Studies on the Conversion of Enzymatically Generated, Microsome-bound Squalene to Sterol*

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We have previously demonstrated that sterol carrier protein1 (SCP1) is required for the conversion of squalene to lanosterol by rat liver microsomes (Srikantaiah, M. V., Hansbury, E., Loughran, E. D., and Scallen, T. J. (1976) J. Biol. Chem. 251, 5496-5505). These studies, however, had the disadvantage that the highly water-insoluble substrate squalene was added exogenously to incubations in small quantities of an organic solvent mixture (dioxan:propylene glycol, 2:1).

Using the techniques described here and in the accompanying article (Gavey, K. L., and Scallen, T. J. (1978) J. Biol. Chem. 253, 5470-5475) radioactive farnesyl pyrophosphate was used to enzymatically generate squalene in situ in the microsomal membranes. This preparation has the advantage that no agent, e.g. organic solvent or detergent, is required for the addition of the substrate squalene. This endogenously generated squalene was firmly bound to the microsomes. Microsomes incubated with cofactors in the absence of SCP1 were only minimally active in converting endogenously generated squalene to sterol. A series of experiments demonstrated that SCP1 was required to activate the conversion of squalene to lanosterol, even when squalene was biosynthesized in situ bound to the microsomal membranes.

Therefore, a requirement for a specific supernatant protein, e.g. SCP1, for the activation of microsomal enzymes in cholesterol biosynthesis is first seen only after the formation of squalene, the first water-insoluble intermediate in the pathway.

The nature of the interaction between SCP1, squalene, and microsomes was investigated, using both endogenously generated squalene and exogenously added squalene as substrates. Incubations begun with the addition of exogenous squalene exhibited a lag phase before the formation of radioactively labeled sterol was observed. This phenomenon was not evident when endogenously generated squalene was converted to sterol; nor was a lag phase evident when radioactively labeled squalene was premixed anerobically with microsomes and SCP1, for 30 min prior to incubation in an atmosphere of oxygen. In addition, microsomes were found to contain a significant amount of endogenous unlabeled squalene that had been biosynthesized in vivo.

The results suggest that a fraction of the squalene bound to microsomes is present as a squalene-SCP1-microsome complex. SCP1 may facilitate the movement of squalene on or within the microsomal membrane and by this mechanism activate the microsomal conversion of squalene to lanosterol.

We have previously demonstrated that sterol carrier protein, (SCP1) is required for the microsomal conversion of squalene to lanosterol, when the squalene substrate is added exogenously to the enzyme incubations (1). These studies, however, had the disadvantage that the highly water-insoluble substrate squalene was added exogenously to incubations in small quantities of an organic solvent mixture (dioxane: propylene glycol, 2:1).

Using the techniques described in the accompanying article (2), radioactive farnesyl pyrophosphate was used to enzymatically generate squalene in the microsomes. This preparation has the advantage that no agent is required for the addition and solubilization of the substrate squalene, e.g. dioxane: propylene glycol or Tween 80 as when squalene is added exogenously to the incubation as described previously (1, 3). This endogenously generated squalene microsome complex was then utilized for studies of the effect of SCP1 and partially purified SCP1 on sterol biosynthesis.

Finally, the present article further defines the role of SCP1 in the microsomal conversion of squalene to lanosterol, using squalene exogenously supplied to the enzyme incubations.

A preliminary description of some of these experiments has appeared previously (4).

EXPERIMENTAL PROCEDURES

Materials—Chemicals, rat liver microsomes, rat liver S100 and sterol carrier protein, were prepared as described in the accompanying article (2).

Preparation of Radioactively Labeled Squalene—DL-[2-14C]-Mevalonolactone was used to prepare [1,5,9,16,20,24-14C]squalene (henceforth referred to as [14C]-squalene) according to the method of Tchen (5) and as previously described (1, 6). In some later experiments, [4,8,12,13,17,21-14C]squalene (henceforth referred to as [14C]-squalene), prepared in the same manner from [2-14C]mevalonic acid, was used. Squalene of low specific activity (1000 cpm/nmol at a counting efficiency of 45% for [14C]-squalene and 85% for [14C]-squalene) was prepared by dilution of radioactive squalene with unlabeled squalene (Eastman) which had been purified as described for radioactively labeled squalene (6).

