Biosynthesis of squalene and cholesterol by cell-free extracts of adult rat brain

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ABSTRACT Cell-free extracts of adult rat brain incubated with mevalonic acid-2-^{14}C synthesize ^{14}C-labeled nonsaponifiable fractions consisting largely of squalene-^{14}C. If the cofactor concentrations of the incubation medium are adjusted, much of the squalene can be induced to undergo turnover, with a resultant increase in ^{14}C-labeled digitonin-precipitable sterols, which include a small amount of cholesterol.

The synthesis of labeled sterols is markedly increased in the presence of Mg^{++} and depressed by nicotinamide. ATP, NADH, GSH, and glucose-6-phosphate are required for optimal synthesis of digitonin-precipitable material but, unlike Mg^{++}, are not essential.

The cofactor-adjusted extracts also synthesize a complex ester mixture containing, in addition to cholesterol-^{14}C, several compounds less polar than cholesterol. The biosynthesis of cholesterol in the extracts is a slow process; at least 12 hr of incubation is required for maximal sterol biosynthesis. A complex mixture of hydrocarbons accompanies squalene in the incubated extracts.

SUPPLEMENTARY KEY WORDS cholesterol companions · biosynthetic hydrocarbons · brain esters · nicotinamide

It is now generally accepted that normal adult mammalian brain can synthesize cholesterol, although in amounts almost negligible compared to those synthesized by immature brain (see references 1 and 2 for reviews). This conclusion, however, has been based largely on in vitro experiments, which with one exception (3) were performed with brain tissue slices or with minced tissue of whole brain from adult animals (4–10). A study of the biosynthesis of brain cholesterol by cell-free extracts of adult brain seemed called for, since neither the in vivo nor in vitro experiments to date give results that agree with those obtained from intracerebral injection of labeled cholesterol precursors (11, 12). The latter results suggest that adult brain has a higher capacity for synthesizing cholesterol than is indicated by other experimental methods. We describe here the biosynthesis of nonsaponifiable products from mevalonic acid-2-^{14}C in cell-free extracts of adult rat brain.

MATERIALS AND METHODS

Animals and Preparation of Brains

The rats we used were the St. Louis University strain of either sex, weighing 250 g or more, and at least 6 months old. Brains (cerebrum, cerebellum, and medulla) were removed, washed in three separate rinses of ice-water after removal of the meninges, and kept in ice until we began homogenization (no more than 15 min after removal from the skull).

Preparation of Cell-Free Extracts and Minced Tissue

Two adult rat brains were blotted and homogenized in 6 ml of 0.15 M phosphate buffer, pH 7.4. A glass homogenizer with a loose-fitting Teflon pestle was used. Not more than six to eight plunges were made by hand at 0°C. Centrifugation of the homogenate at 3000 *g* for 10 min at 0°C yielded 3.5–4 ml of supernatant fluid (for incubation studies) containing about 6 mg/ml of protein as determined by the method of Siekevitz (13), and 0.5 mg/ml of cholesterol as determined by the Sperry–Webb method (14). In what follows, the cell-free extract of adult rat brain will usually be referred to simply as

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Abbreviations: MPD, material precipitated by digitonin; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; MVA-2-^{14}C, mevalonic acid-2-^{14}C; G-6-P, glucose 6-phosphate.

"brain extract." Minced brain tissue was prepared in 6 ml of 0.15 M phosphate buffer pH 7.4 by repeated cutting of whole brain tissue to uniform consistency with scissors.

Incubations

Incubations were performed in 50-ml flasks shaken gently at 37°C for the periods indicated. The flasks were covered with aluminum foil. Generally, we used 1 ml of cell-free supernatant solution, equivalent to 1 g of wet rat brain, and added the cofactors in 1.5 ml of buffer. For incubations longer than 4 hr, 0.1 mg each of streptomycin sulfate and penicillin G were usually added, although without these antibiotics there was no noticeable difference in ^{14}C content or distribution of ^{14}C-compounds. Unless otherwise indicated, all incubations were carried out for 20 hr (for reasons given in Results).

Chemicals

All cofactors were obtained from the Sigma Chemical Co., St. Louis, Mo. except the $MgCl_2 \cdot 6H_2O$, which was Mallinckrodt, A. R. grade. Acid-washed, Merck alumina was used.

All radioactive chemicals were purchased from the New England Nuclear Corp., Boston, Mass., and were used as solutions in distilled water. The mevalonic acid-2-^{14}C (MVA-2-^{14}C) was in the form of the dibenzylethylenediamine salt, which had a specific activity of 4.2 mc/mole.

Pure lanosterol was obtained by the procedure of Bloch and Urech (15). Squalene was obtained from the Sigma Chemical Company. A partially hydrogenated squalene was prepared by shaking squalene for 20 min at room temperature under hydrogen (60 psi) in the presence of Raney's nickel. A major fraction was recovered on distillation in vacuo, bp 258–260°C at 10 mm of Hg.

