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The Conversion of Cholesterol and 20α-Hydroxycholesterol to Steroids by Acetone Powder of Particles from Bovine *Corpus Luteum**

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Acetone-dried powder of "mitochondria" from bovine *corpus luteum* was shown to convert cholesterol and 20α-hydroxycholesterol to pregnenolone and progesterone. Reduced TPN was necessary for this conversion. Interstitial-cell-stimulating hormone *in vitro* was without demonstrable effect on the reaction. Percentage conversion of 20α-hydroxycholesterol to pregnenolone was considerably greater than that of cholesterol to pregnenolone. Evidence is also presented that 20α-hydroxycholesterol is an intermediate in the conversion of cholesterol to pregnenolone by acetone powder of *corpus luteum*.

Present evidence indicates that cholesterol is converted to steroids in the adrenal cortex by way of 20α-hydroxycholesterol which in turn undergoes side-chain cleavage to yield Δ^5-pregnenolone. Halkerston and co-workers (1961) have demonstrated the conversion of cholesterol-4-C^{14} to labeled products which behaved like pregnenolone-C^{14} and progesterone-C^{14} by means of acetone-dried powder of "mitochondria"[1] from bovine adrenal cortex. The conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} by acetone-dried powder of mitochondria from bovine adrenal has also been reported by Constantopoulos and Tchen (1961) and the conversion of 20α-hydroxycholesterol to pregnenolone has been demonstrated by Chanduri *et al.* (1962). These studies also demonstrated that side-chain cleavage of cholesterol requires reduced TPN[2] when intact mitochondria were used, although Constantopoulos and Tchen (1961) reported that soluble preparations derived from mitochondria could use either reduced DPN or reduced TPN. In homogenate of bovine *corpus luteum*, Tamaoki and Pincus (1961) reported that cholesterol is converted to progesterone and that 20α-hydroxycholesterol undergoes side-chain cleavage to yield isocaproic acid. ICSH was without effect on the side-chain cleavage of either cholesterol or 20α-hydroxycholesterol.

It has been shown that ICSH increases the production of progesterone by slices of bovine *corpus luteum in vitro* (Mason *et al.*, 1962). Earlier studies by Stone and Hechter (1954) suggested that ACTH stimulates steroid biosynthesis in the adrenal by promoting the conversion of cholesterol to pregnenolone. Moreover, ICSH causes a fall in the ascorbic acid content of *corpus luteum* in a manner analogous to the well-known adrenal ascorbic acid depletion produced by ACTH (Parlow, 1961). These observations suggest the possibility that the two trophic hormones may stimulate steroid biosynthesis in their respective target organs by means of a mechanism common to both. It was therefore considered important to demonstrate that "mitochondria" from *corpus luteum* convert cholesterol to pregnenolone and that 20α-hydroxycholesterol is an intermediate in this reaction, before detailed studies of the mechanism of action of ICSH upon luteal tissue were undertaken.

The present experiments were designed to demonstrate the capacity of acetone-dried powder of "mitochondria" from bovine *corpus luteum* to convert cholesterol and 20α-hydroxycholesterol to steroids and to characterize the products of this reaction.

Experimental Procedure

Preparation of Tissue.—Bovine ovaries were obtained fresh from a slaughterhouse. *Corpora lutea* were shelled out, stripped of fibrous tissue, and weighed. The tissue was finely chopped and homogenized in two volumes of sucrose (0.44 M) in an all-glass Potter-

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[1] The term "mitochondria" was used by Halkerston *et al.* (1961) to describe the pellet prepared in their experiments. However, these workers did not offer evidence for the exact nature of this pellet. The present authors use the term "mitochondria" in this paper to describe a pellet prepared from *corpus luteum* according to the instructions of Halkerston *et al.* (1961).

[2] The following abbreviations are used: DPN, diphosphopyridine nucleotide; ICSH, interstitial-cell-stimulating hormone; TPN, triphosphopyridine nucleotide; ACTH, adrenocorticotropic hormone; PPO, 2,5-diphenyloxazole; POPOP, 1,4-2-(5-phenyloxazolyl)-benzene.

