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CORTICOSTEROIDS IN HUMAN BLOOD—VI. ISOLATION, CHARACTERIZATION AND QUANTITATION OF SULFATE CONJUGATED METABOLITES OF CORTISOL IN HUMAN PLASMA*

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SUMMARY

Evidence has been obtained for the presence of a total spectrum of sulfate-conjugated metabolites of cortisol in human plasma: 19 monosulfates, two disulfates and six glucuronosulfates. Two hours and 15 min following i.v. administration of a tracer dose of [4-^{14}C]-cortisol to 11 normal subjects, large samples of heparinized blood were obtained. From the separated, deproteinized and defatted plasma, all *conjugated* steroids were extracted by means of an XAD-2 amberlite column. Mono- and di-sulfate conjugated steroids were then separated from other free and conjugated metabolites of cortisol by means of high voltage paper electrophoresis. Individual monosulfate conjugated metabolites and glucuronosulfates were subsequently separated (as seven sub-groups) by paper chromatography. These conjugates were eluted and subjected to cleavage by solvolysis (monosulfates and disulfates) or consecutive solvolysis and β-glucuronidase hydrolysis (glucuronosulfates). The liberated steroid moieties were separated by multiple successive paper chromatographies, and identified by reverse isotope dilution technique. The individual steroids were then quantitated. Sulfate-to-steroid molar ratios were also determined on major conjugates, prior to their cleavage.

The steroid metabolites found in plasma are identical with those isolated by us previously from human urine. The total sulfate fraction constituted 5·3 ± 1·9% of all conjugated metabolites of cortisol in plasma. Of those, 76% were monosulfates, 19% were glucuronosulfates, and 5% were disulfates. A large proportion of monosulfates consisted of steroids conjugated at C-21 (27%), which contrasts with glucuronide conjugates. The steroids found in largest concentrations were: 20β-cortolone-3-sulfate (17%), 5α-tetrahydrocortisol-3-sulfate (14%), 6β-hydroxycortisol-21-sulfate (9%), cortisol-21-sulfate (8%), tetrahydrocortisol-3-sulfate (7%), and 20β-dihydrocortisol-21-sulfate (6%).

From the correlation of plasma and urinary concentration of individual steroid sulfates, the following conclusions are drawn: (1) sulfoconjugation is a consistent, though quantitatively minor, pathway in the peripheral metabolism of cortisol; (2) C-21 sulfates of 4-ene-3-oxo-steroids are formed (and excreted) considerably faster than C-3 sulfates, the formation of which requires a prior reduction of ring-A; (3) C-3 sulfates of steroids with dihydroxyacetone side-chain are formed (and excreted) more slowly than the corresponding sulfates of steroids with glycerol side-chain, *ergo*, the reduction of 20-ketone appears to facilitate sulfoconjugation.

INTRODUCTION

It is generally accepted that steroid conjugation is the final step in the metabolism of these hormones, leading to their deactivation and elimination from the body[2]. However, more recent studies provided experimental data which may be construed as evidence in favor of the concept that certain types of steroid conjugates are biologically active compounds, and that the conjugation serves, in some instances, a purpose other than merely rendering the hormone more water soluble, to facilitate its excretion[3–6]. In particular, sulfate conjugates of steroids were shown to be synthesized in the steroid-producing glands[7–12]. It has been also demonstrated that various "peripheral" tissues possess the ability to form sulfate conjugates[13–16]. The importance of this finding is still unknown.

To gain more insight into the biological role of steroid conjugates, we undertook a systematic study of various conjugated metabolites of adrenal steroids. We have reported previously the isolation and identification of a whole spectrum of sulfate conjugated metabolites of cortisol from human urine[17]. An analogous

* A preliminary report on this study was presented at the *Third International Congress on Hormonal Steroids*, Hamburg, Germany, September, 1970 [1].

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study of sulfate conjugated metabolites of cortisol in plasma has now been completed. This paper describes the results of this study.

MATERIALS AND METHODS

A tracer dose (15 20 μCi) of [4-^{14}C]-cortisol was administered at 8:00 a.m. in a rapid, i.v. injection to 11 normal adult subjects, six men (aged 23–46) and five women (aged 24–55); 2 h and 15 min later, large samples (450–550 ml) of venous blood were drawn into heparinized containers. The blood was centrifuged and the plasma was immediately separated, frozen, and stored at -15°C until processed.

Measurement of radioactivity

Radioactivity was determined in a Packard Tri-Carb-Liquid Scintillation Spectrometer, Model 3375. The scintillator solution used and the method of counting were the same as those described in a previous paper [17].

* The following trivial names and abbreviations are used for steroids: 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; 20α-dihydrocortisol (20-epiE_R) for 11β,17α,20α,21-tetrahydroxy-4-pregnen-3-one; tetrahydrocortisol (THF) for 3α,11β,17α,21-tetrahydroxypregnan-20-one; 5α-tetrahydrocortisol (5αTHF) 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; 20α-cortol for 5β-pregnane-3α,11β,17α,20α,21-pentol; 5α-20α-cortol for 5α-pregnane-3α,11β,17α,20α,21-pentol; 20β-cortol for 5β-pregnane-3α,11β, 17α,20β,21-pentol; 5α-20β-cortol for 5α-pregnane-3α,11β,17α,20β,21-pentol; 20α-cortolone for 3α,17α,20α,21-tetrahydroxy-5β-pregnan-11-one; 5α-20α-cortolone for 3α,17α,20α,21-tetrahydroxy-5α-pregnan-11-one; 20β-cortolone for 3α,17α,20β,21-tetrahydroxy-5β-pregnan-11-one; 5α-20β-cortolone for 3α,17α,20β,21-tetrahydroxy-5α-pregnan-11-one; 11β-hydroxy-aetiocholanolone (11-OH-Aetio) 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-Aetio) for 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-androsterone (11-oxo-Andro) for 3α-hydroxy-5α-androstane-11,17-dione; 11-OH-Δ^4-androstenedione (11-OH-Δ^4A) for 11β-hydroxy-4-androstene-3,17-dione; adrenosterone for 4-androstene-3,11,17-trione; 6-oxo-adrenosterone for 4-androstene-3,6,11,17-tetrone; dehydroepiandrosterone (DHEA) for 3β-hydroxy-5-androsten-17-one; 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; -sulfate for (steroid)-yl-sulfate; -glucuronide for (steroid)-yl-β-D-glucopyranosiduronide.

Quantitation of steroids

Zimmerman color reaction was used for the estimation of C_{19} steroids isolated in this study, and C_{21} steroids with glycerol side chain (cortols, cortolones, 20α- and 20β-dihydrocortisol)* following their oxidation with sodium bismuthate or chromic acid to the corresponding 17-ketosteroids.

Blue tetrazolium (BT) reaction was used for the estimation of C_{21} steroids with γ-ketol side chain, and their acetates.

The details of the methods used were described by us previously[17].

Quantitation of organic sulfates

The sulfate moiety of steroid sulfates was measured by a modification of the method of Roy[18], based on the formation of a chloroform–soluble organic sulfate–methylene blue complex: the dry steroid sulfate was dissolved in 0·5 ml of water and mixed with 0·5 ml of methylene blue soln (2·5 mg of methylene blue, 50 g of sodium sulfate and 10 ml of 98% sulfuric acid, dissolved in 1 liter of water). The ester sulfate–methylene blue complex was then extracted with 5 ml of freshly distilled chloroform. The absorption curve of the colored complex was recorded on a Beckman DK-2 Spectrophotometer in the region between 600 to 700 nm. The Allen correction[19] was applied to the readings at 610, 650, and 690 nm. 11-Oxo-aetiocholanolone-sulfate was used as a standard.

