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IDENTIFICATION OF 3β,21-DIHYDROXY-5-PREGNENE-20-ONE DISULFATE IN HUMAN URINE

By JORGE R. PASQUALINI and MAX-F. JAYLE

(From the Laboratoire de Chimie Biologique, Faculté de Médicine, Paris, France)

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The chromatographic procedure described by Crépy, Jayle and Meslin (1) separates urinary steroid conjugates into two fractions, the less polar comprising the ester sulfates and the more polar, the glucuronides. In the application of this method to extracts of urines from normal and pathological subjects, it was observed (2) that the early glucuronide eluates contained a small fraction that gave a positive Vlitos reaction (3), characteristic of ester sulfates. This fraction was designated S_{II}, to distinguish it from the major sulfate fraction, S_I.

The existence of this fraction was confirmed in a previous publication (4), and it was shown that it contained a steroid of low polarity that reduced blue tetrazolium. The purpose of the present investigation was to isolate and identify this conjugate.

EXPERIMENTAL METHOD

Biological material. Normal subjects were given two intramuscular injections each of 40 U of ACTH-zinc phosphate at 12-hour intervals. Urine was collected for 24 hours after the second injection and approximately 10 L of urine was used for extraction.

Extraction of the free and conjugated steroids. Solid ammonium sulfate was added to the urine to give a 50 per cent (wt/vol) solution, which was extracted three times at pH 7 with an equal volume of ether: ethanol (3:1, vol/vol) (5).

Chromatography. A. Separation of ester sulfate and glucuronide fractions. The dry urine extract was dissolved in 100 ml of water and the free corticosteroids were extracted with dichloromethane. The conjugated corticosteroids corresponding to 1 L of urine were then chromatographed on several columns of 30 g of alumina. The columns were eluted with the following solvents: *1)* 200 ml of butanol: water (98:2); *2)* 250 ml of butanol: water (94:6); *3)* 250 ml of butanol: water (90:10); *4)* 300 ml of butanol: 0.1 per cent ammonium hydroxide (85:15). Ester sulfates were determined in each fraction by the Vlitos reaction, as modified by Crépy (3, 6, 7). After the addition of methylene blue to the aqueous extract, the methylene blue-steroid sulfate complex was extracted with chloroform, and the absorbance at 650 mμ was compared with that obtained with amounts ranging between 5 and 40 μg of sodium dehydroepiandrosterone sulfate. Glucuronides were determined by the Tollens reaction (8). B. Separation of fraction S_{II}. The first eluates obtained with butanol-ammonium hydroxide contained glucuronides but also gave a positive Vlitos reaction, suggesting the presence of a small quantity of polar ester sulfates. These eluates were pooled, evaporated to dryness, and rechromatographed on several columns containing 20 g of alumina. Elution was carried out as follows: *1)* 100 ml of ethanol: water (96:4); *2)* 100 ml of ethanol: water (90:10); *3)* 200 ml of ethanol: water (80:20); *4)* 200 ml of ethanol: water (60:40); *5)* 200 ml of ethanol: water (40:60); *6)* 200 ml of ethanol: water (20:80); *7)* 200 ml of ethanol: water (10:90); *8)* 200 ml of 0.01 per cent ammonium hydroxide in water. (The water was distilled twice and brought to pH 7 with dilute ammonium hydroxide before mixing it with the ethanol.)

Paper chromatography of ester sulfates. The S_{II} fraction, after removal of the glucuronides, and the synthetic steroid sulfate [1] were chromatographed in the two following solvent systems: *1)* butyl acetate: toluene: *n*-butanol/4 N ammonium hydroxide: methanol (60:30:10/50:50) (9); *2)* butyl acetate: *n*-butanol/4 N ammonium hydroxide (25:75/100).

Paper electrophoresis. Fraction S_{II} and the synthetic sulfates were electrophoresed on paper (Carl Schleicher and Schüll, Dassel no. 2043) in an apparatus of the Grassman and Hannig type. Electrophoresis was carried out for 6 hours at 60 v, 1 ma per cm at 20°C. Ammonium carbonate buffer (0.15 N, pH 8.7) was employed.

Detection of ester sulfate on paper. After electrophoresis or chromatography the ester sulfates were detected by an adaptation of the Vlitos reaction (10).

