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Studies on Steroid Conjugates. IV. Demonstration and Identification of Solvolyzable Corticosteroids in Human Urine and Plasma*

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ABSTRACT: The procedure of S. Burstein and S. Lieberman (*J. Biol. Chem. 233*, 331 (1958a)) for solvolytic cleavage of ketosteroid hydrogen sulfates has been modified so as to be applicable to the markedly more polar corticosteroid sulfates. The overall recoveries of the steroid moieties of synthetic corticosteroid mono- and disulfates subjected to this modified procedure were 85–98% with a reproducibility of ±2.5%. This procedure was then incorporated into a fractionated extraction, hydrolysis, and solvolysis method, the application of which to biological fluids resulted in the demonstration of a *consistent presence* of solvolyzable (presumably sulfate) conjugated corticosteroids in human blood and urine. These conjugates were shown to constitute a sizable fraction of urinary (15–33%) and plasma (12–21%) total 17-hydroxycorticosteroids. The steroidal moieties of these conjugates have been identified. It is of interest that along with cortisol, tetrahydrocortisol, and other ring A-reduced steroids, 6β-hydroxycortisol was also isolated from the solvolyzable fraction.

Our interest in conjugated corticosteroids stemming from the results of our studies on corticosteroid metabolism in hypertension (Kornel, 1958, 1960, 1964a; Kornel and Motohashi, 1963) stimulated us to undertake an investigation into the possible biological importance of some steroid conjugates (Kornel, 1963, 1964b; Kornel *et al.*, 1964; Kornel and Lee, 1964). The finding of a substantial fraction of urinary radioactive conjugated metabolites of tracer [4-^{14}C]cortisol, which could not be split by even most exhaustive β-glucuronidase hydrolysis (Kornel and Eik-Nes, 1961), indicated the presence of nonglucuronide conjugated corticosteroids and called for their isolation and identification.

In the first attempts to obtain some clues with regard to the nature of these conjugates, a method was sought which would specifically hydrolyze steroid sulfates. The procedure of solvolysis described by Burstein and Lieberman (1958a) for cleavage of C_{19} steroid sulfates seemed to be well suited for our purpose. However, the extraction methods for steroid conjugates, intrinsic to this solvolysis procedure, were found to be inadequate for work with more polar conjugates (Kornel, 1963). In an attempt to modify the method so as to make it applicable to work with conjugated corticosteroids, a method has been developed which gave 95–100% solvolytic yield of synthetic corticosteroid sulfates.

Application of this procedure to extracts from biological fluids resulted in the demonstration of a consistent presence of solvolyzable (presumably sulfate) conjugated corticosteroids in urine and plasma.[1] The steroidal moieties of these conjugates have been identified.

Experimental Procedure

Materials. All solvents used were J. T. Baker, analytical grade, and were redistilled without further purification, with the exception of diethyl ether which was redistilled over KOH before use, and ethyl acetate which was washed with a saturated aqueous solution of sodium bisulfite, then distilled twice. The latter procedures give peroxide-free solvents, as determined with acidified KI solution (Burstein and Kimball, 1963).

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[1] Preliminary reports of these findings appeared as abstracts (Kornel and Lee, 1962; Kornel and Hill, 1962).

Dehydroepiandrosterone sulfate (DHEA-S)[2] (17-oxo-5-androsten-3β-yl sulfate) was purchased from Steraloids, Inc. (Flushing 52, N.Y.) and was checked by us for its purity by means of column (Crépy *et al.*, 1957) and paper (Schneider and Lewbart, 1959) chromatography, high-voltage paper electrophoresis (Kornel, 1964b), and chromatography of the steroid moiety (Bush, 1961) liberated by solvolysis procedure of Burstein and Lieberman (1958a). Cortisol-21-sulfate (FS) (11β,17α-dihydroxy-4-pregnene-3,20-dion-21-yl sulfate), tetrahydrocortisol-3-sulfate (THFS) (11β,17α,-21-trihydroxy-5β-pregnan-20-on-3α-yl sulfate), and tetrahydrocortisol-3,21-disulfate (THFdiS) (11β,17α-dihydroxy-5β-pregnan-20-one-3α,21-diyl disulfate) were synthesized by us according to a modification of the method by Sobel and Spoerri (1941). Details of their preparation, purification, and characterization are described elsewhere (Kornel *et al.*, 1964). Pregnanediol-3-glucuronide (P-diol-G) (20α-hydroxypregnan-3α-yl-β-D-glucopyranosiduronic acid) was obtained from the Steroid Reference Collection of the Medical Research Council of Great Britain (London) through the courtesy of Prof. W. Klyne. Tetrahydrocortisone-3-glucuronide (THEG) (17α,21-dihydroxy-5β-pregnane-11,20-dion-3α-yl-β-D-glucopyranosiduronic acid) was generously supplied by Dr. J. J. Schneider of the Jefferson Medical College (Philadelphia).