Elvejham apparatus. Acetone-dried powder of "mitochondria" was then prepared according to the directions of Halkerston *et al.*, (1961). It was found that 90 g of *corpus luteum* yielded approximately 600 mg of acetone powder. Before incubation the powder was homogenized in 0.07 M potassium phosphate buffer (17 mg of powder per ml of buffer) at *p*H 6.8. It was found that the supernatant prepared by centrifuging the suspension of homogenized powder at 100,000 × *g* for 60 minutes showed little activity in converting 20α-hydroxycholesterol to pregnenolone, but that the whole suspension was highly active. The whole suspension was therefore used in the experiments to be reported.

Incubation Procedure.—Cholesterol, cholesterol-4-C^{14}, and 20α-hydroxycholesterol were added to 20-ml beakers in 0.02 ml ethanol and the ethanol was evaporated under nitrogen. The suspension of acetone powder and cofactors was then added as shown beneath the accompanying tables. The final volume was 2 ml, *p*H 6.8, and incubation was conducted for 2 hours at 37° with constant agitation in air. In the experiments reported here, three were performed with cholesterol-4-C^{14} as substrate (experiments 1–3) and three with cholesterol and 20α-hydroxycholesterol as substrates. Of the latter, experiment 4 is given in detail in the present paper. The substrates and cofactors for each experiment are described in Tables I and IV.

Extraction and Identification of Products.—(I) Cholesterol and 20α-Hydroxycholesterol as Substrates.—In experiments with cholesterol and 20α-hydroxycholesterol, 1.5 ml of the incubation mixture was removed at the end of incubation and examined colorimetrically for pregnenolone by the furfural procedure as described in detail elsewhere (Koritz, 1962). The minimal amount of pregnenolone which can be measured by this method under the conditions reported here is 0.6 μg.

(II) Cholesterol-4-C^{14} as Substrate.—In experiments with cholesterol-4-C^{14}, at the end of incubation the whole incubation mixture was extracted three times with methylene chloride (three volumes). The combined extracts were washed four times with water, dried over sodium sulfate, and taken to dryness under nitrogen. In order to facilitate identification of the labeled steroids, progesterone (100 μg) was added to the extract which was then applied to paper in the system ligroin–prophylene glycol for 10 hours (Brady, 1951). On either side of the strips containing the extracts, a standard chromatogram of pregnenolone (100 μg) was run. The dry chromatograms of the extracts were examined by means of a Haines ultraviolet scanner to locate the carrier progesterone (Haines and Drake, 1950) and then by means of a thin-window Geiger strip-counter. The strips bearing standard pregnenolone were examined by means of an ethanol–sulfuric acid reaction which produces a brown color in the presence of pregnenolone (Oertel and Eik-Nes, 1959).

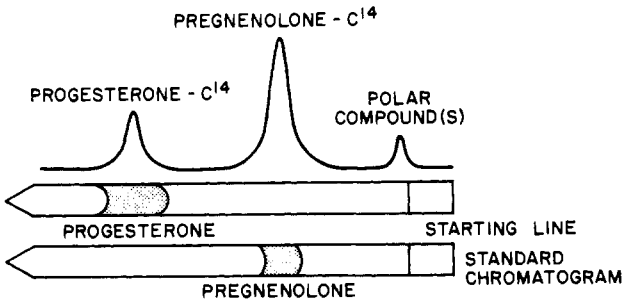

FIG. 1.—Diagram of the recording of radioactivity on a typical paper chromatogram. The relative areas of the three peaks are indicated as well as the position of progesterone on the chromatogram as revealed by the Haines scanner (Haines and Drake, 1950) and that of pregnenolone on an adjacent standard chromatogram as revealed by the ethanol-sulfuric acid reaction. (Oertel and Eik-Nes, 1959).

