The Free and Esterified Sterol present in Bovine Adrenal Cortex and Medulla

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The adrenal gland has been much studied for the mechanism of corticosteroid biosynthesis (Dorfman, 1962). However, the relative amounts of cortex and medulla and their respective compositions vary considerably in different mammals (Bourne, 1958). In these component parts the exact nature of the sterols has not been investigated in detail. The bovine adrenal gland, which was our object of study, is well suited for an investigation of the distribution and chemical nature of the sterols because the cortex is easily separated from the medulla, and moreover a plentiful supply of fresh tissue is readily available. This study has also yielded information on the value and limitations of various forms of chromatography (column, thin-layer and gas-liquid) and of other accepted analytical procedures in determining the nature of the sterols present in the complex lipid extract.

MATERIALS AND METHODS

Adrenal glands and their extraction. Adrenal glands, from newly slaughtered cattle, mainly milking cows (2 years old), were collected from the local abattoir three times a week over a period of 5 weeks. The glands were immediately deep-frozen on collection, transported to the laboratory over solid CO₂ in a vacuum flask and stored in this container. All adhering fat was removed from the still frozen glands, which were then cut into five or six pieces. The cortical and medullary tissues of each piece, which are quite distinct, were separated by dissecting with a scalpel, and weighed. The cortical and medullary tissues from each week's collection of glands (about 600 g.) were stored in separate bottles under ethanol at 4° until extracted, both cortex and medulla being treated in the same manner. After a week's collection of tissue had been combined, the volume of ethanol was made up to 1.5 vol. (v/w) and diethyl ether (0.5 vol., v/w) added, the mixed tissues and solvent being homogenized in a Waring Blender. The resulting homogenate was shaken for 10 min. at 15° and filtered to give a cloudy yellow filtrate. The residue was extracted again with 2 vol. of ethanol-diethyl ether (1:1, v/v) by shaking for 20 min. and filtered, the yellow filtrate being added to the first extract. The residue was finally extracted with 2 vol. (v/w) of boiling ethanol-diethyl ether (1:1, v/v) for 30 min., the suspension filtered and the pale-yellow filtrate added to the other extracts. The residue was discarded and the combined extracts were stored at 4°. Overnight a flocculent white precipitate formed in the extract.

This was removed by filtration and the deep-yellow filtrate concentrated to a yellow paste containing water, which was removed by azeotropic drying with benzene. The extracts from successive weekly collections of glands were combined and stored in the cold room (4°) until required.

Bovine plasma cholesterol ester fatty acids. The cholesterol esters from bovine plasma were isolated as described by Garton & Duncan (1957). The fatty acids were obtained by saponification, then methylated and analysed by gas-liquid chromatography.

Column chromatography. Initial lipid extracts were chromatographed on Florisil (Hermadex Co. Ltd., Richmond, Surrey) to which water (7%, v/w) had been added (Carroll, 1961). The ratio of adsorbent to extract was usually about 10:1. The fractions containing free sterols were chromatographed on a mixture containing 2 parts of silicic acid (100 mesh; Mallinckrodt Chemical Works, New York, U.S.A.) and 1 part of Celite 545 (Johns-Manville Co., New York, U.S.A.) as described by Frantz, Dulit & Davidson (1957) and Kandutsch & Russell (1959). The minor sterols left after crystallization or chemical treatment (below) were chromatographed on alumina (M. Woelm, Eschwege, Germany) or Florisil containing 7% (v/w) of water.

