The Isolation of Urinary 17-Oxo Steroid and Corticosteroid Glucuronides

By FRANCES FOGGITT* AND A. E. KELLIE

Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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Metabolites of endogenous steroid hormones are excreted in the urine mainly in the conjugated form; the C_{21} compounds are conjugated almost exclusively as glucosiduronic acids (glucuronides), and the C_{19} compounds, which are gonadal and adrenal in origin, appear both as glucuronides and as sulphates. Owing to the difficulty of isolation and characterization of water-soluble conjugates, standard methods for the analysis of urinary steroids are usually indirect and involve preliminary hydrolysis. The milder methods of enzyme hydrolysis and solvolysis which have largely replaced hot-acid hydrolysis as a means of liberating the steroid moiety may still be unsatisfactory because they fail to achieve complete hydrolysis or introduce artifacts.

Procedures are now available for the quantitative extraction of steroid conjugates (Edwards, Kellie & Wade, 1953; Bush & Gale, 1957) and the possibility exists of separating and identifying the compounds actually present in urine. Because of the marked difference between glucuronides and sulphates the separation of these two groups presents no great difficulty and has been achieved by many workers (Crepay, Jayle & Meslin, 1957; Pelzer & Staib, 1957; Barlow & Kellie, 1959). The separation of individual glucuronides and sulphates is a greater problem. Individual steroid glucuronides have been isolated from the urine of normal subjects, notably oestriol glucuronide (Cohen & Marrian, 1936) and pregnanediol glucuronide (Venning, 1937) from late-pregnancy urine; from the urine of patients with adrenal hyperplasia Mason & Strickler (1945) obtained the glucuronide of 5β -pregnane- $3\alpha,17\alpha,20\alpha$ -triol. After the administration of large amounts of steroids, tetrahydrocortisone glucuronide (Schneider, Lewbart, Levitan & Lieberman, 1955) and androsterone glucuronide (Lewbart & Schneider, 1956) have been characterized as the glucuronide triacetate methyl esters. Few systematic attempts have been made to achieve the simultaneous separation of the major urinary compounds. Bush & Gale (1960) described a method for separating individual sulphates.

Lewbart & Schneider (1955) and Bush (1957) described chromatographic systems for the separa-

tion of steroid glucuronides; the present work deals with the application of such methods for the isolation and characterization of 17-oxo steroid glucuronides and of corticosteroid glucuronides from human urine. Access to these compounds, with precise knowledge of their variety and structure, is an essential prerequisite to the study of enzyme kinetics on which so many of the present methods of quantitative determination ultimately depend.

A preliminary account of this work has been published (Foggitt & Kellie, 1960).

MATERIALS AND METHODS

Melting points were determined on a Kofler hot-stage microscope (Reichert, Vienna) and are uncorrected. Optical rotations, unless otherwise specified, are in chloroform. Precise measurements of extinction were read with a Unicam SP. 600 by applying Allen's (1950) correction for background colour. Measurements made to follow the elution of steroid material from chromatography columns were less precise and were made against a solvent blank by using a Spekker absorptiometer (micro-cell; 0.4 ml.).

Solvents. All solvents, other than ethanol, were laboratory-grade and were distilled before use. Ethanol, R.R. Grade (James Burrough Ltd.), was used as supplied.

Other chemicals. *m*-Dinitrobenzene 'specially purified' (British Drug Houses Ltd.) was recrystallized three times from ethanol. Blue tetrazolium (BTZ)[3,3'-dianisole-bis-4,4'-(3,5-diphenyl)tetrazolium chloride], synthesized by the method of Bush & Gale (1958), was tested chromatographically for homogeneity, the corresponding formazan being developed with alkaline ascorbic acid solution. Diazald (toluene-*p*-sulphonylmethylnitrosamide), supplied by Aldrich Chemical Co. Inc., was used for the preparation of diazomethane.

The following trivial names have been used throughout the text and Tables; tetrahydro-11-deoxycortisol ($3\alpha,17\alpha,21$ -trihydroxy- 5β -pregnan-20-one); allotetrahydrocortisone ($3\alpha,11\beta,21$ -trihydroxy- 5α -pregnan-20-one); tetrahydrocortisone ($3\alpha,17\alpha,21$ -trihydroxy- 5β -pregnane-11,20-dione); allotetrahydrocortisol ($3\alpha,11\beta,17\alpha,21$ -tetrahydroxy- 5α -pregnan-20-one); tetrahydrocortisol ($3\alpha,11\beta,17\alpha,21$ -tetrahydroxy- 5β -pregnan-20-one); cortolone ($3\alpha,17\alpha,20\alpha,21$ -tetrahydroxy- 5β -pregnan-11-one).

Chromatography

Alumina adsorption columns. Alumina, almost neutral, (M. Woelm, Eschwege, Germany) was deactivated by adding 5% (v/w) of distilled water to the material distributed in shallow trays. After adequate mixing the alumina

* Present address: Frances Thomas (née Foggitt), Medical Professorial Unit, Brisbane General Hospital, Brisbane, Queensland, Australia.

(100 g.) was made into a slurry with ether-ethanol (3:1, v/v) and transferred to a chromatographic column (diam. 3 cm.) fitted with a sintered-glass base (porosity 2). After each addition the column was firmly pressed with a tight-fitting Martin packer. Steroid conjugates were transferred to the column in ether-ethanol solution.