Trapping Experiments.—In two experiments 20α-hydroxycholesterol was used as a trapping agent. This compound (5–60 μg/beaker) was dissolved in ethanol and added to the incubation beaker. The ethanol was evaporated under nitrogen before the remaining contents of the beaker was added.

Measurement of Radioactivity.—Suitable aliquots of samples in which C^{14} was to be measured were evaporated to dryness in 20-ml vials and dissolved in 10 ml of scintillation fluid prepared by dissolving 5 g of 2,5-diphenyloxazole (PPO) and 30 mg of 1,4-2-(5-phenyloxazolyl)-benzene (POPOP) in 1 liter of reagent grade toluene. Radioactivity of samples was counted in a Packard Tri-carb Model 314 liquid scintillation spectrometer. Sufficient counts were allowed to accumulate to give a probable error of less than 5%. The addition of a standard solution of toluene-C^{14} showed that no significant quenching occurred in the measurements to be reported here.

Gas Chromatography.—Samples were examined by gas chromatography on a phase consisting of Hi-Eff-8B[3] (cyclohexane dimethanol polysuccinate) supported by Gas-Chrom P, acid and base washed and silicone-treated (80–100 mesh). The column was 8 feet by 1 inch in an F & M Model 609 chromatograph with a hydrogen flame detector. Column temperature was 220°, flash temperature was 280°, and detector temperature was 250° with argon as carrier gas. Under these conditions pregnenolone showed a retention time of 12 minutes and cholesterol of 24 minutes. Measurement of mass was performed by integration of the area under the appropriate curve and comparison with the areas of peaks corresponding to known amounts of authentic compounds. Determinations were performed in duplicate and in each case the duplicate readings agreed within 5%.

Sources of Substrates and Cofactors.—Cholesterol-4-C^{14} was purchased from New England Nuclear Corporation and aliquots were chromatographed on each of the two following systems of paper chromatography: (1) ligroin–propylene glycol (Brady, 1951); (2) kerosene–60% aqueous *N*-propanol (Halkerston *et al.*, 1961). The dry chromatograms were examined by means of a thin-window Geiger strip-counter which revealed that the aliquots examined showed less than 0.1% of radioactive contaminants.

Cholesterol was purchased from Pfanstiehl Laboratories and showed a melting point of 146.5–148°; this melting point did not change after three recrystallizations.

20α-Hydroxycholesterol was a gift from Dr. Marcel Gut of the Worchester Foundation, Shrewsbury, Massachusetts. The substances TPN, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Company. Interstitial-cell-stimulating hormone was provided by the Endocrine Study Section of the National Institutes of Health in the form of the preparation NIH-LH-S-4.

RESULTS

Cholesterol and Cholesterol-4-C^{14} as Substrates.—When chromatograms prepared as described under Experimental Procedure, following the incubation of acetone powder from bovine *corpus luteum* with cholesterol-4-C^{14}, were examined by means of a strip-counter, three peaks of radioactivity were revealed (Fig. 1):

[3] Applied Science Corporation, State College, Pennsylvania.

TABLE I

THE CONVERSION OF CHOLESTEROL-4-C^{14} TO PREGNENOLONE-C^{14} AND PROGESTERONE-C^{14} BY ACETONE-DRIED POWDER OF *Corpus Luteum*[a]

Experiment	Additions	Pregnenolone-C^{14} (dpm)	Progesterone-C^{14} (dpm)
1	TPN + reducing system	23,600[b]	4,700
	TPN + reducing system	16,800[b]	3,600
	TPN + reducing system + ICSH (10 μg)	17,200	3,900
	TPN + reducing system + ICSH (10 μg)	18,800	4,000
2	—	<20	<20
	—	<20	<20
	TPN + reducing system	37,400	5,000
	TPN + reducing system	40,000	5,600
	TPN + reducing system + 20α-OH cholesterol 5 μg	24,000	2,000
	TPN + reducing system + 20α-OH cholesterol 5 μg	24,800	1,700
	TPN + reducing system + 20α-OH cholesterol 20 μg	6,000	300
	TPN + reducing system + 20α-OH cholesterol 20 μg	4,800	200
3	TPN + reducing system	170,000	32,000[c]
	TPN + reducing system	164,000	32,400[c]
	TPN + reducing system + 20α-OH cholesterol 60 μg	4,200	600
	TPN + reducing system + 20α-OH cholesterol 60 μg	4,800	600