Development of the Procedure. At the time our investigation into the nature of nonglucuronide conjugated corticosteroids was started, nothing was yet known about the existence of corticosteroid sulfates. It was assumed, however, that if such existed, their polarity would be considerably greater than that of C_{19} steroid sulfates; consequently they could not be quantitatively extracted by the extraction methods intrinsic to standard solvolytic procedures[3] (Burstein and Lieberman, 1958a; Segal *et al.*, 1960). Therefore, following an exhaustive hydrolysis of urine with β-glucuronidase and a complete removal of the liberated steroids with dichloromethane and ethyl acetate, the unknown conjugates were extracted with an ethanol-ether mixture, or with 1-butanol, according to the techniques previously shown to extract *all* urinary conjugated Porter-Silber chromogens, as well as *all* radioactive conjugated metabolites of tracer [4-^{14}C]-cortisol (methods F and B, Kornel, 1963). These extracts were evaporated to dryness, and each residue was redissolved in a small volume of ethanol. To mimic the conditions of standard solvolytic procedures, 5 volumes of a solvolyzing organic solvent (ethyl acetate or diethyl ether), saturated with an aqueous solution of NaCl (20% w/v) acidified to *p*H 1,[4] was added to the ethanolic solution of the conjugates[5] and mixed thoroughly. The incubation was then carried out for 18 hours at 37°. Thereafter the solvolysates were neutralized with a few drops of pyridine and evaporated to dryness. The dry residues were redissolved in H_2O, and the solvolyzed steroids were extracted with dichloromethane. Their concentration was then measured by a modified Porter-Silber color reaction (Kornel, 1962a).

It was assumed in the foregoing experiments that the polarity of the ethanol-equilibrated ethyl acetate mixture is sufficiently high to hold even very polar steroid conjugates in the solution. The question which immediately arose was that of a possible interference of the alcohol (necessary for the solubilization of the extract residue containing the nonglucuronide conjugated 17-OH-CS) with the solvolytic process. To answer it the following experiments were performed:

An aqueous solution of standard DHEA-S, acidified to *p*H 1 and containing 20% (w/v) NaCl, was extracted with ethyl acetate as in the solvolysis procedure of Burstein and Lieberman (1958a). The extract was divided into several equal parts to which various volumes of different alcohols were added (see Table II), and the incubation was carried out for 18 hours at 37°. Thereafter the solvolysates were neutralized (with NH_4OH), and were evaporated to dryness. The dry residues were redissolved in H_2O and extracted with CH_2Cl_2. These extracts were filtered (Whatman No. 1) and evaporated to dryness, and the concentration of the liberated DHEA was measured by the Oertel-Eik-Nes (1959) color reaction.

In the next series of experiments standard DHEA-S was extracted from H_2O and from urine with ethanol-ether (method F) or with 1-butanol (method B). These extracts were evaporated to dryness, and the dry residues were redissolved each in 4 ml ethanol; 20 ml of equilibrated ethyl acetate was added to each and mixed thoroughly with the ethanolic solution, and the incubation was carried out for 18 hours. In the experiments concerned with solvolysis of DHEA-S added to urine, urine without added steroid was treated in the identical manner and served as "blank."

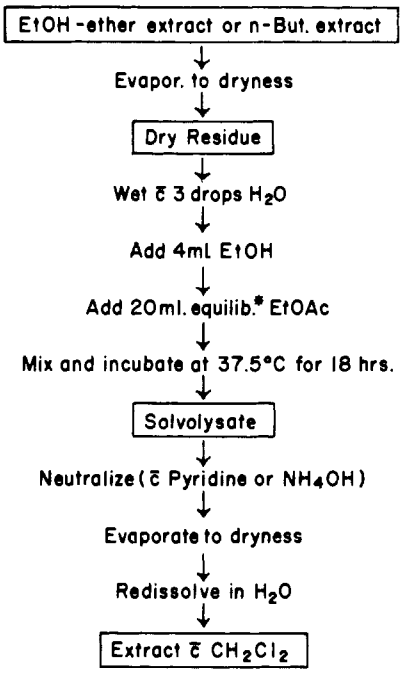
To test our supposition regarding the incomplete removal of corticosteroid sulfates by extraction methods intrinsic to standard solvolytic procedures, reference standard corticosteroid sulfates (FS, THFS, and THFdiS) were synthesized by us (Kornel *et al.*, 1964), and their extractability by various techniques for the

[2] Abbreviations used in this work: DHEA-S, dehydroepiandrosterone sulfate; FS, cortisol-21-sulfate; THFS, tetrahydrocortisol-3-sulfate; THFdiS, tetrahydrocortisol-3,21-disulfate; P-diol-G, pregnanediol-3-glucuronide; THEG, tetrahydrocortisone-3-glucuronide; 17-OH-CS, 17-hydroxycorticosteroids; ACTH, adrenocorticotropic hormone; THF, tetrahydrocortisol; 6β-OH-F, 6β-hydroxycortisol; THE, tetrahydrocortisone; THS, tetrahydrocortexolone.

