The Occurrence and Metabolism of 20α-Hydroxycholesterol in Bovine Adrenal Preparations*

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ABSTRACT: Using tritiated 20α-hydroxycholesterol and its sulfate as internal standards, 5 kg of bovine adrenal glands was processed to determine their content of these compounds. Double-isotope dilution techniques indicated the presence of 37 μg of unesterified 20α-hydroxycholesterol/kg of tissue. The compound was also found in higher concentration (70 μg/kg) in the fatty acid esterified fraction; on the other hand, the sulfate ester fraction was found to be devoid of this sterol. Quantification and identity were achieved using [^{14}C]acetic anhydride and crystallization with carrier material both as the acetate and as the 3β-acetoxy 5,6-oxide. Incubation of the tritium-labeled steroid with sonicated preparations of adrenal mitochondria, resulted in rapid and complete conversion of the substrate into radioactive pregnenolone, 17-hydroxypregnenolone and to more polar products. When incubation experiments were carried out with the tritiated 20α-hydroxycholesterol together with [^{14}C]cholesterol, the recovered, unchanged hydroxysterol was devoid of ^{14}C even though the isolated pregnenolone contained both isotopes. Although the sulfurylated hydroxysterol could not be found in adrenal tissue, the doubly labeled conjugate, when incubated with adrenal mitochondrial preparations that had been disrupted by ultrasound, was found to be converted, in high yield, into pregnenolone sulfate without any alteration of the ^{3}H/^{35}S ratio.

The biosynthetic pathway by which pregnenolone is produced from cholesterol reportedly involves two hydroxylated sterol intermediates, 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol (Figure 1). The evidence for the existence of these intermediates is based mainly on incubation studies in which synthetic samples of these compounds were converted into pregnenolone.[1] Neither of the compounds has been isolated from natural sources in adequately characterized form. It was the objective of this study to attempt to isolate 20α-hydroxycholesterol from adrenal tissue in a well-characterized form and, in so doing, to add further certainty to its intermediacy in the biosynthetic sequence for the steroid hormones.

It was Gurin and his collaborators (Lynn *et al.*, 1954; Staple *et al.*, 1956) who first demonstrated that a six-carbon fragment is cleaved from cholesterol during the process by which the sterol is converted into pregnenolone. This finding led Solomon *et al.* (1956) to attempt to establish that 20α-hydroxycholesterol is an intermediate in the process. These investigators tried to trap the isotopically labeled hydroxysterol in an *in vitro* experiment using homogenates of bovine adrenal glands, [4-^{14}C]cholesterol as substrate and 20α-hydroxycholesterol as a trapping agent. Although the reisolated trap appeared to be associated with ^{14}C radioactivity, they were unable to ensure the radiochemical homogeneity with certitude since the small quantity of available material precluded further manipulation. In 1961, two groups of workers, Constantopoulos and Tchen (1961) and Shimizu and his colleagues (1961) reported further attempts to clarify this problem. The latter group, incubated labeled 20α-hydroxycholesterol with a preparation made from bovine adrenals and showed that it was efficiently converted into pregnenolone. A second compound, compound X, was also formed and it, upon further incubation with the adrenal preparation, was also converted into pregnenolone and isocaproic acid. During the next year, both groups (Shimizu *et al.*, 1962; Constantopoulos *et al.*, 1962) presented evidence that indicated that compound X is 20α,22ξ-dihydroxycholesterol. Neither group of workers succeeded in isolating the intermediate in crystalline form. In the same year, Chaudhuri *et al.* (1962) proved that 22ξ-hydroxycholesterol could also be converted into pregnenolone by a bovine adrenal preparation. This finding complicated the scheme since it made it necessary to postulate more than one pathway between cholesterol and pregnenolone and this, as has been pointed out by Simpson and Boyd (1967), seems unlikely. In 1963, based on a study of the conversion of cholesterol into pregnenolone by acetone

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[1] The following trivial names have been used in the text: pregnenolone, 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate, 3β-hydroxy-5-pregnen-20-one 3-sulfate; 20α-hydroxycholesterol sulfate, 3β,20α-dihydroxy-5-cholestene 3-sulfate; desoxycorticosterone acetate, 21-hydroxy-4-pregnene-3,20-dione 21-acetate; dehydroisoandrosterone, 3β-hydroxy-5-androsten-17-one; 17-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; pregnenetriol, 5-pregnene-3β,17α,20α-triol; androstenediol, 5-androstene-3β,17β-diol.

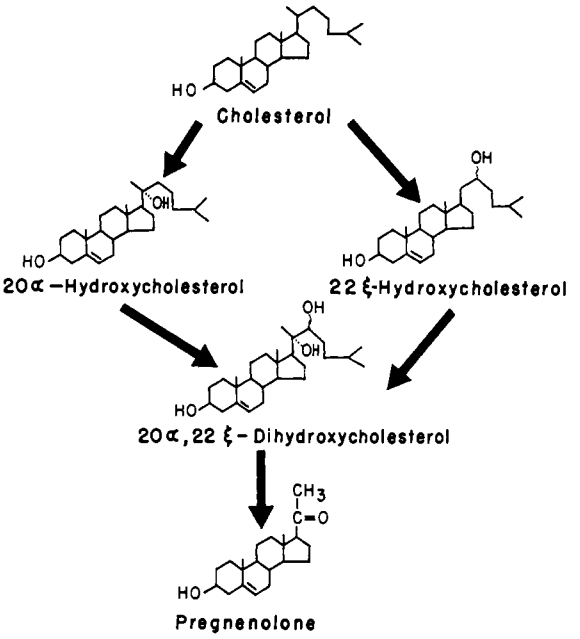

FIGURE 1: Biogenesis of pregnenolone from cholesterol.

powders made from bovine corpus luteum, Ichii *et al.* (1963) reported that both 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol accumulated in the reaction mixture when the incubation was carried out in the presence of added pregnenolone. The identities of the intermediates were established merely by their migration rates when chromatographed on paper in two systems. In 1964, Koritz and Hall reconfirmed that 20α-hydroxycholesterol is efficiently converted into pregnenolone by acetone powders of bovine adrenals. But, unlike Ichii *et al.* (1963), these workers could find no accumulation of 20α-hydroxycholesterol or of 20α,22ξ-dihydroxycholesterol when incubations using [^{14}C]cholesterol as substrate were carried out either in the presence or absence of pregnenolone. Furthermore, they observed that pregnenolone inhibits the reaction, 20α-hydroxycholesterol $\rightarrow$ pregnenolone. Hall and Koritz (1964) also demonstrated that 20α-hydroxycholesterol could inhibit the conversion of [7α-^{3}H]-cholesterol into pregnenolone, by acetone powders of bovine adrenal mitochondria. As before, no radioactive 20α-hydroxycholesterol accumulated during the course of this incubation. Nevertheless, in 1967, Ichii *et al.* described an assay for the enzyme, cholesterol 20α-hydroxylase, which was based on the estimation of [^{14}C]-20α-hydroxycholesterol formed from [^{14}C]cholesterol. The labeled 20α-hydroxysterol was purified by chromatography on thin-layer plates and on paper. The addition of 20α-hydroxycholesterol to the incubation mixture was observed to result in a "product inhibition" of the enzymatic reaction. To confound the problem further, Simpson and Boyd (1967) have reported that a careful search has failed to prove that either 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol are intermediates between cholesterol and pregnenolone. They also cast doubt upon the significance that can be attributed to the observation that 20α-hydroxycholesterol can inhibit the side-chain cleavage of cholesterol. They found that 25-hydroxycholesterol, 26-hydroxycholesterol, and, especially, 24-hydroxycholesterol also inhibited the cleavage. Further, the mechanism of inhibition produced by these hydroxylated cholesterol derivatives was apparently the same as that caused by 20α-hydroxycholesterol, so that the inhibition evoked by the latter compound could not be taken as evidence for its intermediacy.

