Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 1267–1284. Pergamon Press. Printed in Great Britain

STUDIES ON STEROID CONJUGATES—VIII: ISOLATION AND CHARACTERIZATION OF GLUCURONIDE-CONJUGATED METABOLITES OF CORTISOL IN HUMAN URINE[1]

LUDWIG KORNEL and ZENZO SAITO[2]

Steroid Unit, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois 60612,, U.S.A.

(*Received* 25 *July* 1974)

SUMMARY

A total spectrum of glucuronide-conjugated metabolites of cortisol has been isolated from human urine and characterized: 22 steroid monoglucuronides and 7 steroid diglucuronides. Following i.v. administration of a tracer dose of [4-^{14}C]-cortisol to 10 normal subjects, urine was collected for 2 consecutive 24-h periods. Free steroids were removed with ethyl acetate. All conjugated metabolites were extracted by means of an Amberlite XAD-2 column, and were further purified by chromatography on a polyethyleneimine-impregnated cellulose column. *Groups* of mono- and di-glucuronide conjugated steroids were then separated from each other, and from other groups of steroid conjugates by means of high voltage paper electrophoresis. *Individual* monoglucuronide-conjugated metabolites were then separated from each other by means of 15 consecutive paper chromatographies, and their homogeneity was ascertained. Each steroid conjugate was then subjected to the following characterization: (1) identification by *RID* of the steroid moiety released by β-glucuronidase hydrolysis; (2) identification of the glucuronide moiety; (3) determination of the steroid/glucuronide molar ratio; (4) determination of the site of conjugation. The following monoglucuronide-conjugated (-G) metabolites of cortisol were isolated: cortisol-21-G, cortisone-21-G, 20β-dihydrocortisol-21-G, tetrahydrocortisol-3-G, tetrahydrocortisol-21-G,5α-tetrahydrocortisol-3-G,tetrahydrocortisone-3-G,5α-tetrahydrocortisone-3-G,cortol-20α-3-G, 5α-cortol-20α-3-G, cortol-20β-3-G, 5α-cortol-20β-3-G, cortolone-20α-3-G, 5α-cortolone-20α-3-G, cortolone-20β-3-G, 5α-cortolone-20β-3-G, 11-hydroxyaetiocholanolone-3-G, 11-hydroxyandrosterone-3-G, 11-oxoaetiocholanolone-3-G, 11-oxoandrosterone-3-G. In addition, 6β-hydroxy-20β-dihydrocortisol and 6β-hydroxy-20β-cortol were also isolated as monoglucuronides, but their site of conjugation was not determined. The steroids identified as diglucuronide conjugates were: cortol-20α and -20β, cortolone-20α and -20β, tetrahydrocortisol, 5α-tetrahydrocortisol and tetrahydrocortisone. Most of these steroid metabolites have not been heretofore isolated and characterized as conjugates. Three of the isolated metabolites were not known to exist as glucuronide conjugates. The quantitation of individual steroid conjugates revealed interesting relationships between various metabolites, which are discussed.

INTRODUCTION

It is widely accepted that the bulk of C-21 metabolites of adrenocortical steroids appear in urine conjugated with glucuronic acid [1–4]. However, evidence for the existence of the majority of these conjugates is indirect, based on the fact that they can be hydrolyzed with β-glucuronidase, releasing free steroid moieties which have been identified. *Group*-separation of glucuronide-conjugated metabolites of adrenocortical steroids can be readily achieved by means of column [5, 6] or paper chromatography [7], or paper electrophoresis [8]. Paper [7, 9] and column [10] chromatographic systems for the separation of various *individual* steroid conjugates have been also described. Nonetheless, only a few glucuronide-conjugated metabolites of adrenocortical steroids have been isolated *as conjugates* and identified as such [10, 11].

Our interest in conjugated steroids in connection with our work on steroid metabolism in hypertension [12], and also general recent interest in steroid conjugates, stemming from the changing concept of their role and function [13, 14], prompted us to undertake work on the isolation and identification of the total spectrum of plasma and urinary corticosteroid conjugates. We have reported previously the isolation and characterization of sulfate-conjugated metabolites of cortisol [15, 16].

This paper describes the isolation and characterization of glucuronide-conjugated metabolites of cortisol from human urine. The *individual* steroid glucuronides have been isolated *as conjugates* following i.v. administration of a tracer dose of [4-^{14}C]-cortisol. Preliminary results of this study were reported [17].

MATERIALS AND METHODS

Materials

All solvents were J. T. Baker, analytical reagent grade, and were distilled prior to use.

Amberlite XAD-2 was obtained from Rohm–Haas, Philadelphia, Pennsylvania. It was purified by the following procedure: 500g of the resin was suspended in 1·5 l of demineralized water, vigorously stirred with

a glass rod, or shaken, for a few minutes, then let settle down. The supernatant, with floating fine particles of the resin, was removed by decantation. This procedure was repeated five times. The resin was then washed with 500ml methanol three times, and kept in methanol at 4°C until used.

Polyethyleneimine-impregnated (PEI) cellulose was obtained from Brinkman Instruments (MN-Cellulose powder 2100 PEI for column chromatography, manufactured by Macherey, Nagel & Co., D-516 Duren, Germany). Prior to use, it was washed extensively with 1N solutions of sodium hydroxide and hydrochloric acid, and with water, and then reactivated by suspending for 5 min in 1N sodium hydroxide, and then rinsing with water until neutral.

[4-^{14}C]-cortisol (S.A. 51·8 mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass. The sterile tracer solution for i.v. injection was prepared as follows: Benzene–ethanolic solution in which the tracer was supplied, containing 100 μCi of the labeled steroid, was evaporated to dryness under N_2, and the steroid was purified by paper chromatography in B_5 system of Bush (see Table 1), or by thin-layer chromatography on silica-gel in system chloroform–methanol–water (188:12:1) [18], along with cold cortisol standard. Following the scanning of the chromatogram, the area corresponding to the main radioactive peak was eluted, and the eluate was evaporated to dryness *in vacuo* in a sterile tube. The dried tracer was then redissolved in 5 ml of absolute ethanol; 1ml aliquots of this solution were aspirated into a small sterile syringe, passed through a Milipore (Milipore Co., Bedford, Mass.) sterilizing filter, and injected through a sterile rubber cap into a vial containing 15 ml of a sterile solution of normal saline (N.S.). The syringe and the filter were then rinsed twice with 0·5 ml of ethanol; the rinses were also introduced into the vial. A small aliquot of this solution (10μl) was withdrawn for radioactivity counting, and the remainder was drawn quantitatively into a 20 ml syringe. The emptied vial was then rinsed twice, with 2ml and 1 ml, of N.S.; these rinses were aspirated (changing needles) into the syringe containing the tracer. Thus, the final dose of the tracer for i.v. injection was contained in 20 ml of 10% ethanol in N.S.

Free steroids used as reference standards were purchased either from Steraloids, Inc., Pawling, New York, from Sigma Chemical Co., Saint Louis, Missouri, or from Ikapharm, Ramat-Gan, Israel. Steroid glucuronide conjugates used as reference standards, tetrahydrocortisone-3-glucuronide[3], tetrahydrocortisol-3-glucuronide, β-cortol-3-glucuronide and cortisol-21-glucuronide were synthesized and generously donated by Dr. Vernon R. Mattox of the Mayo Clinic and Mayo Foundation, Rochester, Minnesota. Standards cortisone-21-glucuronide and tetrahydrocortisone-3,21-diglucuronide were synthesized by us by the method of Mattox *et al.*[19], and standards cortisol-21-sulfate and tetrahydrocortisol-3,21-disulfate, by the method previously described by us [20].

METHODS

Measurement of radioactivity

Radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3375. Efficiency for C-14 standard (PK 927) was 90% in the ^{14}C quick set counting channel. Two scintillator solutions were used: scintillator 1 (dimethyl-POPOP

Table 1. Paper chromatographic systems

System designation	Solvents (v/v)	Whatman paper No	Running time to front, h	Reference
K_2	petroleum ether–isopropyl alcohol–water (90:80·20 by vol.)	3 MM	3 5–4	24
boric K_2*	(same as K_2)	3 MM	3	
K_3	petroleum ether–benzene–isopropyl alcohol–water (50:50:80:20 by vol.)	3 MM	6	24
boric K_3*	(same as K_3)	3 MM	5	
K_4	benzene–isopropyl alcohol–water (100:70·30 by vol.)	3 MM	6	24
boric K_4*	(same as K_4)	3 MM	5	
K_5	petroleum ether–ethyl acetate–isopropyl alcohol–water (70:30:85:20 by vol.)	3 MM	8	24
CT	isoamyl alcohol–NH_4OH–H_2O (55:27.18 by vol.)	3 MM	18	9
SL_4	toluene–n-butanol–NH_4OH–H_2O (100:100:20:180 by vol.)	3 MM	3	7
SL_7	ethyl acetate–toluene–hexane–tert-butanol–acetic acid–water (60:75:40:25:60:140 by vol.)	3 MM	3	7
SL_8	n-butyl ether–n-butanol–acetic acid–water (130:70:60.140 by vol.)	3 MM	6	7
SL_{10}**	toluene–tert-butanol–methanol–0·02M boric buffer (pH 9·0) (170:40:30.100 by vol.)	2	3 5	25
Y	ethyl acetate–chloroform–methanol–water (25:75:50·50 by vol.)	2 3 MM	5 3·5	26
DP-IV #	benzene–ethyl acetate–methanol–water (70:30:100:100 by vol.)	3 MM	3	27
B_p	benzene–chloroform–methanol–water (50:50:50:50 by vol.)	2	4·5	28
B_1	petroleum ether–toluene–methanol–water (25:25·35 15 by vol.)	2	3·5	29
B_3	petroleum ether–benzene–methanol–water (33:17:40·10 by vol.)	2	3·5	29
B_5	benzene–methanol–water (1000:525:475 by vol.)	2	4	29
boric B_5*	(same as B_5)	2	3	
A	petroleum ether–methanol–water (100:80.20 by vol.)	2	3 5	29
F_3B	iso-octane–tert-butanol–water (100:50:90 by vol.)	2	7	30

* before application, paper was dipped in 5% boric acid and dried in hood.
** before application, paper was dipped in 0·02M boric buffer and dried in hood.
# before application, paper was washed with methanol and EDTA [27].

0·25g, PPO 5g, and toluene 1000ml), for samples soluble in alcohol; scintillator 2 (PPO 11g, POPOP 0·3g, naphtalene 450g, methanol 160ml, ethylene glycol 40ml, *p*-dioxane 2500ml), for samples of low solubility in toluene, such as urine, plasma, or extracts containing a large amount of water. The samples were counted in glass vials of low potassium content in 15ml of the scintillator solution. Sufficient counts were accumulated to afford a standard deviation of 2% or less. D.p.m. values were calculated from a specially constructed quenching curve.

Quantitation of steroids

Blue tetrazolium (BT) reaction was used for the estimation of C_{21} steroids with α-ketol side chain, and their acetates. Dry residues of paper eluates containing the steroids to be quantitated, the corresponding 'paper blanks' and the corresponding steroid standards were dissolved each in 0·25ml of 95% ethanol; 0·25ml of 1% aqueous solution of tetramethylammonium hydroxide in 95% ethanol was added, followed by 0·25ml of 0·5% BT in 95% aqueous ethanol. The tubes were swirled and placed for 25 min in the dark at room temperature. Following the development of color, 1·0ml of glacial acetic acid was added, with mixing, to stop the reaction, and the resulting color was scanned in a Beckman DK-2A spectrophotometer in the region between 400–600nm. The color of the unknown sample was scanned against the corresponding 'paper blank', and the color of the standard was scanned against 'reagent blank'. The Allen[21] correction was applied to the readings taken at 450, 520 and 590nm.