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[17].

Outline of the procedure

The frozen plasma specimens were thawed and the vol. of each was measured. Duplicate 1 ml aliquots were taken for radioactivity counting ("plain plasma count"), and the remaining plasma vol. of each specimen was made up with distilled water to the nearest multiple of 25, mixed well, then divided into a number of 25-ml aliquots, each of which was placed in a 250-ml glass-stoppered centrifuge bottle (Scientific Glass Apparatus Co., Bloomfield, N.J., Cat No. JE-4224).

A. Deproteinization and defatting of plasma

The technique used was a modification of that described by us previously [20,21]: 2 vol. of methanol was added slowly (from a burette) to each centrifuge bottle containing 25 ml of plasma, the bottle being simultaneously gently agitated. It was then stoppered, refrigerated for 15 min, and 100 ml of redistilled carbon

tetrachloride was added to the bottle. The mixture was then vigorously shaken for 90 s. After standing in a refrigerator for 30 min, the bottle was centrifuged for 15 min at 2000 rev./min. The upper, alcoholic layer was carefully transferred to another 250 ml bottle by means of a serum lifter,* without disturbing the precipitated proteins forming a layer between the aqueous methanolic and the CCl_4 phases. Subsequently the carbon tetrachloride layer was discarded and the "cake" of precipitated proteins was saved for further extraction (see below). The aqueous methanolic extract containing free and conjugated metabolites of cortisol, was then acidified to pH 4·5–5·0 by addition of 2–3 ml of 2 M acetate buffer (pH 4·5) in 70% aqueous methanol, and refrigerated for 30 min. The buffered alcoholic extract was washed twice with 100 ml hexane, by shaking for 1 min and centrifuging for 8 min at 2000 rev./min each time. The upper hexane layer was aspired off and traces of hexane remaining on the top of the methanolic extract were removed by blowing air. Subsequently, 0·5 ml of 40% (w/v) aqueous barium acetate was added and the solution was mixed well. It was then allowed to stand for 5 min and 25 ml of ethanol was added. After mixing the contents well, the bottle was placed in a refrigerator for 2 h (or kept overnight). It was then centrifuged for 15 min at 1800 rev./min. The decanted supernatants originating from one plasma specimen were pooled in a 1 l. flask, and were evaporated to dryness under vacuum at 40°C. To the dry residue, water (80 ml/100 ml of original plasma vol.) was added. Two 0·5 ml aliquots were taken for counting radioactivity ("deproteinized and defatted plasma count") and the remaining aqueous solution was kept in a freezer until further processing.

B. Extraction of "free" steroids from deproteinized and defatted plasma

The frozen aqueous solution containing all steroids was thawed and divided into equal portions (40 ml), each of which was placed in a 250 ml centrifuge bottle. The "free" steroids were then extracted twice with 3 vol. of ethyl acetate by shaking each time for 1 min and centrifuging for 15 min at 2000 rev./min. The separated extracts were pooled and their vol. was measured. After saving two 2 ml aliquots for radioactivity counting ("free steroid count"), the ethyl acetate extract was reduced in vol. under vacuum, transferred to a 40 ml conical tube and evaporated to complete dryness by blowing nitrogen. The tube was stoppered, sealed with parafilm, and kept in a dessicator which was placed in a freezer. The remaining aq. phase was also pooled and measured in vol. After taking two 0·5 ml aliquots for radioactivity counting ("conjugate count") it was applied to Amberlite XAD-2 column for the extraction of conjugates, as described below.

C. Extraction of steroids co-precipitated with proteins

To each protein "cake" from 25 ml plasma, remaining in a 250 ml centrifuge bottle, 75 ml of ethanol was added. The bottle was stoppered and shaken vigorously for 5 min. After 15 min centrifugation at 2000 rev./min, the ethanolic extracts were decanted and pooled, and the protein residue was re-extracted with 75 ml of watersaturated butanol, by shaking for 5 min, followed by centrifugation for 15 min at 2000 rev./min. The ethanolic and butanolic extracts were combined and evaporated to dryness. The dry residue was redissolved in 20 ml of water and transferred to a 100 ml centrifuge tube, followed by two successive washings with 10 ml of water. The aqueous solution (40 ml) was washed twice with 16 ml of benzene, by shaking for 5 min and centrifuging for 8 min at 2000 rev./min. The benzene layers were aspirated off and discarded. "Free" steroids were extracted with ethyl acetate (2 × 3 vol.) the same way as they were from deproteinized plasma extract (as described above). Two 1 ml aliquots of the extract were taken for radioactivity counting ("protein–cake free steroid count"); and the remaining ethyl acetate extract was reduced in vol. *in vacuo*, transferred to a 40 ml conical tube and evaporated to drynesss under nitrogen. The remaining aq. phase containing *conjugated steroids* was acidified to pH 1·0 (with a few drops of 25% sulfuric acid), saturated with ammonium sulfate, and extracted consecutively with 4 vol. and 2 vol. of a 3:1 ether–ethanol mixture. The extracts were separated, neutralized with a diluted ammonium hydroxide, and evaporated to dryness. The dry residue was redissolved in 20 ml of 90% aqueous ethanol. Two 1 ml aliquots were taken for counting radioactivity ("protein–cake conjugate count"), and the remaining solution (protein cake extracts from five or six plasma samples) were pooled, evaporated to dryness, and redissolved in 160 ml of water. This aqueous solution was then applied to Amberlite XAD-2 column for the extraction of conjugates.

D. Extraction of steroid conjugates from deproteinized and defatted plasma and from the extract of precipitated plasma proteins ("protein cake")†

This was carried out by means of an Amberlite XAD-2 column according to the method of Bradlow[22] in the following modification: 80 g of Amberlite XAD-2 was suspended in 500 ml of water in a

* Obtainable from the Mechanical Workshop, Presbyterian-St. Luke's Hospital, Chicago, Illinois.

† The results of our previous studies of steroid metabolism in hypertension[32] indicated that plasma levels of certain very polar conjugated corticosteroids are appreciably higher in patients with essential hypertension (H) than in

beaker. After the resin settled down, fine particles were removed by decantation. This procedure was repeated three times. The resin was then washed with 500 ml of methanol three times, and was poured into a column (3·2 × 35 cm, equipped with a coarse fritted disc at the bottom, and a 500 ml reservoir at the top). Glass-wool was packed on the top of the resin bed to prevent the resin from floating up during addition of the eluting solvents. The resin was further washed in the column with 300 ml of methanol followed by 1 l. of water.

A 150–250 ml sample of the aq. soln of deproteinized, defatted, and ethyl acetate-extracted plasma, or a 160 ml sample of the pooled aq. extract of precipitated proteins (containing conjugated steroids), was percolated through the resin under gravity flow (3 ml/min). The column was then washed with 2 vol. of water. The filtrate and the aqueous wash were combined, measured in vol., and two 1 ml aliquots were taken for radioactivity counting. The conjugates adsorbed on the column were than eluted with 2·5 vol. of methanol. In the anticipation of very polar conjugates possibly present in the precipitated protein extracts, additional column elution was carried out with 2 vol. of *n*-butanol 70% aqueous methanol mixture (1:3, v/v). After measuring the vol. of the alcoholic eluates and taking 2 ml aliquots for radioactivity counting, the eluates were reduced *in vacuo*, transferred to 40 ml conical tubes, and evaporated to dryness under nitrogen.