[a] The incubation medium contained 0.5 ml of acetone powder suspension in 0.07 M phosphate buffer, 0.5 ml of 0.07 M phosphate buffer, *p*H 6.8, 5×10^{-4} M TPN, 5×10^{-3} M glucose 6-phosphate, approximately 0.1 Kornberg units glucose 6-phosphate dehydrogenase and 0.154 M KCl to a final volume of 2.0 ml. Incubation was for 2 hours in air. Cholesterol-4-C^{14} (0.77 μg, 3.8×10^5 dpm in experiment 1, and 7.7 μg, 3.8×10^6 dpm in experiments 2 and 3) were added as described under Experimental Procedure. [b] The material from these flasks was recrystallized as shown in Table II. [c] The material from these flasks was recrystallized as shown in Table III.

(1) an unidentified compound(s) at the origin (this was not further examined); (2) a peak opposite the pregnenolone on the standard chromatograms; (3) a peak corresponding to the position of carrier progesterone.

When known amounts of pregnenolone-7α-H^3 and progesterone-4-C^{14} were separately added to heated tissue, recovery of the tritium was 84–86% and of C^{14} 76–82% after extraction and chromatography by the method described above. The data reported here are not corrected for these recoveries.

It can be seen from Table I that cholesterol-4-C^{14} was converted by acetone powder of luteal "mitochondria" to labeled compounds which behaved like pregnenolone-C^{14} and progesterone-C^{14}. The identity and radiochemical purity of these products is established by the data shown in Tables II and III, respectively. These tables show that recrystallization of the labeled compounds in the presence of the authentic steroids reveals a constant specific activity when crystals were allowed to form from four different solvent systems. Moreover, the fact that the specific activities fell very little at the first recrystallization and the specific activities of pregnenolone-C^{14} and progesterone-C^{14} in the respective mother liquors closely approximated those of the associated crystals not only offers strong evidence for the identity of the labeled compounds but demonstrates that they were highly purified after chromatography in ligroin–propylene glycol. The extent of conversion of cholesterol-4-C^{14} to pregnenolone is approximately 2% and to progesterone 0.4%. When incubation was conducted under the same conditions using 60 μg of cholesterol per flask instead of

TABLE II

RECRYSTALLIZATION OF PREGNENOLONE-C^{14} TO CONSTANT SPECIFIC ACTIVITY[a]

Source of Pregnenolone-C^{14}	Stage of Purification	Solvent	Pregnenolone-C^{14} Crystals Total Activity (dpm)	Crystals Specific Activity (dpm/mg)	Mother Liquor Total Activity (dpm)	Mother Liquor Specific Activity (dpm/mg)
Experiment 1 Sample marked[b]	After addition of pregnenolone	—	23,600	998	—	—
	After recrystallization: 1st	Ethyl acetate–ligroin	17,100	1,040	2,200	996
	2nd	Aqueous ethanol	12,600	929	4,500	891
	3rd	Acetone-hexane	9,900	1,000	2,000	972
	4th	Chloroform-hexane	7,200	1,040	400	975
Experiment 1 Sample marked[b]	After addition of pregnenolone	—	16,800	700	—	—
	After recrystallization: 1st	Ethyl acetate–ligroin	11,500	694	1,000	707
	2nd	Aqueous ethanol	6,900	680	4,000	969
	3rd	Acetone-hexane	4,100	692	400	660
	4th	Chloroform-hexane	2,100	713	4,100	667

[a] The areas of the chromatograms from the samples marked as shown in experiment 1 (Table I) were eluted, dried under nitrogen, and recrystallized after addition of authentic pregnenolone (25 mg). After each recrystallization from the solvents shown, mother liquors were carefully removed, and the crystals were washed and dissolved in chloroform. From appropriate aliquots pregnenolone was determined by an ethanol–sulfuric acid color reaction (Oertel and Eik-Nes, 1959) and radioactivity was determined by liquid scintillation spectrometry. [b] See Table I.

