Urinary Excretion of C-20-Reduction Products of Corticosterone

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1. A 200 mg. portion of corticosterone was ingested by a healthy man and the urine collected. Part of the urine was treated with the gastric juice of *Helix pomatia* and extracted with ethyl acetate, and the extract fractionated with Girard T. Paper-chromatographic separation of the non-ketonic fraction in the Bush (1952) system B₅ revealed the presence of two unknown polar components. 2. The unknown compounds did not possess a reducing (blue tetrazolium) or a reducible (potassium borohydride) grouping. Both contained a terminal α -glycollic fragment as shown by the formation of formaldehyde, and of a non-volatile aldehyde on oxidation with sodium bismuthate. 3. Unknown compound (I) had paper-chromatographic mobilities identical with those of 5 β -pregnane-3 α ,11 β ,20 β ,21-tetraol. The oxidation product of compound (I) had a retention time (gas-liquid chromatography) on an SE30 column identical with that of 3 α ,11 β -dihydroxy-21-nor-5 β -pregnan-20-al. The retention times of various derivatives agreed with those produced in an identical manner on the standard, and accordingly compound (I) is formulated as 5 β -pregnane-3 α ,11 β ,20 ξ ,21-tetraol. 4. Unknown compound (II) had a higher R_f than compound (I), and its oxidation product had a longer retention time than that of compound (I). From the group effects observed in paper and gas-liquid chromatography, compound (II) is tentatively formulated as 5 α -pregnane-3 α ,11 β ,20 ξ ,21-tetraol. The 5 α /5 β ratio found was about 2.0.

Three 20,21-glycols, 11 β ,20 β ,21-trihydroxypregn-4-en-3-one (Richardson, Bulaschenko & Dohan, 1958), 11 β ,20 α ,21-trihydroxypregn-4-en-3-one and 20 β ,21-dihydroxypregn-4-ene-3,11-dione (Bulaschenko, Richardson & Dohan, 1960), have been reported in human urine after the ingestion of corticosterone. These C-20-reduction products of corticosterone all contain the 4-en-3-one system and were present only in small quantities. The suggestion that man excretes substantial amounts of as yet unidentified 20,21-glycols (Exley, Ingall, Norymberski & Woods, 1961) stimulated a search for new metabolites of corticosterone. The present study reports the identification of 5 β -pregnane-3 α ,11 β ,20 ξ ,21-tetraol and the tentative identification of 5 α -pregnane-3 α ,11 β ,20 ξ ,21-tetraol in the urine of a healthy man after the ingestion of 200 mg. of corticosterone. The results suggest that relatively large quantities of these new metabolites exist in the urine under these conditions, and that the tentatively identified 3 α ,5 α compound is present in greater amount than the 3 α ,5 β isomer.

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MATERIALS AND METHODS

All solvents but methylene dichloride and ethylene dichloride were of analytical grade, and all but light petroleum (b.p. 60–80°) were redistilled. Colorimetric and ultraviolet-absorption measurements were carried out with a Hilger Uvispek spectrophotometer in microcells of 1 cm. light-path and 0.2 ml. capacity. Infrared-absorption spectra were determined on a Perkin-Elmer Infracord model 137 spectrophotometer. Materials were determined in a 3 mm. sodium chloride cell with methylene dichloride as solvent.

Preparation of reference standards. (a) 5 β -Pregnane-3 α ,11 β ,20 β ,21-tetraol. A 10 mg. portion of 3 α ,21-dihydroxy-5 β -pregnane-11,20-dione was dissolved in 5 ml. of ethanol, and 100 mg. of potassium borohydride in 1 ml. of water added. The material was reduced overnight at room temperature, 2 ml. of 5 N-HCl added and the aqueous phase extracted with 3 $\times$ 50 ml. of ethyl acetate. The organic phase was washed with 15 ml. of 3 N-NaOH and 3 $\times$ 20 ml. of water, dried over anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*. Recrystallization from aqueous methanol gave white crystalline material (9.5 mg.), m.p. 202–204° (Kofler stage). The infrared-absorption spectrum between 3500 and 1200 cm.⁻¹ gave $\nu_{\max}$ at 3600 cm.⁻¹ (hydroxyl absorption) and at 2850 cm.⁻¹ (C—H stretching). Chromatography in the Bush (1952) B₅ and C systems showed the presence of one spot only.