Preparation of Radioactively Labeled, Microsome-bound Squalene—Incubations were routinely carried out with the buffer and cofactor concentrations described in the accompanying article (2) for the assay of squalene synthetase. The total incubation volume was increased (4 ml); microsome concentrations varied (0.5 to 2.5 mg/ml). The incubations were performed in 50-ml Erlenmeyer flasks.

Incubation flasks (containing cofactors plus radioactive farnesyl pyrophosphate) and microsomes were depleted of oxygen separately

1 The abbreviations used are: S100, 105,000 X g supernatant; SCP1, sterol carrier protein; SPF, supernatant protein factor.
and incubation was conducted as described for the assay of squalene synthetase activity (2). At the end of the incubation period the flasks were plunged into a bath of ice water, and chilled buffer (3 to 6 ml) was added. After 10 min, the contents of the flask were transferred to centrifuge tubes and centrifuged (314,000 g for 30 min or 105,000 g for 60 min). Upon completion of the centrifugation, the supernatant was removed with a Pasteur pipette. Chilled buffer (1 to 2 ml) was allowed to flow over the microsomal pellet and was also removed. The pellet was then transferred with a spatula to an appropriate volume of chilled buffer (2 to 5 ml) and rehomogenized with a motor-driven tight-fitting Teflon pestle.

The recovery of microsomal protein during these manipulations was 50 to 75%. The amount of newly synthesized squalene bound to the microsomes was as much as 6 nmol/mg of microsomal protein in some cases..

An alternative technique for concentrating the microsomes and separating them from the incubation media was used in some later experiments. After chilling, the contents of the incubation flask were placed in an Amicon concentration cell over a Diaflo PM-10 membrane, and nitrogen pressure (40 to 50 psi) was applied (20 to 30 min). When the microsomes had been concentrated (1 to 3 ml) the suspension was applied to a Sephadex G-25 column (2.2 x 20 cm) which had been equilibrated with phosphate buffer (20 mm, pH 7.4) containing EDTA (0.1 mm). The passage of the microsomes through the column was visible; the membranes were collected at the void volume (3 to 5 ml fraction). This procedure was conducted at 4°C.

This technique was not more rapid than centrifugation, and the recovery of protein was approximately the same. However, it has the advantage of ensuring the removal of all small molecules not actually bound to the microsomes.

**Incubations Using Endogenously Generated, Microsome-bound Squalene as Substrate**—The incubations routinely contained NADPH (1.2 mm), FAD (0.05 mm), and potassium phosphate buffer (20 mm, pH 7.4) containing EDTA (0.1 mm). Microsomes containing endogenously generated, microsome-bound squalene as a substrate were added as indicated in Figs. 1, 2, 3, and Table I. Incubations were carried out for the periods of time indicated in an atmosphere of nitrogen and redissolved in toluene (0.2 ml) were applied to silver nitrate-impregnated silicic acid chromatography, and the appearance of nonsterol polar products that were also firmly bound to the microsomes when squalene was generated from farnesyl pyrophosphate utilized.

**Extraction of Squalene and Sterols**—All enzymatic reactions were stopped by the addition of an equal volume of 15% KOH in 95% ethanol, followed by saponification (30 to 60 min at 80°C). Extraction of squalene and sterols was performed as previously described (6).

**Squalene Determination by Calorimetric Assay**—Squalene was determined calorimetrically (9). The presence of methyl groups at C-4 of sterols as measured by co-precipitation with unlabeled cholesterol by digitonin in the presence of unlabeled cholesterol, as described below. In most of the experiments recorded here in which endogenously generated squalene was the substrate, the utilization of squalene (as measured by silicic acid chromatography), and the appearance of sterol (as measured by co-precipitation with unlabeled cholesterol by digitonin) were both assayed.

