 9.0 a.m. on the second day to the same time next day and took 100 mg. of

Table 2. *Steroids in plasma after cortisone*

Blood was drawn: 1, 5 min.; 2, 2·08 hr.; 3, 4·08 hr., after ingestion of 100 mg. of cortisone acetate (Expt. *A*).

	Steroids in plasma (μg./100 ml.)		
Sample ...	1	2	3
Cortisol	1·0	15	18
Cortisone	<0·5	?1·0	<1·0

cortisone acetate by mouth at 9.0 a.m. Blood samples were taken at 9.05 a.m., 11.05 a.m. and 1.05 a.m. and plasma was immediately separated by centrifuging. The results are given in Tables 1 and 2.

Identification of metabolites (Chart 1). In view of earlier work, chromatographic identification was carried out by rather fewer degradative reactions than usual. Half of the 24 hr. sample of urine was extracted by procedure 2 of Bush & Mahesh (1959*a*). The extract was pre-fractionated on a 25·4 cm. wide sheet of Whatman no. 3MM paper by a single-length run in system 3*a* and the usual three fractions were eluted. The most polar fraction (no. 3, Bush & Willoughby, 1957) was then run on Whatman no. 3MM in a 25·4 cm. wide lane for 6 hr. in system 1. The main products were detected on strips (3–5 mm.) cut from the main lane by treatment with NaOH, and alkaline blue tetrazolium, and were eluted from the main lane.

Cortisol (I) was identified by its position after a 6 hr. run in system 2 and yellow fluorescence with NaOH, and by its position after a 16 hr. run in system 1 and its reduction of blue tetrazolium. Previous work showed that 95% or more of material in such urine extracts giving a yellow fluorescence in the position of cortisol after a 6 hr. run in system 2 was in fact cortisol, since acetylation gave a homogeneous product with the same R_F as cortisol acetate in systems 2 and 3*a*, and oxidation with bismuthate gave a homogeneous product with the colour fluorescence reactions, and position of 11β-hydroxyandrost-4-ene-3:17-dione (V) in system 3*a*. Similar evidence was obtained with urinary material identified as cortisone (II) via (V) and (VI).

20-Dihydrocortisol (11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one; III) was identified by its position on chromatograms run for 16 hr. in system 1, the mobility of its acetate in system 3*b*, its yellow fluorescence with NaOH, its failure to reduce blue tetrazolium and its oxidation with periodate to 11β-hydroxyandrost-4-ene-3:17-dione, which was obtained in 95% yield and had the R_F, colour reaction with alkaline *m*-dinitrobenzene, and fluorescence with NaOH characteristic of this substance.

20-Dihydrocortisone (17α:20ξ:21-trihydroxypregn-4-ene-3:11-dione; IV) was identified similarly, except that, on oxidation with periodate, androst-4-ene-3:11:17-trione (VI) was obtained.

One-hundredth of the 24 hr. sample was run in system 1 for 16 hr. and the zone from the origin to 2 cm. behind the 'tail' of tetrahydrocortisol eluted. This was then oxidized with sodium bismuthate and the products were estimated by scanning after chromatography in system 3*b* for 14 hr. There were found 11-oxoaetiocholanolone (25 μg.), 11β-hydroxyaetiocholanolone (13 μg.) and 11β-hydroxyandrosterone (8 μg.). After correction for recovery and differences in molecular weight, this gave the 24 hr. excretion rates: cortol + β-cortol, 4 mg.; cortolone + β-cortolone, 6·1 mg.; *allo*cortol (Bush & Mahesh, 1959*a*) [+probably *allo*-β-cortol (Fukushima *et al.* 1955)], 2·4 mg.

The other metabolites were identified by their positions and colour reactions, coupled with absence of fluorescence with NaOH, on the routine chromatograms used for the quantitative estimations. Previous work (De Courcy, Bush, Gray & Lunnon, 1953; Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*) and the considerable amount of

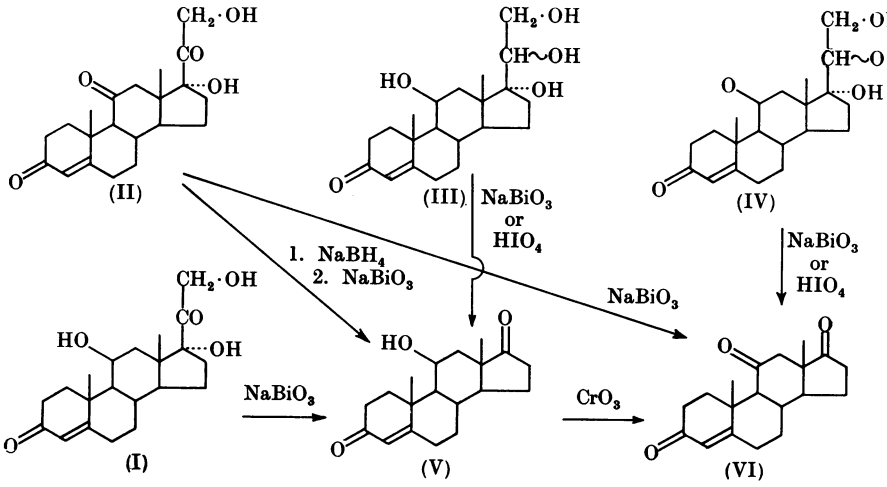


Chart 1. Degradations used to characterize metabolites of cortisone (II) and cortisol (I). (I), (II), (III), (IV) and their acetates, and (V) and (VI) were available as reference substances.

information available on these metabolites (Lieberman & Teich, 1953; Roberts & Szego, 1955) are believed to justify these identifications.

Estimation of plasma steroids. Plasma (5–20 ml.) was diluted with 1 vol. of water and 0·5 vol. of N-NaOH and the solution extracted twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The combined extracts were washed once with 0·05 vol. of 0·1N-NaOH and twice with 0·05 vol. of water. After adding 2–3 drops of acetic acid and filtering through 4–5 g. of anhydrous sodium sulphate, the extract was evaporated at 40° (or below) under reduced pressure with a very fine capillary leak. It was then deposited on a 5 cm. wide strip of Whatman no. 2 paper and run up to the origin in the usual way. The 'band' at the origin thus obtained was then concentrated to a 'spot' 3–4 mm. diam. by 'running in' with ethyl acetate–methanol (2:1, v/v) delivered to the sides of the strip with a syringe-operated pipette. The chromatogram was then run in system 2 for 4 hr. after equilibration for 3 hr. and finally examined with the NaOH fluorescence reaction (Bush, 1952; Bush & Mahesh, 1959*b*). At the time of these experiments, recovery was checked with each batch of plasma by adding three different amounts of cortisol (5–10 μg./100 ml.) to water and extracting 5–10 ml. volumes by the above method; this was because of a period of difficulty with solvents leading to low recoveries (50–60 % instead of the usual 85–95 % at plasma concentrations of 5–10 μg./100 ml.). The results obtained, corrected for the 70 % recovery with this batch of extractions, are given in Table 2. Identification is based solely on chromatographic mobility and NaOH fluorescence reaction.

Metabolism of 2α-methylcortisone (Experiment B, Tables 1 and 3)

A male subject aged 29 years collected a 24 hr. sample of his urine starting at 9.0 a.m. and took 50 mg. of 2α-methylcortisone by mouth at 9.0 a.m. Blood samples were taken at 12.20 p.m. and 3.15 p.m. and the plasma was separated at once. A control sample of urine was collected over the preceding 24 hr.

Preliminary extraction and estimation. One-twentieth of the second urine sample was subjected to extraction procedure 2 (Bush & Mahesh, 1959*a*) and the metabolites of cortisol were estimated as usual. Preliminary estimations of the metabolites of 2α-methylcortisone were also made where their chromatographic positions were characteristic. The very polar metabolites, with mobilities similar to or less than that of cortisol, were estimated by the NaOH fluorescence reaction by visual comparison with 2α-methylcortisol as standard, and given numbers for further identification. Subsequently these metabolites were estimated again, after their structures had been determined, by oxidation with bismuthate to the related 17-ketones and estimating the latter by the scanning method after chromatography and treatment with alkaline *m*-dinitrobenzene (Bush & Mahesh, 1959*a*). The latter estimations are used in Table 3 and are described in full below.

Identification of metabolites. Half (770 ml.) of the urine sample collected during the second day was extracted by procedure 2 (Bush & Mahesh, 1959*a*) by using 75 ml. of resin in chloride form, packed under gravity in a column 2·5 cm. × 15 cm. The urine was percolated at 5 ml./min. and was followed by 100 ml. of glass-distilled water at the same rate. The column was eluted with 500 ml. of methanol–ammonia (sp.gr. 0·880) (19:1, v/v), which was probably excessive in view of later experience. The methanol–ammonia was evaporated under reduced pressure at room temp. (approx. 19°) for 15 min. and then to near dryness at 40–45°. The residue was dissolved in 20 ml. of water and extracted twice with 60 ml. of ether–ethyl acetate (2:1, v/v). The combined extracts were then washed thrice with 6 ml. of N-NaOH and once with 6 ml. of water. The washed extract was partially dried by filtering slowly through 0·5 g. of anhydrous Na_2SO_4 acidified with two drops of acetic acid, and evaporated to dryness under reduced pressure at 40–45°. This extract was called MEU. The aqueous residues were combined and brought to pH 4·8 and 0·5M-acetate by adding 5M-acetic acid and 0·5M-acetic acid–sodium acetate buffer, to reach a final volume of 60 ml. After adding 2·5 ml. of succus entericus of *Helix pomatia* [approx. 200 000 Fishman units of β-glucuronidase, Talalay, Fishman & Huggins (1946)] the solution was incubated for 18 hr. at 45°. The solution was then extracted twice with 180 ml. of ether–ethyl acetate (2:1, v/v) and the extract washed thrice with 18 ml. of N-NaOH and once with 18 ml. of water. It was then dried, acidified, and evaporated as above. This extract was called MEH.

Prefractionation. Extract MEU was deposited over the whole width, 25·4 cm., of a sheet of Whatman no. 3MM paper, and extract MEH over two similar sheets. Small parts of each extract were run similarly on smaller sheets of 3MM paper in parallel with known reference steroids. The sheets were run for 1·8 hr. in system 3*a* after 3 hr. equilibration and three fractions eluted as usual (Bush & Willoughby, 1957): fraction 1 contained the least polar steroids, fraction 2 those of medium polarity such as 11β-hydroxyandrosterone (3α:11β-dihydroxy-5α-androstan-17-one), and fraction 3 the polar steroids such as cortisone and its C_{21} metabolites.

A small part of each fraction was then run in an appropriate system and the chromatograms were examined with the NaOH fluorescence reaction, the blue-tetrazolium reaction, and alkaline *m*-dinitrobenzene. This fractionation is shown in Fig. 1 together with the solvent systems, times of running and types of paper used. With these chromatograms as models, the whole of each fraction was run on a 25·4 cm. wide sheet of no. 3MM paper and fractions were cut and eluted as shown superimposed upon the chromatograms of Fig. 1, with the same solvent systems and other conditions. The positions for cutting off the fractions were determined by inspection of the chromatograms under short-wave light (2537 Å; Chromatolite, Hanovia Ltd., Slough, Bucks). Minor components could not be detected with certainty but were located by using the positions of various fluorescent bands whose relation to the steroids had been determined previously on the preliminary chromatograms of small parts of each fraction. Similar fractions from MEU and MEH were combined for identification.

Fraction 3 (most polar) was run first in system 2 for 3 hr. (Fig. 1). The most polar region of this sheet was then eluted and the subfraction obtained run again in system 1 for 8 hr. One chromatogram each was found sufficient for fractions 1 and 2 and no. 2 paper was used since these fractions were quite clean.