(b) 5 α -Pregnane-3 β ,11 β ,20 β ,21-tetraol was prepared from

3 β ,11 β ,21-trihydroxy-5 α -pregnan-11-one in a manner identical with that described above. The infrared-absorption spectrum was similar to that described above.

Paper chromatography. The Bush (1952) systems B₁ [light petroleum-benzene-methanol-water (5:5:7:1, by vol.)], B₄ [toluene-methanol-water (2:1:1, by vol.)], B₅ [benzene-methanol-water (2:1:1, by vol.)], C [benzene-methanol-water-ethyl acetate (9:5:5:1 by vol.)] and A [light petroleum-methanol-water (5:4:1, by vol.)] were used in conjunction with the Eberlein & Bongiovanni (1955) system E₂B (iso-octane-2-methylpropan-2-ol-water (10:5:9, by vol.)).

Whatman no. 2 paper was used with the descending technique at 22°. All standard and unknowns were run alongside 3 α ,11 β ,17 α ,21-tetrahydroxy-5 β -pregnane-20-one (cortisol), which was detected by inspection in ultraviolet light at approx. 250 m μ . All other compounds were detected by the phosphomolybdic acid test: chromatograms were dipped in ethanolic phosphomolybdic acid (4 g./100 ml.), excess of reagent was removed by blotting and the chromatograms were heated at 100° for 5–10 min.

Chemical procedures. The standard blue tetrazolium reaction was used to indicate the presence of the α -ketolic group. The modified Cifonelli-Smith test for adjacent oxygen functions was performed as described by Bulaschenko *et al.* (1960). The borate test was carried out with an aqueous solution of 0.2 M-sodium metaborate containing 5–10 drops of ethanolic 1% (w/v) phenolphthalein. The paper was dipped in the solution, left to dry for 5–10 min. and then held over strong ammonia solution. This produced a strong white spot on a pink paper background for C₂₁ steroids that had either a glycol or a glycerol side chain. This test appears to be specific for this type of steroid. Oxidation with chromic acid to produce 11-keto compounds was performed according to the method of Leiberman, Katzenellenbogen, Schneider, Studer & Dobriner (1953). Oxidation was carried out for 30 min. only, as suggested by Bush (1961a). Acetates were prepared by treating a solution of the steroid in pyridine (0.1 ml.) and acetic anhydride (0.2 ml.) at room temperature overnight. The reagents were removed by a stream of nitrogen.

The analysis for formaldehydrogenic steroids was essentially as that described by Brooks & Norymberski (1953). Reduction with borohydride were carried out as described by Appleby, Gibson, Norymberski & Stubbs (1955), except that potassium borohydride was used instead of the sodium salt. Analysis for 17-keto steroids was performed by the method of Corker, Norymberski & Thow (1962), for 17-hydroxy corticosteroids by that of Appleby *et al.* (1955) and for 17-deoxy corticosteroids by that of Exley *et al.* (1961).

Outline of isolation procedure. A specimen of urine was treated with the gastric juice of *Helix pomatia* and extracted with ethyl acetate, and the extract fractionated with Girard T reagent. Paper chromatography of the non-ketonic fraction in Bush (1952) system B₅ revealed the presence of two major polar components. Gas-liquid-chromatographic examination of various segments of the paper chromatogram enabled the two components to be separated. Final purification of the respective compounds to remove paper contaminants was made by a further Girard T fractionation and separation on a Celite column.

Ingestion of corticosterone. A 51-year-old male in good health ingested 200 mg. of corticosterone, dissolved in 15 ml. of ethanol, at 8 a.m. Urine was collected for the

periods 8–12 a.m., 12 a.m.–4 p.m., 4–8 p.m. and 8 p.m.–8 a.m. the following morning. The specimens were kept in a refrigerator at –15° until use. Suitable portions of the specimens were assayed for total 17-deoxy corticosteroid content by the method of Exley *et al.* (1961).