The recovery of radioactivity eluted with methanol from the Amberlite XAD-2 column was 101·0 ± 4·6% for the deproteinized and defatted plasma (mean + S.D. of 20 samples), and 97·1% for the "protein-cake" extract (four pooled samples). The amount of radioactivity found in the combined sample-filtrates and aq. column eluates (wash) was 1·7 ± 0·9% for plasma, and 2·4% for the protein extract. No radioactivity was found in the *n*-butanol–70 aqueous methanolic eluate, which followed the methanolic elution (see above).

E. High voltage electrophoresis

The separation of various groups of steroid conjugates was done by means of high voltage electrophoresis using basically the method described by one of us (LK) previously[23]. The dry residue of the methanolic eluate from the Amberlite XAD-2 column was dissolved in a small vol. of the 1:3 mixture of *n*-butanol-70% aqueous methanol and was applied as a streak to Whatman No. 3 MM chromatographic paper. The paper strip was then dipped in a buffer solution from both ends, leaving a narrow dry band around the application line. After gentle blotting, the strip was sandwiched between the two Pyrex glass plates of the H.V.E. chamber, and the buffer solution was allowed to spread over the application line by diffusion from both ends (10–15 min). Following 20 to 30 min of the "equilibration" of the paper under a low current (6mA, 300 V), the high voltage run was performed at pH 2·2 (pyridine–formic–acetic acid buffer) for 1·5 h at 2000 V, 70 mA. After the completion of the run, the electropherogram was dried in a hood. It was then cut into 4 cm-wide strips which were scanned for the distribution of radioactivity in a Nuclear Chicago Actigraph III Radiochromatogram Scanner, Model 4995.

Three peaks of radioactivity were, as a rule, detected in each specimen (Fig. 1, I): a small peak ("N"), which stayed on the application line or moved slightly towards the cathode; a high broad peak (G), corresponding to the glucuronide conjugates which migrated at pH 2·2 only very slowly towards the anode; and a small but fast moving peak migrating towards the anode (S), which corresponded to the sulfate conjugates. However, the separation of each peak (especially the separation of N from G) was not clear-cut during this first run on H.V.E., since recovery experiments revealed that each of the separated groups of conjugates was "contaminated" to some extent with another group. A complete separation of all steroid groups was achieved by five additional successive electrophoretic runs, as depicted in Fig. 1. For a complete separation of sulfate conjugates only, one additional run at pH 2·2 was sufficient (Fig. 1, III).* During that run, the sulfate peak (MS) was clearly separated from the small

normotensive subjects (N); furthermore, some of these polar steroids co-precipitated with plasma proteins more readily than all the other steroid metabolites (unpublished observations). Since the ultimate purpose of the present study has been to compare plasma and urinary levels of individual corticosteroid metabolites in H and N, it was decided to process the extract of precipitated plasma proteins separately from the main extract of deproteinized and defatted plasma, in order not to miss possible differences between H and N, in the levels of these steroids, should they be formed as peripheral metabolites of cortisol. From our previous studies[32, 45, 48], it was not possible to draw conclusions as to their origin, since these studies were not conducted with radioisotopic tracers, and the steroids in question could have been formed either as peripheral metabolites or as direct secretory products of the adrenal cortex (for a pertinent discussion see [45]). However, in the presentation of the quantitative data in this paper (Table 4), pertaining only to normotensive subjects, the values for the concentration of identical steroids present in both extracts (deproteinized plasma and precipitated proteins) were combined.

* Additional electrophoretic runs were performed on S and N steroid metabolite groups pooled from all plasma specimens (Fig 1, VI, VII), to achieve steroid concentrations sufficient for the subsequent chromatographic separation of individual metabolites present in each of these groups; the concentrations of glucuronide conjugates were sufficient for the separation of individual metabolites in individual plasma specimens (to be published).

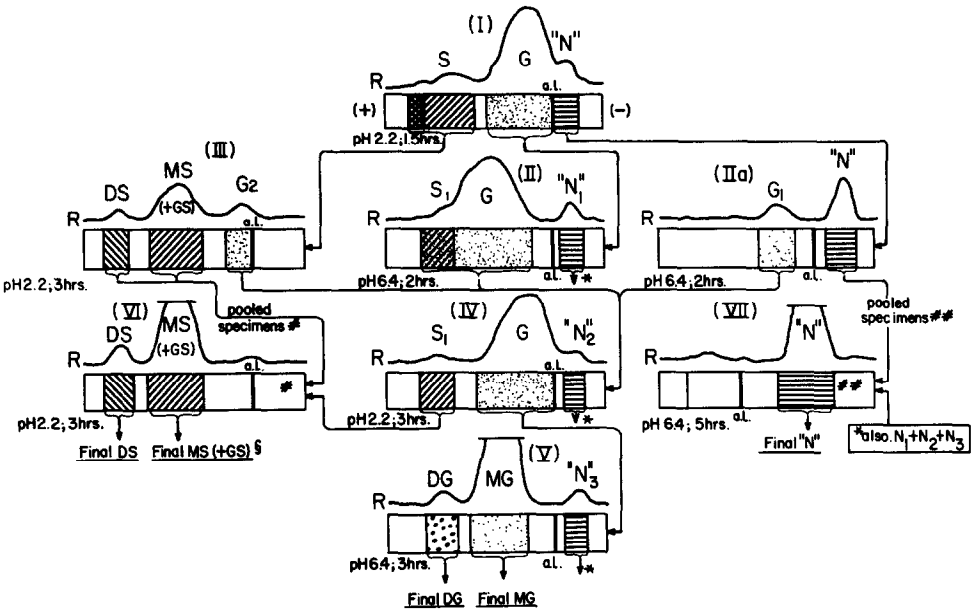

Fig. 1. Separation of various groups of conjugated cortisol metabolites from plasma by means of high voltage paper electrophoresis. All runs performed at 2000 V; at pH 2·2 the current was 70 mA, at pH 6·4, it was 55–60 mA. The prolonged rectangles represent electropherograms, the lines above them (marked R) are the radioactivity scans. Roman numerals (in parentheses) designate numbers of consecutive runs; runs II and IIa were performed simultaneously, on the same paper strip, 23 cm-wide.

G—glucuronide conjugates (MG + DG); MG—monoglucuronides; DG—diglucuronides; S—sulfate conjugates (MS + DS + GS); MS—monosulfates; DS—disulfates; GS—glucuronosulfates; "N"—very polar steroids (6-hydroxy, 20-reduced metabolites) presumably complexed to nucleosides; a subscript number indicates that these conjugates were separated as a minor "contaminating" component from a major group with which they traveled together during a preceding run; a.l.—application line.

Additional purification (by H.V.E. at pH 2·2) of pooled MS and DS from all plasma specimens, prior to paper chromatographic separation and identification of individual steroid metabolites (see text).

§ The separation of glucuronosulfates (GS) from monosulfates (MS) was achieved in the majority of specimens by means of paper chromatography instead of electrophoresis at pH 6·4[17], as it was done in urinary specimens (see text).

