Investigation of the Products of Metabolism after Incubation of Deoxycorticosterone with Rabbit-Liver Homogenate

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(Received 27 November 1958)

Although 21-hydroxypregn-4-ene-3:20-dione (deoxycorticosterone) has been readily available for therapeutic purposes for many years relatively little is known about the metabolic fate of the steroid in animal tissues. The lack of interest in the metabolism of deoxycorticosterone may arise from the fact that the substance is thought to be present in only very small amounts, if at all, in the adrenocortical secretion of higher animals (Hechter & Pincus, 1954). It has been known for some time that administration of deoxycorticosterone results in an increased urinary excretion of 5 β -pregnane-3 α :20 α -diol (pregnanediol) in a number of species including man (Table 1). The isolation of labelled pregnanediol after administration of [21-¹⁴C]deoxycorticosterone, as its acetate, to rabbits has more firmly established that, in rabbits at least, the pregnanediol is produced directly by reduction of the primary alcohol at C-21 and not by some compensatory response to the administered steroid resulting in release of precursors of pregnanediol (Heard *et al.* 1954).

The only other established urinary metabolite of deoxycorticosterone is 3 α :21-dihydroxy-5 β -pregnan-20-one, which has been isolated after administration of deoxycorticosterone to a patient with anterior-pituitary failure (Richardson, Touchstone & Dohan, 1955). Eberlein & Bongiovanni

(1956) isolated the same pregnanediolone from the urine of an 8-year-old girl with the hypertensive form of congenital adrenal hyperplasia, and they have suggested that this disease is characterized by absence or deficiency of adrenocortical '11 β -hydroxylase'. This suggestion is supported by the fact that no 11-oxygenated C₂₁ or C₁₉ steroids could be detected in the blood or urine of the patient. Thus the adrenocortical secretion in this condition is presumed to consist mainly of deoxycorticosterone and 17 β -hydroxydeoxycorticosterone (17 α :21-dihydroxypregn-4-ene-3:20-dione) instead of the corresponding 11 β -hydroxy derivatives, corticosterone and hydrocortisone.

The results of previous investigations of the metabolism of deoxycorticosterone *in vitro*, other than by adrenocortical preparations and micro-organisms (see Dorfman, 1957), are summarized in Table 2. The most comprehensive investigation is that of Schneider (1952), who isolated the products shown in Table 2 after incubation of deoxycorticosterone with rat-liver slices; only steroids of the 5 α -pregnane series were isolated. Taylor (1954) also found that only 5 α -pregnane derivatives could be isolated when progesterone was incubated with rat liver. Rabbit liver, however, converted progesterone into a larger variety of metabolites, with 5 β -pregnane compounds predominating: the prin-

Table 1. *Metabolism of deoxycorticosterone in vivo*

Species	Steroid administered	Urinary metabolite	References
Rabbit	DOCA*	5 β -Pregnane-3 α :20 α -diol	Westphal (1942); Hoffman, Kazmin & Browne (1943); Heard <i>et al.</i> (1954)
Chimpanzee	DOCA	5 β -Pregnane-3 α :20 α -diol	Westphal (1942); Horwitt, Dorfman, Shipley & Fish (1944)
Human (with anterior pituitary failure)	DOC	3 α :21-Dihydroxy-5 β -pregnan-20-one	Richardson, Touchstone & Dohan (1955)
Human (with adrenal hyperplasia)	Nil	3 α :21-Dihydroxy-5 β -pregnan-20-one	Eberlein & Bongiovanni (1956)
Human	DOCA	5 β -Pregnane-3 α :20 α -diol	Horwitt <i>et al.</i> (1944)

* 21-Acetoxypregn-4-ene-3:20-dione.

Table 2. *Metabolism of deoxycorticosterone in vitro*

Species	Tissue preparation	Metabolites	References
Rat	Liver slices	21-Hydroxy-5 α -pregnane-3:20-dione; 3 α :21-dihydroxy-5 α -pregnan-20-one; 3 β :21-dihydroxy-5 α -pregnan-20-one; 5 α -pregnane-3 β :20 ξ :21-triol	Schneider (1952)
Rat	Liver perfusion	3-Oxoeti-4-enic acid (3-oxoandrost-4-ene-17 β -carboxylic acid)	Picha, Saunders & Green (1952)
Rat	Liver perfusion	Not isolated. Large number of α -ketols more 'polar' than DOC	Hechter, Solomon & Caspi (1953)
Rat	Liver perfusion	6 β :21-Dihydroxypregn-4-ene-3:20-dione	Axelrod & Miller (1954)
Pig	Liver brei	20 α :21-Dihydroxypregn-4-en-3-one	Caspi <i>et al</i> (1956)
Bovine	Corpus luteum homogenate	20 β :21-Dihydroxypregn-4-en-3-one	Hayano, Lindberg, Wiener, Rosenkrantz & Dorfman (1954)

principal metabolite was pregnanediol, the major end product of progesterone metabolism in man (Taylor, 1955).

The metabolism of deoxycorticosterone by rabbit-liver homogenate has now been investigated and the following metabolites (as well as unchanged deoxycorticosterone) have been isolated and characterized: 20 α :21-dihydroxypregn-4-en-3-one and its 20 β -epimer; 3 α :21-dihydroxy-5 α -pregnan-20-one and its 3 β -epimer; 3 α :21-dihydroxy-5 β -pregnan-20-one. Evidence is presented for the existence of other metabolites, including: 21-hydroxy-5 α -pregnane-3:20-dione and its 5 β -isomer; 6 β :21-dihydroxypregn-4-ene-3:20-dione; three closely related substances whose properties indicate that they are probably (?) 21-dihydroxy-5 ξ -pregnane-3:20-dione and epimeric 3 α :(?) 21-trihydroxy-5 ξ -pregnan-20-ones; and 5 α - or 5 β -pregnane-3 α :20 ξ :21-triols.

