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NATURALLY OCCURRING LIPOIDAL DERIVATIVES OF 3β-HYDROXY-5-PREGNEN-20-ONE; 3β,17α-DIHYDROXY-5-PREGNEN-20-ONE AND 3β-HYDROXY-5-ANDROSTEN-17-ONE

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SUMMARY

Three steroids have been found to be present in extracts of bovine adrenals as non-polar lipoidal derivatives. The three steroids are: 3β-hydroxy-5-pregnen-20-one, 3β,17α-dihydroxy-5-pregnen-20-one and 3β-hydroxy-5-androsten-17-one. The exact nature of the lipoidal derivatives are as yet unknown but they are less polar than the free compounds and they are converted to the above mentioned steroids by treatment with alkali. Chromatographic analysis on florisil of the non-polar extract containing the lipoidal derivatives resulted in the detection of at least 3 different classes of lipoidal derivatives of 3β-hydroxy-5-pregnen-20-one. Only one of these classes, the most polar, co-chromatographs with that class biosynthesized *in vitro* from radioactive 3β-hydroxy-5-pregnen-20-one.

INTRODUCTION

In a previous paper [1] evidence was presented that indicated that pregnenolone occurs in bovine adrenal tissue in three forms: as the free steroid, as its sulfate and as a previously unrecognized non-polar derivative. Because this latter derivative is much less polar than the parent steroid, it was dubbed the lipoidal derivative of pregnenolone. Treatment with alkali or acid liberates the free steroid from the nonpolar form. In a later presentation [2], we reported that incubation of [^{3}H]-pregnenolone with a homogenate of cortical tissue from bovine adrenals or of rat adrenals results in the conversion of the substrate into a more nonpolar form which resembles the isolated endogenous lipoidal derivative. Chromatographic analysis of the biosynthetically prepared material resolved it into at least two components, each less polar than pregnenolone and each convertible into pregnenolone by alkali. When [^{3}H]-dehydroisoandrosterone was incubated with similar preparations of adrenals, it, too, was converted into two lipoidal derivatives.

This paper describes our continuing efforts in this area. Elaborate isolation procedures, particularly chromatographic techniques, have resulted in the detection in adrenal extracts of lipoidal derivatives of two other steroids, 17-hydroxypregnenolone and dehydroisoandrosterone. The identity of both steroids was established by their chromatographic behavior and by a precise double isotope technique. Dehydroisoandrosterone was also identified by its mass spectrum. While an acceptable mass spectrum was not obtained for 17-hydroxypregnenolone, the data from the analysis made by the double isotope technique from two independent experiments were excellent and clearly established the presence of this steroid as a lipoidal derivative in adrenal tissue.

In addition, chromatographic analysis of extracts of bovine adrenals revealed that at least three different lipoidal derivatives of pregnenolone occur in this tissue. These differ from each other in chromatographic properties but are alike in that each is less polar than the parent steroid and each is converted into pregnenolone by treatment with alkali.

EXPERIMENTAL

Radioactive tracers were purchased from New England Nuclear Corp. [7α-^{3}H]-pregnenolone, [7α-^{3}H]-dehydroisoandrosterone and [7α-^{3}H]-17-hydroxypregnenolone were purified before use by celite partition chromatography as described by Siiteri[3]. High pressure liquid chromatography was carried out

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Abbreviations. Pregnenolone, 3β-hydroxy-5-pregnen-20-one; 17-Hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; Dehydroisoandrosterone, Dehydroepiandrosterone-3β-hydroxy-5-androsten-17-one.

on a Waters ALC-100 liquid chromatograph (Waters Associates, New Milford, MA). Radioactive samples were counted in Packard liquid scintillation spectrometers Models 3255 and 3375. The efficiency of counting for ^{3}H was 51% and for ^{14}C, 88%. When ^{3}H and ^{14}C were counted together, the efficiency for ^{3}H was 42% with negligible overlap into the ^{14}C channel. For ^{14}C, the efficiency was 65% with a 15% overlap into the ^{3}H channel. Corrections were made according to the method of Okita *et al.*[4]. The tritium content of every radioactive sample was at least 10, and sometimes as much as 30 times the background count. Gas chromatography–mass spectrometery (GC–MS) was performed on a Perkin Elmer 3920 gas chromatograph interfaced with a DuPont 21-492B mass spectrometer equipped with a Hewlett Packard 21-094 data acquisition system. Slylon BTZ (a mixture of *N,O*-bis (trimethysilyl) acetamide, tri-methylchlorosilane and trimethylsilylimidazole in 3:2:3, V/V ratio) used for the silylation of steroids was purchased from Supelco, Inc., Bellefonte, PA.

