The Biosynthesis of Squalene, Lanosterol, and Cholesterol by Minced Human Placenta*

Leon Zelewski† and Claude A. Villee

ABSTRACT: Minced human term placenta has been shown to utilize mevalonate and acetate in the synthesis of squalene, lanosterol, and cholesterol when incubated with diphosphopyridine nucleotide (DPN), triphosphopyridine nucleotide (TPN), and adenosine triphosphate (ATP). Acetate-1-^{14}C or mevalonate-2-^{14}C was added at the beginning of the 2-hr incubation and mevalonate-5-^{3}H was added either at the beginning of the incubation or 10, 30, or 60 min later. With this experimental design, comparison of the isotope ratios of the products provides clues as to the sequence of products in the metabolic pathway. The products were isolated and characterized by isotope dilution, by chromatography on alumina columns and thin layer plates of silica gel, by the formation of derivatives, and by crystallization to constant ^{3}H and ^{14}C specific activities and to constant isotope ratios. Cholesterol accounts for only a small fraction, *ca.* 5%, of the total sterols in the placenta. The amount of radioactivity in the crystalline cholesterol present in the placenta after 2 hr of incubation accounted for 0.12% of the labeled mevalonate. The conversion of mevalonate to sterol was linear with time during at least the first hour of the 2 hr of incubation.

The data from the experiments with doubly labeled mevalonate are consistent with the hypothesis that lanosterol is synthesized from one pool of squalene and that cholesterol is synthesized from one pool of lanosterol. The isotope ratios of the three products were identical. In contrast, the isotope ratios in these three products differed markedly in the experiments with acetate-1-^{14}C plus mevalonate-5-^{3}H as substrates. The ^{3}H/^{14}C ratio for lanosterol was much greater and the ratio for cholesterol was much smaller than the ratio for squalene in any given flask. This suggests that in the placenta the metabolic pathway from acetate to cholesterol differs in some respects from that of mevalonate.

Mevalonate has been shown to be a precursor of squalene and sterols in preparations of yeast and rat liver (Amdur *et al.*, 1957; Tavormina *et al.*, 1956; Cornforth *et al.*, 1958). The utilization of mevalonate as a precursor of steroids has been tested in several preparations of steroidogenic tissues. The conversion of mevalonate to steroids has been demonstrated in homogenates of testicular tissue (Rabinowitz, 1959), but other investigators have been unable to demonstrate the synthesis of steroids from mevalonate in testicular slices (Savard *et al.*, 1960) or in homogenates of normal adrenals (Bryson and Sweat, 1962), of an adrenal adenoma (Villee *et al.*, 1962), or of fetal adrenals (Villee *et al.*, 1961).

The biosynthesis of cholesterol in steroidogenic tissues has not been studied in detail. Salokangas *et al.* (1964, 1965) found that rat testicular cell-free preparations can convert mevalonate-5-^{3}H and ^{14}C-isopentenyl pyrophosphate to squalene, lanosterol, and cholesterol. Acetate-1-^{14}C and mevalonate-2-^{14}C were converted to squalene, lanosterol, and cholesterol by slices of testicular tissue (Tsai *et al.*, 1964). The incorporation of DL-mevalonate-5-^{3}H into both free and esterified sterols and the subsequent formation of labeled corticosterone and deoxycorticosterone in adrenal slices was shown by Billiar *et al.* (1965). The conversion of mevalonate-2-^{14}C to squalene, sterols, and progesterone was established by Hellig and Savard (1965) in slices of bovine corpus luteum.

In the present experiments the incorporation of mevalonate and acetate into squalene, lanosterol, and cholesterol has been established in incubations with another steroidogenic tissue, minced human term placenta. The experimental design used mevalonate-2-^{14}C or acetate-1-^{14}C together with mevalonate-5-^{3}H added at different times in the course of 2 hr of incubation. The use of a substrate with two different labels or of two different substrates with different labels has the advantage that the purity of an isolated product can be characterized not only by its specific activity but by the constancy of the ratio of the two isotopes. The ratios of the two isotopes in different metabolites can provide clues as to the sequence of compounds in a metabolic pathway.

Experimental Procedure

Chemicals. Neutral alumina of Woelm grade I was

* From the Department of Biological Chemistry, Harvard Medical School, and the Research Laboratories of the Boston Lying-in Hospital, Boston, Massachusetts. *Received November 23, 1965; revised March 14, 1966.* This research was supported in part by a grant from the Lalor Foundation and by a grant (HD 00404) from the National Institute of Child Health and Human Development.

† International Postdoctoral Fellow, National Institutes of Health.

deactivated by the addition of 7% water. Cholesterol (Fisher) was crystallized twice from methanol and 80% ethanol before use. Lanosterol (Northeastern Chemicals) was purified by bromination followed by regeneration with zinc and by two crystallizations from methyl alcohol–methylene chloride and methyl alcohol–acetone. Squalene (Sigma Chemical) was chromatographed on neutral alumina and eluted with pentane. β-Diphosphopyridine nucleotide, triphosphopyridine nucleotide monosodium salt, and adenosine 5′-triphosphate disodium salt were purchased from Sigma Chemical Co.

Radioactive Compounds. All the compounds used were purchased from New England Nuclear Corp. Sodium acetate-1-^{14}C (2.0 mc/mmole) was used as supplied. DL-Mevalonate-2-^{14}C (2.09 mc/mmole) and DL-mevalonate-5-^{3}H (91.5 mc/mmole) were supplied as the *N,N*-dibenzylethylenediamine salt and were converted to the sodium salt just before use (Hoffman *et al.*, 1957). Both the ^{3}H- and the ^{14}C-labeled mevalonate ran as single peaks with authentic mevalonate on paper chromatography in the system isopropyl alcohol–NH_4OH–H_2O, 8:1:1. The radiochemical purity of the cholesterol was tested by partition column chromatography on a Hyflo supercel column using the system trimethylpentane–90% methyl alcohol and a gradient of dichloroethane (Villee *et al.*, 1961).

