In vitro conversion of squalene from squalene-phospholipid liposomes into sterols by rat liver microsomes and cytosol

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Summary When rat liver cytosol, possessing sterol carrier protein$_1$ (SCP$_1$) activity, was incubated with [^{3}H]-squalene-phospholipid liposomes, cofactors, and rat liver microsomes, squalene from the liposomes was converted into sterols. When cytosol was omitted from the incubation mixture, only insignificant amounts of sterols were produced. Liposomes of squalene with either phosphatidylserine or phosphatidylcholine were equally effective as substrates. The liposomes were stable at 4°C for 3 weeks. The ratio of squalene to phospholipid in the liposomes could be varied over a range of 0.004 to 0.23. Multilamellar liposomes with squalene were not effective as a substrate for the conversion of squalene to sterols. The mechanism for transfer of squalene from the liposomes to the enzymes appears to be initial binding of liposomes to microsomes, with subsequent transfer of the substrate to the enzyme site by the SCP$_1$ in the cytosol. Microsome-liposome complexes prepared in the absence or presence of cytosol are effective in converting squalene to sterols only if cytosol is added again, indicating that cytosol is not required for the binding of liposomes to microsomes.—**Morin, R. J., and M. V. Srikantaiah.** In vitro conversion of squalene from squalene-phospholipid liposomes into sterols by rat liver microsomes and cytosol. *J. Lipid Res.* 1980. **21:** 1143–1147.

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Abbreviations: SCP, sterol carrier protein; S_{105}, 105,000 g supernatant; SCP$_2$, sterol carrier protein$_2$.

It is well established that the hepatic conversion of squalene to cholesterol requires at least two non-catalytic carrier proteins from the cytosol in addition to the microsomal enzymes (1, 2). The first of these carrier proteins, sterol carrier protein$_1$ (SCP$_1$) acts specifically at the oxidation and cyclization of squalene to lanosterol (3). The second, called sterol carrier protein$_2$ (SCP$_2$), is involved in the conversion of lanosterol to cholesterol. SCP$_1$ acts by transferring the substrates within the microsomal membrane (3). In all the in vitro studies the water-insoluble substrate was introduced into the reaction mixture by artificial means such as detergents (4, 5) or dioxane and propylene glycol (6). In the present study we have shown that the substrate squalene can be effectively introduced as squalene-phospholipid liposomes, a technique which has potential application in studies of cholesterol biosynthesis.

MATERIALS AND METHODS

DL-[5-^{3}H]Mevalonic acid was purchased from New England Nuclear, Boston, MA. The following compounds were purchased from Sigma Chemical Co., St. Louis, MO.: NAD, NADPH, bovine brain phosphatidylserine, FAD, and egg yolk phosphatidylcholine.

[^{3}H]Squalene was prepared by anaerobic incubation of DL-(5-^{3}H)mevalonic acid with a 20,000 *g* rat liver supernatant according to the method of Tchen (7). The petroleum ether-extracted radioactive squalene was further purified on a 7-ml volume silic acid column (5:1 Unisil:Supercel) by eluting with carbon tetrachloride–benzene 9:1. This column procedure was repeated to produce pure radioactive squalene. The specific activity of [^{3}H]squalene was determined by

TABLE 1. Squalene to sterol conversion from squalene added in dioxane–propylene glycol 2:1 as compared to squalene in vesicles

Experiment	Mode of Addition	Squalene Converted
		nmol/2 hr
1.	Squalene sonicated in buffer with microsomes alone	0.01
2.	1 + S_{105}	0.04
3.	Squalene added in D–PG[a] with microsomes alone	0.2
4.	3 + S_{105}	0.80
5.	Squalene added as vesicles with phosphatidylserine with microsomes alone	0.4
6.	5 + S_{105}	1.8

[a] D–PG, dioxane–propylene glycol 2:1.

