Biosynthesis of squalene and sterols by rat aorta

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ABSTRACT The synthesis of nonsaponifiable compounds from radioactive mevalonate by segments of adult rat aorta was studied in vitro. The labeled products consisted largely of substances with the chromatographic and chemical behavior of squalene, lanosterol, lathosterol, and cholesterol. Even after 3 or 4 hr of incubation, the incorporation of mevalonate into squalene was higher than its incorporation into C_{27} sterols; cholesterol contained less than 20% of the radioactivity in the total sterols. Lanosterol was the most highly labeled sterol. The level of radioactivity in lathosterol was comparable to the level in cholesterol. Small amounts of radioactivity were found in other sterols. Material with the same mobility on TLC as 7-dehydrocholesterol had less radioactivity than cholesterol, but more than sterols with the mobility of desmosterol. The results of measurements made after short periods of incubation showed that squalene and lanosterol became labeled before the other nonsaponifiable compounds.

SUPPLEMENTARY KEY WORDS cholesterol · lanosterol · lathosterol · 7-dehydrocholesterol · desmosterol

Arterial tissues have the capacity to synthesize cholesterol (1–5). In most previous studies, synthesis has been demonstrated by the incorporation of labeled precursors in vitro into substances that are precipitable by digitonin. When these products are purified, it is found that much of the label has been incorporated into substances other than cholesterol (2–4). The purpose of the present study was to identify other nonsaponifiable compounds synthesized by arterial tissues. The biosynthesis of nonsaponifiable compounds from radioactive mevalonate by rat aorta was studied in vitro. Significant incorporation of the precursor into cholesterol could be demonstrated, but a major fraction of the radioactivity of the nonsaponifiable fraction was in substances with the chromatographic and chemical behavior of squalene, lanosterol, and lathosterol.

MATERIALS AND METHODS

Animals and Tissues

Male rats of the Carworth Farms Nelson Strain (Carworth Farms, New City, N.Y.) weighing 300–400 g were used. The rats were killed by decapitation. The segment of aorta from the heart to the diaphragm was excised immediately. The vessel was placed in ice-cold medium and cleaned of adherent fat and connective tissue. The aorta was opened longitudinally to form a sheet of tissue 0.2–0.3 mm thick. Liver slices approximately 0.5 mm thick were prepared by means of a Stadie-Riggs microtome.

Incubations

Samples of aorta or liver were incubated in Krebs-Ringer bicarbonate medium containing 0.014 M glucose and mevalonate-2-^{14}C or mevalonate-5-^{3}H at the concentrations indicated in the tables. The flasks were flushed with 95% O_2–5% CO_2, stoppered, and shaken in a water bath at 37°C. At the end of the incubation period, ethanol and KOH pellets were added to a final

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Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; C_{27} sterols, 27-carbon sterols; C_{30} sterols, 30-carbon sterols.

Trivial names (in parentheses) have been used for the following sterols: lanost-8-en-3β-ol (dihydrolanosterol); 5α-cholest-7-en-3β-ol (lathosterol); cholesta-5,7-dien-3β-ol (7-dehydrocholesterol); cholesta-5,24-dien-3β-ol (desmosterol); 5α-cholestan-4β-ol (dihydrocholesterol, cholestanol).

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concentration of 5% KOH in 80% ethanol. A mixture of nonradioactive compounds containing 100 μg each of squalene, lanosterol, dihydrolanosterol, lathosterol, desmosterol, and 7-dehydrocholesterol was added to each flask as carrier. The flasks were flushed with nitrogen, stoppered, and heated for 2 hr at 70°C. An aliquot of the digestion mixture was taken for measurement of tissue nitrogen content (6). The balance of the mixture was then diluted with 1 vol of water and extracted twice with 2 vol of hexane. The combined extracts were washed once with water, evaporated to a small volume in a stream of nitrogen, and then applied to thin-layer plates.

Thin-layer Chromatography

Thin-layers, 0.25 mm thick, were prepared from silica gel (Adsorbosil-1, Applied Science Laboratories Inc., State College, Pa.) or aluminum oxide (type E, E. Merck AG, Darmstadt, Germany). With the few exceptions indicated below, thin-layer plates 20 cm long were used. Aluminum oxide plates were sprayed with 25% $AgNO_3$ before activation and with 0.1% rhodamine 6G in ethanol after development. Rhodamine 6G was incorporated into the silica gel layers during their preparation. Both types of plates were activated at 120°C for 20 min before use. Samples containing 100 μg each of individual sterols were applied in a 2 cm streak. All layers were examined under ultraviolet light after development in order to locate the sterols. The zones were scraped from the plates and eluted by washing the adsorbent with chloroform and centrifuging.

