Sterol Biosynthesis in the Bovine Corpus Luteum *in Vitro**

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ABSTRACT: Sterol biosynthesis was studied in slices of bovine corpus luteum incubated with [^{14}C]acetate. When incubations were performed under anaerobic conditions, more than 95% of the total radioactivity in the nonsaponifiable fraction was present as [^{14}C]squalene, the only labeled hydrocarbon formed. Adenosine triphosphate (ATP) and reduced nicotinamide–adenine phosphate (NADPH) were cofactors for squalene biosynthesis from acetate. When O_2 was admitted to a previously anaerobic system, squalene was converted to a mixture of sterols of which lanosterol was the predominant product. When incubations were conducted throughout in the presence of O_2, ^{14}C was incorporated into squalene, all sterol fractions (C_{30}, C_{28} + C_{29}, and C_{27}), and progesterone. [^{14}C]Lanosterol constituted more than 96% of the radioactivity in the C_{30} fraction; small amounts of 24,25-[^{14}C]dihydrolanosterol were present. The percentage of radioactivity in the C_{27} sterol fraction present as [^{14}C]cholesterol increased with time of incubation, but was not more than 50% after 4 hr. Labeled lanosterol and the unidentified C_{28} + C_{29} sterol isolated from these incubations were incubated with rat liver homogenates and were converted to C_{27} sterol. $^{14}CO_2$ was evolved when [^{14}C]lanosterol biosynthesized from [2-^{14}C]acetate by the corpus luteum was utilized as substrate; the ratio of radioactivity in C_{27} sterol to that in CO_2 was 5.06. These findings and the results of time studies on the aerobic incorporation of label from [1-^{14}C]acetate into individual components of the nonsaponifiable fraction are in accord with a pathway sequence in bovine corpus luteum: acetate → squalene → lanosterol → C_{28} + C_{29} sterol → C_{27} sterol → cholesterol → progesterone. Squalene and cholesterol concentrations in bovine corpora lutea varied from 12 to 44 μg/g, and from 2.5 to 3.5 mg/g of wet tissue, respectively. After incubation of corpus luteum slices with [^{14}C]acetate, the squalene isolated had a sufficiently high specific activity to be a precursor of cholesterol and of *de novo* progesterone in the same experiment. The specific activity of cholesterol was too low for this sterol to be a precursor of progesterone unless metabolically distinct pools of cholesterol exist in the tissue.

The incorporation of label from [1-^{14}C]acetate *in vitro* into cholesterol as well as into their respective steroid products has been amply demonstrated in the steroid-producing endocrine tissues, the adrenal (Srere *et al.*, 1948; Zaffaroni *et al.*, 1951; Hechter *et al.*, 1953), the testis (Brady, 1951), and the ovary (Popják, 1954; Rabinowitz and Dowben, 1955). However, in contrast to the now well-documented studies of the intermediary steps between cholesterol and the end-product steroid hormones, relatively little is known of the biosynthetic transformations occurring prior to cholesterol formation in many endocrine organs, including the corpus luteum.

Savard *et al.* (1965) have discussed the possibility that in the corpus luteum luteinizing hormone may act by accelerating more than one step in the biosynthetic pathway to progesterone, and that the gonadotropin may exert an influence at a stage preceding the formation of cholesterol. In order to test this postulate it was found essential to first investigate sterol synthesis in this compartment of the ovary. Earlier studies have shown that under *in vitro* conditions slices of bovine corpus luteum readily incorporate [1-^{14}C]acetate and [2-^{14}C]mevalonate into progesterone, a number of sterol fractions, and squalene (Mason *et al.*, 1962; Savard and Casey, 1964; Hellig and Savard, 1964, 1965). The results of a more detailed investigation of the identity, formation, and precursor relationships of these compounds are presented here. The influence of luteinizing hormone on this biosynthetic pathway is presently being studied.

Materials

ATP,[1] $NADP^+$, and glucose-6-P were obtained from Sigma Chemical Co., St. Louis, Mo. Progesterone, mp 126.5–127.5°, was a gift from Syntex, S. A., Mexico City. Squalene (98% pure) was obtained from Eastman Organic Chemicals, Rochester, N. Y. High purity cholesterol and a 2:1 mixture of lanosterol and dihydrolanosterol were supplied by Applied Science Laboratories, Inc., State College, Pa. The absence of contaminants in these sterols was confirmed by gas–

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[1] Abbreviations used: ATP, adenosine triphosphate; $NADP^+$, nicotinamide–adenine phosphate; NADPH, reduced $NADP^+$.

liquid partition chromatography as described in Methods.

[1-^{14}C]Acetate (sp act. 20.0 μc/μmole) and [2-^{14}C]-acetate (sp act. 8.0 μc/μmole) were obtained from Nuclear Research Chemicals, Inc., Orlando, Fla. The sources of other radioactive compounds have been listed in a previous publication (Hellig and Savard, 1965).

Methods

Measurement of ^{3}H and ^{14}C. Both ^{3}H and ^{14}C were measured by conventional scintillation counting techniques as described previously (Hellig and Savard, 1965). $^{14}CO_2$ was collected in a 40% KOH solution, which was subsequently diluted tenfold and counted by the method of Bray (1960). All radioactivity measurements are presented as disintegrations per minute (dpm).

Tissue Preparation and Incubation Conditions. The acquisition and transportation of the corpora lutea, the slicing of the tissue, and the conventional aerobic incubation conditions used have been described (Hellig and Savard, 1965). Routinely each experiment was performed using slices from a single bovine corpus luteum of pregnancy. Each incubation vessel contained approximately 500-mg slices, 10 μc of [1-^{14}C]acetate (or [2-^{14}C]acetate where specified), and 5 ml o Krebs–Ringer phosphate or bicarbonate buffer, pH 7.4. Aerobic incubations were conducted in an atmosphere of 95% oxygen and 5% carbon dioxide at 37° using the bicarbonate buffer except where comparison was made between aerobic and anaerobic conditions. In such cases the Krebs–Ringer phosphate buffer was used for both aerobic and anaerobic incubations. Anaerobic incubations were conducted in stoppered vessels, flushed with N_2 for 2 min and at hourly intervals throughout the incubation. In each experiment 1 g of corpus luteum slices was frozen for subsequent measurement of the progesterone present in the tissue before incubation, thus permitting the calculation of the net quantity of progesterone formed during the incubation.

Extraction and Analysis of Lipids. Unless noted otherwise, the entire contents of the incubation vessels (tissue slices and medium) were analyzed. The method for the quantitative extraction and preliminary purification of progesterone, sterols, and squalene from a single incubation mixture has previously been reported in detail (Hellig and Savard, 1965). In brief, progesterone was separated from the other lipid components in the initial ether extract of the incubation mixture by countercurrent distribution and purification by two-dimensional thin layer chromatography (Armstrong *et al.*, 1964). After elution of the steroid with methanol, its concentration was obtained by spectroscopic measurement and an aliquot of the solution was assayed for radioactivity. Previous examination of [^{14}C]progesterone samples formed from [^{14}C]acetate has shown that the radiochemical purity of the steroid thus isolated is in excess of 90% (Hellig and Savard, 1965). In this study crystallization of radioactive progesterone with added carrier to constant specific activity was performed only when the total radioactivity in progesterone was less than 10,000 dpm.

20β-Hydroxy-Δ^4-pregnen-3-one, the minor steroid product synthesized by the bovine corpus luteum, was analyzed in the same way. However, as this compound usually comprised less than 10% of the total progestin product, results for this steroid are not reported except when an anomalous tissue produced it in significant amounts.