An outline of the procedures used for the extraction and isolation of the free and lipoidal fractions are shown in Fig. 1. Bovine adrenals glands, 210 g, were brought to the laboratory from the slaughterhouse on ice. As soon as possible, each gland was defatted, demedullated and frozen. When the complete batch was processed, the frozen sample was immersed in 700 ml of methanol. After the tissue thawed, 350 ml of chloroform and 200 ml of water were added and the mixture was thoroughly homogenized in a Waring blender. The homogenate was filtered through a Buchner funnel. The solids were resuspended in 1 l. of a methanol–chloroform (1:1, V/V) mixture and the suspension stirred mechanically for 3 h. The insoluble material was again separated by filtration after which it was resuspended in 700 ml of a methanol–chloroform (1:1, V/V) mixture. After the suspension was stirred overnight, the solids were collected by filtration and were then again mascerated in 350 ml of chloroform. After filtration the precipitate was discarded. The filtrates from all of the above were combined and 600 ml of water was added. The resulting mixture was

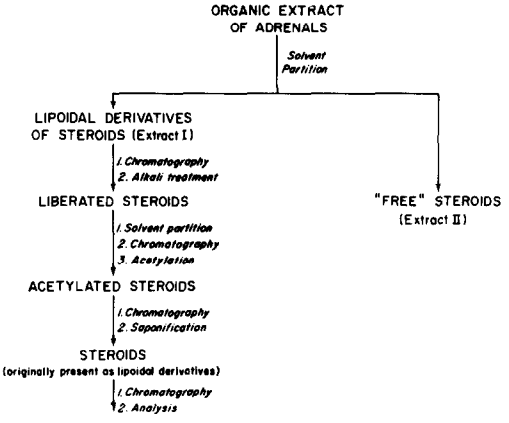

Fig. 1. Outline of the procedures used to isolate lipoidal derivatives of steroids. Extract II was processed in a manner similar to that used for the "liberated steroids". Analysis was carried out by (a) mass spectrometry and (b) double isotope technique.

vigorously shaken in a separatory funnel. When the two phases had separated, the bottom, organic layer was removed and evaporated to dryness, leaving a waxy residue weighing 13.5 g (Extract I). The aqueous fraction was discarded. The following internal standards were added: [^{3}H]-pregnenolone, 1.86×10^{6} c.p.m.; [^{3}H]-17-hydroxypregnenolone, 1.8×10^{6} c.p.m.; and [^{3}H]-dehydroisoandrosterone, 1.63×10^{6} c.p.m. In order to separate the non-polar, lipoidal steroids from the free unconjugated steroids and from the polar conjugates (sulfates), the residue was partitioned between 1 l. of isooctane and 1 l. of a mixture of methanol-water (9:1, V/V). The aqueous methanol extract, *Extract II*, contained virtually all the radioactivity, 5.3×10^{6} c.p.m. whereas the isooctane extract, *Extract I*, was practically devoid of radioactivity.

Lipoidal derivatives. The isooctane fraction was evaporated to dryness, leaving a waxy residue weighing 10 g. This non-polar material was partitioned by chromatography on a 500 g celite column (60 × 750 mm) in System 1 (Table 1). In this system, pregnenolone is eluted in the 5th and 6th holdback

Table 1. Chromatographic systems

Celite

1. Isooctane–Methanol–H_2O (10:9:1, by vol.)
2. Methanol–*n*-Propanol–H_2O–Toluene-Isooctane (4:0.75:2:2:2, by vol.)
3. Benzene–Isooctane–Methanol–H_2O (7:13:7:3, by vol.)
4. Methanol–*n*–Propanol–H_2O–Toluene–Isooctane (4:0.5:2:2:2, by vol.)
5. Benzene–Isooctane–Methanol–H_2O (1:4:2:0.5, by vol.)
6. Benzene–Isooctane–Methanol–H_2O (4:6:3.5:1.5, by vol.)

LH-20 Sephadex

L-1. Isooctane–Methanol–Chloroform (6:1:1, by vol.)
L-2. Isooctane–Benzene–Methanol–*t*-Butanol (9:9:1:1, by vol.)
L-3. Isooctane–Benzene–Ethylacetate–Methanol–*t*-Butanol (4:4:0.5:1:0.5, by vol.)

C_{18}-Corasil (high pressure liquid chromatography)

C-1. Acetonitrile–H_2O (45:55, V/V)
C-2. Acetonitrile–H_2O (30:70, V/V)
C-3. Acetonitrile–H_2O (27:73, V/V)

volumes. This is well after the elution volume for the non-polar materials which are eluted in the first three holdback volumes. The early fractions were combined and further purified by chromatography on 100 g of celite in the reverse phase System 2. In this system, free steroids are eluted at the beginning of the chromatogram, therefore the first two holdback volumes were discarded. The column was washed with benzene to elute compounds less polar than the free steroids. This eluate was found to be devoid of radioactivity. For a second time, internal standards of 1×10^6 c.p.m. of [^{3}H]-pregnenolone, 9×10^5 c.p.m. of [^{3}H]-17-hydroxypregnenolone and 9×10^5 c.p.m. of [^{3}H]-dehydroisoandrosterone were added. The residue was dissolved in 40 ml of benzene and 200 ml of ethanol containing 4 g of KOH were added. The solution was kept at room temperature for 24 h, after which 100 ml of water and enough 6 N acetic acid to bring the mixture to neutrality were added. The ethanol was removed under vacuum and the aqueous residue, approximately 80 ml, was diluted with 720 ml of methanol. The suspension was extracted with 800 ml of isooctane. The isooctane extract was back extracted with 800 ml of a methanol–water (9:1, V/V) mixture. The isooctane extracts were discarded. The combined aqueous methanolic extracts, which contained the steroids liberated from the lipoidal derivatives, were evaporated to dryness *in vacuo*. The residue, weighing 4 g and containing 2.8×10^6 c.p.m. of [^{3}H], was suspended in 200 ml of CH_2Cl_2. A portion remained insoluble and it was removed by filtration. It weighed 2 g, contained no radioactivity and was discarded. The CH_2Cl_2 extract was chromatographed on a silica gel column (Silic AR-Mallinckrodt) (48 × 310 mm) using the following eluents: 3 l. of CH_2Cl_2; 1.5 l. of CH_2Cl_2 containing 1% ethanol and 3 l. of CH_2Cl_2 containing 3% ethanol. All the radioactivity was found in those fractions eluted with the last solvent mixture. These fractions were combined and evaporated to dryness. The residue weighed 43 mg and contained 2.6×10^6 c.p.m. of [^{3}H]. It was further purified by chromatography on a 150 g celite column in System 3. From this process, three fractions were obtained: in hbv 1, pregnenolone, 8×10^5 c.p.m.; in hbv 2, dehydroisoandrosterone, 7×10^5 c.p.m., and hbv 5,17-hydroxypregnenolone, 7×10^5 c.p.m.