Additional purification (by H.V.E. at pH 6·4) of pooled "N" from all plasma specimens, prior to the separation and identification of individual steroids contained in this fraction[6].

amount of the "contaminating" glucuronide conjugates (G_2). In some specimens, the amount of disulfate conjugates (DS) was large enough to give a detectable, clearly separable, second peak of radioactivity. The conjugates (MS + DS) were eluted, the vol. of the eluate was measured, and 2·1 ml aliquots were counted for radioactivity ("sulfate count"). The eluate of the small glucuronide peak was combined with the main glucuronide fraction (Fig. 1, IV). All elutions of the conjugates from paper were done with a 1:3 mixture of *n*-butanol–70% aqueous methanol, followed by absolute methanol (15 ml and 10 ml of each solvent, respectively, per two, jointly eluted, 3 × 10 cm paper strips), in a manner previously described[24].

To achieve steroid concn sufficient for the subsequent separation of individual steroid metabolites (see below), and to obtain a meaningful separation of disulfates from monosulfates, the eluates of both sulfate fractions (MS + DS) from all plasma specimens were pooled and again subjected to H.V.E. at pH 2·2, at 2000 V, 70mA, for 3 h. The pooled disulfates, traveling approx. 1·8 times faster than the monosulfates.* separated distinctly from the latter, and were readily detectable (Fig 1, VI). During this run, a minute amount of "contaminating" glucuronide conjugates still separated from the sulfates. Areas corresponding to each radioactive peak were cut out and eluted separately.

* The monosulfates separated this way also contained glucuronosulfate conjugates; the latter were separated during the subsequent paper chromatography in K_4 system as described on the following pages.

The elutes were measured in vol. and two 1-ml aliquots were taken for radioactivity measurement. (The glucuronide peak was added to the main glucuronide fraction.) The purity of the separated mono- and disulfate conjugates was checked again by rerunning on H.V.E. under similar conditions (pH 2·2, 2000 V, 70 mA, 2·5 h). Standard steroid conjugates (F-S and THF-diS) were applied and run on a parallel strip. Scanning of radioelectrophoretograms showed that each fraction gave a single peak with the same mobility as that of the respective standard.

F. Separation of steroids in the monosulfate conjugates fraction

Monosulfate conjugates eluted from H.V.E. were redissolved in a small vol. of the *n*-butanol–70% aqueous methanol (1:3, v/v) mixture and were chromatographed on paper (Whatman No. 3MM) in the K_4 system (Table 1) for 27 h (5·5 × front) at 28°C. The run-offs were collected in beakers.

The scanning of the strips showed seven well-separated peaks of radioactivity. Each peak was eluted from paper and the eluate was evaporated to dryness under nitrogen. The run-offs were also evaporated. The residues were redissolved in 5 ml of the *n*-butanol–aqueous methanol mixture. Two 0·5-ml aliquots of each eluate were used for counting radioactivity. One 1-ml aliquot was saved for the determination of molar ratios of steroid-to-sulfate moieties.

The remaining solutions were evaporated to complete dryness and subjected to a modified solvolysis procedure[25]: each dry residue was made wet with four drops of H_2O and dissolved in 6 ml of absolute ethanol. Thirty ml of equilibrated* ethyl acetate was added to each ethanolic soln. After 40 h of incubation at 37 C, the solvolysates were neutralized with a few drops of NH_4OH and evaporated to dryness.

Since the residues were not heavy, they were directly chromatographed on paper (Whatman No. 2). A 1:1 v/v methanol–chloroform mixture was used to dissolve the solvolyzed steroids. The systems used for the separation of individual steroids and the sequence in which they were used, are shown in Table 1 and Fig 2. Proper authentic standards (10 to 20 μg each) were chromatographed along with the unknowns. A separate chromatogram with only pure solvent (methanol–chloroform mixture) applied to it was run simultaneously in the same tank, to serve as a "paper blank". Both papers were impregnated with the mobile phase, by equilibration for at least 2 h and were developed for the length of time indicated in Fig 2. The chromatogram was then scanned, and the positions of the radioactive peaks were related to those of authentic standards. The radioactive areas were then eluted, each with 15 ml of 85% aqueous methanol, followed by 10 ml of absolute methanol. The eluates were evaporated to dryness and consecutively chromatographed in other systems, until a complete separation of single steroid moieties was achieved (Fig 2). These were then eluted and used for the identification study by reverse isotope dilution (RID) technique, with derivative formation (see below).

G. Quantitation of steroid and sulfate moieties and determination of steroid:sulfate molar ratio

A 1/5 vol. aliquot of the eluate of peak 3 from K_4 chromatogram was used for this purpose. This fraction was known to contain cortolones, THF and 5α-THF. The study of urinary sulfates clarified that all these compounds were conjugated with sulfate at 3-hydroxyl

* Saturated with 20% aqueous solution of NaCl, acidified to pH 1.

Table 1. Paper chromatographic systems

System designation	Solvents (by vol.)	Whatman paper No.	Running time to front (h)	Ref.
K_4	Benzene–isopropyl alcohol–water (100:70:30)	3 MM	5–6	17
K_5	Petroleum ether–ethyl acetate–isopropyl alcohol–water (70:30:85:20)	3 MM	7	17
Y	Ethyl acetate–chloroform–methanol–water (25:75:50:50)	2	6–7	26
B_P	Benzene–chloroform–methanol–water (50:50:50:50)	2	4	25
B_5	Benzene–methanol–water (1,000:525:475)	2	3–4	27
B_5 (5% boric)	(Same as B-5) Before application, paper was dipped in 5% boric acid and dried in a hood	2	3–4	17
S-L_{10}	Toluene–tert-butanol–methanol–0·02 M boric buffer (pH 9·0) (170:40:30:100)	2	8	28
E_2B	Isooctane–tert-butanol–water (500:250:450)	2	6	29
B_1	Petroleum ether–toluene–methanol–water (25:25:35:15)	2	3·5–4	27
B_3	Petroleum ether–benzene–methanol–water (33:17:40:10)	2	4	27