Zimmermann reaction was used for the estimation of C_{19} steroids, and C_{21} steroids with glycerol side chain (cortols, cortolones, 20β-dihydrocortisol) following their oxidation with sodium bismuthate or chromic acid to the corresponding 17-oxosteroids.

Each of the isolated steroids to be quantitated was dissolved in a small volume of ethanol; this was divided into two halves, placed in two conical test tubes, and evaporated to dryness. To one test tube, 0·2ml 1% *m*-dinitrobenzene (twice recrystallized from ethanol) in absolute ethanol was added, followed by 0·1 ml of 2N KOH in 95% ethanol (*m*-DNB-KOH reagent). To the other tube, 0·2 ml of absolute ethanol (*without m*-DNB) and 0·1ml of 2N KOH solution were added (EtOH-KOH 'blank' reagent). The tubes were swirled and their contents incubated at room temperature for 90min, 0·5ml of 85% aqueous ethanol was then added to each tube to stop the reaction. The 'paper blank' eluates and known amounts of 17-oxosteroid standards were treated in the same way. All determinations were run in duplicates. The developed colors were scanned in a Beckman DK-2A recording spectrophotometer in the region between 400–600 nm: the color produced with *m*-DNB-KOH reagent by the sample, or standard, was read against the color produced by the 'paper blank', or standard blank, respectively; the color produced with EtOH-KOH 'blank' reagent by sample, or standard, was read against that produced by 'paper blank', or standard blank, respectively. From the absorbancy curves, readings were taken at three wavelengths, 460, 520 and 580 nm. At each wavelength, the reading with EtOH-KOH reagent (usually negligible) was then subtracted from the reading with *m*-DNB-KOH reagent. Subsequently, Allen[21] correction was applied, and the concentration of the steroid present in the sample was calculated by relating the corrected optical density of the unknown to that of the standard.

Quantitation of glucuronic acid

This was performed by a modification of the microcarbazol reaction described by Cohn and Bondy[22]: The aqueous sample was divided into 2 halves, each of which was evaporated to dryness (with repeated additions of methanol) under N_2 at 45°C. The dry residue was redissolved, in 0·4 ml of 50% aqueous methanol and hydrolyzed with 1 ml of concentrated sulfuric acid for 20 min in a boiling water bath. The tubes were then cooled and 0·1 ml of 0·1% carbazol solution in methanol was added to one, and 0·1 ml methanol (without carbazol) was added to the second, to serve as a blank. The color was developed for 2h in the dark. Absorbance was recorded in a Beckman DK-2A spectrophotometer against the blank, and readings were taken at 490, 530 and 570nm. The Allen correction [21] was applied and the concentration of glucuronide in the sample was calculated by relating the corrected absorbance to that of standard glucuronic acid, identically processed.

Derivative formation

This was done by means of bismuthate oxidation or chromic acid oxidation, and/or acetylation. The details of the micromethods used were described by us previously [15]. Methylation of glucuronides was performed with diazomethane, as described by Foggitt and Kellie[10].

Column chromatography on Amberlite XAD-2

This was performed by a modification of the method of Bradlow[23]. Two sizes of columns were used: (a) the pooled urine specimen (see below) was chromatographed in a large column (7 × 55 cm.), containing 500g of the washed resin; this column could handle up to 3l of urine; (b) smaller volumes of urine (1/10 vol. of 24-h urinary collections, used for the quantitation of steroids, see below) were chromatographed in a smaller column (3·2 × 35 cm.), containing 80g of the resin. Both columns were equipped with a coarse fritted disc at the bottom. The small column had a 500 ml reservoir attachable at the top. Glasswool was packed on the top of the resin bed to prevent the resin from floating up during the addition of the eluting solvents. The large column was eluted with 3 l. of dist. water, and then with 2·5 l. of methanol; the small column, with 500 ml of water and 420 ml of methanol. The methanolic eluates, containing conjugated steroids, were concentrated *in vacuo*,

transferred to 40 ml conical tubes, and evaporated to dryness under nitrogen.

Column chromatography on PEI-cellulose

The washed PEI-cellulose slurry (containing approximately 300 g of PEI-cellulose) was poured onto a large chromatographic column (5·5 × 65 cm.) and allowed to settle overnight at 4°C. The separated aqueous supernatant was then passed through the column and discarded, and the extract containing conjugated steroids (the methanolic eluate from the Amberlite column, evaporated to almost dryness and redissolved in water) was applied to the PEI-cellulose column. The conjugates were eluted with 2 l. of water and 6 l. of 0·8 M sodium chloride, consecutively. The water eluate contained 60–70% of steroid glucuronide conjugates present in the urinary extract. The remaining 30–40% of the conjugates were eluted with NaCl. Both eluates contained at most 20% (by weight) of the total urinary "pigments" present in the crude extract prior to its application to the column. Approximately 4% of all steroid sulfates present in the extract were also eluted. (The bulk of steroid sulfates stayed on the column and could be eluted with higher concentrations of NaCl [to be published].)

Paper chromatography

The solvent systems used for the chromatographic separation of individual steroid monoglucuronides and of free steroids released from their conjugates by β-glucuronidase hydrolysis are listed in Table 1. In the following text, the length of a chromatographic run is indicated by a number in parentheses after a system designation. *E.g.*: (× 1) = single length run; (× 5) = 5 times single length run (paper overrun 4 times).

Detection of glucuronides on paper

1. PAN-cobalt nitrate method (a modification of the method of Crépy *et al.*[31]). A dry paper chromatogram was drawn quickly through a solution of 1-2-pyridylazo-2-naphthol (PAN) (400mg of PAN dissolved in 100 ml of 95% aqueous ethanol, diluted with 4 vol. of dichloromethane) and then completely dried in the hood; it was subsequently immersed for 2 min in a 1·5% aqueous solution of phenylhydrazine, rinsed briefly in distilled water, and then immersed in a solution of cobalt nitrate (C.N.) (8ml of 0·8% aqueous solution of C.N. mixed with 32 ml of 2M sodium-acetate buffer, pH 4·6, then the volume made-up to 100 ml with dist. water) until pink spots began to appear. It was held in the solution for another 30 s, and then immersed in distilled water until the color of the spots changed to green. The paper was removed, and dried in the hood.

2. Naphthoresorcinol method (a modification of the method of Baldwin *et al.*[32]). Paper chromatogram was drawn slowly through a solution of 0·3% naphthoresorcinol in 20% (w/v) trichloroacetic acid in n-butanol, blotted, dried partially in a hood (for 5–10 min.), then heated at 110°C. until blue spots appeared.

Outline of the procedure

The procedure used was in its general outline similar to that used by us for the isolation and characterization of sulfate conjugated metabolites of cortisol [15, 16].

Tracer [4-^{14}C]-cortisol (15–20 μCi) was administered in a rapid i.v. injection to 10 normal adult subjects, 7 men and 3 women, and urine was collected for the following 48 h. The volume and radioactivity of all urinary samples were measured. One-tenth vol. of each sample was frozen and stored; it was used later for the quantitation of individual steroid metabolites in individual specimens. The remaining 9/10 vol. were pooled and used for the isolation of individual steroid glucuronides *as conjugates*, and for their characterization. The bulk of this steroid glucuronide pool was used up during the development of the chromatographic procedure for the separation of individual steroid glucuronides (see below). However, once the method had been developed, it was established that concentrations of steroid glucuronides present in one 24-h urine collection were sufficient for the separation of all individual steroid metabolites.

A. Extraction and purification of conjugated steroids

Urinary free steroids were removed with ethyl acetate (2 × 3 vol.). The extracted urine, containing conjugated steroids, was chromatographed first on the Amberlite XAD-2 column, then on the PEI-cellulose column, to remove urinary solids and pigments. The recovery of radioactivity eluted from the Amberlite column was 95–99%, that from the PEI-cellulose column, 88–92%. All conjugates present in the final eluate from the PEI-cellulose column were quantitatively extracted with ethanol–ether mixture (method F), as previously described [33].

B. Separation of groups of steroid mono- and di-glucuronides

The glucuronide conjugates were separated from other conjugated metabolites of cortisol, eluted from the PEI-cellulose column, by means of high voltage paper electrophoresis (H.V.E.), as previously described [8, 15, 16]. Owing to the efficient removal of urinary pigments by the PEI-cellulose column, two consecutive H.V.E. runs at pH 2·2 and one at pH 6·4 were sufficient to achieve a complete separation of steroid monoglucuronides and diglucuronides. The latter moved 1·8 times faster than the former. The detection of radioactivity on paper electropherograms (and on paper chromatograms) was done by scanning in a radiochromatogram scanner (Actigraph III Model 4995, Nuclear Chicago Corp., or Model 1200A, Garth–Westenskow Co., Salt Lake City, Utah). Tetrahydrocortisone-3-glucuronide and tetrahydrocortisone-3,21-diglucuronide were used as reference standards. Each group of conjugates was then eluted from electropherograms and subjected to the separation of individual conjugated metabolites and their characterization. The method of the elution of conjugates from paper was previously described [34].

C. Identification of steroid moieties released by β-glucuronidase hydrolysis of total monoglucuronide conjugated steroid metabolite group

To obtain preliminary information about the number and identity of individual monoglucuronide conjugated steroid metabolites present in the total, electrophoretically separated group of steroid monoglucuronides, this group, eluted from paper (1/5 vol. of combined eluates from electropherograms of conjugates from all urine specimens), was subjected to hydrolysis with β-glucuronidase. This procedure was carried out as follows: The dried paper eluate containing monoglucuronides was dissolved in 7ml of water, buffered with 1ml of 2M acetic buffer, pH 4·5, and incubated with 2ml of Ketodase (Warner–Chilcott, 5000 U/ml) at 37·5°C for 48 h. The liberated steroid moieties were then extracted twice with 4 vol. of dichloromethane. Following centrifugation and the separation of the extracts, droplets of dichloromethane remaining in the water-phase were carefully removed by repeated, slow decantations of the aqueous phase, with simultaneous blowing of N_2, until no dichloromethane smell could be detected in the tube containing the extracted hydrolysate. (Traces of dichloromethane, as well as of other organic solvents, present in the hydrolysate, were shown to markedly inhibit the activity of Ketodase [35].) One ml of Ketodase was then added to the tube, and the incubation was continued for 24 h; following this, the cleaved steroid moieties (also those which were cleaved during the first hydrolysis, but were not completely removed with dichloromethane) were extracted twice with 4 vol. of ethyl acetate. Following centrifugation, the extracts were removed, combined with the dichloromethane extracts, and evaporated to dryness at 45°C under N_2. The combined extracts contained 96–99% of radioactivity present in the aqueous phase prior to hydrolysis.