Thin-layer chromatography. Fractions from column chromatography were screened by chromatography on plates of silica gel G (E. Merck A.-G., Germany), activated by heating in an oven at 110° for 10–20 min. The plates were spotted with samples (20–50 µg.), developed by ascending-solvent chromatography and dried and the sterols detected by spraying with colour reagents (see below). Non-polar fractions (e.g. sterol esters) were studied by developing with CCl₄, whereas the free sterols were developed in benzene-ethyl acetate (2:1, v/v). Thin-layer chromatography was also used preparatively to isolate some sterols. In this case the required compound was located by running a standard, the silica gel was then removed carefully by scraping into a 15 ml. centrifuge tube and the compound eluted by shaking with methanol-methylene chloride (1:1, v/v) (4 ml.) for 10 min. and centrifuging. The clear supernatant was decanted off. Three further extractions were made, the combined extracts being evaporated to dryness and the residue used for further experimentation. The movements of individual sterols on thin-layer plates were expressed relative to that of cholesterol taken as 1.00. This value was called movement relative to cholesterol (*R_C*) (Table 1).

Gas-liquid chromatography. Gas-liquid-chromatographic analyses were carried out in a Panchromatograph (W. G. Pye Ltd., Cambridge). Argon was used as the carrier gas, the detector being a ⁹⁰Sr-ionization type.

Sterol analysis. Silicene-treated Celite (100–120 mesh) was prepared by washing the Celite with conc. HCl, water and acetone, drying at 90° for about 4 hr. and treating with

dimethyldichlorosilane (Sjövall, Meloni & Turner, 1961). Glass columns were silane-treated by passing a stream of nitrogen containing dimethyldichlorosilane vapour through them for about 4 hr. They were then washed with methanol and dried at 100° before being packed. The stationary phase [1% (w/w) QF-1 (W. G. Pye Ltd., Cambridge)] was coated on the treated Celite as described by Brooks & Hanaineh (1963). Columns were heated at 250° for 24 hr. with argon passing through them before being connected to the detector. The conditions for chromatography were: column temperature, 210°; detector temperature, 220°; argon flow, 50 ml./min.

Table 1 gives the retention times of standard sterols relative to that of 5 α -cholestane. This value was called relative retention time (R_s).

Fatty acid analysis. The stationary phases used to analyse the methyl esters of the fatty acids from the sterol ester fraction were 10% Apiezon L and 10% polyethylene glycol adipate, both on Celite (100–120 mesh).

Demonstration of minor sterols. In the presence of large amounts of cholesterol conventional chromatography is unsatisfactory for demonstrating the presence of minor ('companion') sterols. This is presumably due to a 'carrier' effect. A useful procedure for the concentration of minor sterols is crystallization from acetic acid, devised by Fieser (1953); the procedure described is a modification for small amounts. The sterol fraction was dissolved in 8 vol. (v/w) of boiling acetic acid with constant stirring. Immediately solution occurred, the flask containing the solution was placed on ice and the contents were stirred vigorously. The crystalline precipitate of cholesterol was removed by filtration and recrystallized from methanol to remove adherent acetic acid. The acetic acid residual liquor was diluted with water (1 vol.) and extracted three times with diethyl ether (1 vol.), and the combined extracts were washed with saturated NaHCO₃, dried over Na₂SO₄ and evaporated to dryness. The material, which still contained some residual cholesterol, was rechromatographed, the fractions being examined by thin-layer and gas-liquid chromatography.

Demonstration of stanols. There is no satisfactory chromatographic method for separating cholestanol from cholesterol; it is necessary to convert the cholesterol into a

derivative with appreciably different properties. This may be done by using bromine in conjunction with thin-layer chromatography (Cargill, 1962). In this procedure, cholesterol fractions containing a small amount of cholesterol are pipetted on to a thin-layer plate. An excess of bromine in chloroform is then added from a micropipette on top of the sample, converting the cholesterol into 5 α ,6 β -dibromcholesterol and to oxidation products, whereas cholestanol is not affected. The dibromide and oxidation products separate easily from cholestanol, thus allowing the latter sterol to be clearly identified if present. Cholesterol may also be oxidized to cholestane-3 β ,5 α ,6 β -triol by perbenzoic acid (Werbin & Chaikoff, 1962) or by performic acid (Fieser & Rajagopalan, 1949; Rosenfeld, Zumoff & Hellman, 1962). Performic acid oxidation was used as follows. The fractions were dissolved in formic acid (5 vol.) and heated to 80° on a hot bath, to give an opaque solution. Then '20 volume' H₂O₂ (4 vol.) was added, and the solution shaken for 4 hr. and left overnight at room temperature. Next day boiling water (10 vol.) was added to the clear solution, which was saponified by boiling with aq. 15% (w/w) KOH (10 vol.) for 30 min. The solution was acidified with HCl, causing it to turn cloudy, and extracted four times with light petroleum (b.p. 40–60°) (1 vol.). The light-petroleum extracts were combined, dried over Na₂SO₄ and evaporated to dryness to give an oily residue. This was chromatographed on acetic acid-deactivated alumina (300:1, w/w). The column was developed with light petroleum-diethyl ether (17:3, v/v), 10 ml. fractions being collected and screened by thin-layer chromatography.