Preliminary accounts of part of this work have been published (Taylor, 1957*a*, 1958).

EXPERIMENTAL

Experimental animals were male and female rabbits, 6–12 months old, of various strains. Any livers which, on superficial examination, appeared to be diseased or abnormal were rejected. The incubation medium (referred to as phosphate-saline) was the calcium-free phosphate-saline described by Krebs & Eggleston (1940), except that the phosphate buffer was 0.25*M*.

In absorption chromatography neutral alumina (L. Light and Co. Ltd.) was deactivated by exposing it to an atmosphere saturated with water vapour at room temperature for 14 days. Silica gel for chromatographic analysis (British Drug Houses Ltd.) was washed with water, ethanol and finally ether and dried for 24 hr. at 110°. Paper chromatography was carried out at 21 $\pm$ 1° by the method of Bush (1951) with the solvent systems shown in Table 3. For large-scale work Whatman no. 3 MM paper was used: satisfactory separations were achieved when up to 7 mg. of material was applied in a zone 0.5 cm. wide and 15 cm. long. Steroids were eluted from the developed papers by cutting the appropriate zones into pieces 2 cm.

Table 3. *Percentage composition (by vol.) of solvent systems used in paper chromatography*

Solvent system	Mobile phase		Stationary phase	
	Hexane	Benzene	Methanol	Water
I	100	—	80	20
II	90	10	80	20
III	40	60	80	20
IV	—	100	65	35

square and allowing them to stand in the dark for 12 hr. covered with ethanol. The ethanol was decanted through a fine sintered-glass filter, and the paper was washed twice more with ethanol with 2 hr. intervals between washings. The combined ethanol solutions were evaporated to 5 ml. by vacuum-distillation at 45° and then passed through a column of silica gel (0.5 cm. $\times$ 5 cm.). The flask and column were washed with 3 $\times$ 2 ml. of ethanol and the combined eluate and washings were evaporated to dryness. This method produced steroids still contaminated with material removed from the paper itself. In preliminary experiments it was found that washing the paper for 48 hr. with benzene and then ethanol by over-running in the chromatography tank did not result in a significantly less contaminated extract, and so this practice was abandoned. Steroids were located on the paper by the blue tetrazolium (BTZ) [3,3'-dianisole bis-4,4'-(3,5-diphenyl)tetrazolium chloride] and NaOH-fluorescence reactions as described by Bush & Willoughby (1957). Infrared spectra were determined on 200–500 μ g. of steroid in the form of a KCl micro-disk in a double-beam Perkin-Elmer 21C spectrophotometer over the range 700–4000 cm.⁻¹.

Reference steroids. Deoxycorticosterone (DOC) was prepared from a commercial preparation of its acetate (Organon Laboratories) by the method of Mattox & Kendall (1951), except that hydrolysis was carried out in an atmosphere of N₂ and the DOC was extracted from the hydrolysate with CHCl₃. The residue obtained from the washed extract was crystallized three times from ether and had m.p. 143–144.5°; [α]_D²¹ + 177 $\pm$ 2° (c, 0.532 in ethanol) (Found: C, 76.3; H, 9.1; calc. for C₂₁H₃₀O₃: C, 76.2; H, 9.1%). 3 β :21-Diacetoxy-5 α -pregnan-20-one was prepared by reduction (with H₂ in the presence of Pt) of an ethanolic solution of 21-acetoxypregn-5-en-3 β -ol-20-one (supplied by Dr W. Klyne), followed by acetylation and crystallization

from methanol. The properties of the product are shown in Table 5. Hydrolysis of this diacetate gave 3β :21-dihydroxy- 5α -pregnan-20-one which had m.p. 170–173°. Other steroids were gifts from the following: 21-hydroxy-5 β -pregnane-3:20-dione, 21-acetoxy-5 β -pregnan-17 α -ol-3:20-dione and 3β :21-diacetoxy-5 β -pregnan-17 α -ol-20-one from Dr W. Klyne; 21-acetoxy-5 α -pregnane-3:20-dione from Dr A. Zaffaroni, the free steroid being obtained by hydrolysis; 3α :21-dihydroxy-5 β -pregnan-20-one from Dr W. R. Eberlein; 20 α :21-dihydroxypregn-4-en-3-one and its diacetate from Dr M. Hayano; 20 β :21-dihydroxypregn-4-en-3-one from Dr D. H. Peterson; hydroxylated derivatives of DOC; 7 α , 15 α and 15 β from Dr A. Wettstein; 14 α from Dr D. H. Peterson; 6 β , 11 β and 17 α from Dr J. K. Grant; 6 β :21-diacetoxy-DOC from Dr M. Ehrenstein. 5 α -Pregnane-3 β :20 β :21-triacetate (3β :20 β :21-triacetoxy-5 α -pregnane) was the preparation described by Taylor (1957b).

Other materials and methods were as described in previous publications (Taylor, 1954, 1955).