Preparation of 3β,21-dihydroxy-5-pregnene-20-one 3,21-disulfate. Ten mg of 3β,21-dihydroxy-5-pregnene-20-one

[1] In this paper the following reference compounds have been used: 11β,17α,21-trihydroxy-4-pregnene-3,20-dione (cortisol); 17α,21-dihydroxy-4-pregnene-3,11,20-trione (cortisone); 11β,21-dihydroxy-4-pregnene-3,20-dione (corticosterone); 21-hydroxy-4-pregnene-3,20-dione (deoxycorticosterone); 3α,21-dihydroxy-5β-pregnane-20-one (tetrahydrodeoxycorticosterone); 3β,21-dihydroxy-5β-pregnane-20-one; 3β,21-dihydroxy-5α-pregnane-20-one; 3β,21-dihydroxy-5-pregnene-20-one (21-hydroxypregnenolone); 3β,17β,dihydroxy-5α-androstane (androstanediol); 3α-hydroxy-5β-pregnane-20-one (pregnanolone); androstanediol disulfate; pregnanolone sulfate; cortisone 21-monosulfate; deoxycorticosterone 21-monosulfate.

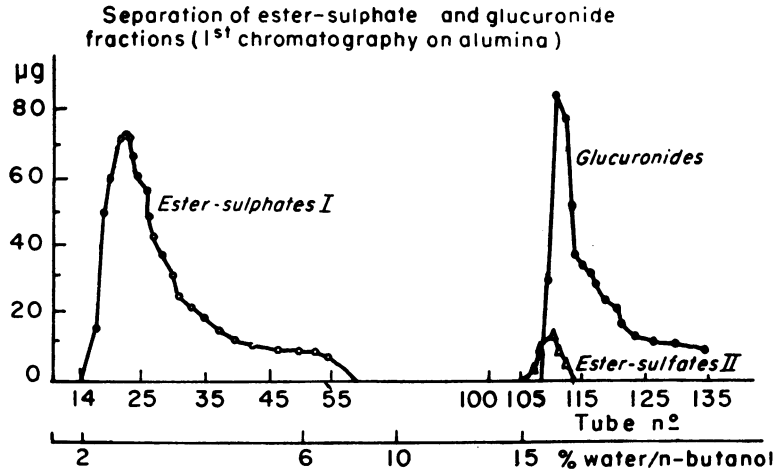

FIG. 1. SEPARATION OF ESTER SULFATE AND GLUCURONIDE FRACTIONS. First chromatography on alumina.

was treated with 25 mg of the pyridine-sulfur trioxide complex as described by Sobel and Spoerri (11), and after evaporation of the pyridine the dry residue was dissolved in a mixture of methanol: chloroform (3:1, vol/vol). The solution was evaporated and the residue chromatographed in the system: butanol acetate: *n*-butanol/4 N ammonium hydroxide (25:75/100). Two compounds giving a Vlitos-positive reaction were detected. The less polar (Rf 0.78) reduced tetrazolium blue; the more polar (Rf 0.28) reduced tetrazolium blue only after solvolysis.[2] The more polar material was eluted; paper chromatography showed it to be more polar than the reference monosulfates and similar to 3β,17β-dihydroxy-5α-androstane 3,17-disulfate in both chromatographic and electrophoretic migration. The quantity of this compound was not sufficient to permit crystallization; however, the positive Vlitos reaction, typical of sulfate conjugates, and the positive rhodizonate test for sulfate ion, after solvolysis, suggest that the synthetic material is 21-hydroxy-pregnenolone 3,21-disulfate.

Hydrolysis of ester sulfates on paper chromatograms. The sulfates were hydrolyzed on paper by the technic of Schneider and Lewbart (13). The dried chromatogram was suspended for 3.5 hours at 40°C in a covered cylindrical glass jar containing a mixture of concentrated hydrochloric acid and dioxane (1:3, vol/vol). The liberated sulfate ion was identified by the rhodizonate test (16) and the free corticosteroid by reaction with tetrazolium blue.

Solvolysis. An aliquot of fraction S_{II} from the chromatogram was hydrolyzed with dioxane-trichloroacetic acid mixture (12).

Enzymatic hydrolysis. A further aliquot of fraction S_{II}

[2] Solvolysis is the hydrolysis of a conjugated steroid in an organic solvent at room temperature (12–15).