Colour tests. Samples (about 0.5 mg.) from chromatographic fractions were tested for 'fast-acting' and 'slow-acting' sterols by the Liebermann-Burchard reaction (Cook, 1961).

Sterols were detected on thin-layer plates by spraying with an ethanolic 10% solution of phosphomolybdic acid and heating at 100° for 5 min. or with a solution of 300 mg. of dinitrophenylhydrazine in 200 ml. of acidified ethanol (Cargill, 1962). The former reagent gives blue spots on a pale-yellow background with hydroxy sterols and the latter gives pale-yellow spots with sterols containing a carbonyl function.

Table 1. *Chromatographic data for reference sterols*

The thin-layer chromatography results are for a benzene-ethyl acetate (2:1, v/v) system, the movement of the compounds being given relative to that of cholesterol (R_C). For gas-liquid chromatography, the retention times are for a 1% QF-1 column. The values quoted are relative to that of 5 α -cholestane (R_s).

Systematic name	Trivial name	R_C	R_s
5 α -Cholestane*	Cholestane	—	1.00
Cholest-5-en-3 β -ol†	Cholesterol	1.00	2.80
4,4,14-Trimethylcholesta-8,24-dien-3 β -ol†	Lanosterol	1.21	4.36
4 α -Methyl-5 α -cholest-7-en-3 β -ol†	Methostenol	1.11	3.29
Cholest-5-en-3-one§	Cholestenone	1.57	7.94
5 α -Cholestan-3 β -ol†	Cholestanol	0.94	3.04
5 α -Cholest-7-en-3 β -ol†	Lathosterol	0.90	3.42
Cholest-5-ene-3 β ,7 α -diol	7 α -Hydroxycholesterol	0.24	—
Cholest-5-ene-3 β ,7 β -diol	7 β -Hydroxycholesterol	0.26	—
Cholest-5-ene-3 β ,24 β -diol§	24 β -Hydroxycholesterol	0.77	6.68
Cholest-5-ene-3 β ,25-diol§	25-Hydroxycholesterol	0.57	4.04

* From L. Light and Co. Ltd., Colnbrook, Bucks. (by purchase).

† Prepared in this Laboratory.

‡ From Professor W. W. Wells, Pittsburgh, Pa., U.S.A.

§ From Professor L. F. Fieser, Harvard University, Cambridge, Mass., U.S.A.

|| From Professor H. B. Henbest, Queen's University, Belfast, Northern Ireland.

Saponification of sterol esters. Methanolic KOH was prepared by dissolving, with shaking, KOH (5.6 g.) in methanol (100 ml.); if cloudy, the solution was filtered before use. The fraction (1 part wt.) was saponified, with the alkali solution (5 vol.) and (to aid solution of the esters) benzene (10 vol.), at the ebullition temperature for 2 hr. under an atmosphere of nitrogen. On completion the solvents were removed *in vacuo* and the residue was dissolved in water (2 vol.). The solution was extracted three times with diethyl ether (1 vol.), and the combined ether extracts were washed with water, dried over Na_2SO_4 and recovered to give unsaponifiable matter consisting mainly of sterols. The aqueous phase and water-washings from the ether extracts were then acidified with 10% (w/w) H_2SO_4 , and the fatty acids extracted three times with diethyl ether (1 vol.), washed with water, dried over Na_2SO_4 and recovered.