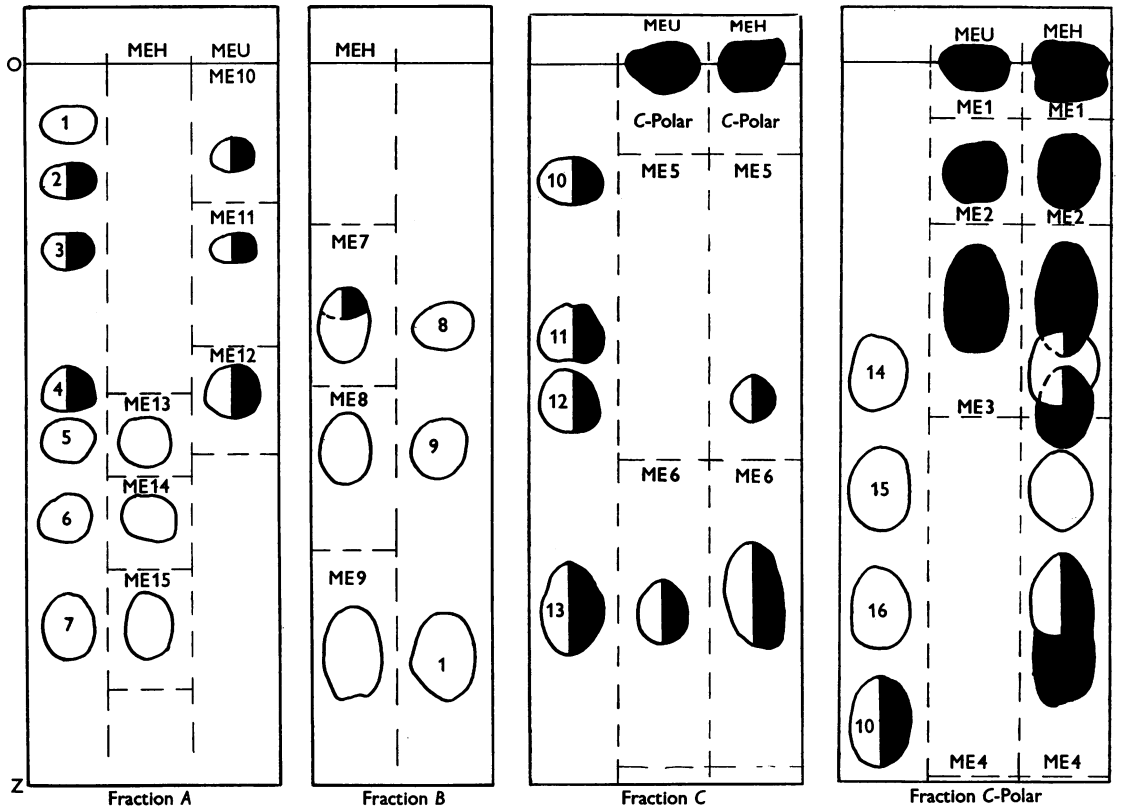


Fig. 1. Second prefractionation of urine extracts from subject who ingested 2α-methylcortisone (semi-diagrammatic). Fractions taken from a first prefractionation as in Bush & Willoughby (1957). ME7–ME15 are those subfractions described in full in the experimental section. O, Origin; Z, solvent front. Fraction *A* (non-polar steroids; Bush & Willoughby, 1957). 1, 11-Oxoaetiocholanolone; 2, adrenosterone; 3, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione; 4, 2α-methyladrenosterone; 5, dehydro*epi*androsterone; 6, aetiocholanolone; 7, androsterone. Run in system 4*b*, 16 hr. MEU, free steroids; MEH, steroids extracted after enzymic hydrolysis. ○, Colour with alkaline *m*-dinitrobenzene; ●, yellow fluorescence with NaOH. Fraction *B* (medium polarity). 8, 11β-Hydroxyaetiocholanolone; 9, 11β-hydroxyandrosterone. Run in system 3*b* for 14 hr. ○, Colour with alkaline *m*-dinitrobenzene; ●, yellow fluorescence with NaOH. Fraction *C* (polar). 10, Cortisol; 11, cortisone; 12, 2α-methylcortisol; 13, 2α-methylcortisone. Run in system 2 for 3 hr. on Whatman no. 3MM paper. Fraction *C*-polar ('*C*-polar' from previous chromatogram). 14, Tetrahydrocortisol; 15, *allo*tetrahydrocortisol; 16, tetrahydrocortisone. Run in system 1 for 8 hr. on Whatman no. 3MM paper. ○, Reducing blue tetrazolium; ●, yellow fluorescence with NaOH.

Each subfraction as numbered in Fig. 1 was then subjected to the sequential degradation procedures described in detail by Bush & Willoughby (1957) and Bush & Mahesh (1959*a*). As expected, many of these subfractions contained a mixture of products. As these were revealed by successive degradations, the derivatives were eluted separately from the chromatograms so that further degradations were carried out with pure fractions. It should be emphasized that each degradation was carried out with a known reference substance, containing the group altered by the reactions, in parallel with the urinary fraction under examination, and the yield of major and minor reaction products carefully compared. To avoid misidentifications, the quantities used in these degradations were such that 15–30 μg. of each major product was obtained: minor products or major products from impurities can usually be detected with the Zimmermann reaction or with the NaOH fluorescence reaction, in amounts of 0·2–0·5 μg. according to the exact nature of the impurity or minor product. In this way it is easy to decide whether a degradation product is that expected from the material in the reaction concerned or whether it must be considered a derivative of an impurity (Bush & Willoughby, 1957) in the original material.

*Subfraction ME*1 (Chart 2). This material was extremely polar (Fig. 1) and on acetylation gave two products having R_F values of 0·09 (X) and 0·24 (IX) in system 3*a*, the latter predominating, suggesting the presence of at least two esterifiable hydroxyl groups and probably three. These compounds gave a yellow fluorescence with NaOH but no reduction of blue tetrazolium.

Oxidation of the original material with bismuthate or periodate gave two 17-ketones which moved 0·4 and 4·7 cm. when run in system 3*a* for 5·25 hr. The latter (XI) predominated and gave a rapidly developing pink–purple colour with alkaline *m*-dinitrobenzene; the former (XII) gave a

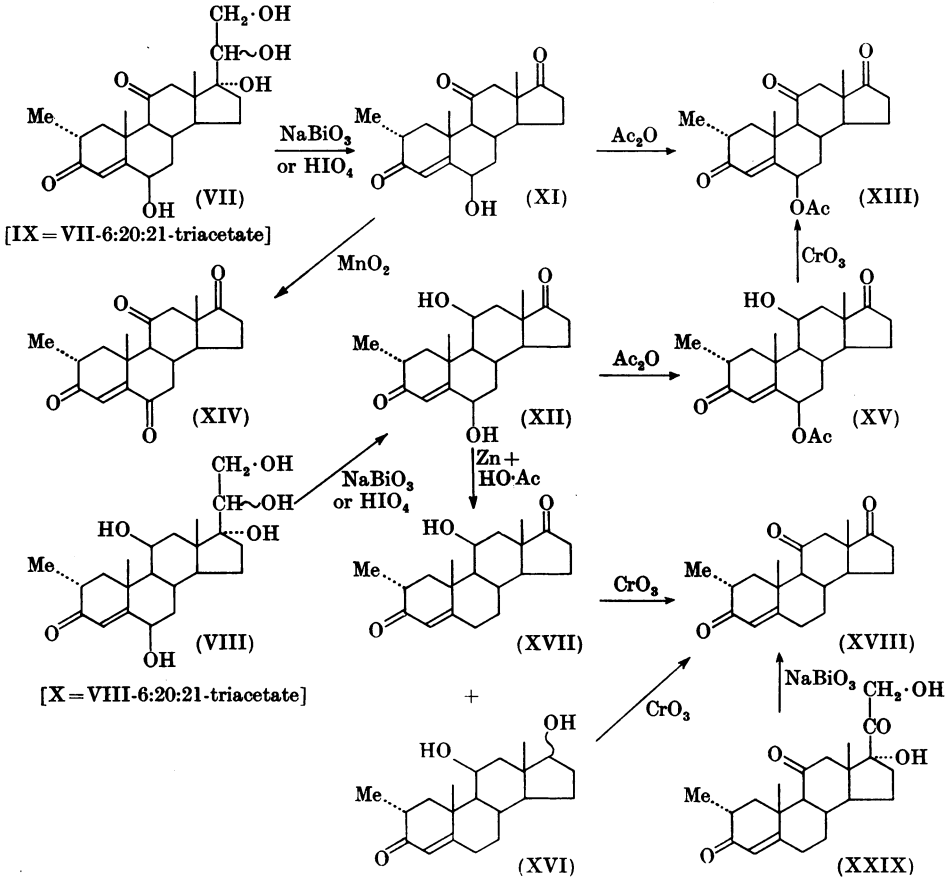


Chart 2. Degradations used to characterize the 6β-hydroxy metabolites (VII and VIII) of 2α-methylcortisone and 2α-methylcortisol. The reference substance was (XXIX; 2α-methylcortisone; see also Chart 1).

slowly developing violet colour. Both gave a fluorescence with NaOH. About 30 μg. of the main bismuthate oxidation product was isolated from another chromatogram after location with ultraviolet light (2537 Å) and dissolved in ethanolic 0·066 N-KOH (3 ml.). The ultraviolet spectrum was measured in the Unicam spectrophotometer (SP. 500) 4 and 24 hr. after mixing, and finally after heating to 60° for 2 hr. The spectra were those characteristic of a 6β-hydroxy-Δ^4-3-oxo steroid (Meyer, 1955) and are given in Fig. 2.

Acetylation of the 17-ketone (XI) gave a new product (XIII) with an R_F of 0·09 in system 3*b*, suggesting the presence of a weakly polar hydroxyl group in the nucleus. Oxidation of (XI) with chromic acid, however, gave some (XIV) and a mixture of products which stayed on or near the origin in system 3*a*, suggesting that ring-opening had occurred. Oxidation of (XIV) with chromic acid for 1 hr. only, however, produced no change in properties, suggesting that the nucleus contained no remaining hydroxyl groups. Oxidation of the acetate (XV) of the minor 17-ketone (XII) yielded a new substance having the same R_F as the acetate (XIII) of the major 17-ketone (XI) and showing the same rapid development of a pink colour with alkaline *m*-dinitrobenzene. This suggested that the minor product (XII) was in fact the 11β-hydroxy analogue of the major product (XI), which was probably an 11-ketone.

The 17-ketone (XI; 50 μg.) isolated from a chromatogram was then oxidized with MnO_2 (1 g.) in refluxing chloroform (50 ml.) for 16 hr. (Amendolla, Rosenkranz & Sondheimer, 1954), the oxide filtered off, and the solution evaporated under reduced pressure at 40–45°. A new product (XIV) was obtained in good yield with an R_F of 0·32 in system 3*b*, and giving an orange colour in the cold with NaOH before giving the yellow fluorescence after heating to dryness (Savard, 1954). This oxidation product was isolated from the rest of the chromatogram and the ultraviolet spectrum examined in ethanol, in ethanolic 0·066 N-KOH, and in the latter solution after neutralizing to pH 6–7 with dilute HCl and finally after bringing to pH 2–3 with acid. The spectra are shown in Fig. 3 and are typical of a 6-oxo-Δ^4-3-oxo steroid (Meyer, 1955).

The product (XIV) was unchanged by chromic acid oxidation for 1 hr. and had an R_F compatible with the structure androst-4-ene-3:6:11:17-tetraone. This suggested that the group oxidized by MnO_2 was the one responsible for ring-opening with chromic acid in the bismuthate oxidation product, and was also the only hydroxyl group in the nucleus. It was also found that very little oxidation of (XI) occurred with MnO_2 in the cold. These properties were compatible only with (XI) having the structure 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, but it was felt desirable to obtain unequivocal degradation to a polyketone which was available as a reference substance, since the 2α-methyl group was present only by inference and was an important group, when interpreting the R_F values. Accordingly, 50 μg. of (XII), which was available in larger amounts from MF 1 (below), was refluxed for 2 hr. with zinc dust (0·1 g.) and acetic acid (10 ml.). After filtering out the zinc dust, water (10 ml.) was added and the mixture brought to pH 5–6 with N-NaOH and extracted twice with 3 vol. of ether–ethyl acetate mixture. The extract was dried over Na_2SO_4 and evaporated under reduced pressure. The extract was run for 3·5 hr. in system 3*b* and, apart from unchanged starting material, contained two substances, one giving a

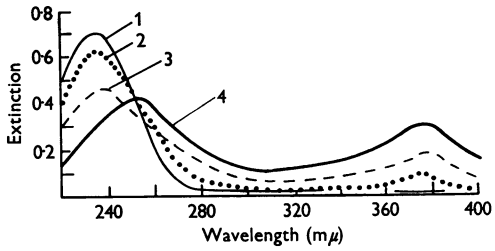


Fig. 2. Ultraviolet spectra of (XI) (bismuthate oxidation product of main component of ME1) (1) (approx. 10 μg./ml.) in ethanol; (2) in ethanolic 0·066 N-KOH after 4 hr.; (3) after 24 hr.; (4) after 24 hr. at room temperature, followed by 2 hr. at 60°. These spectra are characteristic of a Δ^4-6β-hydroxy-3-oxo steroid, and differ from those of 2α-, 6α-, 7α-, 11β-, 11α-, and Δ^4-17β-hydroxy-3-oxo steroids (Meyer, 1955). Similar spectra were obtained with (XXXI) and (XXXIV) (Chart 5).

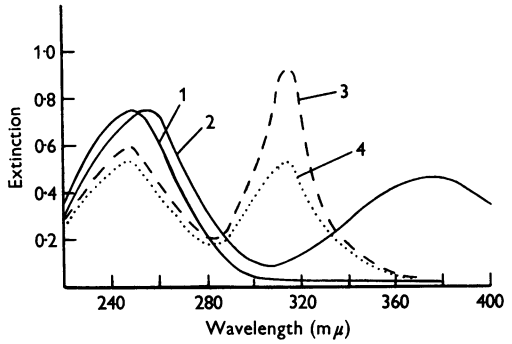


Fig. 3. Ultraviolet spectra of (XIV) (bismuthate oxidation product of ME1, oxidized with manganese dioxide) (1) in ethanol (approx. 10 μg. of steroid/ml.); (2) in ethanolic 0·066 N-KOH; (3) after subsequent acidification to pH 6–7; (4) after further acidification to pH 2–3. These spectra are characteristic of Δ^4-3:6-dioxo steroids (Meyer, 1955). Similar spectra were obtained with (XIV) derived from (XXXI) and (XXXIV) (Chart 5).

yellow fluorescence with NaOH but no colour with alkaline *m*-dinitrobenzene, (presumably XVI), and the other (XVII) giving both reactions. The latter had an R_F identical with that of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (0·46 in system 3*b*). Both substances were oxidized with chromic acid to substances giving both reactions, and having R_F values identical with that of 2α-methylandrost-4-ene-3:11:17-trione (XVIII). This degradation to a polyketone with chromatographic properties identical with those of a known reference steroid via a reaction known to be more or less specific for allylic hydroxyl groups (Fieser, 1953) was regarded as adequate proof that the structure of (XI) was 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, and that of (XII) 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione. The properties of the original material in these fractions were then compatible only with the structures 6β:17α:20ξ:21-tetrahydroxy-2α-methylpregn-4-ene-3:11-dione (VII) for the major, and 6β:11β:17α:20ξ:21-pentahydroxy-2α-methylpregn-4-en-3-one (VIII) for the minor, component.

*Subfraction ME*2. As expected small traces of (VII, Chart 2) were found by bismuthate oxidation to the 6β-hydroxy compound described above. Small amounts of 11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one (III, Chart 1) were identified by its known position in this region of the chromatogram, and its oxidation with bismuthate or periodate to 11β-hydroxyandrost-4-ene-3:17-dione (V); the latter gave no acetate and was oxidized with chromic acid to androst-4-ene-3:11:17-trione (VI). Small amounts of 3α:17α:20ξ:21-tetrahydroxy-5β-pregnan-11-one and 3α:11β:17α:20ξ:21-pentahydroxy-5α-pregnane were identified by bismuthate and periodate oxidation to 3α-hydroxy-5β-androstane-11:17-dione and 3α:11β-dihydroxy-5α-androstan-17-one. These were chromatographically identical with their reference steroids and in turn identified by chromatography of their acetates, and oxidation with chromic acid to the 5α- and 5β-androstane-3:11:17-triones (Bush & Mahesh, 1959*a*).