**Radioactively labeled sterols** were determined by co-precipitation with unlabeled cholesterol as the digitonide by a modification of the method described by Sperry (7). Petroleum ether extracts of the incubations or the sterol-containing fraction from the incubation media was dried in a desiccator for 2 or more days was used as the standard.

**Fractionation of Solvent Mixture**—An alternative technique for concentrating the microsomes and separating them from the incubation media was used in some later experiments. After chilling, the contents of the incubation flask were placed in an Amicon concentration cell over a Diaflo PM-10 membrane, and nitrogen pressure (40 to 50 psi) was applied (20 to 30 min). When the microsomes had been concentrated (1 to 3 ml) the suspension was applied to a Sephadex G-25 column (2.2 x 20 cm) which had been equilibrated with phosphate buffer (20 mm, pH 7.4) containing EDTA (0.1 mm). The passage of the microsomes through the column was visible; the membranes were collected at the void volume (3 to 5 ml fraction). This procedure was conducted at 4°C.

This technique was not more rapid than centrifugation, and the recovery of protein was approximately the same. However, it has the advantage of ensuring the removal of all small molecules not actually bound to the microsomes.

**Incubations Using Exogenously Added Squalene**—Incubations routinely contained NADPH (1.2 mm), FAD (0.05 mm), potassium phosphate buffer (20 mm, pH 7.4) containing EDTA (0.1 mm) and microsomes (2 mg of protein). Reagents were mixed at room temperature. Squalene was added in 2:l ethanol, followed by saponification (30 to 60 min at 80°C). Extraction of squalene and sterols was performed as previously described (6).

**Silyl Acid Chromatography**—Squalene was separated from sterols and other polar compounds by the short silyl acid column (1.2 x 7 cm) method previously described (6). When farnesyl pyrophosphate was the substrate, it was used to be necessary to take a larger volume of the second solvent mixture (20 ml) to ensure that all polar radioactively labeled compounds had been eluted.

**Silver Nitrateg-impregnated Silyl Acid Chromatography**—Petroleum ether extracts that had been dried under a stream of nitrogen and redissolved in toluene (0.2 ml) were applied to silver nitrate-impregnated silyl acid columns (1.2 x 7 cm). Ten to fifteen fractions (7 ml) were collected with toluene as the eluting solvent. These fractions contained sterols. Squalene and other nonsterol compounds containing two or more double bonds remained bound to the column.

**RESULTS**

**Preliminary Investigation of Squalene and Sterol Biosynthesis from Farnesyl Pyrophosphate**—This experiment was carried out in order to demonstrate the biosynthesis of squale
Conversion of Microsome-bound Squalene to Sterol

Although it was only partially successful, in that sterol as well as squalene was formed, a number of important observations were made.

The incubations were carried out in an atmosphere of nitrogen; however, no attempt was made to remove oxygen from the incubation media.

Microsomes alone biosynthesized squalene as can be seen in Table I. Slos slightly increased this activity. Only a trace amount of squalene was formed in the incubation which contained only Slos.

When microsomes alone were incubated with farnesyl pyrophosphate only a trace amount of sterol was formed. The same result was obtained when Slos was incubated in the absence of microsomes. However, when microsomes and Slos were incubated together, a substantial quantity of sterol was produced. This increase in sterol formation was 21-fold when compared to microsomes alone. Thus, while squalene synthesis was vigorous with microsomes alone, sterol synthesis required both microsomes and Slos.