From this short review, it is clear that the question of the identity of the intermediates between cholesterol and pregnenolone remains unclear. Synthetic samples of 20α-hydroxycholesterol, 22-hydroxycholesterol, and 20α,22ξ-dihydroxycholesterol all yield pregnenolone when incubated with preparations of adrenal gland. Some investigators have reported that 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol accumulate in incubation mixtures when the process, cholesterol $\rightarrow$ pregnenolone is inhibited (by pregnenolone or by 20α-hydroxycholesterol). The evidence presented for the existence of these intermediates must be considered to be indirect (mobility on chromatograms and recrystallization with authentic carrier). Other investigators have failed to observe this accumulation. No one has succeeded in isolating either product from these *in vitro* experiments in well-characterized crystalline form.

In this study, an elaborate isolation procedure was employed to process 5 kg of fresh bovine adrenal glands. Tracer amounts of both tritiated 20α-hydroxycholesterol and tritiated 20α-hydroxycholesterol sulfate were added at the start. The hydroxysterol was sought in the free, esterified, and sulfated fractions. The evidence obtained disclosed that the sulfate of 20α-hydroxycholesterol was not present in the extract from these adrenal glands. Results obtained from the analysis of the other two fractions indicated that 20α-hydroxycholesterol was present in both. Although the amounts isolated were insufficient for crystallization or for infrared spectral identification, evidence for the occurrence of the hydroxysterol was provided by tracer techniques (identity of chromatographic mobility with authentic tritiated standards and achievement of constant ^{3}H/^{14}C ratios following repeated crystallization of the acetate prepared from [^{14}C]acetic anhydride and of the 5,6-oxide made from the ^{14}C-labeled acetate) that are generally regarded as affording a high degree of certainty to the identification.

The remaining portion of this report describes the metabolism of cholesterol and both its free and sulfurylated 20-hydroxylated derivatives, by preparations of bovine adrenal mitochondria that had been disrupted by ultrasound. One of the objectives of these studies was to attempt to trap radioactivity associated with the incubated cholesterol in the 20α-hydroxycholesterol fraction in the hope of adding further support for the intermediacy of this hydroxysterol in the biosynthetic sequence between cholesterol and pregnenolone. Although no radioactivity was found to be associated with the reisolated 20α-hydroxysterol, this compound

TABLE I: Systems Used for Partition Chromatography on Celite.

System	Components
A[a]	Methanol 4, *n*-propyl alcohol 1, water 1.3, toluene 2, isooctane 2
B[a]	Methanol 4, *n*-propyl alcohol 1, water 1.5, toluene 2, isooctane 2
C	Isooctane 4, ethyl acetate 1, *t*-butyl alcohol 2, methanol 2, 1 M NH_4OH 3
D[b]	Isooctane 1, methanol–water (9:1) 1
E	Isooctane 5, *t*-butyl alcohol 1.8, methanol 2.8, water 0.4
F[a]	Isooctane 0.5, toluene 0.5, methanol–water (8:2) 1
G[a]	Isooctane 1, toluene 1, water 1, methanol 2
H[a]	Methanol 4, *t*-butyl alcohol 1, water 2, benzene 2, isooctane 2
I[a]	Methanol 4, water 2, isooctane 2, toluene 2
J[a]	Methanol 4, *n*-propyl alcohol 1, water 1.4, toluene 2, isooctane 2
K	Methanol 4, *t*-butyl alcohol 2, water 2, toluene 1, isooctane 3
L	Methanol 4, *t*-butyl alcohol 0.5, water 2, benzene 2, isooctane 2
M	Isooctane 3, ethyl acetate 3, *t*-butyl alcohol 2, methanol 2, 1 M NH_4OH 3
N	Heptane 4, *n*-butyl alcohol 1, methanol 2, 0.3 M pyridinium sulfate containing 5% pyridine 2
P	Isooctane–benzene (8:2) 2, methanol–water (7:3) 1 (first to fourth holdback volumes) Isooctane–benzene (6:4) 2, methanol–water (7:3) 1 (fifth to tenth holdback volumes) Benzene 2, methanol–water (7:3) 1 (tenth holdback volume and beyond)
Q	Isooctane 2, ethyl acetate 4, *t*-butyl alcohol 2, methanol 2, 1 M NH_4OH 3
R	Isooctane 2.66, ethyl acetate 4, *t*-butyl alcohol 2, methanol 2, 1 M NH_4OH 3

[a] Reversed-phase system. 0.3-ml stationary phase (hydrocarbon phase) per g of Celite. [b] 0.7-ml stationary phase/g of Celite.

was found to be metabolized, with high efficiency, to pregnenolone and 17-hydroxypregnenolone.

Experimental Procedures

Preparation and Purification of Tracers. CHOLESTEROL-4-^{14}C. The sterol (0.1 mCi; 0.773 mg) was obtained from New England Nuclear Corp. and was purified by chromatography on 30 g of Celite using the reversed-phase system A (Table I). Preparation of the columns has been described previously (Siiteri, 1963). The labeled material was eluted in the fourth and fifth holdback volumes. Proof of the radioactive homogeneity was established by crystallizing a portion of the label with carrier cholesterol. The crystalline product and the material in the mother liquor possessed identical specific activities. The purified tracer, dissolved in a mixture of benzene and ethanol, was stored prior to use at 5° in an amber flask.

CHOLESTEROL-1,2-^{3}H. The tracer (0.25 mCi; 0.0076 mg) was purchased from New England Nuclear Corp. and purified by chromatography using reversed-phase system B. Cholesterol was eluted in the fourth holdback volume.

CHOLESTEROL-1,2-^{3}H SULFATE-^{35}S. Ammonium cholesterol sulfate-^{35}S was prepared in the following manner. Chlorosulfonic acid-^{35}S (160 mg; 5 mCi) (purchased from New England Nuclear Corp.) was added to 0.7 ml of dry pyridine. Aliquots of the resulting complex were used to prepare sulfates-^{35}S of cholesterol and pregnenolone. One-half of the complex was added to 150 mg of cholesterol (purified *via* the dibromide) dissolved in 1 ml of dry pyridine. After 30 min at 40–50°, the reaction mixture was added dropwise into 20 ml of ether. The precipitate was collected on a Büchner funnel and was washed twice with ether. The product was dissolved in methylene chloride and the solution was then treated with ammonium hydroxide in methanol to form the ammonium salt. The reaction mixture was taken to dryness and a portion of the residue was chromatographed on Celite using system C. Ammonium cholesterol sulfate was eluted in the fifth and sixth holdback volume. Cholesterol-1,2-^{3}H sulfate was prepared in the manner described above using nonradioactive chlorosulfonic acid. The tritium-labeled sterol sulfate was mixed with an appropriate amount of the ^{35}S-labeled material to yield a ^{3}H/^{35}S ratio of approximately 3. Following rechromatography on Celite using system C, the peak fractions were found to have a constant ^{3}H/^{35}S ratio. These fractions were combined and used in subsequent incubations.

20α-HYDROXYCHOLESTEROL-16-^{3}H. The labeled sterol was prepared according to the method of Petrow and Stuart-Webb (1954) using pregnenolone-16-^{3}H (5 mCi; 4.8 Ci/mmole) purchased from Nuclear-Chicago Corp.) as starting material. The tritiated pregnenolone was chromatographed twice on paper using isooctane–90% methanol as the developing solvents. Following localization by radioautography, the material was eluted with methanol. A small aliquot of the purified material was tested for homogeneity by recrystallization with added carrier pregnenolone. The specific activities of the crystals and of the residues in the mother liquor were equal indicating the absence of contaminants. The Grignard reaction was carried out using 25 mg of nonradioactive pregnenolone acetate together with the acetylated labeled pregnenolone. Initial purification of the crude reaction mixture was

achieved using a 5-g Florisil column. The column was developed with heptane, mixtures of heptane, and benzene, and, finally, with pure benzene. The fraction containing the radioactive product with the most tritium (eluted with benzene) was saponified with KOH and the product purified by chromatography on 50 g of Celite using system D. In the fourth holdback volume 20α-hydroxycholesterol containing 1200 × 10^6 cpm was eluted whereas in the seventh to ninth holdback volumes where pregnenolone might be expected, a product having 700,000 cpm was eluted.