The liberated steroids were then separated from each other by several consecutive paper chromatographies, until no further separation could be achieved (Fig. 1). Chromatographic mobilities of each of the separated radioactive steroids were compared with those of pure reference standard compounds. Following elution of the separated steroids and counting of radioactivity present in each eluate, final steroid identification was achieved by means of reverse radioisotope dilution technique (RID), by a modification of

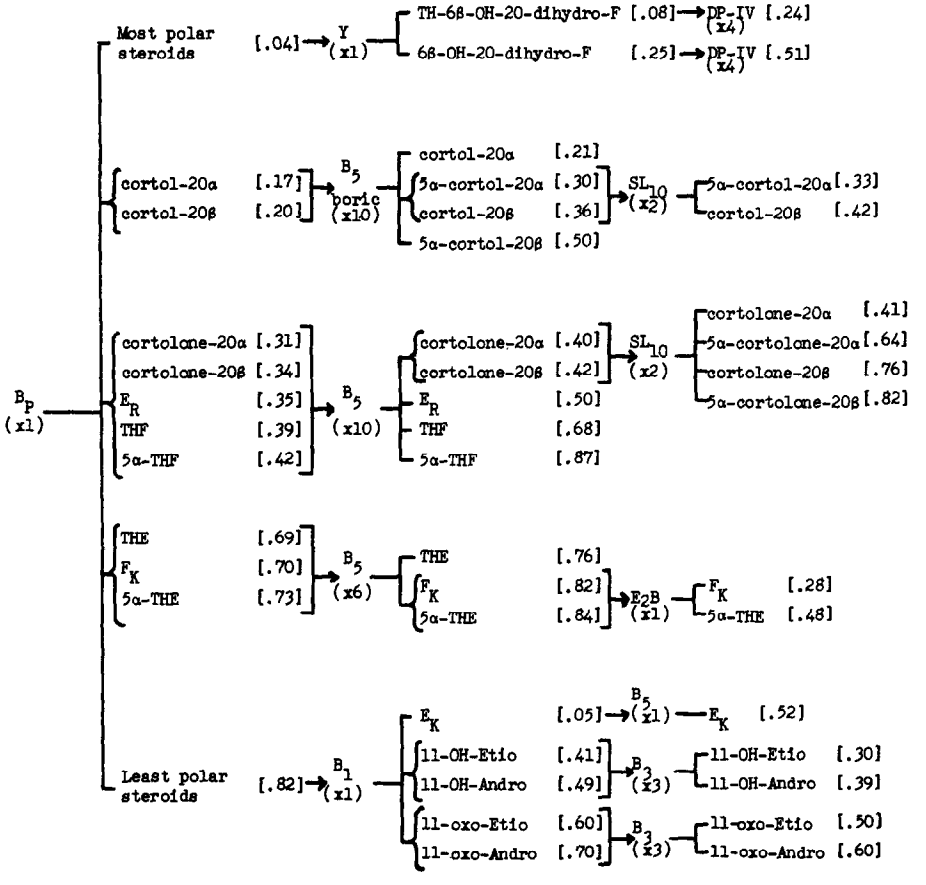

Fig. 1. Paper chromatographic separation of steroid moieties released from the electrophoretically separated *group* of monoglucuronide conjugates by hydrolysis with β-glucuronidase. Flow Sheet of the Method. For steroid abbreviations see footnote 3 in text. For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, e.g., (×1) = single length run; (×3) = 3 times single length run (overrun 2 times); numbers in brackets indicate R_F values or, when systems overrun, steroid mobilities relative to the length of paper chromatogram.

the procedure of Berliner and Salhanick[36]: a non-radioactive steroid carrier with a structure identical with that postulated for a given radioactive metabolite was mixed with the separated radioactive steroid; the mixture was rechromatographed on paper (along with a reference standard), the radioactive areas were eluted, and S.A. of the eluted steroid was determined in a portion of the eluate; the remainder of the eluate was then subjected to formation of derivatives, either by oxidation (with sodium bismuthate and/or chromic acid) or by acetylation, or both. The derivative was chromatographed in an appropriate solvent system, eluted, and its specific activity determined and compared with that of the parent compound (Table 2). Quantitation of steroids was performed by means of microcolor reactions: blue tetrazolium (BT) was used for the determination of C-21 α-ketolic steroids; the Zimmermann reaction for C-19 17-oxosteroids; C-21 steroids with glycerol side chain were first oxidized to the corresponding 17-oxosteroids with sodium bismuthate or chromic acid, then estimated by the Zimmermann reaction. Steroids with a Δ-en-3-oxo grouping were also estimated by U.V. absorbance. All determinations were done in duplicates. All absorbances were read at three equidistant wavelengths, against the reagent plus paper blank, and the Allen correction [21] was applied. The precision was 1·2% for the BT reaction (calculated from 24 duplicated estimations), and 1·7% for the Zimmermann reaction (calculated from 46 duplicates). The high purity of the samples used for the determination of specific acitivites, and the high precision of the

Table 2. Identification of steroid moieties of urinary monoglucuronide conjugated metabolites of cortisol (*RID*)

Isolated steroid†, #	Additional chromatography[θ]	Rechromatography with carrier§ 1st	2nd	Specific activity (d.p.m./μM)	Chemical reaction applied	Derivative formed†	Chromatography of derivative	Specific activity of derivative (d.p.m./μM)
TH-6β-OH-20-dihydro-F[c]	Y (×1) [0·08]	DP-IV (×4) [0·24]	Y (×1) [0·08]	-	acetyl	TH-6β-OH-20-dihydro-F-tetraAc.	A (×1) [0·32] B_3 (×1) [0·88]	
6β-OH-20-dihydro-F	Y (×1) [0·25]	DP-IV (×4) [0·51]	Y (×1) [0·25]	–	acetyl.	6β-OH-20-dihydro-F-triAc.	B_3 (×1) [0·76]	-
					$NaBiO_3$ oxid.	6-keto-11-OH-4-en-androstenedione	B_1 (×1) [0·33]	
cortol-20α	Boric B_5 (×10) [0·21]	SL_{10} (×2) [0·36]	SL_{10} (×2) [0·26]	3246	$NaBiO_3$ oxid.	11-OH-Etio	B_3 (×2) [0·19]	3299
5α-cortol-20α	Boric B_5 (×10) [0·26]	SL_{10} (×2) [0·33]		—*	$NaBiO_3$ oxid.	11-OH-Andro	B_3 (×2) [0·22]	–
cortol-20β	Boric B_5 (×10) [0·36]	SL_{10} (×2) [0·42]	SL_{10} (×2) [0·43]	1036	$NaBiO_3$ oxid.	11-OH-Etio	B_3 (×2) [0·19]	1129
5α-cortol-20β	Boric B_5 (×10) [0·61]	SL_{10} (×2) [0·75]		—*	$NaBiO_3$ oxid.	11-OH-Andro	B_3 (×2) [0·22]	
cortolone-20α	B_5 (×10) [0·40]	Boric B_5 (×6) [0·60]	SL_{10} (×2) [0·41]	6898	acetyl	cortolone-20α-triAc.	B_3 (×1) [0·82]	6902
5α-cortolone-20α	Boric B_5 (×6) [0·90]	SL_{10} (×2) [0·64]	—	—*	$NaBiO_3$ oxid.	11-oxo-Andro	B_3 (×2) [0·43]	–
cortolone-20β	Boric B_5 (×6) [0·84]	SL_{10} (×2) [0·76]	SL_{10} (×2) [0·76]	9190	$NaBiO_3$ oxid.	11-oxo-Etio	B_3 (×2) [0·35]	9187
					acetyl.	cortolone-20β-triAc.	B_3 (×1) [0·86]	
5α-cortolone-20β	Boric B_5 (×6) [0·89]	SL_{10} (×2) [0·80]	SL_{10} (×2) [0·78]	—*	$NaBiO_3$ oxid.	11-oxo-Andro	B_3 (×2) [0·43]	—
E_R	B_5 (×10) [0·62]	SL_{10} (×1) [0·50]	SL_{10} (×1) [0·50]	11053	$NaBiO_3$ oxid	11-OH-4-en-andro-stenedione	B_3 (×1) [0·20]	11220
THF	B_5 (×10) [0·68]	Boric B_5 (×4) [0·70]	B_5 (×6) [0·52]	19887	acetyl	THF-diAc.	B_3 (×1) [0·61]	20119
5α-THF	B_5 (×10) [0·87]	Boric B_5 (×4) [0·79]	B_5 (×6) [0·49]	9530	acetyl.	5α-THF-diAc.	B_3 (×1) [0·57]	9028
THE	B_5 (×6) [0·76]	E_2B (×1) [0·69]	B_5 (×4) [0·49]	5032	acetyl.	THE-diAc.	B_3 (×1) [0·65]	5069
5α-THE	B_5 (×6) [0·84]	E_2B (×1) [0·78]	B_5 (×4) [0·57]	2995	acetyl	5α-THE-diAc.	B_3 (×1) [0·68]	3139
F_R	B_5 (×6) [0·82]	E_2B (×1) [0·28]	B_5 (×4) [0·60]	2851	acetyl.	F-21-Ac	B_1 (×1) [0·51]	2862
E_R	B_5 (×1) [0·53]	E_2B (×1) [0·37]	B_5 (×1) [0·53]	1522	acetyl	E-21-Ac	B_1 (×1) [0·66]	1519
11-OH-Etio	B_1 (×1) [0·41]	B_3 (×3) [0·30]	B_1 (×1) [0·41]	6703	CrO_3 oxid.	actiocholanetrione	B_3 (×1) [0·68]	6718
11-OH-Andro	B_1 (×1) [0·49]	B_3 (×3) [0·32]	B_1 (×1) [0·49]	4404	CrO_3 oxid.	androstanetrione	B_3 (×1) [0·64]	4472
11-oxo-Etio	B_1 (×1) [0·60]	B_3 (×3) [0·50]	B_1 (×1) [0·60]	8173	CrO_3 oxid.	aetiocholanetrione	B_3 (×1) [0·68]	8238
11-oxo-Andro	B_1 (×1) [0·70]	B_3 (×3) [0·60]	B_1 (×1) [0·70]	1666	CrO_3	androstanetrione	B_3 (×1) [0·64]	1701

† For steroid abbreviations and nomenclature see footnote 3 in text.

Postulated configurations based on chromatographic mobilities identical with those of authentic steroids standards; the various metabolites separated by multiple successive paper chromatographies as depicted in Fig. 1.

θ For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, *e.g.*, (×1) = single length run; (×3) = 3 times single length run (overrun 2 times); numbers in brackets indicate R_F values or, when systems overrun, steroid mobilities relative to the length of paper chromatogram.

§ Non-radioactive carrier steroid of structure identical with that postulated for the unknown steroid metabolite.

[c] Tentatively identified, not enough carrier available for reliable determination of specific activity; 20α- and 20β-isomers not separated.

* Carrier not available for determination of S.A.

Table 3. Comparison of the results obtained by the RID method used in this study (method 1) with the classical method involving crystallization to constant specific activity (method 2)

	S.A., d.p.m./μM					
	Method 1		Method 2			
Isolated steroid*	Original Cmpd. #	Derivative§	1st cryst.†	2nd cryst.	3rd cryst.	m.l.†
cortol-20α	3280	3310	3510	3250	3180	3310
cortol-20β	2150	2210	2650	2270	2210	2310
THF	18630	19150	19210	18570	18320	19170
5α-THF	9530	9120	9650	9350	9210	9260
THE	4440	4380	4450	4240	4180	4150

* For steroid abbreviations and nomenclature see footnote 3 in text.

The isolated compound chromatographed twice with non-radioactive steroid carrier, then S.A. determined; *cf.* Table 2.

§ The derivative formed either by acetylation or oxidation, chromatographed, then S.A. determined; *cf.* Table 2.

† Compounds crystallized from methanol–benzene mixture; m.l.—mother liquor.

methods used for colorimetric estimations, assured the desired reliability of the determinations of specific activities, thus obviating the need for the recrystallization of the samples. This was confirmed by comparing results obtained by the method described above with the method utilizing crystallization to constant S.A. [36] applied to 5 of the isolated steroids: cortol-20α, cortol-20β, THF, 5α-THF, THE. The results obtained by the two methods are shown in Table 3.