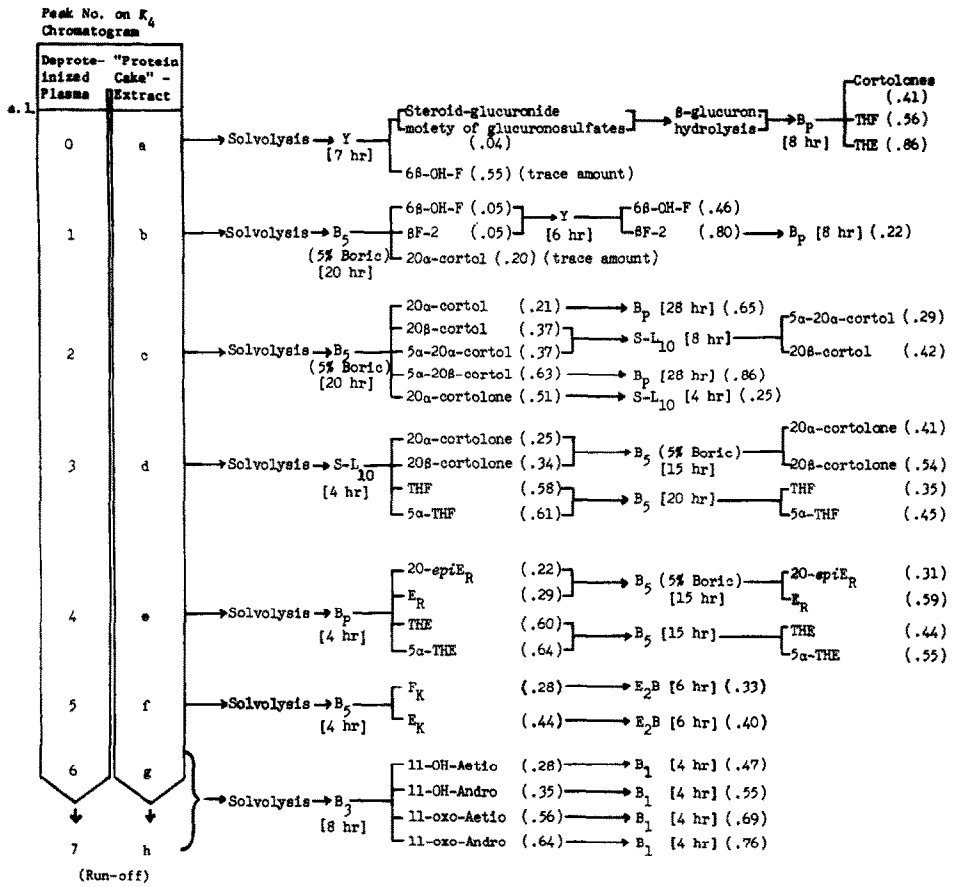

Fig. 2. Paper chromatographic separation of individual monosulfate- and glucuronosulfate-conjugated metabolites of cortisol from plasma. Flowsheet of the procedure. The electrophoretically isolated group of steroid-sulfates was separated into seven sub-groups: glucuronosulfates (0, a) and monosulfates (1–6, b–g) (for details see text). The steroid moieties of monosulfates were then cleaved by solvolysis, those of glucuronosulfates by consecutive solvolysis and β-glucuronidase hydrolysis, and were subjected to multiple paper chromatographies, until a complete resolution of individual steroid metabolites was achieved. For designation of chromatographic systems, see Table 1; numbers in brackets indicate the length of runs; numbers in parentheses indicate R_f values or, when systems overrun, steroid mobilities relative to the length of paper chromatogram; a.l.—application line on K_4 chromatogram.

position[17]. It was therefore expected that after chromic acid oxidation all of these steroid sulfates would be converted to either 11-oxo-androsterone-3-sulfate or 11-oxo-aetiocholanolone-3-sulfate[17]. The sample was oxidized with chromic acid as described in the section "Derivative Formation". To extract the resultant 17-ketosteroid sulfate from chromic acid solution, a small Amberlite XAD-2 (5 g) column chromatography was used: the sample was percolated through the column, followed by 30 ml of water to wash out the acid. The oxidized steroid conjugates were then eluted with 30 ml of methanol. The methanolic eluate was evaporated to dryness and the residue was chromatographed in K_4 system (single length run), along with reference standards 11-oxo-androsterone-sulfate and 11-oxo-aetiocholanolone-sulfate (30 μg of each). The scanning of radioactivity showed a single peak with an R_f (0·32)* identical with that of both standards. The radioactive area was eluted with the *n*-butanol 70% aqueous methanol mixture.

A 1/3 vol. aliquot of this eluate was used for 17-ketosteroid determination by Zimmerman color reaction, using 11-oxo-aetiocholanolone-sulfate as a standard. (Both 11-oxo-aetiocholanolone-sulfate and 11-oxo-androsterone-sulfate have almost the same molecular extinction in Zimmerman color reaction). The

* Steroid sulfates conjugated at C-21 do not oxidize to 17-ketosteroids and, during subsequent chromatography in K_4 system, have an R_f of 0·15[17].

remaining 2/3 vol. of this eluate was analyzed for organic sulfate by a modification of the methylene blue method of Roy[18], see Methods, *Quantitation of organic sulfates*.

The precision of the methods used for determination of the sulfate/steroid ratios, expressed by S.E., calc. from 32 duplicate determinations of monosulfate conjugates isolated from urine[17], was 1·8% for the Zimmerman color reaction (estimation of the steroid moieties) and 2·1% for the methylene blue method (estimation of the sulfate moiety).

H. Identification of steroid moieties

The confirmation of the identity of the separated (by multiple consecutive chromatographies) radioactive steroids was done according to a modification of the method described by Berliner and Salhanick[30]: 0·3–0·9 μM of a nonradioactive steroid carrier of a structure identical with that postulated for a given radioactive metabolite was mixed with the separated radioactive steroid (in ethanolic solution). For the determination of S.A. of this mixture two 1/10 vol. aliquots were taken for radioactivity measurement and two 1/20- to 1/10 aliquots were used for chemical quantitation. The same size aliquots were taken from the solution of the "paper blank" residue.* The remaining steroid material was used for the formation of derivatives: C-20 hydroxy-steroids were oxidized with sodium bismuthate, while C-20 keto-steroids were acetylated and/or oxidized with chromic acid, see Methods, *Derivative formation*. C_{19} steroids (17-keto-metabolites) were oxidized with chromic acid. The derivatives formed were then chromatographed on paper. Whatman No. 2 filter paper was cut in such a way as to give three chromatographic strips with identical width and length, connected through a common head (and a bridge at the bottom, if the chromatograms were not to be overrun). The width of the individual strips depended on the amount of radioactivity and on the amount of residue to be applied. The sample, paper blank residue, and reference standards were applied to each of these strips, and the chromatograms were developed in B_1 or B_3 systems. If a postulated derivative standard was not available, the mother steroid was treated the same way as the unknown and was used as a reference standard. The radioactive peak area corresponding to the standard on the adjacent strip was eluted with 85% aqueous methanol, followed by absolute methanol. The corresponding area of paper blank was also eluted. Both eluates were evaporated to dryness and redissolved in 8 ml of ethanol. Two 2-ml aliquots of this solution were used for color reaction, while two 2-ml aliquots were used for measurement of radioactivity.

* In our study of urinary steroid sulfates[17], the mixture of the unknown compound and the nonradioactive standard was chromatographed again before obtaining S.A.; but in the study of plasma steroids, this chromatography was omitted in view of: (1) the considerably greater purity of the isolated compounds, and (2) the undesirability of a further loss of radioactivity, which would hamper the detection of radioactive peaks on the chromatograms of the derivatives of compounds present in very low concentrations.

I. Separation of steroids in glucuronosulfate fraction

During H.V.E. at pH 2·2, glucuronosulfate conjugates moved together with the monosulfates. They were then separated from the latter, as one group, by paper chromatography in K_4 system. The glucuronosulfates yielded the most polar peak on this chromatogram (Fig. 2; 0,a). They were eluted from paper and subjected to solvolysis by the same procedure as that used for the cleavage of monosulfates. The resulting steroid-glucuronides were subsequently separated from traces of 6α- and 6β-OH-F (released from polar monosulfates) by chromatography in system Y: the conjugates stayed at the origin of the chromatogram (Fig. 2). They were then eluted with the *n*-butanol–70% aqueous methanol mixture and the eluate was evaporated to dryness. The residue was redissolved in 10 ml of water, buffered with 2 ml of 2 M acetate buffer, pH 4·5, and hydrolyzed with 5 ml of β-glucuronidase (Ketodase, 5000 U/ml) for 72 h at 37 C. The hydrolysate was then extracted twice with 3 vol. of ethyl acetate, the extracts were evaporated to dryness, and chromatographed in B_P system. The separated steroid moieties were identified by reverse isotope dilution technique, as described for monosulfate conjugates.

J. Separation of steroids in disulfate fraction

The pooled eluates from high voltage electrophoretograms, containing steroid disulfates, were solvolyzed according to the same procedure as that described above for the monosulfate conjugates. The solvolysate was neutralized, evaporated to dryness and chromatographed in B_5 system for 17 h. The scanning of the chromatogram showed a single radioactive peak with the same mobility as that of authentic cortolones. This chromatogram area was eluted and the steroids present in the eluate were rechromatographed in B_5 system for 15 h, using paper pre-impregnated with 5% boric acid, to separate 20α-cortolone from 20β-cortolone.