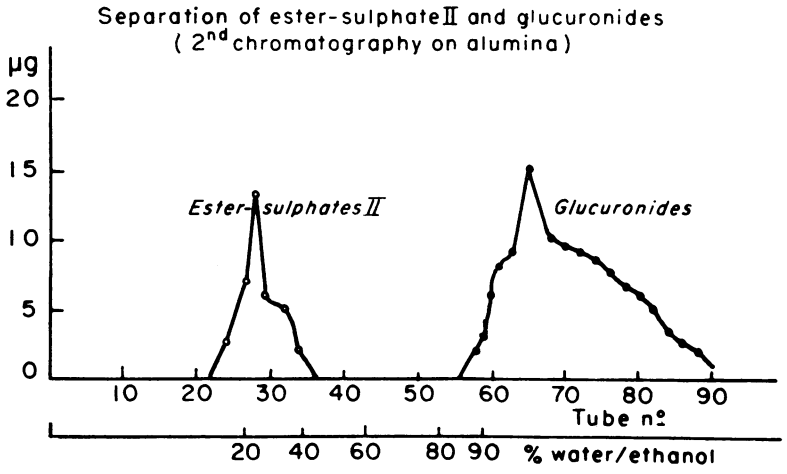

FIG. 2. SEPARATION OF ESTER SULFATE II AND GLUCURONIDES. Second chromatography on alumina.

TABLE I

Rf of the isolated steroid in five different chromatographic systems

Steroids	Structure	Toluene/ propanediol (4 hrs) R_{THDOC}	Isooctane/ methanol: water, 5/3:2 (8 hrs) R_{THDOC}	Isooctane/ propanediol (130 hrs) R_{THDOC}	Decalin/ propanediol (135 hrs) R_{THDOC}	Ligroin/ propanediol (60 hrs) R_{THDOC}
Isolated steroid of S_{II} fraction		0.84	0.88	0.61	0.62	0.72
3β,21-dihydroxy-5-pregnene-20-one	3β,Δ⁵	0.83	0.88	0.60	0.62	0.73
3β,21-dihydroxy-5α-pregnane-20-one	3β,5α	0.85	0.88	0.86	0.87	0.92
3α,21-dihydroxy-5β-pregnane-20-one (THDOC)	3α,5β	1	1	1	1	1
3β,21-dihydroxy-5β-pregnane-20-one	3β,5β	1.25	1.70		1.91	1.66

was subjected to hydrolysis by a purified preparation of sulfatase obtained from *Helix pomatia* (17) and Sigma bacterial glucuronidase.

Paper chromatography. The steroids released by solvolysis and enzymatic hydrolysis were chromatographed in the following systems: chloroform/formamide (18); toluene/propylene glycol (18); isooctane/methanol: water (5/3:2); ligroin/propanediol (19); decalin/propanediol; isooctane/propanediol.

Acetylation. Acetylation was carried out with acetic anhydride and pyridine.

Paper chromatography of the steroid acetates. The steroid acetates of low polarity were chromatographed on paper, without impregnation with the stationary phase as described previously (20). The solvents employed were ligroin (60° to 90°C) and isooctane.

RESULTS

Figure 1 shows the results obtained by initial chromatography of the conjugated steroids on alumina. Fraction S_I, comprising the greater part of the ester sulfates is well separated from the glucuronide fraction. Fraction S_{II}, giving a positive Vlitos reaction, appears in the first eluates of the glucuronide fraction.

Figure 2 shows the separation of fraction S_{II} from the glucuronide fraction by rechromatography on alumina as described above. The S_{II} fraction was eluted with 40 per cent ethanol, the glucuronides with 10 per cent ethanol and water, at pH 8.4.

Composition of fraction S_{II}. Fraction S_{II} was hydrolyzed with dioxane-trichloroacetic acid and the steroids extracted with dichloromethane. After removal of the solvent the dried extract was chromatographed in the chloroform/formamide system, with cortisol, cortisone, and corticosterone as reference steroids. We observed that the bulk of the material, which was blue tetrazolium-reducing, moved with the solvent front in this system. This material was eluted and rechromatographed in five different solvent systems; only one spot was observed after treatment with blue tetrazolium. The results are shown in Table I. In each case the rate of migration of the isolated compound was identical with that of 21-hydroxypregnenolone.