TABLE III

RECRYSTALLIZATION OF PROGESTERONE-C^{14} TO CONSTANT SPECIFIC ACTIVITY[a]

Source of Progesterone-C^{14}	Stage of Purification	Solvent	Progesterone-C^{14} Crystals: Total Activity (dpm)	Crystals: Specific Activity (dpm/mg)	Mother Liquor: Total Activity (dpm)	Mother Liquor: Specific Activity (dpm/mg)
Experiment 3 Sample marked[b]	After addition of progesterone	—	32,400	1,310	—	—
	After recrystallization: 1st	Acetone-hexane	22,800	1,326	11,300	1,218
	2nd	Ethyl acetate–ligroin	9,400	1,286	7,500	1,243
	3rd	Aqueous methanol	4,400	1,294	6,200	1,240
	4th	Chloroform-hexane	2,400	1,263	5,800	1,280
Experiment 3 Sample marked[b]	After addition of progesterone	—	32,000	1,322	—	—
	After recrystallization: 1st	Acetone-hexane	20,000	1,316	8,800	1,321
	2nd	Ethyl acetate–ligroin	13,200	1,294	1,100	1,240
	3rd	Aqueous methanol	8,000	1,274	3,200	1,216
	4th	Chloroform-hexane	6,400	1,272	3,400	1,261

[a] The method of recrystallization is given in Table II. [b] See Table I, footnote *c*.

cholesterol-4-C^{14} as substrate, less than 0.6 μg of pregnenolone per flask was formed (i.e., less than the minimal amount which can be measured by the furfural colorimetric procedure used here). It should be pointed out, however, that the present studies were not designed to explore factors leading to optimal conversion. In the adrenal cortex addition of certain cofactors and changes in the incubation procedure greatly affect the yield of pregnenolone (Halkerston *et al.*, 1961). The present rate of conversion may therefore be well below the maximum possible for such an acetone powder. It is clear that the rate of conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} shown in Table I is too low to produce amounts of pregnenolone detectable by the furfural method of assay used here.

It can also be seen from Table I that reduced TPN is required for the conversion of cholesterol-4-C^{14} to steroids and that ICSH exerted no stimulation upon this conversion. In another experiment, not reported here, it was shown that TPN and fumarate were as effective as TPN and glucose 6-phosphate with glucose 6-phosphate dehydrogenase in stimulating this reaction. It was also shown that Mg^{++} was without significant effect on the conversion of cholesterol-4-C^{14} to pregnenolone-C^{14}.

20α-Hydroxycholesterol as Substrate.—It can be seen from Table IV that acetone-dried powder of luteal "mitochondria" was capable of converting 20α-hydroxycholesterol to pregnenolone. When this substrate (60 μg/flask) was incubated under the same conditions as those used for cholesterol, 8.4–9.2 μg of pregnenolone per flask was formed. It is clear that the conversion of 20α-hydroxycholesterol to pregnenolone is considerably greater than that of cholesterol, since, as indicated above, no pregnenolone (< 0.6 μg) was detected when cholesterol (60 μg) was used as the substrate. Similar results were obtained in two subsequent experiments conducted exactly as described for experiment 4 (Table IV). Mg^{++} had no significant effect on the conversion of 20α-hydroxycholesterol to pregnenolone.