*Subfraction ME*3. Traces of (VII) (Chart 2) and of 11β:17α:20ξ:21-tetrahydroxypregn-4-en-3-one were identified by oxidation with periodate as above, but were not degraded further owing to lack of material. Tetrahydrocortisol was detected with blue tetrazolium and identified by the procedure of Bush & Mahesh (1959*a*). The main component of this fraction (XIX, Chart 3) gave no fluorescence with NaOH and no reduction of blue tetrazolium. It was detected by the production of a new substance (XX), giving rapidly a pink coloration with alkaline *m*-dinitrobenzene, when ME 3 was oxidized with bismuthate or periodate. This presumed 17-oxo steroid had an R_F in system 3*b* of 0·40, compared with 0·26 for 3α-hydroxy-5β-androstane-

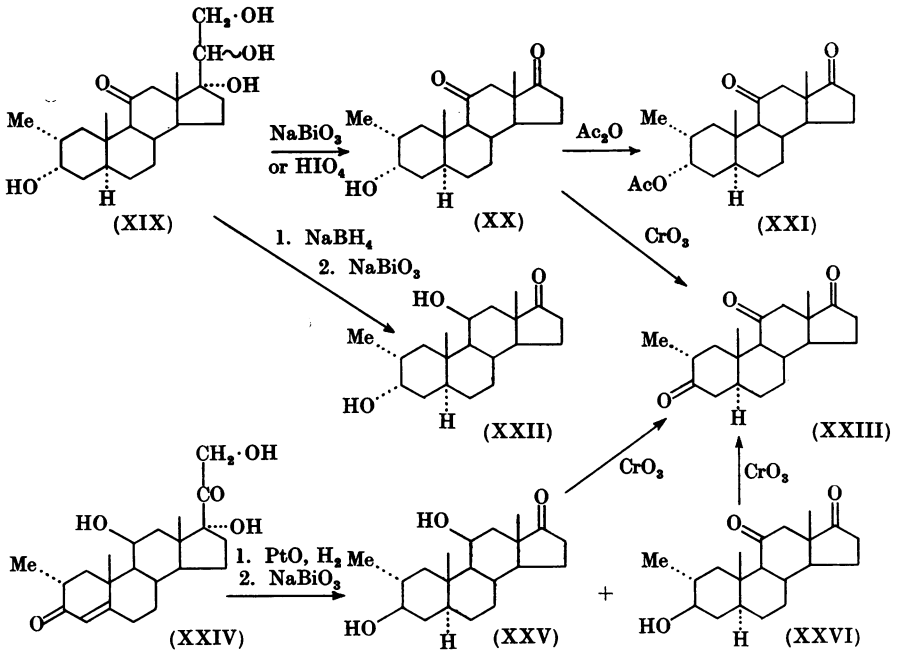


Chart 3. Degradations used to characterize the main component of ME3 (XIX) (2α-methylcortisone experiment). Correlation was achieved by (XXIII) derived via (XXXV) and (XXXVI) from the reference substance (XXIV; 2α-methylcortisol). See text and Fig. 4 for discussion of configuration of 5- H.

11:17-trione (11-oxoaetiocholanolone) and 0·42 for androst-4-ene-3:11:17-trione on the same chromatogram (Fig. 4). Acetylation of the 17-ketone (XX) gave a substance, XXI, with an R_F of 0·61 in system 4*a* compared with 0·56 for 11-oxoaetiocholanolone acetate on the same chromatogram (Fig. 4). The acetate was not altered by oxidation with chromic acid. The original material (XIX; about 20 μg.) was therefore refluxed with sodium borohydride (15 mg.) in tetrahydrofuran (10 ml.) and NaOH (2·57 %, w/v; 1·0 ml.) for 10 hr. Water (10 ml.) was added and the tetrahydrofuran evaporated under reduced pressure. The residue was extracted with ether–ethyl acetate as usual and the reduction product oxidized with sodium bismuthate. A 17-ketone (XXII) was obtained which had an R_F of 0·25 in system 3*b* and gave a slowly-developing violet colour with alkaline *m*-dinitrobenzene. This change in behaviour is characteristic of the reduction of an 11-ketone to an 11β-alcohol (Fig. 4).

Chromic acid oxidation of the 17-ketone (XX) isolated from a chromatogram gave a substance

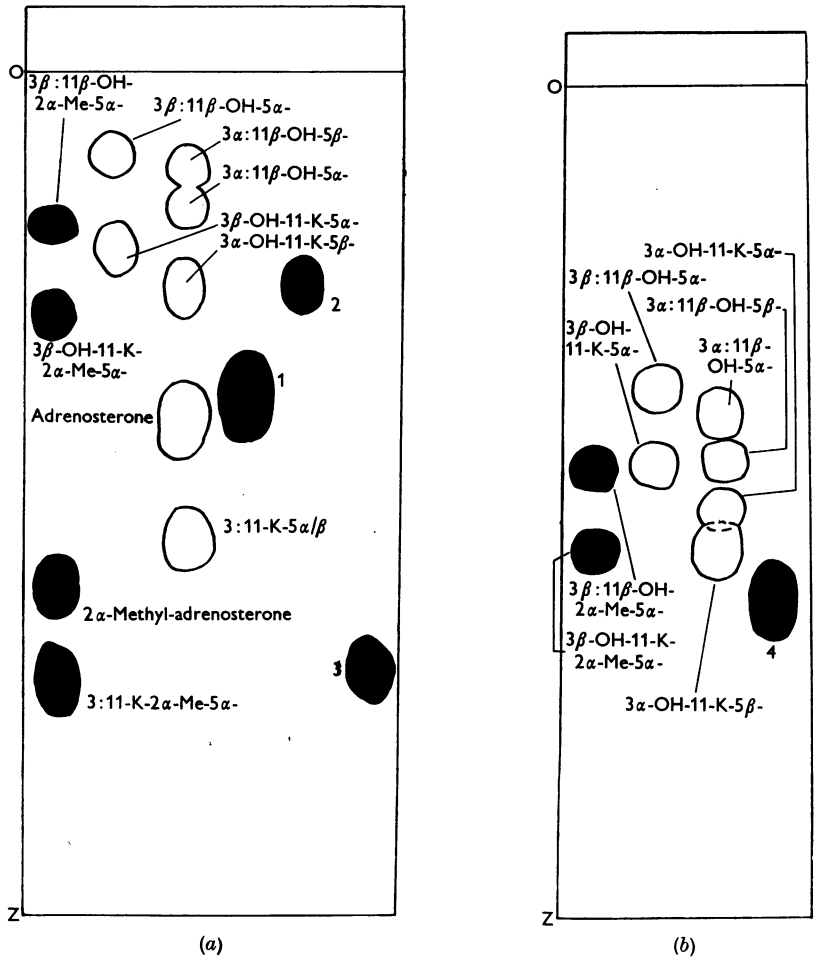


Fig. 4. Chromatographic correlation of degradation products of XIX (Chart 3) (ME3) with saturated 2α-methyl steroids. ●, 2α-Methyl steroids; ○, non-methylated steroids. (*a*) 1, XX; 2, XXII; 3, XXIII. Reference steroids are all derivatives of androstan-17-one, except where full names are given. Run in system 3*b* for approx. 4 hr. O, origin; Z, solvent front. (*b*) 3-Acetates of androstan-17-one derivatives. Run in system 4*a* for approx. 4 hr. 4, Acetate of XX (XXI, suggested as 3α-acetoxy-2α-methyl-5α-androstane-11:17-dione). Note that 5α-H and 5β-H epimers have their relative mobilities inverted on acetylation. In this respect the relationship of 11-oxoaetiocholanolone and its 3-acetate with 1 and 4, and of 3β-hydroxy-2α-methyl-5α-androstane-11:17-dione and its 3-acetate with 1 and 4 strongly suggest the structure 5α-H–3α-OH for 1. Abbreviated nomenclature: Me = methyl; OH = hydroxy or dihydroxy; K = oxo or dioxo.

having an R_F of 0·72 in system 3*b* and giving a bluish violet colour with alkaline *m*-dinitrobenzene. This was identical with 2α-methyl-5α-androstane-3:11:17-trione (XXIII) [the 5α-(H) and 5β-(H)-*cis–trans* epimers of the 3-ketones are not separated in these systems] prepared from 2α-methylcortisol (XXIV) by the route described above (Materials).

The oxidations of the original material in this subfraction are compatible with a side chain having the 17:20-diol or the 17α:20ξ:21-triol structure (XIX). The position of this substance on the original chromatogram is, however, compatible only with the latter structure. The configurations at C-5 and C-3 are discussed below (Discussion).

Subfraction ME 4. This fraction contained traces of 11β:17α:20ξ:21-tetrahydroxy-2α-methylpregn-4-en-3-one (XXVII, Chart 4) and larger quantities of its 11-oxo analogue (XXVIII). It also contained tetrahydrocortisone and *allo*tetrahydrocortisol (3α:11β:17α:21-tetrahydroxy-5α-pregnan-20-one). These were identified by the positions and properties of the original materials, of their acetates (Fig. 5), and by stepwise degradation to the appropriate triones as above, and as in Bush & Mahesh (1959*a*).

Subfraction ME 5. This contained a substance having the mobility of 2α-methylcortisol (XXIV, Chart 4), its reduction of blue tetrazolium, and a rather weak fluorescence with NaOH. About 10 μg. was oxidized with bismuthate and yielded a 17-ketone (XVII), having the R_F, fluorescence with NaOH and colour with alkaline *m*-dinitrobenzene of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione prepared in parallel from 2α-methylcortisol.

Subfraction ME 6. The main component had the same R_F of 0·80 in system 1 as 2α-methylcortisone (XXIX, Chart 4), reduced blue tetrazolium and gave a weak fluorescence with NaOH. Acetylation gave a substance, with the same reactions, which moved 11 cm. on running for 5·25 hr. in system 3*b*, as did the acetate of 2α-methylcortisone (XXX). Oxidation with bismuthate gave a substance with the same reactions and mobility in system 4*a* as 2α-methylandrost-4-ene-3:11:17-trione (XVIII) (13·8 cm. in 16 hr.). This material was unchanged by acetylation and by chromic acid. After preparation of the ethylene ketal, the material was reduced with sodium borohydride, hydrolysed (Bush & Mahesh, 1959*a*), and oxidized with sodium bismuthate. The product gave a fluorescence with NaOH, a violet colour with alkaline *m*-dinitrobenzene, and moved 9·6 cm. in 16 hr. in system 4*a* (XVII, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione: 9·6 cm.). The main component was therefore identified as 2α-methylcortisone.

Subfraction ME 7. Two substances were found on chromatograms in system 3*b*. They and their acetates had the properties of 3α:11β-dihydroxy-5β-androstan-17-one and 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione (XI, Chart 2) and their acetates. The acetate of the latter was unchanged by chromic acid, but there was too little material to carry the identification to completion. The former was identified by the method of Bush & Mahesh (1959*a*).

Subfraction ME 8. This material behaved like 11β-hydroxyandrosterone and was identified by the procedure of Bush & Mahesh (1959*a*).

Subfraction ME 9. This material was identified as 11-oxoaetiocholanolone by the above methods (as ME 8).

Subfraction ME 10. This material gave a rapid pink colour with alkaline *m*-dinitrobenzene; and both an orange colour in the cold with NaOH and a yellow fluorescence after heating with NaOH. It moved 4·3 cm. in 14 hr. in system 4*b* and was not changed by acetylation or by chromic acid. There was too little material to complete the identification but it seems reasonable, on the basis of the more complete degradation of ME 1, to identify this substance as 2α-methylandrost-4-ene-3:6:11:17-tetraone (XIV, Chart 2).

Subfraction ME 11. This fraction contained a substance giving a fluorescence with NaOH and a violet colour with *m*-dinitrobenzene, and moved 7·9 cm. in 14 hr. in system 4*b*, as did 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (XVII, Chart 4). It was unchanged by acetylation and on oxidation with chromic acid gave a substance which moved 13·8 cm. in system 4*a* in 16 hr. as did 2α-methylandrost-4-ene-3:11:17-trione (XVIII, Chart 4). This substance was therefore identified as 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione.

Subfraction ME 12. This material gave a fluorescence with NaOH and a rapidly formed pink coloration with alkaline *m*-dinitrobenzene, and moved 13·1 cm. in system 4*b* in 14 hr., as did 2α-methylandrost-4-ene-3:11:17-trione (XVIII). It was unaltered by acetylation or chromic acid. It was therefore identified as the trione.

Subfractions ME 13, *ME* 14 *and ME* 15. These fractions contained respectively 3β-hydroxyandrost-5-en-17-one (DHA), 3α-hydroxy-5β-androstan-17-one (aetiocholanolone) and 3α-hydroxy-5α-androstan-17-one (androsterone). They were identified by their highly characteristic positions after running in systems 4*a* and 4*b* (Bush & Willoughby, 1957), the mobilities of their acetates in system 4*a*, and the resistance of their acetates to alteration by chromic acid.

Examination for unhydrolysed conjugates. In view of the possibility that 3-glucuronosides of the 2α-methyl steroids might not be hydrolysed by β-glucuronidase, the presence of such conjugates was sought by oxidative fission (Norymberski & Sermin, 1953) via the formates.