Radioactivity

Radioactivity was determined on an Ansitron scintillation spectrometer with an efficiency of 91% for ^{14}C. Aliquots of samples were dried and dissolved in 15 ml of toluene containing 45.0 mg of 2,5-diphenyloxazole (PPO) and 4.5 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP). Incorporation of radioactivity has been calculated on the assumption that only one enantiomorph of DL-mevalonic acid is incorporated.

Thin-layer plates to be radioautographed were covered in the dark with Kodak X-ray film, RP/s Xo'mat (RPS-54). The film was then covered with a sheet of paper and a weight was placed over the entire plate surface. The films were developed after 3 wk.

Saponification and Isolation of Lipid Fractions

To the incubated tissues and cell-free fractions, 30 ml of water–ethanol 1:1 containing 15% KOH (w/v) was added, after which the mixtures were heated on a steam bath for 1 hr. The mixture was cooled and extracted with 5 × 100 ml of petroleum ether. The combined organic phases were washed with 3 × 50 ml of water and distilled to dryness, the last traces of solvent being removed under N_2. The crude nonsaponifiable extracts so obtained were kept in small volumes of benzene at −10°C until used. After saponification, unless otherwise indicated, 1 mg of carrier cholesterol was added to the nonsaponifiable fractions before precipitation of sterol digitonides.

Digitonides were prepared from the nonsaponifiable fractions by the Sperry–Webb method (14). In this paper sterols precipitated by digitonin will be referred to as MPD (material precipitated by digitonin). Free sterol was obtained from the digitonides by the method of Frame (16) and subsequently converted to the dibromides by the Schwenk–Werthessen method (17). Where indicated, free sterols were diluted with nonradioactive cholesterol, converted to the dibromide, and debrominated according to the procedure of Schwenk and Werthessen (17). Squalene hexahydrochlorides were prepared by the method of Heilbron, Kamm, and Owens (18).

Chromatography

Column. Columns 1 cm in I.D., equipped with a coarse fritted glass filter, were filled with 60 g of alumina as a slurry in petroleum ether. Nonsaponifiable fractions dissolved in 40 ml of petroleum ether were applied; the first 40 ml of eluate was collected as fraction 1. Nine 20-ml fractions of petroleum ether were subsequently collected, followed by 110 20-ml fractions of benzene. The column was under 2 psi of nitrogen pressure. Under these conditions squalene is reproducibly recovered in fractions 3–8, triterpenes of the lanosterol type in fractions 33–40, and cholesterol and companions in fractions 60–120. The last traces of sterol were recovered in four 20 ml fractions of absolute ethanol.

Thin-Layer. Silica Gel G (E. Merck A. G., Darmstadt, Germany), 0.30 mm thick, was activated for 0.5 hr at 120°C. The spots or lines placed on the plates were developed in different ascending solvent systems (see figures) at room temperature. After development the plates were sprayed either with 20% $SbCl_5$ in chloroform or 20% phosphomolybdic aid in ethanol, and then heated for 15 min at 120°C. When radioactive substances on the plates were to be further examined, reference spots were sprayed with $SbCl_5$ while the radioactive area was covered with a glass plate. With the reference spots as a guide, the silica gel was scraped off the cor-

responding unsprayed area and eluted by two washes (via centrifugation) with ethyl acetate. The ethyl acetate was removed under a stream of N_2.

Gas–Liquid. A Barber–Colman model 5000 gas chromatograph equipped with a hydrogen flame detector and a radioactive monitoring system (Barber–Colman model 5190) with a 2 meter U-shaped glass column, I.D. 4 mm, was used. Column packings were 1% and 3% QF-1 (fluorosilicone) on Gas-Chrom Q, 100–120 mesh (Applied Science Laboratories Inc., State College, Pa.). The column temperature was 225°C (isothermal), detector 240°C, flash heater 280°C. The carrier gas was argon with a flow rate of 55 ml/min, inlet pressure 24 psi. Samples were injected in benzene solution via a Hamilton microliter syringe equipped with a Chaney adapter.

Intracerebral Injections

Intracerebral injections were performed as previously described (11).

RESULTS

Biosynthesis of Nonsaponifiable Fractions

In preliminary experiments in which the effects of individual cofactors were not known, the following cofactors in the concentration indicated under Table 1 were used in incubations with brain extracts: GSH, ATP, $MgCl_2 \cdot 6H_2O$, NAD, NADH, NADPH, glucose, glucose 6-phosphate (G-6-P), and nicotinamide. Under these conditions the incorporation of MVA-2-^{14}C into nonsaponifiable fractions was rapid and reached its maximum within 2 hr (Table 1). The advantages of using cell-free extracts as opposed to minced tissue are shown in Table 2. The percentage incorporation of mevalonate into nonsaponifiable matter was considerably higher in the cell-free preparations. Presumably this is because the MVA-2-^{14}C has easier access to the subcellular biosynthetic sites in the cell-free preparations.