TABLE IV

THE CONVERSION OF 20α-HYDROXYCHOLESTEROL TO PREGNENOLONE BY ACETONE-DRIED POWDER OF MITOCHONDRIA FROM *Corpus Luteum* (experiment 4)[a]

Additions	Pregnenolone (μg/flask)
20α-Hydroxycholesterol omitted	<0.6
20α-Hydroxycholesterol omitted	<0.6
TPN omitted	<0.6
Complete system	8.4
Complete system	8.8
Complete system	9.2

[a] The conditions of incubation and additions were those given in Table I except that the substrate was 20α-hydroxycholesterol (60 μg/flask) instead of cholesterol-4-C^{14}. Pregnenolone was measured by the furfural procedure (Koritz, 1962).

Proof of the Identity of the Compounds Measured.—(I) Experiment with 20α-Hydroxycholesterol.—In order to establish the identity of the major product of side-chain cleavage (pregnenolone), methylene chloride extracts from thirty flasks each containing 60 μg of 20α-hydroxycholesterol and incubated as described in Table I were pooled, washed four times with water, dried over sodium sulfate, and applied to paper in the system ligroin–propylene glycol as before. On either side of this chromatogram a standard chromatogram of a mixture of pregnenolone (100 μg) and progesterone (100 μg) was run. The positions of the two steroids on the standard strips were determined as before, and the corresponding areas of the strip to which the extract was applied were cut out and eluted with methanol. The eluates were applied to small columns of deactivated aluminum oxide[4] (600 mg/column) and eluted with chloroform. Aliquots of the material corresponding to authentic pregnenolone were examined before and after chromatography by several procedures designed to characterize this material (see Table V) and the remainder was submitted to infrared spectroscopy (Fig. 2). An aliquot of the material eluted from the area of the chromatogram corresponding to progesterone was examined for absorbance at 240 mμ, but no further attempt was made to characterize this material. It was found that 48.3 μg progesterone was recovered from this chromatogram.

(II) Experiments with Cholesterol-4-C^{14}.—From the chromatograms of the methylene chloride extracts the areas corresponding to pregnenolone and progesterone were eluted and an aliquot of the eluate examined by liquid scintillation spectrometry. The specimens were then submitted to recrystallization from a number of different solvent systems following the addition of authentic pregnenolone and progesterone (Tables II and III).

[4] Merck Aluminum oxide for chromatography deactivated according to the procedure of Schneider *et al.* (1957).