Tetrahydrodeoxycorticosterone, 21-hydroxy-

TABLE II

Rf of the isolated steroid acetates (paper adsorption chromatography)

Acetates	Structure	Solvents: Isooctane RDiac. THDOC	Solvents: Ligroin RDiac. THDOC
Steroid acetate from S_{II} fraction		0.66	0.53
3β,21-diacetoxy-5-pregnene-20-one	3β,Δ⁵	0.66	0.54
3β,21-diacetoxy-5α-pregnane-20-one	3β,5α	0.71	0.63
3α,21-diacetoxy-5β-pregnane-20-one (Diac. THDOC)	3α,5β	1	1

TABLE III

Properties of the isolated steroid

Physicochemical reactions	Isolated steroid	3β,21-dihydroxy-5-pregnene-20-one	3β,21-dihydroxy-5α-pregnane-20-one
Blue tetrazolium	pos.	pos.	pos.
Porter-Silber	neg.	neg.	neg.
Zimmermann	neg.	neg.	neg.
Absorption at 254 mμ	neg.	neg.	neg.
Soda fluorescence in Wood's light	neg.	neg.	neg.
Formaldehydogenic test (KIO_4)	pos.	pos.	pos.
Absorption maxima in conc. sulfuric acid (mμ)	320	320	320
Absorption maxima in sulfuric acid: ethyl alcohol (2:1, vol/vol) (mμ)	405	405	No absorption, 400–410 mμ

pregnenolone, 3β,21-dihydroxy-5α-pregnane-20-one, and the unknown compound were acetylated and the acetates chromatographed on paper as described previously (20). Relative rates of migration are shown in Table II.

It is apparent that the diacetate of tetrahydrodeoxycorticosterone (Diac. THDOC) is less polar than is the acetate of the unknown compound, which has the same polarity as the diacetate of the 21-hydroxypregnenolone.

The unknown steroid and its acetate were eluted from paper and purified on silica gel to remove the impurities from the paper and solvent prior to spectroscopy. A column containing 3 g of silica gel was used and sequential elution was carried out with *1*) ethyl ether; *2*) ethyl ether : chloroform (90 : 10); *3*) ethyl ether : chloroform (70 : 30); *4*) ethyl ether : chloroform (50 : 50); *5*) ethyl ether : chloroform (20 : 80); *6*) chloroform; *7*) chloroform : ethyl acetate (95 : 5); *8*) chloroform : ethyl acetate (90 : 10); *9*) chloroform : ethyl acetate (85 : 15); *10*) chloroform : ethyl acetate (80 : 20); *11*) chloroform : ethyl acetate (70 : 30); *12*) chloroform : ethyl acetate (50 : 50).

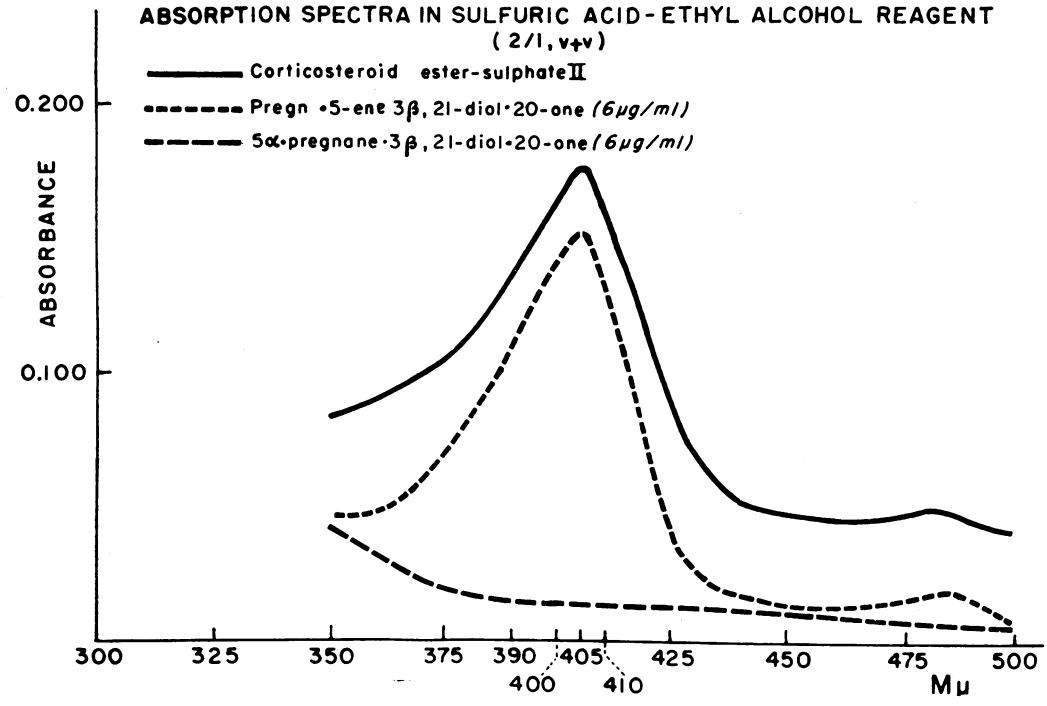

FIG. 3. ABSORPTION SPECTRA IN SULFURIC ACID : ETHYL ALCOHOL REAGENT (2 : 1, VOL/VOL).

