CONVERSION OF 6-HYDROXY-Δ^4-3-KETOSTEROIDS TO STEROID 5α-ANE-3,6-DIONES AND Δ^4-ENE-3,6-DIONES DURING ROUTINE SOLVOLYSIS PROCEDURES.*

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ABSTRACT

It was found that steroids with 6-OH-Δ^4-3-keto grouping are converted to the corresponding 5α-3,6-diones and Δ^4-3,6-diones during any of the solvolytic procedures (including that of continuous ether extraction) for cleavage of steroid hydrogen sulfates. The reaction proceeds considerably faster with the 6β-OH steroids than with the 6α-OH ones. 6β-hydroxycortisol, 6α-hydroxycortisol, 6β-hydroxy-11-deoxycortisol, 6β-hydroxyprogesterone, 6β-hydroxy-Δ^4-cholesten-3-one and 6β-hydroxy-Δ^4-androstene-3,17-dione have been examined — all yielded analogous transformation products during solvolysis. The two compounds formed (5α-3,6-dione and Δ^4-3,6-dione) are derived by independent chemical pathways and are not interconvertible during solvolysis. The first compound is obtained in a 70-80% yield, the second in 10-20%. They can be readily separated by paper or column chromatography. In all the steroids examined the remaining part of the molecule stayed intact. It is concluded that 6-hydroxysteroids should be removed from biological extracts prior to solvolysis, whenever their isolation is also intended. Solvolysis appears to provide a convenient one-step method for the preparation of 5α-3,6-diones and Δ^4-3,6-diones (although the latter in low yield) from corresponding 6β-OH-Δ^4-3-ketosteroids, without necessity of labile group protection.

In a previous publication (1) a method has been described for the solvolytic cleavage of corticosteroid sulfates. During the application of this method to urine and plasma, pre-extracted with dichloromethane, it has been noted that the solvolysable steroid fraction liberates, amongst others, Porter-Silber positive (2) compounds, the polarity of which does not ressemble any of the known free steroid alcohols. These steroids were almost quantitatively extracted with dichloromethane from

the aqueous solvolysate, but were then also quantitatively lost from the extract during its alkaline wash. A possibility was considered that these could be artifacts of 6β-hydroxylated steroids, formed during the solvolysis procedure; 6β-hydroxylated corticosteroids are too polar to be extracted with dichloromethane during the pre-extraction of urine and plasma, and were therefore present, along with the conjugated compounds, in the extracts subjected to solvolysis. Our investigation demonstrated that this was, indeed, the case, and that the steroid-6-hydroxy-Δ^4-3-ones were converted to the corresponding 5α-ane-3,6-diones and Δ^4-ene-3,6-diones by the solvolyzing solvent (ethyl acetate, diethyl ether etc.) under the conditions employed for the solvolytic cleavage of steroid hydrogen sulfates. Furthermore, since continuous ether extraction, a procedure which is still commonly used in many laboratories, is also based on the solvolytic cleavage of solvolysable conjugates, it has been demonstrated that this extraction technique also results in the same kind of transformations of 6-hydroxylated steroids.

The results of these studies are reported in this paper.

EXPERIMENTAL PROCEDURES

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 saturated aqueous solution of sodium bisulfite, then distilled twice. The latter procedures give peroxide-free solvents, as determined with acidified KI solution (3).

The following steroids were subjected to the solvolysis procedure: 6β-hydroxycortisol [6β-OH-F] (6β,11β,17α,21-tetrahydroxy-Δ^4-pregnene-3,20-dione), 6α-hydroxycortisol [6α-OH-F] (6α,11β,17α,21-tetrahydroxy-Δ^4-pregnene-3,20-dione), 6β-hydroxycortisone [6β-OH-E] (6β,17α,21-trihydroxy-Δ^4-pregnene-3,11,20-trione), 6β-hydroxy-11-desoxycortisol [6β-OH-S] (6β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione), 6β-hydroxyprogesterone [6β-OH-Prog] (6β-hydroxy-Δ^4-pregnene-20-one), 6β-hydroxy-Δ^4-androstene-3,17-dione [6β-OH-Δ^4-A], and 6β-hydroxy-Δ^4-cholesten-3-one [6β-OH-Δ^4-Ch]. All the compounds were checked for purity by chromatography in systems outlined below. For the identification of

the corresponding conversion products formed during solvolysis (5α-ane-3, 6-diones and Δ^4-ene-3,6-diones) authentic reference standard compounds were used (4). Allopregnane-3,6,20-trione, Δ^4-pregnene-3,6,20-trione and Δ^4-androstene-3,6,17-trione were also prepared by us using established chemical procedures, as outlined below.

Methods

The solvolysis method used was that described by us previously (1,5). In several instances the solvolysis procedure of Burstein and Lieberman was used (6). Both procedures gave the same results. For the isolation of urinary 6β-hydroxycortisol the method of Frantz et al. (7) was employed. In the experiment concerned with the investigation of 6β-hydroxysteroid artifacts formed during a continuous ether extraction, the extraction apparatus and technique (8) used were those described by us previously (9).

The solvolysates were neutralized with a few drops of NH_4OH and evaporated to dryness under N_2. For experiments on partition coefficients of the conversion products the dry solvolysate residue was redissolved in water and extracted consecutively with CH_2Cl_2 and EtOAc. For the indentification of the transformation products the entire dry solvolysate residue was chromatographed on paper.