Since the purity of the labeled hydroxysterol was of special concern for the incubation experiments described below, a special effort was made to purify the material eluted in the fourth holdback volume. This compound was synthesized from a highly radioactive starting material, pregnenolone, which was the same compound that was sought as a product formed from the sterol in the *in vitro* incubation studies. Consequently, to eliminate the possibility of contamination by tritiated pregnenolone, 4 mg of nonradioactive pregnenolone was added to the sample of the hydroxysterol containing 1200 × 10^6 cpm and the mixture was chromatographed on Celite with system D. In the third and fourth holdback volumes, 20α-hydroxycholesterol-16-^{3}H containing 1100 × 10^6 cpm was eluted. Pregnenolone was detected in the seventh and eighth holdback volumes by a colorimetric assay using cupric acetate reagent.[2] It contained 170,000 cpm of tritium. Recrystallization of this latter compound gave products with constant specific activities, thus providing evidence that most of the radioactivity present in the seventh to eighth holdback volumes was, indeed, associated with the carrier pregnenolone. Further proof that radioactive pregnenolone was present in this fraction was obtained by conversion into the labeled oxime. Radiochemical homogeneity was established by crystallization to constant specific activity. It is especially noteworthy that only one fraction of the Florisil column, that having the most tritium, was processed in this manner. Even superficial analysis of some of the other fractions demonstrated that they, as expected, contained even larger amounts of labeled pregnenolone.

In order to remove all of the pregnenolone from the product, 20α-hydroxycholesterol-16-^{3}H, three additional wash-out Celite partition columns were necessary. The second wash-out chromatogram was carried out on Celite using system E. The 20α-hydroxycholesterol (356 × 10^6 cpm) was eluted in the second holdback volume while approximately 80,000 cpm of tritium was eluted along with the 6 mg of pregnenolone added as carrier prior to chromatography. (Crystallization of the pregnenolone from methanol–acetone resulted in a loss of tritium thus revealing that the major portion of this radioactivity was not associated with that substance.) A third chromatogram using system D was employed in order to ensure the radiochemical homogeneity of the 20α-hydroxycholesterol. In this case, no radioactivity was found to be associated with the additional 5 mg of carrier pregnenolone that had been added before chromatography.

3β-Acetoxy-20α-hydroxycholesterol 5,6-oxide. 3β-Acetoxy-20α-hydroxycholesterol (100 mg; mp 152–155°) was dissolved in 5 ml of chloroform to which was added 200 mg of *m*-chloroperbenzoic acid. The mixture was left overnight at room temperature and then diluted with 100 ml of ether. The solution was washed three times with a saturated sodium bicarbonate solution and then with water to neutrality. The organic layer was dried over anhydrous sodium sulfate and then evaporated to dryness *in vacuo*. Chromatography of an aliquot of the residue on thin-layer silica gel using the system benzene–ethyl acetate (2:1) indicated that the product had been obtained in approximately 80% yield. The remaining weight appeared to be unchanged starting material. The bulk of the reaction products was chromatographed on 150 g of Celite using system H. The 5,6-oxide was eluted in the second to the third holdback volumes while the starting material was eluted only after washing the column with methanol. The substance eluted in the second to the third holdback volumes (80 mg) appeared as light yellow oil. Much of the color was subsequently removed by passing an ethereal solution of the residue through about 0.5 g of alumina. Two crystallizations from methanol–acetone–water yielded 25 mg of crystalline product melting at 125–134°. The unsharp melting point would seem to indicate that the product was a mixture of epimeric oxides. No attempt was made to purify these further. For C–H analysis, the sample required drying at 98° for 16 hr. *Anal.* Calcd for $C_{29}H_{48}O_4$: C, 75.60; H, 10.50. Found: C, 75.48, H 10.22.

20α-Hydroxycholesterol-16-^{3}H sulfate. Initial studies on the preparation of this conjugate indicated that the usual method (*i.e.*, with the pyridine–sulfur trioxide complex) was unsuitable due to extensive elimination of the tertiary hydroxyl group. A second method, using triethylamine–sulfur trioxide complex as the sulfurylating reagent (Dusza *et al.*, 1968), was found to afford much improved yields. Commercial grade triethylamine–sulfur trioxide complex (purchased from Allied Chemical Corp.) was purified by percolation of a methylene chloride solution of the complex through a column (3.5 × 40 cm) of Florisil. Following crystallization of the complex from methylene chloride, the crystals were stored in the dry state in a vacuum desiccator.

Tritum-labeled tracer 20α-hydroxycholesterol (15 × 10^6 cpm) was dissolved in about 0.1 ml of dry pyridine to which was added a few crystals of the above complex. The mixture was left at room temperature for 2 hr. The reaction mixture was diluted with 10 ml of 0.6 M pyridinium sulfate to effect conversion of the sterol sulfate into the pyridinium salt which was then extracted into 60 ml of chloroform. The chloroform extract was dried over anhydrous sodium sulfate and taken to dryness. The residue was chromatographed on 30 g of Celite using system N. Two peaks of radioactivity were located; the first in the 6th–8th holdback

[2] Reagent: 85 mg of cupric acetate in 100 ml of glacial acetic acid; 1.5 ml of this solution together with 1 ml of concentrated H_2SO_4 gave a red solution (maximum at 520 mμ) with pregnenolone. Beer's law was obeyed over a range of 10–100 μg.

volume (6×10^6 cpm of ^3H) and the second, the desired product, in the 14th–16th holdback volume (4.3×10^6 cpm of ^3H). The early peak of radioactivity was assumed to be associated with the product formed by elimination of the tertiary hydroxyl group at C-20. This product has not been further characterized. The identity of the more polar material, 20α-hydroxycholesterol-16-^3H sulfate, after conversion into the ammonium salt, was established by comparison of its chromatographic mobility with that of ^{35}S-labeled material. Since the ^{35}S-labeled hydroxysterol sulfate involved the preparation of milligram quantities of conjugate, an adequate identification was achieved as follows; The infrared spectrum indicated the presence of the sulfate group, the Δ^5 double bond as well as the tertiary hydroxyl group. Specific activity studies indicated the introduction of one labeled sulfate group. Solvolysis in tetrahydrofuran in the presence of 10^{-2} N *p*-toluenesulfonic acid yielded a product which was identical with the starting material, 20α-hydroxycholesterol.

Isolation of 20α-Hydroxycholesterol from Bovine Adrenal Tissue. (A simplified flow sheet for the procedures utilized in the isolation of the hydroxysterol from various fractions of bovine adrenal tissue is presented in Figure 2.)

Fresh bovine adrenal glands (5 kg), obtained from the slaughter house, were freed of surrounding fat. The tissue was homogenized in 12 l. of methanol to which was added 1.6×10^6 cpm of 20α-hydroxycholesterol-16-^3H (35 μg) and 1.6×10^8 cpm of ammonium 20α-hydroxycholesterol-16-^3H sulfate (43 μg) as internal standards. After stirring the mixture overnight in the cold room, the solids were separated by filtration and then resuspended in 8 l. of methanol. Following removal of the solids by filtration, the combined methanol extracts were concentrated under vacuum at low temperature to 1400 ml. The resulting suspension was made 20% with respect to NaCl and extracted twice with 4 l. of freshly distilled tetrahydrofuran. The combined organic extracts (methanol and tetrahydrofuran) were taken to dryness under vacuum (fraction I, 94 g, 2.3×10^6 cpm of ^3H). The solid material remaining after the second methanol extraction was suspended in a mixture of methylene chloride (8 l.) and acetone (5 l.) and stirred for 48 hr in the cold room. After filtration, the solvents were removed under vacuum. The residue weighed 93 g and contained no radioactivity (fraction II).