The steroid was eluted with chloroform: ethyl acetate (85:15). The acetate was purified in the same fashion and eluted with ethyl ether. Table III shows the properties of the unknown steroid, together with those of 21-hydroxypregnenolone and 3β,21-dihydroxy-5α-pregnane 20-one.

Figure 3 shows absorption spectra in sulfuric acid: ethyl alcohol (2: 1, vol/vol) (21) between 350 and 500 mμ for the unknown steroid, 21-hydroxypregnenolone, and 3β,21-dihydroxy-5α-pregnane 20-one. The unknown compound shows the absorption maximum at 405 mμ, characteristic of 3β-hydroxy-Δ^5 steroids.

Infrared spectra. Infrared spectra were obtained on 100-μg quantities of the free steroid and its acetate in a potassium bromide disk. The spectrum was determined on a Perkin-Elmer spectrophotometer (model 13). In the spectrum of the compound, an absorption band occurs at 1,705 cm^{-1} which is typical of the 20-carbonyl function and another band at 1,043 cm^{-1} which is typical of the 3β-hydroxy-Δ^5 grouping.

In the spectrum of the acetate of the isolated steroid the absorption band occurs at 1,740 cm^{-1} (21-acetoxy 20-keto function) and 1,240 cm^{-1} (3-acetoxy function).

Quantitative evaluation. Table IV shows the quantitative evaluation of the steroid isolated from the fraction S_{II} by means of blue tetrazolium (22).

Nature of the conjugate. To investigate the type of conjugation of the principal component of fraction S_{II}, its electrophoretic and chromatographic behavior was compared with that of the reference steroid sulfates.

In system I (butyl acetate: toluene: *n*-butanol/4 N ammonium hydroxide: methanol (60: 30: 10/50: 50), fraction S_{II} did not migrate, but extraneous material was separated. In this system cortisone 21-sulfate has a migration rate of 0.20 cm per hour, and deoxycorticosterone 21-sulfate 1.20 cm per hour. After elution, fraction S_{II} was rechromatographed in system II [butyl acetate: *n*-butanol/4 N ammonium hydroxide (25:75/100], together with the reference steroid sulfate. Table V shows the Rf values obtained.

The steroid sulfates were detected by the chloroform-methylene blue reaction. After solvolysis, a positive rhodizonate reaction was obtained. Table V shows that fraction S_{II} has the same migration rate as 3β,17β-dihydroxy-5α-androstane disulfate and as synthetic 21-hydroxypregnenolone disulfate. Table VI shows the rate of electrophoretic migration of fraction S_{II} and the reference sulfate.

The steroid disulfates migrate more slowly than the monosulfates in the chromatographic system but are relatively faster on electrophoresis. Fraction S_{II} migrates at the same speed as both 3β,17β-dihydroxy-5α-androstane disulfate and 3β,21-dihydroxy-5-pregnene-20-one disulfate.

TABLE IV

Excretion rate of the steroid isolated from the fraction S_{II}

Urine source	Steroid excreted
	μg/L
Normal subjects after ACTH	70
Pregnancy (7 months)	25

TABLE V

Paper chromatography of fraction S_{II} and reference steroid sulfates in butyl acetate: n-butanol/NH_4OH, 4N (25:75/100)

Steroid sulfates	Rf
Fraction S_{II}	0.28
Synthetic 3β,21-dihydroxy-5-pregnene-20-one disulfate	0.27
3β,17β-dihydroxy-5α-androstane disulfate	0.32
Cortisone 21-monosulfate	0.64
Deoxycorticosterone 21-monosulfate	0.78

TABLE VI

Paper electrophoretic migration of fraction S_{II} and reference steroid sulfates