The compounds isolated from solvolysates were indentified using the following criteria: mobility in several paper chromatographic systems (R_f-values), compared to that of the authentic reference standard compounds: color spot tests; absorption maxima in ultraviolet (U.V.) region; absorbancy spectra in Meyer's reagent (10); spectra in concentrated sulfuric acid (11,12); and, in case of 6β-hydroxyprogesterone and its transformed derivatives as a representative series, infrared spectra and melting points. Moreover, to obtain an additional confirmation that the rings A and B of various 6β-hydroxylated steroids undergo the same type of transformation (irrespectively of the substitution groups in other positions of the molecule), 6β-hydroxy-S and its derivatives formed during solvolysis were oxidized with CrO_3, and the physicochemical properties of the resulting C_{19} steroids were compared with those of the corresponding solvolysis transformation products of 6β-OH-Δ^4-androstenedione.

Chromatographic systems. The solvent systems used for paper chromatographic separation of the parent 6β-hydroxylated compounds from the solvolysis transformation products are listed in Table I. All runs in the Bush-type systems (both stationary and mobile phases volatile) were performed at 28° C; runs in the Zaffaroni-type systems were carried out at room temperature.

Color spot tests. The chromatographed compounds were located on paper strips by means of scanning in the U.V. light of the unstained chromatogram (Δ^4-3-keto grouping) and after staining (by dipping) with an aqueous ethanolic solution of KOH (7.5 g in 100 ml 95% aq. ethanol). In the latter procedure steroids with Δ^4-ene-3,6-dione configuration produced an intense yellow color and a strong orange fluorescence in U.V. light immediately after staining; steroids with 5α-ane-3,6-dione configuration stained and fluoresced after drying of paper at room temperature; and steroids with 6β-hydroxy-Δ^4-ene-3-one configuration, became positive only after heating of the chromatogram in the oven (70-80° C). Our 6β-hydroxylated compounds of the corticosteroid series could be spotted by blue tetrazolium (B.T.) color reaction, performed in a fashion previously described by us (18). This, and the fact that they gave a

TABLE I.
Paper chromatographic systems

System Designation	Author	Solvents	Compounds Separated
Y	Frantz et al.(7)	Ethyl acetate/Chloroform/Methanol/H_2O (25:75:50:50)	6β-OH-F* 6α-OH-F*
B_p	-"-	Benzene/Chloroform/Methanol/H_2O (50:50:50:50)	6β-OH-E* 6β-OH-S
$B_{5(2)}$	Bush (13)	Benzene/Methanol/H_2O (1000:525:475)	
B_3	Bush (13)	Petroleum ether/Benzene/Methanol/H_2O (33:17:40:10)	
B_1	-"-	Petroleum ether/Toluene/Methanol/H_2O (25:25:35:15)	
A	-"-	Petroleum ether/Methanol/H_2O (100:80:20)	6β-OH-Prog.* 6β-OH-Δ^4-A*
CB/F	Zander et al.(14)	Cyclohexane/Benzene(1:1)/Formamide (Form-MeOH 1:2)	
T/PG	Savard (15)	Toluene/Propylene glycol (PG-MeOH 1:2)	
H/PC-PG	Motohashi	Hexane/Phenylcellosolve/Propylene glycol (PC-PG-MeOH 1.3:1:5.6)	
H_m	Berliner et al.(16)	Hexane (monophasic)	6β-OH-Ch*
Hep/PC	Neher (17)	Heptane/Phenylcellosolve (PC-MeOH 1:3)	

* and the corresponding derivatives formed during solvolysis.

positive Porter-Silber (P-S) color reaction after elution from paper (19), indicated that the dihydroxy-acetone side chain of these steroids remained intact during solvolysis. The Zimmerman color reaction (17) was used in addition, to visualize 6β-OH-Δ^4-androstenedione and its derivatives.

Absorption maxima in U.V. light. These were obtained with 50-100 μg of the examined steroid in absolute methanol on Beckman DK-2 selfrecording spectrophotometer.

Spectra in Meyer's reagent. This reagent was freshly prepared by mixing 6 ml of 1.1 N aqueous solution of KOH with 94 ml of 95% aqueous ethanol. 3 ml of this reagent were then added to a dry sample of the examined steroid eluted from paper. The elution was performed with 10 ml of abs. methanol by means of an eluting device previously described (20). A corresponding area of a paper-blank strip was also eluted and served a reference "background" color, against which the sample containing steroid was scanned. In several instances eluted steroids were purified by chromatography on alumina column, as described below, prior to being subjected to the alkaline-ethanolic treatment, this, however, proved to be superfluous when paper-blank was used. Following the completion of the color reaction in the alkaline ethanol, and the spectrophotometer scanning, the reaction mixture was acidified with 0.2 ml 1 N aquous HCl (the resulting pH was 6.8), and the sample was rescanned.

Spectra in concentrated sulfuric acid. These were obtained by reacting dry steroid samples, eluted from paper, with 1 or 2 ml of H_2SO_4 (depending of the steroid concentration) for 2 hrs. in the dark, and subsequent scanning of the developed color in a DK-2 spectrophotometer between 220 and 600 mμ, against a corresponding paper-blank eluate reacted with H_2SO_4. In several instances, where the "background" color was intense and the steroid concentration low, a purification of the sample was achieved by means of column chromatography, as described below.

Infrared spectra and melting points. Solvolysis transformation products of 6β-hydroxyprogesterone, separated by paper chromatography, were purified by column chromatography, and twice crystallized from acetone-hexane (allopregnane-3,6,20-trione) and chloroform-hexane (Δ^4-pregnene-3,6,20-trione). Infrared spectra of the crystaline compounds were then obtained in KBr pellets on a Perkin-Elmer (model 21) IR self-recording spectrophotometer (21). These were then compared with those of the authentic standard compounds prepared by us by means of established chemical procedures. Melting points were determined on crystals which were recovered from the KBr pellets.