Each of the three fractions was acetylated by treatment with 0.5 ml of acetic anhydride and 1 ml of pyridine at room temperature overnight. To each reaction mixture was added, 0.5 ml of H_2O and 50 ml of toluene. The solvents were evaporated *in vacuo* (to remove the excess reagents) and then 80 ml of isooctane was added. Evaporation of this solvent removed the last traces of the reagents; a dry, odorless residue remained. The resulting steroidal acetates were chromatographed on 30 g celite columns. Pregnenolone acetate was isolated using reverse phase System 2 where it is eluted in the 2nd–4th hbv; dehydroisoandrosterone acetate was obtained using reverse phase System 4 where it is eluted in the 3rd–4th hbv and 17-hydroxypregnenolone acetate was obtained using System 1 where it is eluted in the 3rd–4th hbv. The fractions containing the radioactive acetates were combined and evaporated to dryness. The amount of ^{3}H in each was: pregnenolone acetate, 5×10^5 c.p.m.; dehydroisoandrosterone acetate, 6×10^5 c.p.m.; and 17-hydroxypregnenolone, 5×10^5 c.p.m.

Each sample was saponified at room temperature overnight with 40 ml of methanol containing 10 ml of a saturated aqueous solution of Na_2CO_3. The next day the alkaline solutions were neutralized with acetic acid and then extracted with 500 ml of ether. The organic extracts were washed with water until neutral and dried over Na_2SO_4. After evaporation of the ether, the pregnenolone fraction was purified by chromatography, first on celite in System 1, then on a LH-20 Sephadex column (24 × 380 mm) with System L-1 where it is eluted in the 15–19th fractions (10 ml fractions were collected). Finally, it was chromatographed on a high pressure liquid chromatograph on a C_{18}-Corasil micro column in System C-1 at a flow rate of 1 ml/min. In this system pregnenolone is eluted in 9–11 ml. Those fractions containing [^{3}H]-pregnenolone were combined and evaporated to dryness leaving a residue having 2×10^5 c.p.m. of tritium (20% recovery) (Sample Ia).

The radioactive dehydroisoandrosterone fraction, after saponification, was chromatographed on a 60 g celite column in System 5 where dehydroisoandrosterone is eluted in the 3rd–4th hbv. The fractions containing the tritiated tracer were combined, the solvents removed and the residue rechromatographed on a column of Sephadex LH-20 (24 × 380 mm) in System L-2 where dehydroisoandrosterone is eluted in the 12–16th fraction (10 ml fractions were collected). The material in these fractions was chromatographed on a high pressure liquid chromatograph on C_{18}-Corasil in System C-2 at 1 ml/min. In this system dehydroisoandrosterone is eluted in 13–16 ml. Evaporation of those fractions containing the radioactive material provided a residue having 3.9×10^5 c.p.m. of tritium (43% recovery) (Sample Ib).

The 17-hydroxypregnenolone sample, after saponification, was chromatographed first on celite in System 6 where the steroid is eluted in the 4th hbv, then on an LH-20 Sephadex column (24 × 380 mm) in System L-3 where it is eluted in the 18–24th fraction (10 ml fractions were collected) and finally on a high pressure liquid chromatograph on C_{18}-Corasil in System C-3 at 1 ml/min. Under these conditions, 17-hydroxypregnenolone is eluted in 17–21 ml. The residue from these fractions contained 3.2×10^5 c.p.m. of [^{3}H] (35.6% recovery) (Sample Ic).

Free steroids. The methanol–H_2O (9:1, V/V) extract from the adrenal gland, *Extract II*, containing 5.3×10^6 c.p.m. of the tritium-labeled internal standards, was evaporated to dryness under vacuum leaving a residue weighing 4 g. This was dissolved in 30 ml of CH_2Cl_2 and the solution applied to a silica gel column (42 × 320 mm) prepared in the same solvent.