Preparation of fatty acid methyl esters. An ethereal solution of diazomethane was prepared (de Boer & Backer, 1954) and added to an ethereal solution of the fatty acids with constant shaking until a persistent yellow colour indicated excess of reagent; excess of reagent and ether were carefully removed under nitrogen at 18° and the esters recovered.

Free and total sterol. These were measured by the modification of the Sperry & Webb method described by Cook & Rattray (1958). Mixing was carried out conveniently by a Vortex mixer.

Optical rotation. This was measured in CHCl_3 solution (concn. 1–2%) in a Hilger M412 polarimeter at $589\text{ m}\mu$.

RESULTS

Table 2 shows the values obtained for the extraction of the two parts of the glands. These findings agree with those of Brown, Knouff, Contin & Schneider (1937). In Scheme 1 the preliminary fractionation of the lipid from the adrenal cortex is shown. As a result of screening the fractions of columns 1 and 2 by thin-layer chromatography the following four combinations were made: (a) fractions containing the sterol esters; (b) fractions containing cholesterol and less polar sterols; (c) fractions containing cholesterol and cholestanol; (d) fractions containing cholesterol and 'companion' sterols.

Investigation of the sterol esters from bovine adrenal cortex. The sterol ester fractions isolated from columns 1 and 2 (Scheme 1) were further purified as

shown in Scheme 2. Fractions 4–13 from column 3 were shown to contain the sterol esters by thin-layer chromatography. A sample of the ester fraction was saponified and found to contain 51% of unsaponifiable material and 49% of fatty acids. The unsaponifiable material was analysed by thin-layer and gas-liquid chromatography. The major compound had R_f 1.00 and R_s 2.80 (i.e. cholesterol); a minor component (R_f 1.11 and R_s 3.29) was also present. This latter compound was methostenol. A 'fast-acting' colour with Liebermann-Burchard reagent confirmed the presence of the methyl sterol. The unsaponifiable material contained 1.3% (5.5 mg.) of methostenol, the remainder being cholesterol. The fatty acids were methylated and analysed by gas-liquid chromatography. Table 3 gives the acids present and their percentage composition.

Cholesterol and less polar sterols. The combined fractions 19–21 from column 1 contained 52.5 mg. of cholesterol and 4.6 mg. of 'fast-acting' sterol. Thin-layer chromatography suggested the presence of lanosterol and methostenol; gas-liquid chromatography showed a large peak at R_s 2.80 (cholesterol) and a small peak at R_s 3.29 (methostenol).

The fractions were chromatographed on a column (178 g.; 20 cm. $\times$ 4.0 cm.) of silicic acid-Celite (2:1), development being carried out by an increasing gradient of benzene in light petroleum (Kandutsch & Russell, 1959); 50 ml. fractions were collected, and screening of these by thin-layer chromatography showed that fractions 4–9 contained compounds with the same R_f values as lanosterol and methostenol. Further attempts to isolate lanosterol were unsuccessful, but visual comparison of the lanosterol spot in these combined fractions with that of a standard suggested about 1% (a total of about 250 $\mu\text{g.}$) was present. Fractions 10–15 were shown to contain compounds with the same R_f values as methostenol and cholesterol, and fractions 16–23 contained only cholesterol. These latter fractions were rechromatographed with other cholesterol-containing fractions in column 4 (see Scheme 4). Preliminary attempts to crystallize methostenol from combined fractions 4–9 and 10–15 were unsuccessful. The fractions were then divided into

Table 2. *Weights, lipid extracts and free and total sterol in bovine adrenal cortex and medulla (220 glands)*