Preparation of allopregnane-3,6,20-trione. This compound was synthesised from 6β-OH-progesterone by a modification of the procedure described by Fieser for the preparation of cholestane-3,6-dione from 6β-hydroxy-Δ^4-cholesten-3-one (22). To 5 ml of 95% aqueous ethanolic solution containing 50 mg of 6β-OH-progesterone, 1 ml of 36% (v/v) HCl and 50 ml absolute ethanol were added and refluxed for 1.75 hrs. Thereafter, the volume of this solution was reduced in vacuo to approx. 3 ml and refrigerated overnight. The resulting white precipitate was dissolved in 1 ml acetone and recrystallized from acetone-hexane (1:10) (23) under N_2. The yield was 33 mg of allopregnane-3,6,20-trione.

Preparation of Δ^4-pregnene-3,6,20-trione. This compound was prepared by two different methods: a) from 3β-hydroxy-Δ^5-pregnen-20-one (pregnenolone) using Jones' reagent (25,26), and b) from 6β-OH-progesterone by oxidation

with chromic acid (27).

a) To 1.7 gm of pregnenolone in 50 ml of acetone, 6.5 ml of Jones' reagent (28) was added dropwise over a period of 15 minutes with simultaneous vigorous electric stirring. When the addition of the reagent was completed, the stirring was continued for another 5 minutes. Thereafter, the acetone layer was decanted from a brownish heavy precipitate formed, and combined with 50 ml H_2O. Following evaporation of the acetone (under N_2), the synthesis product was extracted from the remaining aqueous phase with 100 ml of dichloromethane. The extract was washed consecutively with 1/20 vol. of saturated aqueous solution of $NaHCO_3$ and H_2O), filtered, and evaporated to dryness. The dry residue, containing two less polar compounds besides the main product (Δ^4-pregnene-3,6,20-trione), was chromatographed in B_3 system. The main compound, separated from the contaminations, was then eluted and twice crystallized from $CHCl_3$-hexane (1:10).

b) To 1 mg of 6β-OH-progesterone, 1 ml of glacial acetic acid and 0.7 ml of 2% aqueous CrO_3 solution were added, and the oxidation was carried out overnight in the dark, at room temperature. Thereafter, the oxidation mixture was diluted with H_2O to 10 ml, and extracted with 50 ml CH_2Cl_2. The extract was washed successively with 1/20 vol. of aqueous saturated solution $NaHCO_3$ and H_2O and evaporated to dryness, and the oxidation product was crystallized from $CHCl_3$-hexane.

Experiments with 6β-hydroxy-S. This compound (100 μg) was subjected to a 18 hr. solvolysis procedure at 37° C. The conversion products were then separated by paper chromatography in B_5 system, eluted, and oxidized with CrO_3 (27). The oxidation products were then chromatographed in B_1 system along with the compounds derived from 6β-OH-Δ^4-androstenedione by solvolysis, and their physicochemical properties were compared.

Solvolysis of 6β-hydroxycortisol isolated from human urine. To confirm that transformations seen during solvolysis of pure 6β-hydroxylated compounds take also place with naturally occurring steroids, 6β-OH-F was isolated from 100 ml urine samples of 3 subjects. Urinary extracts obtained by method of Frantz et al. (7) were chromatographed successively in Y and B_5 systems. Areas corresponding to 6β-OH-F reference standard were cut out and eluted with 90% aqueous methanol. The eluates were evaporated to dryness and subjected to solvolysis for 18 hrs. at 37° C. The solvolysis transformation products were paper chromatographed and their physicochemical properties were compared with those of analogous compounds derived from crystalline standard 6β-OH-F.

Transformation of 6β-hydroxyprogesterone during continuous ether extraction procedure. One mg. of 6β-OH-progesterone was dissolved in 1,000 ml H_2O acidified to pH 1 with H_2SO_4; this solution was continuously extracted with 500 ml ether for 5 days, as previously described (9). The separated ether extract was washed successively with 1/20 vol. of 1 N NaOH, 0.04 N acetic acid, and H_2O. The washes were back-extracted with ether, and the ether extracts were combined and evaporated to dryness under N_2. The residue was redissolved in 1 ml ethanol and small aliquots were chromatographed on paper in systems A and B_3, along with products of 6β-OH-progesterone subjected to routine solvolysis procedure.

Quantitative estimation of the reaction yields and rates. To determine the percentage conversion of 6-hydroxylated compounds to the corresponding 5α-ane-3,6-diones and Δ^4-ene-3,6-diones, and the influence of the spacial orientation of the 6-OH group upon the speed of the reaction, the following experiments were performed: 6β-OH-F and 6α-OH-F were

subjected to solvolysis, also varying conditions of the reaction (time of incubation and the concentration of alcohol in solvolysates). The solvolysis products were paper chromatographed in B_p and Y systems, and the three separated compounds (the untransformed portion of the 6-OH-steroid and the two converted compounds) were eluted from paper with 90% aqueous methanol. The eluates were evaporated to dryness at 37° C under N_2, and subjected to the modified Porter-Silber color reaction (19). The relative percentages of steroid present in each spot were then calculated. Since, as it will be seen from the respective part of the section "Results", the results of these experiments also strongly suggested that the two conversion products are formed by independent chemical pathways, and are not simply products of consecutive stages in the reaction, each of the two compounds was separately subjected to solvolysis. The products were chromatographed and identified. To further investigate the influence of the structure of the remaining part of the molecule upon the rates of conversion of Δ^4-3-one-6-ol grouping to 5α-3,6-dione and Δ^4-3,6-dione, 6β-OH-progesterone and 6β-OH-Δ^4-androstene-dione were subjected to solvolysis, the solvolysates were paper chromatographed, and the relative percentages of the conversion products were calculated from the intensity of color at 380 mμ, developed with the Meyer's reagent.