RESULTS

A. Electrophoretic separation and quantitation of various groups of free and conjugated metabolites of cortisol

Steroid monosulfates, disulfates and glucuronosulfates were separated from each other, and from other

Table 2. Radioactivity and relative concentrations of various groups of free and conjugated metabolites of cortisol in plasma
(2·5 h after i.v. administration of 15–20 μC [4-^{14}C]-cortisol to 11 normal subjects)

Steroid Group	Radioactivity	Relative concentration
	d.p.m./100 ml Plasma	Total metabolites (%)
Free steroids	56,730 ± 27,110*	46·1 ± 9·6*
Total conjugates	61,220 ± 14,720	53·9 ± 9·6
Total metabolites	117,950 ± 37,120	100·0
Total radioactivity-plain plasma	122,620 ± 37,190	
		Total conjugates (%)
Sulfates	2360 ± 470	5·3 ± 1·9
Glucuronides	41,820 ± 9830	87·4 ± 2·7
Other conjugates ("N")	3690 ± 910	7·3 ± 1·8
Total conjugates	47,870 ± 10,870	100·0
	d.p.m. Pooled specimens†	Total sulfates (%)
Monosulfates	49,010	76·0
Disulfates	2980	4·7
Glucuronosulfates	12,480	19·3
Total sulfates	64,470	100.0

* Mean ± S.D.
† Pooled electrophoretically separated sulfates from all plasma specimens.

groups of steroid conjugates, by means of H.V. paper electrophoresis, as shown in Fig. 1. It will be seen that it was necessary to subject each of the initially separated groups of steroid conjugates to one or more additional electrophoretic runs, in order to separate from each group a small amount of "contaminating" conjugates of another group (or groups). During the initial separations at pH 2·2, steroid glucuronosulfates ran together with the group of steroid monosulfates (at this pH the glucuronide moiety is only very slightly ionized). Although it was possible to obtain a clear-cut separation of these two groups during a subsequent electrophoresis at pH 6·4, as it was done in our study of urinary steroid sulfates[17], it was found that when this step was not carried out, the glucuronosulfates separated as a group very distinctly from the steroid monosulfates during the subsequent paper chromatography in K_4 system. Therefore, this method of the isolation of glucuronosulfates was finally adopted, as it was time saving and more convenient.

The amount of radioactivity present in each group of the separated "free" and various conjugated metabolites of the injected tracer [4-^{14}C]-cortisol and their relative concn are shown in Table 2. It will be seen that while all conjugates in plasma constituted 44–64% of all cortisol metabolites (which is consistent with the results of our other studies[20, 21, 31–33]),‡ the total sulfate conjugates constituted 3·4–7·2% of all conjugated metabolites, i.e. about 2–3% of *all* metabolites of cortisol. The main bulk of the sulfate conjugates consisted of steroid-monosulfates (76%), while the rest was made up of glucuronosulfates (19%) and disulfates (5%). These proportions correspond well to those found by us in urine[17].

Furthermore, it will be seen from Table 2 that there was an excellent agreement between the total "plain plasma count" and the sums of radioactivity of the free steroids, extracted with ethyl acetate from deproteinized and defatted plasma (including also the steroids re-extracted from the precipitated proteins), and the conjugated steroids extracted by means of the amberlite column. Thus, the average steroid loss during the procedures of deproteinization and defatting of

‡ The agreement of these results with those of our other studies, in which no radioactive tracers were employed, indicates that at 2·25 to 2·5 h after the injection of the tracer, some state of an equilibrium is reached between the rates of formation and elimination (excretion) of the radioactive metabolites.

plasma, the re-extraction of the precipitated proteins, and the extraction of total free and total conjugated steroids was less than 5%.

Furthermore, it will be seen that during all the multiple electrophoretic runs, an average loss of about 20% of radioactivity has occurred. Thus, the overall recovery of all cortisol metabolites during all separation procedures, until the step of paper chromatography, was better than 75%. An additional loss of 20–35% (of the original radioactivity) occurred during all paper chromatographies (including the hydrolytic or solvolytic procedure), this resulting in a final recovery during the entire procedure of 45–50% of the original radioactivity. In view of the considerable complexity of the method, this was considered to be a very good recovery. This was also found to be a very consistent figure for different plasma specimens, and different steroid metabolites.

B. Separation, identification and quantitation of steroid monosulfates

In view of the scarcity of the available material (even though fractions isolated from all plasma specimens were pooled, this being equivalent to 3045 ml of plasma, separation of individual sulfate conjugated steroids, *as conjugates*, was not undertaken, as it was done with the urinary sulfates[17]. Only six subgroups of plasma steroid monosulfates were separated as conjugates by chromatography in K_4 system. A group of glucuronosulfate conjugates was also separated during that chromatography (see below). Each of the separated sub-groups was then subjected to cleavage by solvolysis. The liberated steroid moieties present in the solvolysate of each sub-group were separated by multiple chromatographies and were identified by reverse isotope dilution technique with derivative formation. The results are shown in Table 3. The steroidal composition of each of the separated sub-groups of steroid monosulfates (and also the group of steroid glucuronosulfates [see below]) was found to be identical with that of analogous sub-groups previously isolated by us from urine, where all *individual* monosulfate conjugates were subsequently separated *as conjugates*. This then makes highly plausible the assumption that the sulfate conjugated cortisol metabolites isolated by us from plasma are identical with those isolated and identified from urine[17].

The steroid/sulfate molar ratio, determined on a total sub-group no. 3 of steroid monosulfates (separated by chromatography in K_4 system), containing 20α- and 20β-cortolone, tetrahydrocortisol and 5α-tetrahydrocortisol, was found to be 1:1 (steroid moiety, 0·0707 M; organic sulfate, 0·0687 M). This corresponds very well with the data derived from the determination of sulfate/steroid molar ratios of identical compounds separated from urine as individual conjugates[17]. Furthermore, the chromatographic mobilities of the isolated steroid monosulfates (within the seven sub-groups separated in K_4 system), together with the results of the estimation of steroid/sulfate molar ratios, strongly suggest that the sites of conjugation of these steroids are the same as those of the analogous steroid sulfate conjugates isolated from urine[17].

Table 4 presents the results of the quantitation of individual steroid monosulfates in plasma. It should be kept in mind that the values shown in the Table represent concentrations of the listed steroid metabolites at a definite time, that is 2·25 to 2·5 h, following i.v. administration of the tracer [4-^{14}C]-cortisol. It will be seen that the compounds present in the largest concentrations were: 20β-cortolone-3-sulfate (17% of all monosulfate conjugates), 5α-tetrahydrocortisol-3-sulfate (14%), 6β-hydroxycortisol-21-sulfate (9%), cortisol-21-sulfate (8%), tetrahydrocortisol-3-sulfate (7%), and 20β-dihydrocortisol-21-sulfate (6%). The same steroids were found by us to constitute the major sulfate conjugated metabolites in urine[17]. When groups of metabolites are considered, the following findings are striking: (1) a large proportion of metabolites non-reduced in ring A, conjugated at C-21 (27%); (2) relatively large concentration of 5α-reduced metabolites with dihydroxyacetone side-chain (5α-THF and 5α-THE) (18%); (3) a high proportion of ring-A reduced steroids (both 5α and 5β) with 20-ketone reduced (glycerol side-chain) (31%) vs those with 17,21-dihydroxy-20-keto side chain (29%). All of these findings are in full agreement with the data on the concentrations of urinary sulfoconjugated metabolites of cortisol[17].