Steroid sulfates	Buffer $(NH_4)_2CO_3$, 0.15 N
	cm/hr
Fraction S_{II}	2.00
3β,21-dihydroxy-5-pregnene-20-one disulfate	2.00
3β,17β-dihydroxy-5α-androstane disulfate	2.12
Cortisone 21-monosulfate	1.10
Deoxycorticosterone 21-monosulfate	1.10
Pregnanolone 3α-monosulfate	0.80
Androsterone monosulfate	1.20

DISCUSSION

The identification of the conjugate isolated from human urine as 21-hydroxypregnenolone disulfate appears to be well established on the basis of the chemical procedures employed. Hydrolysis of the conjugate liberated a steroid of low polarity whose chromatographic behavior was identical with that of 21-hydroxypregnenolone, both as the free steroid and after acetylation. The possibility that the unknown steroid was an isomer of tetrahydrodeoxycorticosterone was eliminated by chromatographic comparison. Further evidence of structure is also offered by the absorption spectrum in sulfuric acid-ethanol, which is typical of Δ^5,3β-hydroxysteroids. A negative Porter-Silber reaction would rule out a dihydroxy acetone side chain, whereas the release of formaldehyde after periodic acid oxidation and reduction of blue tetrazolium are evidence for an α-ketol grouping.

The conjugation of the steroid with sulfuric acid is evidenced by its properties, which are typical of steroid sulfates. Thus it is extractable by chloroform after reaction with methylene blue (Vlitos reaction) and is hydrolyzed by a sulfatase preparation. By contrast, bacterial glucuronidase was found to be ineffective. The conjugate undergoes solvolysis in dioxane containing trichloroacetic acid (12), which is considered specific for ester sulfates and after solvolysis gives a positive reaction for sulfate ion with rhodizonate.

It seems reasonable to formulate the compound as a disulfate, on the basis of several observations. First, a 3-monosulfate structure is eliminated, since prior to hydrolysis the compound does not reduce blue tetrazolium. A 21-monosulfate structure also seems unlikely, since the isolated conjugate is more polar than is the 21-monosulfate of corticosteroids and far more polar than 21-hydroxypregnenolone (9), both on paper partition and alumina chromatography. On the other hand, its chromatographic mobility is similar to that of 3β,17β-dihydroxy-5α-androstane disulfate.

Further evidence is offered by observations on electrophoretic migration. It has been shown by Cavina (23), that steroid disulfates migrate more rapidly than do the monosulfates, and this is in agreement with our finding that the monosulfates available to us migrated at only half the speed of androstanediol disulfate. The urinary conjugate studied here moved at an electrophoretic mobility very similar to this latter compound.

Finally, the most polar material obtained by the action of pyridine-sulfur trioxide on synthetic 21-hydroxypregnenolone showed chromatographic and electrophoretic behavior identical with that of the urinary conjugate.

We have shown previously that both 3β-hydroxysteroids (24) and corticosterone and cortisol (25) are excreted in human urine conjugated with sulfuric acid, in the latter case as 21-monosulfates. It therefore seems reasonable to assume that a compound with a 3β,21-dihydroxy structure would appear as a disulfate.

On the basis of those findings there appears to be little doubt that the polar sulfate isolated from the urine of subjects who had received ACTH is 21-hydroxypregnenolone 3,21-disulfate.

SUMMARY

A conjugated steroid has been isolated from the urine of subjects who had received zinc-ACTH. This compound possessed the properties of an ester sulfate and solvolysis or hydrolysis liberated a steroid identified as 21-hydroxypregnenolone. The conjugate showed the characteristics of a steroid disulfate, and its electrophoretic and chromatographic behavior was identical with that of the most polar compound obtained by the action of pyridine-sulfur trioxide on 21-hydroxypregnenolone.

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ADDENDUM

In our recent research we administered orally 280 mg of 21-hydroxypregnenolone 21-monoacetate to a normal subject, and the urine was collected for 24 hours. Using the method described in this work we isolated about 3 mg of 21-hydroxypregnenolone disulfate, identical with the isolated ester sulfate from fraction S_{II} after administration of ACTH; 21-hydroxypregnenolone was found in neither the free nor the glucuronide fraction.

REFERENCES

1. Crépy, O., Jayle, M. F., and Meslin, F. Separation chromatographique des sulfates et des glycuronides

de stéroïdes urinaires. I. Application aux stéroïdes conjugués provenant de l'androstérone et de la déhydroépiandrosterone. Acta endocr. (Kbh.) 1957, **24**, 233.