Fraction I was chromatographed on 2.5 kg of Celite (95×10 cm) using the reversed-phase system A. Three liters of mobile phase eluted approximately 2×10^6 cpm of tritium. This fraction (III) weighed 18 g and contained 1.98×10^6 cpm of ^3H of free and sulfated 20-hydroxycholesterol. Further development of the chromatogram with an additional 9 l. of mobile phase yielded fraction IV (34 g) which contained no radioactivity. The column was washed with methanol and, finally, with ether and the two washes were combined to yield fraction V (50 g) which also was devoid of radioactivity. Fractions IV and V were assumed to contain cholesterol, its fatty acid esters, as well as esterified 20α-hydroxycholesterol, if present. These

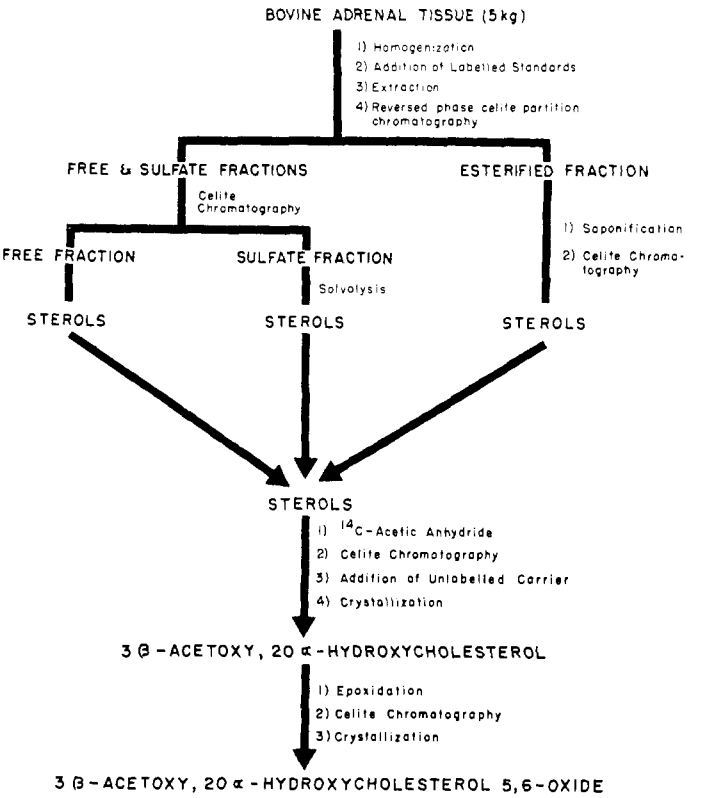

FIGURE 2: Flow chart of the isolation procedure.

were combined with fraction II and were labeled with an additional quantity of radioactive 20α-hydroxycholesterol as internal standard. The mixture was subsequently processed to explore the possibility that 20α-hydroxycholesterol, esterified with fatty acid, was also present.

Since the first reversed-phase column was somewhat overloaded, fraction III was rechromatographed on a second reversed-phase column (system A, 2.5 kg of Celite) collecting 500-ml fractions. Fractions 4–7 were combined (fraction VI, 15 g, 1.8×10^6 cpm) and rechromatographed on 2.5 kg of Celite using system D. In order to conserve Celite, the 2.5 kg of support, which had been used previously and which was still in the column, was washed thoroughly with methanol and methylene chloride. Then, the aqueous methanol stationary phase of system D was applied to the top of the column. When the stationary phase had completely saturated the Celite, mobile phase was passed through the column until aqueous methanol no longer appeared in the eluate. It was estimated that, at this point, 1 g of Celite contained 1 ml of stationary phase. Then the sample (fraction VI), dissolved in 100 ml of stationary phase, was slurried with 200 g of Celite and the mixture was placed at the top of the column. Fractions of 1 l. were collected. Fractions 12–19 (fraction VII) weighed 100 mg and contained 0.99×10^6 cpm of tritium. Following the collection of fraction 20, the mobile phase was changed to one consisting of ethyl acetate–isooctane (2:1, v/v). Fraction VIII, obtained by combining fractions 25–32,

weighed 14 g and contained 0.69×10^6 cpm of ^3H of the added tracer, 20α-hydroxycholesterol sulfate.

Fraction VII was further purified on 50 g of Celite using the reversed-phase system F. The radioactive material (fraction IX) was eluted in the first holdback volume and contained 580,000 cpm of ^3H. Two-thirds of this fraction was rechromatographed on Celite system D and this was followed by another chromatogram on 30 g of Celite using system G. Radioactive material (fraction X, 290,000 cpm of ^3H) was eluted in the fourth and fifth holdback volume and weighed less than 0.5 mg. This residue was acetylated with [^{14}C]acetic anhydride of known specific activity. At the same time, an aliquot (containing 300,000 cpm of ^3H) of the tracer, 20α-hydroxycholesterol, that was used originally for internal standardization was acetylated with a sample of the same acetic anhydride so that a comparison of the $^3H/^{14}C$ ratios could be made. Using this $^3H/^{14}C$ ratio ($^3H/^{14}C$ standard = 82) as a control, any decrease in this value for the biological sample would indicate the presence of endogenous compound. The doubly labeled samples were chromatographed separately on 30 g of Celite, using system A, from which the acetates were eluted in the third holdback volume. Both samples were rechromatographed on Celite using system B and were eluted in the fourth and fifth holdback volumes. At this stage of the purification procedure, the sample extracted from the adrenal glands exhibited a $^3H/^{14}C$ ratio of 5.5. Each sample of acetate was mixed with 15 mg of carrier 20α-hydroxycholesterol acetate and recrystallized from acetone–methanol to constant isotope ratio. The crystallization data are presented in Table II. Before the fourth crystallization, it was necessary to add an additional 20 mg of carrier 20α-hydroxycholesterol acetate.

In order to add further support to the identification, 15 mg of the material from the final crystallization was converted into its 5,6-oxide. The acetate was dissolved in chloroform containing 30 mg of *m*-chloroperbenzoic acid after which the mixture was kept overnight at room temperature. Following the usual work-up, the product was chromatographed on 50 g of Celite using system H. A single peak of radioactive material was eluted in the third to the fourth holdback volume. The curves obtained by plotting the fraction number against the counts of ^3H and the counts of ^{14}C were symmetrical and superimposable. Crystallization of the oxide of the hydroxysterol acetate from methanol–acetone–water failed to change the $^3H/^{14}C$ ratio (Table II).

The possibility that the product was contaminated by the acetate of 25-hydroxycholesterol, a known autooxidation product of cholesterol, was eliminated by the observation that this sterol acetate is easily and completely separated from 20α-hydroxycholesterol by chromatography on Celite with a system used early in the purification procedure. In system A, 25-hydroxycholesterol acetate is eluted in the second holdback volume whereas 20α-hydroxycholesterol acetate is eluted in the third to the fourth holdback volume. The unnatural isomer, 20β-hydroxycholesterol, was also found to be separable, to some degree, from the 20α compound. Chromatography of a mixture of authentic ^3H-labeled 20α-hydroxycholesterol acetate and labeled 20β-hydroxycholesterol acetate-^{14}C on 30 g of Celite, using system A, resulted in a slight separation of the two isomers, the 20α isomer being slightly more polar than the 20β compound. The $^3H/^{14}C$ ratio across the peak fractions ranged from 12 to 0.8; *i.e.*, the presence of the mixture of the two compounds was quite evident from examination of these isotope ratios. It should be noted that only these dihydroxysterols, bearing a tertiary hydroxy group, were of concern since those having secondary hydroxyl groups would have formed diacetates and would have, therefore, been easily separated.

TABLE II: Isotope Ratios of ^{14}C-Acetylated 20α-Hydroxycholesterol Isolated from the Free and Fatty Acid Esterified Fractions of Bovine Adrenal Tissue.