The column was developed with 1.5 l. of each of the following solvent mixtures: CH_2Cl_2, CH_2Cl_2–ethanol (99:1, V/V), CH_2Cl_2–ethanol (97:3, V/V) and CH_2Cl_2–ethanol (96:4, V/V). Then the concentration of ethanol was increased to 20% and finally the column was washed with pure ethanol. All the eluted radioactivity, 3.8×10^6 c.p.m., was found in the fractions obtained with CH_2Cl_2 containing 3% and 4% ethanol. The fractions containing the radioactive tracers were combined, evaporated to dryness and the residue acetylated in pyridine and acetic anhydride as before. The next day the reaction mixture was worked up as described above. The acetylated residue was chromatographed on a 150 g celite column in reverse phase system 4 where 17-hydroxypregnenolone acetate emerges with the solvent front, dehydroisoandrosterone acetate is eluted in the 2nd–4th hbv and pregnenolone acetate is found in the 5–7th hbv. The fractions containing the radioactive steroidal acetates were combined appropriately and each sample was saponified in 50 ml of 2% ethanolic KOH. After standing overnight at room temperature, each alkaline solution was neutralized with 6 N acetic acid and diluted with 10 volumes of water. The aqueous suspensions were each extracted twice with 1 l. portions of ether. The ether extracts were washed with H_2O until neutral, dried over Na_2SO_4 and evaporated to dryness. The three residues were then purified exactly as described above for the steroids obtained from the lipoidal fraction: chromatography on celite; then on LH-20 and finally by high pressure liquid chromatography on C_{18}-Corasil. The tritium recovered as pregnenolone was 8.5×10^5 c.p.m. (46% recovery), (Sample IIa), as dehydroisoandrosterone, 3.9×10^5 (24% recovery), (Sample IIb), and as 17-hydroxypregnenolone, 8.5×10^5 c.p.m. (47% recovery), (Sample IIc).

Mass spectral analysis. Aliquots of each of the steroidal samples, Ia, Ib, Ic, IIa, IIb and IIc were worked up as follows: Each was transferred to a small conical glass tube (0.7 × 3.0 cm), the solvent was removed under a stream of nitrogen at 40° and 3 μl of silylating reagent (pyridine: Sylon BTZ, 3:1, V/V) was added to the dried residue. The small conical tube was immediately enclosed in a vial (1.4 × 4.5 cm) equipped with a Teflon-lined screw cap and containing 50 μl of the silylating reagent mixture. After heating for 20 min at 80°C, the entire sample was removed from the conical tube and injected into the gas chromatograph interfaced with the mass spectrometer. The effluent line of the gas chromatograph was connected to a splitter with permitted one third of the sample to go to a flame ionization detector and two thirds to the source of the mass spectrometer. The all glass chromatograph system consisted of a 6 ft. column packed with 3% SP-2100 on 100/120 Supelcoport (Supelco, Bellefonte, PA), a flash injector and a heated glass line leading to the mass spectrometer through a jet separator. The carrier gas was helium flowing at 35 ml/min. Temperature conditions were as follows: Injector, 260°C; column, isothermal at 220°C for TMS derivatives of pregnenolone or dehydroisoandrosterone and isothermal at 240°C for the bis-TMS derivative of 17-hydroxypregnenolone; detector, 290°C; GC line and jet separator, 220°C, and MS source temperature, 210°C. The electron ionization voltage was 75 eV. Under these conditions, the authentic substances, the TMS derivative of dehydroisoandrosterone, the TMS derivative of pregnenolone and the bis-TMS derivative of 17-hydroxypregnenolone, gave retention times of 20.6, 34 and 23 min, respectively.

The TMS derivative of Fraction Ia emerged from the GC at 34 min. Its mass spectrum was identical to that of the authentic TMS derivative of pregnenolone. The major ion fragments displayed in this spectrum were *m/e* 388 (13%, M^+), *m/e* 373 (4%, M-15), *m/e* 298 (22%, M-90), *m/e* 283 (11%, M-(90 + 15)), *m/e* 259 (15%, M-129), *m/e* 255 (4%, M-(90 + 43)), *m/e* 241 (14%, M-(90 + 57)), *m/e* 129 (90%, A-ring fragment) and *m/e* 43 (100%, base peak).

The TMS derivative of Fraction Ib emerged from the GC at a retention time of 20.6 min. Its mass spectrum was identical to that of the authentic TMS derivative of dehydroisoandrosterone. The spectrum displayed major ions at: *m/e* 360 (13%, M^+), *m/e* 345 (2%, M-15), *m/e* 304 (20%, M-56), *m/e* 270 (10%, M-90), *m/e* 255 (4%, M-(90 + 15)), *m/e* 231 (16%, M-129) and *m/e* 129 (100%, base peak).

The TMS derivative of Fraction Ic was subjected to GC–MS analysis. No material emerged from the chromatogram at the time characteristic for the 17-hydroxypregnenolone derivative nor was the mass spectrum of the suspected compound obtained. The number of ions detected was so small that it seems likely that the sample size was inadequate for the analysis.

The TMS derivatives of Fractions IIa, IIb and IIc were also subjected to GC–MS analysis. The retention times and the mass spectra confirmed the identity of IIa as pregnenolone, IIb as dehydroisoandrosterone and IIc as 17-hydroxypregnenolone.