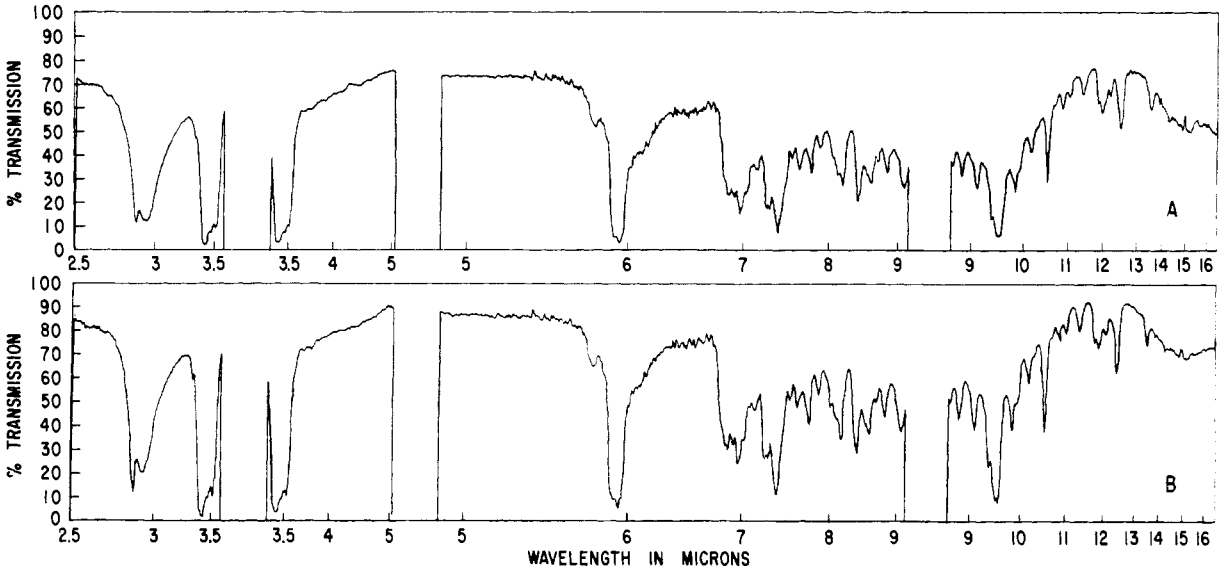

FIG. 2.—Infrared spectra of (A) authentic pregnenolone and (B) material isolated following incubation of 20α-hydroxycholesterol with acetone-dried powder of "mitochrondria" from beef *corpus luteum*. Both the standard and the sample were transferred to vials in chloroform and dried under nitrogen. Small samples were removed from each and the following melting points were determined with the aid of a microscope: authentic pregnenolone, 188°; sample, 186°; mixed melting point, 186°. The sample was prepared in a micro pellet of potassium bromide and the spectra were recorded by means of a Beckman Model 1R-7 infrared spectrophotometer with microbeam attachment by Dr. Henry M. Fales, National Institutes of Health, Bethesda, Maryland.

TABLE V

EXAMINATION AND MEASUREMENT OF PREGNENOLONE ISOLATED FOLLOWING INCUBATION OF ACETONE POWDER OF BOVINE LUTEAL "MITOCHONDRIA" WITH 20α-HYDROXYCHOLESTEROL[a]

Source of Aliquot Examined	Method of Measurement	Total Pregnenolone (μg)
Aliquot of original extract	Furfural reaction[b]	285
Aliquot after 1st chromatogram	Same[b]	248
Same	Ethanol–sulfuric acid reaction[c]	242
Same	Gas-liquid chromatography	238
Aliquot after 2nd chromatogram	Ethanol–sulfuric acid reaction[c]	207
Same	Gas-liquid chromatography	203

[a] Extracts from thirty beakers in which the acetone powder was incubated with 20α-hydroxycholesterol (60 ug/beaker) were pooled and the first aliquot shown in the table was examined for pregnenolone. The extract was then applied to paper in the system ligroin–propylene glycol (first chromatogram) as described in the text, and the 2nd, 3rd, and 4th aliquots were examined. An aliquot of the extract was then applied to paper in the system hexane-formamide (second chromatogram), and the 5th and 6th aliquots were examined as shown in the table. The remainder of the extract was submitted to infrared spectroscopy. [b] Koritz (1962). [c] Oertel and Eik-Nes (1959).

The cholesterol content of the acetone-dried powder was examined by extracting the same volume of the buffer suspension as that used in each beaker with acetone-alcohol-ether (4:4:1, v/v). The dry extract was examined by gas-liquid chromatography. Under the conditions used 0.01 μg of cholesterol could be detected. It was shown that one-half of the extract contained no demonstrable cholesterol (i.e., less than 0.01 μg).

DISCUSSION

The present findings indicate that acetone-dried powder of "mitochondria" from bovine *corpus luteum* is capable of converting cholesterol to pregnenolone and progesterone, thus demonstrating that in this respect luteal tissue behaves like adrenal cortex. The extent of this conversion was limited to 2%, but as mentioned, conditions for optimal conversion have not been explored. However, it is clear from the studies reported here that cholesterol-4-C^{14} was not diluted by endogenous cholesterol to any significant extent, since the acetone powder contained less than 0.02 μg of cholesterol per flask. The present preparation, like that reported by Halkerston *et al.* (1961) from adrenal cortex, requires reduced TPN. Detailed studies of the cofactor requirements and conditions for this reaction are in progress.