TABLE 1 INCORPORATION OF MVA-2-^{14}C INTO NONSAPONIFIABLE FRACTIONS OF CELL-FREE PREPARATIONS OF RAT BRAIN: EFFECT OF INCUBATION TIME

Incubation Time	^{14}C Content of Nonsaponifiable Fractions	^{14}C Incorporation
hr	*cpm*	%
1	707,000	13.8
2	1,555,000	30.8
4	1,583,000	31.4
8	1,330,000	26.4
20	1,288,000	25.6

Each incubation mixture contained 5 μc of MVA-2-^{14}C, cell-free extract equivalent to 4 g (wet wt) of adult brain, and the following cofactors at the concentrations given: G-6-P, 16.5 μM; GSH, 58.7 μM; $MgCl_2 \cdot 6H_2O$, 24.6 μM; ATP, 12.8 μM; NAD, 22.8 μM; NADH, 7.0 μM; NADPH, 2.4 μM; glucose, 111 μM; and nicotinamide, 205 μM. The total volume in each case was 3.5 ml.

TABLE 2 INCORPORATION OF MVA-2-^{14}C INTO NONSAPONIFIABLE FRACTIONS OF ADULT RAT BRAIN: MINCED TISSUE VERSUS CELL-FREE EXTRACTS

Type of Preparation	^{14}C Content of Nonsaponifiable Fraction	^{14}C Incorporation
	cpm	%
Minced	235,000	5.0
Minced	349,000	7.6
Minced	156,000	3.4
Cell-free	1,060,000	22.8
Cell-free	1,307,000	28.0

Each incubation mixture contained either 4 g of minced brain or brain extract equivalent to 4 g of wet adult brain tissue. Each value is for one mixture incubated for 20 hr. MVA-2-^{14}C, cofactors, and the buffer system were the same as in Table 1. Total volume in minced preparations was 7 ml and for cell-free, 3.5 ml.

Chromatography of the nonsaponifiable fraction on alumina showed that the major portion of radioactivity resided in the "squalene fractions" (Nos. 3–8). A much smaller portion occurred in the "lanosterol fractions" (Nos. 33–40) and "cholesterol fractions" (Nos. 60–120), but only after 8 hr or more of incubation. For this reason incubations were usually continued for at least 20 hr.

Detection of Squalene-^{14}C and Other Hydrocarbons

Squalene was identified in fractions 3–8 from the alumina columns in three ways. First, TLC gave a major spot with R_f identical with that of authentic squalene, either chromatographed separately or as a mixture. Second, this major component cochromatographed with squalene on GLC. Third, addition of nonradioactive squalene to fractions 3–8, preparation of the squalene hexahydrochlorides, and crystallization to constant radioactivity gave the values shown in Table 3.

We suspected from the radioactivity in the hexahydrochloride mother liquors that radioactive compounds other than squalene were present. This was substantiated by the radioautograph shown in Fig. 1. Besides radioactivity at the starting line and in squalene, a large number of radioactive spots less polar than squalene are visible after 24 hr of incubation, one of these already being present at 2 hr. In a separate experiment, a "squalene fraction" from a 24 hr incubation was subjected to TLC along with authentic squalene and a partially hydrogenated squalene mixture (Fig. 2). Of the 4.05×10^6 cpm recovered from the eight areas, the majority was associated with squalene (94%) and the starting line area (4%). The less polar hydrocarbons have now been analyzed by GLC and mass spectrometry and found to be a mixture of partially saturated isoprenoid hydrocarbons (19).

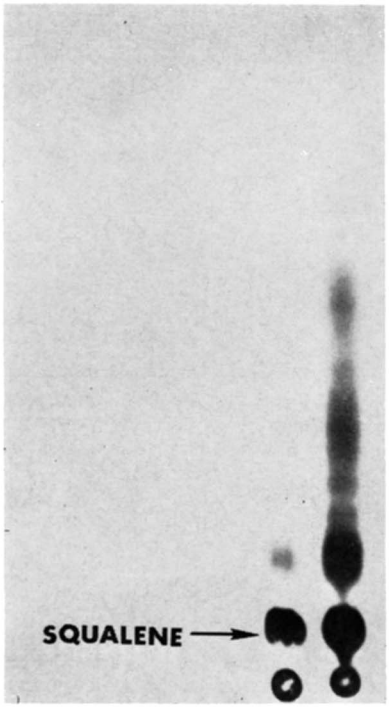

FIG. 1. Radioautograph of a thin-layer plate on which we placed the crude squalene chromatography fractions from incubations for 2 hr (left) and 24 hr (right). A total of 4.2 × 10^6 cpm was initially chromatographed in each fraction. The developing solvent was petroleum ether. Only squalene was visible as a black spot when the plate was sprayed with $SbCl_5$ in $CHCl_3$.