Incubation of deoxycorticosterone with suspension of disintegrated rabbit liver

The experiment was conducted in six parts, a total of 850 mg. of DOC being incubated with 777 g. (wet wt.) of rabbit liver. In a typical experiment 125 mg. of DOC was dissolved in 2.6 ml. of hot ethanol, 150 ml. of phosphate-saline at 95° was added and 15 ml. of the fine suspension of steroid obtained on cooling was added to each of ten 100 ml. conical flasks. The rabbit liver (116 g.) was homogenized for 2 min. at high speed in the Ato-Mix blender (Measuring and Scientific Equipment Ltd., London) with 100 ml. of phosphate-saline containing 2.5 g. of nicotinamide. The resulting suspension was filtered through two layers of gauze, made up to 175 ml. with phosphate-saline and added in 20 ml. portions to the reaction vessels containing the DOC suspension at 37°. The unstopped flasks were shaken for 2 hr. at 37°. A neutral lipid extract was then prepared (Taylor, 1954) and the combined extracts from the six parts were separated into ketonic and non-ketonic fractions with Girard reagent T (Girard & Sandulesco, 1936). After one such separation paper chromatography revealed that the non-ketonic fraction still contained appreciable material reacting with BTZ. Each fraction was treated with Girard reagent a second time, after which the non-ketonic fraction contained no BTZ-positive material. The ketonic and non-ketonic fractions were then treated with digitonin to yield 3β -hydroxy-ketonic and non-ketonic fractions (precipitated by digitonin) and 3α -hydroxy-ketonic and non-ketonic fractions (not precipitated by digitonin). As a control 800 mg. of solid DOC was

added to tissue suspension (containing 800 g. of liver) which had been incubated previously for 2 hr. The neutral lipid extract was then fractionated in the same manner as the incubation extract. The weights of the fractions obtained are shown in Table 4.

RESULTS

α -Hydroxyketonic fraction

Paper chromatography in system III of a portion of this fraction, which weighed 502 mg., from the incubation experiment revealed the presence of at least eight substances giving a positive reaction in the BTZ or NaOH-fluorescence tests or in both (Fig. 1, strip 1). The fraction was dissolved in benzene and applied to a silica-gel column, which was then washed by gradient elution with benzene-ether followed by ether-ethyl acetate mixtures. Paper chromatography of portions of the eluates showed that the silica-gel chromatography had yielded three subfractions, A, B and C (indicated by the dotted lines on strip 1, Fig. 1). These could not be resolved further by adsorption chromatography, and so large-scale chromatography on Whatman no. 3 MM paper was carried out.

Subfraction A. This weighed 163 mg., and was eluted by benzene containing 0–50% of ether. Chromatography in system II indicated that four compounds were present (Fig. 1, strip 2).

Substance A1 had the same R_F as 3α :21-dihydroxy-5 β -pregnan-20-one and weighed 27 mg. Two crystallizations from methanol-ether yielded 14 mg. of needles, m.p. 151–152.5°, unchanged on mixing with 3α :21-dihydroxy-5 β -pregnan-20-one (m.p. 151–153°). The isolated substance and the authentic steroid had identical infrared spectra, and therefore the identity of the metabolite was shown to be 3α :21-dihydroxy-5 β -pregnan-20-one.

Substance A2, which weighed 31 mg., was slightly less polar than A1: it reduced BTZ but did not absorb u.v. light. After acetylation it had the same R_F in system I as 3β :21-diacetoxy-5 α -pregnan-20-one, suggesting that it might be the 3α -epimer of this substance. Crystallization of the acetylated material from methanol yielded 20 mg. of fine needles, m.p. 161–164° [α]_D²⁵ +92±2° (c, 0.523 in ethanol) (Found: C, 71.4; H, 9.0. Calc. for C₂₅H₃₈O₅: C, 71.8; H, 9.1%). Schneider (1952) gives m.p. 164–165° and [α]_D²⁴ +94.5 in ethanol for 3α :21-diacetoxy-5 α -pregnan-20-one. No authentic sample of this steroid was available for comparison, but the infrared spectrum of the isolated acetate showed a major band at 1732 (ester carbonyl) and a complex band at 1262–1232 cm.⁻¹, indicating the presence of a 3-axial hydroxyl group. The isolated substance, therefore was almost certainly 3α :21-dihydroxy-5 α -pregnan-20-one.

Substance A3 (57 mg.) gave positive reactions in the BTZ and NaOH-fluorescence tests and had the same R_F as DOC in systems II and III. Crystallization from ether yielded 35 mg. of crystals, m.p. 141–144°; mixed with authentic DOC (m.p. 143–144.5°) the m.p. was 140–143°: [α]_D²⁵ +175±3° (c, 0.674 in ethanol) (Found: C, 76.1; H, 8.97. Calc. for C₂₁H₃₀O₃: C, 76.2; H, 9.1%). Acetylation and crystallization from methanol yielded fine plates, m.p. 157–159°, unchanged on mixing with 21-acetoxy-pregn-4-ene-3:20-dione, m.p. 158–160°. The u.v. spectra of the isolated substance and of DOC in ethanol and conc.

Table 4. *Weights (mg.) of fractions obtained after treatment of neutral lipid extracts with Girard reagent T and digitonin*

Total neutral lipids	'Incubation'	'Control'
Ketonic fraction		
α -Hydroxy-ketonic	502	642
β -Hydroxy-ketonic	44	Nil
Non-ketonic fraction		
α -Hydroxy-non-ketonic	252	232
β -Hydroxy-non-ketonic	45	53

H_2SO_4 were identical. The isolated substance was therefore characterized as unchanged DOC.