Hydrolysis and extraction. Only two of the above urine specimens were hydrolysed and used for further fractionation. These specimens, the 12 a.m.–4 p.m. and part of the 4–8 p.m. collection, were treated together with 2 M-acetate buffer (0.05 vol.) and sodium benzyl penicillin (1000 units/ml.). The sample was then treated with the enzyme of the digestive tract of *Helix pomatia* (l'Industrie Biologique Française, Gennevilliers, France) (1000 Fishman units/ml.) at pH 5.0 for 60 hr. at 37°. The urine was next extracted with ethyl acetate (3 $\times$ 0.5 vol.), and the extract washed successively with 3 N-NaOH (0.2 vol.) and water (3 $\times$ 0.2 vol.), dried with anhydrous Na₂SO₄ and evaporated to dryness *in vacuo*.

Suitable amounts of this material were assayed for total 17-deoxy corticosteroid content, and the selective assay for 20,21-glycols was made (Exley *et al.* 1961). The material was also analysed for 17-hydroxy corticosteroid content by the method of Appleby *et al.* (1955) and the 17-keto steroid content by the method of Corker *et al.* (1962).

Girard T separation. The remaining material after enzymic hydrolysis was dissolved in 5 ml. of a freshly prepared solution of 5% (w/v) Girard T in acetic acid and left overnight at room temperature. The tube was placed in ice-water and 50 ml. of chilled citrate-phosphate buffer, pH 7.0, was added. The aqueous phase was extracted with 5 $\times$ 50 ml. of chilled ethyl acetate. The ethyl acetate extracts (non-ketonic fraction) were combined, washed with 25 ml. of water, 25 ml. of 1 N-NaOH and again with 25 ml. of water, dried over Na₂SO₄ and filtered through a cotton-wool plug before being evaporated to dryness *in vacuo*.

To the remaining aqueous phase (50 ml.) 10 ml. of conc. HCl was added, and after 2 hr. at room temperature the mixture was extracted and worked up as above. This was the ketonic fraction.

Chromatographic separation of the non-ketonic fraction. The Bush (1952) paper-chromatographic system B₅ was used to separate the glycols in the non-ketonic fraction. The material obtained after Girard T separation was dissolved in methanol-ethyl acetate (1:1, v/v) solution and applied as narrow bands across 5 cm. of the origin of strips of paper (6 cm. $\times$ 100 cm.) together with a small amount of solution containing 5 μ g. of cortisol. Altogether six papers were used. The method was essentially that of Exley & Norymberski (1964b), the papers being suspended in a loop in the chromatographic tanks. After equilibrating overnight the solvent was run for 36 hr.

Removal of the papers from the tank was followed by examination of the ultraviolet band due to cortisol (R_F 0.185).

Cortisol ran to an average distance of 35 cm., and since the suspected glycols have R_{cortisol} values 0.4–0.50 (Exley & Norymberski, 1964b), all material in the 10–20 cm. region was examined. These 10 cm. bands were each cut into ten separate 1 cm. strips.

Elution of chromatographic strips and oxidation of the glycols to 21-norpregnan-20-als. Each 1 cm. strip was cut up into fine pieces and eluted by soaking overnight with 5 ml. of aq. 90% (v/v) methanol. A suitable sample of the

extract (0.25 ml.) was then oxidized with sodium bis-muthate as described in the determination of 17-deoxy corticoids (Exley *et al.* 1961). The 21-norpregnan-20-als were extracted into methylene dichloride and the extracts evaporated to dryness at 40° on a water bath. The dried residues were then dissolved in purified chloroform (0.1 ml.) for gas-liquid-chromatographic examination.

Gas-liquid chromatography. A Pye Argon chromatograph with a ⁹⁰Sr-ionization detector was used. Gas Chrom P (Applied Science Laboratories Inc., State College, Pennsylvania, U.S.A.) (100–120 mesh) was coated with 1% SE30 (Midland Silicones, London), after first treating with 5% (v/v) dichlorodimethylsilane in toluene by the procedure of Horning essentially as described by Brooks & Hanaineh (1963). The packed glass columns were preconditioned by heating at 300° for 18 hr. before use. These columns were 4 ft. long $\times$ $\frac{5}{8}$ in. internal diam.; the upper portion, which projected from the chromatograph housing, was enclosed by an aluminium cylinder (7 cm. $\times$ 9.5 cm. diam.) and maintained 25–30° above the column by a band heater controlled by a Variac transformer. The upper part of the

column was modified to incorporate use of stainless-steel gauzes (Menini, 1963).

