Isolation and Identification of 3 α ,17 α ,21-Trihydroxy-5 α -Pregnan-20-one (Allo-Tetrahydro-Compound S) after Administration of 3 β ,17 α ,21-Trihydroxypregn-5-en-20-one (17 α ,21-Dihydroxypregnenolone)

By J. R. PASQUALINI AND M.-F. JAYLE

Laboratoire de Chimie Biologique, Faculté de Médecine, 45 Rue des Saints-Pères, Paris 6, France

(Received 18 December 1962)

Pasqualini & Jayle (1962) found that 3 β ,21-dihydroxypregn-5-en-20-one (21-hydroxypregnenolone) was excreted in human urine as the 3,21-disulphate. This conjugate was isolated from the urine of subjects who had received adrenocorticotrophic hormone, from pregnancy urine and from urine collected after the oral administration of 21-hydroxypregnenolone 21-monoacetate (Pasqualini, Dutter & Jayle, 1963).

The present study continues our investigation of the influence of the structure of a corticosteroid on its sulphoconjugation (Pasqualini, 1958, 1960; Pasqualini & Jayle, 1961*a*), which revealed that sulphoconjugation at the C₍₂₁₎ hydroxyl group of a corticosteroid diminishes when an α -hydroxyl group is present at C₍₁₇₎. Evidence of this was provided after the administration of 3 β ,17 α ,21-trihydroxypregn-5-en-20-one (17 α ,21-dihydroxypregnenolone 21-monoacetate), which is excreted as the 3 β -monosulphate (Pasqualini *et al.* 1963).

In the present paper we describe the identification of a new corticosteroid in human urine.

MATERIALS AND METHODS

Biological material. 17 α ,21-Dihydroxypregnenolone 21-monoacetate was administered orally to a normal subject in three 100 mg. doses at 1 hr. intervals. The urine was collected for 24 hr. after the first dose.

17 α ,21-Dihydroxypregnenolone 21-monoacetate was obtained from L. Light and Co. Ltd., Colnbrook, Bucks. After saponification (Meyer, 1953) of this compound we found no traces of 3 α ,17 α ,21-trihydroxypregn-5-en-20-one, 3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one (tetrahydro-compound S), or 3 α ,17 α ,21-trihydroxy-5 α -pregnan-20-one (allo-tetrahydro-compound S). The purity of the saponified compound was verified in several chromatographic systems and we found only one spot each time. The mobility was in every case identical with that of the reference steroid (U.S.P. Steroid Reference Standards, 46 Park Avenue, New York, U.S.A.). The steroid was oxidized with sodium bismuthate (Brooks & Norymberski, 1952) and the oxidation product was proved to be identical with dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one). The acetate of the oxidation product also had the same mobility as that of synthetic dehydroepiandrosterone acetate. Not more than 0.01% of other steroids was present in the compound administered.

Extraction of free and conjugated corticosteroids. The urine, to which solid ammonium sulphate had been added to give a 50% (w/v) solution, was extracted three times at pH 7 with an equal volume of ether-ethanol (3:1, v/v) (Edwards, Kellie & Wade, 1953). The extractions were made in separating funnels with continual shaking for 1 hr. The ethereal extracts were combined and evaporated. The dry urine extract was dissolved in water and the free corticosteroids were extracted with dichloromethane. The aqueous solution containing the conjugated corticosteroids was evaporated to dryness.

Alumina chromatography. The conjugated corticosteroids were separated on alumina into ester sulphates and glucuronides (Crepay, Jayle & Meslin, 1957). The glucuronides contained a small fraction of ester sulphates which were separated from the glucuronides by a second alumina-column chromatography (Pasqualini & Jayle, 1962).

Hydrolysis of the steroid conjugates. The ester sulphate fractions of both low and high polarity were hydrolysed with sulphatase from the succus entericus of *Helix pomatia* (500 Roy units/ml. of urine; Roy, 1956). The glucuronides were hydrolysed with β -glucuronidase (500 Fishman units/ml. of urine; Fishman, Springer & Brunetti, 1948). The steroids released were extracted three times with 1 vol. of dichloromethane. The combined extracts were washed once with 0.05 vol. of water.

Paper chromatography. We used the following chromatographic systems: (I) chloroform with formamide-treated paper (Burton, Zaffaroni & Keutmann, 1951); (II) diisopropyl ether-water-formamide (10:1:1, by vol.); (III) benzene with formamide-treated paper; (IV) toluene with propanediol-treated paper; (V) hexane-benzene (1:1, v/v) with propanediol-treated paper; (VI) ligroin with propanediol-treated paper (Savard, 1953); (VII) iso-octane with propanediol-treated paper; (VIII) adsorption paper chromatography without impregnation with the stationary phase: the solvent used was iso-octane (Pasqualini & Jayle, 1961*b*).