[3] As standard reference corticosteroid sulfates were not available for recovery studies this assumption could not be tested at the time; later, when corticosteroid sulfates were synthesized by us and their partition coefficients were examined, the validity of this assumption was confirmed.

[4] It was found advantageous to bring the *p*H slightly below 1, since certain urinary extracts contained large amounts of various "buffering" substances which bound the traces of H_2SO_4 in the equilibrated ethyl acetate, thus making the former unavailable for catalyzing solvolysis.

[5] This saturated ethyl acetate (or ether) will be referred to as *equilibrated* ethyl acetate (or ether).



*The equilibration is done by shaking 50 ml of redistilled ethyl acetate in a separatory funnel with 5ml of 20% aqueous solution of NaCl, acidified to pH 1 with 50% H_2SO_4.

FIGURE 1: Flow sheet of modified solvolysis procedure.

extraction of conjugated steroids was tested (see Table I). Furthermore, cleavage yields of these conjugates by our method and by other solvolytic procedures (Burstein and Lieberman, 1958a; Segal *et al.*, 1960) were compared.

The Final Solvolytic Procedure for Cleavage of Corticosteroid Sulfates. A flow sheet of this technique is shown in Figure 1. Pure corticosteroid sulfates (FS, THFS, THFdiS) or dry residues of urinary or plasma extracts to be solvolyzed were dissolved each in 10 ml distilled water. The aqueous solutions were acidified to *p*H 1 and saturated with ammonium sulfate, and the conjugates were extracted with an ethanol-ether mixture or with 1-butanol, as previously described (extraction methods F and B, respectively, Kornel, 1963). The extracts were quantitatively separated (by means of a "serum lifter"[6]) into another test tube, and were allowed to stand for 10–15 minutes at room temperature to permit droplets of aqueous phase, often removed with the extract and contaminating it, to settle on the bottom of the test tube. The extracts were then decanted into another test tube, neutralized by addition of a few drops of NH_4OH, and evaporated to complete dryness under reduced pressure (and under N_2) at 47°. The dry residues were made thoroughly wet with 2–4 drops of H_2O and were dissolved each in 4 ml absolute ethanol. Twenty ml of equilibrated ethyl acetate was added to each ethanolic solution, and the contents of the tubes were mixed by a brief vigorous shaking. They were then placed on a water bath or in an oven and incubated at 37.5° for 18 hours. (In the experiments concerned with the optimum time and temperature of the incubation these conditions were varied [see Results].) Following the incubation, the solvolysates were neutralized by addition of a few drops of pyridine or NH_4OH, and were evaporated to dryness under reduced pressure and N_2 at 47°. The dry residues were redissolved each in 10 ml of H_2O and were exhaustively extracted with dichloromethane (3 × 4 volumes) to remove the liberated (cleaved) steroids.[7] The remaining aqueous phases were acidified to *p*H 1, saturated with $(NH_4)_2SO_4$, and extracted with 4 volumes ethanol-ether mixture (1:3) to remove this portion of the conjugates, which were not cleaved. Both dichloromethane and ethanol-ether extracts were carefully separated, and each was divided into two equal parts, evaporated to dryness, and subjected to a modified Porter-Silber color reaction (Kornel, 1962a) to determine the amount of split and nonsplit steroid conjugates, respectively.

In a separate series of experiments concerned with the examination of a possible artifact formation during the modified solvolysis procedure, the final dichloromethane extracts of the solvolyzed standard corticosteroid sulfates (FS, THFS, THFdiS) were not divided, but were paper-chromatographed (in B_5 and C systems of Bush) and the liberated steroids were identified by customary procedures (Bush, 1961). The nonsolvolyzed conjugated steroids present in the ethanol-ether extract were identified by means of high-voltage paper electrophoresis (Kornel, 1964b), column (Kornel *et al.*, 1964) and paper (Schneider and Lewbart, 1959) chromatography, and various color reactions (Kornel, 1964b).

In another series of experiments concerned with the determination of yields of the modified solvolysis, aliquots of ethanolic solutions of FS, THFS, and THFdiS were evaporated to dryness (omitting the step of their extraction from the aqueous phase) and the dry steroids were directly subjected to the modified solvolysis. Thereafter their treatment was identical with that described above.

Fractionation and Solvolysis of Urinary 17-Hydroxycorticosteroids (17-OH-CS). Urinary specimens were collected over 24-hour periods. Each freshly obtained portion of urine was immediately frozen to prevent a possible spontaneous hydrolysis of some of the more labile conjugated corticosteroids. Before processing, frozen portions constituting a 24-hour collection were thawed and pooled. Aliquots of 40 ml were extracted twice with 4 volumes of dichloromethane to remove

[6] Obtainable from the Workshop, University of Alabama Medical Center.