Samples (2–5 μ g.) were introduced on to rolls of 50-mesh stainless-steel gauze from polytetraethylene fluoride plates.

Purification of urinary substances. Gas-liquid chromatography revealed the presence of two major peaks. The first three 1 cm. strips contained one of these major peaks (unknown compound I), the next three strips a mixture of the two peaks, and the last four strips the second major peak (unknown compound II) only.

The eluates from the papers containing unknown compound (I) were combined, and similarly the eluates from the papers containing unknown compound (II). Final purification of the separated compounds was made to remove contaminants eluted from the paper. The eluates were evaporated *in vacuo* to remove methanol. The remaining aqueous was extracted with purified ether (3 $\times$ 2 vol.), and the extract was then evaporated to dryness *in vacuo* and subjected to a further Girard T separation (see above). The non-ketonic fraction was extracted with ethyl acetate, and the extract was evaporated to dryness *in vacuo*. The residue was dissolved in 0.5 ml. of ethyl acetate containing 2% (v/v) of ethanol, and chromatographed on a Celite column made as follows: 1 g. of Celite was ground with 0.5 ml. of water, suspended in light petroleum (b.p. 40–60°) and loosely packed in a column (20 cm. $\times$ 1 cm.). The residue for purification dissolved as described above was added to approx. 1 ml. of light petroleum standing over the Celite in the column. This was washed with two further 0.5 ml. portions of ethyl acetate and finally eluted with a further 10 ml. The ethyl acetate eluate was collected and evaporated to dryness *in vacuo*. The material was made up in methylene dichloride, ready for the various identification procedures.

RESULTS

The amount of 17-deoxy corticosteroid material excreted in the urine expressed as a percentage of the dose of corticosterone is shown in Fig. 1. The percentage excreted increased rapidly 4–8 hr. after administration of the corticosterone and thereafter

Fig. 1. Percentage excretion of metabolites of the administered 200 mg. corticosterone dose determined in the urine by the 17-deoxy corticosteroid method of Exley *et al.* (1961), plotted against time after administration.

Table 1. *Yields of 17-deoxy corticosteroid material at the various stages of isolation*

Experimental details are given in the text.

Amount of 17-deoxy corticosteroid (mg.)	Isolation stage	Yield of 17-deoxy corticosteroid (mg.)	Percentage recovery of 17-deoxy corticosteroid	Amount of α -ketolic 17-deoxy corticosteroid (mg.)	Amount of glycollic 17-deoxy corticosteroid (mg.)	Remarks
18.2	Enzymic hydrolysis	17.6	95	9.0	8.6	48% of 17-deoxy corticosteroid is glycollic
7.0	Girard T separation I	5.6	80	2.88	2.7*	—
2.0*	Paper chromatography [Bush (1952) system B ₅]	1.6	80	Nil	1.6*	—
1.5*	Girard T separation II	1.15	77	Nil	1.15*	0.8 mg. is 5 α -steroid, 0.35 mg. is 5 β -steroid†

* Glycollic material only.

† This material was then subjected to the further purification procedures described in the text and no further quantitative analyses were performed.

slowly decreased. Only 22% of the administered dose was excreted during the first 24 hr.

Table 1 shows the amount of 17-deoxy corticosteroid obtained at the various stages of the isolation technique. The starting material con-

tained 18.2 mg. of 17-deoxy corticosteroids, 1.7 mg. of 17-hydroxy corticosteroids and 0.72 mg. of 17-keto steroids. This was subjected to enzymic hydrolysis, but only 7 mg. of 17-deoxy corticosteroid material was used for the subsequent

Table 2. *Identification of 5 β -pregnane-3 α ,11 β ,20 ξ ,21-tetraol, and tentative identification of 5 α -pregnane-3 α ,11 β ,20 ξ ,21-tetraol, in urine extract after the administration of corticosterone*

Experimental details are given in the text.