Celite partition columns. Celite 535 (Johns-Manville Co. Ltd.) was purified by boiling for 2 hr. with conc. HCl, washing with distilled water until free of Cl^- ions and drying at 120° to constant weight. After equilibrating the chromatographic solvents overnight, stationary phase (0.6 ml./g.) was added dropwise with constant stirring to Celite 535. The Celite carrying stationary phase was covered with mobile phase and a firm partition column was built in the conventional manner. For crude urinary glucuronide mixtures 60 g. of Celite (height 36 cm.; diam. 2 cm.) was used; for glucuronide triacetate methyl esters and for free steroids 10 g. of Celite (height 25 cm.; diam. 1 cm.) was adequate. At least four times the retention volume was allowed to flow through a column before use.

Many samples chromatographed were not sufficiently soluble in the mobile or stationary phase to permit transfer in solution to the partition columns; for this reason, material to be chromatographed was dissolved in a small volume of volatile solvent and added dropwise with stirring to Celite (5 g. for a 60 g. column; 1 g. for a 10 g. column). Less Celite was used to receive material known to be clean and reasonably pure. The volatile solvent was removed in a vacuum desiccator and the process repeated until the sample was completely entrained. To the Celite, free of solvent, stationary phase (0.6 ml./g.) was added and the sample was transferred with the minimum volume of mobile phase to the top of the column, where it was firmly packed. The column was run until the mobile phase had reached the surface of the Celite, when a large reservoir of mobile phase was placed on the top of the column. Fractions were collected automatically, 10 ml. fractions from 60 g. columns and 2 ml. fractions from the smaller columns, at a rate that varied with the tightness of packing between 2 and 6 fractions/hr.

Paper chromatography. Solvent systems used were related to those applied to the corresponding Celite partition columns (Table 1). Both free and conjugated steroids were run on Whatman no. 2 paper; whereas with free steroids closely related compounds could usually be separated on the length of paper (40 cm.), resolution of glucuronides of closely related compounds frequently required substantial over-running of the paper. For such high resolution, solvent systems that normally gave R_F values of 0.15–0.25 were over-run for 15–36 hr.

In the text and Tables, partition solvent systems referred to by letter had the following composition (by vol.): (1) based on ethylene dichloride-*tert.*-butyl alcohol-acetic acid-water: E_1 , 8.5:1.5:3:7; E_2 , 8:2:3:7; E_3 , 7.5:2.5:3:7; E_4 , 7:3:3:7; (2) based on toluene-*tert.*-butyl alcohol-acetic acid-water: T_1 , 8:2:3:7; T_2 , 7.5:2.5:3:7; (3) based on light petroleum (b.p. 80–100°)-benzene-methanol-water: L_1 , 10:0:8:2; L_2 , 9:1:8:2; L_3 , 8:2:8:2; L_4 , 7.5:2.5:8:2; L_5 , 6.5:3.5:7:3; L_6 , 5.5:7:3; L_7 , 0:10:5:5.

Location of material in column fractions. 17-Oxo steroid glucuronides in column fractions were located by applying a micro-modification of the Zimmermann procedure. 17-Oxogenic steroid glucuronides were likewise determined after bismuthate oxidation by the method of Norymberski,

Stubbs & West (1953). Corticosteroid metabolites of the α -ketol type were followed by a modification of the method of Chen, Wheeler & Tewell (1957). The BTZ reagent was prepared immediately before use by mixing 0.5% (w/v) BTZ in 95% (v/v) aq. ethanol (0.2 ml.), 1% (w/v) ethanolic tetramethylammonium hydroxide (0.4 ml.) and ethanol (5 ml.). This reagent (0.5 ml.) was pipetted into vials containing small samples of the column fractions. After the vials had stood in the dark for 2 hr. the colours were read in a Spekker absorptiometer at 520 m μ (Spectrum Green) against a cortisol (2 μ g.) standard.

Glucosiduronic acid was determined by the method of Mead, Smith & Williams (1958), with D-glucuronolactone as standard.

On paper chromatograms 17-oxo steroids were located by treatment with Zimmermann reagents. α -Ketolic steroids, not conjugated at C-21, were located with BTZ reagent according to the method of Bush & Willoughby (1957). Papers were dipped in the reagent made by mixing 1% BTZ (w/v) in aq. 95% ethanol (10 ml.), water (25 ml.) and 4N-NaOH (15 ml.). After blotting to remove excess of reagent papers were suspended for 20 min. at room temperature to develop formazan spots and were subsequently suspended in SO_2 for a further 20 min. to preserve a permanent record by preventing the development of background colour.

Identification of steroid conjugates

Enzyme hydrolysis. Portions of the steroid glucuronide solutions containing approx. 10 μ g. of steroid were taken to dryness and incubated at 37° overnight with 1 ml. of a homogenate of limpet β -glucuronidase (2 mg./ml.; 520 000 *p*-chlorophenol units/g.) in 0.5M-acetate buffer (pH 4). The contents of the tubes after incubation were extracted twice with half-volumes of ethyl acetate (corticosteroids) or benzene (17-oxo steroids). The combined extracts were run on paper chromatograms and the free steroids identified. When larger amounts of free steroids were required for column chromatography or for infrared spectroscopy the scale of this procedure was increased accordingly.