D. Separation and characterization of individual *monoglucuronide conjugated steroid metabolites*

The group of monoglucuronide conjugated steroids, eluted from the last electropherogram (pH 6·4), was resolved into 22 individual conjugated steroid metabolites by means of 15 successive paper chromatographies. The solvent systems used and the flow-sheet of the procedure are shown in Table 1 and Fig. 2. The most polar steroid group (I) separated during the first chromatography in the SL_8 system (Fig. 2) contained, besides monoglucuronides, also small amounts of sulfate conjugates and glucurono-sulfate conjugates (less than 0·1% of total radioactivity of the glucuronide group). This "contamination" was due to the fact that the electropherograms were loaded to the fullest capacity, to save time, and only two separations at pH 2·2 were run (cf. procedure described for the separation by H.V.E. of sulfate-conjugated metabolites of cortisol in plasma [17]). These conjugates were completely separated from the monoglucuronides by re-run on H.V.E. at pH 2·2;[4] the monoglucuronides were then chromatographed on paper (Fig. 2). Each of the 22 separated individual steroid monoglucuronides was additionally chromatographed in one or two different solvent systems, to ensure that no further separation could be achieved. Each conjugate was then eluted from paper and subjected to the following characterization studies:

1. *Identification of steroid moieties*

For this purpose 1/3 vol. of the elutate of each separated monoglucuronide was used; the remaining 2/3 vol. of eluate of each conjugate was used for the determination of the steroid/glucuronide molar ratio and for the determination of the site of conjugation, as described below. The steroid moiety was released from each conjugate by hydrolysis with β-glucuronidase, by the procedure described above for hydrolysis of the total, electrophoretically separated, glucuronide conjugated steroid group. The liberated steroids were extracted with ethyl acetate (2 × 4 vol.) and were chromatographed on paper, each steroid in at least two solvent systems, with a corresponding steroid standard. Each steroid was then eluted and subjected to derivative formation by oxidation with sodium bismuthate or chromic acid, and/or acetylation [15]. The derivative obtained was chromatographed on paper and its mobility compared to that of the steroid identically treated and/or that of an authentic derivative standard (Table 4). Thus, the identity of the steroid moiety of each individually separated steroid glucuronide was tentatively established. The final identification was carried out by reverse isotope dilution (RID) on another set of steroid moieties obtained by an identical method from another aliquot of the same original steroid glucuronide pool. The RID method used was the same as that described above for the identification of steroid moieties released by β-glucuronidase hydrolysis of the total electrophoretically separated group of glucuronide conjugates.

2. *Demonstration and identification of glucuronide moiety*

The presence of glucuronic acid on paper chromatograms in locations corresponding exactly to peaks of radioactivity detected by radiochromatogram scanner was demonstrated by color spot tests with PAN–cobalt nitrate, and naphthoresorcinol, as described above.

Furthermore, the presence of glucuronic acid in the eluates of paper chromatograms, containing individually separated steroid glucuronides, was confirmed by a positive micro-carbazol color reaction, used for

Table 4. Characterization of steroid moieties of individually separated monoglucuronide conjugates

Separated steroid conjugate*	Steroid yielded by hydrolysis of the conjugate	Chromatographic systems used for comparing with authentic standard #	Chemical reaction applied to form derivative	Derivative formed	Chromatographic systems used for comparing with authentic standard derivative #
1	TH-6β-OH-20-dihydro-F§	B_P (×10) [0·01] Y (×1) [0·08] DP-IV (×4) [0·24]	Acetylation	TH-6β-OH-20-DHF-tetraAc.	B_3 (×1) [0·88] A (×1) [0·60]
2	6β-OH-20-dihydro-F§	B_P (×10) [0·04] Y (×1) [0·25] DP-IV (×4) [0·51]	Acetylation $NaBiO_3$ oxid.	6β-OH-20-DHF-triAc. 6-oxo-11-OH-4-en-Androstenedione	B_3 (×1) [0·76] B_1 (×1) [0·32]
3	cortol-20α	B_P (×8) [0·67] Boric B_S (×10) [0·30] SL_{10} (×2) [0·26]	$NaBiO_3$ oxid.	11-OH-Etio	B_3 (×2) [0·29] B_1 (×1) [0·41]
4	5α-cortol-20α	B_P (×1) [0·18] Boric B_S (×10) [0·28] SL_{10} (×2) [0·33]	$NaBiO_3$ oxid.	11-OH-Andro	B_3 (×2) [0·32] B_1 (×1) [0·49]
5	cortolone-20α	B_P (×1) [0·31] B_S (×10) [0·40] SL_{10} (×2) [0·41]	$NaBiO_3$ oxid.	11-oxo-Etio	B_3 (×3) [0·67] B_1 (×1) [0·60]
6	cortol-20β	B_P (×1) [0·20] Boric B_S (×10) [0·36] SL_{10} (×2) [0·42]	$NaBiO_3$ oxid.	11-OH-Etio	B_1 (×1) [0·41] B_3 (×2) [0·19]
7	5α-cortol-20β	B_P (×1) [0·21] Boric B_S (×10) [0·50] SL_{10} (×2) [0·75]	$NaBiO_3$ oxid.	11-OH-Andro	B_1 (×1) [0·49] B_3 (×2) [0·22]
8	5α-cortolone-20α	B_P (×1) [0·34] Boric B_S (×6) [0·90] SL_{10} (×2) [0·64]	$NaBiO_3$ oxid.	11-oxo-Andro	B_1 (×1) [0·70] B_3 (×3) [0·60]
9	cortolone-20β	B_P (×1) [0·34] Boric B_S (×6) [0·84] SL_{10} (×2) [0·76]	$NaBiO_3$ oxid.	11-oxo-Etio	B_1 (×1) [0·60] B_3 (×3) [0·50]
10	5α-cortolone-20β	B_P (×1) [0·34] Boric B_S (×6) [0·90]	$NaBiO_3$ oxid.	11-oxo-Andro	B_1 (×1) [0·70] B_3 (×3) [0·60]
11	E_R	B_P (×1) [0·40] B_S (×10) [0·70] SL_{10} (×1) [0·50]	$NaBiO_3$ oxid.	11-OH-4-en Androstenedione	B_3 (×1) [0·20]
12 14	THF	B_P (×1) [0·39] B_S (×10) [0·68] Boric B_S (×4) [0·70]	Acetylation $NaBiO_3$ oxid.	THF-diAc. 11-OH-Etio	B_3 (×1) [0·61] B_1 (×1) [0·40] B_1 (×1) [0·41]
13	5α-THF	B_P (×1) [0·42] B_S (×6) [0·76] E_2B (×1) [0·69]	Acetylation $NaBiO_3$ oxid.	5α-THF-diAc 11-OH-Andro	B_3 (×1) [0·57] B_1 (×1) [0·50] B_1 (×1) [0·49]
15	THE	B_P (×1) [0·69] B_S (×6) [0·76] E_2B (×1) [0·69]	Acetylation $NaBiO_3$ oxid.	THE-diAc. 11-oxo-Etio	B_3 (×1) [0·65] B_1 (×1) [0·60]
16	F_K	B_P (×1) [0·70] B_S (×4) [0·56] E_2B (×1) [0·40]	Acetylation $NaBiO_3$ oxid.	F_K-21-Ac. 11-OH-4-en-Androstenedione	B_1 (×1) [0·51] B_1 (×1) [0·58]
17	5α-THE	B_P (×1) [0·73] B_S (×6) [0·84] E_2B (×1) [0·78]	Acetylation $NaBiO_3$ oxid.	5α-THE-diAc 11-oxo-Andro	B_3 (×1) [0·68] B_1 (×1) [0·70]
18	E_K	B_P (×1) [0·82] B_S (×1) [0·52] E_2B (×1) [0·49]	Acetylation $NaBiO_3$ oxid.	E-21-Ac. Adrenosterone	B_1 (×1) [0·66] B_1 (×1) [0·84]
19	11-OH-Etio	B_1 (×1) [0·41] B_3 (×3) [0·30]	CrO_3 oxid.	Aetiocholane-trione	B_3 (×1) [0·68]
20	11-OH-Andro	B_1 (×1) [0·49] B_3 (×3) [0·32]	CrO_3 oxid.	Androstane-trione	B_3 (×1) [0·64]
21	11-oxo-Etio	B_1 (×1) [0·60] B_3 (×3) [0·50]	CrO_3 oxid.	Aetiocholane-trione	B_3 (×1) [0·68]
22	11-oxo-Andro	B_1 (×1) [0·70] B_3 (×3) [0·60]	CrO_3 oxid.	Androstane-trione	B_3 (×1) [0·64]

* For compound designation see Fig. 2, and compare Tables 6 and 7.

For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, *e.g.*, (×1) = single length run; (×3) = 3 times single length run (overrun 2 times); numbers in brackets indicate R_f values, or, when systems overrun, steroid mobilities relative to the length of paper chromatogram.

§ These compounds isolated in very minute quantities, this permitting only a tentative identification; 20α- and 20β-isomers not separated. For steroid abbreviations and nomenclature, see footnote 3 in text.

the determination of steroid/glucuronide molar ratios, as described below.

3. *Determination of steroid/glucuronide molar ratios*

Individual steroid glucuronide conjugates, separated as described above were hydrolyzed with β-glucuronidase and the liberated steroids were extracted with ethyl acetate. The concentration of a steroid in each of these extracts was measured by a micro BT color reaction (for metabolites with α-ketol group) or by a micro Zimmermann reaction (for metabolites with glycerol side chain, oxidized with sodium bismuthate, and for C_{19} 17-oxo metabolites). The concentration of glucuronic acid of the sample was determined on the aqueous hydrolyzate residue by the micro-carbazol reaction, as described above. The precision of the methods, expressed as standard error, was 2·2% for the micro-carbazol reaction and 1·9% for the BT reaction; it was 2·1% for the Zimmermann reaction without preceding oxidation and 3·2% when a prior oxidation with sodium bismuthate was applied.

The steroid/glucuronide molar ratios were also determined on individual glucuronide conjugates directly, without prior hydrolysis with β-glucuronidase: 1/2 of the dry conjugate sample (eluted from the last paper chromatogram) was reacted, as conjugate, with BT or the Zimmermann reagent (or oxidized with sodium bismuthate and then reacted with Zimmermann reagent); the other half was dissolved in 0·4 ml of 50% methanol and subjected to acid hydrolysis and micro-carbazol reaction, as described above for samples hydrolyzed with β-glucuronidase. The agreement between the two variants of the method was very good, but the procedure which included β-glucuronidase hydrolysis was finally adopted, since steroids conjugated at C-21 did not react with BT unless hydrolyzed.

4. *Determination of the site of conjugation*

This was done by the method of Foggit and Kellie[10] for steroids with dihydroxyacetone side-chain (17,21-diol-20-one). Briefly, the steroid glucuronide under investigation was first converted to the corresponding glucuronoside triacetate methyl ester by consecutive methylation (using diazomethane) and acetylation (using pyridine/acetic anhydride mixture 1:2). The product was then purified by chromatography in K_5 system (×9) and was subjected to consecutive reduction with sodium borohydrate (to reduce the 20-oxo group) and oxidation with sodium bismuthate. The borohydrate reduction was essential, since bismuthate will oxidize the 20,21-diol-20-one side-chain only when 21-hydroxyl group is free. The resulting final product of a steroid-3-glucuronide was 17-oxosteroid-3-glucuronoside triacetate methyl ester, that of steroid-21-glucuronide was either a 17-oxosteroid-3-acetate (for 3-hydroxysteroids) or a free 17-oxosteroid (for 3-oxosteroids). They were then separated from each other by chromatography in B_3 system and quantitated, following elution, by the Zimmermann reaction. The same procedure could not be applied to steroids with a glycerol side-chain, since the 20-hydroxyl group would be acetylated, and consequently the side chain would not be oxidized with bismuthate. Therefore, steroid glucuronides with a glycerol side-chain (cortols, cortolones) were only methylated and then chromatographed in K_5 system (×9). Their R_F values were then compared with the corresponding 20-oxosteroid glucuronides (THE, THF), the site of conjugation of which was already known, which were methylated and reduced with borohydrate, and then chromatographed in K_5 system, along with the methylated glucuronide-conjugates of steroids with glycerol side-chain. This procedure permitted valid information about the site of conjugation of glucuronide conjugated steroid metabolites with glycerol side-chain.[5] Glucuronide conjugated C_{19} metabolites of cortisol were rechromatographed in K_5 (×1) and SL_7 (×6) systems, and their mobility was compared to that of authentic standards 11-hydroxy- and 11-oxo-aetiocholanolone-3-glucuronoside and 11-hydroxy- and 11-oxoandrosterone-3-glucuronoside. The unknowns and the standards were then methylated and acetylated and chromatographic mobilities of the derivatives (in B_3 system) were again compared.