Column chromatography. This was developed with the purpose of purification of the solvolysed and paper chromatographed compounds prior to their crystallization for IR spectra, melting points determinations, and, in some instances, H_2SO_4 spectra. The supportive phase was Woelm alumina No. 1, 2.8 gm, poured as a slurry in benzene into a glass column, 7 mm internal diameter, with a small piece of glass-wool at its bottom. The packing was done under slight positive pressure, the height of the packed alumina being 7 cm. Compounds to be chromatographed were applied in benzene. Elutions were carried out with 30 ml portions of the following solvent mixtures: a) ethanol in benzene, 1 - 10%; b) ethanol in benzene, 0.1 - 5.0%; c) ethanol in benzene-hexane (1:1), 1 - 10%; d) chloroform in benzene, 0 - 100%; e) ethyl acetate in benzene, 0 - 60% (gradient increment 5%); and f) ethyl acetate in benzene, 10 - 30% (gradient increment 2%). The eluates were evaporated to dryness under N_2, at 47° C. The percent recovery and the integrity of the chromatographed compounds were checked by incubating dry eluate aliquots with the Meyer's reagent, and by paper chromatography, respectively. Attempts were also made to resolve by means of column chromatographic separation the mixture of compounds obtained during solvolysis of 6β-OH-progesterone. Total solvolysate residues were applied to the column and the elution was carried out in the same fashion as that used for the purification of individual compounds. Since a transformation of the unchanged portion of the parent compound was found to occur on the column (see section "Results") it was necessary to protect the 6β-OH group prior to chromatography. This was done by acetylating (27) or benzoylating (29) the solvolysate residues.

RESULTS

Table II shows results of pilot experiments conducted with a view to determining whether transformation of 6-hydroxylated steroids to less polar compounds does indeed occur during solvolysis, a possibility

TABLE II.
Changes in the extractability (=polarity) of Δ^4-6-hydroxylated steroids during solvolysis, indicating a modification of the steroid molecule.

Steroid	% of P-S chromogens extracted			
	With CH_2Cl_2	With EtOAc*	With CH_2Cl_2	With EtOAc*
	Before solvolysis (control)		After solvolysis	
6α-OH-F	9.9	90.1	23.0	77.0
6β-OH-F	18.6	81.4	69.0	31.0
6β-OH-E	52.2	47.8	72.4	27.6

* The extraction with 5 vol. of ethyl acetate followed that with 5 vol. of dichloromethane; each extract was evaporated to dryness under N_2 and the concentration of the extracted steroid was measured by a modified Porter-Silber color reaction (30).

suggested by the results of our previous studies (1,31). They confirmed our supposition, and, moreover, suggested that 6α-OH steroids are more resistant to this transformation than their spacial 6β isomers.

Identification of compounds isolated from solvolysates.

The physicochemical properties of the compounds formed during solvolysis from 6β-OH-F, 6α-OH-F, 6β-OH-E and 6β-OH-S are shown in Table III. It will be seen that the characteristics of the compounds derived from 6β-OH-F (βF-2 and βF-3) were identical with those of the corresponding compounds derived from 6α-OH-F (αF-2 and αF-3), suggesting identity of the corresponding derivatives in the 6α- and 6β-series. Moreover, the reactivity characteristics depending on the configuration and substitution groups of rings A and B (U.V. absorbance, staining and fluorescence in ethanolic KOH) of the unknowns were identical in analogous

TABLE III.
Characterization of compounds isolated from solvolysates
(corticosteroid series)

Steroid[a]	R_f - values Chromatogr. systems			Color spot tests			P-S[c]	U.V. λ max. mμ	H_2SO_4 spectra λ max. mμ
	B_5	Y	B_p	UV	BT	EtOH-KOH[b]			
6β-OH-F	0	.50	.06	+	+	±	+	235	388,340,280
βF-1	0	.50	.06	+	+	±	+	235	390,340,280
βF-2	.06	.76	.37	-	+	+	+	(-)	490,382,280
βF-3	.08	.87	.55	+	+	++	+	252	475,382,320,258
6α-OH-F	0	.42	.05	+	+	±	+	241	387,320,276,239
αF-1	0	.42	.05	+	+	±	+	241	388,322,275,239
αF-2	.06	.76	.37	-	+	+	+	(-)	490,380,280
αF-3	.08	.87	.55	+	+	++	+	252	475,382,320,258
6β-OH-E	.03	.78	θ	+	+	±	+	233	420,340,280
E-1	.03	.78	θ	+	+	±	+	233	420,340,280
E-2	.25	.86	θ	-	+	+	+	(-)	θ
E-3	.44	.92	θ	+	+	++	+	250	θ
6β-OH-S	.16	.88	θ	+	+	±	+	238	477,340,283,239
S-1	.16	.88	θ	+	+	±	+	238	477,340,283,239
S-2	.58	.92	θ	-	+	+	+	(-)	θ
S-3	.67	s.f.[d]	θ	+	+	++	+	250	θ

[a] Unknown compounds (which were identified in the course of this work) are marked as spots 1,2 and 3, in decreasing order of their polarity; the authentic standard steroids are indicated by abbreviated trivial name.

[b] ±: EtOH-KOH staining and fluorescence became positive only after heating chromatogram at 60-70° C; +: positive after drying of paper at room temperature; ++: positive immediately after staining.

[c] A modified Porter-Silber color reaction (19) was applied to steroids eluted from paper.

[d] Solvent front.