Identification technique	Standard 5 α -pregnane- 3 β ,11 β ,20 β ,21- tetraol	Standard 5 β -pregnane- 3 α ,11 β ,20 β ,21- tetraol	Unknown compound (I)	Unknown compound (II)	
A. Movement in various solvent systems on Whatman no. 2 paper at 22°					
R_{cortisol} in benzene-methanol-water (Bush system B ₅)	0.35	0.40	0.40	0.49	
R_{cortisol} in toluene-methanol-water (Bush system B ₄)	0.33	0.40	0.41	0.48	
R_{cortisol} in benzene-methanol-water-ethyl acetate (Bush system C)	—	0.51	0.50	0.60	
R_{cortisol} in iso-octane-2-methylpropan-2-ol-water (Eberlein-Bongiovanni system E ₂ B)	—	1.70	1.64	—	
R_F of tri-acetate in light petroleum-benzene-methanol-water (Bush system A)	—	0.87	0.87	0.83	
R_F of tri-acetate in light petroleum-methanol-water (Bush system B ₁)	—	0.66	0.67	0.60	
R_{cortisol} in benzene-methanol-water (Bush system B ₅) after KBH ₄ reduction	0.35	0.40	0.40	0.50	
B. Tests indicating nature of side chain and type of steroid					
Phosphomolybdic acid test	+	+	+	+	
Reaction with alkaline blue tetrazolium	—	—	—	—	
Borate test	+	+	+	+	
KIO ₄ -benzidine test (modified Cifonelli-Smith test)	+	+	+	+	
Analysis for 17-keto steroids	—	—	—	—	
Analysis for 17-hydroxy corticosteroids	—	—	—	—	
Analysis for 17-deoxy corticosteroids	+	+	+	+	
Analysis for formaldehydogenic steroids	+	+	+	+	
C. Gas-liquid chromatographic relative retention times (relative to 5 α -cholestane) after various chemical treatments (1% SE 30 on Gas Chrom P at 220°; Pye Argon instrument)					
NaBiO ₃ oxidation (method of Exley <i>et al.</i> 1961)	2	0.88 $\pm$ 0.01 (<i>n</i> =20)	0.78 $\pm$ 0.01 (<i>n</i> =20)	0.78 $\pm$ 0.01 (<i>n</i> =20)	0.86 $\pm$ 0.01 (<i>n</i> =20)
Reduction by KBH ₄ followed by NaBiO ₃ oxidation	1 and 2	0.88	0.78	0.78	0.85
NaBiO ₃ oxidation followed by KBH ₄ reduction	2 and 3	1.07	0.94	0.94	1.03
Oxidation by CrO ₃ followed by NaBiO ₃ oxidation	4 and 5	0.70	0.62	0.62	0.68
Oxidation by CrO ₃ followed by NaBiO ₃ and KBH ₄ reduction	4, 5 and 6	1.07	0.94	0.94	1.03
Hydrolysis by HCl followed by NaBiO ₃ oxidation	7 and 8	—	0.34	0.34	—
D. Infrared-absorption spectra (in CH ₂ Cl ₂ ; 3 mm. cell of Perkin-Elmer Infracord)					
Prominent peaks (3500-1200 cm.)	3600 and 2850 cm. ⁻¹	3600 and 2850 cm. ⁻¹	3600 and 2850 cm. ⁻¹	3600 and 2850 cm. ⁻¹	

Girard T separation. This yielded 2.7 mg. of glycols, which, after a paper-chromatographic and further Girard T separation, gave 350 μ g. of unknown compound (I) and 800 μ g. of unknown compound (II). This material was then subjected to the Celite-column 'clean-up' before the identification procedures described below were performed.