Substance A4 weighed 27 mg. and reduced BTZ. In systems I, II and III no separation of A4, 21-hydroxy-5 α - and 21-hydroxy-5 β -pregnane-3:20-dione could be achieved, and the acetates of the three substances had the same R_f in system I. Both A4 and its acetate resisted crystallization. This evidence suggested that the material was a mixture of the 5 α - and 5 β -dihydro derivatives of DOC. Alcoholic solutions of A4 and of mixtures of 21-hydroxy-5 α - and 21-hydroxy-5 β -pregnane-3:20-dione were treated with dinitrobenzene-ethanolic KOH for 5 min. as described by Broadbent & Klyne (1954). The absorption spectrum of A4 over the range 360–600 $m\mu$ corresponded closely with that

given by a mixture containing two parts of 21-hydroxy-5 α -pregnane-3:20-dione to one part of 21-hydroxy-5 β -pregnane-3:20-dione. Thus 21-hydroxy-5 α -pregnane-3:20-dione (18 mg.) and its 5 β isomer (9 mg.) were tentatively identified as metabolites.

Subfraction B. This was eluted from the silica-gel column by mixtures containing 55–100% of ether in benzene and weighed 72 mg. Chromatography in system III (Fig. 1, strip 3) showed the presence of three substances positive in the NaOH-fluorescence test, but only one reduced BTZ. The substances were resolved by large-scale chromatography by allowing system III to over-run for 8 hr.

Substance B1 weighed 3.1 mg. and was positive in the BTZ and NaOH-fluorescence tests. In system IV B1 had

Fig. 1. Paper chromatography of 3 α -hydroxy-ketonic fractions. Strip 1. α -Hydroxy-ketonic fraction in solvent system III: vertical dotted lines denote subfractions obtained by chromatography on silica gel. Strip 2. Subfraction A in solvent system II: reference steroids: 3 α -OH-5 β -H₄DOC = 3 α :21-dihydroxy-5 β -pregnan-20-one; DOC = 21-hydroxypregn-4-ene-3:20-dione; 5 α - and 5 β -H₂DOC = 21-hydroxy-5 α -pregnane-3:20-dione and its 5 β epimer. Strip 3. Subfraction B in solvent system III (8 hr. run): reference steroids: 20 α - and 20 β -OH-DOC = 20 α :21-dihydroxy-pregn-4-en-3-one and its 20 β -epimer. Strip 4. Subfraction C in solvent system IV: reference steroids: 15 α - and 15 β -OH-DOC = 15 α :21-dihydroxy-pregn-4-ene-3:20-dione and its 15 β -epimer. Strip 5. Acetate of subfraction C in solvent system I: reference steroids: H₂SA = 21-acetoxy-17 α -hydroxy-5 β -pregnane-3:20-dione; DOCA = 21-acetoxypregn-4-ene-3:20-dione; H₄SA = 3 β :21-diacetoxy-17 α -hydroxy-5 β -pregnan-20-one. Ref. = reference steroids; O = starting line; Z = solvent front. Open spots, NaOH-fluorescence-positive; stippled spots, BZT-positive; black spots, BTZ- and NaOH-fluorescence-positive.

the same R_F as 6β - and 14α -hydroxy-DOC and was separated from 7α -, 11β -, 15α -, 15β - and 17α -hydroxy-DOC. After acetylation at room temperature B1 and 6β -hydroxy-DOC formed diacetates which had the same R_F values in system II, whereas, under the same conditions, 14α -hydroxy-DOC was acetylated only at C-21 and so was readily separated from B1. Further support for the view that B1 might be 6β -hydroxy-DOC was obtained when its $\lambda_{\max}$. (in ethanol) was found to be the same as that of authentic samples before (235 m μ) and after (236 m μ) acetylation (see Dorfman, 1953). The acetylated material resisted attempts at crystallization and appeared to contain contaminating material from the paper. As a result the infrared spectrum did not provide conclusive evidence of its identity, but the spectrum showed complex bands in the ranges 1740–1675 (6β -acetoxy-4-en-3-one) and 1240 cm.⁻¹ (acetoxy) similar to those shown by the authentic steroid. Therefore there is a fair degree of certainty that the isolated substance was 6β :21-dihydroxypregn-4-ene-3:20-dione.

Substance B2 weighed 37 mg. and had the same chromatographic properties in system III as 20α -hydroxy-DOC. Two crystallizations from methanol yielded 19 mg. of material, m.p. 189–193°: mixed with authentic 20α -hydroxy-DOC (m.p. 189–192°) the m.p. was 189–191°. After acetylation the product had the same R_F in system I as the diacetate of 20α -hydroxy-DOC, and crystallization yielded material m.p. 103–106°, unchanged on mixing with the authentic diacetate (m.p. 103–105°). The infrared spectra of the diacetates of B2 and 20α -hydroxy-DOC were identical, and therefore the isolated material was 20α :21-dihydroxypregn-4-en-3-one.

Substance B3 (18 mg.) appeared to be the 20β -epimer of B2, as judged by its chromatographic properties. Crystallization from methanol yielded 10 mg. of material, m.p. 169–171°, unchanged on mixing with 20β -hydroxy-DOC (m.p. 168–171°). The acetylated material, crystallized from ethyl acetate–hexane, had m.p. 154–157°, which was not depressed on mixing with 20β :21-diacetoxypregn-4-ene-3:20-dione, m.p. 155–157°; the infrared spectra of the two diacetates were identical. The isolated material was therefore 20β :21-dihydroxypregn-4-en-3-one.

Subfraction C. This weighed 37 mg., and was eluted from the silica-gel column by ether containing 25–100% of ethyl acetate, and by large-scale paper chromatography was resolved into three BTZ-positive substances.