	Weight (g.)		Percentage (w/w)			
			Cortex		Medulla	
	Cortex	Medulla	Fresh wt. basis	Lipid basis	Fresh wt. basis	Lipid basis
Fresh wt.	2184	868	—	—	—	—
Lipid extract	116	37	5.3	100	4.3	100
Total sterol	6.12	3.37	0.28	5.3	0.39	9.1
Esterified sterol	0.93	0.23	0.04	0.8	0.03	0.6

Scheme 1. Preliminary fractionation of lipid from adrenal cortex. The details of chromatographic fractions given are the eluting solvent, the volume of this solvent, the weight of the material and a description of this material. Fractions marked with an asterisk (*) were not studied further. LP, Light petroleum (b.p. 40-60°); Et_2O , diethyl ether.

Scheme 2. Purification of adrenal-cortical sterol esters. The details of chromatographic fractions given are the eluting solvent, the volume of this solvent, the weight of the material and a description of this material. Fractions marked with an asterisk (*) were not studied further. LP, Light petroleum (b.p. 40-60°); Et_2O , diethyl ether.

2.5 mg. portions and each portion was chromatographed on a thin-layer plate in a benzene-ethyl acetate (2:1, v/v) system. The 'methostenol' band was located by running a standard compound, and the band scraped off, eluted with solvent and the solvent evaporated, leaving an oily residue.

This oil showed only one component when chromatographed in three different thin-layer-chromatographic systems. The systems and R_c values were: benzene-ethyl acetate (2:1, v/v), R_c 1.11; chloroform-ethanol (19:1, v/v), R_c 1.03; light petroleum-diethyl ether (17:3, v/v), R_c 1.58. The same R_c values were obtained with a standard sample of methostenol in these systems. It was, therefore, concluded that the 'fast-acting' sterol in these fractions was methostenol (4 α -methylcholest-7-en-3 β -ol).

Fractions containing cholesterol and cholestanol. The treatment of these fractions is shown in Scheme 3.

Fractions containing cholesterol and 'companions'. These fractions were separated as shown in Scheme 4. Cholesterol was the major component. Fractions 14-19 from column 4 were shown to contain about 4 mg. of cholestanol [i.e. the compound

obtained after performic acid oxidation had R_c 0.94 in thin-layer chromatographic system benzene-ethyl acetate (2:1, v/v) and R_s 3.04 when analysed by gas-liquid chromatography]. Fractions 20-27 contained three compounds, the major one being cholesterol. Thin-layer chromatography showed a trace of a polar sterol (R_c 0.24) that gave an immediate colour with phosphomolybdic acid at room temperature. 7 α -Hydroxycholesterol gave the same colour on immediate spraying with phosphomolybdic acid and had the same R_c value. These fractions also contained a compound, with R_c 1.35, that gave a yellow colour with dinitrophenylhydrazine but no reaction with phosphomolybdic acid. This suggests that the compound is a keto sterol, without any hydroxyl function. Gas-liquid chromatography confirmed that the main component was cholesterol (peak at R_s 2.80) with a minor constituent at R_s 7.92. This latter peak was probably the keto sterol, as the diol was present only in trace amounts. Cholestenone has R_s 7.94, but is less polar than the suspected keto sterol by thin-layer chromatography.

These fractions did not absorb in the ultraviolet range 200-400 m μ , showing that there was no conjugated ketone present in the molecule. Visual estimation of the colour produced by the dinitrophenylhydrazine suggested there was about 600 μ g. of this compound in these fractions. Thin-layer chromatography showed that fractions 28-36 contained cholesterol and a more polar compound (R_c 0.55). This was similar to the R_c values of 25-hydroxy- and 26-hydroxy-cholesterol. Gas-liquid chromatography showed a peak at R_s 2.81 (cholesterol) and a peak at R_s 4.22. This latter peak was most probably due to the polar compound, but its R_s was greater than that of either 25-hydroxy- or 26-hydroxy-cholesterol. However, the thin-layer and gas-liquid chromatographic data suggest a stenediol. The Liebermann-Burchard reaction did not give a 'fast-acting' colour, showing that the diol did not have a Δ^7 double bond, or a hydroxyl group that could be dehydrated to give such a structure. The normal 'slow-acting' green colour produced suggests that the hydroxyl group is in the side chain. Visual estimation of the colour produced by phosphomolybdic acid suggests that there was about 1 mg. present in these fractions.