The chromatographic steps are summarized in Fig. 1. Squalene, C_{30} sterols, and C_{27} sterols were separated on silica gel developed in hexane–ether–acetic acid 83:17:1 (TLC system 1). The C_{27} sterols were eluted and applied to aluminum oxide–silver nitrate TLC plates that were developed in chloroform (TLC system 2). Mixtures of lanosterol plus dihydrolanosterol, and of dihydrocholesterol plus lathosterol, were acetylated by the micro procedure of Johnson, Gautschi, and Bloch (7), and then resolved on silica gel plates developed in hexane–benzene 10:4 (TLC system 3). TLC systems 2 and 3 were based on methods developed by Kammereck, Lee, Paliokas, and Schroepfer (8) and by Avigan, Goodman, and Steinberg (9). The recovery of material carried through the three chromatographic systems was determined on a sample of cholesterol-4-^{14}C. The cumulative recovery of cholesterol carried through the three TLC systems was 77%. Since the other lipids were not available as highly purified radioactive materials, recovery was measured by means of GLC with the use of an internal standard. The cumulative recovery of the other sterols after passage through TLC systems 1, 2, and 3 was not significantly different from that of cholesterol.

We occasionally achieved separation of dihydrocholesterol and lathosterol in TLC system 2. In most experiments, however, these sterols were not separated on aluminum oxide–silver nitrate layers. We found that the acetates of dihydrocholesterol and lathosterol could always be separated in TLC system 3, although it was often necessary to develop the chromatogram more than once.

Sterols isolated by means of TLC systems 2 and 3 were precipitated with digitonin in order to eliminate extraneous radioactive materials that might have been carried through the chromatographic steps. A diagram

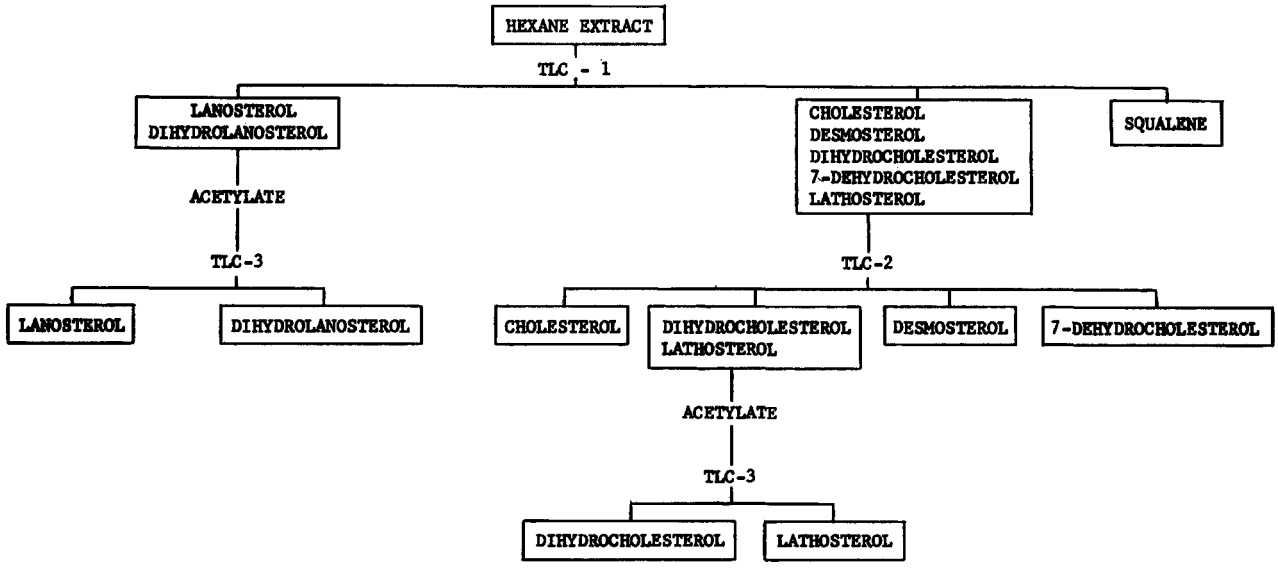

FIG. 1. Flow sheet for analysis of nonsaponifiable lipids. TLC systems: *1*, silica gel plates developed in hexane–ether–acetic acid 83:17:1; *2*, aluminum oxide plates (treated with silver nitrate) developed in chloroform; *3*, silica gel plates developed in hexane–benzene 10:4.