	$^3H/^{14}C$ Ratio			
	Free Sterol Fraction		Fatty Acid Esterified Fraction	
Acetate	Crystals	Mother Liquor	Crystals	Mother Liquor
1	8.3	2.5	4.9	3.8
2	9.8	6.2	4.9	4.8
3	10.0	9.8	5.3	4.6
4	9.7	8.2		
Epoxy Acetate				
1	10.1	9.6	4.3	4.3
2	9.9	10.2		

The amount of 20α-hydroxycholesterol present in the original batch of bovine adrenals was calculated from the $^3H/^{14}C$ ratio of the third crystallization ($^3H/^{14}C$ = 10 or, calculated from dpm = 19) using the equation: weight (μg/kg) = $(dpm)_{IS}/(dpm)_2 \times M_1/M_2 \times (^3H/^{14}C)_U \times 1/5$, where dpm_{IS} = dpm of tritium in internal standard, 20α-hydroxycholesterol-^3H added, dpm_2 = specific activity in dpm of desoxycorticosterone-^{14}C acetate prepared from the sample of acetic anhydride-^{14}C used to acetylate 20α-hydroxycholesterol standard (S) and unknown (U), M_1 = molecular weight of 20α-hydroxycholesterol = 402, M_2 = molecular weight of desoxycorticosterone acetate = 370, and $(^3H/^{14}C)_U$ = ratio of isotopes in product of third recrystallization (see Table I) when expressed as disintegrations per minute. $(4.8 \times 10^6)/1208 \times (402/370) \times 1/19 \times 1/5 = 44$ μg/kg. By substracting the amount of internal standard known to be present in the sample (7 μg/kg of internal standard was originally added to 5 kg of tissue) from this value (44 − 7), it appears that 37 μg of 20α-hydroxycholesterol was isolated from each kilogram of bovine adrenal tissue processed.

Isolation of 20α-Hydroxycholesterol from the "Fatty Acid Ester" Fraction. To the combined fractions II,

IV, and V (177 g) was added 1.5×10^6 cpm of 20α-hydroxycholesterol-^{3}H as internal standard and then the mixture was saponified overnight with 1% methanolic KOH. After the reaction mixture was neutralized with dilute acetic acid, the methanol was removed under vacuum. The remaining aqueous suspension was extracted twice with three volumes of methylene chloride. After the organic extract was washed twice with water, the solvent was evaporated leaving a residue weighing 150 g. This was chromatographed on 2 kg of Celite using system I and collecting 1-l. fractions. Fractions 6–10 contained 1×10^6 cpm of ^{3}H and weighed 2 g. This product was rechromatographed on 250 g of Celite using system D (0.5-ml stationary phase/g of Celite). The radioactive fraction, eluted in the third to the fifth holdback volumes, weighed 130 mg. This material was rechromatographed on the same system using 50 g of Celite. The radioactive fraction, eluted in the third holdback volume, weighed 50 mg. Rechromatography on 50 g of Celite, using system I, yielded radioactive material (672,000 cpm of ^{3}H) in the fourth holdback volume which weighed 22 mg. Still another chromatogram on 50 g of Celite using system D provided a radioactive (490,000 cpm of ^{3}H) residue (20 mg) eluted in the fourth and fifth holdback volumes. An aliquot (300,000 cpm of ^{3}H) of this fraction was acetylated with [^{14}C]acetic anhydride and the hydroxysterol 3-acetate was twice chromatographed on 50 g of Celite using system J. The doubly labeled acetate was eluted in the fourth holdback volume. Rechromatography on 50 g of Celite, using system K, yielded a material in the second and third holdback volumes having a ^{3}H/^{14}C ratio of 1.7.

At this stage of the purification procedure, the residue was analyzed by infrared spectroscopy using the micro KBr technique. While several bands in the fingerprint region were similar to those present in authentic 3β-acetoxy-20α-hydroxycholesterol, identity could not be established with certainty due to the presence of impurities which were later attributed to contaminants from the solvents. Consequently, the residue was subjected to two additional chromatograms, one on Celite using system A and the other on 0.5 g of Florisil. The radioactive material, a colorless oil, was eluted with a solution of benzene–ether (1:1, v/v). Again, due to solvent contaminants, its identity could not be established by infrared spectroscopy even though approximately 25 μg (calculated from the ^{3}H/^{14}C ratio of 20α-hydroxycholesterol acetate) was estimated to be present in the sample. In an attempt to effect further purification, the oil was saponified, reacetylated with [^{14}C]acetic anhydride, and the product was chromatographed on 30 g of Celite using system A. Those fractions exhibiting a similar ^{3}H/^{14}C ratio were combined and rechromatographed on 0.5 g of Florisil. The solvents used during these latter steps were redistilled before use. The doubly labeled acetate, eluted with a solution of benzene–ether (1:1, v/v), contained 56,000 cpm of ^{3}H. This represents a recovery of approximately 4% of the initial radioactivity added as internal standard. The ^{3}H/^{14}C ratio was 3.9. A third attempt to establish identity by infrared spectroscopy gave results which again were inconclusive. To one-half of the radioactive sample was added 25 mg of carrier 20α-hydroxycholesterol acetate and the mixture crystallized from acetone–methanol and acetone–benzene to constant ^{3}H/^{14}C ratio. The crystallization data are presented in Table II.

The material remaining (12 mg) from the second and third crystallizations and mother liquors were combined and epoxidated as described previously. The oxide was chromatographed on 50 g of Celite, using system L, from which it was eluted in seventh and eighth holdback volumes. The ^{3}H/^{14}C ratio showed no appreciable change following the formation and chromatography of this derivative.

Using the equation given previously, the concentration of 20α-hydroxycholesterol from the "ester fraction" was calculated as follows

$$\frac{4.54 \times 10^6}{5 \times 1453} \times \frac{402}{370} \times \frac{1}{8.7} = 77 \ \mu g/kg$$

$$77 \ \mu g/kg - 7 \ \mu g \text{ (internal standard)} = 70 \ \mu g/kg$$

Search for the Presence of Cholesterol Sulfate and 20α-Hydroxycholesterol Sulfate in Bovine Adrenal Tissue. To fraction VIII (14 g containing 0.69×10^6 cpm of ^{3}H from internal standard) was added the ammonium salt of [4-^{14}C]cholesterol sulfate (5 μg, 0.48×10^6 cpm). The mixture was chromatographed on 2 kg of Celite using system M. The fraction containing cholesterol sulfate (fraction XI) was eluted in the first and second holdback volumes (2.4 g of residue, 0.42×10^6 cpm of ^{14}C) while the tritiated 20α-hydroxycholesterol sulfate (fraction XII) was eluted in the third and fourth holdback volumes (1.9 g of residue, $0.55 \times 10_t$ cpm of ^{3}H).

CHOLESTEROL SULFATE. Fraction XI was chomatographed on 500 g of Celite using system C and then again on 150 g of the same absorbant. A 700-mg residue containing 0.4×10^6 cpm of ^{14}C was eluted in the third and fourth holdback volumes. Following extraction of the residue from aqueous 0.3 M pyridinium sulfate into chloroform, the pyridinium salt of cholesterol sulfate was chromatographed on 100 g of Celite using system N from which the sterol sulfate was eluted in the third and fourth holdback volumes. The pyridinium salt was converted into the ammonium salt and this was rechromatographed on 50 g of Celite using system C. A white solid (3.5 mg, 0.29×10^6 cpm of ^{14}C) was eluted in the fourth and fifth holdback volumes. The product was leached with ether to give 3 mg of ammonium cholesterol sulfate having an infrared spectrum identical with that of the authentic conjugate. After correction for losses, 1–1.5 mg of cholesterol sulfate/kg of tissue was estimated to be present; this value agrees well with that reported previously by Drayer *et al.* (1964). A more accurate quantification was not possible since the internal standard was added after the first stages of the purification procedure had been completed.