Quantification with [^{14}C]-acetic anhydride. Aliquots of each of the steroidal samples, Ia, Ib, Ic, IIa, IIb and IIc, were acetylated with [1-^{14}C]-acetic anhydride (specific activity 1 mCi/10.2 mg) as previously described [1]. In addition, a standard consisting of [^{3}H]-pregnenolone of known specific activity was also acetylated with an aliquot of the same batch of [1-^{14}C]-acetic anhydride in order to confirm its specific activity. The reaction mixtures were kept at room temperature for 48 h and then each was dissolved in 100 ml of ether. The ether solutions were washed four times with 50 ml of 1 N NaOH and then with H_2O until neutral. The ethereal extracts were dried over sodium sulfate and evaporated to dryness. Each residue was purified by chromatography on celite: pregnenolone acetate in System 2; 17-hydroxypregnenolone acetate in System 1; and dehydroisoandrosterone acetate in System 2. Those fractions of the

Table 2. Crystallization data ($^3H/^{14}C$ ratios) on steroids isolated from bovine adrenals

Steroids	X_1	ML_1	X_2	ML_2	X_3	ML_3	X_4	ML_4	X_5	ML_5
As *Lipoidal* derivatives										
Pregnenolone (Ia)	1.05	0.86	1.07	0.91	1.08	1.09	1.08	1.09		
Dehydroisoandrosterone (Ib)	14.1	3.35	16.3	10.0	16.1	14.2	16.5	16.3	16.4	15.0
17-Hydroxypregnenolone (Ic)	18.0	0.44	37.4	12.1	41.3	17.6	42.0	34.7	41	43.0
As *Free* steroids										
Pregnenolone (IIa)	0.85	0.83	0.87	0.97	0.82	0.87	0.83	0.84		
Dehydroisoandrosterone (IIb)	12.5	3.13	11.1	6.8	11.3	10.1	11.0	12.0		
17-Hydroxypregnenolone (IIc)	5.43	2.79	6.5	4.0	7.0	5.2	7.0	6.7		

X_n is the crystalline product obtained from the n^{th} crystallization. ML_n is the residue remaining on the mother liquor from the n^{th} crystallization. The following solvents were used for the crystallizations. X_1, methanol; X_2, acetone–hexane (for 17-hydroxypregnenolone acetate, chloroform–hexane was used); X_3, ethyl acetate–petroleum ether; X_4, chloroform–hexane; X_5, ethanol–hexane.

chromatogram containing the tritiated steroidal acetates were appropriately combined and the solvents removed by distillation. To each sample, 80 to 100 mg of the appropriate carrier steroidal acetate was added. The samples were then recrystallized four or five times, each time from a different solvent, (legend to Table 2), and the specific activities with respect to ^3H and ^{14}C as well as the $^3H/^{14}C$ ratios were determined for both the crystalline products and for the residues in each mother liquor. The steroids from both the free and lipoidal fractions were all recrystallized to constant $^3H/^{14}C$ ratios (Table 2). In this way the occurrence of the three steroids in both fractions, as free and as lipoidal derivatives, was established.

From the $^3H/^{14}C$ ratios, corrected for the amount of tritiated internal standard added, and the specific activity of the ^{14}C-acetic anhydride, it was possible to calculate the amount of each steroid originally present in the adrenal gland [1]. The data in Table 3 show the results of these estimations.

Detection of several lipoidal derivatives of pregnenolone. Intact frozen bovine adrenal glands (433 g) were dried by lyophilization. The dessicated glands (133 g) were homogenized with 1.5 l. of isooctane in a Waring blender. The resulting homogenate was then mechanically stirred for 24 h. The solids were removed by filtration and discarded. The filtrate was evaporated under vacuum yielding 52 g of a yellow oil which solidified at room temperature. To this fraction was added 22,500 c.p.m. of a tritium-labeled tracer of a lipoidal derivative of pregnenolone, which had been prepared by incubating [^3H]-pregnenolone with a homogenate of 200 mg of cortical tissue from bovine adrenal glands [2]. The tritiated lipoidal derivative was separated from the substrate and other steroids by chromatography on silica gel. It was eluted from the column by a 4:1, V/V mixture of benzene–isooctane. The lipoidal derivative prepared biosynthetically has characteristics similar to those of the endogenous derivative in that it is less polar than pregnenolone (as indicated by its solvent distribution and chromatographic properties) and is converted into pregnenolone by alkali treatment.

The adrenal extract, 52 g, now labeled with a tritium-labeled lipoidal derivative of pregnenolone, was chromatographed on a 600 g column (5.4 × 48 cm) of Florisil (60–100 mesh). The column was developed first with pure isooctane and then with increasing amounts of benzene in isooctane. This was followed by pure benzene and then by mixtures of ethyl acetate and benzene. The sequence of elution is shown in Fig. 2. Each fraction was checked for radioactivity. The labeled biosynthetic lipoidal derivative of [^3H]-pregnenolone was found in Fraction 8B which was eluted with a mixture of ethyl acetate in benzene (2:98, V/V). Cholesterol was detected by thin layer chromatographic analysis in Fractions 6A, 6B and 7A. Those fractions were eluted with benzene–isooctane (4:1, V/V) and pure benzene.

Each fraction of the chromatogram was also analyzed by radioimmunoassay for both free pregnenolone and its lipoidal derivative. To a 1% aliquot of each fraction was added 200 c.p.m. of [^3H]-pregnenolone. Each fraction was then chromatographed on a silica gel (Woelm) column (8 × 100 mm) prepared in benzene. The column was eluted first with 50 ml of benzene and then with 50 ml of benzene containing 20% ethyl acetate. The benzene eluent contained the lipoidal derivatives. The mixture of benzene and ethyl acetate eluted the labeled free pregnenolone.