The sterols (and their approximate amounts) demonstrated to be present in the bovine adrenal cortex by this work are summarized in Table 4.

Adrenal medulla lipid. The lipid extract (37.0 g.) from the adrenal medullary tissue was fractionated and treated essentially as described for the cortical extract. Table 4 gives a summary of the 'companion' sterols demonstrated in this tissue and the approximate amount of each one. The fatty acids esterified to cholesterol are shown in Table 3.

Table 3. *Sterol ester fatty acids in bovine adrenal cortex and medulla*

The percentage distributions of the fatty acids in the sterol esters from bovine adrenal cortex, adrenal medulla and blood plasma are given; these values were obtained from gas-liquid chromatography on a polyethylene glycol adipate column. Fatty acids are designated $x:y$, where x is the no. of carbon atoms and y is the no. of double bonds. Trace, < 0.3 %.

Fatty acid	Sterol ester fatty acids		
	Cortex	Medulla	Blood plasma
6-10:0	Trace	Trace	Trace
12:0	0.3	Trace	Trace
13:1	Trace	Trace	Trace
14:0	2.5	4.0	1.0
14:1	1.2	0.7	0.5
14:2	1.2	1.1	1.5
14:3	0.5	Trace	0.3
15:0	0.9	0.9	1.0
15:1	1.3	Trace	Trace
15:3	4.0	—	1.0
16:0	20.0	19.0	11.4
16:1	4.0	5.5	3.7
16:3	1.3	—	Trace
17:0	1.0	1.3	1.3
17:1	0.9	—	0.8
18:0	12.8	9.4	3.5
18:1	21.1	14.8	6.6
18:2	21.7	28.2	50.0
18:3	7.3	6.3	3.9
18:4	—	—	0.8
19:1	1.0	1.3	0.7
20:0	Trace	—	3.9
20:1-3	Trace	—	6.7
20:4	Trace	4.4	Trace

DISCUSSION

The adrenal gland of the bovine (as with other ruminants), in contrast with those of most mammals, particularly man, has a lower ratio of cortex to medulla (3:1; cf. 10:1 in man). The bovine gland represents a 'non-fatty' type, the lipid content of the cortex and the medulla being about the same and relatively low in amount (about 5 % fresh wt.; cf. 17–20 % in man). The total sterol in each part is small (about 0.3 % fresh wt.) and about 10 % of this is esterified. Glick & Ochs (1955) report, however, that the bovine adrenal contains 2.5 % of total cholesterol, of which 50 % is esterified, and suggest that the stress during slaughter may be responsible for the low value of sterol generally reported and also that post-mortem hydrolysis decreases the esterified sterol; there has been no confirmation of these results.

The present analytical study can only suggest the origin of the sterol in the two parts of the gland. Because of the same general sterol composition and lack of intermediate sterols normally associated with cholesterol biosynthesis our results indicate that the cholesterol in the blood is the source of most of the cholesterol in both parts of the bovine adrenal gland. This agrees with the experimental findings of Werbin & Chaikoff (1961) for guinea-pig adrenal cholesterol and with those of Morris & Chaikoff (1959) for rat adrenal cholesterol. However, it is known that bovine adrenal tissue can synthesize cholesterol from acetate (Srere, Chaikoff & Dauben, 1948; Hechter, Solomon, Zaffaroni & Pincus, 1953), so that some adrenal cholesterol is likely to arise from synthesis.