[7] When urinary or plasma extracts were solvolyzed, the dichloromethane extraction was followed by one with ethyl acetate (2 × 4 volumes), to assure a complete removal of more polar steroids (e.g., 6β-OH-cortisol), should such have been liberated from solvolyzable conjugates.

the "free" steroids. The extracts were separated by means of a pressure-operated liquid separator (Scientific Glass Apparatus Co., Bloomfield, N.J.), filtered (Whatman No. 1 paper), and combined, and their volume was reduced *in vacuo* to 50 ml (at 47°). The purification of these extracts and the estimation of "free" 17-OH-CS was carried out by the methods previously described (Kornel, 1959a, 1962a). The aqueous urinary residue left after the extraction with CH_2Cl_2 was extracted consecutively with ethyl acetate (2 × 4 volumes) (this extract contained "polar free" 17-OH-CS) and with an ethanol-ether mixture, according to the technique previously described (method F, Kornel, 1963), to remove *all* the conjugated 17-OH-CS. The ethyl acetate extract was filtered (Whatman No. 1), divided into two parts, evaporated to dryness (47°), and subjected to a modified Porter-Silber color reaction (Kornel, 1962a). The separated ethanol-ether extract was allowed to stand for 10–15 minutes at room temperature to permit droplets of the aqueous phase, often removed with the extract and contaminating it, to settle on the bottom of the test tube. The extract was then decanted into another test tube, neutralized by addition of a few drops of NH_4OH, and evaporated to complete dryness under N_2 at 47°. The dry residue was then redissolved in 14 ml of distilled water; 2 ml of 2 M sodium acetate buffer, *p*H 4.5, and 4 ml of β-glucuronidase (Ketodase,[8] Warner-Chilcott, 5000 units/ml) were added, and incubation was carried out for 48 hours at 37°. The liberated steroids ("glucuronides") were then exhaustively extracted with dichloromethane; 4 ml of Ketodase was added (traces of CH_2Cl_2 were removed by blowing N_2 through the aqueous phase) to the hydrolysate and the incubation was repeated for 36 hours. The hydrolysate was then extracted consecutively with dichloromethane, ethyl acetate, and ethanol-ether mixture, the same way as described above for the "free," "polar free," and conjugated 17-OH-CS. The dichloromethane extracts (first and second β-glucuronidase hydrolysis) and the ethyl acetate extract of the hydrolysate were processed identically with the "free" and "polar free" 17-OH-CS extracts.[9] *The ethanol-ether extract, containing all the conjugates which were not split by β-glucuronidase, was then used for solvolysis* according to the procedure for the cleavage of synthetic corticosteroid sulfates described in the preceding section. Following evaporation to dryness of the solvolysate, the dry residue was taken up in 10 ml H_2O and extracted consecutively with dichloromethane, ethyl acetate, and again with ethanol-ether mixture; the latter extract contained all the remaining conjugates which were not split by either hydrolysis or solvolysis. All the three solvolysate extracts were processed the same way as the corresponding extracts of the β-glucuronidase hydrolysate, with the exception that the Porter-Silber chromogen content of the *final* ethanol-ether extract was determined as described in the method for unhydrolyzed conjugated 17-OH-CS (Kornel, 1963).

Fractionation and Solvolysis of Plasma 17-OH-CS. The method for plasma was identical with that described previously (Kornel, 1962b). In all subjects blood was drawn between 8:30 and 9:00 A.M. Corticotropin (ACTH) was then administered intramuscularly as gel, 80 units every 8 hours, for a total of five doses; blood specimens were drawn again 4 hours after the administration of the last dose.

Identification of the Steroid Moieties of Solvolyzable Corticosteroids in Urine and Plasma. The procedures of extraction and cleavage were the same as those described above for the fractionation and solvolysis of extracts from urine and plasma. However, larger urine aliquots were used[10] (0.5 volume of the total 24-hour collection), and the CH_2Cl_2 and ethyl acetate extracts of solvolysates were not divided (as for the Porter-Silber color reaction) but were washed succesively with 0.05 volume of 0.1 N NaOH and H_2O, dried, and applied to an alumina-silica partition column (a modified Wilson's column [Wilson *et al.*, 1958]) as previously described by us (Starnes *et al.*, 1963), with the following modification: In addition to fractions AB and CD, the elution from the column of compounds more polar than tetrahydrocortisol (THF) was carried out with plain chloroform (traction E). Fractions CD and E were then pooled and chromatographed on paper in the B_5 system of Bush (1961) with overrunning for 24 hours. The runoff was collected and rechromatographed in the B_3 system of Bush. The position of the separated compounds was then located by scanning chromatograms in ultraviolet light, and by staining a pilot strip with blue tetrazolium. Compounds from the corresponding areas of the unstained portion of each chromatogram were eluted, and subjected to further characterization by rechromatography (alone and with authentic carriers), formation of derivatives (oxidation and acetylation) and their chromatography, various color reactions (blue tetrazolium, Porter-Silber, Zimmerman for the oxidation products), absorbance of ultraviolet light, and spectra in concentrated sulfuric acid (see Results).