20α-HYDROXYCHOLESTEROL SULFATE. Fraction XII (1.9 g, 0.55×10^6 cpm of ^{3}H) was chromatographed on 500 g of Celite using system M and then again on 150 g of the same absorbant. The radioactive product

from the last chromatogram was extracted into chloroform from an aqueous 0.3 M pyridinium sulfate solution. The residue from the chloroform extract was chromatographed on 150 g of Celite using system N. This procedure yielded 65 mg of oil which contained 0.45×10^6 cpm of ^3H. One-half of the sample was then solvolyzed by standing in tetrahydrofuran solution overnight at room temperature. Under these conditions and without addition of acid, pyridinium salts of the steroid sulfates are readily cleaved. The free sterol was first chromatographed on 30 g of Celite using system I and then on a column of 30 g of Celite using system D. The radioactivity (139,000 cpm of ^3H) was eluted in the second and third holdback volumes as a colorless oil weighing less than 0.5 mg. The sample was acetylated with [^{14}C]acetic anhydride and the product was chromatographed on 30 g of Celite with system B. Radioactivity was located in the third and fourth holdback volumes where the $^3H/^{14}C$ ratio was 24. However, following the addition of carrier 20α-hydroxycholesterol 3-acetate and recrystallization, essentially all the ^{14}C was removed. The traces of ^{14}C remaining in the crystallisate were assumed to be associated with the added internal standard and, therefore, it appears that bovine adrenal tissue is devoid of 20α-hydroxycholesterol sulfate.

Metabolism of Cholesterol-4-^{14}C and 20α-Hydroxycholesterol-16-^3H by Sonicated Preparations of Bovine Adrenal Mitochondria. EXPERIMENT 1. The adrenal cortices from eight bovine glands were chopped finely in a Waring blender and then homogenized in 0.25 M sucrose in a glass homogenizer. The method used for the isolation and sonication of the mitochondria has been reported by Cooper *et al.* (1965) and by Simpson and Boyd (1966). The composition of the incubation medium, containing 0.1 M phosphate buffer at pH 7.4, magnesium ion, and a TPNH-generating system, was identical with that described by Simpson and Boyd (1966).

The washed mitochondrial pellet was resuspended in distilled water and sonicated for 10 min at 5° with a Sonifer cell disrupter (Heat Systems Ultrasonics, Inc., Melville, N. Y.) operating at 20 kcps. The supernatant, obtained by centrifugation of the sonicate at 105,000*g* for 30 min, was divided into three equal portions. To one aliquot was added 45 μg of 20α-hydroxycholesterol-16-^3H (1.92×10^6 cpm) (incubation A); to the second aliquot was added 10 μg of cholesterol-4-^{14}C (1.96×10^6 cpm) (incubation B); and to the third portion was added a mixture consisting of 11 μg of cholesterol-4-^{14}C (2.07×10^6 cpm) and 7 μg of 20α-hydroxycholesterol-16-^3H (0.3×10^6 cpm) (incubation C). In each case, the substrates were added to the incubation mixture in 0.2 ml of acetone. The final volume of each incubate was approximately 50 ml. Each flask was incubated for 15 min at 37° in a Dubnoff shaker with constant bubbling of air. At the end of the incubation, the contents of each flask were poured into 200 ml of ethanol and the resulting suspension was filtered. The filtrate was evaporated under vacuum to remove the alcohol and the remaining aqueous solution was extracted exhaustively with ether. The ether extracts were washed with 0.05 N NaOH and water and then dried over anhydrous sodium sulfate.

Isolation of Pregnenolone. From incubation A, the residue left after removal of the ether was chromatographed on 30 g of Celite using the reversed-phase system I (0.3-ml stationary phase/g of Celite). Most of the radioactivity (1.4×10^6 cpm) was eluted in the first holdback volume (more polar than 20α-hydroxycholesterol). In addition, 70,000 cpm of ^3H was eluted in the region corresponding to the substrate.

The more polar fraction was chromatographed on 30 g of Celite using system D. A peak of radioactivity (957,000 cpm of ^3H) was eluted in the fifth and sixth holdback volumes (fraction I) and a second quantity of radioactive material (436,000 cpm) was found in the methanol washes (fraction 2). To a 10% aliquot of fraction I was added 20 mg of carrier pregnenolone and the mixture was crystallized from methanol-acetone to constant specific activity. The crystallization data are presented in Table III. From the isotope data, it appears that the labeled 20α-hydroxycholesterol had been converted into the product, pregnenolone, in almost 50% yield.

TABLE III: Isotopic Data of Pregnenolone Formed from Sterol Precursors by Mitochondria.

Incubation	Specific Activity (cpm/mg)			
	A ^3H	B ^{14}C	C ^3H	C ^{14}C
Crystallization 1	4380	4850	2840	4760
Mother liquor 1	4490	4590	2950	4730
Crystallization 2	4340	4600	2920	4770
Mother liquor 2	4630	4710	2980	4910

Fraction 2 (436,000 cpm of ^3H) was rechromatographed on 30 g of Celite using system P. This system is of somewhat different design in that a stepwise gradient elution pattern is employed. From previous experience, pregnenolone was found to be eluted in the first holdback volume, dehydroisoandrosterone in the second and third holdback volumes, 17-hydroxypregnenolone in the sixth and seventh holdback volumes, while pregnenetriol and androstenediol are eluted with pure benzene. Radioactive materials were found in the first, second, sixth, seventh, and twelfth holdback volumes but no effort was made to identify any of these. As later experiments revealed, most of the radioactivity appearing in the sixth and seventh holdback volumes was probably associated with 17-hydroxy pregnenolone.

From incubation B, the residue remaining after removal of the ether was chromatographed on 30 g of Celite using the reversed-phase system A. The polar metabolites were eluted in the first holdback volume (0.31×10^6 cpm of ^{14}C) while the unreacted substrate (1.5×10^6 cpm of ^{14}C) appeared in the third and fourth holdback volumes. The polar products were rechromatographed on Celite system D (0.3 ml of stationary

phase/g of Celite) from which radioactive material (0.23×10^6 cpm of ^{14}C, presumably pregnenolone, was eluted in the sixth and seventh holdback volumes. In the methanol washes, 44,000 cpm of ^{14}C was eluted. Carrier pregnenolone (20 mg) was added to one-half of the radioactive material eluted in the sixth and seventh holdback volumes and the mixture was crystallized to constant specific activity (Table III). The data showed that cholesterol had been converted into pregnenolone in approximately 10% yield.

The methanol washes, when chromatographed on Celite system P, exhibited a pattern of polar metabolites similar to that obtained when 20α-hydroxycholesterol was the substrate (incubation A). The metabolites were not identified.

For incubation C, the residue remaining after removal of the ether was chromatographed on 30 g of Celite using reversed-phase system A. Those metabolites that are more polar than cholesterol were eluted in the first holdback volume (277,000 cpm of ^3H, 332,600 cpm of ^{14}C, $^3H/^{14}C = 0.83$ (fraction C1)). The unconsumed cholesterol was eluted in the third and fourth holdback volumes (1.47×10^6 cpm of ^{14}C). The polar fractions from the reversed-phase system (fraction C1) were chromatographed on system D (0.5 ml of stationary phase/g of Celite) from which radioactive material corresponding to 20α-hydroxycholesterol (6250 cpm of ^3H and 675 cpm of ^{14}C) was eluted in the second and third holdback volumes (fraction C2). To determine whether the ^{14}C was, in fact, associated with the reisolated tritiated 20-hydroxy compound, the eluate was acetylated and rechromatographed on Celite system A. The hydroxysterol acetate, eluted in the third holdback volume, was found to be devoid of the ^{14}C label.

A second radioactive compound, corresponding in polarity to pregnenolone, was eluted in the fifth and sixth holdback volumes (fraction C3) (147,760 cpm of ^3H and 239,260 cpm of ^{14}C, $^3H/^{14}C = 0.62$). To a 50% aliquot of this pregnenolone fraction was added 20 mg of authentic compound. This mixture was crystallized to constant specific activity with respect to both isotopes and the data are also presented in Table III. From these data, it is evident that when [^{14}C]cholesterol alone was incubated with sonicated mitochondria, pregnenolone was obtained in a yield four times that achieved when the mixture, cholesterol and 20α-hydroxycholesterol served as substrates. Thus, under identical incubation conditions and using identical methods of isolation, the conversion of cholesterol into pregnenolone was inhibited approximately 75% in the presence of a tracer quantity (7 μg) of 20α-hydroxycholesterol. This finding is in agreement with the data of Hall and Koritz (1964) who reported that minute quantities of 20α-hydroxycholesterol inhibited the conversion of cholesterol into pregnenolone by some 70%. These authors also reported that they were unable to reisolate labeled 20α-hydroxycholesterol when labeled cholesterol was used as substrate, even though radioactive pregnenolone was obtained in good yield.