θ Not run.

compounds of each series, as was also the order of their polarity. This, in conjunction with the apparent intactness of the side chain (all compounds were BT and P-S positive) strongly suggested that transformations during solvolysis pertain only to the 6-OH-Δ^4-3-one grouping, and proceed essentially uninfluenced by the structure of the remaining part of the molecule. This probability was tested by subjecting to the solvolytic reaction 6β-OH-Δ^4-androstenedione, 6β-OH-progesterone and 6β-OH-Δ^4-cholestenone (Table IV). The results obtained not only confirmed this supposition, but also permitted identification of the solvolysis transformation products by comparing their characteristics with those of the authentic reference standard compounds (32). In order to reconfirm our identification independently of the reference standards, absorbancy spectra of our compounds in Meyer's reagent were investigated. The results are shown in Table V. The α,β-unsaturated 3,6-diketosteroids (Δ^4-ene-3,6-diones) produced an intense yellow color immediately after contact with the alkaline ethanol, exhibiting one absorption maximum at 260 mμ, and a second, of approximately 3/4 intensity of the first peak, at 380 mμ. The saturated 3,6-diketones, did not give an immediate color reaction with the color reagent, but gave a graduate rise to the same two absorbancy peaks, reaching their maximum after 10 hrs. at room temperature. These compounds produced the same absorptivity pattern as the corresponding 6α-hydroxylated parent compounds after 12 hrs. at room temperature, or 3 hrs. at 60° C; and the 6β-hydroxylated compounds only after incubation at 60° C, for 4 hrs.(33). Following acidification, a shift of the two absorption maxima was observed in all samples to 253 mμ and 315 mμ, respectively. These reverted to the previous absorptivity pattern after renewed addition of alkali. These results confirmed our conclusion as to the nature of the transformations of the 6-OH-Δ^4-3-keto

TABLE IV.
Characterization of compounds isolated from solvolysates
(Androstenedione, Progesterone and Cholestenone series)

Steroid[a]	R_f - Values Chromatographic systems				Color spot tests			U.V. λ max. mμ	H_2SO_4 spectra λ max. mμ
	B_3	B_1	CB/F	T/PG	U.V.	EtOH-KOH[b]	ZIM.[c]		
6β-OH-Δ^4-A	.12	.37	.07	.31	+	+	+	235	445,343,290
A-1	.12	.37	.07	.32	+	+	+	235	445,343,290
Androstane-3,6,17-trione	.26	.72	.28	.83	-	+	+	(-)	398,319,258
A-2	.26	.72	.28	.82	-	+	+	(-)	395,320,258
Δ^4-Androstene-3,6,17-trione	.34	.81	.39	s.f.[d]	+	++	+	250	520,344,235
A-3	.34	.81	.39	s.f.	+	++	+	250	520,344,235
6β-OH-Prog.	.38	.59	.18	.44	+	±	-	235	475,346,290
P-1	.38	.59	.18	.45	+	±	-	234	475,346,288
Allopregnane-3,6,20-trione	.63	.85	.59	.78	-	+	-	(-)	380,314,282,236
P-2	.63	.86	.59	.78	-	+	-	(-)	382,316,282,236
Δ^4-Pregnene-3,6,20-trione	.71	.90	.68	.85	+	++	-	250	347,234
P-3	.71	.90	.68	.84	+	++	-	250	347,237
6β-OH-Ch	.28		.23		+	±	θ	237	400,296
Ch-1	.28		.24		+	±.	θ	237	400,297
Cholestane-3,6-dione	.60		.41		-	+	θ	(-)	395,310
Ch-2	.60		.41		-	+	θ	(-)	395,310
Δ^4-Cholestene-3,6-dione	.69		.48		+	++	θ	249	490,346,270
Ch-3	.69		.48		+	++	θ	250	490,346,270

a Unknown compounds (which were identified in the course of this work) are marked as spots 1,2 and 3, in decreasing order of their polarity; the authentic standard steroids are indicated by abbreviated trivial name.
b ±: EtOH-KOH staining and fluorescence became positive only after heating chromatogram at 60-70° C; +: positive after drying of paper at room temperature; ++: positive immediately after staining.
c Zimmerman color reaction (17).
d Solvent front.
θ Not run.

grouping during solvolysis.

Furthermore, infrared spectra of the two solvolysis transformation products of 6β-hydroxyprogesterone (P-2 and P-3) were compared with those

TABLE V.
Absorbancy spectra in Meyer's reagent

Steroid[a]	Treatment	Incubation time and temperature[c]	Absorbancy, mμ		
			Max.(I)	Min.	Max.(II)
6β-OH-F	KOH-EtOH	4 hrs. 60 C	260	300	379
	→ pH 6.8[b]	-	252	272	312
βF-1	KOH-EtOH	4 hrs. 60 C	260	300	379
	→ pH 6.8	-	252	272	312
βF-2	KOH-EtOH	10 hrs.room t.	260	302	380
	→ pH 6.8	-	253	273	314
βF-3	KOH-EtOH	2 min.room t.	260	302	380
	→ pH 6.8	-	248	273	313
6α-OH-F	KOH-EtOH	12 hrs.room t. or 3 hrs. 60 C	260	300	379
	→ pH 6.8	-	252	272	312
αF-1	KOH-EtOH	12 hrs.room t. or 3 hrs. 60 C	260	300	379
	→ pH 6.8	-	252	272	312
αF-2	KOH-EtOH	10 hrs.room t.	260	302	380
	→ pH 6.8	-	253	274	314
αF-3	KOH-EtOH	2 min.room t.	260	302	380
	→ pH 6.8	-	248	272	313
6β-OH-Prog.	KOH-EtOH	4 hrs. 60 C	260	298	381
	→ pH 6.8	-	251	280	311
P-1	KOH-EtOH	4 hrs. 60 C	260	298	381
	→ pH 6.8	-	251	280	311
Allopregnane-3,6,20-trione	KOH-EtOH	10 hrs.room t.	259	302	380
	→ pH 6.8	-	251	273	312
P-2	KOH-EtOH	10 hrs.room t.	259	302	380
	→ pH 6.8	-	250	273	312
Δ^4-Pregnene-3,6,20-trione	KOH-EtOH	2 min.room t.	259	303	380
	→ pH 6.8	-	249	273	314
P-3	KOH-EtOH	2 min.room t.	259	304	380
	→ pH 6.8	-	249	272	314

[a] βF-1,-2,-3 and αF-1,-2,-3 designate unknowns isolated from solvolysates of 6β-OH-F and 6α-OH-F, respectively (F-1: unchanged portion of parent compound; F-2: allopregnane-3,6-dione; F-3: Δ^4-pregnene-3,6-dione). P-1,-2,-3 — analogous derivatives of 6β-OH-Prog.