Methylation of steroid glucuronides. Methylation was carried out with diazomethane prepared from Diazald as described by de Boer & Backer (1954). The steroid glucuronide (1–3 mg.) was dissolved in ethanol (10 ml.) in a round-bottomed flask and the ethereal solution of fresh diazomethane produced from 1 g. of Diazald was added. The mixture was kept in an ice bath for 1–2 hr. and was then taken to dryness on a rotary evaporator. The methyl ester was transferred in methanol to a small vial to be taken to dryness before acetylation. Acetylation of free glucuronides resulted in the formation of more than one product, possibly because of lactone-ring formation; methylation was always carried out before acetylation.

Acetylation of steroid glucuronide methyl esters. Steroids or steroid glucuronide methyl esters were kept overnight in the presence of pyridine (1 vol.) and acetic anhydride (2 vol.). The mixture was then diluted with methanol and taken to dryness with a jet of nitrogen on a water bath below 60° . The glucuronide triacetate methyl esters were purified by partition chromatography.

Oxidation of glucuronide triacetate methyl esters. Information about the structure of corticosteroid glucuronide

acetate methyl esters was obtained by oxidation to the corresponding 17-oxo compounds. Reference to the alternative structures for tetrahydro-11-deoxycortisol glucuronide (Fig. 1), according as conjugation takes place on the 3 α -hydroxyl or the 21-hydroxyl position, shows that after methylation and acetylation the 21-position is substituted. 17 α -Hydroxy corticosteroids with a 20,21- α -ketol structure are oxidized by sodium bismuthate only if the 21-position is free (Appleby, Gibson, Norymberski & Stubbs, 1955), and it was necessary first to reduce the 20-carbonyl group, thus forming a 17,20- α -glycol as a preliminary to bismuthate oxidation. When 17-oxogenic steroid glucuronide triacetate methyl esters were reduced overnight at room temperature with sodium borohydride in aqueous *tert*.-butyl alcohol according to the method of Appleby *et al.* (1955) they could then be oxidized with sodium bismuthate. The products of bismuthate oxidation under these conditions included free 17-oxo steroids and compounds that travelled on paper chromatograms as 17-oxo steroid formates, but did not include any glucuronide triacetate methyl esters. It was apparent that the alkaline conditions produced by the use of NaBH₄ had hydrolysed

the acetate groups, leaving the glucuronide residue exposed to oxidation by sodium bismuthate. Conditions under which BH₄⁻ ions reduced the 20-ketone without removing the acetate groups were achieved by shortening the reaction time, lowering the temperature and decreasing the concentration of BH₄⁻ ions (Dodson & Sollman, 1957). A solution of the steroid glucuronide triacetate methyl ester (50 μ g.) in methanol (2 ml.) was cooled in an ice bath and treated with NaBH₄ (1 mg.). After 10 min. acetic acid (8 drops) was added to remove excess of BH₄⁻ ions, the solution was taken to dryness and the residue dissolved in *tert*.-butyl alcohol (0.5 ml.) and aq. 50% acetic acid (15 ml.). Sodium bismuthate (2 g.) was added and the tube was shaken in the dark for $\frac{1}{2}$ hr. After centrifugation the supernatant fluid was removed to a tube containing 7.5% sodium metabisulphite (disulphite) solution (2 ml.). Distilled water (3 ml.) was added and the mixture was extracted twice with benzene (10 ml.). The combined extracts, after evaporation to dryness, were ready for chromatography.

Under these conditions the 20-carbonyl group of the α -ketol side chain was reduced, as shown by the suppression of the BTZ reaction, while the glucuronide acetate ester

Fig. 1. Location of glucuronide residue by reduction with borohydride and subsequent oxidation with bismuthate.

structure survived (infrared-absorption spectrum). Fig. 1 illustrates how subsequent oxidation by sodium bismuthate may be used to differentiate between conjugates formed at the 3-hydroxyl position or at the 21-hydroxyl position. The former give rise to 17-oxo steroid glucuronide triacetate methyl esters, and the latter to 17-oxo steroid acetates.

RESULTS

Preparation of the urinary glucuronide fraction

Urine (48 hr.) from a normal subject treated with corticotrophin (80 units daily by intramuscular injection) was brought to pH 2.0, partially saturated with ammonium sulphate (50 g./100 ml.) and extracted three times with half volumes of ether-ethanol mixture (3:1, v/v). The ether-ethanol extract was poured directly on an alumina adsorption column (100 g., 31 cm. $\times$ 3 cm. diam.; Barlow & Kellie, 1959), the sulphates were eluted with aq. 50% ethanol (500 ml.) and the glucuronides with 0.5M-acetate buffer, pH 5.0 (1 l.). After adjusting the buffer solution to pH 2.0 the glucuronides were re-extracted as before into ether-ethanol mixture and the extract was taken to dryness on a rotary evaporator. The residual dark-brown gum was still quite crude and contained non-steroidal as well as steroidal glucuronides.