For the identification of the solvolyzed compounds from plasma, heparinized blood specimens were obtained from two patients with Cushing's syndrome who received ACTH (the dosage and the way of its administration was the same as that described before). The techniques of the separation and identification of the individual compounds extracted from plasma solvolysates were essentially the same as those applied to steroids isolated from urine; the partition column chromatography was here omitted, as owing to the

[8] We are indebted to Mr. Stephen J. Koziol, Manager, Diagnostic Division, Warner-Chilcott Lab., for the generous supply of this enzyme.

[9] For the estimation of 17-OH-CS in the dichloromethane extracts after the first β-glucuronidase hydrolysis, extract aliquots equivalent to only 2 ml of urine were used, in view of a high 17-OH-CS concentration in this fraction.

[10] The volumes of solvents and reagents were proportionately increased.

TABLE I: Extraction of Synthetic Corticosteroid Sulfates by Methods Intrinsic to Various Solvolytic Procedures.

Method of Extraction			FS Extracted (%)[a]				THFdiS Extracted (%)[a]			
Aqueous Phase[b]		Solvent	From H_2O		From Urine		From H_2O		From Urine	
*p*H	Salt		1st[c]	2nd[c]	1st	2nd	1st	2nd	1st	2nd
1	NaCl (20%, w/v)	EtOAc	51	26	46	25	2.5	1.5	2	1
1	NaCl (20%, w/v)	Ether	49	23	40	21	2	1	1	1
5% H_2SO_4[d]		EtOAc	52	24	46	26	3	3	3	2
5% H_2SO_4[d]		Ether	50	22	44	20	3	2	2	1.5
7	NaCl (3 M)	EtOAc	40	27	34	17	2	1.5	2	1
7	NaCl (3 M)	Ether	41	24	30	16	2	1.5	2	1

[a] Average values from five experiments, each carried out in quadruplicate. [b] The *p*H of the aqueous phase and the salt added, prior to the extraction; the salt concentration is indicated in parentheses. [c] First and second extractions, respectively, each with 4 volumes of solvent. [d] Concentration of the acid in the aqueous phase brought to 5%.

TABLE II: Influence of Various Alcohols Added to Solvolysates[a] upon Solvolytic Yield and Recovery of the Liberated Steroid.

Alcohol Added	Alcohol Concentration (% of solvolysate volume)	DHEA-S[b] Cleaved (%) 2 hr	18 hr
None		98	96
Ethanol	3	90	100
	5	88	100
	10	78	100
	20	75	99
Methanol	3	90	100
	5	87	98
	10	78	98
	20	70	96
1-Butanol	3	85	96
	5	80	90
	10	70	88
	20	65	85

[a] Extracts for solvolysis were obtained according to the method of Burstein and Lieberman (1958a). [b] Aqueous solution of standard DHEA-S was used for these experiments.

efficient plasma deproteinization and defatting technique (Kornel, 1959b), the dichloromethane and ethyl acetate extracts of the solvolysates (washed with 0.1 N NaOH and H_2O as described) could be directly chromatographed on paper.

Results

Development of the Procedure. The results of the experiments concerned with the development of the modified solvolysis procedure are summarized in Tables I, II, and III. It will be seen that the extraction techniques intrinsic to the standard solvolytic procedures were found to be grossly inadequate for the extraction of *corticosteroid* sulfates (Table I). It was therefore necessary to extract these conjugates with other methods, better suited for the extraction of this class of compounds (Kornel, 1963). However the extracts obtained with these methods yielded, after evaporation to dryness, residues which were not soluble in ethyl acetate or ether. It was possible to redissolve them in alcohol and to add a solvolyzing solvent to this alcoholic solution, but it was first necessary to investigate to what extent the presence of alcohol in the solvolysates would affect the solvolytic process. It will be seen from Table II that the addition of alcohol to solvolysates did *not* inhibit solvolysis of DHEA-S, although it delayed the rate of cleavage of this conjugate; with 20% (v/v) of ethanol added, the solvolysis was completed in 18 hours. When standard corticosteroid conjugates were synthesized, the developed procedure could be properly tested and compared with other solvolytic methods. These results are shown in Table III.

A series of subsequent experiments was concerned with the optimum time and temperature necessary for a complete cleavage of FS, THFS, and THFdiS, and of urinary extracts. The results are presented in Tables IV and V. It is of interest that a considerably longer time was necessary for cleavage of THFdiS than that of FS. Dichloromethane extracts of solvolysates were also examined for a possible presence of steroid artifacts. None were detected. THEG and P-diol-G were also subjected to the modified solvolysis; they were *not* split by this procedure.