C. Separation, identification and quantitation of steroid disulfates and glucuronosulfates

Steroid disulfates were separated by means of H.V. paper electrophoresis at pH 2·2. Their electrophoretic mobility at two different pH's (2·2 and 6·4) was identical with that of standard tetrahydrocortisol-3,21-disulfate, synthesized by us[35], and with that of disulfate conjugated metabolites of cortisol isolated from urine[17]. The disulfates eluted from electropherograms were solvolyzed; the two steroids cleaved (20α- and 20β-cortolone) were then identified by reverse isotope dilution with derivative formation (Table 5). The same two steroids were identified following cleavage of urinary disulfate conjugated metabolites of cortisol[17]. The latter contained also tetrahydrocortisol-3,21-disulfate; this steroid however was not present

Table 3. Identification of steroid moieties of monosulfate conjugates (*RID*)

Separated steroid moiety following cleavage of the conjugate by solvolysis (cf. Fig. 2)*,†	Paper chromatography‡	Nonradioactive carrier added§	S.A. (d.p.m./μM)	Chemical reaction applied	Derivative formed*	Chromatography of derivative, system‡ (R_f)‖	Specific activity of derivative (d.p.m./μM)
6β-OH-F	B_5 (5% Boric),	6β-OH-F	808	CrO_3 oxidation	6-oxo-Adrenost.	B_1 (0·65)	781
βF-2¶	Y, B_P	βF-2¶	708	CrO_3 oxidation	11-oxo-A-2¶	B_1 (0·48)	702
20α-cortol		20α-cortol	821	$NaBiO_3$ oxidation	11-OH-Aetio	B_1 (0·41)	831
20β-cortol	S-L_{10},	20β-cortol	1103	$NaBiO_3$ oxidation	11-OH-Aetio	B_1 (0·41)	1121
5α-20α-cortol	B_5 (5% Boric)	5α-20α-cortol	—	$NaBiO_3$ oxidation	11-OH-Andro	B_1 (0·53)	—
5α-20β-cortol		5α-20β-cortol	—	$NaBiO_3$ oxidation	11-OH-Andro	B_1 (0·51)	—
20α-cortolone**	S-L_{10},	20α-cortolone	417	$NaBiO_3$ oxidation	11-oxo-Aetio	B_1 (0·65)	405
20β-cortolone**	B_5 (5% Boric)	20β-cortolone	1670	$NaBiO_3$ oxidation	11-oxo-Aetio	B_1 (0·65)	1651
THF		THF	559	acetylation	THF-diacetate	B_1 (0·77)	569
5α-THF	S-L_{10}, B_5	5α-THF	1335	acetylation	5α-THF-diacetate	B_1 (0·77)	1297
20-*epi*E_R	B_P,	20-*epi*E_R	103	$NaBiO_3$ oxidation	11-OH-Δ⁴A	B_1 (0·59)	111
E_R	B_5 (5% Boric)	E_R	424	$NaBiO_3$ oxidation	11-OH-Δ⁴A	B_1 (0·59)	410
THE	B_P, B_5	THE	2332	acetylation	THF-diacetate	B_1 (0·78)	2360
5α-THE	B_P, B_5, then acetylated & chromatographed in B_1	5α-THE-diacetate	1366	CrO_3 oxidation	11-oxo-andro-acetate	B_1 (0·89)	1398
F_K	B_5, E_2B	F_K	886	acetylation	F_K-acetate	B_1 (0·28)	860
E_K		E_K	170	acetylation	E_K-acetate	B_1 (0·41)	162
11-OH-Aetio		11-OH-Aetio	629	CrO_3 oxidation	aetiocholanetrione	B_3 (0·69)	642
11-OH-Andro	B_3, B_1	11-OH-Andro	168	CrO_3 oxidation	androstanetrione	B_3 (0·64)	160
11-oxo-Aetio		11-oxo-Aetio	795	CrO_3 oxidation	aetiocholanetrione	B_3 (0·69)	778
11-oxo-Andro		11-oxo-Andro	195	CrO_3 oxidation	androstanetrione	B_3 (0·64)	178

* For steroid abbreviations and nomenclature see Methods, *Quantitation of steroids*, footnote*.

† Postulated configurations based on chromatographic mobilities identical with those of authentic steroid standards; chromatographic systems used listed in the next column.

‡ For designation of chromatographic systems, see Table 1.

§ The mobility of the unknown identical with that of the carrier added.

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

¶ βF-2: 11β,17α,21-trihydroxy-5α-pregnane-3,6,20-trione, an artifact of 6β-OH-F formed during solvolysis [34]. 11-oxo-A-2: 5α-androstane-3,6,11,17-tetrone.

** The 5α isomers of cortolones were not detectable on chromatograms.

Table 4. Quantitation of individual steroid monosulfates

Steroid	Presumed Site of conjugation*	(d.p.m.†)	Total monosulfate conjugates (%)
6β-OH-F	21-yl-sulfate	4448	9·1
20α-cortol	3α-yl-sulfate	1595	3·3
20β-cortol	3α-yl-sulfate	732	1·5
5α-20α-cortol	3α-yl-sulfate	1473	3·0
5α-20β-cortol	3α-yl-sulfate	705	1·4
20α-cortolone	3α-yl-sulfate	2252	4·6
20β-cortolone	3α-yl-sulfate	8578	17·5
THF	3α-yl-sulfate	3617	7·4
5α-THF	3α-yl-sulfate	6921	14·1
20-*epi*E_R	21-yl-sulfate	344	0·9
E_R	21-yl-sulfate	3154	6·4
THE	3α-yl-sulfate	2329	4·8
5α-THE	3α-yl-sulfate	1680	3·4
F_K	21-yl-sulfate	4073	8·3
E_K	21-yl-sulfate	909	1·9
11-OH-Aetio	3α-yl-sulfate	1705	3·4
11-OH-Andro	3α-yl-sulfate	1189	2·4
11-oxo-Aetio	3α-yl-sulfate	2053	4·1
11-oxo-Andro	3α-yl-sulfate	1255	2·5

* Partly indirect evidence based on the analogy with urinary monosulfate conjugates (see text).

† Each value in this column represents a sum of radioactivity count of a given steroid isolated from deproteinized and defatted plasma, and that of an identical steroid isolated from the extract of precipitated proteins [steroids co-precipitated with proteins were re-extracted with ethanol and water-saturated *n*-butanol, and were processed separately from those contained in the main deproteinized plasma extract (see text)]. All values corrected for losses during chromatographies.

in plasma in concentrations sufficient for identification studies.

Steroid glucuronosulfates were readily separated from monosulfates by means of chromatography in K_4 system. The reasons for choosing this method for their separation, rather than H.V.E. at pH 6·4, were presented on the preceding pages. Table 5 lists steroid moieties released from these conjugates by means of successive solvolysis and β-glucuronidase hydrolysis, and identified by RID. They were identical with those isolated from urine[17]. Presumptive evidence has been recently obtained, using glucuronosulfate conjugates isolated from urine, that the sulfate moiety is attached to 21-hydroxyl group (to be published).

Plasma concn of individual glucuronosulfate-conjugated metabolites of cortisol are shown in Table 6. The two major steroids in this group were, like in urine[17], tetrahydrocortisol and tetrahydrocortisone. The concn of steroid moieties of disulfate conjugates, 20α- and 20β-cortol, were approx. equal.