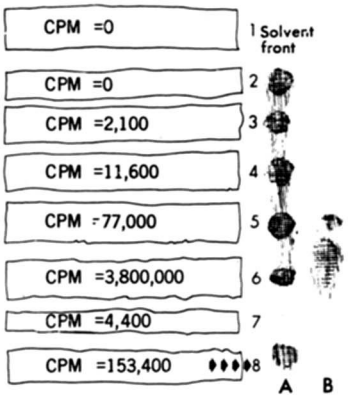

FIG. 2. Distribution of radioactivity on a thin-layer plate after application as a streak (about 4.5 × 10^6 cpm) of a crude squalene fraction from a 24 hr incubation. The plate was developed at room temperature with petroleum ether. Controls on the right [partially hydrogenated squalene (A) and squalene (B)] were made visible by spraying with $SbCl_5$ in $CHCl_3$. Arrows show starting line.

Lanosterol and Cholesterol Fractions

Detection of Lanosterol-^{14}C. Fractions 33–40 from the alumina column should contain lanosterol-^{14}C. Except for the 24 hr incubation, the incorporation of labeled material in the lanosterol region was small. Inspection by GLC failed to reveal any evidence of lanosterol-^{14}C; the major ^{14}C peak had a longer retention time than that of lanosterol. Only a small amount of lanosterol-^{14}C formed before 24 hr was shown by isotope dilution (Table 4). On TLC the product(s) in the "lanosterol fractions" had the mobility of lanosterol but stained brown with $SbCl_5$, whereas lanosterol under similar conditions turns light yellow. The "lanosterol-like" material has not yet been identified. We concluded that labeled lanosterol is formed under these circumstances so slowly that the formation of cholesterol will be extremely slow. The major "block" in the sequence therefore seems to be between squalene and lanosterol.

Evidently, the major component of the nonsaponifiable fractions of adult brain extracts incubated as described is squalene. Only trace amounts of ^{14}C-sterols precipitable by digitonin were formed.

Cofactors and Biosynthesis of MPD. The amounts of nicotinamide, G-6-P, GSH, ATP, Mg^{++}, glucose, NAD, and NADH in the incubation medium were varied. The concentrations of nicotinamide and Mg^{++}

TABLE 3 IDENTIFICATION OF SQUALENE SYNTHESIZED FROM MVA-2-^{14}C BY CELL-FREE EXTRACTS OF ADULT RAT BRAIN

Incubation Time	Specific Activity of Squalene-^{14}C Hexahydrochloride	
	3rd Crystallization	6th Crystallization
hr	*cpm/mg*	
0.5	306	319
1	691	703
2	1274	1353
4	859	878
8	1169	1196
24	945	1000

Crude squalene fractions 3–8 from the alumina column were diluted with 100 mg of nonradioactive squalene, converted to the hexahydrochloride, and crystallized to constant radioactivity from acetone.

TABLE 4 BIOSYNTHESIS OF LANOSTEROL FROM MVA-2-^{14}C BY CELL-FREE EXTRACTS OF ADULT RAT BRAIN

Incubation Time	Total ^{14}C in Original Sample*	^{14}C-Lanosterol Specific Activity†		Weight of Lanosterol 6th Crystallization
		3rd Crystallization	6th Crystallization	
hr	*cpm*	*cpm/mg*		*mg*
0.5	10,720	20	14	5.71
1	6,420	30	28	4.77
2	42,160	80	78	6.00
4	98,720	80	78	4.00
8	97,040	60	62	4.45
24	240,920	360	365	6.12

Crude lanosterol fractions 33–40 from the alumina column were diluted with 40.0 mg of nonradioactive lanosterol, then crystallized twice from acetone–ethanol (and four times from acetone).

* From alumina column, fractions 33–40.

† Specific activity was determined only after the 3rd and 6th crystallizations.

TABLE 5 EFFECT OF NICOTINAMIDE AND Mg^{++} ON ^{14}C-MPD BIOSYNTHESIS IN CELL-FREE EXTRACTS OF ADULT RAT BRAIN

Cofactor Added (μM)	Total Incorporation of ^{14}C	
	Nonsaponifiable	MPD
	cpm	
Nicotinamide*		
0	—	24,400
41.0	—	22,300
82.0	—	15,200
164.0	—	8,400
328.0	—	1,400
656.0	—	50
Mg^{++}		
0	296,000	70
2.4	446,000	360
4.9	854,000	200
9.8	799,000	14,200
24.6	1,477,000	23,000
49.2	1,621,000	16,500

Each incubation mixture contained cell-free extract (1 ml) equivalent to one rat brain, all from one preparation. Also present were 1 μc of MVA-2-^{14}C in 0.02 ml of water, and the following (in 1.5 ml of phosphate buffer, pH 7.4): ATP, 12.8 μM; GSH, 16.5 μM; NAD, 22.8 μM; NADH, 7.0 μM; NADPH, 2.4 μM; glucose, 111 μM; G-6-P, 16.5 μM; $MgCl_2 \cdot 6H_2O$, 24.6 μM (in incubation mixtures containing nicotinamide). Total volume of each mixture was 3 ml.