Demonstration and Identification of Solvolyzable Corticosteroids in Human Urine. The application of the

TABLE III: Cleavage of Synthetic Corticosteroid Sulfates by Various Solvolytic Procedures (cf. Table I).

Extraction Method[a]				FS Cleaved[b] (%)		THFdiS Cleaved[b] (%)		DHEA-S Cleaved[b] (%)	
Aqueous phase[c]			Footnote						
*p*H	Salt	Solvent	ref.[d]	1st[e]	2nd[e]	1st	2nd	1st	2nd
1	NaCl (20%, w/v)	EtOAc	[f]	47	23	2.5	1.5	90	0
1	NaCl (20%, w/v)	Ether	[f]	45	20	2	1	88	1
5%[g] H_2SO_4		EtOAc	[f]	42	22	2.5	2.5	86	1.5
7	NaCl	EtOAc	[h]	37	21	2	1.5	78	5
1	$(NH_4)_2SO_4$	E-E[i]	[j]	93	4	82	4	94	0

[a] Extraction method intrinsic to a given solvolysis procedure. [b] Average values from five experiments, each carried out in quadruplicate; solvolyses were performed at 37.5° for 24 hours. [c] *p*H of the aqueous phase and salt dissolved in it to give the indicated concentration; the aqueous phase in these experiments was distilled water. [d] Reference to the corresponding solvolysis procedure. [e] Extracts obtained from the first and second extractions (each with 4 volumes of solvent) were separately solvolyzed. [f] Burstein and Lieberman (1958a). [g] Concentration of the acid in the aqueous phase brought to 5%. [h] Segal *et al.* (1960). [i] Ethanol-ether mixture, 1:3; extraction method F (Kornel, 1963). [j] This is the solvolysis procedure described in this paper.

TABLE IV: Solvolysis of Synthetic Corticosteroid Sulfates. Influence of Incubation Time and Temperature upon Solvolytic Yield.

Corticosteroid Sulfate	Temp. (°C)	Per Cent Corticosteroid Sulfates Cleaved at: (hours of incubation) 0.5	1	2	4	6	8	16	24	30	45	60
FS	24	8	40	65	82	87	89	92	94	96		
	37	25	63	87	94	96	97	99	100			
	47	36	74	92	93	94	92	90				
THFS	24	7	16	30	54	68	75	87	91	93	97	98
	37	10	20	38	59	73	82	95	98	98	100	
	47	25	60	78	86	92	95	96				
THFdiS	24		4	12	26	37	44	56	59	61	62	63
	37	2	9	23	47	65	73	81	84	86	89	90
	47	19	55	76	85	87	89	85				

developed procedure to urine samples from twenty-seven normal subjects ranging in age from nineteen–forty revealed a consistent presence of solvolyzable Porter-Silber chromogens in all the samples examined (Table VI). It can be seen that the solvolyzable corticosteroids constitute a sizable fraction (15–33%) of the total urinary Porter-Silber chromogens. The steroidal nature of the solvolyzed compounds (isolated from urine specimens of ten subjects) was further proved by a chromatographic separation of the individual compounds and their identification by customary procedures (see Methods). Tetrahydrocortisol (THF), 6β-hydroxycortisol (6β-OH-F), cortisol, tetrahydrocortisone (THE), and tetrahydrocortexolone (THS) were identified (Table VII) among the Porter-Silber positive steroids. A regular presence of compounds even more polar than 6β-OH-F was noted. Their identification is under way at present. Moreover, in three subjects who received an intravenous injection of a tracer dose of [4-^{14}C]cortisol, an appreciable amount of radioactivity was found in the solvolyzable steroid fraction. This investigation will be reported in detail elsewhere.

Demonstration and Identification of Solvolyzable Corticosteroids in Human Plasma. The demonstration of solvolyzable corticosteroids in urine stimulated search for a corresponding fraction of corticosteroid conjugates in blood. In twenty-five normal subjects (age range twenty-four–fifty-four) and in four patients with Cushing's syndrome a consistent presence of solvolyzable Porter-Silber (17-OH-CS) chromogens has been demonstrated (Table VIII). It will be seen that the solvolyzable 17-OH-CS constitute a sizable fraction of plasma conjugated corticosteroids. Interest-

TABLE V: Solvolysis of Urinary Extracts. Influence of Incubation Time and Temperature upon the Cleavage of Solvolyzable Porter-Silber Chromogens (P-S).

Incubation Time (hr)	P-S Cleaved[a] (%) at 24°	at 37°	at 47°
1	10	45	56
2	12	57	68
6	36	72	86
12	55	89	96
18	64	98	99
24	70	100	100
48	80	100	98
60	88	100	97

[a] The highest value for the concentration of "free" P-S chromogens released by solvolysis in these experiments was considered as 100%.

TABLE VI: Fractionation of Urinary 17-OH-CS (Porter-Silber Chromogens). Demonstration of Solvolyzable Corticosteroids in Human Urine.