[b] Following complete development of color in alkaline ethanol, the reaction mixture was acidified with 0.2 ml of 1 N aqueous HCl.

[c] Length of time and temperature necessary for complete development of color.

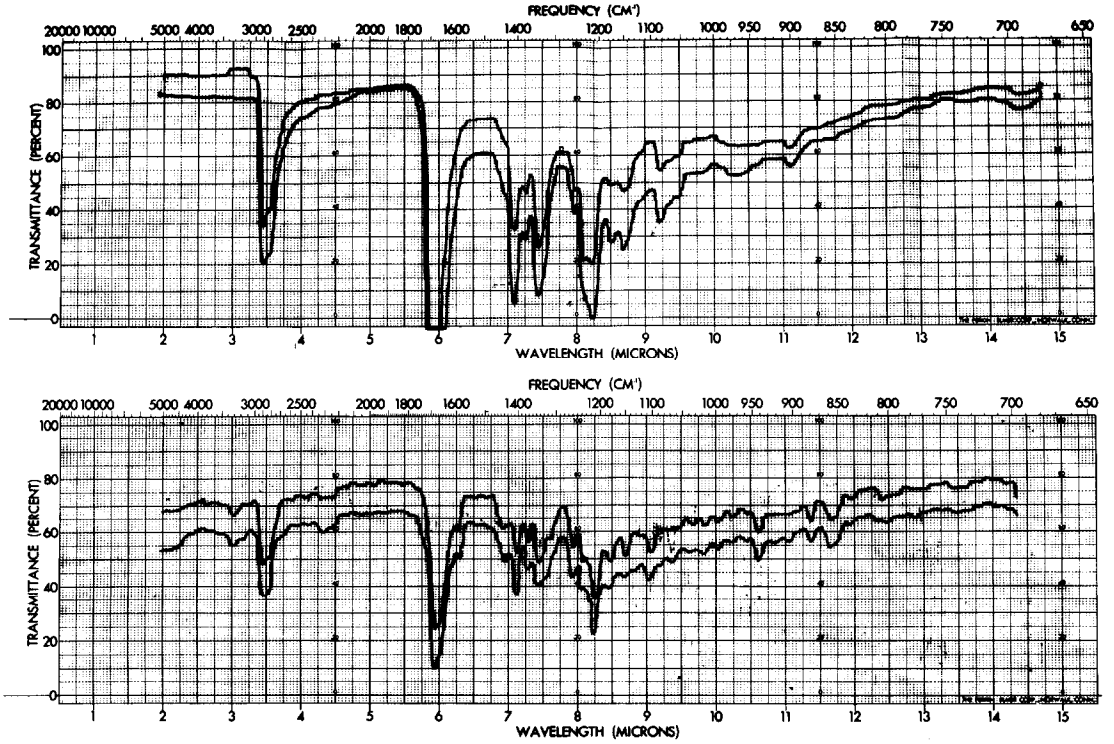

Fig. 1. IR spectra of 6β-hydroxyprogesterone conversion products formed during solvolysis. The top record: allopregnane-3,6,20-trione; the bottom record: Δ^4-pregnene-3,6,20-trione. In each record the upper tracing was obtained with a reference standard compound, the lower tracing — with the unknown isolated from solvolysate.

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of the presumably identical compounds (allopregnane-3,6,20-trione and Δ^4-pregnene-3,6,20-trione) prepared by us using established chemical procedures (see section "Experimental Procedures"). They were indeed identical, as shown in Fig. 1. The melting points of the same compounds were as follows: P-2, 231-233° C; allopregnane-3,6,20-trione, 232-233° C; P-3, 192-195° C; Δ^4-pregnene-3,6,20-trione, 192-194° C.

A general scheme for conversion of 6-hydroxy-Δ^4-3-ketosteroids to 5α-ane-3,6-diones and Δ^4-ene-3,6-diones by various procedures, including solvolysis, is shown in Fig. 2.

To obtain an additional confirmation that the transformation of

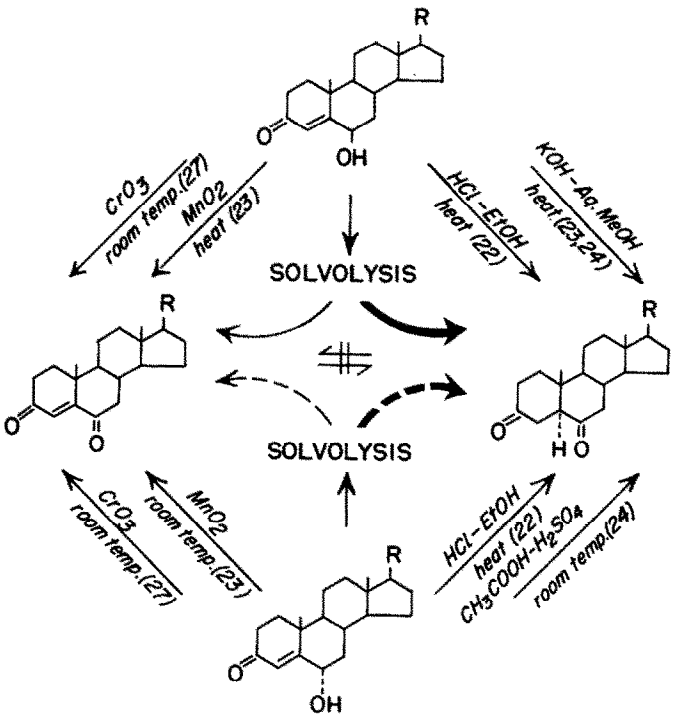

Fig. 2. A scheme of procedures for conversion of 6-OH-Δ^4-3-keto-steroids to 5α-ane-3,6-diones and Δ^4-ene-3,6-diones. Broken line indicates slower reaction; solid line, faster reaction. # indicates: not interconvertible during solvolysis.