Preliminary separation of the glucuronide fraction

The crude glucuronide fraction from the adsorption column was rechromatographed on a column of Celite (60 g.) with the solvent system E_3 , and elution from the column was followed by measuring the 'total 17-oxo steroid' content and the BTZ-reducing capacity of the serial fractions. The results, plotted graphically (Fig. 2), show a rough subdivision into four main groups of compounds (groups A, B, C and D). Selected fractions from these regions were hydrolysed by β -glucuronidase and the free steroids were examined by paper chromatography. Group A was complex and consisted largely of a mixture of 11-oxy- and 11-deoxy-17-oxo steroids. Fractions of group B were almost free of 17-oxo steroid and contained mainly tetrahydrocortisone. Fractions of group C yielded mainly 5 β -tetrahydrocortisol. By implication, the glucuronides of these steroids were present in the unhydrolysed fraction and these fractions were combined in groups for further chromatography and examination. The material in group D has been combined but not extensively investigated; as the 'total 17-oxo steroid' content of the pooled material was substantially greater than the BTZ-reducing capacity it seems probable that this material contains hexahydro corticosteroid metabolites with the glycerol type of side chain, e.g. cortolone.

Conjugates of group A were rechromatographed on the slower solvent system E_1 , and gave partial

resolution of the 17-oxo steroid glucuronides; the separation of androsterone glucuronide and aetiocholanolone (3 α -hydroxy-5 β -androstan-17-one) glucuronide from each other was just discernible. The resolution of the glucuronides of the 11-hydroxy-17-oxo steroids, 11-oxoaetiocholanolone, 11 β -hydroxyaetiocholanolone and 11 β -hydroxyandrosterone from each other was more complete, but each was still contaminated with BTZ-reducing material (Fig. 3). Subsequently androsterone and aetiocholanolone glucuronides were resolved on the solvent system T_1 . Later fractions from the group A column, containing a mixture of 11 β -hydroxyaetiocholanolone and 11 β -hydroxyandrosterone glucuronides and α -ketolic steroid glucuronides, were resolved in a similar way with the solvent system T_2 in a slow-running column which ran to 350 fractions. Separation of the 11 β -hydroxy-17-oxo steroids conjugates was good, and the peaks of BTZ-reducing material were shown by enzyme hydrolysis to contain the glucuronides of tetrahydro-11-deoxycortisol and of allotetrahydrocorticosterone (Fig. 4).

Combined fractions of group B from the preliminary separation were rechromatographed on the solvent system E_3 and yielded tetrahydrocortisone glucuronide as the main component. In an analogous manner combined fractions from group C were resolved in the solvent system E_4 into tetrahydrocortisol glucuronide and a small amount of material which behaved chromatographically as the glucuronide of the 5 α -epimer, allotetrahydrocortisol. The isolated steroid glucuronides were

Fig. 2. Preliminary chromatography (with solvent mixture E_3) of crude glucuronide fraction. A, Mainly 17-oxo steroid glucuronides; B, mainly tetrahydrocortisone glucuronide; C, mixture of 5 α - and 5 β -tetrahydrocortisol glucuronides; D, not investigated (see text). —, 'Total 17-oxo steroid'; - - -, BTZ-reducing material.

Fig. 3. Rechromatography (with solvent system E_1) of fraction *A* (see Fig. 2), showing separation of 11-hydroxy-17-oxo steroid glucuronides and the presence of corticosteroid glucuronides. —, 17-Oxo steroids; - - -, BTZ-reducing material. 1, Androsterone; 2, aetiocholanolone; 3, 11-oxo-aetiocholanolone; 4, 11 β -hydroxyaetiocholanolone; 5, 11 β -hydroxyandrosterone.

Fig. 4. Further rechromatography (with solvent system T_2) of fraction *A* (see Fig. 2), showing separation of 11 β -hydroxy-17-oxo steroid glucuronides and of the corticosteroid glucuronides of tetrahydro-11-deoxycortisol and allotetrahydrocorticosterone. —, 17-Oxo steroids; - - -, BTZ-reducing material. 1, 11 β -Hydroxyandrosterone; 2, 11 β -hydroxyaetiocholanolone; 3, tetrahydro-11-deoxycortisol; 4, allotetrahydrocorticosterone.

separately rechromatographed to exclude trace impurities; after hydrolysis with β -glucuronidase, the 17-oxo steroids and corticosteroids were rechromatographed on Celite partition columns and characterized by paper chromatography as free steroids and as acetates and also by recording the infrared-absorption spectra. The chromatographic systems used are shown in Table 1. Although the 'fingerprint' region of the infrared-absorption spectra (1200–800 cm^{-1}) of the free steroids is characteristic of each compound, the absorption spectra of the steroid glucuronides and their triacetate methyl esters in this spectral region, in solution and as KBr disks, were very similar to each other and quite unsatisfactory as a means of identification.

Preparation of steroid glucuronide triacetate methyl esters

Although the 17-oxo and 17-oxogenic steroid glucuronides isolated from urine were substantially pure, as indicated by their 17-oxo steroid and 17-oxogenic steroid contents respectively, only with androsterone and aetiocholanolone has it been possible to crystallize the urinary glucuronides. Subsequent experience with synthetic glucuronides suggests that these compounds recrystallize more readily as the sodium salts from water and aqueous ethanol; but here too, possibly owing to the retention of molecules of solvent of crystallization, analytically pure samples have been difficult to prepare.