The role of adrenal-gland cholesterol esters has been the subject of speculation. Comparative studies of cholesterol ester fatty acids have been

Scheme 3. Fractionation of adrenal-cortical fractions containing cholesterol and cholestanol. The details of the chromatographic fractions given are the eluting solvent, the volume of this solvent, the weight of the material and a description of this material. Fractions marked with an asterisk (*) were not studied further. Total recoveries were: cholesterol, 398 mg.; cholestanol, about 600 µg.

made by Dailey, Swell, Field & Treadwell (1960) in several species with fatty-type glands, and Riley (1963) has studied the cholesterol ester fatty acids of human adrenal glands. Our results show that cholesterol oleate and linoleate are present in both parts of the bovine gland in approximately equally high amounts; cholesterol linoleate is the major sterol ester of the blood. This suggests a mainly plasma origin for the adrenal sterol esters, which agrees with the results of Longcope & Williams (1963). They found negligible cholesterol-esterase activity in bovine adrenal homogenates. Recent results indicate that cholesterol linoleate is a metabolically important source of the corticosteroids. Dailey, Swell & Treadwell (1963) found that cholesterol linoleate was converted into corticosteroids in canine adrenal homogenates twice as efficiently as were esters of saturated fatty acids, and Riley (1963) has noted a decrease in human adrenal cholesterol linoleate after stimulation with adreno-

corticotrophic hormone, suggesting that this ester is preferentially used for corticosteroid biosynthesis.

The lengthy analytical procedures necessary in the present study demonstrate the difficulties of isolating small amounts of 'companion' sterols, and our results are of interest in showing which sterols are not present (or not demonstrable) in the normal adrenal gland. Similar techniques have demonstrated the presence of at least four minor sterols in addition to cholesterol in bovine corpora lutea (D. I. Cargill & C. H. L. Shackleton, unpublished work). There is little information on possible precursors of the corticosteroids, other than cholesterol, which appears to be the main adrenal sterol in all species (Dorfman, 1962). Table 4 shows the C_{27} sterols that have been demonstrated in the adrenal tissue. Our results do not throw light on the pathway of corticosteroidogenesis from cholesterol. Although 'fatty' and 'non-fatty' glands differ histologically in the number of zones that can

Scheme 4. Fractionation of adrenal-cortical fractions containing cholesterol and 'companion' sterols. The details of the chromatographic fractions given are the eluting solvent, the volume of this solvent, the weight of the material and a description of this material. Fractions marked with an asterisk (*) were not studied further. LP, Light petroleum (b.p. 40-60°); Et₂O, diethyl ether. Total recoveries were: cholesterol, 5253 mg.; cholestanol, about 4 mg.; possible keto sterol, about 600 µg.; possible stenediol, about 1 mg.

Table 4. *Sterols of the adrenal gland*

Sterol	Wt. (per 100 g. fresh wt.)		Remarks
	Cortex	Medulla	
Cholesterol	286 mg.	415 mg.	—
Esterified	44 mg.	27 mg.	—
Cholestanol	230 µg.	231 µg.	Guinea-pig adrenal contains 600 mg. of cholestanol/100 g. (Werbin & Chaikoff, 1962)
Esterified	None	None	
Methostenol	460 µg.	36 µg.	This 'fast-acting' sterol is probably the same as the SeO ₂ -positive compound described by Nakanishi, Bhattacharyya & Fieser (1953)
Esterified	252 µg.	Trace	
Lanosterol	12 µg.	392 µg.	—
Esterified	Trace	Trace	—
7α-Hydroxycholesterol	Trace	Trace	—
7β-Hydroxycholesterol	Trace	Trace	—
'Stenediol'	46 µg.	Trace (esterified)	The relation of this 'stenediol' to these polar sterols, which were detected (in small amounts) on incubation of adrenal tissue, is uncertain (see Solomon, Levitan & Lieberman, 1956; Shimizu, Hayano, Gut & Dorfman, 1961; Shimizu, Gut & Dorfman, 1962)
Cholest-5-ene-3β,20α-diol	—	—	
Cholest-5-ene-3β,20α,22x-triol	—	—	—
'Keto sterol'	27 µg.	—	—
Cholestane-3,6-dione	—	—	Found in human adrenal adenoma (Keller, 1956)

be seen in the normal cortex (Symington, 1962), it seems that the pathways of corticosteroid biosynthesis are the same in all glands. However, the high amount of cholestanol (16 % of the adrenal 'cholesterol'; Werbin & Chaikoff, 1962) in guinea-pig adrenal glands suggests that there may be species differences in the metabolism of C₂₇ sterols.