Methods. Samples were assayed for ^{3}H and ^{14}C using the dual channel ratio method (Veal and Vetter, 1958) in a Packard TriCarb liquid scintillation spectrometer Model 3014. The efficiency of counting for each set of experiments was calculated from counts of appropriate ^{14}C and ^{3}H standards. All measurements of radioactivity are presented as disintegrations per minute (dpm). Samples were dissolved in 10 ml of scintillation fluid containing 4 g of 2,5-diphenyloxazole and 0.2 g of *p*-bis-2′(5′-phenyloxazolyl)benzene/l. of toluene. With samples of low radioactivity an aliquot was chosen to give at least five times the background count and enough counts were accumulated to reduce the probable error of counting to <5%. Squalene was measured iodometrically (Wheatley, 1953). Crystals of the hexahydrochloride derivative of squalene were weighed on a Cahn electrobalance. Sterols were determined colorimetrically with the Liebermann–Burchard color reagent (Schoenheimer and Sperry, 1934).

Human term placenta obtained immediately after delivery was cut in small pieces weighing *ca.* 0.5 g, and washed with Krebs–Ringer phosphate buffer, pH 7.4 (Umbreit *et al.*, 1957). Tissue (10 g) was placed in an incubation flask containing 10 ml of Krebs–Ringer phosphate buffer with 0.5 mM DPN,[1] 1.0 mM TPN, 1.0 mM ATP, 30 mM nicotinamide, 11.1 mM glucose, and the radioactive substrate. The tissues were incubated in erlenmeyer flasks with shaking at 37° in an atmosphere of 95% O_2–5% CO_2. The incubations were terminated by freezing the contents of the flasks.

The incubation of experiment I was continued for 2 hr with 10 μc of DL-mevalonate-2-^{14}C as substrate. After the incubation the tissue was removed, transferred to a homogenizer, and 0.14 μc of cholesterol-7α-^{3}H was added. The tissue was homogenized three times in 20 ml of an ethyl alcohol–acetone–ether (4:4:1) mixture and the homogenate was filtered through Whatman No. 1 paper. The filtrate was evaporated and the residue was saponified for 1 hr at 60° in 5 ml of 15% potassium hydroxide and 50% methyl alcohol. Using phenolphthalein, the pH was adjusted with glacial acetic acid. The volume was reduced to 1 ml by evaporation. The extract was partitioned between hexane and 90% methyl alcohol by a five-tube countercurrent distribution (Weisiger, 1954). The hexane fraction was evaporated and a digitonide precipitation was performed (Sperry, 1963). The sterols from the original digitonin precipitation were released by treatment with pyridine, extracted with ether, and assayed for radioactivity. The supernatant fraction from the digitonide precipitate was not examined further in this experiment. To the sterol fraction was added 35 mg of carrier cholesterol and the mixture was purified by bromination. Some of the crystals of cholesterol bromide were counted to determine the ratio of ^{3}H/^{14}C and the remaining crystals were regenerated with zinc (Johnston *et al.*, 1957). These were then recrystallized from methyl alcohol, acetone–water, 80% ethyl alcohol, and acetic acid.

In experiment II four separate flasks were prepared as in experiment I; each contained 10 μc of DL-mevalonate-2-^{14}C in the incubation medium. The incubations of all four flasks were begun at the same time and ended after 2 hr. DL-Mevalonate-5-^{3}H, 18 μc in 0.6 ml of incubation mixture, was added to each of the four flasks but at different times. It was added to the first flask at the beginning of the incubation and to the second, third, and fourth flasks at 10, 30, and 60 min, respectively, after the beginning of the incubation. At the end of the incubation the tissue was transferred to a glass homogenizer and 5 mg of carrier cholesterol was added. The extraction and analysis of cholesterol was carried out as described for experiment I. To measure the ratio of ^{3}H/^{14}C in squalene, an aliquot of the supernatant fraction from the digitonide precipitation from each flask was evaporated and partitioned between a mixture of 10 ml of chloroform, 5 ml of ether, and 5 ml of water made to pH 9 by the addition of KOH. The chloroform–ether fraction was evaporated and the residue was extracted with 25 ml of pentane. Carrier squalene (10 mg) was added. The pentane fraction was concentrated to 3 ml and applied to an alumina column (0.7 × 8.0 cm). The squalene was eluted with 30 ml of pentane. The squalene fraction was placed on a thin layer plate of silica gel G plus rhodamine 6G and chromatographed in the benzene–ethyl acetate (5:1) system (Avigan *et al.*, 1963a). The squalene zone, identified by use of authentic squalene in an adjacent lane of the chromatogram, was recovered by scraping the powder from the plate and eluting the powder with 25 ml of chloroform. To one part (5 ml) of this was added 240 mg of squalene and successive thiourea

[1] Abbreviations used: DPN and TPN, di- and triphosphopyridine nucleotides; ATP, adenosine triphosphate.