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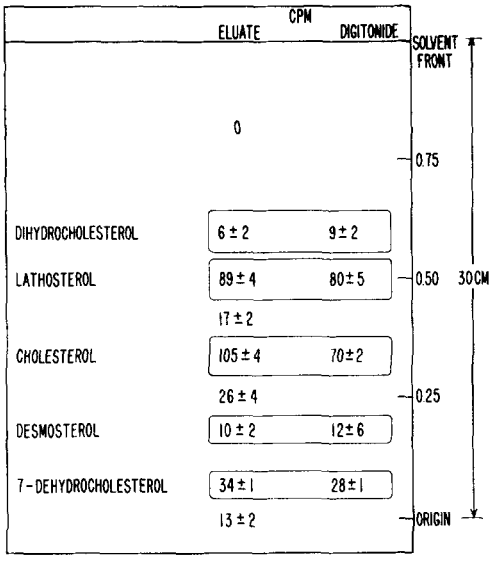

FIG. 2. Distribution of radioactivity on a thin-layer plate after development in TLC system 2 (aluminum oxide–silver nitrate developed in chloroform). Aortas from three rats were divided longitudinally and distributed among three flasks. Each flask contained 2.5 μCi (0.39 μmole) of DL-mevalonate-2-^{14}C in 1 ml of medium and was incubated for 3 hr. The C_{27} sterol fraction was applied to the thin-layer plate. The cpm ± SEM are shown for the material eluted from the plate before and after precipitation with digitonin.

of the separation of C_{27} sterols in TLC system 2 is shown in Fig. 2. Three samples of aorta were incubated with mevalonate-2-^{14}C for 3 hr. The nonsaponifiable fraction was extracted, fractionated in TLC system 1, and the C_{27} sterols were separated from each other in TLC system 2. A thin-layer plate 40 cm long, instead of the usual 20-cm plate, was used for the separation shown in the diagram in order to increase the separation between zones. The radioactivity in and between sterol zones is shown, as well as radioactivity in material precipitated by digitonin. 80% of the radioactivity on the plate is associated with known compounds, and 83% of the radioactivity in the zones corresponding to sterols is precipitable by digitonin.

The mobility of materials isolated by the procedure outlined in Fig. 1 was studied in other chromatographic systems in order to confirm the association of radioactivity with specific sterols. The mobility of free sterols was studied in two systems described by Avigan et al. (9): thin-layer plates prepared from silica gel (20 cm long) developed in benzene–ethyl acetate 5:1 (TLC system 4), and thin layer plates of silica gel (40 cm long) developed in benzene–ethyl acetate 20:1 (TLC system 5). The mobility of steryl acetates was studied by a method adapted from procedures for column and thin-layer chromatography described by Kammereck et al. (8) and Lee, Kammereck, Lutsky, McCloskey, and Schroepfer (10). The acetates were chromatographed on thin-layers of alumina impregnated with silver nitrate, with hexane–benzene 90:10 as solvent (TLC system 6).

Gas–Liquid Chromatography

A Barber-Colman model 5000 gas chromatograph equipped with a hydrogen flame detector was used. A U-shaped glass column (6 ft × 6 mm I.D.) packed with 1.5% SE-30 methyl silicone on Gas-Chrom Q (100–120 mesh) (Applied Science Laboratories Inc.) was used. The temperature of the column was 230°C, the detector 270°C, and the flash heater 260°C. The carrier gas was nitrogen with a flow rate of 50 ml/min. Peak areas were measured with a disc chart integrator (Disc Instruments, Inc., Santa Ana, Calif.).

Purification of Squalene

200 mg of nonradioactive squalene was added as carrier to the squalene eluted from the gel after separation in TLC system 1. Squalene was purified by preparation of the hexahydrochloride, which was crystallized to constant specific activity (11). The results of the purification of a group of samples isolated from incubation mixtures are shown in Table 1. All samples reached constant specific activity after three crystallizations.

Preparation of Digitonides

Digitonides of all sterols except lanosterol and dihydrolanosterol were precipitated by the procedure of Sperry (12) after the addition of 1 mg of cholesterol as carrier. As reported by Gaylor (13), lanosterol is not completely precipitated under conditions satisfactory for the C_{27} sterols. We used the following procedure, by which at least 80% of lanosterol or dihydrolanosterol is precipitated. To 0.5–1 mg of lanosterol in 2.5 ml of acetone–ethanol 1:1 is added 2.5 ml of 2% digitonin in 80% ethanol and 1 ml of water; the mixture is allowed to stand overnight. The digitonide is thus precipitated from a solvent mixture in which the free sterol is soluble at the concentration employed. The lanosteryl digitonide

TABLE 1 PURIFICATION OF SQUALENE

Number of Crystallizations	Specific Activity of Squalene Hexahydrochloride			
	I	II	III	IV
	cpm/mg			
Sample 1	9.37	8.93	8.57	8.56
2	11.0	11.8	11.5	
3	7.30	7.60	7.11	7.42
4	3.19	3.34	3.38	3.42

After incubation of four samples of aorta with mevalonate-2-^{14}C, squalene was isolated in TLC system 1, diluted with 200 mg of nonradioactive squalene, converted to the hexahydrochloride, and crystallized from ethyl acetate.

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is washed three times with 0.8% digitonin in the same solvent mixture, dissolved in warm methanol, and reprecipitated by the addition of an equal volume of water.