The nonpolar portion of the nonsaponifiable fraction was partitioned on an alumina column (Langdon and Bloch, 1953) to yield the hydrocarbon fraction, eluted with petroleum ether (bp 40–50°), and the total sterol fraction, eluted with diethyl ether–acetate (1:1, v/v). All the radioactive material applied to the column was recovered in these two eluates. The total sterols were then separated into three distinct fractions by thin layer chromatography on silica gel G impregnated with Rhodamine 6G as described by Avigan *et al.* (1963). When benzene–ethyl acetate (5:1, v/v) is used as solvent, C_{30} sterols (R_F 0.69) are separated from the C_{27} sterols (R_F 0.49). The zone intermediate between C_{27} and C_{30} sterols was presumed to contain C_{28} and possibly C_{29} sterols (Goodman *et al.*, 1963). On each 20 × 20 cm plate, lanosterol and cholesterol were run as reference substances in parallel with the sample mixtures. These were applied to the thin layer plate in bands 3 cm long. Fluorescent sterol zones were detected by viewing under ultraviolet light and each zone was separately eluted by scraping the powder off the plate and eluting with methylene chloride. After the initial chromatography each individual zone was chromatographed in the same system and the radioactivity measured. The hydrocarbon fraction and the three sterol fractions thus obtained served as the starting material for the more detailed and specific analytical procedures described in the text.

All quantitative data were corrected to 1.0 g, wet weight, of tissue. Cholesterol and progesterone measurements were corrected to 100% recovery by radioactive assay of tracer amounts of the respective tritium-labeled isotopes added at the start of the analysis and recovered in the eluates from the thin layer chromatograms. The correction for losses in the other sterol fractions, separated from the C_{27} sterols by the initial thin layer chromatographic procedure, was estimated from the value for the recovery of [7α-^{3}H]cholesterol at this stage. Generally recoveries of sterols ranged from 85 to 95% and of progesterone from 70 to 85%. The values for squalene were not corrected for losses during the analytical procedure. However, as the recovery of cholesterol was high, and due care was exercised during procedures such as evaporation of extracts *in vacuo*, losses in squalene were considered minimal.

Gas–Liquid Partition Chromatography. A Research Specialities 600 Series gas–liquid partition chromatogram, equipped with a ^{90}Sr ionization detector, was used. Lanosterol and dihydrolanosterol were chromatographed on a 6 ft × 0.25 in. column packed with

acid-washed and silanized Gaschrome S (100–200 mesh) coated with 2% XE-60. Column temperature was 240°, input pressure was 28 lb, retention time for lanosterol was 46 min, and the separation factor lanosterol–dihydrolanosterol was 1.25. Squalene and cholestane were chromatographed on a 4 ft × 0.25 in. column using the same support packing coated with 3% QF-1. Column temperature was 223°, input pressure was 12 lb, retention time for cholestane was 7.5 min, and the separation factor cholestane–squalene was 1.39. An accurately measured amount of cholestane was added to all squalene solutions as an internal standard to correct for variations in injection volume. This procedure avoided the loss attendant on concentrating squalene solutions to dryness.

The individual compounds were identified by comparison with retention times of authentic reference substances. Mass was measured by triangulation and measurement with a compensating polar planimeter of the areas under the peaks shown on the recorder which received the entire effluent, as no effluent splitter was used. Separate calibration curves were plotted for each compound. For measurement of specific activity, fractions of the effluent were collected by freezing the carrier argon in U tubes partially submerged in liquid nitrogen. The U tube was attached to the heated column exit by a 1-in. Teflon connector. After the argon had been allowed to evaporate slowly, the residue in the tube was quantitatively transferred to a counting vial for assay of radioactivity. The efficiency of the trapping procedure for [^{14}C]squalene, known to be radiochemically pure, was evaluated by assay of radioactivity in the trapped effluent. The percentage of lanosterol recovered was measured by rechromatographing an aliquot of the compound and measuring the mass difference. Recoveries of squalene ranged from 30 to 43%, of lanosterol from 60 to 80%.

Experimental and Results Section

Influence of Anaerobic Condition. As squalene is known to be the ultimate precursor of the sterols, it was decided to study first the requirements for its biosynthesis. Although the radioactive labeling of squalene formed from [1-^{14}C]acetate under aerobic conditions was considerable, after 3 hr of incubation most of the radioactive label was in the sterol fraction; [^{14}C]squalene constituted only a minor portion of the total radioactivity of the nonsaponifiable fraction. However, when O_2 was excluded from the incubation mixture, the labeling of the hydrocarbon fraction was diminished, no synthesis of progesterone occurred, and only very small amounts of radioactivity (never more than 5% of that in the hydrocarbon fraction) were detected in the diethyl ether–acetone eluate from the alumina column. Virtually only the hydrocarbon fraction became labeled (Table I).

Analysis of the Hydrocarbon Fraction. The hydrocarbon formed anaerobically was identified as squalene by the following criteria. Samples of the hydrocarbon fraction obtained from ten anaerobic experiments were individually subjected to thiourea clathrate formation by the method of Goodman and Popják (1960). Invariably more than 98% of the radioactivity was recovered in the clathrate. The hydrocarbon fractions obtained from six anaerobic experiments were pooled and 50 mg of carrier squalene was added to an aliquot (219,000 dpm). Both isomers of the hexahydrochloride were prepared and crystallized by the method of Langdon and Bloch (1953). Table II shows that the specific activity was unchanged from that of the calculated value. The melting points of both isomerides were in agreement with reported values (Langdon and Bloch, 1953). Thus [^{14}C]squalene is the only radioactive compound present in the hydrocarbon fraction isolated from anaerobic as well as

TABLE I: Influence of Anaerobic Condition on Sterol Synthesis.[a]

Expt	Gas Phase (%)	(dpm/g of tissue) × 10^{-3} Hydrocarbon[b]	Total Sterols[b]	Progesterone	Progesterone (μg/g of tissue)
21	O_2 (95) and CO_2 (5)	958	10,002	755	95
	Nitrogen	1670	50	0	0
24	O_2 (95) and CO_2 (5)	318	11,600	263	23
	Nitrogen	1510	25	0	0

[a] The standard incubation mixture consisted of 0.5-mm thick slices and 10 μc of [1-^{14}C]acetate in 5 ml of Krebs–Ringer phosphate buffer, pH 7.4. In expt 24 the anaerobic incubation medium also contained 1 mM ATP, 2 mM NADP, and 2 mM glucose-6-P. The tissue weights in both experiments varied from 320 to 380 mg. The incubations were performed for 3 hr at 37°. [b] Fractions obtained by alumina column chromatographed as described in Methods.

TABLE II: Crystallization of [^{14}C]Squalene Hexahydrochloride.[a]

Crystn Step	Sp, Act. of Isomerides Crystallized (dpm/μmole)			
	Acetone	Mother Liquor	Ethyl Acetate	Mother Liquor
	Crystals		Crystals	
After addn of carrier	1836[b]			
First	1760	...	1975	1905
Second	1867	1760	1804	1880
Third	1886	1867	1835	1842
Mp (°C)	110–111		143–144	

[a] Carrier squalene (98% pure, 50 mg) was added to an aliquot (219,000 dpm) of the pooled hydrocarbon fractions obtained from six anaerobic incubations. [b] Calculated on the assumption that all radioactivity in the hydrocarbon fraction is associated with squalene. [c] Uncorrected.

from aerobic (Hellig and Savard, 1965) incubations of bovine corpus luteum with [^{14}C]acetate.