TABLE I: Incorporation of DL-Mevalonate-2-^{14}C into Cholesterol by Human Placenta.[a]

Purification Step	$^{3}H/^{14}C$	dpm/mg
Digitonide pptn	0.3	
Bromination	9.0	
Debromination	8.9	380
Crystn I	9.6	290
Crystn II	9.9	320
Crystn III	10	320

[a] The incubation medium consisted of 10 g of minced human placenta and 10 μc of mevalonate-2-^{14}C in 10 ml of Krebs–Ringer phosphate buffer, pH 7.4, with 5 μmoles of DPN, 10 μmoles of TPN, 10 μmoles of ATP, 300 μmoles of nicotinamide, and 111 μmoles of glucose. The incubation was continued for 2 hr at 37° in an atmosphere of 95% O_2 and 5% CO_2. After the incubation, 0.14 μc of cholesterol-7α-^{3}H was added as labeled carrier before the tissue was extracted.

adducts were prepared (Goodman and Popjak, 1960). To another part of the chloroform fraction (10 ml) 400 mg of squalene was added and the hexahydrochloride derivative of squalene was prepared (Loud and Bucher, 1958).

In experiment III four flasks were incubated as in experiment II but 8 μc of DL-mevalonate-2-^{14}C was present in each flask initially and 18 μc of DL-mevalonate-5-^{3}H was added at 0, 10, 30,and 60 min of incubation to flasks 1, 2, 3, and 4, respectively. After the incubation the tissue was transferred to a homogenizer and 5 mg of cholesterol, 5 mg of lanosterol, and 10 mg of squalene were added. The samples were extracted, saponified, and partitioned as in experiment I. The hexane fraction was concentrated to small volume and applied to an alumina column, 0.7 × 8.0 cm (Salokangas *et al.*, 1964). Fractions (20 ml) were collected. Fractions 1 and 2 were eluted with pentane, fractions 3–9 were eluted with 95% pentane–5% benzene, fractions 10–14 with 80% pentane–20% benzene, and fractions 15 and 16 with 70% pentane–30% benzene. The elution pattern was determined with the aid of appropriate standards and by analyzing aliquots of each fraction on thin layer chromatographic plates. Fractions 1 and 2, containing squalene, were transferred to thin layer plates of silica gel G plus rhodamine 6G and chromatographed in the system benzene–ethyl acetate (5:1). The squalene zone was scraped from the plate and eluted with 25 ml of chloroform. To one part (5 ml) of the eluate 270 mg of squalene was added and successive thiourea adducts of squalene were made. To 10 ml of the eluate, 680 mg of squalene was added and the hexahydrochloride derivative of squalene was prepared. Fractions 3–9, containing lanosterol, were

TABLE II: Incorporation of DL-Mevalonate-2-^{14}C and DL-Mevalonate-5-^{3}H into Cholesterol by Human Placenta.[a]

			Cholesterol Crystn				
	Digitonide Pptn	Debromination	I	II	III	IV	V
Flask a							
^{3}H	28,000	450	290	230	240	240	240
^{14}C	17,000	210	180	130	140	140	140
$^{3}H/^{14}C$	1.6	2.1	1.6	1.8	1.7	1.7	1.7
Flask b							
^{3}H	23,000	410	300	200	180	180	190
^{14}C	16,000	270	230	190	200	180	190
$^{3}H/^{14}C$	1.4	1.5	1.3	1.1	0.95	1.0	1.0
Flask c							
^{3}H	16,000	290	190	110	100	100	100
^{14}C	16,000	340	270	210	250	250	250
$^{3}H/^{14}C$	1.0	0.85	0.70	0.52	0.40	0.40	0.40
Flask d							
^{3}H	8,900	96	66	24	39	26	21
^{14}C	17,000	340	270	200	220	200	200
$^{3}H/^{14}C$	0.52	0.28	0.24	0.12	0.18	0.13	0.10

[a] The incubations were carried out in four flasks, each containing 10.3 μc of DL-mevalonate-2-^{14}C. DL-Mevalonate-5-^{3}H, 17.7 μc, was added to flask a at the beginning, to flask b after 10 min, to flask c after 30 min, and to flask d after 60 min of the incubation. The incubations of all four flasks were terminated after 2 hr. The incubation medium and incubation conditions were given with Table I. The values for specific radioactivity are expressed as disintegrations per minute per milligram.

TABLE III: Incorporation of ^{3}H and ^{14}C from Mevalonate into Squalene by Minced Term Human Placenta.[a]

	Pentane Extn	Alumina Column Chromatography	Thin Layer Chromatography	Thiourea Adduct				Hexahydrochloride Deriv			
				Calcd	I	II	III	Calcd	I	II	III
Flask a											
^{3}H	82,000	23,000	25,000	160	170	160	160	130	130	130	130
^{14}C	52,000	15,000	16,000	100	110	100	100	82	85	87	82
$^{3}H/^{14}C$	1.6	1.5	1.6	1.6	1.5	1.6	1.6	1.6	1.5	1.5	1.6
Flask b											
^{3}H	110,000	30,000	30,000	190	220	200	220	150	150	150	160
^{14}C	78,000	21,000	20,000	130	150	170	150	100	100	110	110
$^{3}H/^{14}C$	1.4	1.4	1.5	1.5	1.5	1.4	1.5	1.5	1.5	1.4	1.5
Flask c											
^{3}H	58,000	26,000	23,000	130	150	140	140	110	130	120	120
^{14}C	44,000	19,000	18,000	100	120	110	110	83	93	90	92
$^{3}H/^{14}C$	1.3	1.4	1.3	1.3	1.3	1.3	1.3	1.3	1.4	1.3	1.3
Flask d											
^{3}H	73,000	26,000	24,000	150	160	150	150	120	120	120	120
^{14}C	89,000	26,000	25,000	150	160	150	140	120	130	130	130
$^{3}H/^{14}C$	0.83	1.0	0.96	1.0	1.0	1.0	1.1	1.0	0.92	0.92	0.92