Radioactivity

Solutions of lipids were dried in counting vials and dissolved in 15 ml of toluene containing 60 mg of 2,5-diphenyloxazole and 1.5 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene. Digitonides were prepared for counting by solution in methanol or 1% H_3PO_4 in 1,4-dioxane (14). Radioactivity was measured in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) with an efficiency of 82% for ^{14}C and 24% for ^{3}H under the conditions employed for counting.

Chemicals

The compounds used as chromatographic standards and as carriers were obtained from the following sources: squalene, Eastman Organic Chemicals, Rochester, N.Y.; lanosterol, Calbiochem, Los Angeles, Calif.; desmosterol and lathosterol, Mann Research Laboratories, N.Y.; dihydrocholesterol and 7-dehydrocholesterol, Aldrich Chemical Co., Inc., Milwaukee, Wis.; and cholesterol, Nutritional Biochemical Corp., Cleveland, Ohio. 4,4-Dimethylcholest-5-en-3β-ol, 4,4-dimethyl-5α-cholest-7-en-3β-ol, and 4α-methyl-5α-cholest-7-en-3β-ol were generous gifts from Dr. J. L. Gaylor of Cornell University. Lanosterol was separated from dihydrolanosterol in the commercial preparation by chromatography (7). Cholesterol was purified through the dibromide (15). Dihydrocholesterol was purified by the procedure of Anderson and Nabenhauer (16). 7-Dehydrocholesterol was recrystallized twice from ethanol. Each lipid showed one major peak or spot and less than 5% impurities when analyzed by GLC and TLC as described above.

DL-Mevalonic acid-2-^{14}C, and DL-mevalonic acid-5-^{3}H were obtained from New England Nuclear Corp., Boston, Mass. as the dibenzylethylenediamine salts. The organic base was removed by dissolving the salt in water, adding a slight excess of sodium bicarbonate, and extracting the solution with ether three times. Nitrogen was bubbled into the aqueous solution in order to remove dissolved ether. The incorporation of mevalonate into products was calculated with the assumption that only one enantiomorph is utilized for biosynthesis. Cholesterol-4-^{14}C was obtained from New England Nuclear Corp. All samples of radioactive cholesterol, including those isolated from incubation mixtures, were purified through the dibromide by a slight modification of the procedure of Fieser (15). 150 mg of unlabeled cholesterol was added as carrier to each sample, and the scale of the procedure was reduced 1000-fold.

RESULTS

Incorporation of Mevalonate-2-^{14}C into Nonsaponifiable Compounds by Rat Aorta

When samples of rat aorta were incubated with mevalonate-2-^{14}C for 3 hr, small, but significant, amounts of ^{14}C were incorporated into nonsaponifiable lipids. Values obtained for the radioactivity in the total nonsaponifiable fraction after incubation of one aorta (70 mg wet wt, 2.5 mg of nitrogen) with 2.5 μCi (0.43 μmole) of DL-mevalonate-2-^{14}C in 1 ml of medium were in the range of 500–1000 cpm. These values correspond to the incorporation of 20–40 pmoles/mg of tissue nitrogen in 3 hr.

It will be demonstrated that most of the radioactivity in the nonsaponifiable lipids is associated with compounds comprising minute fractions of the tissue mass. All of the compounds considered here, with the exception of cholesterol, were isolated after the addition of unlabeled carrier compounds. The nonsaponifiable compounds were fractionated according to the scheme shown in Fig. 1. After separation in TLC system 1, lipids were eluted from the zones corresponding to squalene, C_{27}, and C_{30} sterols. Squalene was purified as the hydrochloride, and aliquots of the C_{27} and C_{30} sterols were precipitated with digitonin. The distribution of radioactivity among these fractions is shown in Table 2. The incorporation of mevalonate into nonsaponifiable lipids increased when the concentration of mevalonate in the medium was raised from 0.4 to 14 mM. At all concentrations employed, however, squalene was a major labeled component of the nonsaponifiable lipids, and accounted for more radioactivity than the C_{27} sterols.

The C_{27} and C_{30} sterols were fractionated by TLC systems 2 and 3. Several C_{27} sterols contained significant amounts of label, but the major radioactive C_{27} sterols were cholesterol and lathosterol. Experiments were conducted to confirm the identity of the most highly labeled sterols. Synthesis of cholesterol by isolated rat aorta had been previously demonstrated, but the association of radioactivity with cholesterol was confirmed, as in previous experiments (4), by purification of

TABLE 2 INCORPORATION OF MEVALONATE-2-^{14}C INTO SQUALENE AND STEROLS BY RAT AORTA

Experiment	Squalene	C_{30} Sterols	C_{27} Sterols
		dpm	
1	310	366	134
2	113	120	58
3	395	666	305

In each experiment, one aorta (avg wt, 80 mg; avg N, 2.5 mg) was incubated for 3 hr in 1 ml of medium containing 2.5 μCi (0.43 μmole) of DL-mevalonate-2-^{14}C.