An internal standard of 200 c.p.m. of [^3H]-pregnenolone was added to the benzene eluates. These were then evaporated to dryness and each residue was dissolved in 1 ml of benzene, 1.6 ml of methanol and

Table 3. Steroidal content (μg/kg) of bovine adrenals

	As free steroid	As lipoidal derivatives	Lipoidal/Free
Pregnenolone	624	275	0.41
Dehydroisoandrosterone	42	14	0.33
17-Hydroxypregnenolone	68	7	0.10

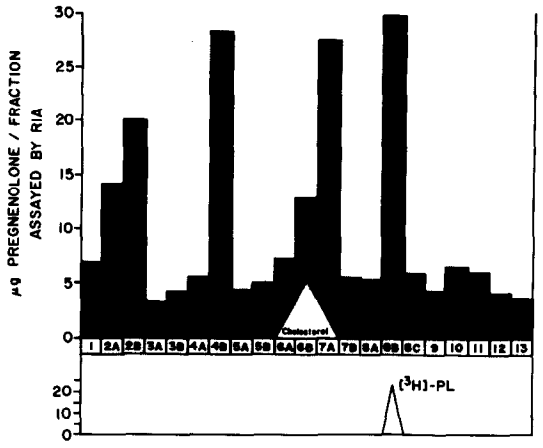

Fig. 2. Profile of chromatographic analysis of adrenal extract on florisil. Pregnenolone (after hydrolysis of lipoidal derivatives) was estimated by radioimmunoassay. The solvents used were: isooctane (i), benzene (b) and ethyl acetate (e). The following code indicates, in order: the fraction number, the eluting solvent or solvent mixture, the volume of eluent and the weight of the eluted material.

1—i. 3 l., 5.5 g; 2A—i:b (8:2), 3 l., 12.4 g; 2B—i:b (8:2), 3 l., 4.4 g; 3A—i:b (6:4), 3 l., 5.4 g; 3B—i:b (6:4), 3 l., 3.1 g; 4A—i:b (5:5), 3 l., 4.9 g; 4B—i:b (5:5), 3 l., 3 g; 5A—i:b (4:6), 3 l., 1.6 g; 5B—i:b (4:6), 3 l., 2.2 g; 6A—i:b (2:8), 2.5 l., 1.2 g; 6B—i:b (2;8), 2.5 l., 1.3 g; 7A—b, 2.5 l., 1 g; 7B—b, 2.5 l., 0.2 g; 8A—b:e (98:2), 1.5 l., 0.2 g; 8B—b:e (98:2), 2 l., 1.3 g; 8C—b:e (98:2), 1 l., 0.6 g; 9—b:e (95:5), 2 l., 0.7 g; 10—b:e (9:1), 2 l., 0.1 g; 11—b:e (8:2), 2 l., 0.1 g; 12—b:e (6:4), 2 l., 0.1 g; 13—e, 2 l., 0.1 g.

The tracing at the bottom of the figure indicates the elution pattern of a lipoidal derivative of tritiated pregnenolone prepared biosynthetically. The units on the ordinate are c.p.m. of tritium $\times 10^{-3}$ per fraction.

0.4 ml aqueous 1 N NaOH. The solutions were heated at 90° for 1 h, then cooled and neutralized with 5 N HCl. The organic solvents were stripped, 1 ml of water was added to each residue and the resulting suspensions extracted with 10 ml of ether. The ether extracts were washed three times with 5 ml of water, dried over Na_2SO_4 and evaporated to dryness. The residues were chromatographed on silica gel columns as described above. The sample of pregnenolone liberated from its lipoidal derivative by the alkali treatment as well as that added as a [^{3}H]-marker was eluted in the benzene:ethyl acetate (4:1, V/V) fraction. Radioactivity present in aliquots of appropriate fractions were used to estimate procedural losses. Aliquots of the remainder of each fraction was then analyzed quantitatively for pregnenolone by radioimmunoassay. The antibody employed was preprred in rabbits using pregnenolone succinate conjugated to bovine serum albumin as hapten. The RIA technique was essentially the same as that described by Nishina *et al.*[5]. With this assay, 200–300 pg can be easily measured. The results are shown in Fig. 2. If present, in these non polar extracts, free pregnenolone would have been found in fractions 11–13 of the chromatogram. Assay by RIA showed that at least four different areas of the chromatogram contained lipoidal derivatives of pregnenolone, Fractions 1–2B, 4B, 6B–7A and 8B. Only one of these areas, 8B, also contained the biosynthetically prepared [^{3}H]-labeled lipoidal derivative of pregnenolone.