Cofactor Requirements for Squalene Biosynthesis under Anaerobic Conditions. Time studies of the rate of squalene synthesis from [1-^{14}C]acetate under anaerobic conditions revealed that this synthesis ceased or was greatly slowed after 1 to 2 hr of incubation. Figure 1A shows that the addition of 1 mM ATP (5 μmoles) to the incubation medium every hour maintained squalene synthesis. Figure 1B shows that preincubation under N_2 for 1 hr prior to the addition of [1-^{14}C]acetate resulted in a greatly diminished biosynthesis of [^{14}C]-squalene, and the addition of ATP restored this impaired synthesis. The addition to the medium of an NADPH-generating system (2 mM $NADP^+$ and 2 mM glucose-6-P) further increased the extent of [1-^{14}C]acetate incorporation into [^{14}C]squalene, but only in the presence of ATP (Table III).

TABLE III: Cofactor Requirements for Squalene Biosynthesis from [1-^{14}C]Acetate under Anaerobic Conditions.[a]

Addn (mM)[b]	Squalene Synthesis (dpm/g of tissue) × 10^{-3}		
	Expt 25	Expt 45	Expt 46
None	1353	137	577
ATP (1)	2615	494	926
NADP (2) + glucose-6-P (2)	1053	152	876
ATP (1) + NADP (2) + glucose-6-P (2)	4840	720	1220

[a] The incubation mixture was as described in Table I. A different corpus luteum was used for each experiment. The tissue weights varied from 243 to 390 in expt 25, 579 to 680 in expt 45, and 314 to 321 mg in expt 46. The incubations were performed for 3 hr at 37° in an atmosphere of N_2. [b] ATP (1 mM) was added at the end of the first and second hour of incubation. The NADPH-generating system was added at the beginning of the incubation.

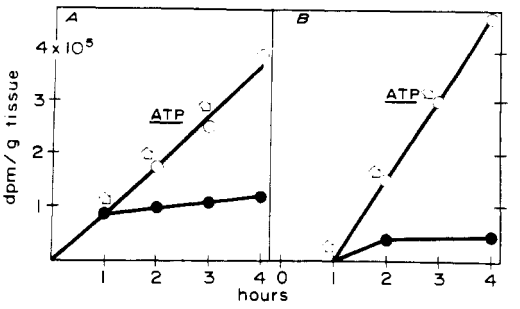

FIGURE 1: Requirement of ATP for anaerobic synthesis of squalene. The incubation mixture was as described in Table I; tissue weights in both expt A and B varied from 350 to 480 mg. [1-^{14}C]Acetate was added at zero time in A, and after 1 hr of anaerobic incubation in B. O, squalene, 1 mM ATP (5 μmoles) added at the times indicated by the arrow; ●, squalene, no ATP added.

TABLE IV: Conversion of Exogenous [^{14}C]Squalene to Sterols and Steroids.[a]

Substrate (μc)	Radioactivity (dpm × 10^{-3})			Mass Progestin[b] (μg)
	Squalene	Total Sterols	Progestin[b]	
[1-^{14}C]Acetate (10)	147.5	4370	217	67
[^{14}C]Squalene[c] (0.63)	36	225	1.41	70

[a] The incubation mixture consisted of 0.5-mm thick slices and 5 mg of bovine plasma albumen in 5 ml of Krebs–Ringer bicarbonate buffer, pH 7.4. The tissue weights were 600 and 620 mg. The incubations were performed for 3 hr at 37° in an atmosphere of 95% O_2 and 5% CO_2. In this experiment only the tissue slices were analyzed. [b] Progestin = progesterone + 20β-hydroxy-Δ^4-pregnen-3-one. [c] Biosynthesized from [1-^{14}C]acetate and purified by alumina column chromatography.

TABLE V: Conversion to Sterols of [^{14}C]Squalene Synthesized *in Situ*.[a]

Substrate	No.	Time (min) in N_2	Time (min) in N_2 + 95% O_2, 5% CO_2	Radioactivity in Nonsapon Fraction (dpm/g of tissue) × 10^{-3} Total	Squalene	Sterols	Sterols (%)[b]
			Experiment 34				
[1-^{14}C]Acetate (0.5 μmole, 10 μc)	1	180	...	1825	1730	94.5	5.2
	2	200	...	2106	2015	91.2	4.3
	3	180	20	2420	1475	945.0	39.0
	4	180	20	2105	1275	830.0	39.2
			Experiment 36				
[2-^{14}C]Acetate (1.25 μmoles, 10 μc)	1	180	...	281.3	272.5	8.8	3.1
	2	200	...	296.0	286.5	9.5	3.2
	3	180	20	317.2	187.7	129.5	40.8
	4	180	20	264.7	128.2	135.5	49.0
	5	180	60	284.0	112.0	172.0	60.5
			Experiment 39				
[2-^{14}C]Acetate (1.25 μmoles, 10 μc)	1	200	...	1291	1268	23.0	1.8
	2	180	20	1319	1167	151.8	11.5
	3	180	20	1228	1022	205.5	16.7
	4	180	20	1148	995	153.9	13.3

[a] The incubation conditions for these experiments were the same as those given for expt 20 (Figure 2). A separate corpus luteum was used for each experiment. The tissue weights varied from 1084 to 1160 mg (expt 34), from 479 to 651 mg (expt 36), and from 1000 to 1214 mg (expt 39). The radioactivity in the sterol fraction is expressed as a percentage of the total radioactivity in the nonsaponifiable fraction.

Metabolism of Squalene. Since squalene was not significantly converted to any other nonsaponifiable product under anaerobic conditions, we attempted to investigate its metabolism by adding biosynthetically prepared [^{14}C]squalene to an aerobic slice incubation. As a check on the synthetic capability of the tissue, slices were also incubated with [1-^{14}C]acetate. In this experiment only the tissue slices were analyzed. Table IV shows that the incorporation of radioactivity from squalene into sterol and steroid fractions was significant but low, a result expected from similar data obtained by Mason and Savard (1964) on the utilization of exogenous [^{14}C]cholesterol for steroid biosynthesis in the same type of slice preparation. The utilization of squalene appears to be limited by its poor penetration into the slice.

Because of this difficulty the problem was approached by studying the metabolism of [^{14}C]squalene which had been biosynthesized *in situ* in the slice preparation. One of these experiments is depicted in Figure 2; the data of three similar experiments are given in Table V. All incubation mixtures were first incubated anaerobically in the presence of [^{14}C]acetate and ATP for 3 hr. At this point, air was introduced into all the flasks, except for two used to measure the extent of anaerobic synthesis at 3 hr and the residual [^{14}C]-squalene synthesis during the final period of incubation (shown by the broken line in Figure 2). The data show that in the presence of O_2, the newly synthesized [^{14}C]-squalene was quantitatively converted to [^{14}C]sterols. At each of the time intervals measured during the last hour of incubation, the total radioactivity in the nonsaponifiable fraction was constant under both aerobic and anaerobic conditions (Figure 2 and Table V). This indicated that the radioactive sterols and progesterone had indeed been formed from [^{14}C]squalene which had been synthesized *in situ* during the prior incubation under N_2. Very little [^{14}C]progesterone was synthesized in the experiments cited in Table V, although up to 30 μg of the steroid was formed, apparently from an unlabeled precursor already present in the tissue when air was admitted to the incubation. Thin layer chromatographic analysis of the total sterol fraction formed in the experiments depicted in Table V revealed that it was composed of a mixture of C_{30}, C_{28} + C_{29}, and C_{27} sterols, and that the C_{30} fraction was the most highly labeled component (Table VI). This finding constitutes an important difference from the labeling profile of the sterols obtained from a conventional aerobic incubation, where most of the sterol radioactivity was associated with the C_{27} sterol fraction (Table VI, expt 2-2).