Oxidation with sodium bismuthate. This reaction (Brooks & Norymberski, 1952; Norymberski, Stubbs & West, 1953) was carried out as follows. A sample (25 μ g.) of steroid was dissolved in 2 ml. of 50% (v/v) acetic acid, and 30 mg. of sodium bismuthate was added. The suspension was shaken mechanically for 1 hr. The reaction was stopped with 2 ml. of aq. 6% (w/v) sodium disulphite (metabisulphite), and after the addition of 2.5 ml. of water the solution was extracted three times with 1 vol. of dichloromethane. The dichloromethane was washed with 0.05 vol. of 9% (w/v) NaHCO₃ and with 0.05 vol. of water.

Acetylation. A sample (20 μ g.) of the compound studied and 20 μ g. of the product of its oxidation were dissolved in

4 drops of pyridine and 4 drops of acetic anhydride, and left for 18 hr. in the dark at room temperature.

Detection of the steroids. The corticosteroids were detected by the tetrazolium blue reaction (Mader & Buck, 1952) and the 17-oxo steroids by the Zimmermann (1935) reaction.

Tetrazolium blue microreaction. A portion (0.5–3 μ g.) of the compound studied was dissolved in 0.3 ml. of ethanol, and then 0.1 ml. of 0.5% (w/v) blue tetrazolium in aq. 96% (v/v) ethanol and 0.1 ml. of 1% (v/v) tetramethylammonium hydroxide in aq. 90% (v/v) ethanol were added. The mixture was left for 30 min. at room temperature, and then 0.2 ml. of acetic acid was added. The extinction was measured at 510 $m\mu$. Cortisone was used as the reference steroid.

Phenylhydrazine microreaction. This reaction (Porter & Silber, 1950) was carried out as follows. A sample (1–4 μ g.) of the compound studied was dissolved in 0.6 ml. of phenylhydrazine in acid ethanol [65 mg. of phenylhydrazine hydrochloride was dissolved in a mixture of 33 ml. of ethanol and 66 ml. of 62% (v/v) sulphuric acid]. The mixture was left for 30 min. at 60°. The extinction was then read at 410 $m\mu$. From this value the extinction obtained when the same quantity of the compound studied had been treated with ethanol–62% (v/v) sulphuric acid (1:2, v/v) was subtracted. Cortisone was used as reference steroid.

Reference compounds. In the present work the following reference compounds were used: 17 α ,21-dihydroxypregn-4-ene-3,20-dione (compound S); 11 β ,21-dihydroxypregn-4-ene-3,20-dione (corticosterone); 17 α ,21-dihydroxypregn-4-ene-3,11,20-trione (cortisone); 3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one (tetrahydro-compound S); 3 α ,17 α ,21-trihydroxy-5 α -pregnan-20-one (allo-tetrahydro-compound S); 3 β ,17 α ,21-trihydroxypregn-5-en-20-one (17 α ,21-dihydroxypregnenolone); 3 α ,11 β ,21-trihydroxy-5 β -pregnan-20-one (tetrahydrocorticosterone); 3 β ,21-dihydroxypregn-5-en-20-one (21-hydroxypregnenolone); 17 α ,21-dihydroxy-5 β -pregnane-3,20-dione (5 β -dihydro-compound S); 3 α ,17 α ,20 ξ ,21-tetrahydroxy-5 β -pregnane (20 ξ -hexahydro-compound S); 3 α -hydroxy-5 α -androstane-17-one (androsterone); 3 α -hydroxy-5 β -androstane-17-one (etiocholanolone); 3 β -hydroxy-5 α -androstane-17-one (epiandrosterone); 3 β -hydroxy-5 β -androstane-17-one (epietiocholanolone); 3 β -hydroxyandrost-5-en-17-one (dehydroepiandrosterone); 3 α -hydroxyandrost-5-en-17-one; 3 α -hydroxy-5 α -androstane-3,17-dione; androst-5-ene-3,17-dione.

EXPERIMENTAL AND RESULTS

Qualitative studies. The corticosteroids released by hydrolysis of the ester sulphate and the glucuronide fractions were chromatographed in the chloroform–formamide system (I). The zone with a mobility range of R_{TBS} 1.00–1.25 (R_{TBS} is the mobility with respect to tetrahydro-compound S) was eluted from the chromatogram and rechromatographed in the di-isopropyl ether–formamide–water system (II). The zone with a mobility range of R_{TBS} 1.35–1.60 in system (II) was eluted and rechromatographed in the benzene–formamide system (III). The zone with a mobility range of R_{TBS} 1.35–1.65 in system (III) was eluted and the

eluate was evaporated to dryness. The residue was chromatographed on a column containing 2 g. of silica gel made up in dichloromethane and eluted successively with dichloromethane containing 5, 10, 15, 20, 25, 35, 50, 70 and 90% (v/v) of ethyl acetate, and ethyl acetate itself.