For androsterone, aetiocholanolone, 11 β -hydroxyandrosterone, 11 β -hydroxyaetiocholanolone, 11-oxoaetiocholanolone, tetrahydro-11-deoxycortisol and tetrahydrocortisone glucuronides sufficient material was available from the urinary

Table 1. *Column-chromatography systems used for separating steroids, steroid glucuronides and steroid glucuronide triacetate methyl esters*

	Steroids (free)	Glucuronides	Glucuronide triacetate methyl esters
Androsterone	L_1	T_1	L_1
Aetiocholanolone	L_1	T_1	L_1
11 β -Hydroxyandrosterone	L_6	T_1	L_4
11-Oxo-aetiocholanolone	L_6	T_1	L_2
11 β -Hydroxyaetiocholanolone	L_6	T_1	L_2
5 α -Tetrahydrocorticosterone	L_6	T_2	L_3
5 β -Tetrahydro-11-deoxycortisol	L_6	T_2	L_3
5 β -Tetrahydrocortisone	L_7	E_3	L_6
5 β -Tetrahydrocortisol	L_7	E_4	L_6

source to prepare crystalline derivatives in the form of the triacetate methyl esters. (The tetrahydro-11-deoxycortisol glucuronide triacetate methyl ester isolated came not from the urine of normal subjects but from that of a patient with an adrenal tumour producing tetrahydro-11-deoxycortisol as the main corticosteroid excretion product.) After methylation and acetylation the derivatives were purified by partition chromatography on Celite 535 with systems containing benzene–light petroleum (b.p. 80–100°)–aqueous methanol with benzene concentrations ranging from 0 to 25% (v/v) (Bush, 1952) (Table 1). The steroid-conjugate derivatives were usually obtained in crystalline form directly from the column fractions and could be recrystallized from mixtures of light petroleum and benzene.

Androsterone, aetiocholanolone, 11 β -hydroxyandrosterone, 11 β -hydroxyaetiocholanolone and 11-oxoaetiocholanolone glucuronide triacetate methyl esters obtained in this way gave a normal reaction with Zimmermann reagent, showing that the 17-carbonyl group is free and unhindered. The corresponding derivatives of tetrahydro-11-deoxycortisol and tetrahydrocortisone readily reduced BTZ reagent and, indeed, this reagent was used to follow the elution of these compounds from the partition columns. Chromatography of the tetrahydrocortisol glucuronide fraction after methylation and acetylation produced more than one crystalline product. Early fractions from the column contained a white crystalline material which was very weakly BTZ-positive (F_1); fractions following this also contained a crystalline compound that strongly reduced BTZ and which was

subsequently identified as the triacetate methyl ester derivative of the 3 α -glucuronide of tetrahydrocortisol (F_2). A comparison of the properties of the isolated materials with some synthetic compounds is shown in Table 2.

Synthetic steroid glucuronide triacetate methyl esters

For comparison with derivatives prepared from urinary steroid glucuronides, where adequate amounts of the corresponding free steroid were available, steroid glucuronide triacetate methyl esters have been synthesized. The route to these derivatives has been made possible by the preparation of methyl 2,3,4-tri-*O*-acetyl-1-bromo-1-deoxy-D-glucuronate (bromo ester) (I) (Goebel & Babers, 1935); this compound has been used by Schapiro (1938, 1939) and others (Huebner, Overman & Link, 1944; Schneider & Lewbart, 1959; Pelzer, 1959; Wotiz, Smakula, Lichtin & Leftin, 1959; Brooksbank & Haslewood, 1961) for the synthesis of steroid glucuronide triacetate methyl esters. The method is based on a Koenigs–Knorr reaction coupling the steroid hydroxyl group with bromo ester (I) in an inert anhydrous solvent in the presence of silver carbonate or oxide as hydrogen bromide acceptor.

*Methyl (17-oxo-5 α -androstan-3 α -yl 2,3,4-tri-*O*-acetyl- β -D-glucosid)uronate*; androsterone glucuronide triacetate methyl ester (II). Androsterone (1 g.) and bromo ester (I) (3 g.) were dissolved in anhydrous ether (50 ml.), freshly prepared dry Ag_2CO_3 (3 g.) was added and the mixture shaken in the dark at room temperature for 5 hr. The silver salts were removed by centrifugation and the super-

Table 2. *Physical constants of urinary and synthetic steroid glucuronides and steroid glucuronide triacetate methyl esters*

For the composition of the systems, see text. —, Not determined.