Experiments 1, 3, and 5 contained 1.0 ml reaction mixture with microsomes (2 mg), NADPH (1.2 mM), NAD+ (3 mM), and FAD (0.1 mM). Experiments 2, 4, and 6, in addition, contained 3 mg of S_{105}. [^{3}H]Squalene (3 μM) was added as described in the Table. In experiments 1–4, where liposomes were not used, phosphatidylserine (0.13 mM) was added separately. Incubations were carried out for 2 hrs.

liquid scintillation counting and colorimetric assay (8). Different specific activity [^{3}H]squalene solutions were prepared by mixing with cold squalene. This was stored in toluene at −20°C. Male Sprague Dawley rats (200–300 g) were used for preparation of liver microsomes and liver 105,000 *g* supernatant (S_{105}). Livers were homogenized in three times their volume of buffer (0.02 M potassium phosphate buffer pH 7.4 containing 10^{-4} M EDTA) with a Polytron homogenizer. From this homogenate the S_{105} and microsomes were prepared as previously described (3). The microsomes were washed once by suspending them in buffer equivalent to the initial volume of homogenate and then rehomogenizing with the Polytron. After centrifugation at 105,000 *g* for 1 hr, the pellet was suspended in the buffer to give a protein concentration of 10 mg/ml. Protein was determined by the method of Lowry et al. (9). Vesicles were prepared by sonicating a mixture of [^{3}H]squalene, unlabeled squalene, and phospholipid in 1 ml of the above buffer for 10 min in an ice bath using a Biosonic IV sonicator at 15 kh with a needle probe. The molar ratio of squalene to phospholipid was varied from 0.004 to 0.23. The final concentrations of [^{3}H]squalene were 0.5 μM–30 μM. Radioactivity was kept constant at 0.1 μCi. Squalene concentration was varied by adding unlabeled squalene. The multilamellar vesicles were prepared by first drying the squalene-phospholipid mixture under nitrogen, adding buffer, and then mixing on a vortex stirrer.

The formation of sterols from squalene was assayed in a reaction mixture of 1 ml buffer, microsomes (2 mg), NADPH (1.2 mM), NAD^+ (3 mM), FAD (0.1 mM), S_{105} as a source of SCP_1 (3.0 mg), and 100 μl of vesicles containing 0.1 μCi of [^{3}H]squalene (3 to 10 μM) and phospholipid (0.13 to 0.26 mM). The incubations were carried out for 2 hr at 37°C in a Dubnoff Shaker under oxygen. After stopping the reaction with alcoholic potassium hydroxide, squalene and products were separated by the procedure of Scallen, Dean, and Schuster (10) to determine SCP_1 activity.

RESULTS

Assay conditions were established for microsomal concentrations, S_{105} concentration, and linearity of the assay with time. The squalene to sterols activity by microsomes in the absence of S_{105} reached saturation at 1.0 mg final concentration. Therefore in all subsequent experiments 2.0 mg of microsomal protein concentration was employed. With 2.0 mg of microsomes, the activity reached maximum at 7.0 mg of S_{105} added. In subsequent experiments 3.0 mg of S_{105} protein was used, which was in the linear range of activity. With saturating microsomal concentration and S_{105} concentration in the linear range, the assay system was linear for up to 2 hr.

Squalene dispersed in buffer with phosphatidylserine functions as well in the reaction mixture as when added in the previously used nonphysiological dispersing medium of dioxane and propylene glycol (**Table 1**). Sonicating squalene alone in the buffer failed to disperse the substrate; aliquots of squalene sonicated in this manner contained only a fraction of the radioactivity as compared to squalene dispersed with phospholipid. Sonicated phosphatidylserine added separately did not have any effect in dispersing the substrate (Experiments 1 and 2). The sonicated squalene prepared in this manner separated out from the buffer medium on storage for 24 hr at 4°C. Squalene to sterol conversion activity occurred over a wide range of concentrations of squalene relative to phospholipid (**Table 2**). Phospholipids kept the squalene dispersed over ratios of 0.004 to 0.23 (about a 60-fold range of variation). Microsomes used in saturating concentrations possessed low activity in the absence of S_{105}. Microsomal activities with any single concentration of squalene when done in duplicate were essentially identical. Activities with microsomes plus S_{105} were done in triplicate and means and standard deviations were calculated. The increase in activity with microsomes and S_{105} using liposomes from a squalene/phospholipid ratio 0.004 to 0.061 was highly significant ($P < 0.001$), whereas activities remained constant using liposomes with ratios higher than 0.061. Data in Table 2 also indicate that the squalene sub-