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cholesterol through the dibromide (15). Material isolated from the cholesterol zone after chromatography in TLC system 2 retained 80% of its radioactivity after purification through the dibromide.

The identification of radioactive lathosterol is based upon the following evidence. Lathosterol was isolated by the scheme shown in Fig. 1. Radioactivity remained associated with lathosterol carrier when the migration of the sample was tested in TLC systems 4–6. The radiopurity of lathosterol isolated by TLC systems 1–3 was checked in the following way. The sample of lathosteryl acetate was hydrolyzed, and the free sterol was applied to an aluminum oxide–silver nitrate TLC plate which was then developed in chloroform (TLC system 2). The distribution of radioactivity on the plate is shown in Fig. 3. The only significant radioactivity is that associated with the spot corresponding to the lathosterol carrier. The lathosterol spot was divided into three parts as shown in the figure. Material was eluted from each portion of the gel with chloroform. An aliquot of each eluate was counted. The mass of lathosterol in each portion was determined by means of GLC with the use of lanosterol as an internal standard. The retention times for lathosterol and lanosterol were 11 and 17 min, respectively. The specific activities of material eluted from regions *A*, *B*, and *C* were 135, 123, and 123 cpm/μg, respectively. The fact that material in region *A* had a higher specific activity than *B* or *C* suggests that other unidentified sterols, possibly Δ^8 sterols, were contaminating the slowest portion of the lathosterol peak. The material in regions *B* and *C* had the same specific activity, however. These results suggested that radioactivity coincided with mass for the major portion of the peak, and that most of the radioactivity could probably be attributed to lathosterol. The presence of radioactive lathosterol was confirmed by testing the sensitivity of material in the "lathosterol" region to oxidation by selenous acid at 0°C (17). In two experiments, samples eluted from the lathosteryl acetate zone showed 82 and 65 cpm before oxidation, and 12 and 0 cpm, respectively, after oxidation. The disappearance of most of the radioactivity after oxidation confirms the presence of radioactive lathosterol, but small amounts of other radioactive sterols may have been present in the sample showing persistence of radioactivity after oxidation.

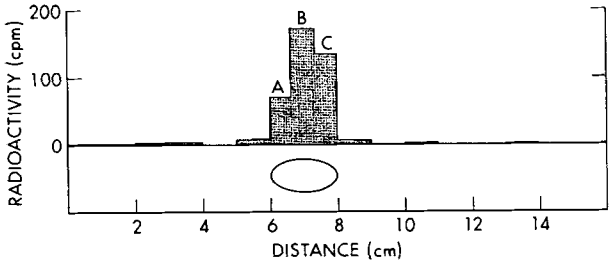

FIG. 3. Thin-layer chromatographic analysis of radiopurity of lathosterol. Aluminum oxide–silver nitrate; chloroform. Radioactivity of material eluted from the plate is presented above a diagram showing the position of lathosterol on the plate as an oval. The specific activities of lathosterol eluted from regions *A*, *B*, and *C* were 135, 123, and 123 cpm/μg, respectively.

The C_{30} sterols were fractionated by TLC system 3, and the bulk of the radioactivity in this fraction was associated with material having the mobility of lanosterol. Radioactivity remained associated with lanosterol when the sample migrated in TLC systems 2, 4, 5, and 6. Chromatography of the acetates in TLC system 6 also eliminated the possibility that certain C_{28} and C_{29} sterols contributed to the radioactivity of the "lanosterol" fraction. The R_F of lanosteryl acetate in that system was 0.42, while the R_F values of the acetates of 4,4-dimethyl-5α-cholest-7-en-3β-ol and 4α-methyl-5α-cholest-7-en-3β-ol were 0.72 and 0.65, respectively. The procedure may not have eliminated the possibility that C_{28} or C_{29} sterols with a second double bond in position 24 were present. The radiopurity of the lanosterol was checked by a procedure similar to that used for lathosterol. Lanosteryl acetate, isolated by the procedure outlined in Fig. 1, was hydrolyzed and chromatographed in TLC system 2. The distribution of radioactivity on the plate is shown in Fig. 4. The only significant radioactivity on the plate was associated with the spot corresponding to lanosterol. The specific activity of material eluted from different parts of the lanosterol zone was determined. The radioactivity was measured, and the mass of sterol in the samples was determined by GLC, using cholesterol as internal standard. The retention times for cholesterol and lanosterol were 9 and 17 min, respectively. The specific activities of material

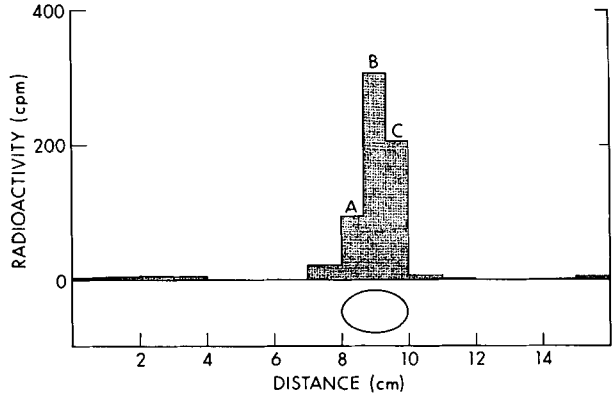

FIG. 4. Thin-layer chromatographic analysis of radiopurity of lanosterol. Aluminum oxide–silver nitrate; chloroform. Radioactivity of material eluted from different regions of the plate is presented above a diagram showing the position of lanosterol on the plate as an oval. The specific activities of lanosterol eluted from regions *A*, *B*, and *C* were 2900, 3036, and 2904 cpm/μg, respectively.