	Glucuronide triacetate methyl ester						Glucuronide R_F		
	R_F			Melting point					
	System	Urinary	Synthetic (3 α)	Urinary	Synthetic (3 α)		System	Urinary	Synthetic (3 α)
Androsterone	L ₁	0.71	0.71	97–101°	97–103°		T ₁	0.16	0.16
Aetiocholanolone	L ₁	0.69	0.69	174.5–175.5	175.5–176		T ₁	0.11	0.11
11-Oxo-aetiocholanolone	L ₁	0.25	0.25	166–168	168.5–170		T ₁	0.21*	0.21*
	L ₄	0.75	0.76						
11 β -Hydroxy-aetiocholanolone	L ₁	0.25	0.24	172–176	177–178.5		T ₁	0.12*	0.12*
	L ₄	0.46	0.49						
11 β -Hydroxyandrosterone	L ₁	0.25	—	226.5–228.5	—		T ₁	0.15*	—
	L ₄	0.47	—						
21-Acetoxytetrahydro-11-deoxycortisol	L ₅	0.81	—	165–167	—		E ₂	0.53	—
21-Acetoxytetrahydrocortisone	L ₅	0.59	0.58	202–208	210–212		E ₂	0.27	—
	L ₆	0.77	0.77						
21-Acetoxytetrahydrocortisol	L ₅	0.55	—	—	—		E ₂	0.25	—
	L ₆	0.74	—						

* R_F relative to androsterone glucuronide on an over-run (36 hr.) paper.

natant fluid together with washings (chloroform, 2×10 ml.) filtered and evaporated to dryness under 40° . The residual pale-yellow gum was purified by partition chromatography on Celite 535 with light petroleum (b.p. 80 – 100°)–methanol–water (10:8:2, by vol.). Androsterone glucuronide triacetate methyl ester (II), located in the eluate by Zimmermann reaction and by infrared spectroscopy (androsterone $\nu_{\text{max}}^{\text{CS}}$ 1742 cm^{-1} ; II, $\nu_{\text{max}}^{\text{CS}}$ 1753 , 1742 , 1241 , 1217 cm^{-1}), weighed 625 mg. (30% yield). After recrystallization (twice) from acetone–light petroleum (b.p. 60 – 80°) the yield was 547 mg., m.p. 175.5 – 176° , $[\alpha]_{\text{D}}^{21.5} + 12 \pm 2^\circ$ (c 0.409) (Found: C, 63.0; H, 7.6. $\text{C}_{32}\text{H}_{44}\text{O}_{11}$ requires C, 63.4; H, 7.6%). Lewbart & Schneider (1955) reported the formation of this compound but quoted no data.

17-Oxo-5 α -androstan-3 α -yl β -D-glucopyranosiduronic acid; androsterone glucuronide (III). The triacetate ester (II) (480 mg.) dissolved in methanol (10 ml.) was treated under reflux with 3N -NaOH (0.53 ml.) for 15 min. The reaction mixture was cooled and acidified with HCl (pH 2) and evaporated to dryness under 40° . The residue was chromatographically purified on Celite in the solvent system T_1 ; column fractions containing androsterone glucuronide (III) were combined and recrystallized from aqueous methanol (10% methanol, v/v). The product had m.p. 148.5 – 149.5° , $[\alpha]_{\text{D}}^{21.5} + 20 \pm 4^\circ$ (c 2.0) (Found: C, 64.2; H, 8.2. $\text{C}_{25}\text{H}_{38}\text{O}_8$ requires C, 64.4; H, 8.2%). Yield 262 mg. (71% yield).

Methyl (17-oxo-5 β -androstan-3 α -yl 2,3,4-O-acetyl- β -D-glucosid)uronate; aetiocholanolone glucuronide triacetate methyl ester (IV). Aetiocholanolone (502 mg.) by a method analogous to the formation of (II) using Ag_2CO_3 (1500 mg.) and bromo ester (1500 mg.) yielded 420 mg. of aetiocholanolone glucuronide triacetate methyl ester (IV). Recrystallized from light petroleum (b.p. 60 – 80°), this new compound had m.p. 176.5 – 178° , $[\alpha]_{\text{D}}^{21.5} + 29 \pm 2^\circ$ (c 0.39) (Found: C, 63.8; H, 7.4. $\text{C}_{35}\text{H}_{46}\text{O}_{11}$ requires C, 63.4; H, 7.6%). Yield, 40%.

17-Oxo-5 β -androstan-3 α -yl β -D-glucopyranosiduronic acid; aetiocholanolone glucuronide (V). The triacetate ester (IV) (312 mg.) on hydrolysis by the method used to prepare (III) yielded aetiocholanolone glucuronide (154 mg.). Recrystallized slowly from acetone–light petroleum (b.p. 60 – 80°) this new compound had m.p. 175 – 177° , $[\alpha]_{\text{D}}^{21.5} + 33 \pm 4^\circ$ (c 1.6 in ethanol) (Found: C, 64.5; H, 7.9. $\text{C}_{25}\text{H}_{38}\text{O}_8$ requires C, 64.3; H, 8.2%). Yield, 64%.

3 α -Hydroxy-5 β -androstan-11,17-dione; 11-oxo-aetiocholanolone (VI). This 17-oxo steroid was prepared from 21-acetoxy-3 α ,17 α -dihydroxy-5 β -pregnane-11,20-dione (VII) (1 g.) by the method of Appleby *et al.* (1955) but by allowing the reduction with NaBH_4 to proceed for only 15 min. The crude product obtained was purified by gradient elution from alumina (Kellie & Wade, 1957) and by re-

crystallization from acetone–light petroleum (b.p. 60 – 80°) to yield 659 mg. of (VI) (yield 89%) with m.p. 187 – 189° . Literature m.p. 188 – 189° .