E. Quantitation of individual steroid monoglucuronides

This was performed on the non-pooled 1/10 vol. of each urine specimen. The separation procedure for individual glucuronide-conjugated steroid metabolites, *as conjugates*, was the same as that used for the characterization of steroid glucuronides in the pooled specimens, with the exception that the PEI-cellulose column was omitted, since the amount of the urinary pigment contained in a 1/10 vol. of a 24-h urine collection was small enough to permit a direct application to H.V.E. of the conjugated steroids eluted from the Amberlite column. Each finally separated glucuronide conjugate was eluted from paper and the eluate was divided into 2 equal parts. One was used for counting radioactivity present in this compound, the other was hydrolyzed with β-glucuronidase, as described above. The liberated steroid was extracted with ethyl acetate and identified by chromatography and derivative formation, according to the methods described for the identification of steroid moieties of individual steroid monoglucuronides.

F. Characterization of steroid-diglucuronides

As already described, these conjugates were separated from steroid monoglucuronides by H.V. paper electrophoresis at pH 6·4. Their electrophoretic mobility was identical with that of standard tetrahydrocortisone-3,21-diglucuronide. The glucuronide moiety was detected on paper by the PAN color spot test (as described for monoglucuronide conjugates): its location corresponded exactly to the radioactive peak detected by radioactivity scanning of the electropherogram.

The separated diglucuronides were eluted from paper with n-butanol–70% aqueous methanol mixture [34]. Two 1ml portions of each eluate were taken for radioactivity counting and the remaining eluates were pooled to obtain steroid concentrations sufficient for the following characterization studies:

1. *Identification of steroid moieties*

Steroid diglucuronides contained in the 1/3 vol. of the pooled eluate were subjected to 2 consecutive β-glucuronidase hydrolyses, according to the procedure described above for hydrolysis of monoglucuronide conjugates. The liberated steroids were extracted with ethyl acetate and identified by the methods described for the identification of the steroid moieties of monoglucuronide conjugates.

2. *Determination of steroid/glucuronide molar ratio*

For this purpose, 1/3 vol. of the pooled diglucuronide conjugates was used. The methods used for the estimation of the steroid and the glucuronide moieties were the same as those used for the determination of steroid/glucuronide ratios of individual monoglucuronide conjugated metabolites.

3. Quantitation of individual steroid diglucuronides

The remaining 1/3 vol. of the pooled diglucuronide conjugates was subjected to 2 consecutive β-glucuronidase hydrolyses. The liberated steroids were extracted with ethyl acetate and were separated by multiple successive paper chromatographies according to the scheme shown in Fig. 1. The technique used was the same as that described for the separation of steroid moieties yielded by β-glucuronidase hydrolysis of the *total* monoglucuronide conjugated steroid metabolite group (separated electrophoretically). The separated steroids were eluted from paper chromatograms, and the eluates were evaporated to dryness directly in counting vials under nitrogen, at 40°C. The dry residues were redissolved in 1ml of methanol to which 15ml of toluene scintillator solution was added, and the samples were counted. The values obtained were corrected for losses incurred during paper chromatography, the amount of radioactivity contained in the total diglucuronide conjugates group eluted from H.V. electropherogram being taken as 100%.

RESULTS

A. High voltage electrophoretic separation and quantitation of various groups of conjugated metabolites of cortisol

Glucuronide conjugated metabolites of cortisol were separated from other conjugates by means of high voltage paper electrophoresis [8, 15, 16]. For the separation of *all* groups of various conjugates, several consecutive runs at different pH values were necessary to obtain a complete separation of each group, since during initial separations each group of conjugates carried a small amount of contaminating conjugates of another group. The method of the separation was presented in detail elsewhere [16]. In the present study two successive runs at pH 2·2 and one at pH 6·4 were sufficient for a complete separation of glucuronide conjugates, owing to the efficient purification of the extract by chromatography on the PEI-cellulose column prior to electrophoresis. The separation of monoglucuronides from diglucuronides was accomplished during the run at pH 6·4. The recovery of radioactivity following the three runs was 78–88% of the original amount present in the sample prior to its application to the paper. The recovery of standard THE-3-glucuronide was 93%. These figures also include losses incurred during the elution of the separated compounds from paper. The reproducibility of the method was within 7% of standard error of means. This value was calculated from a series of 15 quadruplicate runs at pH 2·2 and 10 at pH 6·4.

The relative urinary concentrations of the various groups of free and conjugated metabolites of cortisol excreted over a 24-h period, following tracer administration, are shown in Table 5. Monoglucuronides constituted 81–96% of all conjugated metabolites of cortisol, but diglucuronides, only 1–3%. We have previously demonstrated [15] that glucuronosulfate conjugates constitute 0·3–0·6% of all conjugated metabolites of cortisol (or 8–13% of all sulfate conjugated metabolites). The isolation and characterization of these conjugates has been described [16, 17].

B. Isolation and characterization of individual monoglucuronide conjugated metabolites of cortisol

The electrophoretically separated group of steroid monoglucuronides was resolved into 22 *individual* steroid conjugates by means of 15 consecutive paper

Table 5. Radioactivity and relative concentrations of various groups of urinary free and conjugated metabolites of cortisol, in 10 normal subjects, excreted over a 24-h period

Steroid Group	Radioactivity, d.p.m., 10^3	Relative Concentration
		% of Total Metabolites
Total Free Metabolites (F)	2577 ±978*	8·3 ±4·6
Total Conjugated Metabolites (C)	27,679 ±3244	91·7 ±2·4
F + C	30,256 ±3979	
		% of Total Conjugates
Monoglucuronide Conjugates (MG)	25,181 ±3675	89·7 ±6·7
Diglucuronide Conjugates (DG)	436 ±252	1·6 ±1·0
Sulfate Conjugates (S)#	1544 ±959	5·5 ±3·2
Other Conjugates ("N")§	911 ±758	3·2 ±3·2

* Mean ± S. D.

Include monosulfates, disulfates and glucuronosulfate conjugates (15).

§ This group consists mainly of 20α- and 20β-reduced 6α- and 6β-hydroxycortisol, "complexed" to various urinary nucleosides; the nature of the bond has not been elucidated. These conjugates move on H.V.E., unlike other conjugates, towards the cathode (at pH 2·2 and 6·4).

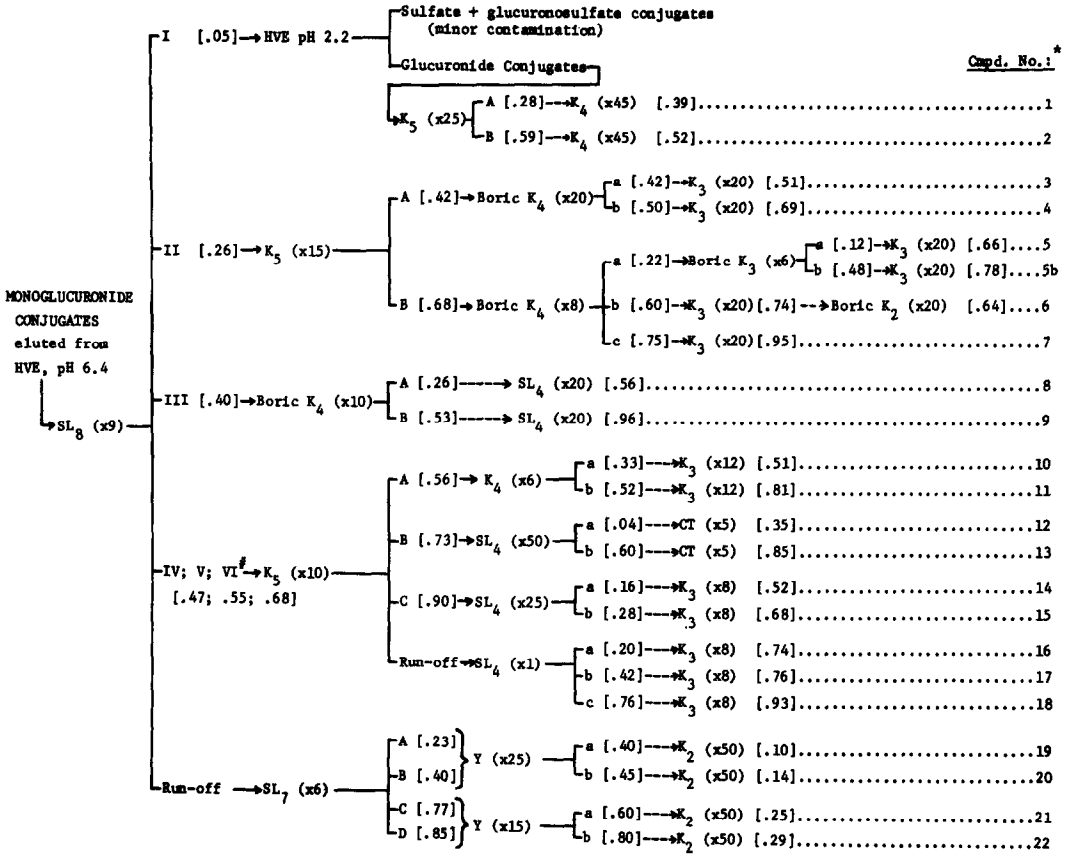

Fig. 2. Paper chromatographic separation of individual monoglucuronide-conjugated metabolites of cortisol. Flow Sheet of the Method. For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, *e.g.*, (× 1) = single length run; (× 10) = overrun 9 times. Numbers in brackets indicate R_F values, or, when systems overrun, steroid mobilities relative to the length of the chromatogram. A dotted line indicates an additional chromatography of the separated conjugate, in a different system, carried out to ascertain that it is a single compound. # These groups eluted together because of incomplete separation. * For identification of the separated steroid monoglucuronides, see Tables 4, 6, and 7. Cmpd. 5b is identical with cmpd. 6 (cortol-20β-3-glucuronide).

chromatographies, as schematically depicted in Fig. 2. This pattern of separation was very reproducible from run to run, and also from specimen to specimen. Most of the solvent systems used for the separation of individual conjugates were developed by us especially for this purpose [24]. Each of the separated steroid monoglucuronides was then additionally chromatographed in one or two solvent systems to confirm its chromatographic homogeneity. This was further confirmed by the demonstration of a single steroid moiety released by β-glucuronidase hydrolysis (see below), from each conjugate, following its elution from the last paper chromatogram, and by a constant glucuronide/steroid ratio which did not change following acetylation of the separated conjugate and rechromatography in a less polar system (see below).

Preliminary information about the number and identity of individual steroid metabolites present in the electrophoretically separated group of steroid monoglucuronides was obtained by hydrolyzing this group *in toto* with β-glucuronidase, and resolving the mixture of steroids present in the hydrolysate extract by a number of consecutive paper chromatographies. Table 2 lists the steroids identified.