EXPERIMENT 2. The previous experiment failed to provide evidence for the intermediacy of 20α-hydroxycholesterol in the conversion of cholesterol into pregnenolone since little of the substrate, the 20-hydroxysterol, was recovered from the incubation mixture. Since the time of incubation in expt 1 was 15 min, expt 2 was conducted for shorter times so that the substrate could be recovered in amounts sufficient for the accurate estimation of ^{14}C. The method of preparation of the mitochondria, obtained from sixteen adrenal glands, and the added cofactors were the same as those used in expt 1.

TABLE IV: Isotopic Data of 17-Hydroxypregnenolone Formed from Sterol Precursors by Mitochondria.

Incubn Time (min)		Sp Act. (cpm/mg)		
		^3H	^{14}C	$^3H/^{14}C$
3	Crystallization 1	1680	110	15.3
	Mother liquor 1	1880	130	14.5
	Crystallization 2	1670	110	15.2
	Mother liquor 2	1730	110	15.7
6	Crystallization 1	2720	260	10.5
	Mother liquor 1	3110	280	11.1
	Crystallization 2	2690	270	10.0
	Mother liquor 2	2850	270	10.6

Incubation 2A was carried out with half of the mitochondrial preparation and with 20α-hydroxycholesterol-16-^3H (45 μg, 1.9×10^6 cpm) and cholesterol-4-^{14}C (22 μg, 4.2×10^6 cpm) as substrates. The time of incubation was 3 min and the volume of each incubate was approximately 35 ml.

Incubation 2B was carried out under the same conditions except that the incubation time was 6 min. The initial purification procedures for each incubate were identical with those described previously. Each sample was chromatographed on 30 g of Celite using the reversed-phase system F; approximately 75 ml of mobile phase was collected. This fraction contains those metabolites that are more polar than cholesterol.

In incubation 2A the material eluted from the reversed-phase column with 80% methanol was rechromatographed on 150 g of Celite using system D. A peak of radioactive material (743,000 cpm ^3H and 1100 cpm of ^{14}C), corresponding to unused substrate, was eluted in the third holdback volume. Crystallization of this material with 50 mg of carrier 20α-hydroxycholesterol completely eliminated all traces of the ^{14}C label. A second peak of radioactive material (457,000 cpm of ^3H, and 32,700 cpm of ^{14}C), representing doubly labeled pregnenolone, was eluted in the fifth and sixth hold back volumes. Its identity was not further ensured by additional procedures. The more polar metabolites, obtained by washing the column with methanol, were chromatographed on 30 g of Celite using system P. A radioactive compound (115,000 cpm of ^3H and 7400 cpm of ^{14}C), corresponding to 17-hydroxypregnenolone, was eluted with benzene 40%; ligroin 60%. Following the addition of 50 mg of carrier, this substance was recrystallized twice from methanol. The

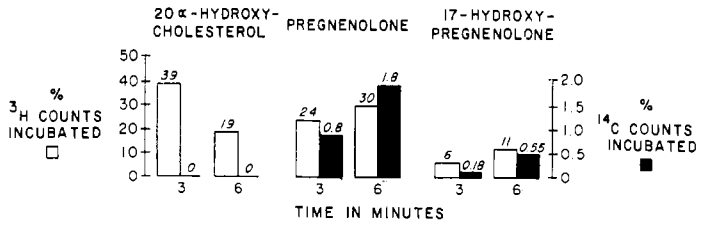

FIGURE 3: Radioactive products formed by incubation of sonicated bovine adrenal mitochondria with 20α-hydroxycholesterol-16-^{3}H (1×10^{6} cpm) and cholesterol-4-^{14}C (1×10^{6} cpm).

crystallization data, presented in Table IV, appear to establish the identity of the doubly labeled metabolite. In addition, at least seven other radioactive metabolites, more polar than pregnenolone, were located by analysis of the fractions from system P but no attempts were made to identify any.

In incubation 2B isolation and purification of the radioactive products were achieved by procedures identical with those used in incubation 2A. Crystallization data of the isolated 17-hydroxypregnenolone are also recorded in Table IV. Figure 3 summarizes the radioactivity associated with the reisolated substrate and its two metabolites following Celite partition chromatography. It is important to note that in each case, the ^{14}C associated with the reisolated 20α-hydroxycholesterol was completely removed by crystallization following the addition of authentic carrier.

Incubation of 20α-Hydroxycholesterol-16-^{3}H Sulfate-^{35}S with Sonicated Bovine Adrenal Mitochondria. Fresh bovine adrenal glands, after removal of the surrounding fat, weighed 28 g (wet weight). The sample was homogenized twice, once in 80 ml of 0.25 M sucrose solution in a Waring blender and then in 10-ml portions in an all-glass homogenizer. The procedures for isolation, purification, and sonication of the mitochondrial fraction were identical with those described previously. Doubly labeled 20α-hydroxycholesterol-16-^{3}H sulfate-^{35}S (approximately 230 μg, 2.25×10^{6} cpm of ^{3}H and 1.21×10^{6} cpm of ^{35}S, ^{3}H/^{35}S = 1.86) was added to the mitrochondrial preparation and this mixture, along with added cofactors (Simpson and Boyd, 1966) was incubated for 70 min in phosphate buffer at pH 7.4 (volume of incubate = 42 ml). The incubate was poured into three volumes of ethanol and the precipitated proteins were removed by filtration. The residue remaining after removal of the solvent was chromatographed on the reversed-phase Celite (50 g) partition system A. The substrate and polar metabolites were eluted in the first holdback volume. The combined fractions comprising the first holdback volume were chromatographed on Celite (50 g) system M from which the unreacted substrate (approximately 10% of original radioactivity incubated) was eluted in the the fourth and fifth holdback volumes. After collection of the tenth holdback volume the mobile phase was changed to that of system Q in an attempt to elute the polar metabolites without eluting the colored impurities. Immediately following the change to the more polar mobile phase, a peak of radioactive material containing 1.67×10^{6} cpm of ^{3}H and 0.925×10^{6} cpm of ^{35}S (^{3}H/^{35}S = 1.80) was eluted. This material was rechromatographed on Celite system Q yielding a major peak of radioactivity in the second and third holdback volumes (1.42×10^{6} cpm of ^{3}H and 0.793×10^{6} cpm of ^{35}S; ^{3}H/^{35}S = 1.79). Rechromatography of this fraction on Celite system R yielded a peak of radioactivity in the fourth and fifth holdback volumes (1.12×10^{6} cpm of ^{3}H and 0.66×10^{6} cpm of ^{35}S; ^{3}H/^{35}S = 1.7). To a 50% aliquot of this radioactive material was added 50 mg of ammonium pregnenolone sulfate and the mixture was crystallized twice from methanol and ether. The specific activities with respect to both ^{3}H and ^{35}S of the products of the two crystallizations were identical (second crystallization: 10,190 cpm of ^{3}H/mg and 5080 cpm of ^{35}S/mg and ^{3}H/^{35}S = 2.01; mother liquor from second crystallization: 9954 cpm of ^{3}H/mg and 4918 cpm of ^{35}S/mg; ^{3}H/^{35}S = 2.02). These ^{3}H/^{35}S ratios agreed well with the isotope ratio (2.05) of an aliquot of the substrate that had been set aside as a control to determine the degree of decay of the ^{35}S during the course of the experiment. The approximate yield of pregnenolone sulfate from 20α-hydroxycholesterol sulfate was calculated to be over 50% without taking into account the losses incurred during the purification procedures.