3 α ,11 β -Dihydroxy-5 β -androstan-17-one; 11 β -hydroxyaetiocholanolone (VIII). This compound was prepared in an analogous manner from (VII) (1 g.), with the exception that the reduction with NaBH_4 was allowed to proceed overnight. The residual material from the preparation of (VII) was added to the product before purification by gradient elution from alumina. Recrystallized from acetone the product (606 mg.) had m.p. 239 – 241° . Literature m.p. 236 – 237° . Yield 81%.

Methyl (11,17-dioxo-5 β -androstan-3 α -yl 2,3,4-triacetyl- β -D-glucosid)uronate; 11-oxoaetiocholanolone glucuronide triacetate methyl ester (IX). This compound was prepared by the method of Schapiro (1939) from (VI) (600 mg.) by using anhydrous benzene as solvent. The resulting yellow gum was purified by chromatography on Celite with benzene–light petroleum (b.p. 80 – 100°)–methanol–water (2:8:7.5:2.5, by vol.) and the product was located by infrared spectroscopy ($\nu_{\text{max}}^{\text{CS}}$ 1753 , 1742 , 1712 , 1241 , 1217 cm^{-1}) and recrystallized from acetone–light petroleum (b.p. 60 – 80°). This new compound had m.p. 168.5 – 170° , $[\alpha]_{\text{D}}^{20} + 58 \pm 4^\circ$ (c 1.86). (Found: C, 61.9; H, 7.2. $\text{C}_{32}\text{H}_{44}\text{O}_{16}$ requires C, 62.0; H, 7.1%).

Methyl (11 β -hydroxy-17-oxo-5 β -androstan-3 α -yl 2,3,4-triacetyl- β -D-glucosid)uronate; 11 β -hydroxy-aetiocholanolone glucuronide triacetate methyl ester (X). Because of the insolubility of (VIII) in cold ether and benzene, dioxan was used as solvent in a conventional synthesis by the Schapiro method. The product purified on Celite with benzene–light petroleum (b.p. 80 – 100°)–methanol–water (33:66:75:25, by vol.) yielded white crystalline rods which on recrystallization from a small volume of aq. methanol had $\nu_{\text{max}}^{\text{CS}}$ 1753 , 1742 , 1241 , 1217 cm^{-1} and m.p. 198 – 199° . The yield, however, was poor (15%) and the synthesis was repeated with the conditions of Meystre & Miescher (1944). In this synthesis, which is carried out in boiling benzene solution, the yield as measured by Zimmermann reaction was higher (57%), but even after purification by chromatography the material was difficult to crystallize, forming a water-clear gel from most solvents. From 600 mg. of (VIII) used in the hot method 183 mg. (29%) of crystalline material was obtained by repeated recrystallization from aqueous methanol. The new compound had m.p. 198 – 199° , $[\alpha]_{\text{D}}^{20} + 35 \pm 4^\circ$ (c 2.8) (Found: C, 62.0; H, 7.4. $\text{C}_{32}\text{H}_{46}\text{O}_{12}$ requires C, 61.7; H, 7.4%).

Methyl (21-acetoxy-17 α -hydroxy-11,20-dioxo-5 β -pregnan-3 α -yl 2,3,4-tri-O-acetyl- β -D-glucosid)uronate; 21-acetoxytetrahydrocortisone glucuronide triacetate methyl ester (XI). 21-Acetoxy-3 α ,17 α -dihydroxy-5 β -pregnane-11,20-dione (VII)

(250 mg.) was treated with bromo ester (750 mg.) by the method of Meystre & Miescher (1944). The yellow gum obtained by evaporating the final reaction mixture to dryness below 40° was chromatographically purified on the system benzene–light petroleum (b.p. 80–100°)–methanol–water (5:5:8:2, by vol.). The main product, located by reduction of BTZ, was recrystallized from chloroform–light petroleum (b.p. 80–100°) to give white needles, m.p. 210–212°, $[\alpha]_D^{20} + 32 \pm 4^\circ$ [Schneider *et al.* (1955) obtained m.p. 209–212°]. Yield 53 mg. (11 %).

Structure of the corticosteroid glucuronides

The corticosteroid glucuronide acetate methyl esters obtained from urine were submitted to oxidative degradation to determine the position of the glucuronoside linkage. After treatment with NaBH₄ (10 min. at 0°) and with sodium bismuthate, tetrahydro-11-deoxycortisol glucuronide acetate ester was converted into aetiocholanolone glucuronide acetate ester and tetrahydrocortisone glucuronide acetate ester into 11-oxoaetiocholanolone glucuronide acetate ester. In an analogous manner, the BTZ-positive material from the tetrahydrocortisol derivative (F₂) yielded 11 β -hydroxy-aetiocholanolone glucuronide acetate ester (with some of the corresponding 11-oxoaetiocholanolone ester, presumably by reverse oxidation by bismuthate). This evidence, together with the agreement in chromatographic running properties and melting points with the synthetic 3-conjugates available, suggested that the compounds isolated were, in fact, also 3 α -glucuronides.