DISCUSSION

This paper reports isolation and identification of a whole spectrum of sulfate conjugated metabolites of cortisol in plasma: 19 monosulfates, two disulfates and six glucuronosulfates. The steroid metabolites found in plasma are identical with those isolated from urine[17]. This reaffirms the consistent presence of these compounds as metabolites of cortisol, and points to the importance of sulfation as a significant, though quantitatively minor, pathway in the peripheral metabolism of glucocorticoids.

While the question of the biological importance of sulfate conjugated steroids in general remains unsettled, it is now well-established that certain steroid sulfates are the actual secretion products of steroid-producing glands[3, 4]. It has been also demonstrated that steroid sulfates may undergo "direct" metabolism of the steroid moiety, without prior cleavage of the sulfate moiety[36–39]. Furthermore, certain steroid sulfates, have been shown to be markedly more effective precursors in steroid biosynthetic pathways than the corresponding "free" steroids[5, 40]. It has also been suggested that steroid sulfates may have a regulatory role in steroid binding and transport[4, 41].

The data presented in this paper reveals significant quantitative differences in the steroidal composition of sulfate vs glucuronide conjugated cortisol metabolite fractions:

Table 5. Identification of steroid moieties of glucuronosulfate and disulfate-conjugated metabolites of cortisol (*RID*)

Postulated steroid metabolite*,†	Chromatography‡	Nonradioactive carrier added	S.A. (d.p.m./μM)	Chemical reaction applied	Derivative formed*	Chromatography of derivative‡,§	S.A. of derivative (d.p.m./μM)
Glucuronosulfates							
Cortols‖	B_P (×2) (0·16)	20α-cortol	432	$NaBiO_3$ oxidation	11-OH-Aetio	B-1 (×1) (0·41)	450
Cortolones‖	B_P (×2) (0·39)	20β-cortolone	480	$NaBiO_3$ oxidation	11-oxo-Aetio	B-1 (×1) (0·64)	491
THF	B_P (×2) (0·56)	THF	1421	acetylation	THF-diAc	B-1 (×1) (0·84)	1401
THE	B_P (×2) (0·86)	THE	1561	acetylation	THE-diAc	B-1 (×1) (0·89)	1590
Disulfates							
20α-Cortolone	B-5 (5% Boric) (×4) (0·40)	20α-cortolone	354	$NaBiO_3$ oxidation	11-oxo-Aetio	B-1 (×1) (0·62)	367
20β-Cortolone	B-5 (5% Boric) (×4) (0·56)	20β-cortolone	354	$NaBiO_3$ oxidation	11-oxo-Aetio	B-1 (×1) (0·62)	367

* For steroid abbreviations and nomenclature see Methods. *Quantitation of steroids*, footnote*.

† 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. (×1) = single length run; (×4) = four times single length run (overrun three times); second number in brackets indicate R_f value or, when systems overrun, steroid mobility relative to the length of paper chromatogram.

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

‖ Because of the very small amount of glucuronosulfate conjugates available, separation of α- and β-isomers of these steroids was not carried out.

Table 6. Quantitation of individual glucuronosulfate (GS) conjugated metabolites of cortisol

Steroid	(d.p.m.*)	Total GS (%)
Cortols	225	2·5
Cortolones	1367	11·0
THF	5272	42·3
THF	5608	44·9

* See footnote † to Table 4.

(1) Steroids with both ring-A and 20-ketone reduced (cortols and cortolones) constitute the largest group amongst the sulfate-conjugated cortisol metabolites (32%), whereas the bulk of glucuronide conjugates consists of ring-A reduced steroids with non-reduced 20-ketone (tetrahydrocortisol, tetrahydrocortisone, and their 5α-epimers). This finding is in full agreement with the results of our study of various cortisol metabolites in urine[17]. Furthermore, the results of our study of urinary steroid sulfates demonstrated that, following i.v. administration of tracer [4-^{14}C]-cortisol, the sulfate conjugated metabolites with glycerol side-chain were excreted much faster than those with dihydroxyacetone side-chain. The correlation of the findings in plasma and urine permits the deduction that sulfates of steroids with reduced 20-ketone are formed faster than those of the corresponding metabolites with dihydroxy-acetone side-chain. The possible explanation of this finding is that the metabolites with glycerol side-chain are better substrates for the pertinent sulfokinases than the corresponding compounds with dihydroxyacetone side-chain, whereas the reverse appears to be true with regard to glucuronyl-transferases. The implication of this finding is that, sterically, the side-chain is not indifferent to the enzyme, even when the latter is effecting the conjugation at C-3 position, that is, at the opposite end of the longitudinal axis of the steroid molecule. An alternative explanation is that differences in the intracellular compartmentalization of ring-A reductases vs 20-ketone-reducing enzymes, and sulfokinases vs glucuronyl-transferases are responsible for this finding. A possibility also exists that the 3-sulfates of steroid metabolites with non-reduced 20-ketone are formed not at a slower rate but are retained longer intracellularly than the corresponding conjugates of steroids with the glycerol side-chain.

(2) A large portion (27%) of plasma steroid monosulfates consists of steroids conjugated at C-21 (F_K, E_K, E_R, 20-epiE_R, 6β-OH-F). This contrasts with the glucuronide conjugates, only a very minute fraction of which consists of metabolites conjugated at C-21[42, 43]. From the correlation of this data with that on the urinary excretion of various monosulfate conjugated metabolites of cortisol[17, 44], it can be deduced that the production of C-21 sulfated metabolites* is a much faster process than the production of C-3 sulfates: this is not surprising in view of the fact that for the latter conjugates to be formed, the steroid molecule has first to undergo ring-A reduction (prior to the conjugation), a step known to be a rather slow, rate-limiting reaction[2].

(3) Another striking difference between glucuronide and sufate-conjugated metabolites of cortisol is a much higher ratio of 5α- to 5β-reduced steroids (5α-THF/THF; 5α-THE/THE) amongst the sulfate conjugates than amongst the glucuronide conjugates[2, 43, 45].

Finally, the presence of 6β-hydroxylated cortisol as a sulfate conjugate, and in relatively large concentration (9% of all monosulfates), deserves special comment. This steroid has been generally considered to exist only as a "free" metabolite[26, 46]. The high polarity conferred to the molecule by 6-hydroxylation was thought to assure its ready excretability with urine. In teleological terms, therefore, there appears to be no need for its conjugation. We have, however, already previously reported the isolation from urine of 6β-hydroxycortisol as a conjugate with sulfate[17, 25]. Moreover, we have obtained recent evidence that also 6α-hydroxycortisol, and both 20α- and 20β-dihydroderivatives of 6α- and 6β-hydroxycortisol, are present as sulfate conjugates (to be published). Concentrations of 6-hydroxylated steroids *reduced* at C-20 have been found by us previously to be very markedly increased in patients with essential hypertension[6], and by Pennington in hypertension occurring during pregnancy[47]. The meaning of these findings is not known at present.

In conclusion, the existence of so many steroid metabolites in the sulfate conjugated fraction which, *in toto*, constitutes only 2·3% of all metabolites of cortisol, and certain quantitative and qualitative aspects of the composition of this fraction provoke a conjecture that the sulfoconjugated metabolites, or at least some of them, may have a specific, as yet unknown, biological function.

* These metabolites were found in the highest concn in the first 4 h urine collections following the administration of the tracer[44].

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