[a] The incubations were carried out in four flasks, each containing 10.3 μc of DL-mevalonate-2-^{14}C. DL-Mevalonate-5-^{3}H, 17.7 μc, was added to flask a at the beginning, to flask b after 10 min, to flask c after 30 min, and to flask d after 60 min of the incubation. The incubations of all four flasks were terminated after 2 hr. The incubation medium and incubation conditions were given with Table I. The values for specific radioactivity are expressed as disintegrations per minute per milligram.

transferred to thin layer plates of silica gel G plus rhodamine 6G and chromatographed in the system benzene–ethyl acetate (5:1). The lanosterol zone was scraped from the plate and eluted with 25 ml of chloroform. The lanosterol was finally crystallized from methyl alcohol–methylene chloride and from methyl alcohol–acetone. Fractions 10–14, containing cholesterol, were transferred to thin layer plates of silica gel G plus rhodamine 6G and chromatographed in the system benzene–ethyl acetate (5:1). The cholesterol zone was scraped from the plate and eluted with 25 ml of chloroform. This fraction was taken to small volume under reduced pressure and again chromatographed on thin layer plates using plates 40 cm long and the system benzene–ethyl acetate (20:1) (Avigan *et al.*, 1963b). The cholesterol zone was eluted with chloroform and the eluate was taken to dryness. The sterol was acetylated overnight with 1 ml of pyridine and 1 ml of acetic anhydride. The acetyl derivative was extracted with 20 ml of hexane and chromatographed for 24 hr on thin layer plates 40 cm long in the system hexane–benzene (5:1). The cholesterol acetate was eluted, saponified, and extracted with hexane. The cholesterol was then purified further by bromination followed by regeneration with zinc, and by recrystallization from methyl alcohol, acetone–water, 80% ethyl alcohol, and acetic acid.

The incubations in experiment IV were carried out as described for experiment II but each flask contained 500 μc of acetate-1-^{14}C instead of DL-mevalonate-2-^{14}C. DL-Mevalonate-5-^{3}H (25 μc) was added to flasks 1–4 at 0, 10, 30, and 60 min, respectively, after the beginning of the incubation. Squalene, lanosterol, and cholesterol were extracted, purified, and analyzed as in experiment III.

Results

The data from experiment I (Table I) indicate that minced human placenta can utilize mevalonic acid in the synthesis of sterols. Using cholesterol-7α-^{3}H as carrier it was possible to crystallize cholesterol to constant $^{3}H/^{14}C$ ratio and to constant specific activity. The low ratio of $^{3}H/^{14}C$ in the total sterol fraction precipitating with digitonin and the relatively high ratio for the final crystalline cholesterol indicates that cholesterol constitutes a rather small fraction of the total sterols present in the placenta.

The incorporation of label from mevalonate-2-^{14}C and mevalonate-5-^{3}H into the sterol fraction in experiment II is summarized in Table II. The ratio of $^{3}H/^{14}C$ in the sterol fraction precipitated with digitonide was proportionately less in the flasks to which the mevalonate-5-^{3}H was added subsequent to the beginning of the incubation. The specific activity of the ^{14}C in the sterol digitonide was the same in all four

TABLE IV: The Incorporation of ^{3}H and ^{14}C from Mevalonate into Squalene and Lanosterol by Minced Term Placenta.[a]

	Squalene									Lanosterol		
	Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Thiourea Adduct				Hexahydrochloride Deriv				Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Crystn		
		Calcd	I	II	III	Calcd	I	II	III		I	II	III
Flask a													
^{3}H	230,000	690	750	720	740	370	360	380	360	380,000	56,000	55,000	56,000
^{14}C	110,000	320	370	350	370	170	180	190	180	180,000	28,000	27,000	28,000
$^{3}H/^{14}C$	2.1	2.2	2.0	2.0	2.0	2.2	2.0	2.0	2.0	2.1	2.0	2.0	2.0
Flask b													
^{3}H	200,000	640	640	630	650	350	320	330	320	350,000	54,000	53,000	51,000
^{14}C	100,000	320	330	330	330	170	170	170	170	190,000	30,000	29,000	28,000
$^{3}H/^{14}C$	2.0	2.0	1.9	1.9	2.0	2.0	1.9	1.9	1.9	1.9	1.80	1.8	1.8
Flask c													
^{3}H	160,000	520	510	480	500	270	240	260	240	280,000	44,000	45,000	45,000
^{14}C	90,000	300	300	290	300	160	150	160	150	200,000	31,000	32,000	31,000
$^{3}H/^{14}C$	1.8	1.7	1.7	1.7	1.7	1.7	1.6	1.6	1.6	1.4	1.4	1.4	1.5
Flask d													
^{3}H	100,000	240	260	240	250	130	140	150	140	180,000	23,000	21,000	20,000
^{14}C	84,000	200	220	210	210	110	120	130	120	210,000	28,000	24,000	24,000
$^{3}H/^{14}C$	1.3	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	0.86	0.82	0.88	0.83

[a] The incubations were carried out in four flasks as described in Table II except that 8.3 μc of DL-mevalonate-2-^{14}C and 18 μc of DL-mevalonate-5-^{3}H were used.