Following the isolation of *individual* steroid monoglucuronides, each conjugate was also hydrolyzed with β-glucuronidase, and the steroid moiety released from each conjugate identified as shown in Table 4. These steroids were identical with those listed in Table 2. It will be seen that tetrahydrocortisol was identified in two chromatographically distinctly different monoglucuronide conjugates. These conjugates were shown to have different sites of conjugation (see below). Both 5α- and 5β-isomers of THF and THE monoglucuronides were isolated as conjugates. Furthermore, monoglucuronides of all 4 isomers of cortols and cortolones (5α 20α 20β, 5β 20α, and 5β 20β) were also isolated as conjugates. The isolation of monoglucuronides of 4-ene-3-oxosteroids, i.e. cortisol, cortisone and 20-dihydrocortisol (compound E_R), as conjugates, also deserves special mention.

The presence of glucuronic acid in each of the isolated conjugates was demonstrated by means of two different color spot tests on paper chromatograms (PAN-cobalt nitrate and naphthoresorcinol). The location of the glucuronide moiety on chromatograms corresponded exactly to the location of the steroid moiety detected by radioactivity scanning. In addi-

Table 6. Determination of glucuronide/steroid molar ratio of monoglucuronide conjugated metabolites of cortisol

Cmpd. No.#	Steroid Released by β-glucuronidase*	Glucuronide Moiety (as glucuronic acid), μM	Steroid Moiety, μM	Glucuronide/Steroid Molar Ratio
1	TH-6β-OH-20-dihydro-F	.180	.195	.92
2	6β-OH-20-dihydro-F	.064	.058	1.10
3	cortol-20α	3.115	2.925	1.06
4	5α-cortol-20α	1.323	1.485	.89
5	cortolone-20α	2.914	2.825	1.03
6	cortol-20β	2.821	2.624	1.08
7	5α-cortol-20β	.065	.081	.80
8	5α-cortolone-20α	.124	.096	1.29
9	cortolone-20β	.218	.231	.94
10	5α-cortolone-20β	.102	.098	1.04
11	E_R	.144	.138	1.04
12	THF	.180	.201	.90
13	5α-THF	.316	.321	.98
14	THF	.270	.281	.96
15	THE	.272	.274	.99
16	F_K	.104	.104	1.00
17	5α-THE	.102	.108	.94
18	E_K	.113	.109	1.04
19	11-OH-Etio	.203	.204	1.00
20	11-OH-Andro	.197	.185	1.07
21	11-oxo-Etio	.175	.180	.97
22	11-oxo-Andro	.163	.170	.96
			Mean ratio:	1.00

For compound designation see Fig. 2, and compare Tables 4 and 7.

* For steroid abbreviations and nomenclature see footnote 3 in text.

tion, the presence of glucuronic acid in the individual monoglucuronide conjugates eluted from paper chromatograms was confirmed by the micro-carbazol color reaction with a characteristic absorption spectrum on scanning in a recording spectrophotometer.

In each of the isolated monoglucuronide conjugates, the steroid-to-glucuronic acid molar ratio was determined. The relevant data are shown in Table 6. It will be seen that this ratio was uniformly 1:1, allowing for the standard error of the methods used for the determination of both glucuronic acid and steroid moieties.

Table 7 shows the results of the determination of the site of conjugation. Monoglucuronides of 4-en-3-oxosteroids were found, as expected, to have the glucuronic acid moiety attached at C-21. Of steroid monoglucuronides reduced in ring A, only THF was found to have the site of conjugation at C-21. However, a larger proportion of THF-monoglucuronide was found to be conjugated at C-3. Sites of conjugation of very polar metabolites, 6β-OH-20-dihydro-F and TH-6β-OH-20-dihydro-F were not determined, in view of the scarcity of the available material, and the need for the separation of the individual isomers of these compounds which has not yet been accomplished.

C. Isolation and characterization of diglucuronide conjugated metabolites of cortisol

The presence of diglucuronide conjugates was first detected by means of H.V.E. These conjugates exhibited an electrophoretic mobility at pH 6·4, which was almost twice that of monoglucuronides, and were completely hydrolyzable with β-glucuronidase. The amount of radioactivity present in the steroid moieties was equal to that of the unhydrolysed conjugates. The presence of glucuronic acid on paper chromatograms, in a position exactly corresponding to the radioactivity peak obtained by chromatogram-scanning, was demonstrated by two color spot tests (PAN-cobalt nitrate and naphthoresorcinol). Furthermore, a characteristic absorption spectrum of the chromogen produced with the carbazol reagent, which was identical with that produced by a standard glucuronic acid, was demonstrated in diglucuronide conjugates eluted from paper. Individual steroid diglucuronides were not separated as conjugates, because of extremely minute differences in their relative polarity. The group *in toto* was hydrolyzed with β-glucuronidase and the liberated steroid moieties were separated by a number of consecutive paper chromatographies, as described for the electrophoretically separated group of steroid-monoglucuronides (cf. Fig. 1). The

Table 7. Determination of the site of conjugation of monoglucuronide-conjugated metabolites of cortisol

Cmpd. No.#	Steroid released by β-glucuronidase hydrolysis of the conjugate, and identified by RID§	*Group I* *Steroids with 17,21-diol-20-one side-chain*		Determined site of Conjugation
		Chromatography in B_3 (x1) of methylated, acetylated, reduced and oxidized conjugate, R_f	Derivative formed	
12	THF	.03	11-OH-Etio-3-GTM*	C-3
13	5α-THF	.04	11-OH-Andro-3-GTM	C-3
14	THF	.78	11-OH-Etio-3-ac.	C-21
15	THE	.06	11-oxo -Etio-3-GTM	C-3
16	F_K	.38	11-OH-4-en-A-dione	C-21
17	5α-THE	.07	11-oxo-Andro-3-GTM	C-3
18	E_K	.42	Adrenosterone	C-21

		Group II *Steroids with 17,20,21-triol side-chain*			
		Chromatography of methylated conjugate in K_5 (x9), R_f	Conjugate of analogous 20-oxosteroid with determined site of conjugation, used for comparison	R_f in K_5 (x9) of derivative formed by methylation & reduction of this conjugate	
3	cortol-20α	.36	THF-3-G	.36 (.21)+	C-3
4	5α-cortol-20α	.36	5α-THF-3-G	.36 (.20)	C-3
5	cortolone-20α	.40	THE-3-G	.39 (.29)	C-3
6	cortol-20β	.20	THF-3-G	.21 (.36)	C-3
7	5α-cortol-20β	.20	5α-THF-3-G	.20 (.36)	C-3
8	5α-cortolone-20α	.40	5α-THE-3-G	.40 (.28)	C-3
9	cortolone-20β	.28	THE-3-G	.28 (.40)	C-3
10	5α-cortolone-20β	.29	5α-THE-3-G	.29 (.40)	C-3
11	E_R	.51	F_K-21-G	.50 (.62)	C-21

		Group III - 17-oxosteroids				
		Chromatographic mobility of the isolated conjugate (c) compared to that of the authentic standard (std)¢				
		R_f in SL_7 (x6)		R_f in B_3 (x8) following methylation & acetylation		
		c	std	c	std	
19	11-OH-Etio	.23	.23	.18	.18	C-3
20	11-OH-Andro	.40	.39	.22	.23	C-3
21	11-oxo-Etio	.76	.75	.38	.36	C-3
22	11-oxo-Andro	.85	.86	.43	.42	C-3

For compound designation see Fig. 2, and compare Tables 4 and 6; the site of conjugation of cmpds. No. 1 and 2 was not determined.

§ cf. Table 4; for steroid abbreviations and nomenclature see footnote 3 in text.

* GTM: Glucuronoside-triacetate-methyl ester.

\+ During borohydrate reduction, a mixture of 20α- and 20β-OH isomers was formed; the number in parentheses indicates R_f of the isomer.

¢ 11-OH-Etio-3-G, 11-OH-Andro-3-G, 11- oxo-Etio-3-G and 11- oxo-Andro-3-G, respectively.

Table 8. Identification of steroid moieties of diglucuronide-conjugated metabolites of cortisol (*RID*)

Postulated steroid metabolite*,#	Chromatography¢	Nonradioactive carrier added	Specific activity dpm/μM	Chemical reaction applied	Derivative formed*	Chromatography of derivative¢,Θ	Specific activity of derivative dpm/μM
cortol-20α+	B_P (x1) [.16] Boric B_5(x10)[.22]	cortol-20α	520	$NaBiO_3$ oxid.	11-OH-Etio	B_3 (x2) [.19] B_1 (x1) [.41]	498
cortol-20β+	B_P (x1) [.20] Boric B_5(x10)[.36]	cortol-20β	850	$NaBiO_3$ oxid.	11-OH-Etio	B_3 (x2) [.19] B_1 (x1) [.41]	820
cortolone-20α	B_P (x1) [.31] Boric B_5(x6) [.60]	cortolone-20α	878	$NaBiO_3$ oxid.	11-oxo-Etio	B_3 (x2) [.35] B_1 (x1) [.60]	860
cortolone-20β	B_P (x1) [.34] Boric B_5(x6) [.82]	cortolone-20β	346	$NaBiO_3$ oxid.	11-oxo-Etio	B_3 (x2) [.67] B_1 (x1) [.60]	310
THF	B_P (x1) [.39] B_5 (x10) [.68]	THF	1020	acetylation	THF-diAc.	B_3 (x1) [.60] B_1 (x1) [.40]	1008
5α-THF	B_P (x1) [.42] B_5 (x10) [.87] E_2B (x1) [.69]	5α-THF	648	acetylation	5α-THF-diAc.	B_3 (x1) [.56] B_1 (x1) [.50]	610
THE	B_P (x1) [.70] B_5 (x6) [.76]	THE	1160	acetylation	THE-diAc.	B_3 (x1) [.65] B_1 (x1) [.58]	1090

* For steroid abbreviations and nomenclature see footnote 3 in text.

Chromatographic mobility of radioactive steroid identical with that of steroid standard listed in the column.

¢ For designation of chromatographic systems, see Table 1; numbers in parentheses indicate the length of the run, e.g., (x1) = single length run; (x4) = 4 times single length run (overrun 3 times); numbers in brackets indicate R_f values or, when systems overrun, steroid mobilities relative to the length of paper chromatogram.

Θ Chromatographic mobility of radioactive derivative identical with that of steroid standard listed in preceding column.

\+ Compounds with chromatographic mobilities of 5α-isomers of cortol-20α and -20β noted on chromatograms; however, their identification by *RID* was not carried out, in view of the very small amounts of these compounds comprised in the diglucuronide-conjugated steroid group.

isolated steroid moieties of diglucuronide conjugates, identified by RID technique, are shown in Table 8. The 5α isomers of the cortolone-20α and -20β, and the 5α isomer of THE were not detected, probably because of their very small concentrations.

The determination of the glucuronide-to-steroid molar ratio was carried out on a total electrophoretically separated group of steroid diglucuronides, additionally purified by chromatography in K_5 system. It was found to be 2:1 (glucuronic acid 1·204 μM; steroid, following β-glucuronidase hydrolysis and oxidation with bismuthate, 0·586 μM).

D. Urinary excretion and relative concentrations of individual glucuronide-conjugated metabolites of cortisol in normal subjects

These are shown in Table 9. It will be seen that the largest fraction (47% of all monoglucuronide conjugates) consisted of the monoglucuronides of steroids reduced in ring A, with the dihydroxyacetone side-chain preserved (THF, THE and their 5α-epimers). Approximately 1/6 of this steroid metabolite fraction consisted of THF conjugated at C-21. Other compounds conjugated at C-21 were those with the 4-en-3-oxo configuration intact (E_K, F_K, E_R). All of them together constituted only 3% of total monoglucuronide conjugates. Monoglucuronides of steroids with reduced 20-ketone (cortols, cortolones) were present in appreciably lower concentrations than those of the corresponding metabolites with a dihydroxyacetone side-chain. In contrast, amongst diglucuronide conjugates, steroids with a glycerol side-chain constituted over 50% of all of these conjugates. Steroids with a dihydroxyacetone side-chain made up only 32%. The lowest concentration in this group was that of 5α-THF.