TABLE 2. Squalene to sterol conversion activity using vesicles with different squalene to phospholipid ratios

Squalene/ Phospholipid Ratio	Squalene Concentration	Experiment	Squalene converted
			nmol/2 hr
0.004	0.5 μM	Microsomes alone	0.1
		Microsomes + S_{105}	0.29 ± 0.01
0.008	1.0 μM	Microsomes alone	0.15
		Microsomes + S_{105}	0.6 ± 0.1
0.023	3.0 μM	Microsomes alone	0.18
		Microsomes + S_{105}	1.95 ± 0.12
0.061	8.0 μM	Microsomes alone	0.21
		Microsomes + S_{105}	4.4 ± 0.21
0.115	15.0 μM	Microsomes alone	0.25
		Microsomes + S_{105}	4.5 ± 0.1
0.230	30.0 μM	Microsomes alone	0.3
		Microsomes + S_{105}	4.75 ± 0.15

Vesicles were prepared by sonicating a mixture of [^{3}H]squalene, unlabeled squalene, and phosphatidylserine in buffer for 10 min. The final concentration of phosphatidylserine was 0.13 mM. Experiments with microsomes and S_{105} were done in triplicate, and means and standard deviations were calculated. The 1.0 ml reaction mixture contained microsomes (2 mg), S_{105} (3 mg) when used, FAD, NAD, and NADPH as given in Table 1. Incubations were carried out for 2 hr.

strate is saturating at 8.0 μM concentrations. Conversion of squalene from the vesicles into sterols was unrelated to the specific phospholipid moiety (**Table 3**). Squalene-phosphatidylserine vesicles are as good a substrate as squalene-phosphatidylcholine. Multilamellar liposomes were not active as substrate. The vesicles prepared by sonication were effective in dispersing squalene in the buffer for 3 weeks or longer at 4°C. Results of experiments shown in **Table 4** indicate that the vesicles first bind to microsomes or that transfer of squalene occurs by collision. Centrifugation at 105,000 *g* for 1 hr did not sediment the vesicles, as indicated by only 12% of the added radioactivity in the pellet (Table 4). There was 68% of the total radioactivity in the pellet when the vesicles were incubated with the microsomes. This radioactivity did not increase when S_{105} was included in the incubation mixture, indicating that S_{105} is not needed for binding of vesicles to microsomes. When this microsome-liposome complex prepared in the presence of S_{105} was incubated under oxygen with the squalene substrate, it resulted in very low activity (1.8 nmol product/2 hr), comparable to that of similar microsome-liposome complexes prepared in the absence of S_{105} (1.6 nmol/2 hr). Activities

TABLE 3. Squalene to sterol conversion activity in different vesicles

Vesicles	Experiment	Squalene Converted
		nmol/2 hr
Squalene – phosphatidylserine (sonicated)	Microsomes alone	0.35
	Microsomes + S_{105}	3.20
Squalene – phosphatidylcholine (sonicated)	Microsomes alone	0.20
	Microsomes + S_{105}	2.80
Squalene – phosphatidylcholine multilamellar (vortexed)	Microsomes alone	0.40
	Microsomes + S_{105}	0.75
Squalene – phosphatidylserine (aged 3 weeks at 4°C) (sonicated)	Microsomes alone	0.40
	Microsomes + S_{105}	3.10

Vesicles were prepared by using [^{3}H]squalene, unlabeled squalene, and phosphatidylserine (0.13 mM) or phosphatidylcholine (0.26 mM) as above. The 1 ml reaction mixture contained microsomes (2 mg), S_{105} where mentioned (3.0 mg), NADPH, NAD, and FAD as in Table 1. Incubations were carried out for 2 hr.