Identification of 5 β -pregnane-3 α ,11 β ,20 ξ ,21-tetraol. Table 2 shows the characteristics of the unknown compound (I). The reactions involved in the gas-liquid-chromatographic method used in the identification are shown in Scheme 1. The characteristics of the reference standard are given in Table 2 for comparison. Chromatography of the free compounds in four solvent systems (section A in Table 2) demonstrated mobilities similar to those of the reference standard. Running rates in Bush (1952) systems A and B₁ of the unknown after acetylation were similar to that of the reference standard and compatible with the presence of 3 acetyltable groups/mol. The absence of the 4-en-3-one group was indicated by lack of an ultraviolet-absorption peak at 240 m μ in ethanol, and the fact that no fluorescence was observed under

ultraviolet light at 250 m μ . The lack of reaction with blue tetrazolium indicated the absence of the α -ketolic side chain. The formation of a 21-norpregnan-20-al and production of formaldehyde after bismuthate oxidation, coupled with a positive potassium periodate-benzidine test on paper (modified Cifonelli-Smith test for adjacent oxygen groups) and a positive borate test, are all compatible with the presence of a glycolic side chain. Chemical analysis by established methods indicated that the unknown was 17-deoxy corticoid in nature.

The absence of reducible groups in the unknown was confirmed by two observations. First, there was no change in the mobility of the compound in Bush (1952) paper-chromatographic system B₅ when it was subjected to extensive reduction with potassium borohydride. Secondly, the chemical sequence potassium borohydride reduction followed by sodium bismuthate oxidation (according to the 17-deoxy corticoid method) gave a 21-norpregnan-20-al with a gas-liquid-chromatographic retention time identical with that produced by bismuthate oxidation only (reactions 1 and 2 in Scheme 1).

Mild chromic acid oxidation produced a change

Scheme 1. Reactions used to identify 5 β -pregnane-3 α ,11 β ,20 ξ ,21-tetraol in extracts of urine, by using gas-liquid chromatography. In each case the appropriate reference compound was treated in a manner identical with that used for the material from urine. In this way relative yields of major and minor products can be compared with confidence. The same scheme was used in the tentative identification of 5 α -pregnane-3 α ,11 β ,20 ξ ,21-tetraol.

consistent with the oxidation of an 11β -hydroxyl group. A derivative was obtained which was identical with that produced by the reference standard (section C in Table 2). Very mild oxidation [2% (w/v) chromium trioxide for 30 min.] was used, and oxidation was carried out without the usual protection by acetylation Bush (1961a). The gas-liquid-chromatographic characteristics of the compound produced after chromic acid and bismuthate oxidations was consistent with that of 3α -hydroxy-21-norpregnan-20-al-11-one (reactions 4 and 5 in Scheme 1).

Additional evidence for the presence of the 11β -hydroxyl group is supported by the fact that both reference compound and unknown compound (I) produced $3\alpha,11\beta$ -dihydroxy-21-norpregnan-20-al by direct oxidation with bismuthate. The two compounds produced identical gas-liquid-chromatographic retention times. Taken through the sequence hydrochloric acid hydrolysis and bismuthate oxidation (Bush 1961b) the 11β -hydroxyl group was eliminated to give the $\Delta^{9(11)}$ -anhydro compound, and oxidation produced identical compounds from both the reference compound and the unknown compound (I) (section C in Table 2) (reactions 7 and 8 in Scheme 1). Further proof that the reference compound and the unknown compound (I) were identical (with reservation on the configuration of the 20-position) was produced when gas-liquid chromatography was used to examine the product produced by the oxidation with sodium bismuthate followed by potassium borohydride reduction, both with (reactions 4-6 in Scheme 1) and without (reactions 1-3 in Scheme 1) chromic acid oxidation. This compound was presumably 17β -hydroxymethylandrosterane- $3\alpha,11\beta$ -diol. The reference compound and the unknown compound (I) again gave compounds with identical gas-liquid-chromatographic retention times. Finally, examination of infrared-absorption spectra of reference compound and unknown compound (I) gave identical infrared-absorption peaks in the 3500 – 2850 cm^{-1} region (section D in Table 2). The spectral region below 1200 cm^{-1} ('fingerprint' region), which establishes identity of the compound rather than the presence of its constituent functional groups, was unfortunately not very definite.

Tentative identification of 5α -pregnane- $3\alpha,11\beta,20\xi$,21-tetraol. Tentative identification of compound (II) as 5α -pregnane- $3\alpha,11\beta,20\xi,21$ -tetraol was made despite the lack of authentic 5α -pregnane- $3\alpha,11\beta,20\xi,21$ -tetraol as reference standards. This identification was based primarily on the following evidence. Tests indicating the nature of the side chain (section B in Table 2) were compatible with the presence of a glycollic side chain; this was based on the same reasoning as that presented in the preceding section for 5β -pregnane- $3\alpha,11\beta,20\xi,21$ -tetraol.