The results presented in Table VI thus indicate that prolonged anaerobic incubation of the tissue produced an impairment in the subsequent conversion of C_{30} to C_{27} sterol in the presence of O_2. The small extent of labeling of progesterone was considered to be a consequence of this impaired incorporation of

TABLE VI: Influence of Partially Anaerobic Conditions on the Composition of the [^{14}C]Sterol Fraction.

Expt[a]	Substrate	Time (min) in N_2	Time (min) in N_2 + 95% O_2, 5% CO_2	Percentage Distribn of ^{14}C Labeling in Sterol Fraction: C_{30}	$C_{28} + C_{29}$	C_{27}
34-3	[1-^{14}C]Acetate	180	20	76.0	11.2	12.8
36-3	[2-^{14}C]Acetate	180	20	62.0	24.1	13.9
36-5	[2-^{14}C]Acetate	180	60	54.4	25.2	20.4
39-2	[2-^{14}C]Acetate	180	20	75.0	14.6	10.4
2-2	[1-^{14}C]Acetate	...	180	3.2	25.2	71.6

[a] Experiments 34, 36, and 39 given in Table V. The data of expt 2-2 were reported in a previous publication (Hellig and Savard, 1965).

^{14}C-label into the C_{27} sterol fraction.

Analysis of the C_{30} Sterol Fraction. The C_{30} sterol fraction, formed from squalene when air was introduced into a previously anaerobic incubation, was identified as lanosterol by the following criteria. A solution of approximately 30 μg (estimated by the size and intensity of the fluorescent zone on a thin layer chromatogram) in 3 ml of ethanol exhibited no ultraviolet absorption in the range 230–285 mμ using a cell with a 1-cm light path. This eliminated the possibility that conjugated dienes (agnosterol or dihydroagnosterol, E_{244} 17,400) were present in this fraction in measurable amounts.

Aliquots of the ^{14}C-labeled C_{30} sterol isolated from expt 34 and from expt 36 (Table V) were acetylated in the presence of 400 μg of a 2:1 carrier mixture of lanosterol and dihydrolanosterol by the procedure of Johnston *et al.* (1957). The acetates were then separated by thin layer chromatography on silica gel G, previously sprayed with $AgNO_3$ solution and dried before use. The solvent used was petroleum ether–benzene (5:1, v/v) (Avigan *et al.*, 1963). In this system the dihydrolanosterol acetate zone moved faster than the lanosterol acetate zone and was separated from it by a distance of 2–3 cm. The acetates were eluted with methylene chloride after being detected in ultraviolet light. A typical example is quoted. When 60,400 dpm of the acetylated mixture was chromatographed on the $AgNO_3$-impregnated plate, 5540 dpm was recovered at the origin, 43,390 dpm was associated with the lanosteryl acetate zone and 2370 dpm with the dihydrolanosteryl acetate zone. The latter was rechromatographed in the same system with additional carrier lanosteryl acetate. Elution of the second thin layer chromatogram now revealed 400 dpm at the origin, 1200 dpm associated with the lanosteryl acetate zone, and only 450 dpm with the dihydrolanosteryl acetate zone. The radioactivity at the origin was thought to be an artifact produced by $AgNO_3$ oxidation, as usually 10% of the radioactivity in the various samples analyzed on $AgNO_3$ plates was retained at the origin. However, if trace amounts of acetylated samples were chromatographed in the absence of carrier sterol acetate, the proportion retained at the origin could rise to 30%.

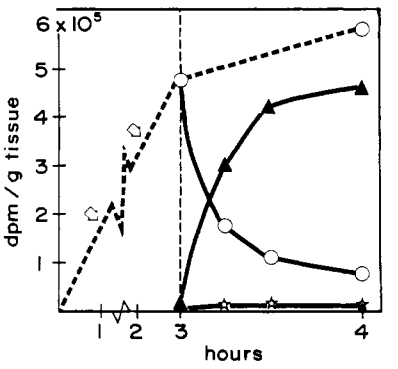

FIGURE 2: The conversion of squalene to sterols when oxygen is admitted to a previously anaerobic system. The incubation mixture was as described in Table I, except that 1 mM ATP was added at the times indicated by the arrow; tissue weights varied from 390 to 550 mg. ○, squalene; ▲, sterols; ☆, progesterone. Upper curve, broken line, incubation in an atmosphere of N_2. Lower curves, solid lines, incubation in an atmosphere of 95% O_2 and 5% CO_2 after 3 hr of anaerobic incubation.

Another sample of the C_{30} sterol fractions, isolated from expt 39 (Table V) by the usual thin layer chromatography in the benzene–ethyl acetate (5:1) system, was chromatographed twice in the same system and then analyzed by gas–liquid partition chromatography. Initially a trace amount (8000 dpm) was chromatographed together with 4 μg of lanosterol and 2 μg of dihydrolanosterol. As 7000 dpm was recovered in the combined lanosterol and dihydrolanosterol fractions, it was apparent that the radioactivity in the sample was associated only with these two C_{30} sterols. A larger portion of the C_{30} sterol fraction was then applied to the column and the individual effluents corresponding to the lanosterol and dihydrolanosterol peaks were collected and counted. The radioactivity coincident with lanosterol was 128,000 dpm while only 4350 dpm

TABLE VII: Purification of [^{3}H,^{14}C]Cholesterol to Constant Specific Activity.[a]

Stage of Purifcn	Mass (mg)	Radioactivity (cpm) ^{3}H	Radioactivity (cpm) ^{14}C	Sp Act. (cpm/μmole) ^{3}H	Sp Act. (cpm/μmole) ^{14}C	Ratio ^{3}H/^{14}C
After addn of carrier	10.195	14,404	15,824	546	601	0.910
After bromination						
1st crystals	5.4	5,094	1,268	525	131	4.018
mother liquor residue		3,485	1,557			2.238
2nd crystals	3.0	3,098	651	564	119	4.759
mother liquor residue		1,682	576			2.923
3rd crystals	1.2	1,203	261	548	119	4.601
mother liquor residue		1,259	278			4.540

[a] After addition of a trace of [^{3}H]cholesterol as internal recovery standard, the C_{27} sterol fraction from aerobic incubations was isolated, diluted, and purified as described in the text. The data are from the purification of a single sample. [b] After bromination the precipitated dibromide was collected and the reaction mixture containing bromine was discarded. Crystallization of the precipitated dibromide from ethyl acetate and methanol yielded "1st crystals" and "1st mother liquor residue."

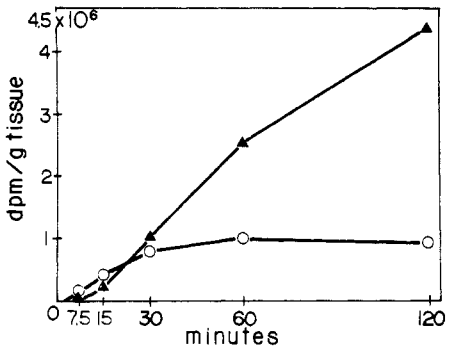

FIGURE 3: Time course of aerobic incorporation of [1-^{14}C]acetate into squalene and sterols (2 hr). The incubation mixture was as described in Table I; tissue weights varied from 382 to 451 mg (expt 48). The incubations were performed in an atmosphere of 95% O_2 and 5% CO_2. O, squalene; ▲, sterols.

of ^{14}C was associated with the emergence of dihydrolanosterol from the column.