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eluted from regions *A*, *B*, and *C* were 2900, 3036, and 2904 cpm/μg, respectively. The correspondence between mass and radioactivity in the "lanosterol" zone was within 3%, and confirmed the identification of lanosterol as the major sterol in the "lanosterol" zone.

The results of 10 experiments in which rat aortas were incubated with mevalonate-2-^{14}C for 3 hr are summarized in Fig. 5. Aortas of two animals were pooled in each experiment. The nonsaponifiable fraction was isolated and fractionated by the procedures already described. The radioactivity in each sterol is expressed relative to the radioactivity in cholesterol set at 100. Lanosterol is the most highly labeled sterol. The levels of radioactivity in lathosterol are comparable to those in cholesterol. Levels of radioactivity in 7-dehydrocholesterol are lower than those in cholesterol, but significantly higher than the levels in desmosterol and dihydrocholesterol.

TABLE 3 INCORPORATION OF MEVALONATE INTO SQUALENE AND STEROLS BY LIVER AND AORTA

Experiment	Liver		Aorta	
	1	2	1	2
	dpm/mg tissue N			
7-Dehydrocholesterol	417	452	5	11
Desmosterol	3,237	4,222	6	10
Lathosterol	1,321	1,265	22	65
Lanosterol	1,232	2,582	24	43
Dihydrolanosterol	166	84	4	6
Dihydrocholesterol	209	1,240	1	1
Cholesterol	96,783	87,605	18	34
Squalene	1,912	4,179	105	312

In each experiment, 0.3 g (wet wt; 9 mg of tissue N) of liver slices was incubated with 1.25 μCi (0.09 μmole) of DL-mevalonate in 2 ml of medium. 90 mg (wet wt; 3.3 mg of tissue N) of aorta was incubated with 2.5 μCi (0.19 μmole) in experiment 1, and 5 μCi (0.37 μmole) in experiment 2, in 1 ml of medium. Incubation time, 3 hr.

Comparison of Aorta and Liver

The incorporation of mevalonate into nonsaponifiable compounds was compared in aorta and liver incubated for 3 hr under the same conditions except for the concentration of radioactive mevalonate in the medium. The results are shown in Table 3. The absolute incorporation of mevalonate into nonsaponifiable compounds by liver is markedly higher than aorta. The comparison is not completely valid, since we do not know whether the cells of the two tissues are equally permeable to mevalonate. Moreover, the fact that over 50% of the dry weight or nitrogen content of the aorta represents extracellular proteins (18) should be considered when comparisons are made. It is clear, however, that the distribution of radioactivity among the nonsaponifiable compounds isolated from liver and aorta is entirely different. Cholesterol isolated from liver slices had 90–94% of the total sterol radioactivity; that isolated from aorta, 20–30%. Squalene accounts for a major portion (57–65%) of the radioactive nonsaponifiable compounds isolated from the aorta, but for a minor portion (2–4%) of those isolated from the liver.

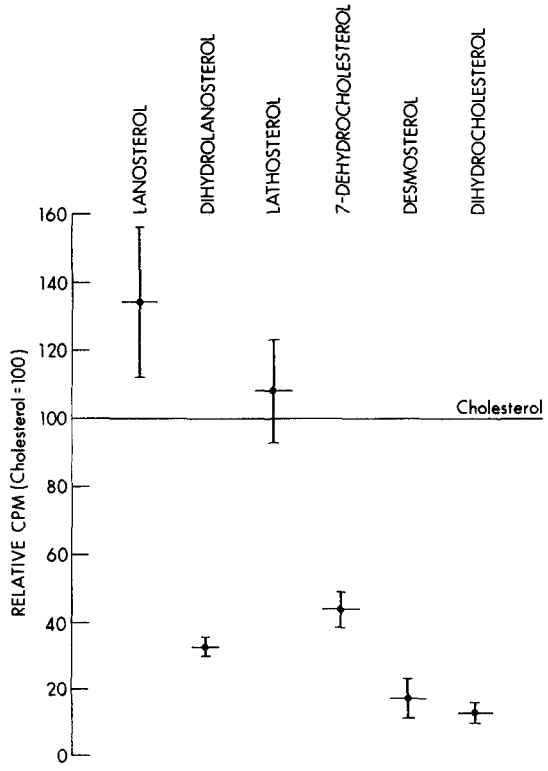

FIG. 5. Distribution of radioactivity among sterols relative to cpm in cholesterol, set at 100. Sterols were fractionated by the scheme shown in Fig. 1. Means ± SEM are presented, n = 4–10. In each experiment, two aortas were pooled and incubated with 5 μCi (0.8 μmole) of mevalonate-2-^{14}C in 2 ml of medium for 3 hr.