Substances C1 and C2, 3.0 and 2.1 mg. respectively, were more polar in system IV than was 15α -hydroxy-DOC, and after acetylation were less polar in system I than 3β :21-diacetoxy-5 β -pregnan-17 α -ol-20-one (Fig. 1, strips 4 and 5). The residues were dissolved in 0.5 ml. of warm acetone and allowed to stand at –15° for 48 hr. The supernatant fluid was removed from the semicrystalline solids, which were washed with 2 $\times$ 0.2 ml. of cold acetone. Portions of the solids were removed for infrared analysis, the supernatant fluids were returned to their corresponding solids and the whole was evaporated to dryness. After acetylation the residues were treated with methanol–hexane and portions of the solid residues so obtained were taken for infrared analyses. The spectrum of C1 had major bands at 3280 (–OH), 2900 (C–H), 1720 (carbonyl), 1050 (C–OH) and 755 cm.⁻¹. The acetate of C1 showed no absorption due to free hydroxyl groups, but major bands were seen at 2900 (C–H), 1737 (21-acetoxy-20-oxo), 1378,

and a simple band at 1243 cm.⁻¹ indicative of a 3-equatorial acetate. The spectrum of C2 was very similar to that of C1 except for a more pronounced shoulder on the carbonyl band at 1713 and the absence of the band at 755 cm.⁻¹. The spectrum of C2 acetate also closely resembled that of C1 acetate; there were major bands at 1735 (21-acetoxy-20-oxo) (with a definite smaller band at 1678 which was absent from C1 acetate), 1378 and a simple band at 1245 cm.⁻¹ (3-equatorial acetate). Substance C3 (0.8 mg.) was less polar than 15β -hydroxy-DOC in system IV, and the acetate was less polar than 21-acetoxy-17 α -hydroxy-5 β -pregnane-3:20-dione and slightly more polar than 21-acetoxypregn-4-ene-3:20-dione in system I (Fig. 1, strips 4 and 5). As a result of contamination from the paper the infrared spectra were poor, but C3 showed major bands at 3340 (–OH), 2900 (C–H) and 1700 cm.⁻¹ (20- and other oxo). The acetate showed no absorption due to free hydroxyl groups, but had major bands from 1700 to 1750 (3, 20 and possibly 6-oxo) and smaller bands at 1469, 1385 and 1236 cm.⁻¹. The absence of significant absorption at 1245 cm.⁻¹ suggests that no 3-acetoxy group was present.

The lack of reference compounds prevents any definite conclusions being drawn about the identity of these substances, but the properties described are consistent with the view that: (a) C1 and C2 were tetrahydro derivatives of DOC having (i) an α -ketol side chain, (ii) 3α -hydroxy-5 β configuration in ring A, and (iii) an extra hydroxyl group and forming an epimeric pair, with C1, the more polar, having the equatorial conformation (cf. Savard, 1953); (b) C3 was a dihydro derivative of DOC having (i) an α -ketol side chain, (ii) an oxo group at C-3, and (iii) an extra oxo group (possibly at C-6) or a readily acetylated hydroxyl group. It can also be seen that C1, C2 and C3 appear to bear the same chromatographic relationship to the hydroxy-DOCs as do A1, A2 and A4 to DOC.

Paper chromatography of the α -ketonic fraction from the 'control' showed only one BTZ-positive and NaOH-fluorescence-positive spot with the same R_F as DOC. Chromatography on silica gel yielded 379 mg. of DOC, which was identified as follows: m.p. 141–143°, unchanged on mixing with authentic DOC (m.p. 143–144.5°), $[\alpha]_D^{25} + 172 \pm 2^\circ$ (c, 0.832 in ethanol) (Found: C, 76.2; H, 9.23. Calc. for $C_{21}H_{30}O_3$: C, 76.2; H, 9.1%). The acetate melted at 156–159°, unchanged on mixing with 21-acetoxypregn-4-ene-3:20-dione, m.p. 158–160°

α -Hydroxy non-ketonic fraction

This fraction from the 'incubation' experiment was a dark-brown gum weighing 252 mg., which contained no substances giving a positive reaction in the BTZ and NaOH-fluorescence tests. One-tenth of the fraction was dissolved in toluene and divided into two equal parts A and B. To part B was added 50 μ g. of pregnanediol; then both parts were treated as toluene extracts of acid-hydrolysed urine in the method of Kloppe, Michie & Brown (1955), which is sufficiently sensitive to measure 50 μ g. of pregnanediol with reasonable accuracy. No pregnanediol was detected in part A, whereas 42 μ g. of the steroid was recovered from part B. A one-tenth portion of the α -non-ketonic material from the 'control', a dark-brown gum weighing 232 mg., was treated in the same way; 43 μ g. of pregnanediol was recovered from part B, but none from part A. Therefore it

can be concluded that no significant amount of pregnane-diol was present in this fraction.

The remainder of the 'incubation' and 'control' fractions were dissolved in benzene and eluted from silica-gel columns with benzene-ether mixtures, and finally with methanol. Portions of all fractions were examined for formaldehydogenic substances by the method of Paterson & Marrian (1953). Only dark-brown gums containing no formaldehydogenic substances spread over a large number of fractions were eluted by benzene-ether. Each series of fractions was combined ('incubation', 163 mg.; 'control', 172 mg.) and acetylated, and chromatography on silica gel showed that no significant change in chromatographic properties had occurred. Chromatography on alumina resulted in no resolution, the material appearing as brown gums spread over all fractions.