Thus, on the basis of analysis both by thin layer chromatography after acetylation and by gas–liquid partition chromatography of the free sterols, the radioactive C_{30} sterol fraction, obtained from three separate experiments conducted under initially anaerobic conditions, appeared to consist almost entirely of [^{14}C]lanosterol. The small amount of radioactivity found associated with lanosterol was never more than 4% of the radioactivity in lanosterol.

Identical results were obtained on two analyses of the C_{30} sterol fraction isolated from incubations conducted throughout in an atmosphere of 95% O_2 and 5% CO_2. Thus [^{14}C]lanosterol was found to be virtually the only radioactive component of the C_{30} sterol fraction obtained after both aerobic and partially anaerobic incubations of slices of corpus luteum with [^{14}C]-acetate.

Purification and Measurement of [^{14}C]*Cholesterol.* Although both the hydrocarbon and C_{30} sterol fractions each contained only a single radioactive component, the C_{27} sterol fraction isolated from conventional aerobic incubations was found to consist of a mixture of labeled sterols. Accordingly, a stringent purification procedure was required to separate [^{14}C]cholesterol from "high-counting companions" (Schwenk and Werthessen, 1952) present in the C_{27} sterol fraction. [^{14}C]Cholesterol in each individual sample was therefore purified and measured as described. For subsequent calculation of cholesterol specific activity, the mass of endogenous cholesterol present in an aliquot of the C_{27} sterol fraction was measured by the Liebermann–Burchard reaction, and an equal aliquot was diluted with 10 mg of carrier cholesterol. This mixture was then brominated according to the method of Kabara and McLaughlin (1961) and the precipitated dibromide was repeatedly crystallized from ethyl acetate and methanol. The data on a representative sample reported in Table VII show that constant specific activity was achieved after the second crystallization. As [7α-^{3}H]cholesterol had been added to measure losses during the analytical procedure, a ratio of ^{3}H/^{14}C in the mother liquor equal to that in the crystals (third crystallization) provided a convenient index of purity. Table VII also shows that only 20% of the radioactivity in this particular C_{27} sterol fraction, obtained from 30 min of incubation, was present as [^{14}C]cholesterol. However, this percentage varied in

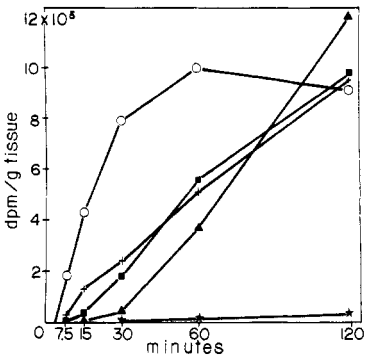

FIGURE 4: Time course of aerobic incorporation of [1-^{14}C]acetate into individual components of the nonsaponifiable fraction (2 hr). Incubation conditions for this experiment are described in Table I. ○, squalene; +, lanosterol; ■, C_{28} + C_{29} sterol; ▲, cholesterol; ★, progesterone.

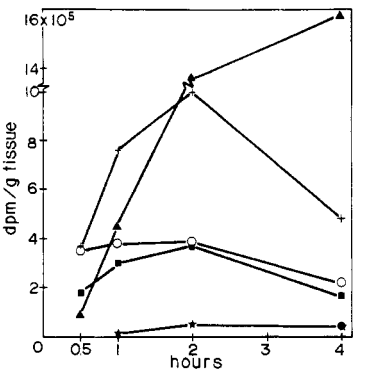

FIGURE 5: Time course of aerobic incorporation of [1-^{14}C]acetate into individual components of the nonsaponifiable fraction (4 hr). The incubation mixture was as described in Table I, except that the phosphate buffer was replaced by bicarbonate buffer; tissue weights varied from 426 to 489 mg (expt 54). The incubations were performed in an atmosphere of 95% O_2 and 5% CO_2. ○, squalene; +, lanosterol; ■, C_{28} + C_{29} sterol; ▲, cholesterol; ★, progesterone.

different samples, increasing to 50% with longer periods of incubation.

Aerobic Incorporation of Label from [1-^{14}C]Acetate into Sterols. The precursor–product relationships of the sterols biosynthesized from [1-^{14}C]acetate by the bovine corpus luteum under aerobic conditions were investigated by measuring the changes with time in the ^{14}C labeling of the various components of the nonsaponifiable fraction. Figure 3 illustrates the incorporation of label from [1-^{14}C]acetate into total sterols and squalene over a 2-hr period of conventional aerobic incubation (expt 48). During the first 15 min of incubation [^{14}C]squalene was the major radioactive component of the nonsaponifiable fraction. By 30 min the labeling of the total sterol fraction exceeded that of squalene, and continued to increase for the duration of the incubation. The data of Figure 4, showing the incorporation of label from [1-^{14}C]acetate into the individual sterol components, illustrates the sequential formation of labeled squalene, lanosterol, "intermediate" (C_{28} + C_{29}) sterols, cholesterol, and progesterone in the same experiment. The difference between the total radioactivity associated with the individual sterol components (Figure 4) and the total sterol radioactive labeling plotted in Figure 3 is due to the incorporation of radioactivity into the "high-counting" components of the C_{27} sterol fraction, not plotted in Figure 4. The results of a similar time study performed over a 4-hr period (Figure 5, expt 54) illustrate the gradual loss of radioactivity from squalene, lanosterol, and "intermediate" (C_{28} + C_{29}) sterols during the last 2 hr of incubation. During this time the incorporation of [1-^{14}C]acetate into cholesterol continued to increase slowly.

Incubation of Labeled Sterols with Homogenates of Rat Liver. The time course of the ^{14}C labeling of the components of the nonsaponifiable fraction (Figures 4 and 5) suggests a pathway sequence

acetete ⟶ squalene ⟶ lanosterol ⟶ C_{28} + C_{29} sterols ⟶ cholesterol

The C_{28} + C_{29} sterol fraction isolated from bovine corpus luteum was therefore incubated with a rat liver preparation to determine whether the crude fraction could act as a precursor for cholesterol in a known sterol synthetic system. [^{14}C]Lanosterol biosynthesized by corpus luteum was also incubated with the liver preparation in order to investigate the isotopic labeling of this sterol.

Rat liver homogenates were made in 100 mM potassium phosphate buffer, pH 7.4, containing 4 mM $MgCl_2$ and 30 mM nicotinamide (Popják *et al.*, 1958; Goodman and Popják, 1960), by the method of Bucher and McGarrahan (1956). The homogenates were centrifuged at 10,000*g* for 25 min, and the 10,000*g* supernatant solution was immediately used for the incubations. Glucose-6-P (2 mM) and $NADP^+$ (1 mM) were added to 5 ml of the 10,000*g* supernatant before addition of the substrate sterols dissolved in 100 μl of acetone. The incubations were performed in Warburg flasks containing filter paper strips (Whatman No. 54) saturated with 0.3 ml of 40% KOH in the center well in order to trap CO_2. The flasks were gassed with pure O_2 before being tightly stoppered and incubated for 1 or 2 hr at 37°. The incubations were then terminated by the injection of 0.25 ml of 5 N HCl through a rubber cap in the side arm of the flasks and the acidified mixture was shaken gently for 1 hr. The KOH solution containing CO_2 was quantitatively removed and an aliquot was assayed for radioactivity.