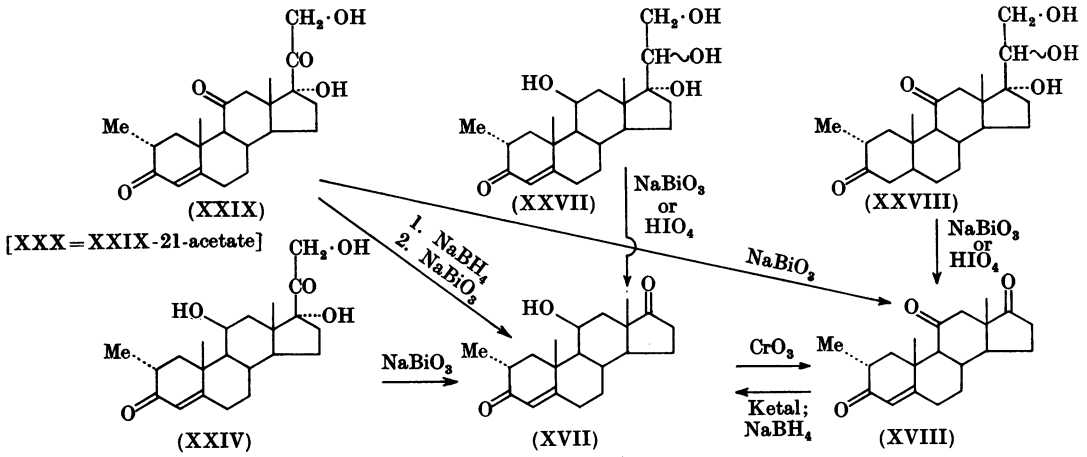


Chart 4. Degradations used to characterize metabolites of 2α-methylcortisone (XXIX) and 2α-methylcortisol (XXIV). Reference substances (XXIX) and (XXIV).

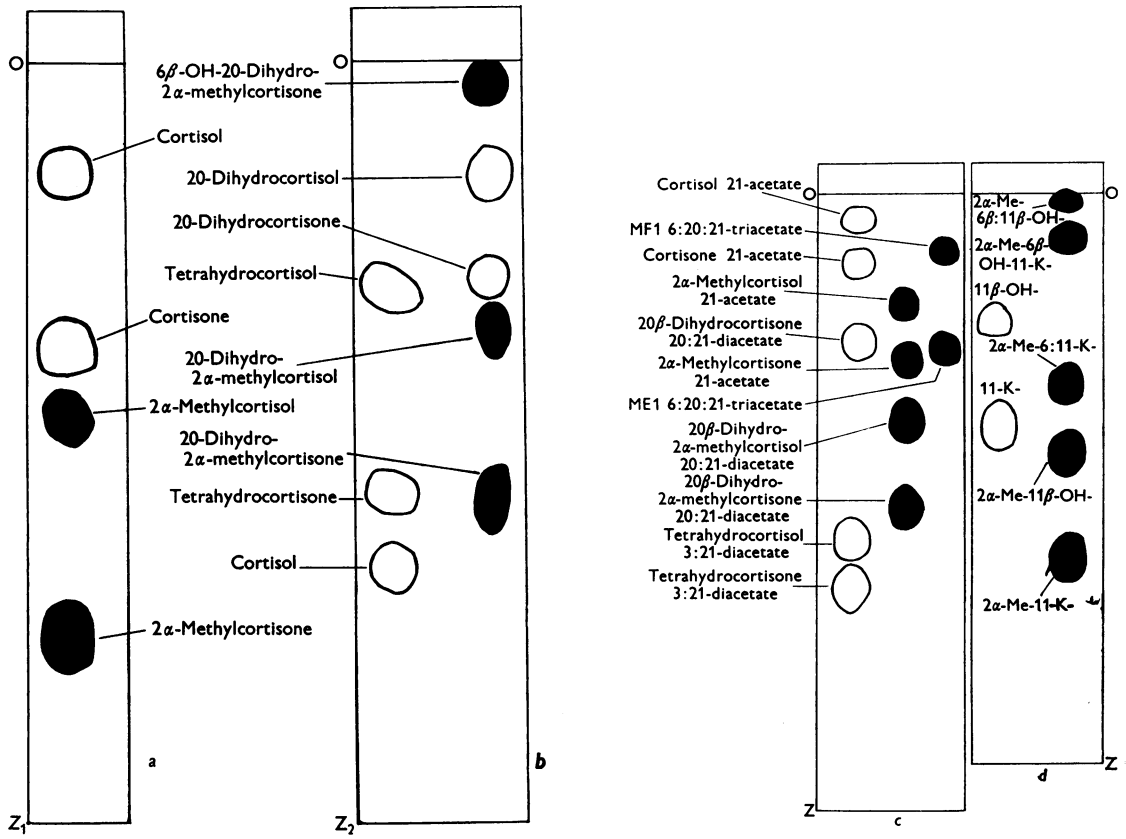


Fig. 5. Chromatographic correlations of 2α-methyl steroids (●) with non-methylated analogues (○) (semi-diagrammatic). O, Origin; Z, bottom of sheet. (*a*) System 1, 3 hr. run on Whatman no. 3MM paper. (*b*) System 1, 16 hr. run. (Slight taper not shown.) (*c*, *d*) System 3*b*, approx. 3·5 hr. Unless full names are given, the compounds are the derivatives indicated of androst-4-ene-3:17-dione. Other correlations are shown in Fig. 4. Abbreviations as in Fig. 4.

One-twentieth of the 24 hr. urine sample was extracted by the resin method and the extract dissolved in buffer and hydrolysed with succus entericus of *H. pomatia* as usual. The aqueous solution was then extracted with ether–ethyl acetate (2:1, v/v) and the extract washed with N-NaOH and water as usual. The aqueous residue and washes were then combined and brought to pH 3 with HCl and re-extracted by the resin method. The dry extract was oxidized with sodium bismuthate according to the general procedure (Bush & Mahesh, 1959*a*). The oxidation products were dissolved in benzene (10 ml.) and run on a column of alumina (10 g., Savory and Moore, grade II–III). After 24 hr. of contact with the alumina, the column was eluted with ethyl acetate–methanol (1:1, v/v; 100 ml.) and the eluate evaporated to dryness under reduced pressure at 40–45°. It was then run in system 3*b* for 3·5 hr. and the chromatogram treated with alkaline *m*-dinitrobenzene. No 17-oxo steroids were detected, suggesting that less than 0·5 μg. of any single component of this type was present in the oxidized extract. As a control, a concentrate of the glucuronosides of tetrahydrocortisol, *allo*tetrahydrocortisol and tetrahydrocortisone from another urine sample, prepared by the methods of Bush & Gale (1957) and Bush (1957), was treated with bismuthate and alumina

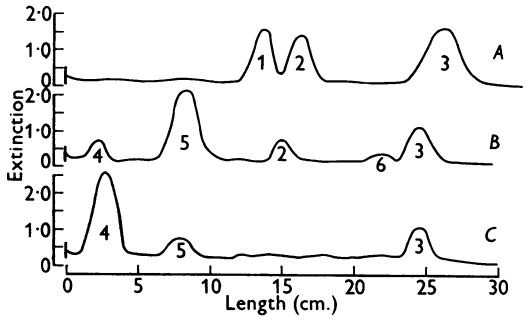


Fig. 6. Scanning records of chromatograms used for estimation of very polar compounds by their oxidation to 17-ketones with bismuthate. Strips run in system 3*b* for 14 hr. and scanned after treatment with alkaline *m*-dinitrobenzene. The extinction was read on an EEL scanner. *A*, Standard reference steroids: 1, 11β-hydroxyaetiocholanolone; 2, 11β-hydroxyandrosterone; 3, 11-oxoaetiocholanolone. *B*, Oxidation products from experiment *B* (2α-methylcortisone metabolites). 4, 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione derived from (VIII) (Chart 2); 5, 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione derived from (VII) (Chart 2); 6, unidentified: probably 11β-hydroxyandrost-4-ene-3:17-dione derived from traces of 20-dihydrocortisol. *C*, Oxidation products from experiment *C* (2α-methylcortisol metabolites). Other details are in experimental section.

Table 3. *Excretion of metabolites of 2α-methylcortisol and 2α-methylcortisone*

Methods of estimation: 1, oxidation with $NaBiO_3$, chromatography of oxidation products, and scanning the chromatogram after treatment with alkaline *m*-dinitrobenzene; 2, NaOH fluorescence, visual matching; 3, scanning of chromatogram after treatment with alkaline *m*-dinitrobenzene. All figures were corrected by assuming the recovery values of the last column. Figures for method 1 were corrected for losses in $NaBiO_3$ oxidation step and for molecular-weight difference due to oxidation.

Metabolites	Method of estimation	Expt. *B* 2α-Methylcortisone (mg.)	Expt. *B* 2α-Methylcortisone (% of dose)	Expt. *C* 2α-Methylcortisol (mg.)	Expt. *C* 2α-Methylcortisol (% of dose)	Fraction no. of major occurrence	Formula no. and (chart)	Recovery value used (%)
6β:11β:17α:20ξ:21-Pentahydroxy-2α-methylpregn-4-en-3-one	1	0·28	0·56	6·8	13·6	MF 1	VIII (2)	60
6β:17α:20ξ:21-Tetrahydroxy-2α-methylpregn-4-ene-3:11-dione	1	4·8	9·6	1·2	2·4	ME 1	VII (2)	60
11β:17α:20ξ:21-Tetrahydroxy-2α-methylpregn-4-en-3-one	2	0·071	0·14	1·1	2·2	MF 3	XXVII (4)	70
17α:20ξ:21-Trihydroxy-2α-methylpregn-4-ene-3:11-dione	2	0·86	1·7	0·071	0·14	ME 4	XXVIII (4)	70
11β:17α:21-Trihydroxy-2α-methylpregn-4-ene-3:20-dione (2α-methylcortisol)	2	0·11	0·22	1·7	3·4	MF 5	XXIV (4)	70
17α:21-Dihydroxy-2α-methylpregn-4-ene-3:11:20-trione (2α-methylcortisone)	2	1·1	2·2	0·36	0·72	ME 5	XXIX (4)	70
3α:17α:20ξ:21-Tetrahydroxy-2α-methyl-5α-pregnan-11-one	1	2·0	4·0	<0·05	—	ME 3	XIX (3)	70
6β:11β-Dihydroxy-2α-methylandrost-4-ene-3:17-dione	2	~0·03	0·06	—	—	MF 7	XII (2)	80
6β-Hydroxy-2α-methylandrost-4-ene-3:11:17-trione	2	~0·03	0·06	—	—	ME 7	XI (2)	80
11β-Hydroxy-2α-methylandrost-4-ene-3:6:17-trione	2	~0·03	0·06	—	—	MF 11	—	80
2α-Methylandrost-4-ene-3:6:11:17-tetraone	2	~0·03	0·06	—	—	ME 10	XIV (2)	80
11β-Hydroxy-2α-methylandrost-4-ene-3:17-dione	3	0·047	0·09	0·23	0·46	MF 12	XVII (4)	85
2α-Methylandrost-4-ene-3:11:17-trione	3	0·29	0·58	0·059	0·12	ME 12	XVIII (4)	85
		Total	19·33		23·04			

in the same way and gave an 80–90% yield of the expected 11β-hydroxyaetiocholanolone, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone. It was concluded that apart from subfraction ME 3 no 3-hydroxy metabolites of 2α-methylcortisone were excreted in significant amounts in the form of glucuronosides.

Quantitative estimation of steroids. After identification of the steroids in this urine sample had been completed, the more polar metabolites were estimated again by oxidation with bismuthate to the 17-oxo steroids and chromatography of the latter. After treatment of the chromatograms with alkaline *m*-dinitrobenzene, they were scanned. 2α-Methylcortisol and 2α-methylcortisone, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione and 2α-methyladrenosterone were not re-estimated by this method since they had been shown to be homogeneous on the original chromatograms.

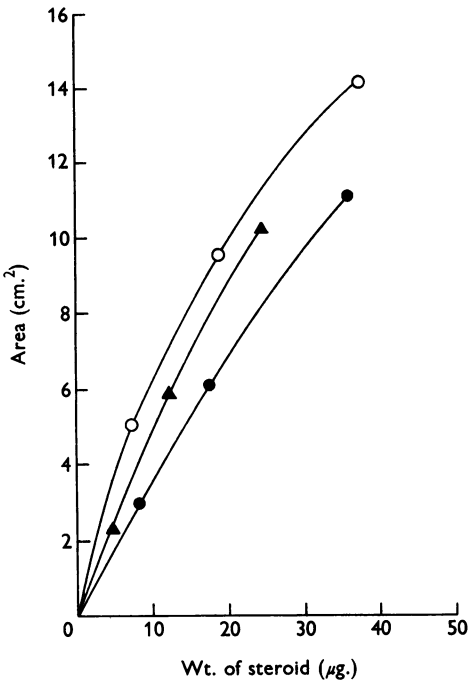


Fig. 7. Calibration curves for 2α-methylsteroids using bismuthate oxidation followed by chromatography of the 17-ketones, and scanning after treatment with alkaline *m*-dinitrobenzene. ○, 11-Oxoaetiocholanolone standard; ▲, 2α-methyladrenosterone from oxidation of 2α-methylcortisone; ●, 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione from oxidation of 2α-methylcortisol. Ordinates: area under peaks measured by the conventions of Bush & Willoughby (1957). Abscissae: μg. of original steroid (i.e. not the bismuthate oxidation product, e.g. the curve for 2α-methyladrenosterone itself would lie above that shown (▲) by an amount determined by the molecular-weight ratio 372/312 and the losses during bismuthate oxidation).

One-hundredth of the 24 hr. sample (15·4 ml.) was extracted by procedure 2 (Bush & Mahesh, 1958*a*) and the extract prefractionated by running for 14 hr. on a 5 cm. wide strip of Whatman 3MM in system 1. The material, more polar than tetrahydrocortisol, was eluted and dissolved in 2 ml. of 50% (w/v) acetic acid and 50 mg. of sodium bismuthate added. After shaking for 1 hr. the suspension was filtered, treated with sodium metabisulphite (10%, w/v; 1 ml.) and extracted twice with 3 vol. of ether–ethyl acetate (2:1, v/v). The extract was dried with anhydrous Na_2SO_4 (0·5 g.) and evaporated under reduced pressure at 40–45°. It was then run in system 3*b* for 14 hr. on a 2·5 cm. strip of Whatman no. 2 paper treated with alkaline *m*-dinitrobenzene and scanned. The main products of oxidation were 6β:11β-dihydroxy-2α-methylandrost-4-ene-3:17-dione, 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione, 11β-hydroxyandrosterone and 11-oxoaetiocholanolone being derived from VIII, VII (Chart 2), 3α:11β:17α:20ξ:21-pentahydroxy-5α-pregnane and 3α:17α:20ξ:21-tetrahydroxy-5β-pregnan-11-one respectively. In addition, 3α:17α:20ξ:21-tetrahydroxy-2α-methyl-5α-pregnan-11-one gave rise to 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione, which was estimated similarly but with a 14 hr. run in system 4*a*.