The materials eluted from the first silica-gel columns by methanol were light-brown formaldehydogenic resins weighing 70 mg. ('incubation') and 52 mg. ('control'). Each was dissolved in 50 ml. of ethanol-water (7:3, v/v) and extracted three times with 5 ml. of hexane, the hexane being back-washed with 5 ml. of aqueous ethanol. The hexane extracts yielded brown gums from which no solids could be obtained by chromatography on silica gel or, after acetylation, on alumina. Evaporation of the aqueous ethanol yielded 33 mg. from the 'incubation' extract and 23 mg. from the 'control'. These were white resins containing formaldehydogenic substances.

Chromatography of the 'incubation' 70% aqueous ethanol extract on silica gel gave two fractions containing formaldehydogenic substances: one was eluted by benzene containing 35-50% of ethyl acetate and the other by benzene containing 70-100% of ethyl acetate. When the 'control' extract was chromatographed in the same way only one formaldehydogenic fraction, eluted by benzene containing 35-55% of ethyl acetate, was obtained. This material had the same properties as the corresponding fraction from the 'incubation' extract. Both were white waxes which were formaldehydogenic before, but not after, acetylation; their absorption spectra in conc. H_2SO_4 were identical and showed no features characteristic of steroids. These properties suggest that the substances were monoglycerides but no further characterization was attempted.

The material from the 'incubation' extract eluted by benzene containing 70-100% of ethyl acetate was a white solid weighing 4.7 mg. which was formaldehydogenic

before, but not after, acetylation. After acetylation, chromatography on alumina yielded two fractions, α -NK1 and α -NK2, eluted by benzene. The spectra of the two substances in conc. H_2SO_4 were identical with that of 3 β :20 β :21-triacetoxy-5 α -pregnane. α -NK1 was a white solid weighing 2.7 mg. which resisted crystallization: its infrared spectrum showed major bands at 2940 (C-H) and 1735 (ester carbonyl) and a complex band in the region of 1224 indicative of a 3-axial acetate; minor bands were present at 1450, 1375 and 1045 cm^{-1} . α -NK2 was a colourless gum which weighed 0.7 mg.; the infrared spectrum was very similar to that of α -NK1 except that the major band at 1240 cm^{-1} was simple, indicating the presence of a 3-equatorial acetate. These spectra were similar to that of 3 β :20 β :21-triacetoxy-5 α -pregnane, which had major bands at 2920, 1715 and 1232 (simple) and minor ones at 1457, 1360, 1068, 1017 and 955 cm^{-1} . These findings suggest that the isolated substances were impure samples of 3:20:21-triacetates probably possessing the α -configuration at C-3 (not precipitated by digitonin), and that α -NK1 was the 5 α -isomer and α -NK2 the 5 β .

β -Hydroxy-ketonic fraction

This fraction weighed 44 mg. and was white and semi-crystalline. Paper chromatography in systems II and III showed the presence of one major component which reacted with BTZ and had the same R_f as 3 β :21-hydroxy-5 α -pregn-20-one. Two very faint BTZ-positive spots more slowly moving than the main spot were also seen, but by visual inspection these were considered to amount to less than 0.5% of the fraction and so isolation was not attempted. After acetylation of the fraction followed by crystallization from methanol, fine needles (25.5 mg.), m.p. 150-152°, were obtained. Further treatment of the mother liquors yielded 3 mg. of less-pure material, m.p. 148-151°. A comparison of the properties of the isolated material, of the substance prepared by hydrogenation of 21-acetoxy-3 β -hydroxypregn-5-en-20-one (see Reference steroids, p. 443) and of the reported properties of 3 β :21-dihydroxy-5 α -pregnan-20-one is shown in Table 5. The infrared spectra of the isolated and prepared steroid diacetates were identical in every respect. Therefore the major component of the β -ketonic fraction was 3 β :21-dihydroxy-5 α -pregnan-20-one.

No β -hydroxy-ketonic fraction was obtained from the 'control'.

Table 5. Comparison of properties of substance isolated from β -hydroxy-ketonic fraction, of steroid obtained by reduction of 21-acetoxy-3 β -hydroxypregn-5-en-20-one and of reported properties of 3 β :21-dihydroxy-5 α -pregnan-20-one

Free steroid	Isolated	Prepared	Reported
M.p.	—	170-173°	170-171°* 171-176†
Diacetate			
M.p.	150-152°	151-152°	151-153°
Mixed m.p.		150-152°	
C, H analysis	C, 71.7 H, 9.0 +86 $\pm$ 2° (c, 0.620)	C, 71.8 H, 9.2 +87 $\pm$ 1° (c, 0.783)	Calc. for $C_{25}H_{38}O_5$: C, 71.7; H, 9.1% +82.6† +88.3§
$[\alpha]_D$ in $CHCl_3$			

* Schneider (1952).

† Reichstein & Euw (1939).

‡ Kahnt, Meystre, Neher, Vischer & Wettstein (1952).

§ Plattner, Heusser & Anglikler (1946).

β-Hydroxy-non-ketonic fraction

This fraction from the 'incubation' experiment weighed 45 mg. and was white and semicrystalline. A quantitative Lieberman-Burchard reaction (Kerr & Bauld, 1953) showed that the material consisted entirely of cholesterol. Crystallization from ethanol yielded 36 mg. of irregular plates, m.p. 145–146°: mixed with an authentic sample of cholesterol (m.p. 145–147°) the m.p. was 144–146°. The acetate, m.p. 112–114°, did not depress the m.p. of authentic cholesteryl acetate (m.p. 113–114.5°). The absorption spectra in conc. H_2SO_4 over the range 230–550 $m\mu$ of the isolated material and of cholesterol were identical. The isolated substance was therefore identified as cholesterol, and no metabolites of DOC were present.