In Table 10, values for 24-h urinary excretion of various monoglucuronide conjugated metabolites of cortisol obtained in this study are compared with those obtained by other techniques. It will be seen that there is a very good agreement between the results yielded by different methods. No significant differences were noted between male and female subjects in the urinary concentrations of various glucuronide conjugated metabolites of cortisol. However, the small number of subjects does not permit drawing definite conclusions.

Table 9. Quantitation of individual glucuronide-conjugated metabolites of cortisol excreted over a 24-h period in 10 normal subjects

Steroid Moiety	Relative Concentration, % of Total MG
MONOGLUCURONIDES (MG)	
TH-6β-OH-20-DHF	0.8 ± 0.6
6β-OH-20-DHF	0.4 ± 0.1
Total very polar metabolites	1.2 ± 0.6
cortol-20α	2.4 ± 1.4
5α-cortol-20α	1.1 ± 0.8
cortol-20β	4.3 ± 1.2
5α-cortol-20β	1.4 ± 1.2
cortolone-20α	6.3 ± 0.8
5α-cortolone-20α	0.6 ± 1.2
cortolone-20β	8.2 ± 1.5
5α-cortolone-20β	0.7 ± 0.7
Total cortols & cortolones	25.0 ± 4.8
THF (3-G)	12.1 ± 6.4
5α-THF	4.9 ± 1.9
THF (21-G)	8.1 ± 6.1
THE	19.8 ± 7.9
5α-THE	2.2 ± 1.2
Total THF & THE (5α + 5β)	47.1 ± 8.5
E_R	0.9 ± 0.2
F_K	1.1 ± 0.3
E_K	1.1 ± 0.6
Total 4-en-3-oxo metabolites	3.1 ± 0.8
11-OH-Etio	4.8 ± 2.9
11-OH-Andro	5.0 ± 4.3
11-oxo-Etio	9.1 ± 6.9
11-oxo-Andro	4.7 ± 3.0
Total 17-KS	23.6 ± 7.2
*DIGLUCURONIDES (DG)**	
	% of Total DG
cortol-20α	13.5
cortol-20β	16.0
cortolone-20α	12.8
cortolone-20β	8.9
THF	13.6
5α-THF	7.4
THE	11.2
Unidentified	16.6

* Individual steroid metabolites separated from pooled diglucuronide conjugates from urine specimens of all subjects.

DISCUSSION

This paper describes the isolation and characterization of a whole spectrum of glucuronide-conjugated metabolites of cortisol in human urine: 22 steroid monoglucuronides and 7 steroid diglucuronides. All individual monoglucuronide-conjugated metabolites have been isolated *as conjugates* and characterized as such. Only a few of these steroid conjugates have been isolated and characterized previously.

Tetrahydrocortisone-3-monoglucuronide was isolated fom human urine after oral administration of 2g of tetrahydrocortisone, and was characterized *as a conjugate* by Schneider *et al.*[11], and both tetrahydrocortisol- and tetrahydrocortisone-3-monoglucuronide were isolated from urine, and characterized, by Foggit and Kellie, following administration of ACTH [10]. The latter investigators developed an elegant and useful method for the determination of the site of conjugation of glucuronide conjugates; this method was used in a part of the present study. Of cortisol metabolites with a glycerol side-chain, only cortolone-20β has been isolated as conjugate, but was not characterized as such (Schneider *et al.*[7, 11]).

Of other urinary C_{21}-steriod glucuronides isolated and characterized as conjugates, 5β-pregnane-3α,20α-diol-3-monoglucuronide was the first (isolated by Venning and Browne[40]), followed by 3α,17α-dihydroxy-5β-pregnan-20-one(21-desoxy-THS)-3-monoglucuronide (isolated by Mason and Strickler[41]). More recently, tetrahydrocorticosterone- and 5α-tetrahydrocorticosterone-monoglucuronide were also isolated and characterized as conjugates [10], as was tetrahydro-11-desoxycortisol(THS)-monoglucuronide [42].

Evidence for the existence of glucuronide conjugates of the 4-en-3-oxo-steroids, cortisol and cortisone, has been deduced by several investigators [4, 43, 44]. A more extensive study of these conjugates was conducted by Brouillet and Mattox [45], who presented evidence for the conjugation of these compounds at C-21.

In the present study, both cortisol- and cortisone-21-glucuronides have been isolated as conjugates and characterized as such. In addition, 21-glucuronides of 20β-dihydrocortisol (E_R) and of tetrahydrocortisol (THF) have been also isolated and characterized. Indirect evidence has been found by Kellie [42] for the existence of THF-21-glucuronide, but not for the existence of 21-glucuronides of other ring-A reduced corticosteriods. This is compatible with our findings. The results of the study of Brouillet and Mattox [45] suggested the possibility that a portion of urinary THE is conjugated at C-21. The conclusion was derived from partial resistance of the steroid conjugate to side-chain oxidation by active aeration in the presence of alcoholic alkali. It is likely that under these conditions the 11β-hydroxyl group was also oxidized, thus THF was converted to THE. We did not detect appreciable amounts of THE-21-glucuronide in the urines examined.

Table 10. Comparison of values for 24-h urinary excretion of various glucuronide conjugated metabolites of cortisol in normal subjects, obtained by different methods

Steroid(s)	% of all cortisol metabolites excreted over 24-h period*			
	1 Present Method	2 β-Glucur. Hydrolysis of E-E Extract #	3 Direct β-Glucur. Hydrolysis of Urine§	4 β-Glucur. Hydrolysis + Continuous Ether Extraction†
Very polar metabolites	1·2	1·8	0·8	—
THF	20·2[c]	18·5	15·7	17·8
5α-THF	4·9	7·7	6·8	9·5
THE	19·8	20·8	21·9	24·1
5α-THE	2·2			
E_R	0·9	—	—	—
F_K	1·1	0·9	3·0	1·8
E_K	1·1	1·0	2·5	1·7
cortol-20α	2·4	—	—	1·9
5α-cortol-20α	1·1	—	—	0·5
cortol-20β	4·3	—	—	4·6
5α-cortol-20β	1·4	—	—	0·7
cortolone-20α	6·3	—	—	10·4
5α-cortolone-20α	0·6	—	—	0·3
cortolone-20β	8·2	—	—	8·7
5α-cortolone-20β	0·7	—	—	0
C_{19} metabolites	23·6	—	21·4	12·3

* Mean values.

Urine was extracted with ethyl-acetate to remove free steroids. The conjugates were then extracted with a 3:1 ethanol–ether mixture (E-E) from aqueous phase acidified to pH 1 and saturated with ammonium sulfate. This extract was evaporated to dryness, redissolved in water and hydrolyzed with β-glucuronidase. The liberated steroids were extracted with dichloromethane and ethyl acetate, purified by a partition column and separated by paper chromatography [37].

§ Urine was hydrolyzed with β-glucuronidase for 5 days; it was then extracted with ethyl ether. The extract was purified by column chromatography on an alumina-silica mixture, and the steroids were separated by paper chromatography [38]. Thus the values obtained by this method include also free steroids, which were not removed by pre-extraction of urine; this is particularly apparent in the values for the concentrations of cortisol and cortisone.

The values presented in columns 2 and 3 were calculated from mg. values given in the pertinent papers, assuming that all the steroids with dihydroxyacetone side-chain constituted 50% of total cortisol metabolites.

† All steroids were isolated following i.v. administration of tracer [4-^{14}C]-cortisol. Urine was hydrolyzed with β-glucuronidase, then acidified to pH 1 and continuously extracted with ethyl ether [39]. Thus the values obtained by this method include also steroids released from their sulfates, but these constitute less than 10% of total cortisol metabolites [15]. Very polar metabolites were not estimated. The relatively high concentrations of cortisol and cortisone are due to the contribution of free steroids which were not removed prior to hydrolysis or urine.

[c] 3-gluc. + 21-gluc.

Each of the isolated 4-en-3-oxo-steroid-21-monoglucuronides (F_K, E_K, E_R) constituted approximately only 1% of the total monoglucuronide-conjugated steroid group. Therefore, it is very likely that the THF-21-glucuronide, found to constitute 8% of all urinary monoglucuronides, was derived by conjugation of the free THF *and* not by direct metabolism of cortisol-21-glucuronide. Such direct metabolism has been demonstrated for other steroids by Baulieu[46] and Pasqualini[47].

The ring-A reduced metabolites with a non-reduced 20-oxo group (THF, THE, 5α-THF, 5α-THE) constituted the largest portion of all monoglucuronide conjugates (47%). This is compatible with the findings of many other investigators who identified free steroid moieties following β-glucuronidase hydrolysis of urine, urinary extracts containing conjugated steroids, or total glucuronide conjugates separated by column or paper chromatography, or paper electrophoresis [1, 4, 22, 37–39, 48]. The site of conjugation of the majority of these conjugates was at C-3.

The second largest group (25%) of monoglucuronide-conjugated metabolites of cortisol consisted of ring-A reduced steroids with the 20-oxo group reduced: cortols and cortolones. This is compatible with the findings of Fukushima *et al.*[49] who were the first to identify these steroids after consecutive enzymatic hydrolysis and solvolysis of urine. All isomers of these steroids have been isolated and characterized as conjugates in the present study. Metabolites with the side-chain oxidized to 17-oxo were also isolated and characterized as conjugates.

Of the monoglucuronide-conjugated metabolites of cortisol isolated by us, E_R, 6β-OH-20-DHF and the ring-A reduced metabolite of the latter, were not known until now to occur as glucuronide conjugates. We have recently isolated and characterized sulfate conjugates of these steroids [15, 16, 50]. The impor-

tance of these findings remains to be elucidated. These very polar steroids were thought to represent products of an "alternative metabolic pathway" for cortisol [26, 27, 51], which could be increased when the activity of the ring-A reducing system was constrained (as in liver cirrhosis). The polarity of these metabolites, as *free* steroids, is sufficiently great to permit their excretion in urine. Therefore, the finding of the existence of sulfate- and glucuronide-conjugated metabolites of these polar steroids is surprising, unless one assumes that these conjugates are formed for a purpose other than ready clearance by the kidney.

The diglucuronide-conjugated metabolites of cortisol have not been heretofore isolated and characterized. We have previously reported evidence for the presence of such conjugates in human plasma [52, 53]. The total diglucuronide-conjugate group constitutes less than 2% of total conjugated metabolites of cortisol, whereas the monoglucuronides comprise almost 90%. It is interesting to note the reverse quantitative relationship in the ratio of steroids with glycerol side-chain to those with dihydroxyacetone side-chain in the diglucuronide-conjugated steroid group, as compared with this ratio in the monoglucuronide group. This could suggest that steroids with glycerol side-chain have a higher affinity for C-21-glucuronyl transferase. Although we were not able to detect any 21-monoglucuronides of cortols and cortolones, it is possible that the concentrations of these conjugates were so small as to escape detection by the methods used. This seems likely, since all isomers of cortols and cortolones conjugated as diglucuronides comprised less than 0·9% of the total glucuronide conjugates.

With regard to the methodology used, it should be pointed out that the chromatographic separation of a large number of conjugated steroid metabolites with only minute differences in relative polarities necessitated development of a technique based on very long chromatographies (up to 300 h per run); this was particularly necessary in view of the fact that the new solvent systems developed did not contain any acids or alkali to prevent formation of steroid artefacts.