The significance of medullary cholesterol is difficult to assess. Rosenfeld (1955) has shown that, though stimulation with adrenocorticotrophic hormone lowers the adrenal-cortical cholesterol concentration with concomitant increase in corticosteroid secretion, there is no change in the concentration of the medullary cholesterol. There seems therefore to be no interchange between cholesterol in the cortex and that in the medulla.

SUMMARY

1. Extracts (diethyl ether-ethanol) of bovine adrenal cortex and medulla were made and fractionated on Florisil to give free and esterified sterol fractions.

2. The minor sterols were concentrated by crystallization from boiling acetic acid and investigated by thin-layer and gas-liquid chromatography.

3. The cholesterol ester fatty acids of the cortex and medulla were studied. The major fatty acids in the cortex are (values as percentages of the total): linoleic acid, 21.7; oleic acid, 21.1; palmitic acid, 20. The same major acids appear in the medulla, their

respective percentage weights being 28.2, 14.8 and 19.

4. The cortex contains (mg./100 g. fresh wt.): cholesterol, 286, of which 15.4 % is esterified; cholestanol, 0.230; methostenol, 0.460, of which 55 % is esterified; and trace amounts of other 'companion' sterols.

5. The medulla contains (mg./100 g. fresh wt.): cholesterol, 415, of which 6.5 % is esterified; cholestanol, 0.231; lanosterol, 0.392; and trace quantities of other sterol 'companions'.

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Synthesis of 3-Hydroxy-19-nor[21-¹⁴C]pregna-1,3,5(10)-trien-20-one from [¹⁴C]Methyl Iodide

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It is widely accepted that C₁₈ oestrogens are formed *in vivo* from C₁₉ steroids by steps that involve the aromatization of the A ring of the cyclopentenophenanthrene nucleus and the loss of the C-19 angular methyl group (Breuer, 1962). Corresponding changes in the pregnane series (C₂₁) would lead to the formation of C₂₀ steroids having the aromatic A-ring structure of the oestrogen series and the D-ring side-chain structures of progesterone and its derivatives. No compounds of this type have yet been found in mammalian sources.

The conversion of androgens (C₁₉) into oestrogens (C₁₈) *in vitro* in high yield has been reported with a system consisting of human placental microsomes, NADPH and oxygen (Ryan, 1959; Gual, Morato, Hayano, Gut & Dorfman, 1962). No phenolic products were detected by these workers with this system with either progesterone or 17 α -hydroxyprogesterone (C₂₁) as substrate. Nevertheless, if aromatization of the A ring of progesterone does

occur *in vivo*, it is reasonable to suppose that the compound 3-hydroxy-19-norpregna-1,3,5(10)-trien-20-one would be formed.

This C₂₀ steroid, a structural hybrid between oestrone and progesterone, has now been synthesized from oestrone by two independent routes. Although gram quantities have been prepared by following the route of Mills, Ringold & Djerassi (1958), this method did not lend itself to the introduction of ¹⁴C, and a second synthesis based on the formation of a cyanohydrin at C-17 (Ruggieri & Ferrari, 1959) has been devised. By this method 3-hydroxy-19-nor[21-¹⁴C]pregna-1,3,5(10)-trien-20-one has been prepared for metabolic studies. A preliminary account of this work has been presented (Schulster, Whitehead & Kellie, 1964).

MATERIALS AND METHODS

Ethanol, R.R. grade (James Burrough Ltd.), was used as supplied; all other solvents were distilled before use. Ether and benzene were dried over sodium. Acetone cyanohydrin (British Drug Houses Ltd.) was freshly distilled im-

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