* In preliminary experiments (unpublished) we found that nicotinamide does not affect total ^{14}C-nonsaponifiable values.

proved to be important; they are the only ones we shall present (Table 5). At concentrations higher than 41 μM, nicotinamide is quite detrimental to MPD biosynthesis, and it was accordingly not used in further incubations. Increasing quantities of Mg^{++}, within the limits investigated, proved favorable for both total ^{14}C-nonsaponifiable biosynthesis (probably largely squalene) and MPD biosynthesis. In the absence of Mg^{++}, the biosynthesis of MPD was negligible.

With a new incubation mixture containing cofactors at their optimal concentrations as a standard, we determined the effects (Table 6) of eliminating each in turn on the synthesis of MPD. NADPH was found to be inessential and was eliminated, and the final incubation mixture adopted contained the following cofactors (at the following concentrations shown) for brain extracts equivalent to 1 g of wet brain: G-6-P, 6.6 μM; GSH, 16.3 μM; $MgCl_2 \cdot 6H_2O$, 24.6 μM; ATP, 1.7 μM; and NADH, 14.1 μM.

Effect of Incubation Time on Formation of MPD. Brain extract, cofactors at optimal concentrations, and MVA-2-^{14}C were incubated for 2, 4, 8, 12, and 20 hr. Nonsaponifiable extracts were prepared after the addition of 1 mg each of squalene and cholesterol, and cholesterol was precipitated as the digitonide. The results, given as incubation time plotted against total ^{14}C-incorporation and percentage incorporation, are shown in Fig. 3. Although there was a slight increase in MPD biosynthesis at 20 hr, 12 hr of incubation seemed sufficient to

TABLE 6 EFFECT OF COMBINED "OPTIMUM COFACTORS" ON ^{14}C-NONSAPONIFIABLE AND ^{14}C-MPD BIOSYNTHESIS

Added	Total Incorporation of ^{14}C	
	Nonsaponifiable	MPD
	cpm	
All cofactors	59,500	19,000
No cofactors	7,850	—(0)
No ATP	63,500	17,900
No G-6-P	63,000	17,800
No GSH	62,000	10,700
No Mg^{++}	13,800	—(0)
No NADH	52,500	6,100
No NADPH	59,700	23,250

Each incubation mixture contained brain extract (1 ml) equivalent to 1 g of wet brain (all from the same extract), 2×10^5 dpm of MVA-2-^{14}C in 0.1 ml of water, and the following cofactors, except where indicated, in 1.5 ml of phosphate buffer, pH 7.4: ATP, 1.7 μM; GSH, 16.3 μM; $MgCl_2 \cdot 6H_2O$, 24.6 μM; G-6-P, 6.6 μM; NADH, 14.1 μM; and NADPH, 1.2 μM. Total volume of each mixture was 2.6 ml.

demonstrate definite biosynthesis. The total ^{14}C-nonsaponifiable biosynthesis at 20 hr was 80% (not shown).

Biosynthesis of Cholesterol-^{14}C. It has been well established in experiments with liver that digitonin precipitates "labeled companions" along with cholesterol (17). Accordingly, a nonsaponifiable extract from a 20 hr optimal incubation with MVA-2-^{14}C was chromatographed on alumina. Fig. 4, in which fraction number is plotted against ^{14}C-content, is to be contrasted with Fig. 5, a similar chromatogram of the nonsaponifiable fraction from adult brain tissue 20 hr after intracerebral injection of MVA-2-^{14}C. In the latter no squalene is present (fractions 3–8); some triterpene is evident in the "lanosterol region" (fractions 33–45), but most of the radioactivity is in cholesterol (fractions 80–90) (cf. reference 12). Fig. 4 shows that the sterol fractions (60–120) from the alumina column consisted of a complex mixture.

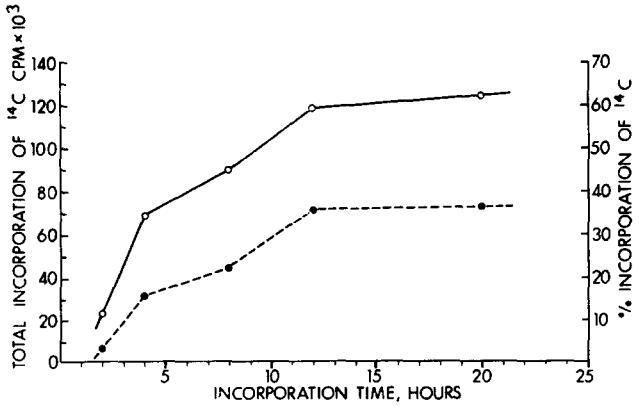

FIG. 3. Incorporation of MVA-2-^{14}C into MPD by cell-free preparations of adult rat brain. Each point on the curves represents an incubation containing 1 ml (≡1 g of wet tissue) of cell-free extract and 7.3×10^5 cpm of MVA-2-^{14}C. Solid line, total incorporation; broken line, percentage incorporation.