6-OH-Δ^4-3-one grouping takes place irrespectively of the character of the substitution groups in other positions of the molecule, compound 6β-hydroxy-S was subjected to solvolysis and the derivatives formed (presumably 17α,21-dihydroxy-5α-pregnane-3,6,20-trione and 17α,21-dihydroxy-Δ^4-pregnene-3,6,20-trione) were oxidized overnight with CrO_3. The physicochemical properties (chromatographic mobility, U.V. absorbance, staining properties, reaction in ethanolic KOH, spectra in H_2SO_4) of the oxidation products were compared with those of the analogous compounds formed from 6β-OH-Δ^4-androstenedione during solvolysis. They were respectively identical. This also permitted a final identification of the 6β-OH-S derivatives formed during solvolysis. The sequence of the reactions followed is depicted in Fig. 3.

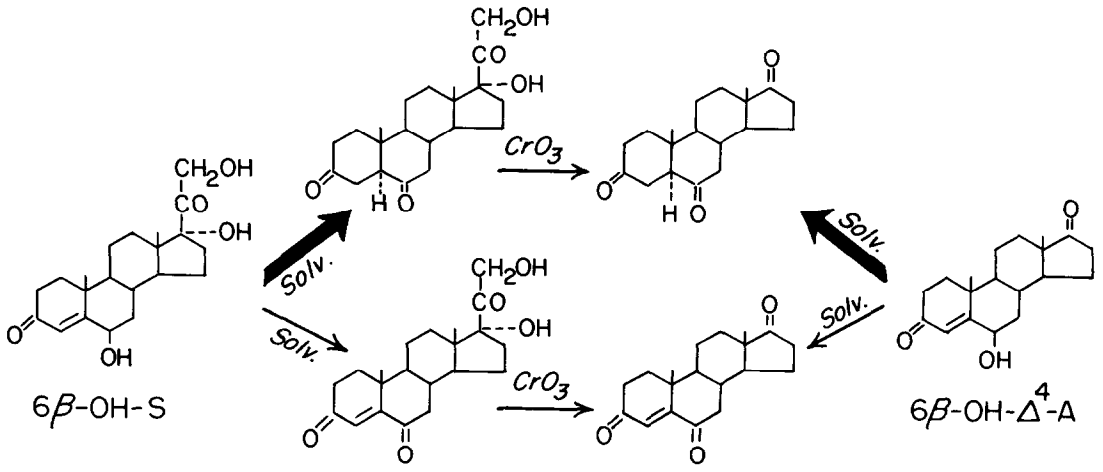

Fig. 3. Identification of 6β-OH-S derivatives formed during solvolysis.

Quantification of the reaction yields.

The solvolyzed and chomatographed compounds were eluted from paper strips and their concentrations were estimated by color reactions as described in the respective part of the section "Experimental Procedures". The results obtained are shown in Table VI. It will be seen that the ring-A saturated compounds consistently constituted the major conversion products; that, furthermore, the presence of ethyl alcohol in the reaction mixture slowed down the velocity of the reaction; and that the latter was also influenced to a certain degree by the structure of the side chain (possibly due to different solubility characteristics of various 6β-OH-compounds in the solvolysing mixture). The greater resistance of 6α-hydroxysteroids to the described transformations was here quantitatively confirmed. Furthermore, from the interrelation of the concentrations of the derivatives formed and the lengths of the incubation time necessary for their formation, a conclusion was drawn that the two conversion products (5α-ane-3,6-dione and Δ^4-ene-3,6-dione) were formed from the parent compound (6-OH-Δ^4-3,6-dione) by independent chemi-

TABLE VI.
Quantitation of the conversion yields.

Parent Compound	Solvolysis Conditions[a] Time,hr.	EtOH[b]	Conversion yields (as % of total products)[c] # 1	# 2	# 3
6β-OH-F	18	+	24	65	11
	60	+	0.5	80.7	18.8
	140	+	0	81	19
	18	(-)	0	87	13
6α-OH-F	18	+	89	11	0
	60	+	58	34	8
	140	+	25	58	17
	18	(-)	24	62	14
6β-OH-P	18	+	47	40	13
6β-OH-Δ^4A	18	+	55	30	13

[a] The temperature of the incubation in all these experiments was 37° C.

[b] + in this column indicates 20% (v/v) of ethyl alcohol present in solvolysates [as in the modified solvolysis procedure (1,5)]; (-) indicates absence of alcohol.

[c] # 1: unchanged portion of the parent compound; # 2: 5α-ane-3,6-dione; # 3: Δ^4-ene-3,6-dione.

cal pathways, and were not simply products of consecutive stages in the reaction. This was unequivocally confirmed by subjecting separately each of the conversion products to solvolysis. No interconversion either way was observed.

Solvolysis of 6β-OH-F isolated from urine.

This steroid was isolated from urine of 3 subjects by the method of Frantz et al. (7) and was, without further purification, subjected to

solvolysis. Three compounds were isolated from each solvolysate; they were identical with those isolated from solvolysates of pure crystalline 6β-OH-F.

Transformations during continuous ether extraction.