After addition of 100 μg of mixed lanosterol–dihydrolanosterol carrier and saponification (4% KOH in 50% methanol at 60°), the sterols were quantita-

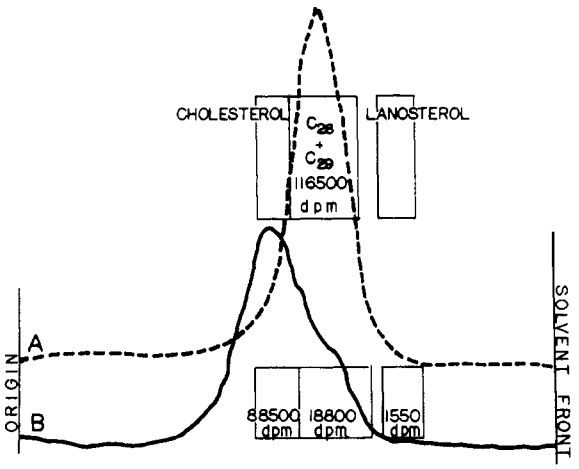

FIGURE 6: Thin layer chromatography of the sterols isolated from incubations of rat liver homogenate with ^{14}C-labeled C_{28} + C_{29} sterol. This labeled sterol was synthesized from [1-^{14}C]acetate by slice preparations of bovine corpus luteum. The experimental procedure is described in the text. The curves are tracings of radioactivity scanner records. A, broken line, control incubation with boiled homogenate; B, solid line, test incubation. The enclosed areas show the location on the chromatograms of reference sterols and of the zones eluted and assayed for radioactivity by liquid scintillation counting.

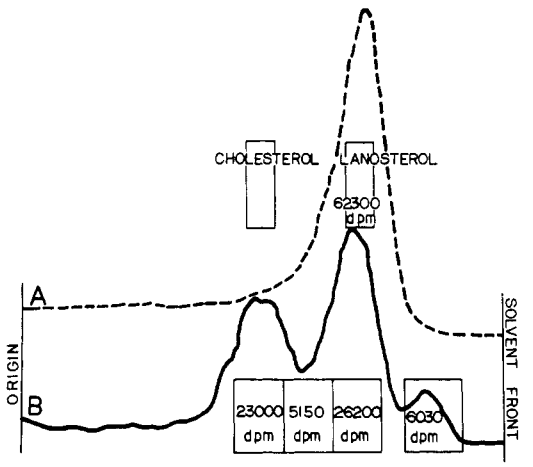

FIGURE 7: Thin layer chromatography of the sterols isolated from incubations of rat liver homogenate with [^{14}C]lanosterol. The labeled lanosterol was synthesized from [1-^{14}C]acetate by slice preparations of bovine corpus luteum. Designations are given in legend to Figure 6.

tively extracted with petroleum ether and analyzed by thin layer chromatography using benzene–ethyl acetate (5:1), as solvent. Plates measuring 4 × 20 cm were used. After development the plates were scanned for radioactivity using a Vanguard 880 Autoscanner equipped with the 885 glass-plate attachment with slit width adjusted to 1 cm. The cholesterol and lanosterol zones were located by viewing in ultraviolet light. The relevant zones on the chromatograms were then eluted and counted.

The results of two such experiments, where incubation with boiled liver extract served as controls, are depicted in Figures 6 and 7. In both these experiments the sterol substrates had been synthesized *in vitro* from carboxyl-labeled acetate by corpus luteum slices.

Figure 6 shows that 86% of the ^{14}C-labeled C_{28} + C_{29} sterol fraction was converted to C_{27} sterol, thus confirming its suggested role as a precursor of cholesterol. [^{14}C]Lanosterol was converted to C_{27} sterol in 37% yield (Figure 7). This relatively low conversion of [^{14}C]lanosterol to C_{27} sterol was a result of carrying out the incubation in the presence of approximately 100 μg of carrier lanosterol and dihydrolanosterol. In incubations performed with only trace amounts of sterol higher conversions were observed.

In the second experiment, shown in Figure 7, 10% of the [^{14}C]lanosterol was converted to an unknown substance which has not been characterized. This radioactive product had a chromatographic mobility (R_F 0.89) greater than that of lanosterol (R_F 0.53) or dihydrolanosterol when run on thin layer chromatograms in benzene–ethyl acetate (11:1, v/v). The substance was not an esterified sterol since its R_F was unchanged after repeated saponification. It did not appear to be the related 3-ketone, lanosta-8,24-dien-3-one, because treatment with $NaBH_4$, in pyridine and methanol (Bush, 1961) under conditions for the specific reduction of 3-ketones (Soloway *et al.*, 1953), did not alter its chromatographic mobility in the same system.

The CO_2 collected from both these incubations (Figures 6 and 7), in which the ^{14}C label in the substrates was derived from [1-^{14}C]acetate, was devoid of radioactivity. However, when [^{14}C]lanosterol (44,000 dpm) biosynthesized by the corpus luteum from *methyl*-labeled acetate was used as a substrate in a similar incubation, [^{14}C]C_{27} sterol (14,400 dpm) was synthesized and $^{14}CO_2$ (2850 dpm) was evolved. The ratio of radioactivity in C_{27} sterol to that in CO_2 was therefore 5.06. Lanosterol biosynthesized from [2-^{14}C]acetate in the liver contains 18 labeled carbon atoms, including label in all three "extra" methyl groups (at positions 4, 4′, and 14) which are lost in the transformation of a C_{30} sterol to a C_{27} sterol. Hence the agreement of the observed ratio of 5.06 with the ratio of 5 expected (Olson *et al.*, 1957; Gautschi and Bloch, 1957) for the conversion of lanosterol, labeled as described, indicates that the distribution of the labeled carbon atoms in the C_{30} sterol, biosynthesized by the corpus luteum from [^{14}C]acetate, is the same as in the liver sterol synthetic system.

Measurement of Tissue Content and Specific Activity of Nonsaponifiable Compounds. The probability that the corpus luteum contains a number of pools of cholesterol, and possibly of other sterol intermediates, has recently been discussed by Savard *et al.* (1965). In order to investigate this possibility, squalene, choles-

terol, and lanosterol concentrations in the corpus luteum were measured and the specific activities of these compounds and of *de novo* progesterone were assessed.

The cholesterol content, as measured by the Liebermann–Burchard reaction on aliquots of the C_{27} sterol fractions isolated from three different corpora lutea, ranged from 2.5 to 3.5 mg/g wet tissue. Table VIII shows

TABLE VIII: Squalene Content of Bovine Corpora Lutea.[a]

Total Wt of Corpus Luteum (g)	Wt Equiv of Aliquot Analyzed[b] (g)	Squalene Mass		
		μg	μg/ Corpus Luteum	μg/g of Tissue
4.713	0.943	11.6	58	12
6.388	1.278	17.9	90	14
5.014	1.003	17.7	83	18
2.241	0.831	32.9	89	40
4.663	0.993	40.8	204	44

[a] The squalene content was determined by gas–liquid partition chromatography as described in the text. Duplicate determinations on each sample were within 3–8% of the mean value. The extent of deviation from the mean was in inverse proportion to the amount of squalene present. [b] Obtained from fresh unincubated corpora lutea by procedure described in the text; $^2/_5$ or $^1/_5$ volume of the total hydrocarbon fraction in petroleum ether carefully concentrated in a stream of N_2 and 5-μl aliquots were employed. Cholestane (250 μg) added to the original extract as internal standard.

that the squalene concentration, as measured in five separate corpora lutea, ranged from 12 to 44 μg/g of wet tissue.

