Biosynthesis of 5α-cholestan-3β-ol in rat and guinea pig liver in vitro

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SUMMARY The in vitro conversion of DL-mevalonate-2-C^{14} and cholesterol-4-C^{14} to 5α-cholestan-3β-ol was studied in liver homogenates of rats and guinea pigs. On the average 0.01–0.05% of the substrate radioactivity was converted to 5α-cholestan-3β-ol by the livers of both species during the 4 hr incubation period. The biosynthesis of 5α-cholestan-3β-ol by cell-free liver homogenates in vitro demonstrates that the presence of this saturated sterol is at least partly due to biosynthesis by mammalian tissues, and cannot be ascribed exclusively to bacterial action upon cholesterol in the intestine.

KEY WORDS 5α-cholestan-3β-ol · biosynthesis · in vitro · liver homogenate · rat · guinea pig · adrenal · mevalonate-2-C^{14} · cholesterol-4-C^{14} · triparanol

VARYING AMOUNTS (0.1–10%) of the saturated sterol 5α-cholestan-3β-ol have been detected in all sterol samples of mammalian origin examined so far (1). The biological origin of tissue cholestanol has not been elucidated, although its relatively large concentration in adrenal tissue suggests an important biologic function (2). Previous studies showed that following the intracardial administration of DL-mevalonate-2-C^{14} to guinea pigs, labeled cholestanol could be isolated from liver, intestinal wall, and adrenals at the end of 24 hr. These studies indicated that cholestanol was synthesized in vivo, and the data obtained provided good evidence that the cholestanol did not arise from bacteriological transformation in the intestinal tract (3). However, in order to eliminate the effect of the intestinal flora completely, the biosynthesis of cholestanol from mevalonate-2-C^{14} and cholesterol-4-C^{14} was studied in liver homogenates in vitro.

It was found that the biosynthesis of cholestanol takes place in liver homogenates of rats and guinea pigs, suggesting that this saturated sterol is synthesized by liver enzymes and does not arise exclusively by bacterial action upon cholesterol in the intestinal tract.

EXPERIMENTAL PROCEDURE

Labeled Substrates

DL-Mevalonic-2-C^{14} acid lactone (11.3 μc/mg) was obtained from the Nuclear-Chicago Corp., Des Plaines, Ill., and was used without further purification. The labeled compound did not contain labeled cholestanol, as shown by an isotope dilution experiment (3). Cholesterol-4-C^{14} (Nuclear-Chicago Corp.) was purified by chromatography on $AgNO_3$–silicic acid columns in the presence of 3 mg of unlabeled cholestanol (3, 4). The purified cholesterol contained less than 0.002% cholestanol-C^{14}. The labeled sterol was added to the incubation mixture in a Tween 20–water emulsion (5).

Experimental Animals

Male or female guinea pigs (200–250 g; Hartley strain) or rats (50–150 g; Wistar strain) were maintained on Rockland mouse and rat diet; the guinea pigs received supplements of vitamin C. When required, triparanol (1 - [*p* - (β - diethylaminoethoxy) - phenyl] - 1 - (*p*-tolyl) - 2 - (*p* - chlorophenyl) ethanol), kindly supplied by the Wm. S. Merrell Co., Cincinnati, Ohio, was administered in the diet in the concentration of 0.25% (guinea pigs) or 0.125% (rats) for 2–3 weeks.

Preparation of Tissues

The animals were killed by decapitation and the livers were removed rapidly, chilled on cracked ice, and extruded through a tissue press (Harvard Instrument Co.). The tissue was then homogenized in a loose fitting Potter-

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TABLE 1 PURIFICATION OF CHOLESTANOL-C^{14} ISOLATED FROM RAT AND GUINEA PIG LIVER HOMOGENATES, INCUBATED WITH MEVALONATE-2-C^{14} AND CHOLESTEROL-4-C^{14}

Cholestanol	Rat		Guinea Pig	
	Mevalonate-2-C^{14}	Cholesterol-4-C^{14}	Mevalonate-2-C^{14}	Cholesterol-4-C^{14}
	cpm/mg		*cpm/mg*	
After first peroxidation and chromatography on $AgNO_3$–silicic acid*	157	458	83	870
After second peroxidation and chromatography on Ag NO_3–silicic acid	158	460	81	855
Oxidized with CrO_3 and cholestanone chromatographed on silicic acid	160	436	83	860

* Three milligrams of carrier cholestanol added in each case.