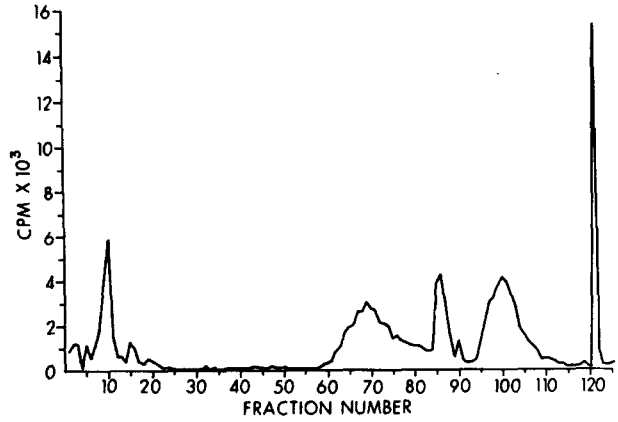

FIG. 4. Alumina chromatogram of a nonsaponifiable fraction from a 20 hr incubation of 1 ml (≡1 g of wet tissue) of cell-free extract of adult rat brain, "optimum cofactors," and 7.3 × 10^6 cpm of MVA-2-^{14}C. Carrier squalene and cholesterol (1 mg each) were added before the nonsaponifiable material was extracted. Squalene region, fractions 3–8; sterol region, fractions 60–120.

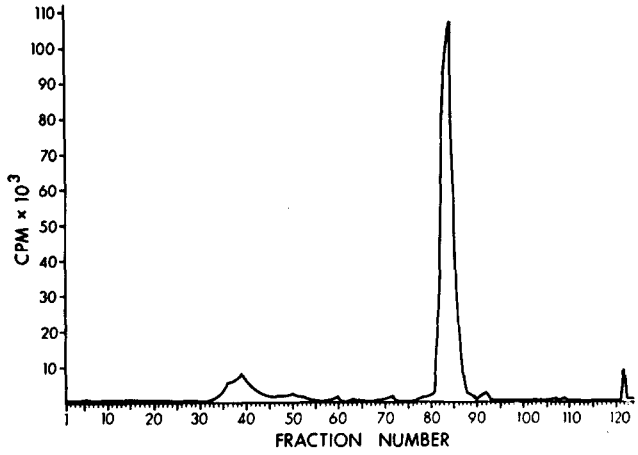

FIG. 5. Alumina chromatogram of total nonsaponifiable fraction from one-half of an adult rat brain (1 g wet weight) 20 hr after intracerebral injection of 5 μc of MVA-2-^{14}C. Same chromatographic conditions as in Fig. 4. Cholesterol region, fractions 80–90.

The presence of radioactive cholesterol in this mixture was established in three ways as follows, as well as by GLC with a radioactive monitoring system.[1]

(*a*) The sterol fractions from incubations for 2, 4, 8, 12, and 20 hr, isolated by chromatography on alumina, were treated with digitonin. The free sterols obtained by cleavage of the digitonides were diluted with nonradioactive cholesterol. Isolation through a dibromide gave free cholesterol (17). The specific activity values for each sample plotted against incubation time are shown in Fig. 6. The amount of cholesterol-^{14}C in the ^{14}C-MPD from the above incubations, calculated from the specific activities of the regenerated cholesterol, was 2–3%. These low values are probably not truly representative of the capacity of the tissue to synthesize this sterol in vivo since there are some unidentified ^{14}C-sterols more polar than cholesterol in the incubated material.

(*b*) The MPD fraction from a nonsaponifiable fraction obtained by incubating brain extract for 20 hr with MVA-2-^{14}C was converted to free sterol and subjected to TLC (Fig. 7). The ^{14}C distribution was determined by scraping areas from the plate, extraction, and subjecting the extracts to liquid scintillation counting. The radioactivity was distributed almost equally between the cholesterol and the more polar regions, with a small

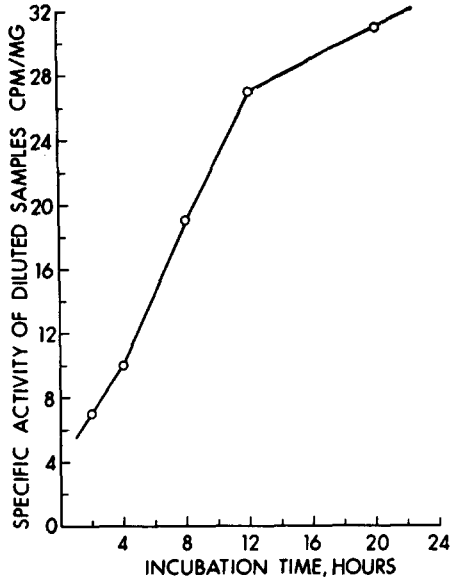

FIG. 6. Relation between specific activity of "regenerated cholesterol" and time of incubation of cell-free brain extracts with MVA-2-^{14}C. Digitonides from each interval in Fig. 3 were converted to free sterol and diluted with 100 mg of nonradioactive cholesterol. Each sample was brominated and free cholesterol was recovered from the dibromides.