In one experiment, the specific activity of [^{14}C]-squalene after anaerobic incubation with [1-^{14}C]-acetate (expt 39-1, Table V) was 24,700 dpm/μg and the concentration was 50 μg/g of wet tissue. The [^{14}C]-lanosterol from the paired incubation vessels of the same experiment (expt 39-2,3,4, Table V) had a specific activity of 3880 dpm/μg. Hence the newly formed [^{14}C]-lanosterol had been diluted at least five- to sixfold by unlabeled lanosterol already present in the tissue. Utilizing these specific activity data, the weight of slices incubated (3.488 g), and the total radioactivity recovered in [^{14}C]lanosterol (408,000 dpm), the lanosterol concentration in this particular tissue was estimated to be 29–31 μg/g of wet tissue (of which a maximum of 5 μg/g of tissue could have been derived from the cyclization of squalene during the 20-min period of aerobic incubation). Thus, although the concentrations of these compounds are low compared to that of cholesterol, the corpus luteum contains appreciable amounts of both squalene and lanosterol.

Relevant specific activity measurements on compounds isolated from aerobic incubations with [1-^{14}C]-acetate, expt 48 (Figure 4) and expt 54 (Figure 5), are presented in Table IX. Squalene and progesterone

TABLE IX: Specific Activities of Compounds Isolated from Aerobic Incubations.[a]

Expt	Incubn Period (hr)	Compd	Specific Activity	
			dpm/μg	(dpm/μmole) × 10^{-5}
48	2	Squalene	18,000	74.0
		Cholesterol	391	1.51
		Progesterone	1,118	3.52
54	2	Squalene	8,000[b]	32.8[b]
		Cholesterol	438	1.69
		Progesterone	4,400	13.8
	4	Squalene	4,000[b]	16.4[b]
		Cholesterol	596	2.30
		Progesterone	4,130	13.0

[a] Incubation conditions given with Figures 3 and 5. Method of measurement of specific activities is described in the text and in Table VII. [b] In expt 54 the mass of squalene was not measured; the specific activity was estimated by dividing the measured radioactivity of [^{14}C]squalene (Figure 5) by 50, the highest concentration of squalene, microgram per gram of tissue, measured in any experiment. The estimated value is therefore the lowest possible specific activity.

specific activities were assessed as described in Methods. The section on the purification and measurement of [^{14}C]cholesterol explains how the specific activity of this compound was measured. The implications of these findings are discussed later in the text.

Discussion

A consideration of the time course of ^{14}C labeling of the nonsaponifiable fraction (squalene plus total sterols) formed from [1-^{14}C]acetate in the corpus luteum (Figure 3) reveals that after an initial lag period, the ^{14}C label accumulated in the nonsaponifiable fraction at a constant rate during the first 7–60 min of incubation. During the second hour of incubation the extent of labeling of this fraction continued to increase, though at a slightly slower rate. This observation, confirmed in other time studies not presented here, implies that the [1-^{14}C]acetate (0.5 μmole) added to the incubation mixtures was diluted by endogenous acetate to an indetermined extent sufficient to provide [^{14}C]acetate in excess for about 2 hr. This consideration is pertinent to the following discussion of our data.

Under aerobic conditions, the incorporation of label from [1-^{14}C]acetate into the total nonsaponifiable fraction varied in different corpora lutea, but was in the range of 5.3–12 $\times$ 10^6 dpm/g of tissue in 2- and 3-hr incubations (Figure 3, Table I). Thus 23–55% of the [1-^{14}C]acetate (10 μc, 22.2 $\times$ 10^6 dpm) added to the incubation mixture was incorporated into squalene, sterols, and progesterone by 1 g of tissue. Comparison of these values with data reported by Popják (1954) on [1-^{14}C]acetate incorporation into squalene and sterols by rat liver slices similarly incubated *in vitro* reveals that the corpus luteum utilizes [^{14}C]-acetate for sterol synthesis with an efficiency fully comparable to that of liver. This finding is in agreement with the work of Popják and Beeckmans (1950), who showed that after *in vivo* injection of [1-^{14}C]acetate, the digitonin-precipitable sterol in the ovaries excised from pregnant rabbits had a higher specific activity than either the serum or the liver cholesterol in the same animals, thus indicating that the ovary of the pregnant animal was an active site of extrahepatic synthesis of cholesterol from acetate.

The data presented in this report also indicate that, on a qualitative basis, the cholesterol biosynthetic pathway in the corpus luteum closely parallels that in liver. Thus the following results are all in accord with established concepts of sterol biosynthesis (reviewed by Popják and Cornforth, 1960).

ATP and NADPH are cofactors required for the biosynthesis of squalene from acetate (Figure 1, Table III). However, it is possible that additional cofactors are involved in this biosynthesis, as the ^{14}C labeling of the total nonsaponifiable fraction, isolated from aerobic incubations, far exceeded that of squalene, formed in slices of the same tissue under anaerobic conditions in the presence of ATP and NADPH (Table I, expt 24). Squalene can be synthesized under anaerobic conditions (Table I, Figure 1), but oxygen is required for the subsequent cyclization of squalene to lanosterol (Figure 2, Tables V and VI). The partial block in the metabolism of lanosterol, manifest when air was admitted to a previously anaerobic incubation (Table VI), has also been reported by Bucher *et al.* (1959), who performed a similar experiment using homogenates of rat liver. Tchen and Bloch (1957) have shown that the squalene–lanosterol conversion requires a lower concentration of reduced pyridine nucleotide than does the lanosterol–cholesterol conversion, and proceeds at a good rate when the latter processes are no longer operative in crude enzyme preparations of rat liver. A preliminary experiment was performed in which NADPH was added to an anaerobic–aerobic incubation of corpus luteum slices synchronously with the admission of oxygen.[2] The results indicated that a deficiency of this nucleotide may account in part for the impaired ability of the tissue to convert lanosterol to cholesterol. The results obtained from the incubation with rat liver homogenate of labeled lanosterol (synthesized in corpus luteum either from [1-^{14}C]- or [2-^{14}C]acetate) prove that this C_{30} sterol can be converted to C_{27} sterol (Figure 7), and that the "extra" methyl groups eliminated during this transformation all have their origin in the methyl carbon of acetate. The ratio of radioactivity in C_{27} sterol to that in carbon dioxide, found to be 5.06 after incubation with [^{14}C]lanosterol labeled from [2-^{14}C]-acetate, is consistent with the hypothesis that this lanosterol isolated from the corpus luteum contained 18 labeled carbon atoms, three of which were eliminated as carbon dioxide in the transformation of C_{30} to C_{27} sterol.

The time course (Figures 4 and 5) of the incorporation of label from [1-^{14}C]acetate into the individual components of the nonsaponifiable fraction, under aerobic conditions, suggests that the biosynthetic pathway in the corpus luteum is: squalene $\rightarrow$ lanosterol $\rightarrow$ C_{28} + C_{29} sterol $\rightarrow$ cholesterol $\rightarrow$ progesterone. Thus Figure 4 illustrates the sequential appearance of ^{14}C label in these compounds in the order cited. The curves in Figure 4 also show that the maximal slope of each product curve occurs after the maximum slope of the curve of its suggested precursor, a dynamic requisite of the proposed precursor–product relationships. The 4-hr time study (Figure 5) revealed that during the latter 2 hr of incubation the radioactivity in squalene, lanosterol, and the C_{28} + C_{29} sterol fraction declined, while [^{14}C]cholesterol continued to accumulate. This finding most probably reflects the depletion of the labeled acetate pool prior to the cessation of turnover of the intermediary compounds. The continued accumulation of label in [^{14}C]cholesterol supports this suggestion.