In addition, from the tetrahydro-11-deoxycortisol glucuronide acetate methyl ester column, a small amount of material was found which on reduction with BH₄⁻ and oxidation with BiO₃⁻ gave aetiocholanolone acetate. The possibility that this was aetiocholanolone formate was excluded by running all three compounds on paper in the chromatographic system formic acid–light petroleum (b.p. 80–100°)–methanol (10:9:1, by vol.) (Edwards, 1957) in which the formate and acetate are just separated. The acetate could have been formed from the 21-glucuronide of tetrahydro-11-deoxycortisol. Similarly, reduction and oxidation of the weakly BTZ-positive compound (F₁) from the tetrahydrocortisol glucuronide triacetate ester column produced 11 β -hydroxyaetiocholanolone acetate (with some 11-oxoaetiocholanolone acetate) which could arise from the 21-glucuronide of tetrahydrocortisol. Moreover, substantial amounts of this compound were present, and when a portion of the original tetrahydrocortisol glucuronide (group C) was hydrolysed with β -glucuronidase there was a substantial increase in BTZ-reducing capacity and very much more tetrahydrocortisol was liberated than could be accounted for on the basis of the

BTZ reaction given by the unhydrolysed material. This indirect evidence implies that in tetrahydro-11-deoxycortisol and tetrahydrocortisol some conjugation also occurs at C-21. Conjugation in this position as the sulphate has been reported for cortisol by Pasqualini & Jayle (1961) and for 3 β ,21-dihydroxypregn-5-en-20-one as the 3 β ,21-disulphate by Pasqualini, Dutter & Jayle (1963).

SUMMARY

1. Urinary steroid conjugates extracted from the urine of normal subjects after intramuscular administration of corticotrophin have been separated into sulphate and glucuronide conjugates by adsorption on alumina.

2. The glucuronide fraction, further purified by repeated partition chromatography on Celite, has been separated into the glucuronides of the 17-oxo steroids, androsterone, aetiocholanolone (3 α -hydroxy-5 β -androstan-17-one), 11 β -hydroxyandrosterone (3 α ,11 β -dihydroxy-5 α -androstan-17-one), 11 β -hydroxyaetiocholanolone (3 α ,11 β -dihydroxy-5 β -androstan-17-one) and 11-oxoaetiocholanolone (3 α -hydroxy-5 β -androstan-11,17-dione), and of the corticosteroids, allotetrahydrocorticosterone (3 α ,11 β ,21-trihydroxy-5 α -pregnan-20-one), tetrahydro-11-deoxycortisol (3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one), tetrahydrocortisone (3 α ,17 α ,21-trihydroxy-5 β -pregnane-11,20-dione) and tetrahydrocortisol (3 α ,11 β ,17 α ,21-tetrahydroxy-5 β -pregnan-20-one).

3. Androsterone glucosiduronic acid and aetiocholanolone glucosiduronic acid have been isolated in crystalline form. With the exception of tetrahydrocortisol, sufficient material was available to convert all the urinary glucuronides into crystalline triacetate methyl esters.

4. The glucuronide triacetate methyl esters of androsterone, aetiocholanolone, 11-oxoaetiocholanolone, 11 β -hydroxyaetiocholanolone and tetrahydrocortisone have been synthesized and their properties compared with derivatives formed from the corresponding urinary glucuronides. This evidence suggests that all the steroids identified are conjugated as glucuronides at the 3 α -hydroxyl position.

5. Circumstantial evidence suggests that in tetrahydro-11-deoxycortisol and tetrahydrocortisol some conjugation also occurs at the 21-hydroxyl group of the corticosteroid.

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Coenzyme A in the Livers of Young Lambs, Adult Sheep and Rats

By I. G. JARRETT AND O. H. FILSELL

C.S.I.R.O. Division of Biochemistry and General Nutrition, University of Adelaide, South Australia

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Adult sheep derive the greater part of their energy requirements from the lower fatty acids which are absorbed from the rumen where they are produced by microbial degradation of cellulose and related carbohydrates. Young lambs, however, rely largely on constituents of milk as the ultimate source of energy, and during the immediate post-natal period many aspects of carbohydrate and fatty acid metabolism differ from those characteristic of adult sheep and more closely resemble those of monogastric animals.

The size of the glucose pool and the rate of utilization of glucose is much greater in the young lamb than in the adult (Jarrett, Jones & Potter, 1964), and when either glucose or acetate is injected into the circulating blood stream the rate of removal of these substances is faster in lambs than in adult sheep (McCandless & Dye, 1950; Jarrett & Potter, 1952; Jarrett & Filsell, 1960).

Jarrett & Filsell (1958), McLean (1958), Gallagher & Buttery (1959) and Filsell, Jarrett, Atkinson, Caiger & Morton (1963) have reported that quantitative differences occur in the concentrations of certain enzymes associated with carbohydrate metabolism between tissues of lambs, adult sheep and rats. Caiger, Morton, Jarrett & Filsell (1962) have shown that higher concentrations of NAD are present in the livers of adult sheep than in the livers of lambs and rats. They have suggested that this relatively higher activity may be related to the metabolism of the lower fatty acids, important in sheep nutrition, via the tri-carboxylic acid cycle which is largely NAD-dependent.

CoA also plays an important role in the metabolism of intermediaries through the tricarboxylic acid cycle and in the synthesis of higher fatty acids. In view of known differences in other enzymes and