The chromatogram of these derivatives is shown in Fig. 6. The results of this estimation are given in Table 3, where they are corrected for both the differences in molar extinctions with alkaline *m*-dinitrobenzene (in the scanning method) and for the differences in the molecular weights of the C_{21} steroids and their bismuthate oxidation products. Calibration curves obtained by the bismuthate oxidation method with 2α-methylcortisol and 2α-methylcortisone are given in Fig. 7.

Distribution of metabolites between MEU and MEH. One-twentyfifth of the 24 hr. sample was extracted with ether–ethyl acetate in the usual way but without enzyme hydrolysis. The aqueous residues were combined, brought to pH 4·8 and 0·5M concentration of sodium acetate–acetic acid buffer and incubated for 16 hr. at 45°. The extracts of the hydrolysate and the unhydrolysed urine were then run for 6 hr. in system 2 on 2·5 cm. lanes on Whatman no. 2 paper and examined by the NaOH fluorescence method. The results are given in Table 4.

Estimation of steroids in plasma. Plasma was extracted and the Δ^4-3-oxo steroids estimated by the NaOH fluorescence method as above. The results (corrected for 70% recovery) are given in Table 5.

Table 4. *Distribution of metabolites between free and conjugated forms*

The figures are for one-twentyfifth of the 24 hr. urines. One-twentyfifth of a 24 hr. sample was used for the free steroids, but the figures for conjugated steroids are derived from smaller fractions with which more accurate estimates were possible. Expt. *B*, 2α-methylcortisone as precursor. Expt. *C*, 2α-methylcortisol as precursor. The 6β- and 20ξ-hydroxy metabolites were just detectable in the free steroid fraction; hence the approximation for this figure.

	Free (μg.)		Conjugated (μg.)	
Experiment	*B*	*C*	*B*	*C*
Metabolite				
2α-Methylcortisone	4·3	0	53	11
2α-Methylcortisol	0	8·6	5·0	86
6β-Hydroxy-2α-methylsteroids and 20ξ-Hydroxy-2α-methylsteroids	Approx. 8·0	Approx. 8·0	240	370

Table 5. *Steroids in plasma after 2α-methylcortisone*

Blood was drawn: 1, 3·33 hr.; 2, 6·25 hr. after ingestion of 50 mg. of 2α-methylcortisone (Expt. *B*).

	Steroids in plasma (μg./100 ml.)	
Sample	1	2
Steroid		
Cortisol	0·4	0·4
2α-Methylcortisol	1·5	<0·6
2α-Methylcortisone	15	<1·2

Table 6. *Steroids in plasma after 2α-methylcortisol*

Blood drawn: 1, 2·1 hr.; 2, 4·75 hr. after ingestion of 50 mg. of 2α-methylcortisol (Expt. *C*).

	Steroids in plasma (μg./100 ml.)	
Sample	1	2
Steroid		
Cortisol	<0·2	<0·2
2α-Methylcortisol	5·9	37·5
2α-Methylcortisone	<1·0	<1·0

Metabolism of 2α-methylcortisol (Experiment C, Tables 1 and 3)

A male subject aged 24 years collected two 24 hr. samples of urine beginning at 9.0 a.m. and took 50 mg. of 2α-methylcortisol by mouth at 9.0 a.m. on the second day. Blood samples were drawn at 11.06 a.m. and 1.45 p.m. on the second day. The preliminary extraction and estimation was carried out as with Expt. *B* (above).

Identification of metabolites. Half (520 ml.) of the second urine sample was extracted and fractionated as in Expt. *B* (Fig. 1). Each subfraction (MF) obtained contained the same metabolites as in Expt. *B* but in this experiment the 11β-hydroxyl member of each pair was excreted in much greater amounts than the 11-ketone. The identification of these metabolites was carried out by the same routes of degradation as were used for comparable subfractions (ME) in Expt. *B* except that the preponderant 11β-alcohols were used for carrying out the more extensive degradations to known polyketones. In the case of 6β:11β:17α:20ξ:21-pentahydroxy-2α-methylpregn-4-en-3-one, however, elimination of the 6β-hydroxyl group with zinc and acetic acid was carried out before oxidation of the 11β-hydroxyl group, in order to avoid a mixture of 11α- and 11β-ols. Neither (XIX) (ME 3) nor its 11β-hydroxyl analogue was found in this urine. These degradations are summarized in Charts 2 and 4.

Quantitative estimation. As in Expt. *B*, preliminary estimation was followed by oxidation of the main polar metabolites with sodium bismuthate and estimation of the derived 17-oxo steroids by scanning a chromatogram of these steroids after treatment with alkaline *m*-dinitrobenzene. The scanning trace of this chromatogram is shown in Fig. 6 and the results of these estimations are given in Table 3.

Examintion of unhydrolysed conjugates. This was carried out by the same method as in Expt. *B* with the same negative results.

Distribution of metabolites between free and conjugated fractions. Extracts of untreated urine and of enzymic hydrolysates of the aqueous residues were made as in Expt. *B*, and the steroid content estimated similarly with the NaOH fluorescence reaction. The results are given in Table 9.

Plasma steroids. These were estimated as above on 20 ml. samples. The results are given in Table 6.

Metabolism of adrenosterone (androst-4-ene-3:11:17-trione) (Experiment D, Table 1)

A male subject aged 33 years took 5 mg. of prednisolone by mouth at approximately 8.0 a.m. and 8.0 p.m. for 3 days. On the second day he collected a 24 hr. specimen of urine ending at 10.30 a.m. on the third day. On the third day he took in addition 50 mg. of adrenosterone at 10.30 a.m. and collected a second 24 hr. specimen of urine. At 1.30 p.m. a sample of antecubital venous blood was drawn from which 25 ml. of plasma was separated.

Estimation of steroids in urine. The routine method was applied (Methods) with the results shown in Table 1. In addition, the toluene layer from the urine bottle was separated and evaporated under reduced pressure at 40–45°, after which the residue was run in system 3*b* as for fraction 2 of the usual prefractionation chromatogram. No detectable 17-oxo steroids were found; with the limits of sensitivity of the reaction with alkaline *m*-dinitrobenzene, this means that the toluene layer contained less than 0·05 mg. of any individual 17-oxo steroid (cf. Expt. *E*).

Identification of metabolites. The three principal metabolites were identified by elution from a chromatogram in system 3*b* and preparation of the derivatives given by Bush & Mahesh (1958*b*). In each case the derivatives were chromatographically identical with those derived from the known reference compounds.

Estimation of plasma steroids. This was carried out with 25 ml. of plasma separated from a blood sample drawn 3·0 hr. after ingestion of the steroid under test. The method was the same as above except that the chromatogram was run for 3·5 hr. in system 3*b*. The content of 11β-hydroxyandrost-4-ene-3:17-dione and adrenosterone was 9·0 and 1·0 μg./100 ml. (corr.) respectively.

Metabolism of 2α-*methyladrenosterone* (2α-*methylandrost*-4-*ene*-3:11:17-*trione*) (*Experiment E, Table* 7)

A male subject aged 25 years took 5 mg. of prednisolone at 8.0 a.m. and 8.0 p.m. by mouth for 4 days. On the fourth day he took in addition 40 mg. of 2α-methyladrenosterone by mouth at 9.0 a.m. Urine was collected from 9.0 a.m. until 9.0 a.m. the next day. At 12 noon blood was drawn from the antecubital vein from which 25 ml. of plasma was separated.

Identification of metabolites. A quarter of the 24 hr. sample was extracted by the resin method (procedure 2, Bush & Mahesh, 1959*a*) and the extract run for 1·8 hr. in system 3*b* on a 25 cm. wide sheet of Whatman no. 3MM paper. Three fractions corresponding to those taken in the routine prefractionation step were eluted with ethyl acetate–methanol and concentrated with a jet of air.

Fraction 3 (polar steroids) was run on a 12·5 cm. wide sheet of Whatman no. 2 paper for 4 hr. in system 1. Strips 3–5 mm. wide were cut from the sheet and treated with alkaline blue tetrazolium, alkaline *m*-dinitrobenzene, and NaOH. Apart from faint bands with the first two reagents, the only compounds found were two with R_F values of 0·81 and 0·91 which gave a yellow fluorescence with NaOH but no colour with alkaline *m*-dinitrobenzene. The areas containing these substances were eluted and called subfractions MA 1 and MA 2 respectively.

Fraction 2 was run as above, but in system 3*b* for 14 hr. Two substances, 15·3 and 26·7 cm. from the origin, were detected which gave a yellow fluorescence with NaOH and weak colours with alkaline *m*-dinitrobenzene. The region behind the first was eluted and called subfraction MA 3; the former substance was eluted as subfraction MA 4, and the latter as MA 5.

Fraction 1 was run for 14 hr. in system 4*a* as above and three substances reacting with alkaline *m*-dinitrobenzene were detected at positions 4·7, 21·3 (aetiocholanolone 21·3 cm. on the same sheet) and 25·2 cm. (androsterone 25·2 cm. on the same sheet) from the origin. These three areas were eluted as subfractions MA 6, MA 7 and MA 8.

The toluene from the urine bottle was evaporated under reduced pressure at 40–45° and the residue run in system 4*a* as above for 14 hr. Two substances, 8·3 and 14·4 cm. from the origin, were detected on this chromatogram which gave both a yellow fluorescence with NaOH and a violet colour with alkaline *m*-dinitrobenzene (11β-hydroxy-2α-methylandrost-4-ene-3:17-dione 8·3 cm., and 2α-methyladrenosterone 14·4 cm. on the same sheet). These substances were eluted as subfractions MA 9 and MA 10.

Subfraction MA 1. Acetylation gave a substance (XXXII, Chart 5), detected by the NaOH fluorescence reaction, which had an R_F of 0·17 in system 4*a*, showing the presence of at least two esterifiable hydroxyl groups. Oxidation of the acetate gave a substance (XXXIII) with an R_F of 0·29 in system 4*a*, showing the presence (in XXXII) of an unesterified hydroxyl group. Oxidation of the original free steroid (XXXI) with chromic acid for 1 hr. at room temp. gave two main products, and material at the origin when run in system 3*b* for 4 hr. The first had an R_F of 0·09 and gave both the NaOH fluorescence reaction and a violet colour with alkaline *m*-dinitrobenzene; the second (XIV) had an R_F of 0·32 and gave both these reactions. In addition, the latter gave an orange colour in the cold with NaOH and had the same R_F as 2α-methylandrost-4-ene-3:6:11:17-tetraone [isolated from urine of Expt. *B* as a degradation product (XIV, Chart 2)]. These properties were compatible with the structure 6β:11β:17ξ-trihydroxy-2α-methylandrost-4-en-3-one (XXXI) for the original substance and incompatible with any other reasonable steroid formula. About 30 μg. of MA 1 was dissolved in ethanolic 0·06 N KOH acid and examined as above (subfraction ME 1, Expt. *B*, Fig. 2). The spectral changes characteristic of a 6β-hydroxy-4-ene-3-oxo steroid were found (Meyer, 1955).

Subfraction MA 2. Acetylation gave a substance (XXXIII) giving the NaOH fluorescence reaction

with an R_F of 0·29 in system 4*a* (same sheet as with MA1 and derivatives thereof). Oxidation of the acetate with chromic acid yielded the material unchanged. Oxidation of the original free steroid with chromic acid yielded the same products (XIV) obtained from similar oxidation of MA1. The original structure was therefore identified as 6β:17ξ-dihydroxy-2α-methylandrost-4-ene-3:11-dione (XXXIV). The main oxidation products from MA1 and MA2 (believed to be XIV) were combined (30 μg.) and dissolved in ethanolic 0·066N-KOH. The ultraviolet spectra showed the changes characteristic of steroid 3:6-dioxo-4-enes (Meyer, 1955) (see Fig. 3).