It has also been shown in these experiments that 20α-hydroxycholesterol is converted to pregnenolone and a compound behaving like progesterone. Moreover, the trapping experiments reported in Table I (experiments 2 and 3) show that the conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} and progesterone-C^{14} is considerably decreased by addition of 20α-hydroxycholesterol. This suggests that not only is 20α-hydroxycholesterol converted to pregnenolone, but that this compound is an intermediate between cholesterol and pregnenolone in steroid biosynthesis by bovine *corpus luteum*.

The conversion of cholesterol to pregnenolone is considerably less than the conversion of 20α-hydroxycholesterol, since 60 μg of cholesterol yields less than 0.6 μg of pregnenolone per flask, while 60 μg of 20α-hydroxycholesterol yielded 8–9 μg of pregnenolone per flask. Although it is not possible to make a strict comparison between these two substrates, since in both cases it is likely that 60 μg is far in excess of the maximal amount of substrate which can be converted by the tissue under the conditions used, it is nevertheless clear that conversion of 20α-hydroxycholesterol is considerably greater than that of cholesterol. It seems that three explanations for the observed differ-

ence in conversion can be offered, namely: (1) greater solubility of 20α-hydroxycholesterol than cholesterol in the incubation medium; (2) selective destruction during the preparation of acetone-dried powder of the enzyme system responsible for the conversion of cholesterol to 20α-hydroxycholesterol, leaving the systems involved in the conversion of 20α-hydroxycholesterol to pregnenolone relatively intact; (3) the conversion of cholesterol to 20α-hydroxycholesterol is rate-limiting for steroid synthesis, at least in luteal tissue.

The first possibility cannot be excluded at present. However, the production of pregnenolone from 20α-hydroxycholesterol (9.2 μg/beaker, Table IV), is at least fifteen times greater than the production of pregnenolone from cholesterol (<0.6 μg/beaker). Therefore, unless 20α-hydroxycholesterol is at least fifteen times as soluble in the medium used as is cholesterol, the present findings cannot be completely attributed to a difference in solubility of the two substrates. Moreover, the conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} was 2% when only 0.77 μg of cholesterol-4-C^{14} was used (Table I), whereas when 20α-hydroxycholesterol (60 μg/beaker) was used as substrate under conditions in which the solubility of this compound in the medium was exceeded, 9.2 μg of pregnenolone was found, representing a minimal conversion of 15%.

Although the second alternative cannot be entirely excluded by the present findings, the conversion of cholesterol-4-C^{14} to pregnenolone-C^{14} and progesterone-C^{14} shows that some enzyme activity remains. With respect to the third alternative, Halkerston *et al.* (1961) and Koritz (1962) have both suggested that 20α-hydroxylation of cholesterol is rate limiting for steroid biosynthesis from cholesterol in the adrenal.

The acetone powder used in these experiments was capable of converting some pregnenolone to progesterone. In experiments with cholesterol-4-C^{14} as substrate (Table I), it will be seen that the ratio of pregnenolone to progesterone in the extract is approximately 5. In the experiment described under Results, in which extracts from 30 flasks in which 20α-hydroxycholesterol was used as substrate were pooled, the ratio of pregnenolone to progesterone was also approximately 5 (pregnenolone 248 μg–progesterone 48.3 μg), which is in keeping with the presumed identity of the "progesterone" formed from 20α-hydroxycholesterol in these experiments. The conversion of pregnenolone to progesterone requires 3β-hydroxy-steroid dehydrogenase and DPN. The findings of Beyer and Samuels (1956) demonstrated that in the rat adrenal the dehydrogenase was found for the most part in microsomes with, however, some mitochondrial activity (presumably due to the presence of microsomes in the mitochondrial pellet). It is therefore likely that the present acetone powder includes some microsomal protein. It must also be assumed that the acetone powder used in these experiments also contained some DPN.

It is therefore concluded that the conversion of cholesterol to pregnenolone occurs in "mitochondria" from *corpus luteum* by way of 20α-hydroxycholesterol as in the adrenal cortex. This conversion is not increased by addition of ICSH *in vitro*, which is entirely in keeping with the findings of Tamaoki and Pincus (1961) using homogenate of *corpus luteum*.

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