Steroid Fraction	Per Cent of Total Porter-Silber Chromogens[a] Mean	Range	Steroids Identified in Each Fraction[b]
Free	4	3–7	*F*, *E*, *THF*, *allo*-THF, THE, THS
Polar free	10	8–14	*THF*, *allo*-THF, *6β-OH-F*, 6α-OH-F
Glucuronides	61	56–80	*THF*, *allo-THF*, *THE*, THS, DHF
Solvolyzable	22	15–33	*F*, *6β-OH-F*, *THF*, THE, THS
Nonhydrolyzed	3	1–4	

[a] These determinations were performed on twenty-seven healthy subjects. [b] From the conjugated fractions the steroid moieties were liberated by means of an appropriate hydrolytic procedure (see Methods). Steroids making up the bulk of each fraction are shown in italics. The following abbreviations are used: F (cortisol) for 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; E (cortisone) for 17α,21-dihydroxy-4-pregnene-3,11,20-trione; THF (tetrahydrocortisol) for 3α,11β,17α,21-tetrahydroxy-5β-pregnan-20-one; THE (tetrahydrocortisone) for 3α,17α,21-trihydroxy-5β-pregnane-11,20-dione; *allo*-THF for 3α,11β,17α,21-tetrahydroxy-5α-pregnan-20-one; THS (tetrahydrocortexolone) for 3α,17α,21-trihydroxy-5β-pregnen-20-one; DHF (dihydrocortisol) for 11β,17α,21-trihydroxy-5β-pregnane-3,20-dione; 6β-OH-F (6β-hydroxycortisol) for 6β,11β,-17α,21-tetrahydroxy-4-pregnene-3,20-dione; 6α-OH-F for 6α,11β,17α,21-tetrahydroxy-4-pregnene-3,20-dione.

ingly enough, the increase in this fraction after administration of ACTH was markedly smaller than the corresponding increase in the glucuronide fraction. This is in perfect agreement with our previous findings (Kornel and Hill, 1961), where a mild acid hydrolysis at 37° was used to cleave the nonglucuronide conjugated corticosteroids.

The steroidal nature of these solvolyzable conjugates was subsequently proved in plasma samples of two patients with Cushing's syndrome (Kornel, 1962c). THF, allo-THF, and THE were identified by the same techniques as those applied to steroids isolated from urine. Moreover, in two normal subjects, one patient with Cushing's syndrome, and two Addisonian patients on maintenance cortisol therapy in whom kinetic studies of the metabolism of tracer [4-^{14}C]-cortisol were performed, measurements of the rate of appearance of radioactivity in various conjugated metabolites proved beyond doubt the steroidal nature of the solvolyzable fraction. The results of this study will be reported in a subsequent publication.

Discussion

In our investigation of the nature of nonglucuronide conjugated corticosteroids, a need was encountered for a method which would hydrolyze corticosteroid sulfates. While it was possible to cleave standard cortisone-21-sulfate by one of the procedures of Burstein and Lieberman (1958b), neither this nor similar methods gave satisfactory results when applied to biological fluids. This was shown to be mainly owing to the unsuitability of the extraction methods intrinsic to these solvolytic procedures for the extraction of more polar steroid conjugates. In an attempt to modify the available techniques so as to make them applicable to work with conjugated corticosteroids, a method was developed which gave 95–100% solvolytic yield of synthetic corticosteroid sulfates: FS, THFS, and THFdiS. With this method the recovery of these standard compounds added to urine and plasma was in the range of 86–98%.

In our previous study (Kornel and Hill, 1961) evidence was presented for existence of urinary conjugated corticosteroids, which could not be split with β-glucuronidase, but were cleaved by a prolonged mild acid (*p*H 1) hydrolysis at 37°. Pasqualini (1962) reported presence of corticosteroid conjugates in urine, which could be cleaved enzymatically by a sulfatase; his study was carried out on a pooled-urine specimen from subjects receiving large doses of depo-ACTH. Since prior to hydrolysis these conjugates were eluted from a chromatographic column (Crépy *et al.*, 1957) with a solvent mixture which would elute synthetic steroid monosulfates, it was assumed that these conjugates are "sulfates." The concentration of these steroids was found to be extremely low (2.2% of the total

TABLE VII: Identification of the Steroid Moieties of Urinary Solvolyzable Conjugated Corticosteroids.