In order to validate the results of the radioimmunoassay, appropriate fractions of the chromatogram were combined (see legend to Fig. 3) and an aliquot of each was analyzed for pregnenolone (after alkali treatment) by the double isotope dilution assay using [^{14}C]-acetic anhydride to prepare doubly labeled pregnenolone acetate. Each fraction (I–XI, Fig. 3) was evaporated, and dissolved in 1 l. of isooctane. The solutions were extracted twice with equal volumes of aqueous methanol (1:9, V/V). The extractions were carried out in order to ensure the removal of any free pregnenolone. The aqueous methanol extracts were discarded. The isooctane solutions were evaporated to dryness under vacuum. Each of the residues were dissolved in 40 ml of methanol containing 2.8×10^5 c.p.m. of [^{3}H]-pregnenolone. To release pregnenolone from its lipoidal derivatives, 10 ml of 1 N NaOH was added and each solution was boiled for 1 h. After neutralization with acetic acid, the solvents were evaporated. The residues were dissolved in 200 ml of a methanol–H_2O (9:1, V/V) mixture. Each solution was extracted twice with equal volumes of isooctane. The hydrocarbon extracts were discarded and each aqueous methanol extract was evaporated to dryness. The resulting residues were purified first by chromatography on celite in System 1 and then by high pressure liquid chromatography in System C-1. Those fractions of the last chromatogram containing [^{3}H]-pregnenolone were acetylated with [^{14}C]-acetic anhydride as described above. The resulting acetates were chromatographed on celite in System 2. Those areas of the chromatogram containing [^{3}H]-pregnenolone acetate were combined and evaporated to dryness. To each was added about 50 mg of authentic carrier pregnenolone acetate. The mixtures were recrystallized four times and the $^3H/^{14}C$ ratios determined for each of the crystalline products and the residues in the mother liquors. The results are given in Table 4. Except for fraction V all the other fractions had a constant ($^3H/^{14}C$) ratio providing proof for the presence of pregnenolone. Although radioimmunoassay had detected the lipoidal derivative of pregnenolone, in fraction V, the $^3H/^{14}C$ ratio of this fraction did not reach constancy. As a result, it has been assumed that no pregnenolone was present in this fraction.

As described above, the $^3H/^{14}C$ ratio together with the amount of tritiated pregnenolone added as internal standard were used to calculate the amount of the lipoidal derivative of pregnenolone (expressed as pregnenolone) in each fraction. The results are shown in Table 4 and Fig. 3. As can be seen in Fig. 3, the distribution of lipoidal derivatives of pregnenolone as measured by the double isotope method is in reasonable agreement with that indicated by radioimmunoassay (Fig. 2). One important exception is

Table 4. Pregnenolone isolated as lipoidal derivative, crystallization data and content*

Fraction	X_1†	X_2	X_3	X_4	ML_4	Pregnenolone (μg/fraction)
I	3.2	3.3	3.2	3.2	3.1	10.2
II	13.9	17.9	18.7	18.2	17.3	2.3
III	42.0	46	50	41	45	
IV	3.7	3.6	3.5	3.5	3.5	15.6
V	90	101	130	108	89	
VI	17.6	17.1	17.9	17.3	17.4	2.0
VII	37.4	38.0	36.8	38.9	38.0	1.4
VIII	9.7	10.4	9.8	10.4	10.2	3.2
IX	2.9	2.9	2.9	2.9	2.9	18.8
X	5.7	5.7	5.5	5.5	5.5	6.0
XI	15.0	15.6	16.9	16.8	16.8	1.9

* Crystallization data ($^3H/^{14}C$) ratio) and content (μg/fraction) of pregnenolone isolated from lipoidal derivatives present in fractions obtained from the chromatographic analysis profiled in Fig. 3. † The symbols, (X, X_1, etc.) are explained in the legend to Table 2. The following solvents were used for the crystallizations: X_1, methanol; X_2, chloroform–hexane; X_3, acetone–petroleum ether; X_4, ethyl acetate–petroleum ether. The content of each fraction was calculated by the method described in [1]. The fractions of the samples to which the tritiated material standard was added are given in the legend to Fig. 3.

apparent. Fraction 7A (Fig. 2) appeared to contain a considerable amount of pregnenolone (as a lipoidal derivative) as estimated by RIA, but, when assayed by the more reliable double isotope dilution technique, it was shown to contain much smaller amounts of the steroid. Nevertheless, in spite of the fact that the two methods of analysis do not give identical quantitative results, it is clear that there are present in the extracts examined at least three different lipoidal derivatives of pregnenolone. Only one of these (IX) possesses the same chromatographic properties as the derivatives biosynthesized from tritiated pregnenolone by homogenates of bovine adrenal glands.

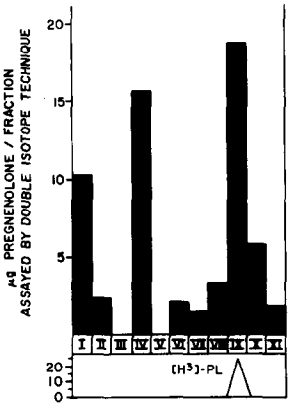

Fig. 3. *Profile of chromatographic analysis of adrenal extract on florisil.* Pregnenolone (after hydrolysis of lipoidal derivatives) was estimated by double isotope technique. The roman numerals correspond to the fractions or combination of fractions as indicated in Fig. 2 as follows: I—1; II—2A and 2B; III—3A, 3B and 4A; IV—4B; V—5A and 5B; VI—6A and 6B; VII—7A; VIII—7B and 8A; IX—8B; X—8C, 9 and 10; and XI—11, 12 and 13. Aliquots of Fractions I–XI were taken for quantitative analysis by the double isotope technique. The fractions and the aliquots are: I—50%; II—40%; III—50%; IV—30%; V—50%; VI—50%; VII—30%; VIII—50%; IX—30%; X—50% and XI—50%. The tracing at the bottom is the same as that in Fig. 2.