The corticosteroid studied was eluted with dichloromethane containing 70% (v/v) of ethyl acetate.

Table 1 shows the R values of the substance studied compared with those of tetrahydro-compound S, allo-tetrahydro-compound S, corticosterone, compound S, 17 α ,21-dihydroxypregnenolone and tetrahydrocorticosterone.

The isolated compound gave a positive tetrazolium blue reaction and a positive phenylhydrazine reaction, did not absorb at 240 $m\mu$ in ethanol solution, and was not fluorescent after treatment with 2N-sodium hydroxide (Bush, 1953). After oxidation with bismuthate a steroid giving a positive Zimmermann reaction was obtained. Table 2 shows the R values of this oxidized steroid compared with those of dehydroepiandrosterone, androsterone,

Table 1. R values of the isolated compound in four different systems

The isolation of the compound and the details of the chromatographic systems are given in the text.

System	R relative to compound S			
	(I)	(II)	(III)	(IV)
Steroids				
Compound isolated	0.70	4.95	0.82	0.53
Allo-tetrahydro-compound S	0.72	4.98	0.83	0.52
Tetrahydro-compound S	0.64	3.40	0.56	0.32
17 α ,21-Dihydroxy-pregnenolone	0.61	2.80	0.48	0.27
Tetrahydrocorticosterone	0.59	1.35	0.53	0.45
Compound S	1.00	1.00	1.00	1.00
Corticosterone	0.92	0.60	1.04	1.49

Table 2. R values of the oxidation product of the compound isolated

The isolation and oxidation of the compound and the details of the chromatographic systems are given in the text.

System	R relative to androsterone	
	(V)	(VI)
Steroids		
Oxidation product	1.00	1.00
Androsterone	1.00	1.00
Epietiocholanolone	0.97	0.95
Etiocholanolone	0.83	0.58
Epiandrosterone	0.74	0.53
Dehydroepiandrosterone	0.67	0.50

Scheme 1

etiocholanolone, epietiocholanolone and epiandrosterone.

The acetate of the compound isolated has the same migration as the 3,21-diacetate of allo-tetrahydro-compound S in chromatographic systems (VI) and (VII).

The acetate of the compound obtained after oxidation of the compound isolated had the same migration as androsterone monoacetate in chromatographic system (VIII). This system allows the differentiation of the axial isomer with 3 α ,5 α -structure from its 3 β ,5 β -isomer (Pasqualini & Jayle, 1961b).

Scheme 1 shows the steps taken in the identification of allo-tetrahydro-compound S after the administration of 17 α ,21-dihydroxypregnenolone.

Quantitative determinations. We studied the distribution of the corticosteroids in the different urinary fractions and found 240 $\mu\text{g.}/24$ hr. of the isolated compound in the least-polar ester sulphate fraction and 160 $\mu\text{g.}/24$ hr. in the glucuronide fraction, but none was traced in either the free or the most-polar ester sulphate fractions.

DISCUSSION

These results show that, after the administration of 17 α ,21-dihydroxypregnenolone to a normal subject, part of it is metabolized as a steroid with a

mobility identical with that of allo-tetrahydro-compound S in four different chromatographic systems.

The possibility of the unknown steroid having a 3 β ,5 β -structure was ruled out since the chromatographic behaviour of the 17-oxo steroid obtained by oxidation was the same as that of androsterone whose migration is also similar to that of epietiocholanolone. Comparison by paper adsorption chromatography in system (VIII) of the acetate of the oxidation product of the unknown steroid, androsterone acetate, and epietiocholanolone acetate provided evidence that the 17-oxo steroid obtained after oxidation with bismuthate of the corticosteroid studied was androsterone.

On the basis of these findings there appears to be little doubt that the corticosteroid isolated is 3 α ,17 α ,21-trihydroxy-5 α -pregnan-20-one (allo-tetrahydro-compound S).

Richardson *et al.* (1955) demonstrated that compound S (17 α , 21-dihydroxypregn-4-ene-3,20-dione) is metabolized to tetrahydro-compound S (3 α ,17 α ,21-trihydroxy-5 β -pregnan-20-one); Gandy, Keutmann & Izzo (1960) identified 5 β -dihydro-compound S in a patient with adreno-genital syndrome; Eberlein & Bongiovanni (1956) identified 20 ξ -hexahydro-compound S (3 α ,17 α ,20 ξ ,21-tetrahydroxy-5 β -pregnane). It therefore seems reasonable to assume that hydrogenation of the C₄-C₅,

Scheme 2

double bond of compound S takes place more readily to give the 5β -pregnane metabolite. By contrast hydrogenation at the $C_{(5)}-C_{(6)}$ double bond of $17\alpha,21$ -dihydroxypregnenolone takes place more readily to give the 5α -pregnane derivative.