The 'control' β -hydroxy-non-ketonic fraction (53 mg.) yielded 41 mg. of cholesterol, identified as described above.

DISCUSSION

For convenience the results are summarized in Table 6; the amounts of the substances isolated as a percentage of the total DOC added in the 'incubation' and 'control' experiments are also given. Because of the large number of steps in the purification procedure quantitative recovery of metabolites was not attempted.

Large-scale incubation experiments with rat liver have been used for the study of the metabolism of DOC by slices (Schneider, 1952) and of progesterone by homogenate (Taylor, 1954). The metabolites isolated were closely related in being of the 5 α -pregnane series only and in consisting almost entirely of dihydro and tetrahydro derivatives of the substrates reduced in ring A. Rabbit liver, on the other hand, converted progesterone into both

5 α - and 5 β -pregnane compounds, with the latter predominating, and the principal metabolite was pregnanediol, a hexahydro derivative of progesterone reduced in ring A and at C-20 (Taylor, 1955). This greater metabolic versatility of rabbit liver has now been shown to extend to DOC also (Table 6).

There are, however, major differences in the types of compounds produced by rabbit liver from progesterone and DOC, the most striking being the almost complete absence of hexahydro reduced derivatives of DOC. That this is not the result of the lack of enzymes able to reduce the C-20 oxo group ('C-20 oxo reductase') is shown by the isolation of appreciable amounts of 20 α - and 20 β -hydroxy-DOC. The possibility that the C-20 oxo reductases act only on DOC itself and not on reduced derivatives requires further investigation. Also the total weight of 5 α -pregnane derivatives isolated exceeds that of the 5 β , whereas the reverse situation is found in the case of progesterone (Taylor, 1955). Others have found that closely related steroids may be metabolized to different products by the same tissue preparation. Thus Hubener, Fukushima & Gallagher (1956) found that the C-20 oxo group of 3 α :11 β :17 α :21-tetrahydroxy-5 β -pregnan-20-one and 11 β :21-dihydroxy-pregn-4-ene-3:20-dione were converted predominantly into the 20 β -hydroxy isomers by rat-liver homogenate, and 3 α :17 α :21-trihydroxy-5 β -pregnane-11:20-dione and 17 α :21-dihydroxy-pregn-4-ene-3:20-dione mainly into the 20 α -hydroxy derivatives (see also de Courcy & Schneider, 1956; Recknagel, 1957).

Table 6. *Steroids isolated from 'incubation' and 'control' experiments*

Steroid isolated	From 'incubation'		From 'control'	
	Wt. (mg.)	As percentage of added DOC	Wt.	As percentage of added DOC
21-Hydroxy-5 α -pregnane-3:20-dione	18	2.1*	—	—
21-Hydroxy-5 β -pregnane-3:20-dione	9	1.0*	—	—
20 α :21-Dihydroxypregn-4-en-3-one	19	2.2	—	—
20 β :21-Dihydroxypregn-4-en-3-one	10	1.2	—	—
3 α :21-Dihydroxy-5 α -pregnan-20-one	20	2.3	—	—
3 β :21-Dihydroxy-5 α -pregnan-20-one	28.5	3.3	—	—
3 α :21-Dihydroxy-5 β -pregnan-20-one	14	1.6	—	—
6 β :21-Dihydroxypregn-4-ene-3:20-dione	3.1	0.4†	—	—
21-Hydroxypregn-4-ene-3:20-dione	35	4.1	379	44.5
Unidentified				
α -Hydroxy-ketonic C ₁	3.0	0.4	—	—
C ₂	2.1	0.2	—	—
C ₃	0.8	0.1	—	—
α -Hydroxy-non-ketonic 1	2.7	0.3	—	—
2	0.7	0.1	—	—
Totals	165.9	19.5	379	44.5

* Not separated: amounts of isomers estimated by method of Broadbent & Klyne (1954).

† Impure: not fully characterized.

Other differences exist between the metabolism of DOC in rat and rabbit liver and between the metabolism of DOC and progesterone in rabbit liver, but interpretation of these would be speculative, for in the present investigation the use of paper chromatography and infrared spectroscopy has facilitated the isolation and partial or full characterization of a number of minor metabolites which would have escaped detection by the methods used in the earlier investigations. Furthermore, recent studies have shown that the livers of animals of different sexes differ in their metabolic activity in relation to the proportions of 3 β - and 3 α -hydroxy metabolites formed from androst-4-ene-3:17-dione and 5 α -androstane-3:17-dione (Rubin 1957). Also Forchielli & Dorfman (1956) have reported that 17 α :21-dihydroxypregn-4-ene-3:20-dione can be converted into 5 α or 5 β C₁₉ and C₂₁ steroids by two enzymes which can be separated from rat liver. Since in large-scale experiments with rat liver only 5 α -compounds have been isolated, it would seem that such studies do not necessarily reflect the full potential metabolic activity of a tissue.

The absence of pregnanediol from the metabolic products found in the present investigation is surprising, since this steroid is a metabolite of DOC in the rabbit *in vivo*, and especially since pregnanediol has been found to be a major metabolite of progesterone in the rabbit *in vivo* and in rabbit liver *in vitro*. Possible explanations of this are that the liver of the animal does not contain the enzyme systems responsible for the reduction of the C-21 hydroxyl group, or that pregnanediol is formed but is metabolized further. Taylor (1956) found that incubation of pregnanediol, as its dihemisuccinate, with rabbit liver resulted in the formation of 5 β -pregnane-3:20-dione and 3 α -hydroxy-5 β -pregnan-20-one. Neither of these compounds would have been detected in the present investigation unless present in large amounts.