6β-hydroxyprogesterone subjected to the continuous ether extraction procedure for 5 days yielded two compounds which were identified as allopregnane-3,6,20-trione and Δ^4-pregnene-3,6,20-trione. This confirmed our supposition that the continuous ether extraction, being in essence a specific case of solvolysis, will result in the same kind of transformations of 6-OH-Δ^4-3-keto-grouping, as does the standard solvolytic procedure. An absence of the unchanged portion of the parent compound in the ether extract was noted, indicating a complete conversion of 6β-OH-progesterone to its two derivatives.

Column chromatography of the solvolysis transformation products.

Purification of allopregnane-3,6,20-trione was satisfactorily achieved employing either of the two following solvent systems: system b), in which the steroid was quantitatively eluted with 0.5% - 2.0% ethanol in benzene; system f), in which a complete elution was obtained with 12% - 14% ethyl acetate in benzene. Purification of Δ^4-pregnene-3,6,20-trione was achieved in the same two systems, the eluting fractions being 1% - 2% ethanol in benzene, and 16% - 40% ethyl acetate in benzene, respectively.

A direct separation by column chromatography of the mixture of compounds from solvolysates of 6β-OH-progesterone was only partly successful. Although it was possible to satisfactorily separate allopregnane-3,6,20-trione from Δ^4-pregnene-3,6,20-trione in the f) solvent system (the first compound was completely eluted with 12% - 14% ethyl acetate in benzene, the second — with 16% - 30% fractions),

the intact portion of the solvolysed 6β-OH-progesterone could not be recovered from the column, even after desintegration of the column, and extraction of alumina with ethanol. Further investigation demonstrated that 6β-OH-progesterone was quantitatively converted to allopregnane-3,6,20-trione during chromatography in this solvent system (benzene-ethyl acetate). Since this was not seen in the other solvent systems, it was concluded, that a reaction similar to that taking place during solvolysis was occuring during chromatography in the ethyl acetate containing sytem, the alumina probably acting as a catalyst. It is of interest to note that, unlike during solvolysis, Δ^4-pregnene-3,6,20-trione was not formed during column chromatography in ethyl acetate containing solvent systems. Acetylation or benzoylation of 6β-OH-progesterone, although successful in preventing the transformation of this compound during column chromatography, did not decrease its polarity enough for a satisfactory separation from the solvolysis conversion products in this system (34). It was possible, however, to modify the condition of solvolysis so (cf. Table VI) as to obtain a complete conversion of 6β-OH-progesterone to the two derivatives, which could then be satisfactorily separated on the column, as described above.

Finally, since it has been recently demonstrated that certain batches of ethyl acetate may generate appreciable amounts of hydrogen peroxide (3), a possible role of H_2O_2 in the mechanism of the transformations described was investigated. Various amounts of H_2O_2 were added to the solvolysing mixture, prior to the incubation. They neither increased the yield nor the rate of the reactions observed during solvolysis. To the contrary, there was a marked slowing down of the reaction rate. Two other, more polar artifacts were also noted when

H_2O_2 was added to solvolysates.

DISCUSSION

The results of the work described in this paper clearly demonstrated that 6-hydroxylated steroids undergo an appreciable artifact formation during solvolysis procedures (including that of continuous ether extraction). Since these procedures are very frequently used for splitting of steroid sulfates prior to chromatography of urinary or plasma steroids, it becomes obvious that, whenever a total profile of a class of steroids is the aim of an investigation, the 6-hydroxylated steroids should be removed from the extracts before solvolysis.

The importance of 6β-hydroxysteroids has been brought to focus by work of Burstein et al. (35), and Frantz et al. (7) who demonstrated that 6β-hydroxycortisol constitutes a major urinary free 17-hydroxycorticosteroid in guinea pig and man. The recent isolation by us of 6β-hydroxycortisol from the solvolysable fraction of conjugated corticosteroids in human urine (after a prior exhaustive removal of free 6β-OH-F) leads to interesting speculations regarding a possible physiological significance of this steroid (1), in addition to what is known about it (7,36-40). The findings reported in this paper pose a new problem — how to protect the 6β-OH-F, liberated by solvolysis from its sulfate, from the molecular rearrangement and dehydrogenation, to both of which this compound is object during solvolysis. Work along these lines is in progress.

Regarding the mechanism of the described reactions, steps involved in the formation of 5α-ane-3,6-dione most probably are: 1) protonation of 3-ketone followed by enolization ($\Delta^4 \rightarrow \Delta^3$); 2) shift of 6α-H to 5α position; 3) deprotonation of 6β-OH group to 6-ketone; 4) rearrangement

of the formed enol to 4β-H-3 ketone. Ethyl acetate, due to its available pairs of unshared electrons, probably facilitates the transport of protons. It is more difficult to envisage the mechanism of the formation of the minor conversion product, Δ^4-3,6-dione. Here the net change involves a loss of two protons and two electrons. In our preliminary experiments concerned with the elucidation of the mechanism of this reaction, the role of atmospheric oxygen as a possible electron acceptor has been excluded. Since the reaction involves only steroids and pure solvents, it is likely that one of the ethyl acetate degradation products acts as electron acceptor. Further work on the elucidation of these reactions, with the aid of tritiated compounds, is in progress.

Besides the discovery of the fact that 6-hydroxysteroids undergo during solvolysis transformations described in this paper, the results of this study help to elucidate the nature of certain polar steroids which have been noted by various investigators on paper chromatograms following continuous ether extraction of urine (9,27).

Finally, a simple one-step method is hereby offered for the preparation of steroid 5α-ane-3,6-diones and Δ^4-3,6-diones (although the latter in poor yield) from the corresponding 6β-OH steroids, this being of particular value when compounds with labile substitution groups (e.g. side chain of 6β-OH-F) are considered: the "solvolytic" method does not require any protection of these groups. A quantitative separation of the two synthesis products can be readily achieved by means of column chromatography. Moreover, our experience with alumina column chromatography in ethyl-acetate containing systems indicates that during a base catalyzed reaction only the α,β-saturated compounds are formed.

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