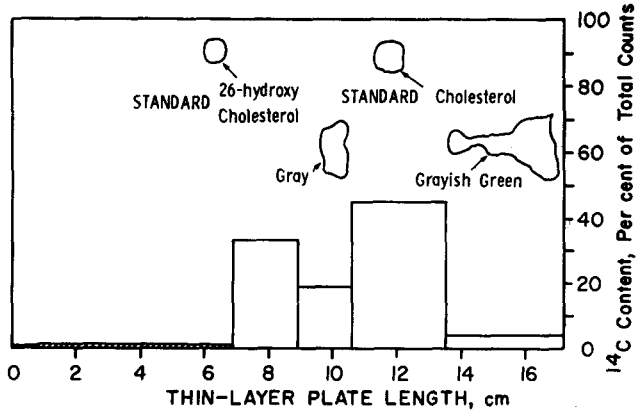

FIG. 7. TLC of standard sterols and of the digitonin-precipitable sterol in benzene–ethyl acetate 2:1. Cholesterol in the experimental sample was barely visible on staining with $SbCl_5$ in $CHCl_3$. The identity of the other products evident is unknown.

[1] Unpublished observations.

percentage in the less polar region. None of the known cholesterol precursors between cholesterol and squalene is more polar than cholesterol (20). On this account the products shown here may be autoxidation products. It seems reasonable to suppose that the long periods required to demonstrate cholesterol biosynthesis might result in autoxidation of the sterol. However, 7α- and 7β-hydroxy cholesterol, 7-ketocholesterol, and cholestane-3β,5α,6β-triol, the most likely autoxidation products, do not form a part of the radioactive impurities coprecipitating with cholesterol in biosynthetic experiments with liver (21, 22). Furthermore, these compounds are more polar than the products indicated in Fig. 7. The identity of these products remains unknown.

(*c*) A nonsaponifiable fraction was obtained from a brain extract incubated for 20 hr with MVA-2-^{14}C. It was chromatographed on alumina as previously described except that after elution with petroleum ether, the column was washed with absolute ethanol to elute all other material. A radioautograph of the cholesterol fractions (60–120), plus the material eluted with ethanol, subjected to TLC is shown in Fig. 8. Radioactive cholesterol, some less polar material and at least four products more polar than cholesterol are evident in the mixture. Thus Fig. 4 and 7 lead to the same conclusion, that most of the radioactive products accompanying cholesterol in the incubation medium after 12 hr are products more polar than cholesterol.

Biosynthesis of Sterol and Triterpene Esters. In Fig. 4, a pronounced radioactive peak is present in fractions 7–11, slightly more polar than squalene. The combined fractions gave no precipitate with digitonin. On additional saponification (1 hr *reflux* in 15% KOH in 50% ethanol) a nonsaponifiable fraction was obtained. This fraction *did* give a precipitate with digitonin. TLC indicated the presence of a small quantity of radioactive cholesterol accompanied by several products less polar and some more polar than this sterol (Fig. 9). We have preferred to consider these collectively as "sterol–triterpenes," since their collective identity is not known. Thus the original fractions contained esters that had not been saponified by the more gentle steam-bath treatment. In view of previous observations that adult brain contains no cholesteryl esters or cholesterol esterase (23), this finding deserves further exploration, which is under way.

Further proof that the resaponified fraction contained radioactive cholesterol was obtained as follows. A resaponified fraction containing 15,000 cpm of ^{14}C was diluted with 50.0 mg of nonradioactive cholesterol and brominated, and free cholesterol was regenerated. The specific activity of the latter was 18 cpm/mg. The amount of cholesterol-^{14}C in the "ester fraction" may be calculated as 6.0% of this fraction.

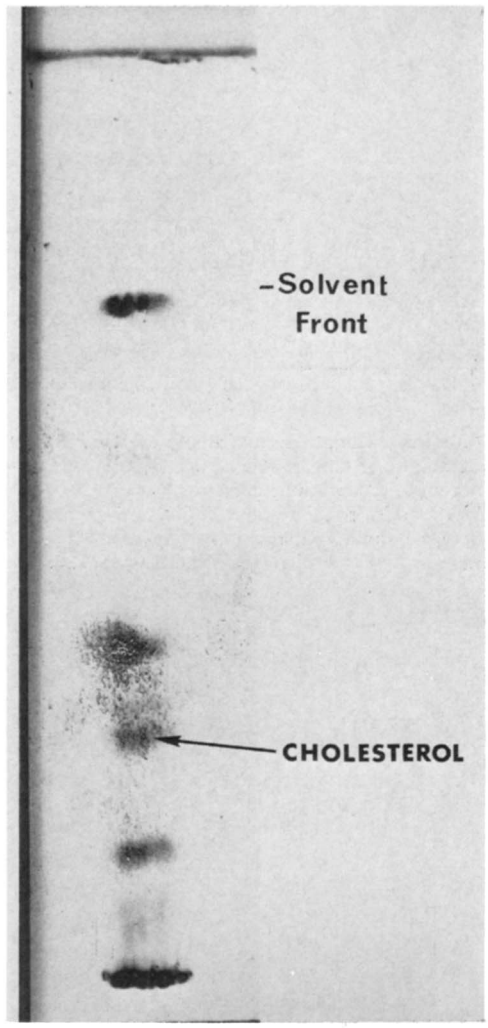

FIG. 8. A TLC radioautograph of "cholesterol fractions" (60–120, Fig. 4) in chloroform–acetone 95:5 on Silica Gel G containing 25% $AgNO_3$.