Subfraction MA3. This fraction gave neither a fluorescence with NaOH nor a colour with alkaline *m*-dinitrobenzene. Oxidation of the material with chromic acid, however, gave a single substance giving rapidly a violet colour with alkaline *m*-dinitrobenzene and having an R_F of 0·75 in system 3*b* [2α-methyl-5α-androstane-3:11:17-trione (XXIII) 0·75 on the same sheet]. A part of the fraction was run in system 3*b* for 14 hr., in parallel with another part which had been previously

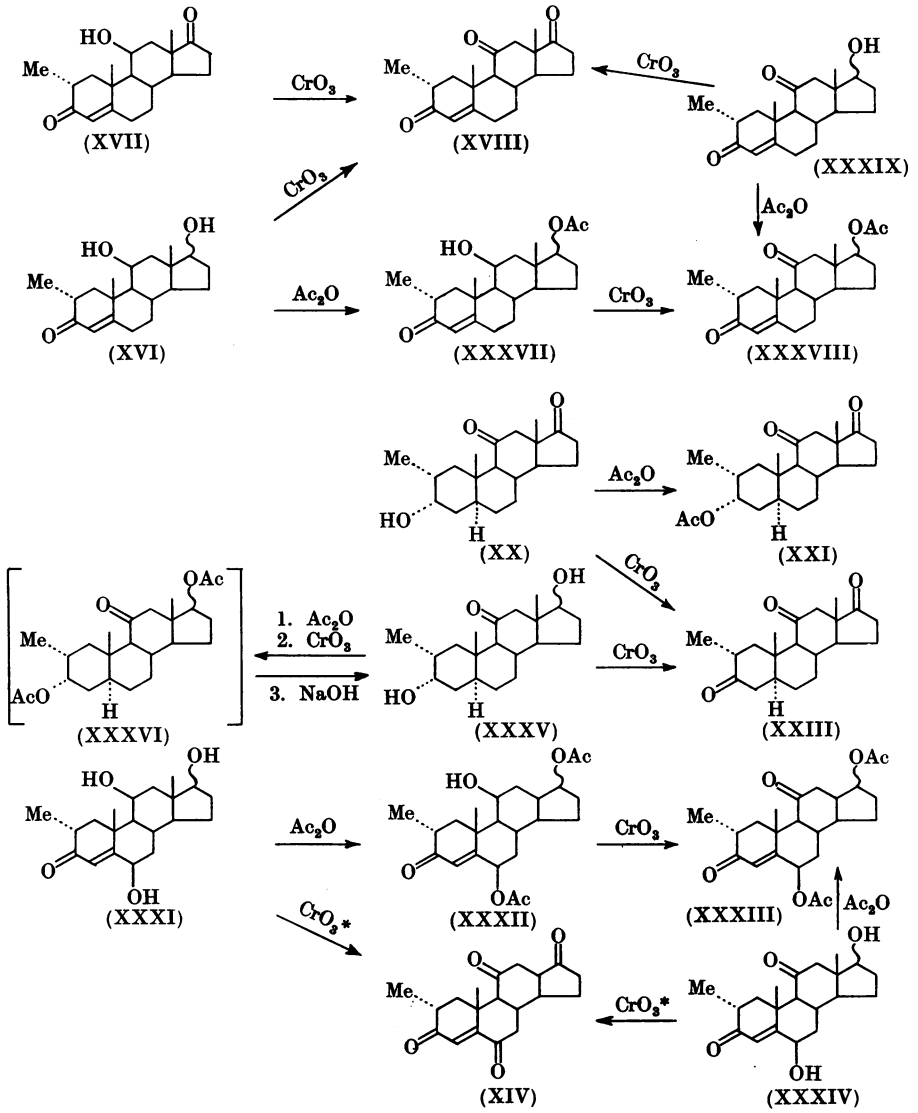


Chart 5. Degradations used to characterize the metabolites of 2α-methyladrenosterone. (XXXVI) was not isolated. Demonstration of the 6β-hydroxyl group is better achieved with MnO_2 oxidation (see XI→XIV, Chart 2). To avoid ring-opening, the oxidations marked (*) were shortened to 1 hr.

acetylated, oxidized with chromic acid and saponified. The chromatogram was dipped in boiling 0·4% chromic acid in 90% acetic acid, dried on a hot plate in an electric oven at 80° and heated with alkaline *m*-dinitrobenzene (Axelrod, 1955). Both fractions gave a bluish pink colour, the spots being 10·1 cm. from the origin. This showed that the oxygen function at C-11 was a ketone group in MA 3. The most probable structure for this substance was 3α:17ξ-dihydroxy-2α-methyl-5α-androstan-11-one (XXXV).

Subfraction MA 4. Acetylation gave a substance (XXXVII) with an R_F of 0·13 in system 4*a*, showing the presence of at least one esterifiable hydroxyl group. Chromic acid oxidation of the acetate gave a new substance (XXXVIII) with an R_F of 0·24 in system 4*a*, showing the presence of an unesterified hydroxyl group. Chromic acid oxidation of the original material gave a substance which moved 13·7 cm. when run in system 4*a* for 16 hr. [2α-methyladrenosterone (XVIII) 13·7 cm. on the same sheet]. Only the last degradation product of MA 4 gave a colour with alkaline *m*-dinitrobenzene, the rapidly developing pinkish purple characteristic of 11:17-dioxo steroids. These properties were compatible only with the structure 11β:17ξ-dihydroxy-2α-methylandrost-4-en-3-one for MA 4 (XVI).

Subfraction MA 5. Acetylation gave a new substance (XXXVIII) with an R_F of 0·24 in system 4*a*. The acetate was unchanged on treating with chromic acid. Oxidation of the original material with chromic acid gave 2α-methyladrenosterone (XVIII), which moved 13·7 cm. when run in system 4*a* for 16 hr. (same sheet as for the oxidation product of MA 4 above) and gave a pink–purple colour with alkaline *m*-dinitrobenzene. The original steroid was therefore identified as 17ξ-hydroxy-2α-methylandrost-4-ene-3:11-dione (XXXIX).

Subfraction MA 6. This material gave a pink–purple colour with alkaline *m*-dinitrobenzene, but no fluorescence with NaOH. It had the same R_F as the bismuthate oxidation product of the main component of subfraction ME 3 (Expt. *B*) and was therefore suspected to be 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione (XX). Acetylation gave a substance with an R_F of 0·61 in system 4*a*, identical with that of presumed 3α-acetoxy-2α-methyl-5α-androstane-11:17-dione (XXI) on the same sheet. Oxidation of the original material with chromic acid, however, gave a substance with an R_F of 0·75 in system 3*b*, identical with that of 2α-methyl-5α-androstane-3:11:17-trione (XXIII) run on the same sheet. The original material was therefore identified as 3α-hydroxy-2α-methyl-5α-androstane-11:17-dione (see Discussion).

Subfraction MA 7. This was identified as aetiocholanolone by its position on the chromatograms run in system 4*a* for 16 hr., the symmetry of the peak obtained on scanning the strip and chromatographic properties of its acetate (Bush & Willoughby, 1957).

Subfraction MA 8. This was identified as androsterone by the same criteria as used for MA 7.

Subfraction MA 9. This material had the R_F, fluorescence with NaOH, and colour with alkaline *m*-dinitrobenzene of 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione (XVII). Acetylation left the material unchanged. On oxidation with chromic acid a single substance was obtained which moved 13·7 cm. when run in system 4*a* for 16 hr. as did 2α-methyladrenosterone (XVIII) on the same sheet. The material was therefore identified as 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione.

Subfraction MA 10. This material had the chromatographic mobility and above two reactions characteristic of 2α-methyladrenosterone (XVIII). Since it was unchanged by acetylation or by chromic acid, it was identified as this substance.

Quantitative estimation of urinary metabolites. None of the metabolites interfered with the routine method of estimating the metabolites of endogeneously produced steroids and these were estimated in the usual way (Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*). Compounds MA 1 and MA 2 were estimated visually with 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione as standard by using the NaOH fluorescence reaction (95% confidence limits approximately ± 15% in the range used). Compounds MA 3, MA 4, and MA 5 were estimated by scanning the chromatogram of their chromic acid oxidation products, after treatment with alkaline *m*-dinitrobenzene and using 2α-methyladrenosterone as standard to allow for the enhancement of extinction due to the 11-oxo group. [The Δ^4-3-oxo group appears to make very little contribution to this colour reaction in the 2α-methyl compounds, whereas the 11-oxo group has its usual effect (Wilson, 1954; fig. 7).] Compounds MA 6, MA 9 and MA 10 were estimated by direct scanning of chromatograms run in system 4*a* for 16 hr. and treated by the standard method with alkaline *m*-dinitrobenzene. 2α-Methyladrenosterone was used as standard for the two 11-ketones, and 11β-hydroxy-2α-methylandrost-4-ene-3:17-dione for MA 9. The results are given in Table 7.

Estimation of plasma steriods. Plasma (12·5 ml.) was extracted with ether–ethyl acetate (Bush & Mahesh, 1959*a*) and the extract run in system 3*b* for 4 hr. by the method of Bush & Mahesh (1959*b*) to concentrate the extract at the origin of the chromatogram in a spot approximately 2 mm. in diameter. The steroids were estimated visually using the NaOH fluorescence reaction and 2α-methyladrenosterone and the related 11β-ol as standards. These two compounds were estimated to

Table 7. *Excretion of metabolites of 40 mg. of 2α-methyladrenosterone (Experiment E)*

Methods of estimation: 1, scanning after treatment of chromatogram with alkaline *m*-dinitrobenzene; 2, NaOH fluorescence; 3, separation on chromatograms, oxidation with CrO_3, chromatography of products and then method 1.

Metabolites	Excretion (mg.)	Excretion (% of dose)	Method of estimation	Fraction no.	Formula no. (Chart 5)	Recovery value assumed (%)
6β:11β:17ξ-Trihydroxy-2α-methylandrost-4-en-3-one	1·0	2·5	2	MA1	XXXI	80
6β:17ξ-Dihydroxy-2α-methylandrost-4-ene-3:11-dione	1·5	3·7	2	MA2	XXXIV	80
11β-Hydroxy-2α-methylandrost-4-ene-3:17-dione	2·0	5·0	1	MA9	XVII	85
2α-Methylandrost-4-ene-3:11:17-trione (2α-methyladrenosterone)	2·8	7·0	1	MA10	XVIII	85
11β:17ξ-Dihydroxy-2α-methylandrost-4-en-3-one	2·4	6·0	3	MA4	XVI	80
17ξ-Hydroxy-2α-methylandrost-4-ene-3:11-dione	4·1	10·0	3	MA5	XXXIX	80
3α:17ξ-Dihydroxy-2α-methyl-5α-androstan-11-one	1·0	2·5	3	MA3	XXXV	80
3α-Hydroxy-2α-methyl-5α-androstane-11:17-dione	0·5	1·3	1	MA6	XX	80
Total	15·3	38·0				

be present in amounts (uncorrected) of 0·5 μg., which is close to the limit of detection and therefore subject to considerable error (± 30 %). After correction for 80 % recovery, this is equivalent to a plasma concentration of 5 μg./100 ml.

DISCUSSION

Reliability of methods of identification and estimation

Chromatographic methods of identifying steroids are still controversial since the number and variety of structures based on the steroid nucleus is enormous and numerous possibilities of confusion exist in the identification of any given steroid. However, our method of stepwise degradation in parallel with known reference steroids is believed to overcome these difficulties and the arguments supporting identification by this method have been given fully in previous papers (Bush, 1954; Bush & Willoughby, 1957; Bush & Mahesh, 1959*a*). Only a few points therefore need attention in the experiments reported here.

The effects of substituents at carbon atoms 3, 11, 17, 20 and 21 were shown to be entirely similar in the 2α-methyl steroids to those found with steroids unsubstituted at C-2 by preparation of derivatives of 2α-methylcortisol by unequivocal reactions. The influence of the 2α-methyl group was the expected one (Martin, 1950) of reducing the polarity of the steroid in which it was a substituent, the effect being similar in the Δ^4-3-ketones and in the saturated 3-ketones. The effect of this group in the 3β-alcohols was, however, rather less than in the 3-ketones. These results are summarized in the chromatograms shown in Figs. 4 and 5.

Identification of the Δ^4-2α-methyl-3-ketones described in this paper is therefore considered to be established beyond reasonable doubt. After the estensive investigations of the structure of 6β-hydroxy-2α-methylandrost-4-ene-3:11:17-trione derived from the bismuthate oxidation of ME1 and MF2 (Expts. *B* and *C*) it was thought sufficient to demonstrate the structures of MA1 and MA2 (Expt. *E*) by carrying their degradation as far as the fully oxidized product 2α-methylandrost-4-ene-3:6:11:17-tetraone (XIV) which had the correct mobility and also gave the characteristic orange colour in the cold with NaOH (Savard, 1954) and spectral changes in ethanolic KOH (Meyer, 1955). A further characteristic was the production of more polar oxidation products, probably with ring A opened, during the oxidation of the related 6β-alcohol with chromic acid. This effect was reduced by shortening the period of oxidation to 1 hr.

Identification of the saturated 3-hydroxy metabolites of these 2α-methyl steroids is, however, still doubtful as regards the configuration at position 5. Inferences based on the characteristic

behaviour of such isomers and their acetates in the series of non-methylated steroids (Bush & Willoughby, 1957) cannot be used with certainty, although they are reasonable, until a complete set of isomers of the 2α-methyl steroids has been prepared. The correlation of these derivatives is summarized in the chromatograms shown in Fig. 4 and compared with the behaviour of the related steroids unsubstituted at C-2. The reason why these compounds cannot be identified with certainty is that the characteristic differences in mobility of the four isomeric saturated 3-alcohols (which holds for the series based on androsterone, 11-oxoandrosterone, 11β-hydroxyandrosterone, tetrahydrocortisone and tetrahydrocortisol), and the still more characteristic changes in relative mobility on acetylation, are likely to be altered by the presence of the large methyl group on the adjacent C-2, particularly the latter changes which most probably depend on the 'effective polar group' of the acetoxyl group having the opposite conformation to that of the hydroxyl group of the original steroid. However, the identification of the 11-substituent in these steroids, with which we are mainly concerned, is as reliable as with the other steroids considered here.

The reliability of our methods of estimation depends largely on three sources of error. The first is the error in hydrolysis and extraction of the conjugated steroids in the urine, which is unknown for most of the 2α-methyl steroid metabolites. All the figures obtained have been corrected therefore by factors based upon experience with the natural steroid metabolites. These factors are largely determined by the partition coefficient of each steroid between the extracting solvent and water, which in turn parallels closely the polarity of each steroid as judged by its behaviour on chromatograms. Correction factors have therefore been applied to each 2α-methyl metabolite which match those found by experiment to correct for the extraction losses of a steroid having a similar mobility on these chromatograms. These factors are given in the tables to enable back-calculation of the actual amounts found. The assumption that glucuronosides of 2α-methyl steroids would be completely hydrolysed by our technique was confirmed by our failure to obtain any further 17-oxo steroids by applying Norymberski's technique of oxidative fission to aqueous residues which had been extracted previously by the standard technique using enzymic hydrolysis.

The second error is in the method of estimation by scanning chromatograms. The 95% confidence limits of this method have been approximately ±3% with blue tetrazolium and ±6% with alkaline *m*-dinitrobenzene over the last 3 years with the usual steroid metabolites, and there is no reason to suppose that results with the 2α-methyl steroids are less accurate. An additional error is introduced when this method is combined with bismuthate oxidation (Norymberski, 1952). This has been allowed for by using 2α-methylcortisol and 2α-methylcortisone as models for the whole process; a calibration curve is shown in Fig. 7.