TABLE V: The Incorporation of ^{3}H and ^{14}C from Mevalonate into Cholesterol by Minced Term Placenta.[a]

	Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Thin Layer Chromatography (Benzene–Ethyl Acetate, 20:1)	Thin Layer Chromatography (Hexane–Benzene, 5:1)	Debromination	Crystn			
					I	II	III	IV
Flask a								
^{3}H	4200	1800	670	470	450	380	400	420
^{14}C	1700	750	290	200	190	160	170	170
$^{3}H/^{14}C$	2.5	2.4	2.3	2.4	2.4	2.4	2.4	2.5
Flask b								
^{3}H	5300	1600	690	480	420	370	370	380
^{14}C	2600	1600	460	300	230	240	250	240
$^{3}H/^{14}C$	2.0	1.0	1.5	1.6	1.8	1.5	1.5	1.6
Flask c								
^{3}H	5900	980	280	210	220	140	160	160
^{14}C	3800	950	430	290	300	250	250	260
$^{3}H/^{14}C$	1.6	1.0	0.65	0.72	0.73	0.56	0.64	0.62
Flask d								
^{3}H	1800	180	*b*	*b*	*b*	*b*	*b*	*b*
^{14}C	2700	780	290	200	240	180	190	190
$^{3}H/^{14}C$	0.67	0.23						

[a] The incubations were carried out in four flasks as described in Table II, but in these experiments 8.3 μc of DL-mevalonate-2-^{14}C and 18 μc of DL-mevalonate-5-^{3}H were used. [b] The counts did not exceed twice the background.

flasks as one would expect, since the mevalonate-2-^{14}C was added to all four flasks at zero time. The specific activity of the ^{3}H in these sterol digitonides decreased from flask a to d since the mevalonate-5-^{3}H was added at 0, 10, 30, and 60 min, respectively. When the $^{3}H/^{14}C$ ratios of the sterol digitonides were plotted *vs.* time, the points yielded a straight line which was evidence that the conversion of mevalonate to sterol was linear during at least the first hour of 2 hr of incubation.

As the cholesterol was purified by bromination and debromination and successive crystallizations, the specific activity of the ^{14}C in the product decreased sharply. Comparison of the specific activity of the final crystals of cholesterol with that of the total digitonide-precipitable fraction indicated that the radioactive cholesterol was a small fraction, *ca.* 5%, of the total sterols. The $^{3}H/^{14}C$ ratio for the final crystals decreased with time to a greater extent than did the ratio for the digitonide-precipitable material, which suggested that cholesterol is later in the sequence of metabolites than some of the other compounds that were precipitated by digitonin. The ^{14}C-specific activity of the cholesterol in flasks b–d was higher than that of flask a, although the mevalonate-2-^{14}C was added at zero time in all four flasks. The flasks differed only in the time of addition of the mevalonate-5-^{3}H, the mass of which was $^{1}/_{25}$ of that of mevalonate-2-^{14}C (0.2 μmole *vs.* 5 μmoles). Perhaps when mevalonate-5-^{3}H was present from time zero along with mevalonate-2-^{14}C it competed with the ^{14}C-labeled substrate at some rate-limiting step in the sequence of enzyme reactions between precursor and product. That this difference is observed in the purified cholesterol but not in the total digitonide-precipitable fraction nor in the squalene and lanosterol (Table IV) suggests that the rate-limiting step is in the latter part of the sequence and involves the conversion of some precursor sterol into cholesterol.

The purification of squalene from experiment II is summarized in Table III. The radiochemical purity of the squalene from the alumina column was confirmed by thin layer chromatography and by the addition of carrier squalene and formation of thiourea adducts and hexahydrochloride derivatives of squalene. These gave constant specific radioactivities for both ^{3}H and ^{14}C and constant ratios of $^{3}H/^{14}C$ in the materials counted. The ratio of $^{3}H/^{14}C$ in the squalene decreased proportionately with the time of addition of the mevalonate-5-^{3}H. In each flask except the one in which the two mevalonates were added simultaneously the ratio of $^{3}H/^{14}C$ in squalene was higher than that in cholesterol. This reflects the fact that squalene is earlier in the biosynthetic sequence from mevalonate than is cholesterol.

The purification of squalene and lanosterol from

TABLE VI: The Incorporation of Acetate-1-^{14}C and DL-Mevalonate-5-^{3}H into Squalene and Lanosterol by Minced Term Placenta.

	Squalene					Lanosterol			
	Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Thiourea Adduct			Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Crystn		
		Calcd	I	II	III		I	II	III
Flask a									
^{3}H	62,000	630	660	660	670	360,000	31,000	30,000	32,000
^{14}C	1,100	12	18	20	18	4,400	410	400	400
^{3}H/^{14}C	56	53	37	33	38	82	76	75	80
Flask b									
^{3}H	57,000	590	600	590	590	340,000	31,000	29,000	31,000
^{14}C	1,100	11	17	18	19	5,600	530	480	500
^{3}H/^{14}C	52	53	35	33	31	61	59	60	62
Flask c									
^{3}H	62,000	420	390	430	420	250,000	27,000	27,000	27,000
^{14}C	1,100	7	12	13	14	6,600	670	680	710
^{3}H/^{14}C	56	60	33	33	30	38	40	40	38
Flask d									
^{3}H	27,000	300	340	320	330	130,000	7,000	6,400	7,100
^{14}C	1,200	14	22	23	25	5,900	230	230	230
^{3}H/^{14}C	23	22	15	14	13	22	30	28	31

[a] The incubation conditions and procedure were as described in Table II except that 500 μc of acetate-1-^{14}C and 25 μc of DL-mevalonate-5-^{3}H were used.

experiment III is summarized in Table IV. In this experiment as in experiment II the ratio of ^{3}H/^{14}C in squalene decreased in proportion to the time of addition of the mevalonate-5-^{3}H. The ratio of ^{3}H/^{14}C in the lanosterol also decreased proportionately with the time of addition of the mevalonate-5-^{3}H. In flask a, to which the mevalonate-5-^{3}H was added at zero time, the squalene and lanosterol isolated had identical ratios of ^{3}H/^{14}C, 2.0. In the other flasks the ratio in lanosterol was less than the ratio in the squalene from the same flask. The difference was most marked in flask d, in which the mevalonate-5-^{3}H was added only after 60 min of incubation.