Elvehjem homogenizer exactly as previously described (6). The homogenization mixture consisted of potassium phosphate buffer, magnesium chloride, nicotinamide, and Versene.

Incubation Mixture

The complete system prepared and incubated as described above contained the following in a volume of 3.0 ml: homogenate supernatant fluid; potassium phosphate buffer pH 7.4, 80 mM; magnesium chloride, 4.8 mM; nicotinamide, 30 mM; Versene, 1 mM; DPN, 0.8 mM; ATP, 4 mM; glutathione, 4 mM; TPN, 0.6 mM; glucose-6-phosphate, 1.1 mM; sodium fluoride, 2 mM; and labeled substrate in the molarities shown in Table 2 (Results section). The incubations were carried out in 50-ml Erlenmeyer flasks, which were first flushed with a stream of oxygen for 2 min and sealed with a rubber cap. The water bath–shaker was kept at 37° for the 4 hr experimental period. An aliquot of the homogenization mixture was boiled for 10 min, then cooled to room temperature. The sample was incubated after addition of cofactors and substrate to serve as boiled enzyme control.

Radioactive Assay

Sterol samples were counted in a gas flow counter by direct plating or in a scintillation counter, exactly as described previously (3).

Isolation of Biosynthetic Cholestanol

At the end of the incubation, 15 ml of 10% (w/v) KOH in 95% ethanol were added to each flask and the contents were saponified at 60° for 1 hr. The nonsaponifiable material was extracted, esterified with formic acid, and oxidized with peroxyformic acid as described previously (1, 3). The cholestanol formate was hydrolyzed in 5% (w/v) KOH in 95% ethanol at 60° for 1 hr; 3 mg of carrier cholestanol was added and the free sterols were extracted with 20% (v/v) ethyl ether in *n*-hexane. After evaporation of the solvent the residue was chromatographed on a silver nitrate–silicic acid column (3, 4), the fraction containing cholestanol was evaporated, and the cholestanol weighed and counted.

Radioactive Purity of Biosynthetic Cholestanol-C^{14}

If the cholestanol isolated from the incubation mixture as described above contained radioactivity it was subjected to a second peroxidation, hydrolyzed, and again chromatographed on a $AgNO_3$–silicic acid column. If this twice-purified cholestanol still contained label it was oxidized with CrO_3 to 5α-cholestan-3-one (3, 7) and chromatographed on silicic acid (3). If the C^{14} label remained with the cholestanol through all of these steps without change in specific radioactivity, this was considered proof that the labeled substrate had been converted to cholestanol.

RESULTS

Table 1 summarizes specific radioactivities of biosynthetic cholestanol-C^{14} isolated from liver homogenates of rats and guinea pigs incubated with DL-mevalonate-2-C^{14} and cholesterol-4-C^{14}, respectively. These results prove that mevalonate and cholesterol radioactivity was transferred to cholestanol in vitro, since the specific radioactivity of the biosynthetic cholestanol remained constant during the purification procedures. All of the samples of biosynthetic cholestanol referred to in Table 2 were subjected to identical purification procedures.

Table 2 summarizes data on the biosynthesis of cholestanol in rat and guinea pig liver homogenates in vitro, using mevalonate and cholesterol as the labeled precursors. The table shows that both substrates were converted to cholestanol, although the order of conversion was never higher than 0.05% on the basis of radioactivity. It is, nevertheless, believed that the biosynthesis of cholestanol took place by enzymatic processes, since boiled enzyme controls consistently resulted in the isolation of cholestanol carrier of very low radioactivity. In addition, in experiments with livers of triparanol-fed rats it was observed that triparanol lowered the conversion of mevalonate to cholestanol but did not inhibit the conversion of cholesterol to cholestanol.