Effect of Incubation Time on Incorporation of Mevalonate into Nonsaponifiable Compounds by Aorta

For studies of the time course of the incorporation of mevalonate into nonsaponifiable compounds, aortas were divided longitudinally into two or three strips. Tissues from two or more animals were distributed into replicate flasks so that each flask in a series contained tissue from the same animals. In Table 4 the results of incubation of tissue with mevalonate-2-^{14}C for periods of 90 min to 4 hr are shown. The radioactivity of the total nonsaponifiable fraction was measured on an aliquot of the hexane extract. The radioactivity labeled "total sterols" was the cpm in the digitonide precipitated from an aliquot of the hexane extract by the procedure used for C_{27} sterols, and includes part of the lanosterol radioactivity. The radioactivity in the "C_{27} sterols" was measured on the digitonide prepared from the eluate of the C_{27} sterol zone in TLC system 1. The

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TABLE 4 EFFECT OF TIME ON THE INCORPORATION OF MEVALONATE INTO NONSAPONIFIABLE COMPOUNDS BY RAT AORTA

Experiment	Incubation Time	Total Nonsaponifiable Lipids	"Total Sterols"	C_{27} Sterols	Cholesterol
	hr		*dpm*		
1	1.5	2,805	183		13
	3	2,988	634		39
2	2	2,932	329		
	4	3,780	506		
3	2			228	50
	4			626	135

For each time period, tissue equivalent to one aorta (80 mg wet wt; 3.0 mg of N) was incubated in 2 ml of medium with 5 μCi (0.8 μmole) of DL-mevalonate-2-^{14}C.

results show that the radioactivity in the sterol fraction increased from 90 min to 4 hr. Cholesterol accounts for an increasing percentage of the radioactivity in the nonsaponifiable lipids with time, but there is little change in the radioactivity of cholesterol relative to the "total" or C_{27} sterols with time under these conditions.

The time course of the distribution of radioactivity among certain components of the nonsaponifiable lipids was also studied after shorter periods of incubation. In these experiments, the aorta was incubated with a tracer dose of mevalonate-5-^{3}H of high specific activity to permit the detection of small amounts of precursor after short periods of incubation. Average results of two experiments in which the distribution of radioactivity is compared at 10, 20, and 120 min are shown in Table 5. Under these conditions, the radioactivity in squalene reached a maximum at or before 10 min. Lanosterol was significantly labeled at 10 min, and its radioactivity increased slowly thereafter. Lathosterol and cholesterol were not significantly labeled before 20 min of incubation. These data indicate that squalene and lanosterol become labeled before lathosterol and cholesterol.

TABLE 5 TIME COURSE OF DISTRIBUTION OF RADIOACTIVITY AMONG NONSAPONIFIABLE COMPOUNDS

Lipid	Incubation Time (min)					
	10	20	120	10	20	120
	dpm			*% of total dpm*		
Squalene	692	475	550	82	75	55
Lanosterol	129	125	304	15	20	31
Lathosterol	13	17	92	1	3	9
Cholesterol	8	13	46	1	2	5
Total	842	629	992			

Tissue equivalent to one aorta (80 mg wet wt; 3.0 mg of N) was incubated with 12.5 μCi (0.06 μmole) of DL-mevalonate-5-^{3}H in 1 ml of medium for each time period. The figures are averages of data from two experiments.

Biosynthesis in Different Layers of the Vessel

In the experiments that have been reported thus far, a preparation of vessel that included intima, media, and part of the adventitia was used. The bulk of this preparation by weight is media, and it seemed probable that most of the biosynthesis took place in the media. Some information concerning the layers involved was obtained from three experiments in which aortas were incubated with radioactive mevalonate for 3 hr and then divided into a preparation of intima plus inner media, which included most of the media, and one of adventitia plus residual media (19). In three experiments the media–intima portions contained 91, 94, and 96% of the total sterol radioactivity. The media–intima portions incorporated, respectively, 4, 7, and 12 times as much label into sterols per unit weight as the adventitia–media layers. The results indicate that most of the biosynthetic activity can be attributed to the media–intima layers of the vessel.