Discussion

The evidence presented in this paper strongly indicates that 20α-hydroxycholesterol is a constituent of adrenal tissue. Proof is based on radiochemical techniques that are generally regarded as acceptable since they approach the certainty of identification by the elimination of other reasonable possibilities. Obviously, such indirect proof is less satisfying than traditional methods of identification such as spectral data (mass, infrared, or nuclear magnetic resonance) but the microgram quantities present in the 5000-g sample of adrenal glands processed precluded the use of these techniques. Attempts were made to obtain the mass spectra and the infrared spectra of some of the fractions but none of these was sufficiently uncontaminated with impurities to permit unequivocal identification by these spectroscopic methods. Nevertheless, the isotope dilution techniques used in this method are powerful tools and have made possible the detection of trace amounts of this compound.

In recent years, two other groups of investigators have also presented evidence for the presence of unsaturated dihydroxy sterols in extracts obtained from bovine adrenal cortex. In neither case, however, is it possible to state expressly that these investigators were

dealing with 20α-hydroxycholesterol. Cargill and Cook (1964) isolated an enediol which was completely separable from 7α-hydroxycholesterol, 25-hydroxycholesterol, and 26-hydroxycholesterol. The amounts found, however, were about ten times the concentration of 20α-hydroxycholesterol found in our study. In addition, Riley (1963) also reported the occurrence of a hydroxysterol in the fatty acid ester fractions obtained from human adrenal glands.

Although suggestive, the isolation of 20α-hydroxycholesterol from extracts of adrenal glands does not, itself, constitute proof that it is an intermediate in the conversion of cholesterol into pregnenolone. The case for its intermediacy would have been immeasurably strengthened had the C_{27} enediol been recovered in radioactive form, labeled with both tritium and ^{14}C following the incubation of tritium-labeled 20α-hydroxycholesterol and [^{14}C]cholesterol with adrenal mitochondria. The compound was, however, isolated devoid of ^{14}C and this failure to "trap" the isotope confirms the results of Hall and Koritz (1964) and of Simpson and Boyd (1967). On the other hand, Ichii *et al.* (1967) recently reported that doubly labeled 20α-hydroxycholesterol had been recovered from acetone powders of hog adrenal mitochondria when incubated with [^{14}C]cholesterol as substrate and ^{3}H-labeled 20α-hydroxycholesterol as trapping agent. Radiochemical homogeneity was established solely on the basis of constant $^{3}H/^{14}C$ ratios following thin-layer and paper chromatography. An earlier report by Ichii *et al.* (1963) also described the accumulation of radioactivity within the 20α-hydroxycholesterol "zone" following incubation of labeled cholesterol with bovine corpus luteum. In this experiment, also, paper chromatography was the method of purification. In our experience, some ^{14}C, derived from ^{14}C-labeled cholesterol, is usually associated with the reisolated 20α-hydroxysterol during the initial stages of purification but this isotope is removed by the application of more rigorous purification procedures. Because of the discrepant results, it remains questionable whether such trapping experiments have provided evidence for the intermediacy of this hydroxysterol. It has been suggested that the failure to accumulate isolable amounts of 20α-hydroxycholesterol or 20α,22ξ-dihydroxycholesterol, in the experiment cited above, is due to the fact that these intermediates are bound to enzymes and, therefore, are not free to mix with these sterols when they are added exogenously (Hall and Koritz, 1964; Simpson and Boyd, 1967). Support for this idea came from the observation that no radioactivity was found in the reisolated 20α-hydroxycholesterol following the incubation of tritium-labeled cholesterol with acetone-dried powders of bovine adrenal mitrochondria using nonradioactive 20α-hydroxycholesterol as a trap. The trapping agent was devoid of tritium although the product, pregnenolone, was labeled. The existence of enzyme-bound intermediates which do not accumulate and which cannot be trapped has been invoked by Lynen (1961) and Jones and Gutfreund (1964) to explain observations made on other biosynthetic processes.

Additional proof that 20α-hydroxycholesterol is an intermediate in the process, cholesterol → pregnenolone, is provided by those *in vitro* experiments which showed the facile conversion of this 20-hydroxysteroid into pregnenolone. From Figure 3, it appears that the formation of pregnenolone from this precursor occurred much more rapidly than it did when [^{14}C]cholesterol was the substrate. However, these results must be interpreted cautiously since they reflect differences only in the rate constants of the two processes. It is quite likely that large differences in the pool sizes of cholesterol and 20α-hydroxycholesterol exist and these alone could account for the observed differences in rates. It is clear, however, that 20α-hydroxycholesterol is, under these *in vitro* conditions, an excellent precursor of pregnenolone.

There are other data that have been obtained in this study that seem to be in conflict with previously published findings. The results of some of the incubations described above indicate that adrenal mitochondria are capable of effecting hydroxylation at C-17 and this is in contrast to previous observations. For example, in 1954, Plager and Samuels observed that 17-hydroxylase, present in bovine adrenal, is located in the "soluble" fraction, *i.e.*, the supernatant obtained by centrifugation of the homogenate at 20,000*g*. Hayano *et al.* (1956) were able to demonstrate conversion of cholesterol into cortisol using adrenal mitochondrial preparations but these workers believed that the 17 hydroxylation required for this conversion occurred as a result of contamination of the particulate fraction by the soluble 17-hydroxylase. Grant (1962) has reported the location of this enzyme in the endoplasmic reticulum. After the separation of the various cellular components of bovine adrenal cortical tissue by centrifugation, Young *et al.* (1965) had also reported that the steroid 17-hydroxylating system resides primarily in the particle-free cytoplasm. Activity was found neither in the mitochondria nor in the microsomal fraction.

The discrepancy between our findings and those of others may be due to the fact that the 17-hydroxylase substrate used in this study was a 3β-hydroxy Δ^5-steroid, pregnenolone (derived from another such steroid, cholesterol), whereas the other investigators who had studied this enzyme and had found it to be in the soluble fraction, had used an α,β-unsaturated ketone, progesterone, as substrate. Thus, there may be two enzymes one residing in the cytoplasm and using progesterone as substrate and the other associated with mitochondria and using pregnenolone as substrate. Alternately, this discrepancy may be related to the homogenization procedure to which the tissue had been subjected. It is likely that mitochondria could be sufficiently damaged by homogenization so that mitochondrial enzymes would be released into the soluble fraction. In our study, the integrity and purity of the mitrochondrial preparation was checked by electron microscopy prior to sonication. Although it cannot be stated that the preparation was devoid of microsomal contamination, the amount of contamination did not seem to be sufficiently large to account for the high yield (11%) of 17-hydroxypregnenolone. Repeated washing of the

mitrochondrial pellets, with sucrose solution and with isotonic KCl solution, was employed to remove the soluble enzymes. Our results, therefore, suggest that adrenal mitochondria do contain 17-hydroxylase activity.

Although the sulfate of the 20-hydroxysterol could not be found in bovine adrenal tissue, the metabolism of this conjugate by the mitochondrial preparation could be readily demonstrated. As described in a previous publication concerning the metabolism of cholesterol sulfate (Roberts *et al.* 1967), the disruption of the mitochondrial membrane by ultrasound was required for the efficient utilization of the sulfurylated derivative. It is especially noteworthy that, with these preparations, over 50% of the sulfurylated hydroxysterol was converted into pregnenolone.

Finally, mention should be made of a technical point in the preparation of labeled radioactive 20α-hydroxycholesterol. Previous publications by Shimizu *et al.* (1961), Tamaoki and Pincus (1961), Shimizu (1966), and by Ichii *et al.* (1963, 1967) all report the purification of synthetic labeled 20α-hydroxycholesterol by one or two paper chromatography systems. It has been our experience that the tritiated product, prepared by the method of Petrow and Stuart-Webb (1954) from labeled pregnenolone, is purified to radiochemical homogeneity only with great difficulty. As might be expected, the labeled hydroxysterol was contaminated with tritiated pregnenolone and the separation of the radioactive starting material from the product requires extraordinary care. Obviously, the presence of tracer amounts of tritiated pregnenolone in the sample of synthetic 20α-hydroxycholesterol would provide sufficient radioactivity to label carrier pregnenolone which, in incubation experiments, is added to facilitate the detection of the formation of the C_{21} product from the C_{27}-hydroxysterol.

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