The purification of cholesterol from this experiment is summarized in Table V. The ratio of ^{3}H/^{14}C in cholesterol decreased with the time of addition of mevalonate-5-^{3}H. The ^{3}H/^{14}C ratio of the cholesterol in flask a, 2.5, was greater than the ratio of isotopes in squalene and lanosterol from the same flask. In the conversion of lanosterol (C_{30}) to cholesterol (C_{27}) one of the labeled carbons from mevalonate-2-^{14}C, the methyl carbon attached to position 4, would be lost. When the ^{14}C specific activity of the final crystals of cholesterol was multiplied by 1.2, the ^{3}H/^{14}C ratio calculated with this corrected ^{14}C specific activity became 2.0, identical with that of the squalene and lanosterol. During the purification of cholesterol from flask a the ^{3}H/^{14}C ratio remained constant even though the specific activity of the final crystals was only one-tenth of that of the material recovered from the first thin layer system. This suggested that the contaminants in the initial material were substances, presumably other sterols, with the same ^{3}H/^{14}C ratio. In flasks b and c the purification process led to the isolation of cholesterol with a ^{14}C specific activity of *ca.* 10% of that of the material in the first thin layer systems but the specific activity of the ^{3}H decreased even more, so that the ^{3}H/^{14}C ratio decreased with purification. This suggested that the contaminants in the initial material were substances, presumably other sterols, with somewhat higher ^{3}H/^{14}C ratios, compounds occurring somewhat earlier in the biosynthetic sequence than cholesterol itself. The fact that the decrease in ratio with purification is much greater in flasks c and d than in flask b is consistent with this hypothesis. To compare the ^{3}H/^{14}C ratio of the cholesterol with that of the squalene and lanosterol from the same flask, the ^{14}C specific activity was multiplied by 1.2 and the ratio was then calculated. This gave a value of 1.3 for the cholesterol in flask b and 0.50 for the cholesterol in flask c.

TABLE VII: The Incorporation of Acetate-^{14}C and DL-Mevalonate-5-^{3}H into Cholesterol by Minced Term Placenta.[a]

	Thin Layer Chromatography (Benzene–Ethyl Acetate, 5:1)	Thin Layer Chromatography (Benzene–Ethyl Acetate, 20:1)	Thin Layer Chromatography (Hexane–Benzene, 5:1)	Debromination	Crystn			
					I	II	III	IV
Flask a								
^{3}H	3300	690	250	310	260	270	260	260
^{14}C	340	100	58	68	60	59	60	61
^{3}H/^{14}C	9.7	6.9	4.3	4.6	4.3	4.6	4.3	4.3
Flask b								
^{3}H	3200	440	250	380	280	280	280	300
^{14}C	780	96	57	85	69	69	70	70
^{3}H/^{14}C	4.1	4.6	4.4	4.5	4.1	4.1	4.0	4.3
Flask c								
^{3}H	2500	330	160	190	160	120	120	120
^{14}C	790	170	99	99	96	94	95	100
^{3}H/^{14}C	3.2	1.9	1.6	1.9	1.7	1.3	1.3	1.2
Flask d								
^{3}H	1100	150	30	37	32	36	38	31
^{14}C	700	120	75	90	80	67	77	78
^{3}H/^{14}C	1.6	1.3	0.40	0.40	0.40	0.50	0.50	0.40

[a] The incubation conditions and procedure were as described in Table II except that 500 μc of acetate-1-^{14}C and 25 μc of DL-mevalonate-5-^{3}H were used.

The purification of squalene and lanosterol from experiment IV is summarized in Table VI and the purification of cholesterol is summarized in Table VII. It is clear that, as in the earlier experiments, tritium from mevalonate was incorporated into squalene, lanosterol, and cholesterol. It is also clear that carbon-14 from acetate was incorporated into squalene, lanosterol, and cholesterol. The ratio of ^{3}H/^{14}C in all three products decreased as the mevalonate-5-^{3}H was added later, *e.g.*, compare flasks b–d with flask a. The decrease in the ratio resulted primarily from the lesser incorporation of tritium. When the ratios for the three substances isolated from any given flask were compared, the ^{3}H/^{14}C ratio for lanosterol was much greater and the ratio for cholesterol was much smaller than the ratio for squalene. This was in marked contrast to the results of experiment III (Tables IV and V) in which the substrates were mevalonate-2-^{14}C and mevalonate-5-^{3}H.

Discussion

The experimental results presented here provide evidence of the biosynthesis of squalene, lanosterol, and cholesterol from mevalonate and acetate by minced human term placenta. These results are in contrast to those of Levitz *et al.* (1964) who was unable to demonstrate the conversion of mevalonate to cholesterol in homogenized human term placenta. The specific activity of cholesterol decreased markedly during its purification from the alumina columns, the digitonide fraction had a high specific activity compared to that of the purified cholesterol, and in the experiment to which cholesterol-7α-^{3}H was added as carrier there was a marked increase in the ratio of ^{3}H/^{14}C during the bromination of the digitonide fraction. From the specific activity of the final crystals of cholesterol from experiment I, it can be calculated that 0.12% of the labeled mevalonate was recovered as cholesterol. In a comparable experiment with labeled acetate as substrate, 0.004% of the acetate was recovered as cholesterol (Van Leusden and Villee, 1965). This evidence leads to the conclusion that the incorporation of mevalonate into cholesterol is small relative to its incorporation into other sterols. The previous experiments with acetate as substrate led to a similar conclusion.