The third error is in the extinction values used with the 17-oxo steroids (Wilson, 1954). Since it has been found that the main source of variation here is due to presence of 11-oxo or 11β-hydroxy groups which exert opposite effects, that other groups exert much smaller effects in the scanning method, and that the 2α-methyl group produced a slight fall in extinction, 2α-methyl-11β-hydroxyandrost-4-ene-3:11-dione and 2α-methyladrenosterone have been used as standards for the 2α-methyl metabolites, the former for the 11β-alcohols and the latter for the 11-ketones. It is unlikely therefore that this factor contributes an error of more than about ±5% to the corrected figure given in this paper; furthermore, such errors would be in one direction and would not affect the comparisons we wish to make.

In Expts. *B* and *C* two determinations of the naturally excreted steroid metabolites are given. These were carried out with 4 weeks' interval between them. As expected from earlier work, the second set of figures are all rather lower than the first, the relative proportions remaining very similar. Duplicate determinations carried out at the same time have agreed to within ±10% for each individual steroid during the last 3 years. As with most work of this type, the corrected figures given here have 95% confidence limits of approximately ±10% for the scanning methods and ±20% for the NaOH fluorescence method. This is sufficient for the argument of this paper.

The pattern of metabolites observed

The metabolites of cortisone and adrenosterone were entirely similar to those expected from earlier work (Dorfman, 1954; Burstein, Savard & Dorfman, 1953; Savard, Burstein, Rosenkrantz & Dorfman, 1953; Bradlow & Gallagher, 1958), although the ratio of 5α/5β metabolites of adrenosterone was lower than expected (2·1 instead of 4·0). While this may be due to the concomitant administration of prednisolone, the contrast between this steroid and cortisone (Dorfman, 1954) is still pronounced. Thus the ratio 11β-hydroxyandrosterone/(11β-hydroxyaetiocholanolone + 11-oxoaetiocholanolone) (after subtraction of the probable endogenous contribution) was 2·1 for adrenosterone (Expt. *D*) and 0·14 for (Expt. *A*).

*allo*Cortol was found as a metabolite of cortisone; this was expected from the finding of *allo*cortol as a metabolite of *allo*tetrahydrocortisone (Bush & Mahesh, 1959*a*). The ratio of 5α-H/5β-H steroids in

the 4:5-dihydro-3:20-diol group of hexahydro metabolites was of the same order as in the 4:5-dihydro group of tetrahydro metabolites (24 and 22·5% respectively). These and earlier results (Bush & Willoughby, 1957) give further support to the suggestion of Fukushima & Gallagher (1957) that 5α-H-3α-OH metabolites of cortisol will be of greater importance than was suspected previously.

The metabolites of the 2α-methyl steroids were remarkable for the large numbers and amounts of steroids retaining the Δ^4-3-oxo group compared with the very small amounts of saturated 3-hydroxy metabolites. This was expected, and the apparent inhibition of the reduction of the Δ^4-3-oxo group, which is the main reaction responsible for inactivation of the natural hormones, has previously been suggested as one reason for the enhanced biological activity of 2α-methylcortisol (Liddle *et al.* 1956). Despite this fact, it was remarkable that the greater part of all these metabolites, with the exception of 2α-methyladrenosterone and the related 11β-alcohol, were apparently conjugated, since very little could be extracted before enzymic hydrolysis of the urine, or before hydrolysis of the extract obtained by the resin method, which contains both free and conjugated steroids (Bush & Gale, 1957). This suggests that conjugation at positions 6, 20 or 21 must have occurred, which is interesting since the greater part of the 21-hydroxysteroid tetrahydrocortisone is excreted normally as the 3-monoglucuronoside (Schneider, Lewbart, Levitan & Lieberman, 1955) and similar evidence has been obtained for β-cortolone (J. J. Schneider, personal communication) and for tetrahydrocortisol and *allo*tetrahydrocortisol (I. E. Bush & M. Gale, unpublished work). It seems likely that conjugation at positions other than 3 in the natural steroid metabolites with a 3α-hydroxyl group is a slow reaction compared with the excretion of monoglucuronosides by the hepatic cell. This supposition is based upon the fact that it has been found that the yield of unchanged precursor excreted as monoglucuronoside is about twice as large when the precursor is a 3α-hydroxy steroid as with the related Δ^4-3-oxo steroids (e.g. compare Bush & Mahesh, 1959*a*, with the above; J. J. Schneider, personal communication). This suggests that most dehydrogenations and hydroxylations of the steroid molecule *in vivo* are reactions which are slow compared with the formation and excretion of 3-monoglucuronosides from hepatic cells. In the absence of a 3α-hydroxyl group, however, the slower formation of conjugates with 6-, 20- or 21-hydroxyl groups could occur to a considerable extent if the excretion of free steroid by hepatic cells were a slow reaction: this appears to be so, since more than 98% of the total 2α-methyl steroid metabolites were in conjugated form.

The other striking feature of the metabolism of the 2α-methyl steroids was the far greater percentage yield of 20-hydroxy metabolites of the C_{21} precursors, and of 6β-hydroxy metabolites of both the C_{21} and C_{19} precursors than is obtained with the related non-methylated steroids. This contrast seems also to be due, with the natural steroids, most probably to the predominance of the reactions: Δ^4-3-ketone → saturated 3α-alcohol → 3-monoglucuronoside → hepatic excretion. It seems likely that, as with the position of conjugation, the almost complete absence of these reactions with the 2α-methyl steroids allows the slower reactions of 20-hydrogenation and 6β-hydroxylation to proceed to a far greater extent. Although changes in enzyme–substrate affinity almost certainly play a part in these findings (Tomkins, 1956), the role of competing reactions suggested here seems of greater probable importance in view of the similar results in this respect obtained with steroids as different as 2α-methylcortisone, 2α-methylcortisol, prednisone, prednisolone and 2α-methyladrenosterone.

Liddle *et al.* (1956) studied the metabolic fate of 2α-methylcortisone and 2α-methylcortisol in terms of the rates of disappearance of Porter–Silber-reactive material from plasma, and the appearance of such material which was released from plasma by treatment with β-glucuronidase. They found only minor amounts of conjugated material after intravenous injection of these precursors, compared with the large amounts which appeared after administering hydrocortisone itself. This might appear at first to be in disagreement with our finding that most of the 2α-methyl steroid metabolites were in conjugated form. This, however, is not so, since the Porter–Silber reaction depends upon the presence of the 20-oxo group of the dihydroxyacetone side chain, and one of the main features of the 2α-methyl steroids is that the great majority of their metabolites have been reduced at position 20 to the related 20ξ-alcohols. Such metabolites would not have been measured by Liddle *et al.* (1956) with their methods. These authors also found that the ring-A reducing system of rat liver (Tomkins, 1956) would reduce 2α-methylcortisone but not 2α-methylcortisol. This would agree with the total lack of reduction in ring A that we observed with 2α-methylcortisol, but the figures given in the table of Liddle *et al.* would not explain the almost equally inefficient reduction of ring A of 2α-methylcortisone in our experiments, since they found no difference between cortisone and 2α-methylcortisone with this enzyme system. However, qualitative agreement between human metabolism *in vivo* and that of the rat *in vitro* is indicated, since only with 2α-methylcortisone were we able to find a saturated 3-hydroxy metabolite (ME3; XIX, Chart 3),

although this made up only 21% of the total recognized metabolites, or 4% of the ingested precursor. Such metabolites made up 99% or more of the total recognized metabolites of cortisone, or 45% of the ingested steroid.

The pattern of 11-*oxo and* 11β-*hydroxy metabolites*

It is clear from the results that the pattern of metabolites of cortisone and adrenosterone are those expected from previous work. Thus the 5α-H saturated 3α-OH metabolites are largely or entirely in the 11β-hydroxy form, as are the Δ^4-3-ketones themselves. On the other hand, their 5β-H saturated metabolites are predominantly in the 11-oxo form, the ratio 11-ketone/11β-alcohol being approximately 2/1 in each pair. In contrast, the metabolites of 2α-methylcortisone and 2α-methylcortisol are largely in one or other form according to the nature of the 11-oxygen function in the precursor. Thus with 2α-methylcortisone the ratio total 11-ketones/total 11β-alcohols was 15·8/1, while with 2α-methylcortisol this ratio was 0·16/1. Similarly, the plasma steroids were in similar ratios after oral administration, 2α-methylcortisone contrasting strongly with cortisone in this respect; the latter, as shown here and previously (Bush, 1956), was almost entirely reduced to cortisol in the plasma after oral administration, the small amount of material which might have been unchanged cortisone being too small to be identified with certainty.

With 2α-methyladrenosterone the same general effect was noticed, but to a smaller degree than with 2α-methylcortisone. Thus the ratio total 11-ketones/total 11β-alcohols was 1·8/1 in the urinary metabolites. The contrast with adrenosterone itself was well shown in the plasma, where the ratio adrenosterone/11β-hydroxyandrost-4-ene-3:17-dione was 1/9 after oral adrenosterone, while the comparable ratio for the 2α-methyl analogues after 2α-methyladrenosterone was 1/1. Reduction of the 11-oxo group in 2-methyladrenosterone is therefore clearly less complete than in adrenosterone itself, but the inhibition of the reduction is not so pronounced as in the cortisone analogue.

Reduction of 11-*oxo groups*

In the previous paper (Bush & Mahesh, 1959*a*) we showed that reduction of steroid 11-oxo groups occurred readily with steroids of the 5α-H-3α-OH but not with those of the 5β-H-3α-OH type. This is readily understood on stereochemical grounds, since the buckled A/B ring system of the 5β-H steroid would be expected to hinder the approach of the 11-oxo group to the active site of an enzyme which associates with the steroid by the α-surface of the latter. This is the most likely mode of association, since reduction to the 11β-hydroxy form requires that the α-bond of the carbonyl group be attacked by hydrogen, and this is most likely to be in the form of reduced coenzyme attached to the enzyme surface (Vennesland & Westheimer, 1954). Whether this reaction is carried out by one enzyme or several, it seems that the active region of such enzymes is similar in requiring a flat α-surface to the steroid substrate for efficient attack upon the α-carbonyl bond.

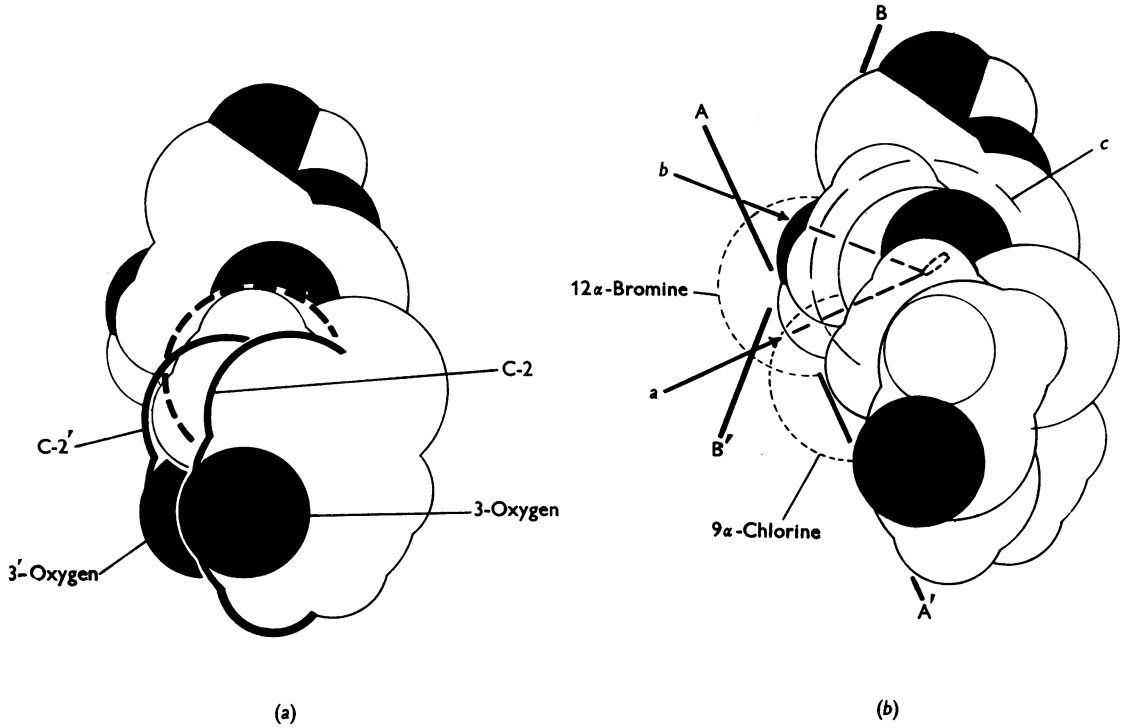
While this simple conception suffices to explain the results with non-methylated steroids, it does not explain the behaviour of 2α-methyl steroids, nor does it entirely explain the behaviour of the 9α-chloro steroids. In Fig. 8 two sets of projections are shown for typical steroids examined in these studies. These have been constructed by using the positions of carbon atoms in rings B, C and D derived from X-ray studies (Crowfoot, 1944; Shoppee, 1946), and the conformations of ring A derived from X-ray studies for 5α-(H) and 5β-(H) steroids, and from models for the unsaturated A rings. The van der Waals radii are taken directly from Pauling (1940), since the larger values given by him are more appropriate for this type of problem, which involves molecular associations similar to those of the solid state, rather than the smaller values, which are better for explaining intramolecular steric hindrance (Braude & Sondheimer, 1955). The former situation is largely governed by repulsions in the line of the bonds; the latter largely by repulsions normal to the bonds involved, and complicated by the consideration of excited states and larger energy barriers. It is easily seen that the 2α-methyl group of steroid Δ^4-3-ketones, which is equatorial, projects only a small distance beyond the plane of the α-surface of rings C and D. It projects, in fact, 1·4 Å less than C-2 and C-3, and 1·6 Å less than O-3, of prednisone, which is known to be reduced to prednisolone, although less completely than cortisone is reduced to cortisol (Bush & Mahesh, 1958*b*). Again, the reduction of 2α-methyladrenosterone is more complete (35% of all metabolites) than that of 2α-methylcortisone (6% of all metabolites), suggesting that the 17β-side chain of the latter is involved in hindering association with the active region of the enzyme. These findings could be explained by assuming that the active site of the steroid 11β-dehydrogenase resembled a shallow pit with a sharp quarry-like edge closely apposed to the outer edge of the steroid at C-2, C-16, C-17, C-20 and C-21. The absence of the side chain at C-17 would then allow 2α-methyladrenosterone to associate more closely with the active region by rotation about the 11-oxo group on an axis normal to the main plane of ring C, anti-clockwise as the formula is usually drawn, to let the 2α-methyl group slip down 'over the edge' at C-2. However,

this explanation will not allow for the apparently quite good apposition of 9α-chlorocortisone (I. E. Bush & V. B. Mahesh, unpublished work). The only explanation that covers all the findings satisfactorily at present is that hydrogen attack on the α-carbonyl bond with this enzyme occurs from the 'north-west' direction in the projection (Fig. 8) rather than from 'due west' and that the steroid associates with the active region of the enzyme with the two surfaces at an angle (projection *b*, BB′) rather than with the face-to-face apposition assumed in the original simple theory (projection *b*, AA′; Fig. 8).