It is well established that the adrenal cortex contains a number of enzyme systems responsible for the introduction of hydroxyl groups into C₁₉ and C₂₁ steroids mainly at the 11 β , 17 α and 21 positions and, to a lesser extent, at positions 6 β , 18 and 19 (see Grant, 1955, for references). Other tissues, including liver, are also able to hydroxylate steroids. Schneider & Mason (1948) showed that 3 β -androst-5-en-17-one is hydroxylated in the 16 α position when incubated with rabbit-liver slices, and Ofner (1955) has suggested that hydroxylation of testosterone occurs in rat-liver mince. A number of C₁₉ and C₂₁ steroids (including DOC) have been shown to undergo hydroxylation at positions 2 β , 6 β or 16 α when perfused through rat or dog liver (see Dorfman, 1957, for references). Whereas hydroxylations at positions 11 β , 17 α and 21, and

possibly at 2, 18 and 19, are considered to be anabolic reactions, those at 6 and 16 are thought to be catabolic, preceding ring-opening and further degradation of the steroid nucleus. In the present investigation evidence has been presented which suggests that approximately 1% of the incubated DOC was converted into hydroxylated derivatives, one of which was tentatively identified as 6 β -hydroxy-DOC while the remainder (C1-C3) were probably A-ring reduced derivatives of oxo- or hydroxy-DOC with the α -ketol side-chain intact. The failure to isolate such hydroxy compounds from the 'control' experiment does not necessarily prove that these were formed by the enzymic activity of the liver, for one would expect those compounds to be more readily detectable in the mixture of steroids from the 'incubation' extract than from the large excess of DOC present in the 'control' extract. Therefore the possibility that the hydroxylations occurred non-enzymically during the incubation or isolation procedures cannot be ruled out.

A wide variety of micro-organisms have been shown to possess steroid-hydroxylating enzymes (see Dorfman, 1957, for references), and therefore it is possible that the hydroxylated compounds isolated in the present study were produced as a result of microbiological contamination of the incubation mixture.

Only about 20% of the incubated DOC was recovered as metabolites and unchanged DOC from the 'incubation' experiment, whereas 45% was obtained from the 'control'. It is probable that this discrepancy is the result of the greater manipulative losses involved in the purification of the separate metabolites. It is possible that other compounds were formed which were not detected by the isolation procedures used. Thus etienic or etianic acids formed by removal of the C₁₇ side chain, as stated by Picha, Saunders & Green (1952) to occur in rat liver, would have been removed during the preparation of the neutral lipid extract. Compounds not possessing the α -ketol side chain or the $\alpha\beta$ unsaturated oxo group, such as 20:21-dihydroxypregnan-3-ones, would not have been detected unless present in large amounts.

Richardson *et al.* (1955) and Eberlein & Bongiovanni (1956) isolated 3 α :21-dihydroxy-5 β -pregnan-20-one from urine previously treated with β -glucuronidase, and therefore it is possible that water-soluble conjugates of this type were formed during the 'incubation' experiment. That such conjugates can be formed in liver *in vitro* has been shown by Sie & Fishman (1957), and de Meio, Lewycka, Wizerkaniuk & Salciunas (1958) have demonstrated the formation of sulphuric acid esters of steroids in liver. No search for conjugated metabolites was made in the present investigation.

The results of experiments *in vitro* do not necessarily bear any relation to the metabolic fate of steroid hormones in the intact animal. Nevertheless the present results show that the liver of the rabbit is able to convert DOC into a wide variety of substances, few of which have as yet been shown to be metabolites of the hormone *in vivo*. A comprehensive study of the metabolism of DOC in man and animals involving analyses of urine and bile for metabolites of the type isolated in experiments *in vitro* might provide results of value in the investigation of disorders of the adrenal cortex involving absence or deficiency of adrenocortical '11 β -hydroxylase.'

SUMMARY

1. In a large-scale experiment in which deoxycorticosterone was incubated in air with a suspension of disintegrated rabbit liver with added nicotinamide, about 20 % of the added steroid has been accounted for by isolated metabolites and unchanged substrate.

2. The following substances were isolated and fully characterized: 20 α :21-dihydroxypregn-4-en-3-one and its 20 β -epimer; 3 α :21-dihydroxy-5 α -pregnan-20-one and its 3 β -epimer; 3 α :21-dihydroxy-5 β -pregnan-20-one; unchanged substrate.

3. Evidence is presented for the formation of other metabolites: 21-hydroxy-5 α -pregnane-3:20-dione and its 5 β -isomer; 6 β :21-dihydroxypregn-4-ene-3:20-dione; three closely related substances which were probably hydroxylated derivatives of dihydro and tetrahydro reduced deoxycorticosterone; 5 α - or 5 β -pregnane-3 α :20 ξ :21-triols.

4. In a 'control' experiment 45 % of the added steroid was recovered unchanged.

5. The significance of these results is discussed.

The author wishes to thank the many colleagues for the generous gifts of steroids, particularly Dr C. L. Hewett, Organon Laboratories Ltd., Newhouse, Lanarkshire, and Dr W. Klyne for making so readily available the resources of the Reference Steroid Collection of the Medical Research Council. A deep debt of gratitude is owed to Dr R. K. Callow, F.R.S., National Institute for Medical Research, London, for determining and interpreting the infrared spectra.

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