DISCUSSION

The data presented in this manuscript show that cell-free extracts from adult rat brain can synthesize sterols from mevalonic acid, although cholesterol biosynthesis is heavily blocked between squalene and its cyclization to lanosterol. Squalene was first detected in adult brain by tracer methods and by a colorimetric procedure in 1959 (12). Since then, the triterpene has been identified in adult human brain (24) and more recently in adult human meninges and meningiomas (25).[2] If the proper cofactors in the proper concentrations are provided in vitro, the biosynthesis of digitonin-precipitable sterols can be increased. This increase is accompanied by increased squalene turnover.

[2] Dr. Smith has informed us that the meninges and meningiomas were from 40–50-yr old adults.

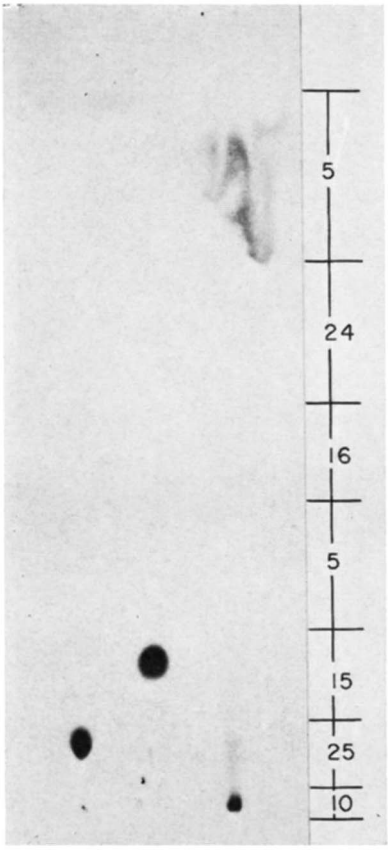

FIG. 9. TLC of resaponified "ester fraction" (fractions 8–11, Fig. 4) in benzene–petroleum ether–ethyl acetate 75:23:2. To the plate we applied, from left to right, 100 μg each of cholesterol, lanosterol, and the unknown. The identity of the products associated with cholesterol is not known. Numbers of the right give ^{14}C content of areas as % of total.

Nicotinamide, which evidently (Table 5) should not be included in the incubation media, probably exerts its inhibitory effect on the cyclization of squalene, in which case the activity is similar to that of nicotinic acid inhibiting the cyclization of squalene in yeast (26). The mechanisms for these inhibitions are not known. Nicotinic acid, but not nicotinamide, reduces serum cholesterol levels (27).

In general, the cofactor requirements for sterol biosynthesis we describe here are not markedly different from those required by liver microsomal–supernatant fractions. Thus for liver either Mg^{++} or Mn^{++} must be supplied as a metallic ion, a reducing agent such as GSH must be present, and ATP and pyridine nucleotides are requisites (28).

We would like to mention the possibility, however remote, that some of the resultant radioactive products might be due to contamination with yeasts or bacteria not inhibited by the antibiotics used in the incubations.

Several aspects of the present work will require more extensive investigation. The biosynthesis of sterols by the incubation of extracts of adult brain is slow. No clearcut reason for this is known at present. The turnover of squalene and cholesterol biosynthesis in liver is considerably more rapid (29). More perplexing is the fact that although the formation of ^{14}C-labeled sterols from MVA-2-^{14}C was enhanced by the adjustment of cofactor concentrations, only a small portion of the total sterols formed was found to be cholesterol. Two additional observations may have some significance for the over-all phenomenon. First, some unpublished studies in our laboratory have shown that there is a factor (or factors) present in microsomes and supernatant fractions from adult rat brain that inhibits the biosynthesis of cholesterol by liver in vitro. Something of this nature could retard or inhibit cholesterol biosynthesis in the brain itself. Second, a large number of sterols other than cholesterol occur in brain tissue (30). Perhaps it will be necessary to study the biosynthesis of these compounds in cell-free extracts from brain before the question of maximum cholesterol biosynthesis can be clarified.

A mixture of sterol–triterpene esters is formed after lengthy incubation of adult brain extracts with optimum cofactors. Although cholesteryl esters are alleged to be absent from adult brain (23), failure to demonstrate an esterase by histochemical means in adult brain may be due to inability to devise a suitable method.

Finally, it should be pointed out that in the biosynthesis of cholesterol by adult brain in vitro, mevalonic acid formation itself may be rate limiting. Labeled nonsaponifiable material may also be obtained from the extracts described if sodium acetate-2-^{14}C and glucose-U-^{14}C are used as the radioactive substrates (unpublished observations). We made no detailed study of sterol formation, however, and further study is suggested.

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