Although this transformation is inferred from the time studies, the direct conversion of the C_{28} + C_{29} sterol fraction to cholesterol has not been proven in the corpus luteum. However, the crude sterol fraction can act as a precursor for C_{27} sterol in the rat liver homogenate (Figure 6). The composition of the C_{28} + C_{29} sterol fraction has not been determined, but the chromatographic behavior of the unidentified sterol (Figure 6), closer to that of cholesterol than to lanosterol, suggests a C_{28} rather than a C_{29} sterol structure. This suggestion is supported by the work of Goodman *et al.* (1963), who presented evidence that a labeled sterol of similar chromatographic mobility, isolated from rat liver after brief exposure to [2-^{14}C]acetate, was a C_{28} compound.

The proportion of the total radioactivity in the C_{27} sterol fraction present as [^{14}C]cholesterol increased from 20 at 30 min to 40–50% after 2 hr of incubation in both the experiments depicted in Figures 4 and 5. In expt 48 (Figure 4), where the C_{27} sterol fraction was analyzed after shorter intervals of incubation, no radioactivity could be detected in cholesterol at 7.5 min and [^{14}C]cholesterol comprised only 7% of the total radioactivity in this fraction at the 15-min interval. This labeling of the "high-counting" companions prior to the labeling of cholesterol is in accord with the assumption that these unidentified components of the C_{27}

[2] H. R. Hellig and K. Savard, unpublished experiment.

fraction are precursors of cholesterol. In accord with the well-documented evidence that cholesterol is converted to progesterone by corpus luteum preparations (Tamaoki and Pincus, 1961; Ichii *et al.*, 1963; Hall and Koritz, 1964), the time studies illustrate that the incorporation of ^{14}C label into progesterone did not occur until after [^{14}C]cholesterol was formed. However, it remains to be rigidly established that cholesterol is an obligatory intermediate in the biosynthesis of progesterone.

Previous work has shown that radioactive progesterone becomes a *major* product of [^{14}C]acetate utilization by the corpus luteum only when the tissue is incubated in the presence of luteinizing hormone (Savard and Casey, 1964; Hellig and Savard, 1965). This finding was confirmed in the studies presented here, where all incubations were performed with tissues not subjected to the stimulatory action of gonadotropin *in vitro*. Since [^{14}C]progesterone synthesis was at a low "control" level, cholesterol could, therefore, be considered to be virtually the end product of [1-^{14}C]acetate utilization in these experiments.

The similarity of the sterol synthetic systems of corpus luteum and liver has been discussed. However, a major difference between corpus luteum and liver utilization of [^{14}C]acetate for sterol synthesis is revealed in the relative amounts of radioactivity recovered in either total sterols or cholesterol compared to that in squalene.

After incubating liver slices for 4 hr with [1-^{14}C]-acetate, Popják (1954) has reported that the radioactive labeling of the digitonide-precipitable sterol fraction was 100–150 times greater than the labeling of squalene formed in the same experiment. Representative values for the ratio, total [^{14}C]sterol/[^{14}C]squalene, in bovine corpora lutea were 4.7 (Figure 3, 2 hr, expt 48), 10.4 and 35.5 (Table I, 3 hr), and 23 (found in expt 54, 4 hr).

The [^{14}C]cholesterol/[^{14}C]squalene ratio in expt 48 (Figure 4) was 1.33 and in expt 54 (Figure 5) was 3.4 at the 2-hr and 8.0 at the 4-hr time period. These ratios are much lower than the values reported for liver and are of the same order as the ratio of 13 found by Popják (1954) after incubating slices of the ovaries of laying hens with [1-^{14}C]acetate. The lower ratio found in corpus luteum is a result of a proportionately much higher incorporation of the ^{14}C label into squalene than occurs in liver.

Figure 2 shows the accumulation of radioactive label from [1-^{14}C]acetate in squalene under anaerobic conditions; it also shows that 80% of the newly formed squalene was cyclized to sterol within 1 hr when air was admitted to the incubation. The data of three similar experiments (Table V) show that in two other tissues 40–50% of [^{14}C]squalene was converted to sterol within 20 min. In the third experiment the conversion was somewhat lower. These results suggest that a large proportion of newly formed squalene is readily available for transformation to sterol, and therefore the accumulation of radioactivity in squalene under aerobic conditions is unlikely to be due solely to a transfer of *de novo* squalene to an inert pool which fails to undergo conversion to sterol. The existence of such metabolically distinct pools of squalene has been demonstrated in liver by Popják (1954) and by Loud and Bucher (1958), who have shown that only a portion of the total hepatic squalene could have a specific activity high enough to be a precursor of cholesterol. The data given in Table IX, however, show that the squalene isolated from corpus luteum incubations appears to have a high enough specific activity to function as precursor for both cholesterol[3] and, more significantly, for *de novo* progesterone, even allowing for some dilution of the label by nonlabeled lanosterol already present in the tissue. These findings do not preclude the existence of metabolically distinct pools of squalene in the corpus luteum. Whether such pools do in fact exist remains to be established by a different experimental approach.

The same is not true for cholesterol. If this sterol is indeed an obligatory precursor of progesterone, the relatively low specific activity of the total cholesterol (Table IX) indicates that there must be an inhomogeneous distribution of labeled molecules in the tissue cholesterol. Only a portion of the cholesterol could have a high enough specific activity to act as a progesterone precursor. A separation of cholesterol into different metabolic pools in other steroidogenic tissues has been inferred from the results of similar experiments with the adrenal and the testis (discussed by Hechter, 1958).

Recent reports on sterol synthesis in whole cell suspensions and homogenates of testis tissue (Tsai *et al.*, 1964; Gaylor and Tsai, 1964; Salokangas *et al.*, 1964, 1965) enforce the concept that cholesterol synthesis in steroidogenic tissues is qualitatively similar, but quantitatively different, to that in liver. Tsai *et al.* (1964) and Salokangas *et al.* (1964, 1965) have also reported the synthesis and accumulation in testis of relatively large amounts of squalene from small-molecule precursors, and Tsai *et al.* (1964) have shown that [^{14}C]-lanosterol is a major component of the sterol fraction isolated from whole cell suspensions of testis, incubated with [^{14}C]acetate. On the basis of (a) the above findings in testis, (b) the accumulation of newly formed squalene in corpus luteum previously noted, and (c) the appreciable concentrations of endogenous squalene and lanosterol in this tissue, one might speculate that in both testis and corpus luteum these precursors may act as reserves for rapidly replenishing a small "steroidogenic" cholesterol pool. The recent work of Gaylor and his associates (Gaylor and Tsai, 1964; Ying *et al.*, 1965; Gaylor *et al.*, 1965) on control of lanosterol demethylation in testis tissue is in accord with this concept.

Acknowledgments

The authors are indebted to Dr. D. Sandberg for

[3] Since the average tissue content of cholesterol is 3 mg/g, increases of the order of 5–10% would be within the limit of experimental error of the method used to measure cholesterol concentration. Thus *de novo* cholesterol synthesis could not be assessed in these experiments.

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