DISCUSSION

This work has revealed two important facts. One bears on the fact that two other steroids, in addition to pregnenolone [1], have now been shown to exist in adrenal tissue as lipoidal derivatives. All three compounds, pregnenolone, 17-hydroxypregnenolone and dehydroisoandrosterone, are 3β-hydroxy-5-ene-steroids. The methods of isolation and identification used in this study would not detect steroids with other structural features and therefore it is not possible to assert from the findings reported in this paper that only β, γ-unsaturated-3β-hydroxy steroids exist as lipoidal derivatives in steroidogenic tissues. Examples of this class of steroid were sought because the parent free compounds are known to be intermediates in processes that are involved in the biosynthesis of steroid hormones. Each of the parent steroids can be formed via enzymatic processes from cholesterol, which is also a 3β-hydroxy-5-ene-steroid. Whether lipoidal derivatives of these steroids can, in fact, serve as substrates for biosynthetic processes is still unknown. In this regard, it may be pointed out that cholesterol acetate, as well as other esters of cholesterol, can be oxidized by the side-chain cleavage enzyme system present in mitochondria from bovine adrenals to the corresponding pregnenolone esters [6]. These cholesterol esters are, at least with respect to their polar qualities and behavior towards alkali, similar to the naturally occurring lipoidal derivatives detected in this study. Moreover, kinetic evidence recently obtained in this laboratory [7] suggests that more than one side-chain cleavage enzyme system exists in adrenal mitochondria. The data further suggest that these systems appear to differ from each other in their substrate specificities: cholesterol, cholesterol sulfate (a naturally occurring constituent of adrenals) and cholesterol acetate (chosen because it may resemble the endogenous lipoidal de-

rivatives) are each oxidized by what may be its own specific enzyme system.

The second important fact disclosed by these studies is that there are several different lipoidal derivatives of pregnenolone in adrenal tissue. The three (fractions I, IV and IX) differ from each other in their chromatographic properties. At present, there is no reason to believe that any one of the materials isolated is a homogeneous entity. The resolving power of the florisil chromatogram is relatively poor and probably is insufficient to separate compounds that are structurally similar. The fact that three distinctly different fractions were separated from each other suggests that the differences in their structures are not small. If the *in vitro* experiments quoted [2] are any indication, it may be anticipated that each of the three fractions is still a mixture of components. The presumption is that the lipoidal derivatives of 17-hydroxypregnenolone and dehydroisoandrosterone isolated in this study are also mixtures of structurally related components. The significance of this heterogeneity is unknown.

Three techniques were used in this study for the qualitative analysis of the steroidal moieties of the lipoidal derivatives. They were: radioimmunoassay, double isotope dilution technique and mass spectroscopy. The first two methods were also used for quantitative analysis. It is not surprising that these two were not in perfect agreement. The possibility that the labeled steroid bound to its specific antibody is displaced by structurally similar compounds, particularly if these are present in amounts much larger than the specific ligand, is so likely that qualitative identification by means of RIA always entails considerable uncertainty. With the double isotope technique used in this investigation the uncertainty is much less. When constancy in the $^{3}H/^{14}C$ ratios of the derivatives (acetates) is achieved after several recrystallizations (from different solvent mixtures), the probability that the unknown compound (as its acetate) is identical with the added carrier of known structure is extremely high. The unknown compound may be present in only trace amounts but so are contaminants. These impurities are removed either during chromatography or even more efficiently as a result of the successive recrystallization. In this latter process, the partition properties between the crystals and its mother liquor of the unknown compound, which by now has been diluted with several thousand times its amount of carrier, are very different from those of the contaminants which are still present in trace quantities. By measuring the specific activities of both the crystalline products and of the residues in the mother liquors, a quantitative evaluation of purity can be made. Homogeneity is highly probable when the $^{3}H/^{14}C$ ratios of crystals and residues in mother liquors over several recrystallizations are constant. Moreover with reagents of high specific radioactivity and counting equipment of great accuracy, the isotope dilution technique permits precise quantitative estimations of trace amounts of compounds.

Confirmation of identity by mass spectral analysis is obviously desirable and, in the case of dehydroisoandrosterone, this was obtained. With 17-hydroxypregnenolone, the mass spectral analysis was inconclusive. Possibly too small a sample was taken for analysis. In addition, the sample may have deteriorated during the several weeks that elapsed between the analysis by the double isotope technique and that by GC–MS. Recent experience has shown that special precautions must be taken to prevent decomposition of submicrogram quantities of compounds that are stored for long periods of time. The double isotope technique is a gentle procedure and the results obtained with it clearly prove the identity of 17-hydroxypregnenolone as the steroid moiety of a lipoidal derivative isolated from adrenal tissue.

Table 3 lists the amounts of the lipoidal derivatives found in an extract of bovine adrenal glands. These must be considered to be minimal values because recovery, particularly from the initial extraction process, could not be monitored. Moreover, there is reason to believe that the values for 17-hydroxypregnenolone are especially low because its lipoidal derivative(s) probably does not have partition properties (between isooctane and 90% methanol) that are as favorable as those of the lipoidal derivative(s) of pregnenolone. In spite of these uncertainties, the values in Table 3 reveal that the lipoidal derivatives are not present in adrenal tissue in inconsequential amounts. These newly discovered, naturally occurring compounds are present in quantities that are of the same order of magnitude as are those of the free steroids.

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