TABLE 4. Binding of vesicles to microsomes

Incubation Mixture	Percent Radioactivity in 105,000 *g* Pellet[a]
Vesicles alone	12.3 ± 2.6
Vesicles + microsomes	68.3 ± 4.6
Vesicles + microsomes + S_{105}	71.5 ± 3.4

[a] Means ± standard deviations of 4 determinations.

[^{3}H]Squalene (10 μM, 100,000 cpm) – phosphatidylcholine (0.26 mM) vesicles were incubated with microsomes (2 mg) or microsomes (2 mg) + S_{105} (3 mg) for 15 min under N_2 in the presence of FAD and cofactors. After 15 min, they were immediately cooled at 0°C and centrifuged at 105,000 *g* for 1 hr. Radioactivities were counted in aliquots of the supernatants or pellets separately, using an Amersham ACS liquid scintillation mixture. The percent of the total radioactivity in the pellet was then determined.

of both these preparations were significantly stimulated when further S_{105} was added just prior to incubation (6.0 and 5.9 nmol/2 hr).

DISCUSSION

It is now well established from work in different laboratories that some or all of the reactions between the conversion of squalene to cholesterol by the liver require one or more proteins from the cytosol in addition to microsomal enzymes (1, 2, 4, 11–13). Scallen et al. (1) first demonstrated that the liver cytosol contained at least two proteins which are required by the microsomal enzymes. The involvement of two cytosolic proteins was also demonstrated by Johnson and Shah (2). Scallen et al. (1) showed additionally that SCP_1 acted on the microsome-squalene complex by using squalene physiologically synthesized while bound to the microsomal membrane and then adding exogenous crude SCP_1. Saat and Bloch (14) further substantiated this mechanism of action of SCP_1. Since squalene is water insoluble, the medium for adding squalene in the in vitro assay of squalene to cholesterol conversion poses a problem. Scallen, Schuster, and Dhar (6) chose a mixture of dioxane and propylene glycol (2:1) for adding squalene into the reaction mixture. Tai and Bloch (4) used Tween 80 for dissolving squalene prior to addition. In the present experiment, we have shown that squalene from liposome-microsome complexes is converted to sterols, and this requires S_{105}. The presence of S_{105} does not seem to increase the binding of radioactive squalene to microsomes, but only after addition of S_{105} is the squalene from the microsome-liposome complex converted into sterols. The process by which squalene from liposomes is converted into sterols by the microsomes, therefore, appears to be an initial binding of liposomes to microsomes, or simply a transfer of squalene by collision, following which the squalene is translocated by SCP_1 to the sites of sterol synthesis. This mechanism will be more definitively investigated using purified SCP_1. Adding squalene as liposomes is preferable because they contain physiological components and these liposomes are stable. Also it is known that purified SCP_1 requires phosphatidylserine for its maximum activity (3, 4).

Recently, Nakamura and Sato (5) showed that both rat liver cytosolic protein and phosphatidylinositol were required for binding of squalene (added as mixed micelles with Tween 80) to microsomal membranes in vitro. Phosphatidylcholine was less effective. Our experiments show that SCP_1 is not required for binding of squalene to microsomes and also that squalene was effective as a substrate when added in phosphatidylcholine liposomes. The differences observed could be due to Nakamura's and Sato's method of adding squalene (ie., in Tween 80) (5). The effect of Tween 80 in binding of squalene to microsomes is not known. We have shown that when 7-dehydrocholesterol was added in Tween 80 to study rabbit liver microsomal reductase activity, the stimulating effect of cytosolic SCP_2 was abolished (15).

Use of liposomes as a vehicle for adding squalene will also be of value because many inhibitors of squalene to sterol conversion (which we are presently studying in our laboratory) are lipophilic and can be incorporated into the liposomes along with the substrate. The possibility of adding other intermediates between squalene to cholesterol as liposomes is also currently being investigated.

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