DISCUSSION

The conversion of mevalonate and cholesterol to cholestanol observed in these experiments was of the order

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of 0.05% of the substrate radioactivity during the 4 hr incubation period. This low order of cholestanol biosynthesis is not surprising (*a*) since cholestanol is present in relatively low concentration (approximately 1% of total sterol) in the livers of these animals (3), and (*b*) since the conditions chosen for the incubation may not have been optimal for the reduction of the cholesterol double bond even though they were such as to ensure a high percentage conversion of mevalonate to sterol.

The total sterol fractions isolated from guinea pig adrenals contain 5–15% cholestanol (2, 3) while the liver sterol fractions of these animals usually contain less than 5% of the stanol. It might have been expected therefore that adrenal homogenates would be more suitable for studies of cholestanol biosynthesis than liver preparations. However, we have so far been unable to demonstrate the conversion of mevalonate-2-C^{14} and cholesterol-4-C^{14} to cholestanol-C^{14} in adrenal tissue in vitro. This suggests either that the conditions of the experiments were not suitable for demonstrating cholestanol biosynthesis or that adrenal tissue may have the ability to concentrate cholestanol and not necessarily to synthesize it.

Although previous experiments have shown that mevalonate-2-C^{14} was converted to cholestanol in rabbits and guinea pigs in vivo, the possibility still existed that cholestanol was formed from cholesterol by bacterial action in the intestinal tract. The experiments of Werbin et al. (2), who fed labeled cholesterol to guinea pigs for 2–7 weeks and isolated labeled cholestanol from their tissues, are strongly suggestive of a role of the intestinal microorganisms in the biosynthesis of cholestanol. The present experiments suggest, however, that tissue cholestanol can arise, at least in part, by the action of liver enzymes on cholesterol. These experiments do not provide evidence concerning the intermediate steps of this transformation, but they are in accord with the hypothesis that tissue cholesterol can be converted to Δ^4-cholestenone, which is known to be a precursor of cholestanol both in vivo and in vitro (3, 8).

The experiments were not carried out under sterile conditions so that the presence of bacterial contamination is not excluded. However, adrenal homogenates incubated under the same conditions as the liver homogenates did not convert mevalonate or cholesterol to cholestanol to a measurable extent. Presumably, if the biosynthesis of cholestanol was carried out by contaminating microorganisms, labeled stanol would have been found in our experiments with adrenal tissue.

TABLE 2 Biosynthesis of 5α-Cholestan-3β-ol in Rat and Guinea Pig Liver in Vitro

Species	Substrate	Substrate Concentration*	Substrate Radioactivity* × 10^{-6}	Radioactivity Nonsaponifiable Fraction × 10^{-6}	Total Radioactivity in Cholestanol	Per Cent of Substrate Radioactivity in Cholestanol × 10^2	Comment
		μmoles/vessel	*cpm/vessel*	*cpm*	*cpm*		
Rat	DL-Mevalonate-2-C^{14}	1.05	0.80	0.42	344	4.3	
		1.05	0.65	0.20	91	1.4	Triparanol-fed†
		1.05	0.65	0.0004	11	0.16	Boiled enzyme‡
		2.10	1.30	0.25	440	3.4	
		2.10	1.30	0.21	351	2.7	
	Cholesterol-4-C^{14}	0.12	2.14	—§	1000	4.7	Triparanol-fed†
		0.20	9.10	—	2100	2.3	
		0.20	9.10	—	2300	2.5	
		0.10	4.55	—	57	0.13	Boiled enzyme‡
		1.21	9.10	—	2100	2.3	
Guinea pig	DL-Mevalonate-2-C^{14}	1.1	0.8	0.24	107	1.3	
		2.1	1.3	0.13	377	2.9	
		2.1	1.3	0.11	243	1.9	
		2.1	1.3	0.0005	19	0.15	Boiled enzyme‡
	Cholesterol-4-C^{14}	0.20	9.1	—§	3000	3.3	
		0.10	4.5	—	75	0.18	Boiled enzyme‡
		1.2	16.0	—	2390	1.5	
		1.2	9.1	—	3440	3.8	

* Calculated on the basis of the L-isomer only.
† The rats received 0.125% triparanol in their diet for 3 weeks.
‡ Boiled enzyme controls were included in every incubation. The example shown here is to be compared with the experiment immediately above it in the Table.
§ Not determined.

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