Sterol Content of Rat Aorta

The cholesterol content of preparations of rat aorta similar to those used in the present study has been measured (4). In order to determine whether other sterols were present, nonsaponifiable lipids were isolated from a pool of aortas obtained from 10 rats. The lipids were fractionated by the procedure outlined in Fig. 1. Since the amounts of sterols other than cholesterol were so small, they were located on thin-layer plates with the aid of standards placed in adjacent lanes. The gel was eluted from regions other than that occupied by the predominant cholesterol zone. Sterols were detected in the eluate by means of GLC. The amounts of individual sterol fractions were not determined, but the total content of these sterols was 1% of the cholesterol content of the tissue sample.

DISCUSSION

The data presented in this paper show that the nonsaponifiable lipids synthesized by rat aorta in vitro from labeled mevalonate consist largely of substances with the chromatographic and chemical behavior of squalene, lanosterol, lathosterol, and cholesterol. In contrast to the results of experiments with human arterial intima reported by Chobanian (5), the amount of label in dihydrocholesterol was insignificant. The two studies, however, differ with respect to the species studied, the layers of vessel employed, and the experimental conditions. The present study is in agreement with the report of St. Clair, Lofland, Pritchard, and Clarkson, who demonstrated synthesis of squalene by arterial tissues of other animals (20).

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Squalene, lanosterol, and lathosterol have been shown to be intermediates in the pathway of cholesterol biosynthesis in other tissues (21). It is probable that they became labeled in the present experiments because they are intermediates in the aorta as well. Consistent with their probable roles as intermediates, squalene and lanosterol were shown to become labeled before cholesterol. Information about the time course of appearance of label in the other sterols is not yet available.

It is not clear why such large amounts of radioactivity persist, after 3 hr of incubation, in substances that are potentially precursors of cholesterol, and that do not comprise a large fraction of the tissue mass. There are other tissues, such as skin, which show a similar tendency for the accumulation of label in sterols that are intermediates in cholesterol biosynthesis. In these tissues, however, sterols other than cholesterol comprise a major fraction of the total sterol mass (22, 23). The presence of squalene and traces of sterols other than cholesterol has been reported in arterial tissues (24, 25), but, to our knowledge, the presence of lanosterol and lathosterol has not been reported previously. The concentrations of endogenous squalene, lanosterol, and lathosterol were not measured in the tissues used in the present study, but they must have been small. The total content of sterols other than cholesterol is approximately 1% of the cholesterol content. The squalene content was not measured in this preparation, but it has been found to be low in other arterial tissues (24). The accumulation of radioactivity in intermediates may be related to the fact that, in comparison with tissues such as liver, cholesterol biosynthesis is extremely slow in the aorta. In the experiments reported here, radioactivity may have accumulated in compounds located immediately before slow steps in the biosynthetic pathway.

It is known that further reactions of squalene, lanosterol, and lathosterol in the pathway of biosynthesis of cholesterol are dependent upon oxygen (21). These results, therefore, raise questions concerning the adequacy of the oxygen supply under the conditions of incubation. It seems unlikely that supplies of oxygen were limiting in the present experiments, however. The aorta was opened to form a sheet of tissue 0.2 to 0.3 mm thick, and the rate of oxygen consumption of comparable preparations of rat aorta is known (26). The limiting thickness permitting adequate oxygenation of the tissue during incubation was estimated by the method described by Cohen (27). The result indicated that the atmosphere of 95% oxygen provided in the present experiments should have been sufficient to supply adequate oxygen to a sample of rat aorta 1.6 mm thick. Kirk and Laursen have calculated a limiting thickness of 1 mm for human aorta in vivo based on their measurements of the diffusion coefficient for oxygen in that tissue (28). The samples of rat aorta used in the present experiments, therefore, appear to have been well below estimates of critical thickness. Furthermore, samples of rat aorta, ranging from 0.16 to 0.4 mm in thickness, have shown no significant differences in the distribution of radioactivity among nonsaponifiable compounds after incubation with radioactive mevalonate.[1] These results may reflect other artifacts of the experimental conditions. It is possible that access of mevalonate to biosynthetic sites is difficult in this intact preparation. Cell-free systems should provide the substrate with easier access to these sites, but such preparations have not yet been studied. It should be pointed out, however, that in studies of cholesterol synthesis from mevalonate-2-^{14}C by mature rat brain, a major fraction of the label is found in squalene even with the use of cell-free preparations (29).

In the present experiments, a consistent pattern of labeling among sterols was observed after 3 hr of incubation. The amounts of radioactivity in lanosterol and lathosterol are comparable to or somewhat higher than the amount in cholesterol. 7-Dehydrocholesterol contained less label than cholesterol, but significantly ($P < 0.01$) more than desmosterol. The distribution of radioactivity among sterols may have a bearing on their importance as intermediates in cholesterol biosynthesis in the tissue. If so, the results suggest that the pathway of cholesterol biosynthesis in the aorta proceeds through lathosterol, and that pathways involving desmosterol are unimportant. This conclusion must remain tentative until information about the intermediary pool sizes and more detailed studies of the time course of sterol biosynthesis are available.

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