The absence of the 17-hydroxyl group and proof of its 17-deoxy nature are also based on the same reasoning as above. The infrared-absorption spectrum gave peaks at 3600 and 2850 cm^{-1} only in the 3500 – 1200 cm^{-1} region (section D in Table 2). On the other hand, mild chromic acid oxidation followed by bismuthate oxidation gave a compound with gas-liquid-chromatographic properties compatible with the presence of a 21-norpregnan-20-al-11-one (section C of Table 2), but which had a retention time different from those of both 3α -hydroxy-21-norpregnan-20-al-11-one and its $3\beta,5\alpha$ -isomer.

The compound thus possessed a glycollic side chain and an 11β -hydroxyl group. Acetylation produced a derivative with paper-chromatographic properties consistent with that of 3 acetylatable groups/mol. The 11β -hydroxyl group would not be acetylated under the conditions used, and therefore the compound appears to possess an additional hydroxyl group other than the two hydroxyl groups of the side chain, and this was naturally suspected of being at position 3, since it was considered to be a metabolite of the administered corticosterone. However, the unknown compound (II) had paper-chromatographic (section A in Table 2) and gas-liquid-chromatographic properties (after sodium bismuthate oxidation) (section C in Table 2) that were different from the equatorial 3α -substituted 5β -steroid and 3β -substituted 5α -steroid reference standards. By process of elimination the compound must then be one of the axial epimers, i.e. the 3α -substituted 5α -steroid or the 3β -substituted 5β -steroid. The tentative identification of the 3α -substituted 5α -steroid rests on the following evidence and reasoning.

(i) The paper-chromatographic properties in the various solvent systems are more consistent with that of $3\alpha,5\alpha$ - rather than a $3\beta,5\beta$ -epimer [compare analogous hydrocortisone series at 22° with Bush (1952) solvent B₅ (Neher & Wettstein, 1956; Neher, 1958)].

(ii) The gas-liquid-chromatographic retention times suggest that it is the $3\alpha,5\alpha$ -isomer. Brooks & Hanaineh (1963) have shown that the epimeric separation factor (ratio of retention time of the 5α -steroid to its 5β -epimer on 1% SE30 gas-liquid-chromatographic columns at 220°) is 1.10 ± 0.02 ; this has been confirmed in this Laboratory, the 5α -steroid being retained more readily on the column. Brooks & Hanaineh (1963) have also shown that the introduction of the 3α - or 3β -hydroxyl group into the pregnane molecule has scarcely any discernible influence on this epimeric separation factor under these conditions on 1% SE30. Section C in Table 2 gives the relative retention times (relative to 5α -cholestane) of the 21-norpregnan-20-als produced by bismuthate oxidation of the two reference standards and that produced by similar treatment

of unknown compound (II). The results are given as means $\pm$ s.d. for 20 determinations of each. The relative retention time of the unknown compound (II) (0.86 ± 0.01) is much nearer to that of the relative retention time (0.88 ± 0.01) of the $3\beta,5\alpha$ -steroid reference standard than to that of the $3\alpha,5\beta$ -steroid reference standard (0.78 ± 0.01). This suggests the compound is a 5α -steroid, because calculation shows that the change in the retention time from the $3\alpha,5\beta$ -steroid to the $3\alpha,5\alpha$ -steroid would be from 0.78 to 0.86 when calculated for an epimeric separation factor 1.10. Again, all the derivatives prepared from the unknown compound (II) possess a mean epimeric separation factor when compared with their respective $3\alpha,5\beta$ -steroid reference standards of 1.10 ± 0.003 . On the other hand, the change in epimeric separation factor for the reference standards and their respective derivatives is 3.5% greater (i.e. that from $3\alpha,5\beta$ -steroid to $3\beta,5\alpha$ -steroid is 1.135 ± 0.005). It is obvious the compound is not the $3\beta,5\alpha$ -steroid (this is particularly noticeable when considering its paper-chromatographic properties) and yet the above reasoning shows it is not the $3\beta,5\beta$ -steroid: hence it must be the $3\alpha,5\alpha$ -steroid.