These speculations may seem too bold for results obtained from experiments *in vivo*, but we have already given reasons for supposing that they are justifiable and that the many other factors that might be involved in producing our results largely cancel one another when they are evaluated for the wide variety of precursors that have been studied (Bush & Mahesh, 1958*b*). However, they require confirmation by experiments with other steroid precursors and by experiments *in vitro*.

Correlation of 11*-oxygen functions with biological activity*

In a previous paper (Bush, 1956) it was suggested that the biological effects of the 11-oxygen function of adrenocortical steroids could not be explained on the basis of simple solubility effects or of an activation of some other part of the steroid molecule. One had instead to suppose that this oxygen function was itself involved in a highly specific interaction with the 'receptor site' for such



(*a*) (*b*)

Fig. 8. (*a*) Composite projection on a plane normal to a line joining C-8 and C-10 of cortisone, prednisone and 2α-methylcortisone. C-2, C-2 of cortisone and 2α-methylcortisone; C-2′, C-2 of prednisone; 3-oxygen, as in cortisone and 2α-methylcortisone; 3′-oxygen, as in prednisone. The heavy line is the α-side profile of C-2 and C-3 and of the 4-H atom. The heavy dashed line is the profile of the 2α-methyl group. Because of conjugation the repulsion between the 2α-methyl and 3-oxo groups has been assumed to lead only to bending and slight lengthening of the 2α-methyl–C-2 bond. This repulsion was calculated with 'small' van der Waals radii: all other van der Waals radii are taken from Pauling (1940) (see text). The conformation of the side chain is not necessarily the preferred one. (*b*) Hypothetical projection of possible association between cortisone and active region of the 11β-dehydrogenase. AA′, plane of enzyme 'surface' of simplified theory (Bush & Mahesh, 1958*b*) with direction of hydrogen attack on α-carbonyl bond, *a*; BB′, more probable orientation of enzyme 'surface' and hydrogen attack, *b*, to account for relative hindrance of 2α-methyl group, the A ring of prednisone, the influence of the 17β-side chain and of the 9α-chloro and 12α-bromo groups (see text); *c* is the circumference within which a hydrogen atom or hydride ion must approach C-11 before hydrogenation is more likely than recoil.

hormones. In the light of evidence available at that time, two possibilities were raised: either the known oxidation–reduction reaction of the 11-oxygen function was the basis of the biological actions of such hormones, being linked with some essential redox system; or the 11β-hydroxy forms were active at the receptor site but not the related 11-ketones, which were active after systemic administration only because of their rapid reduction to the former. The results given above clearly suggest that the latter hypothesis is correct, thus placing considerable limitations on the type of chemical process underlying the biological actions of these hormones.

The original biological findings, with the exceptionally large difference between the biological activities of 2α-methylcortisone and 2α-methylcortisol, strongly supported this hypothesis (Liddle *et al.* 1956; Peterson *et al.* 1957) but were not a satisfactory proof by themselves. Since we have been able to show that all the metabolites of 2α-methylcortisone are largely unreduced at C-11, and that the absorption and distribution of 2α-methylcortisone is not greatly different from that of 2α-methylcortisol, it seems clear that this exceptionally low activity of the 11-ketone is not easily accounted for except by supposing that it is itself inactive at the receptor site. The small activity actually possessed by 2α-methylcortisone is reasonably attributed to the very small amount of 2α-methylcortisol to which it gives rise in the plasma.

Such results not only favour the hypothesis that biological activity is confined to the 11β-hydroxy forms of the 11-oxygenated adrenal steroids, but seem to exclude the alternative possibility that the reversible 11β-dehydrogenase reaction is itself the basis of their biological activity. Since Talalay & Williams-Ashman (1958) have recently tentatively favoured this very alternative, on the basis of a penetrating analysis of Villee's (1955) findings with oestradiol and placental *iso*citric dehydrogenase, it is essential to consider just how far our evidence, however suggestive, can be accepted as exclusive of such hypotheses. The most powerful argument against our interpretation at first sight is the usual objection to metabolic studies of this type being used to infer connexions between overall metabolism and pharmacological effects; since only 19·33 % of the dose of hormone has been accounted for as metabolites, one does not know what reactions the remainder has undergone. In this sense our interpretation cannot be considered exclusive. However, it is reasonably certain that the active hormone in the tissues is rapidly exchanged with the circulating hormone, and the steady-state ratio 11-ketones/11β-alcohols in plasma must be a definite function of the same ratio in the tissues. If the basic mechanism of action of this type of hormone depended on the 11β-dehydrogenase reaction, then the activity would depend on the turnover rate of the reaction which, for a hormone such as 2α-methylcortisol which is more active than cortisol (Dulin *et al.* 1957), would have to be at least comparable with that of cortisol. In this case the steady-state ratio 11-ketone/11β-alcohol in tissues and in plasma, with the known time factors involved (Peterson *et al.* 1957; Ayres *et al.* 1957), would be the same whether the steady state were approached from the direction of the ketone or the alcohol. In actual fact, 4·75 hr. after administering 2α-methylcortisol, this ratio was 1/37·5, and 3·33 hr. after administering 2α-methylcortisone it was 10/1. Unless one invokes the theory that hitherto undiscovered modifications of such hormones are actually active at receptor sites, these results are incompatible with the theory that these two steroids were undergoing rapid interconversion of the type required by the hypothesis of Talalay & Williams-Ashman (1958).

Various other attempts can be made to reinterpret our results to fit the redox theory of biological action, but they will not be explored here since they all involve unproven or unlikely complications which make them unacceptable on Occam's principle. Two other examples reinforce the argument given here. First, we have found that prednisone (15 mg./24 hr.) is relatively inefficient in suppressing adrenal secretion in man, whereas prednisolone (10 mg./24 hr.) is as efficient as cortisone (37·5 mg./24 hr.). Hodges & Vernikos (1958) have found the same thing with rats. This is correlated with a partial inhibition of the reduction of prednisone such that the plasma concentration ratio prednisone/prednisolone is approximately 1/1. (Bush & Mahesh, 1958*b*). Secondly, Herz, Fried & Sabo (1956) have found that 12α-bromo-11β-hydroxyprogesterone has about one-third the activity of cortisone in the liver-glycogen deposition test, whereas the related 11-ketone is inactive. These compounds are at present under study, but it can be seen from the projection *b* (Fig. 8) that the large bromine atom, with an axial conformation, presents the largest steric hindrance to association with the 11β-dehydrogenase of all the steroids so far considered in these studies. Again, the inactivity of the 11-ketone is probably due to its failure to undergo reduction to the 11β-alcohol *in vivo*.

Thus three exceptional pairs of steroids are known in which the 11-ketonic forms are less active, compared with the 11β-hydroxy forms, than is found with the natural adrenal steroids, although the 11β-hydroxy forms are more active than their parent hormones.

In two of these cases a partial or almost complete inability to undergo reduction of the 11-oxo

group is associated with a partial or almost complete loss of biological activity; in the third the inactivity of the ketone is associated with the possession of a group almost certain to prevent this reduction from occurring. One is left with the almost inescapable conclusion that the biological activity of 11-oxygenated steroids as glucocorticoids is due to the specific interaction of an 11β-hydroxyl group with the receptor sites for such hormones, and that this interaction does not involve oxidation–reduction at C-11. While it is possible that this interaction is with an enzyme carrying out a redox reaction at C-20 (no other group capable of such reactions is common to all known glucocorticoids), this does not seem likely at present.

Although these considerations leave much to be done before the mode of action of the glucocorticoids is understood on a molecular level, they provide reasonable grounds for excluding a large number of mechanisms that have been postulated previously. Thus, in agreement with Hechter (1955), it seems less and less profitable to pursue the 'coenzyme' theory of hormone action, at any rate for redox reactions. Rather, one must consider the physical actions of such hormones that might result from their specific interaction with receptor sites. This is being investigated.

SUMMARY

1. The metabolism of 2α-methylcortisone, 2α-methylcortisol and 2α-methyladrenosterone was compared with that of cortisone and adrenosterone after oral administration to young men, with special reference to oxidation–reduction of the 11-oxygen function of these steroids.

2. The 2α-methyl steroids showed four main features of interest: There was an almost complete lack of metabolites reduced in ring A; there was a much higher proportion of 6β-hydroxy and 20ξ-hydroxy metabolites; there was an inhibition of the usual interconversion of 11-ketones and 11β-alcohols, which was far more complete with 2α-methylcortisone than with 2α-methyladrenosterone and, despite the lack of 3α-hydroxy metabolites, at least 98% of the recognized metabolites were in conjugated form.

3. The extent of reduction of the 11-oxo groups of the precursors suggested by the pattern of urinary metabolites was paralleled in each case by the 11-ketone/11β-alcohol ratio in plasma collected up to 3 hr. after taking each precursor. Furthermore, there was little oxidation of the 11β-hydroxyl group of 2α-methylcortisol.

4. Arguments are given suggesting that association of 11-oxo steroids with the 11β-dehydrogenase occurs with the steroid tilted on the active region of the enzyme, so that contact is mainly over the α-surface of the molecule in the region of carbon atoms 1, 2, 11, 12, 17 and 21.

5. The exceptionally high ratio of biological activities of 2α-methylcortisol and 2α-methylcortisone appears to be correlated with the exceptionally poor reduction of the latter to the former. It is concluded that biological activity of the 11-oxygenated adrenal steroids is confined to the 11β-hydroxy forms, except when the 11-ketones can be reduced to the former, and that the oxidation–reduction of the 11-oxygen function occurring with the natural adrenal hormones of this type is not directly involved in their mode of action. Alternative theories are discussed.

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Studies on the Biosynthesis of Riboflavin

5. GENERAL FACTORS CONTROLLING FLAVINOGENESIS IN THE YEAST *CANDIDA FLARERI**

By T. W. GOODWIN and D. McEVOY
Department of Biochemistry, The University, Liverpool 3

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Among the flavinogenic fungi most studied from the point of view of the biosynthesis of riboflavin are the Ascomycetes *Eremothecium ashbyii* (Schopfer, 1944; MacLaren, 1952; Goodwin & Pendlington, 1954; McNutt, 1954, 1956; Brown, Goodwin & Pendlington, 1955; Goodwin & Jones, 1956; Brown, Goodwin & Jones, 1958) and *Ashbya gossypii* (Tanner, Vojnovich & van Lanen, 1949; Plaut, 1954*a*, *b*; Plaut & Broberg, 1956). Certain yeasts of the genus *Candida*, e.g. *C. guilliermondia* (Burkholder, 1943) and *C. flareri* (Tanner, Vojnovich & van Lanen, 1945), are also good riboflavin producers, but have not been examined in such detail. The present investigation was undertaken to define more precisely the general factors controlling flavinogenesis in *C. flareri* and to compare the results with those already obtained in this Laboratory with *E. ashbyii*. *C. flareri* was preferred to *C. guilliermondia* because it synthesizes considerably more riboflavin (Tanner *et al.* 1945).

The most critical constituent of the medium for flavinogenesis in *Candida* spp. is undoubtedly iron; the optimum concentration is 0·005–0·01 μg./ml. and higher concentrations are strongly inhibitory (Tanner *et al.* 1945; Levine, Oyaas, Wasserman, Hoogerheide & Stern, 1949). This permissible concentration of iron can be increased ten times by controlling the amount of cobalt in the medium (10 μg./ml.) (Enari, 1955). According to Levine *et al.* (1949), other trace elements (manganese, copper, zinc, tin, nickel and aluminium) up to 0·10 μg./ml. have no inhibitory effect on flavinogenesis, whereas zinc at slightly higher concentrations (0·2–0·8 μg./ml.) is stimulatory (Schopfer & Knüsel, 1956).

Because the significance of iron was not known when the original investigations on *Candida* spp. were carried out (Burkholder, 1943, 1944) the results obtained must be considered quantitatively suspect.

Both *C. guilliermondia* and *C. flareri* will produce riboflavin on a medium containing ammonium sulphate or urea as the sole nitrogen source, but urea is rather more effective in *C. flareri* (Levine *et al.* 1949). Supplementation of a medium containing ammonium sulphate as its sole nitrogen source with asparagine, glycine or urea stimulated further flavin production in *C. guilliermondia* and *C. flareri* (Burkholder, 1944; Levine *et al.* 1949).

Aeration or agitation or both is required for good yields of riboflavin, which reach its peak 3–5 days after inoculation (Levine *et al.* 1949).

Some of the present results have already been briefly reported (Goodwin & McEvoy, 1957).

* Part 4. Brown, Goodwin & Jones, 1958.