Steroid	Chromatographic Systems[b] B_5 R_F Values	B_p R_F Values	B_5-OR[c] R_{sF}	B_1 R_F Values of Oxidized Derivatives[a]	B_3 R_F Values of Oxidized Derivatives[a]	Color Reactions UV	BT[d]	P-S[e]	Zimm.[f]	Ultraviolet λ_{max} (mμ)	Spectra in H_2SO_4[g] λ_{max} (mμ)
F_x[h]	0.34	0.76	1.00	0.61	0.55	+	+	+	+	241	475, 390, 280, 240
F std	0.34	0.76	1.00	0.61	0.55	+	+	+	+	241	475, 390, 280, 240
					MCB						
THE_x	0.28	0.76	0.88	0.67	0.78	−	+	+	+		410, 335
THE std	0.28	0.76	0.88	0.66	0.78	−	+	+	+		410, 335
THF_x	0.18	0.55	0.58	0.43	0.35	−	+	+	+		510, 415, 330
THF std	0.18	0.56	0.58	0.43	0.34	−	+	+	+		510, 415, 330
	Y				B_5						
6β-OH-F_x	0.50	0.10	0.05	0.18	0.30	+	+	+	+	235[i]	388, 340, 280
6β-OH-F std	0.50	0.10	0.05	0.18	0.31	+	+	+	+	235[i]	388, 340, 280
	B_5	B_3			MC						
THS_x	0.72	0.32	RO[j]	0.86	0.69	−	+	+	+		410, 315
THS std	0.71	0.32	RO	0.86	0.68	−	+	+	+		410, 315

[a] Oxidized with $NaBiO_3$ (Bush, 1961). [b] B_5, B_1, B_3, systems of Bush (1952); Y, system of Frantz *et al.* (1961); MCB, MC, modified systems of Neher (1959, as used by Starnes *et al.*, 1963); B_p, benzene-chloroform-methanol-water (1:1:1:1). [c] OR, overrun for 24 hours; in this column, R_{sF} values (relative mobilities to standard cortisol) are given, instead of R_F values. [d] Blue tetrazolium color spot test, performed as previously described (Kornel, 1964b). [e] A modified Porter-Silber color reaction (Kornel, 1964c) was applied to steroids eluted from paper chromatograms. [f] A modified Zimmerman color reaction (Axelrod, 1953) applied to the oxidized derivatives on paper chromatograms. [g] Two-hour incubation in the dark. [h] Subscript *x* denotes compounds isolated from solvolysates; std, reference standard compounds. [i] Absorbancy spectrum of this compound in Meyer's reagent (1955) was also investigated. After incubation for *4 hours at 60°* it was as follows: max I, 260 mμ; min, 300 mμ; max II, 379 mμ, this being compatible with 6β-OH-F. [j] RO, runoff.

TABLE VIII: Conjugated 17-OH-CS (Porter-Silber Chromogens) in Human Plasma. Demonstration of Solvolyzable Corticosteroid Fraction.

Subjects	No.	Per Cent of Total Conjugated 17-OH-CS Glucuronides Control	Glucuronides After ACTH	Solvolyzable Control	Solvolyzable After ACTH
Normal	25	66–80	82–85	20–33[a]	15–18
Cushing's syndrome	4	82–85	84–85	15–18	15–16[b]

[a] This represents 12–21% of plasma *total* 17-OH-CS. [b] In this fraction the following steroids were identified: THF, *allo*-THF, and THE (ratio 2:1:1); two other unidentified metabolites were also noted.

urinary Porter-Silber positive steroids). Isolation of FS and corticosterone-21-sulfate was subsequently accomplished by Pasqualini (1962) from urines of subjects who received very large doses of the respective free steroids.

With the aid of our modified solvolysis procedure it was possible to demonstrate a consistent presence of *endogenous* solvolyzable conjugated corticosteroids in plasma and urine, even under conditions of normal cortisol secretion rates. Since it has been shown that

solvolysis will not cleave steroid glucuronides[11] or phosphates (Hudson and Oertel, 1961), one can assume with a considerable degree of certainty that the solvolyzable conjugates *are* sulfates. In addition, our solvolysis procedure was applied to urine and plasma after an exhaustive extraction of "free" steroids and an exhaustive β-glucuronidase hydrolysis, followed by a complete extraction of the liberated steroids. The concentration of the solvolyzable corticosteroids was found to be markedly higher than that of the sulfatase-hydrolyzable conjugates reported by Pasqualini.

The steroidal moieties of solvolyzable corticosteroid conjugates have been identified. Moreover, the presence of radioactivity in these conjugates, after intravenous administration of a tracer dose of [4-^{14}C]cortisol, constitutes an additional proof of their steroidal nature. It is of interest that along with cortisol, THF, and other ring A–reduced steroids, 6-hydroxylated compounds were also isolated from the solvolyzable fraction.

The biological importance of corticosteroid sulfates is at present totally obscure. However, in the light of a recent demonstration by Baulieu and Dray (1963) and Siiteri and MacDonald (1963) of the important role of DHEA-S as an effective natural precursor of placental estrogens, and our own findings that production of solvolyzable corticosteroids is increased in essential hypertension (Kornel and Motohashi, 1965), it is not unreasonable to believe that these conjugates, or at least some of them, are not merely metabolites of cortisol, formed with the purpose of its inactivation and elimination, but that they may well possess a specific biologic activity.

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