2. Crépy, O. Personal communication.
3. Vlitos, A. J. Biological activation of sodium 2-(2,4-dichloro-phenoxy) ethyl sulfate. Contr. Boyce. Thomson Inst. 1953, **17**, 127.
4. Pasqualini, J. R. Formas de conjugación de los corticosteroides urinarios. Acta physiol. lat.-amer. 1960, **10**, 135.
5. Edwards, R. W. H., Kellie, A. E., and Wade, A. P. The extraction and oxydation of urinary steroid conjugates. Mem. Soc. Endocr. (Lond.) 1953, **2**, 53.
6. Crépy, O., and Rulleau-Meslin, F. Détermination quantitative et qualitative des sulfates de stéroïdes à l'aide du bleu de méthylène. I. Microdosage des stéroïdes sulfates. Rev. franç. Ét. clin. biol. 1960, **5**, 283.
7. Roy, A. B. The steroid sulphatase of *Patella vulgata*. Biochem. J. 1956, **62**, 41.
8. Tollens, C. Über einen einfachen Nachweis der Glucuronsäure mittels Naphthoresorcin, Salzsäure und Äther. Ber. dtsch. chem. Ges. 1908, **41**, 1788.
9. Pasqualini, J. R., Zelnik, R., and Jayle, M. F. Séparation par chromatographie sur papier des 21-sulfates de corticostéroïdes. Experientia 1960, **16**, 317.
10. Crépy, O., and Judas, O. Détermination quantitative et qualitative des sulfates de stéroïdes à l'aide du bleu de méthylène II. Détection des sulfates de stéroïdes en chromatographie sur papier. Rev. franç. Ét. clin. biol. 1960, **5**, 284.
11. Sobel, A. E., and Spoerri, P. E. Steryl sulfates. I. Preparation and properties. J. Amer. chem. Soc. 1941, **63**, 1259.
12. Cohen, S. L., and Oneson, I. B. The conjugated steroids. IV. The hydrolysis of ketosteroid sulfates. J. biol. Chem. 1953, **204**, 245.
13. Schneider, J. J., and Lewbart, M. L. Enzymatic synthesis of steroid sulfates. J. biol. Chem. 1956, **222**, 787.
14. Burstein, S., and Lieberman, S. Hydrolysis of ketosteroid hydrogen sulfates by solvolysis procedures. J. biol. Chem. 1958, **233**, 331.
15. Burstein, S., and Lieberman, S. Kinetics and mechanism of solvolysis of steroid hydrogen sulfates. J. Amer. chem. Soc. 1958, **80**, 5235.
16. Burma, D. P. Separation of lithium, sodium and potassium present as sulfates and citrates by means of paper chromatography. Analyt. Chim. Acta 1953, **9**, 513.
17. Jarrige, P., and Lafoscade, G. Élution des protéines après séparation par électrophorèse à travers un gel d'amidon. Bull. Soc. Chim. biol. (Paris) 1959, **41**, 1197.
18. Zaffaroni, A., and Burton, R. B. Identification of corticosteroids of beef adrenal extract by paper chromatography. J. biol. Chem. 1951, **193**, 749.
19. Savard, K. Paper partition chromatography of C_{19}- and C_{21}-ketosteroids. J. biol. Chem. 1953, **202**, 457.
20. Pasqualini, J. R., and Jayle, M. F. Chromatographie d'adsorption sur papier des acétates de stéroïdes peu polaires. J. Chromatog. 1961, **5**, 336.
21. Oertel, G. M., and Eik-Nes, K. B. Determination of Δ^5-3-beta-hydroxysteroids. Analyt. Chem. 1959, **31**, 98.
22. Mader, W. J., and Buck, R. R. Colorimetric determination of cortisone and related ketol steroids. Analyt. Chem. 1952, **24**, 666.
23. Cavina, G. Elettroforesi e chromatografia su carta di coniugati di steroidi: Applicazione alla preparazione ed all'isolamento di alcuni esteri solforici. Est. Rend. Ist. Sup. Sanita (Rome) 1957, **20**, 923.
24. Jayle, M. F. Données nouvelles sur la glucuroconjugaison et la sulfoconjugaison. Thérapie 1958; **13**, suppl. 37.
25. Pasqualini, J. R., and Jayle, M. F. Corticosteroid 21-sulphates in human urine. Biochem. J. 1961, **81**, 147.