When DL-mevalonate-2-^{14}C and DL-mevalonate-5-^{3}H were added to the incubation mixture at the same time, the ratio of ^{3}H/^{14}C was greater in the purified cholesterol isolated at the end of the incubation than in the lanosterol and squalene, which had identical ratios. This would appear to result from the loss of one ^{14}C atom, the methyl group attached to position 4, as lanosterol was converted to cholesterol. The alternative, that the altered ratio resulted from reduction of the lanosterol with ^{3}H derived from the mevalonate,

seems unlikely but is not excluded by the present data.

In the purification of the cholesterol fraction from the alumina column (Table V) the material at each step had a higher ratio of $^{3}H/^{14}C$ than that of the lanosterol fraction. This indicates that the substances removed during the purification of cholesterol also had a high ratio of $^{3}H/^{14}C$ and suggests that they were sterols that had undergone demethylation at position 4.

The experiments in which DL-mevalonate-2-^{14}C was added at time zero and DL-mevalonate-5-^{3}H was added at zero time and at 10, 30, and 60 min of incubation permit the following conclusions. The incorporation of additional substrate into both squalene and lanosterol decreased proportionately with time of incubation. The fact that the $^{3}H/^{14}C$ ratios in squalene and lanosterol were the same in flask a, in which the tritium and ^{14}C-labeled substrates were added at the same time, whereas the ratio for lanosterol was lower than that in squalene in flasks b–d, suggests that lanosterol is synthesized from one pool of squalene. The incorporation of DL-mevalonate-5-^{3}H into cholesterol also decreased as the tritium-labeled material was added at subsequent times. The fact that the $^{3}H/^{14}C$ ratio for cholesterol, when corrected for the loss of the labeled carbon atom from position four, was the same as the $^{3}H/^{14}C$ ratio for lanosterol in flask a but was less than the ratio for lanosterol from the corresponding flask in flasks b–d, suggests further that cholesterol is synthesized in the placenta from one pool of lanosterol.

There is some evidence which suggests that the incorporation of acetate and mevalonate into steroids may occur by pathways that differ in some respects in different tissues and tissue preparations. In slices of bovine ovaries, Hellig and Savard (1965) showed that the incorporation of acetate into progesterone was greater than the incorporation of mevalonate into progesterone. Using adrenal homogenates, Bryson and Sweat (1962) could demonstrate the conversion of acetate-1-^{14}C but not mevalonate-2-^{14}C into steroids. However, Billiar *et al.* (1965) were subsequently able to demonstrate the conversion of mevalonate-5-^{3}H into steroids in adrenal slices. In the present experiments labeled atoms from both acetate and mevalonate were incorporated into squalene, lanosterol and cholesterol by minced human placenta. However, the incorporation of label from acetate into cholesterol was relatively greater than the incorporation of label from mevalonate into cholesterol. In contrast, the incorporation of label from mevalonate into lanosterol was relatively greater than the incorporation of label from acetate into lanosterol.

It does not seem likely that the differences in the incorporation of label from ^{14}C-acetate and ^{3}H-mevalonate can be ascribed to isotope effects. Isotope effects might be expected to occur in the multiple step condensations of isoprenoid units to squalene but no such differences were observed in the experiments with ^{14}C- and ^{3}H-labeled mevalonates. In those experiments the ratio of $^{3}H/^{14}C$ in the three products isolated was similar to that in the substrate. Isotope effects occurring in the reactions between acetate and mevalonate should lead to the production of squalene, lanosterol, and cholesterol with similar $^{3}H/^{14}C$ ratios, that of their common precursor. However, the ratios in the products are notably different, 38 for squalene, 80 for lanosterol, and 4.3 for cholesterol. It does not seem possible to explain the results by assuming that endogenous mevalonate made from acetate and exogenous mevalonate are not in equilibrium and that the former is a more effective precursor of sterols, for although it is relatively a better precursor of cholesterol than mevalonate, it is a poorer precursor of lanosterol than mevalonate. The simplest explanation for the markedly different $^{3}H/^{14}C$ ratios of squalene, lanosterol, and cholesterol is that the metabolic pathways in the placenta from acetate through squalene and lanosterol to cholesterol differ in some respects from those from mevalonate. The placenta is composed of several cell types and the different kinds of cells could differ in their enzymatic complements and hence in their metabolic sequences.

References

Amdur, B. H., Rilling, H., and Bloch, K. (1957), *J. Am. Chem. Soc. 79*, 2646.

Avigan, J., Goodman, D. S., and Steinberg, D. (1963a), *J. Biol. Chem. 238*, 1283.

Avigan, J., Goodman, D. S., and Steinberg, D. (1963b), *J. Lipid Res. 4*, 100.

Billiar, R. B., Oriol-Bosch, A., and Eik-Nes, K. B. (1965), *Biochemistry 4*, 457.

Bryson, M. J., and Sweat, M. L. (1962), *Arch. Biochem. Biophys. 96*, 1.

Cornforth, J. W., Cornforth, R. H., Popjak, G., and Gore, T. Y. (1958), *Biochem. J. 69*, 146.

Goodman, D. S., and Popjak, G. (1960), *J. Lipid Res. 1*, 286.

Hellig, H. R., and Savard, K. (1965), *J. Biol. Chem. 240*, 1957.

Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W., and Folkers, K. (1957), *J. Am. Chem. Soc. 79*, 2316.

Johnston, J. D., Gautschi, F., and Bloch, K. (1957), *J. Biol. Chem. 224*, 185.