The homogeneity of the separated individual steroid monoglucuronides was judged by the following criteria: (1) presence of only one steroid moiety in each conjugate following its hydrolysis with β-glucuronidase; (2) a 1:1 molar ratio of glucuronide-to-steroid moieties, which remained constant during 2 additional chromatographies and following methylation and acetylation of the individual glucuronide conjugate and its chromatography; (3) the specific activity of the conjugate based on the quantitative determination of the glucuronic acid moiety was very close (within 8%) to the specific activity based on the quantitative determination of the steroid moiety released by β-glucuronidase and chromatographed on paper.

Chromatographic separation of individual diglucuronide conjugates was not attempted in view of negligible differences in relative polarities of these conjugates. The determination of the steroid/glucuronide molar ratio was carried out on the total, electrophoretically-separated group of steroid diglucuronides. The steroid moieties of these conjugates were identified following hydrolytic cleavage of the group *in toto*.

Acknowledgements—Mrs. Judith Miller and Sandra Holmes provided excellent secretarial assistance in the preparation of this paper.

FOOTNOTES

[1] Supported by NIH Grants HE 11651, and in part GRS 5SO1RR05477.

[2] Research Fellow, on leave from 2nd Department of Internal Medicine, University of Kanazawa, Japan.

[3] The following trivial names and abbreviations are used for steroids and their conjugates: cortisol (F_K) for 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisone (E_K) for 17α,21-dihydroxy-4-pregnene-3,11,20-trione; 6β-hydroxycortisol (6β-OH-F) for 6β,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione; 20β-dihydrocortisol (E_R) for 11β,17α,20β,21-tetrahydroxy-4-pregnen-3-one; 6β-hydroxy-20α-dihydrocortisol (6β-OH-20α-DHF) for 6β,11β,17α,20α,21-pentahydroxy-4-pregnen-3-one; 20α-dihydrocortisol (20-*epi*E_R) for 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one; tetrahydrocortisol (THF) for 3α,11β,17α,21-tetrahydroxy-pregnan-20-one; 5α-tetrahydrocortisol (5α-THF) for 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one; tetrahydrocortisone (THE) for 3α,17α,21-trihydroxypregnane-11,20-dione; 5α-tetrahydrocortisone (5α-THE) for 3α,17α,21-trihydroxy-5α-pregnane-11,20-dione; cortol-20α for 3α,11β,17α,20α,21-pentahydroxy-5β-pregnane; 5α-cortol-20α for 3α,11β,17α,20α,21-pentahydroxy-5α-pregnane; cortol-20β for 3α,11β,17α,20β,21-pentahydroxy-5β-pregnane; 5α-cortol-20β for 3α,11β,17α,20β,21-pentahydroxy-5α-pregnane; cortolone-20α for 3α,17α,20α,21-tetrahydroxy-5β-pregnan-11-one; 5α-cortolone-20α for 3α,17α,20α,21-tetrahydroxy-5α-pregnan-11-one; cortolone-20β for 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one; 5α-cortolone-20β for 3α,17α,20β,21-tetrahydroxy-5α-pregnan-11-one; tetrahydro-6β-hydroxy-20β-dihydrocortisol (TH-6β-OH-20β-DHF) or 6β-OH-cortol-20β for 5β-pregnane-3α,6β,11β,17α,20β,21-hexol; 11β-hydroxy-aetiocholanolone (11-OH-Etio) for 3α,11β-dihydroxy-5β-androstan-17-one; 11β-hydroxy-androsterone (11-OH-Andro) for 3α,11β-dihydroxy-5α-androstan-17-one; 11-oxo-aetiocholanolone (11-oxo-Etio) for 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-androsterone (11-oxo-andro) for 3α-hydroxy-5α-androstane-11,17-dione; 11-OH-4-en-androstenedione (11-OH-4-en-A-dione) for 11β-hydroxy-4-androstene-3,17-dione; adrenosterone for 4-androstene-3,11,17-trione; 6-oxo-11-OH-4-en-androstenedione for 11β-hydroxy-4-androstene-3,6,17-trione; androstanetrione for 5α-androstane-3,11,17-trione; aetiocholanetrione for 5β-androstane-3,11,17-trione; -Ac. for (steroid)-yl-monoacetate; -diAc. for (steroid)-diyl-diacetate; -triAc. for (steroid)-triyl-triacetate; -tetraAc. for (steroid)-tetrayl-tetraacetate; -sulfate for (steroid)-yl-sulfate; -glucuronide for (steroid)-yl-β-D-glucopyranosiduronide.

[4] Subsequently, the monosulfates could be separated readily from glucuronosulfates either by means of H.V.E. at pH 6·4 [15] or by paper chromatography in K_4 system [16].

[5] Another method, discriminating more directly between conjugation at the C-3 and C-21 positions of steroids with glycerol side-chain is under development at present.

REFERENCES

1. Samuels L. T.: In *Metabolic Pathways* (Edited by D. M. Greenberg) Vol. 1 (1960) pp. 431–480. Academic Press, New York.

2. Dorfman R. I. and Unger F.: *Metabolism of Steroid Hormones* (1965). Academic Press, New York.
3. Bongiovanni A. M. and Eberlein W. R.: *Proc. Soc. exp. Biol. Med.* **89** (1955) 281–285.
4. Kornel L. and Hill S. R. Jr.: *Metabolism* **10** (1961) 18–26.
5. Crépy O., Jayle M. F. and Meslin F.: *Acta endocr., Copenh.* **24** (1957) 233–248.
6. Barlow J. J. and Kellie A. E.: *Biochem. J.* **71** (1959) 86–91.
7. Schneider J. J. and Lewbart M. L.: *Rec. Prog. Horm. Res.* **15** (1959) 201–230.
8. Kornel L.: *J. clin. Endocr. Metab.* **24** (1964) 956–964.
9. Cavina G. and Tentori L.: *Clin. Chim. Acta* **3** (1958) 160–164.
10. Foggitt F. and Kellie A. E.: *Biochem. J.* **91** (1964) 209–217.
11. Schneider J. J., Lewbart M. L., Levitan P. and Lieberman S.: *J. Am. chem. Soc.* **77** (1955) 4184–4185.
12. Kornel L., Miyabo S., Saito Z. and Wu F.-T.: In *Research on Steroids* (Edited by M. A. Finkelstein, A. Klopper, P. Jungblut and C. Conti) Vol. 5 (1973) pp. 413–427. Societa' Editrice Universo, Rome, Italy.
13. Baulieu E.-E., Milgrom E., Lebeau M.-C., Weintraub H. and Robel P.: *Progress in Endocrinology* (Edited by C. Gual) Proc. 3rd Int. Cong. of Endocrinology, Mexico City. Excerpta Med. Int. Congr. Ser. 184 (1969) 763–769.
14. Lieberman S.: Proc. II Intern Congress on Hormonal Steroids (Edited by L. Martini, F. Fraschini and M. Motta) Milan Italy. Excerpta Medica Intern Series 132, (1966) 22–36.
15. Kornel L., Miyabo S. and Takeda R.: *Steroidologia* **2** (1971) 197–236.
16. Miyabo S. and Kornel L.: *J. steroid Biochem.* **5** (1974) 233–247.
17. Saito Z. and Kornel L.: *Fedn. Proc.* **31** (1972) 283.
18. Kornel L., Ezzeraimi E. and Economou P.: *Clin. Res.* **22** (1974) 633.
19. Mattox V., Goodrich J. and Vrieze W.: *Biochemistry* **8** (1969) 1188.
20. Kornel L., Kleber J. and Conine J.: *Steroids* **4** (1964) 67.
21. Allen W. M.: *J. clin. Endocr. Metab.* **10** (1950) 71–83.
22. Cohn G. L. and Bondy K.: *J. biol. Chem.* **234** (1959) 31–34.
23. Bradlow L.: *Steroids* **11** (1968) 265–272.
24. Kornel L. *et al.*: *J. Chromatog.* **111** (1975) 200.
25. Schneider J. J. and Lewbart M. L.: *Tetrahedron* **20** (1964) 943–956.
26. Frantz A. G., Katz F. H. and Jailer J. W.: *J. clin. Endocr. Metab.* **21** (1961) 1290–1303.
27. Dixon R. and Pennington G. W.: *J. Endocr.* **34** (1966) 281–288.
28. Kornel L.: *Biochemistry* **4** (1965) 444–452.
29. Bush I. E.: *Biochem. J.* **50** (1952) 370–378.
30. Eberlein W. R. and Bongiovanni A. M.: *Archs Biochem.* **59** (1955) 90–96.
31. Crépy O., Judas O. and Lachese B.: *J. Chromatog.* **16** (1964) 340–344.
32. Baldwin B. C., Robinson D. and Williams R. T.: *Biochem. J.* **71** (1959) 638–642.
33. Kornel L.: *J. clin. Endocr. Metab.* **23** (1963) 1192–1202.
34. Kornel L.: *Analyt. Chem.* **36** (1964) 443–444.
35. Gautney M. C., Barker S. B. and Hill S. R. Jr.: *Science* **129** (1959) 1281.
36. Berliner D. and Salhanick M.: *Analyt. Chem.* **28** (1956) 1608–1610.
37. Kornel L., Starnes W. R., Hill S. R. Jr. and Hill A.: *J. clin. Endocr. Metab.* **29** (1969) 1608–1617.
38. Starnes W. R., Partlow T., Grammer M., Kornel L. and Hill S. R. Jr.: *Analyt. Biochem.* **6** (1963) 82.
39. Fukushima D. K., Bradlow H. L., Hellman L., Zumoff B. and Gallagher T. F.: *J. biol. Chem.* **235** (1960) 2246–2252.
40. Venning E. M. and Browne J. S. L.: *Proc. Soc. exp. Biol. Med.* **34** (1936) 792–793.
41. Mason H. L. and Strickler H. S.: *J. biol. Chem.* **171** (1947) 543–549.
42. Kellie A. E.: In *Structure and Metabolism of Corticosteroids* (Edited by J. R. Pasqualini and M. F. Jayle) (1964) pp. 21–30. Academic Press, New York.
43. Oertel G. W.: In *Structure and Metabolism of Corticosteroids* (Edited by J. R. Pasqualini and M. F. Jayle) (1964) pp. 65–76. Academic Press, New York.
44. Pasqualini J. R.: *Bull. Soc. chim. Biol.* **45** (1963) 277–300.
45. Brouillet J. C. and Mattox V. R.: *J. clin. Endocr. Metab.* **26** (1966) 453–458.
46. Baulieu E.-E., Corpechot C. and Emiliozzi R.: *Steroids* **2** (1963) 429–451.
47. Pasqualini J. R. and Faggett J.: *J. Endocr.* **31** (1964) 85–86.
48. Bernstein S. and Solomon S.: *Chemical and Biological Aspects of Steroid Conjugation* (1970). Springler-Verlag, New York.
49. Fukushima D. K., Leeds N. S., Bradlow H. L., Kritchewsky T. H., Stokem M. B. and Gallagher T. F.: *J. biol. Chem.* **212** (1955) 449–460.
50. Kornel L. and Yuan L.: *Steroids* (in Press).
51. Ulstrom R. A., Colle E., Burley J. and Gunville R. J.: *J. clin. Endocr. Metab.* **20** (1960) 1080–1094.
52. Kornel L. and Lee J. P.: Progr. 46th Meeting of the Endocrine Society (1964) 108.
53. Kornel L.: Proc. 6th Pan American Congress of Endocrinology, Mexico City. Excerpta Med. Int. Congr. Ser. **99** (1965) E89.