It is possible that during its metabolism $17\alpha,21$ -dihydroxypregnenolone passes through the $3\text{-oxo-}\Delta^5$ compound (Talalay, 1957), and this hypothetical compound would have its 3-oxo group reduced to 3α -hydroxyl and its double bond Δ^5 reduced to give the 5α -compound, as proved by Fukushima, Williams, Zumoff & Gallagher (1962), who demonstrated that after administration of androst-5-en-3,17-dione part of it is transformed into 3α -hydroxy-androst-5-en-17-one and into 3α -hydroxy- 5α -androst-17-one. Scheme 2 shows the steps of this possible pathway. Of the allo-tetrahydro-compound S isolated in this research 60 % was sulpho-conjugated and 40 % glucuroconjugated.

SUMMARY

1. $3\alpha,17\alpha,21$ -Trihydroxy- 5α -pregnan-20-one (allo-tetrahydro-compound S) has been isolated and identified in human urine after the administration of $3\beta,17\alpha,21$ -trihydroxypregn-5-en-20-one ($17\alpha,21$ -dihydroxypregnenolone) to a normal subject.

2. Its identification was established on the basis of chromatography in four different systems and in two systems after acetylation. Its oxidation product was proved to be androsterone by chromatography of the free compound and of the acetate.

3. Of this steroid 60 % was found in the least-polar ester sulphate fraction and 40 % in the glucuronide fraction.

The authors are grateful to Dr R. V. Brooks for reading this paper and to Dr E. Forchielli for giving us a sample of allo-tetrahydro-compound S.

REFERENCES

- Brooks, C. J. W. & Norymberski, J. K. (1952). *Chem. & Ind.* p. 804.
- Burton, R. B., Zaffaroni, A. & Keutmann, E. H. (1951). *J. biol. Chem.* **188**, 763.
- Bush, I. E. (1953). *Ciba Found. Colloq.: Endocrinology*, **5**, 203.
- Crepy, O., Jayle, M.-F. & Meslin, F. (1957). *Acta endocr., Copenhagen*, **233**, 24.
- Eberlein, W. R. & Bongiovanni, A. M. (1956). *J. biol. Chem.* **223**, 85.
- Edwards, R. H. W., Kellie, A. E. & Wade, A. P. (1953). *Mem. Soc. Endocrin.* **2**, 53.
- Fishman, W. H., Springer, B. & Brunetti, R. (1948). *J. biol. Chem.* **173**, 449.
- Fukushima, D. K., Williams, K. I. H., Zumoff, B. & Gallagher, T. F. (1962). *Acta endocr., Copenhagen*, **41**, 391.
- Gandy, H. M., Keutmann, E. H. & Izzo, A. J. (1960). *J. clin. Invest.* **39**, 364.
- Mader, W. J. & Buck, R. R. (1952). *Analyt. Chem.* **24**, 666.
- Meyer, A. (1953). *J. biol. Chem.* **203**, 469.
- Norymberski, J. K., Stubbs, R. D. & West, H. F. (1953). *Lancet*, **i**, 1276.
- Pasqualini, J. R. (1958). *C.R. Acad. Sci., Paris*, **246**, 2945.
- Pasqualini, J. R. (1960). *Acta physiol. lat.-amer.* **10**, 135.
- Pasqualini, J. R., Dutter, F. & Jayle, M. F. (1963). *Biochim. biophys. Acta*, **69**, 331.
- Pasqualini, J. R. & Jayle, M.-F. (1961a). *Biochem. J.* **81**, 147.
- Pasqualini, J. R. & Jayle, M.-F. (1961b). *J. Chromat.* **5**, 336.
- Pasqualini, J. R. & Jayle, M.-F. (1962). *J. clin. Invest.* **41**, 981.
- Porter, C. C. & Silber, R. H. (1950). *J. biol. Chem.* **185**, 201.
- Richardson, E. M., Touchstone, J. C., Dohan, F. C., Bulaschenko, H., Landolt, I. & Applin, W. (1955). *J. clin. Invest.* **34**, 285.
- Roy, A. B. (1956). *Biochem. J.* **62**, 41.
- Savard, K. (1953). *J. biol. Chem.* **202**, 457.
- Talalay, P. (1957). *Physiol. Rev.* **37**, 362.
- Zimmermann, W. (1935). *Hoppe-Seyl. Z.* **233**, 281.