Levitz, M., Emeran, S., and Dancis, J. (1964), Meeting on Biological and Chemical Aspects of Placental Steroidogenesis, Baltimore, Md., Williams and Wilkins, p 4.

Loud, A. V., and Bucher, N. L. R. (1958), *J. Biol. Chem. 233*, 37.

Rabinowitz, J. L. (1959), *Atompraxis 5*, 1.

Salokangas, R. A., Rilling, H. C., and Samuels, L. T. (1964), *Biochemistry 3*, 833.

Salokangas, R. A., Rilling, H. C., and Samuels, L. T. (1965), *Biochemistry 4*, 1606.

Savard, K., Goldweber, M., and Goldzieher, J. W. (1960), *Federation Proc. 19*, 169.

Schoenheimer, R., and Sperry, W. M. (1934), *J. Biol. Chem. 106*, 745.

Sperry, W. M. (1963), *J. Lipid Res. 4*, 221.

Tavormina, P. A., Gibbs, M. H., and Huff, J. W. (1956), *J. Am. Chem. Soc. 78*, 4498.
Tsai, S., Ying, B. P., and Gaylor, J. L. (1964), *Arch. Biochem. Biophys. 105*, 329.
Umbreit, W. W., Burris, R. H., and Stauffer, J. F. (1957), Manometric Techniques, 3rd ed, Minneapolis, Minn., Burgess, p 149.
Van Leusden, H., and Villee, C. A. (1965), *Steroids 6*, 31.
Veal, N., and Vetter, M. (1958), Radioisotope Techniques in Clinical Research and Diagnosis, London, Butterworth and Co., p 105.
Villee, D. B., Engel, L. L., Loring, J. M., and Villee, C. A. (1961), *Endocrinology 69*, 354.
Villee, D. B., Villee, C. A., Engel, L. L., and Talbot, N. B. (1962), *J. Clin. Endocrinol. Metab. 22*, 481.
Weisiger, J. R. (1954), *in* Organic Analysis, Vol. 2, Mitchell, J., Ed., New York, N. Y., Interscience, p 277.
Wheatley, V. R. (1953), *Biochem. J. 55*, 637.

The Structure of Neoxanthin and the Trollein-like Carotenoid from *Euglena gracilis**

B. P. Schimmer† and N. I. Krinsky

ABSTRACT: Neoxanthin and the trollein-like carotenoid from *Euglena gracilis* were subjected to acetylation with [1-^{14}C]acetic anhydride, treatment with acidic chloroform, and treatment with acidic ethanol in order to elucidate their structures. Results of acetylation of neoxanthin, a monoepoxy triol, demonstrate that only two hydroxyl groups can be acetylated, indicating that one of the hydroxyl groups is on a tertiary carbon atom.

Treatment of neoxanthin with acidic chloroform results in the immediate rearrangement of neoxanthin to its furanoid isomer, neochrome, and the subsequent *exo* elimination of the tertiary hydroxyl group to form the $\Delta^{5'(18')}$ derivative. In addition, *endo* elimination occurs, leading to the formation of the $\Delta^{2',4'}$- (or $\Delta^{3',5'(18')}$-) monofuranoid monohydroxyl derivative. As concluded from the results of acetylation and from the course of dehydration with acidic chloroform, neoxanthin is 3,3′,5′-trihydroxy-6′-hydro-5,6-epoxy-β-carotene. The course of dehydration with acidic ethanol confirms this proposed structure for neoxanthin. The trollein-like polyol carotenoid from *E. gracilis* also has only two hydroxyl groups which can be acetylated. Results of treatment with the acidic chloroform and acidic ethanol indicate that an additional hydroxyl group is on a tertiary carbon atom, but that the trollein-like carotenoid is more complicated than one with hydroxyl groups on the 3,3′,5′ (or 6′) positions.

Strain (1938) first isolated neoxanthin from green leaves and proposed a $C_{40}H_{56}O_4$ formula for this pigment. From his analyses, one can conclude that, along with lutein and violaxanthin, neoxanthin is one of the major xanthophylls of green leaves. Curl and Bailey (1957) examined the effect of concentrated hydrochloric acid on ethereal solutions of neoxanthin and found that a blue color, associated with the presence of cyclic ethers (Karrer and Jucker, 1950), was formed. Based on the formation of the blue color and on the results of countercurrent distribution, they proposed a trihydroxyl 5,6-epoxide structure for this pigment, and this structure was independently supported by the observations of Goldsmith and Krinsky (1960). The latter authors suggested that Strain's original chemical analyses could not distinguish between compounds with 56 or 58 hydrogens, and that the formula for neoxanthin might be more correctly written as $C_{40}H_{58}O_4$. The spectral similarity of neoxanthin to violaxanthin and the possibility that neoxanthin might be an intermediate in violaxanthin biosynthesis prompted Goldsmith and Krinsky (1960) to propose that neoxanthin is 3,3′,5′- (or 6′-) trihydroxy-6′- (or 5′-) hydro-5,6-epoxy-β-carotene (A).

The results reported by Krinsky (1963) for the relative polarity of neoxanthin (3.24 units) were in complete accord with the epoxy triol structure. Furthermore, as evidenced by the lack of change of relative polarity after treatment with dilute acidic methanol, neoxanthin reportedly contained only nonallylic hydroxyl groups. As shown by Petracek and Zechmeister (1956), carotenoids with allylic hydroxyl groups are

* Department of Pharmacology, Tufts University School of Medicine, Boston, Massachusetts. *Received February 10, 1966.* Supported in part by Public Health Service Research Grant GM-07984 from the National Institute of General Medical Science.

† Predoctoral fellow in Biochemical Pharmacology supported by Public Health Service Training Grant 5T1 GM-765.