Compound (II) is therefore tentatively identified as 5α -pregnane- $3\alpha,11\beta,20\xi,21$ -tetraol.

DISCUSSION

Although reduction of the 20-keto group of α -ketolic steroids is a recognized metabolic pathway (Zaffaroni, 1955), and fully hexahydrogenated metabolites of hydrocortisone and cortisone have been found in urine (Fukushima *et al.* 1955), this is the first time any hexahydrogenated metabolites of corticosterone have been reported in urine. The corticosterone released into the circulation after the ingestion of large doses might have a different metabolic pathway from that of the endogenous hormone. Swamping of enzyme systems and factors due to the gut and liver may cause this different pathway, and therefore, until it is proved that similar metabolites exist in the normal, the present results must be taken only for the conditions stated. The three unsaturated glycols, $11\beta,20\beta,21$ -trihydroxypregn-4-en-3-one (Richardson *et al.* 1958), $11\beta,20\alpha,21$ -trihydroxypregn-4-en-3-one and $20\beta,21$ -dihydroxypregn-4-ene-3,11-dione (Bulaschenko *et al.* 1960), which were found after ingestion of large amounts of corticosterone, are the only C-20-reduction products of corticosterone and 11 -dehydrocorticosterone reported in urine to date. No quantitative evidence was presented by these authors, however large amounts of urine were used for the isolations, and it is inferred that the glycols they found were present only in small quantities.

The present work, on the other hand, though not strictly quantitative, suggests the presence of large amounts (approx. 20 mg.) of the new hexahydrogenated C-20-reduction products in the first 24 hr. after the oral administration of 200 mg. of corticosterone.

The presence of larger amounts of the tentatively identified 3α -substituted 5α -glycollic steroid than its 3α -substituted 5β -epimer is expected from the known corticosterone α -ketolic pattern in urine. The major α -ketolic metabolite of corticosterone in the urine of normal subjects, with and without oral administration of corticosterone, is $3\alpha,11\beta,21$ -trihydroxy- 5α -pregnan-20-one (allo-THB) (Peterson & Pierce, 1960). These workers reported that they found 9 mg. of $3\alpha,11\beta,21$ -trihydroxy- 5α -pregnan-20-one after the oral administration of 100 mg. of corticosterone and 6 mg. of its 5β -epimer, i.e. the $5\alpha/5\beta$ ratio was 1.5. The present work suggests that the 5α -glycol/ 5β -glycol ratio is about 2.0.

No results on the presence of the respective 11-keto-20,21-glycols are reported in the present paper, which is solely concerned with the identification of the new glycols. However, Pasqualini (1962), reviewing the results of past workers, has stated that the presence of the 11-keto group greatly influences the configuration of metabolites at position 5, and that little 11-keto- 5α -steroids are found in the urine. Thus the expected 11-keto metabolite of this corticosterone series would be $3\alpha,20\xi,21$ -trihydroxy- 5β -pregnan-11-one rather than its 5α -epimer. Pasqualini (1962) has suggested that this 5β -steroid would be preferably sulphoconjugated.

Proof of configuration at C-20 in the two new metabolites in the urine has not been definitely established. The fact that the paper-chromatographic properties of compound (I) in the various solvent systems were similar to those of the reference standard 5β -pregnane- $3\alpha,11\beta,20\beta,21$ -tetraol certainly suggests that it has the 20β -configuration, but it is considered that this was not proof enough of certainty of configuration. The 20α -hydroxy compounds were not available for reference. The chromatographic spots were a little streaky, and the presence of the 20α -configuration is not excluded.

Evidence for the presence of hexahydrogenated metabolites of corticosterone in the urine of normal subjects was suggested in a method for the determination of 17-deoxy corticosteroids (Exley *et al.* 1961). A clinical survey of 17-deoxy corticosteroids (Exley & Norymberski, 1964a) and a paper-chromatographic fractionation technique (Exley & Norymberski, 1964b) suggest that these new compounds may exist in substantial quantities in the urine of pregnant subjects and newborn babies.

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