Activation of Sterol Biosynthesis from Microsome-bound Squalene by Slos—Microsome-bound squalene was generated from [3H]farnesyl pyrophosphate, using a method which depletes the oxygen from the incubation media, as previously described (2). Microsome-bound [3H]squalene (5.3 nmol/mg of microsomal protein) was then incubated either in the presence or in the absence of Slos for various periods of time as shown in Fig. 1. The results show that the conversion of squalene to sterols by microsomes alone does not represent the total capacity of the system. The addition of Slos substantially increases squalene disappearance (Fig. 1A) and sterol formation (Fig. 1B). A plot of log, nmol of squalene/mg of microsomal protein versus time, is shown in Fig. 1C. For incubations which included Slos the apparent first order rate constant was 0.013/min and the apparent half-life of the reaction was 53 min (linear correlation coefficient = 0.997).

The equivalent transformation of data from the incubations containing only microsomes was not linear (Fig. 1C).

Activation of Sterol Biosynthesis from Microsome-bound Squalene by Partially Purified SCP—Microsome-bound [14C]squalene (1.3 nmol/mg of microsomes) was generated from [1,5,9,14C]farnesyl pyrophosphate as previously described (2).

Microsomes alone were capable of converting squalene to sterols (a maximum of 0.37 nmol/mg of microsomal protein) as measured by the disappearance of squalene (Fig. 2A). Most of this activity took place during the first time interval examined (15 min). In this same time interval, microsomes plus SCP1 converted twice as much squalene to sterols (0.73 nmol/mg of microsomal protein) as measured by the disappearance of squalene. In the presence of SCP1 the reaction continued so that at a later time (45 min) almost 4 times as much squalene had disappeared (1.15 nmol/mg of microsomal protein). At this point in time the microsome-bound [14C]squalene substrate was almost exhausted. Fig. 2B shows sterol formation as a function of time for the same experiment. The findings described above for squalene disappearance are confirmed in terms of sterol formation.

Effect of SCP1 Concentration on Conversion of Microsome-bound Squalene to Sterol—Microsome-bound [3H]squalene was generated from [1,5,9,14C]farnesyl pyrophosphate as previously described (2). The microsomal control converted essentially the same amount of squalene to sterols (0.38 nmol) as that recorded at the lowest concentration of SCP1 (calculated from the disappearance of squalene). Fig. 3A shows that with increasing the amount of SCP1 added, increasing

<table>
<thead>
<tr>
<th>Table I</th>
<th>Preliminary investigation of squalene and sterol biosynthesis from [1,1-3H]farnesyl pyrophosphate</th>
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<tr>
<td>Incubations contained microsomes in the amounts shown, NADPH (1.2 mM), magnesium chloride (5 mM), ATP (3 mM), potassium phosphate buffer (20 mM, pH 7.4) containing EDTA (0.1 mM) and [1,1-3H]farnesyl pyrophosphate (190 µM, 10 mCi/mmol) in a total volume of 2 ml. Slos was added as indicated. Incubations were conducted for 30 min under a stream of nitrogen, however, no attempt was made to remove oxygen from the incubation media.</td>
<td></td>
</tr>
</tbody>
</table>
| Total radioactivity recovered from squalene and sterol biosynthesis | Nonsterol polar compounds
cpm | cpm |
<table>
<thead>
<tr>
<th>mg protein</th>
<th>Squalene</th>
<th>Sterol*</th>
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<tr>
<td>0.50</td>
<td>202,550</td>
<td>29,580</td>
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<tr>
<td>0.50</td>
<td>478,160</td>
<td>195,570</td>
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<td>0.00</td>
<td>95,430</td>
<td>88,560</td>
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</table>

* Calculated on the basis of polar radioactivity recovered from squalene and sterol biosynthesis.
amounts of squalene are metabolized. The same result is apparent also for sterol formation. An Eadie-Hofstee plot for the disappearance of squalene (Fig. 3B) exhibits a straight line at the higher SCP, concentrations. V_{\text{max}} (apparent) is 0.63 nmol of squalene utilized/30 min/0.5 mg of microsomal protein. The Eadie-Hofstee plot for the appearance of sterols (Fig. 3B) is biphasic. A possible explanation for the deviation from linearity at low concentrations of added partially purified SCP, (18 and 36 \mu g of SCP, incubation) is the presence of a small quantity of endogenous SCP, bound to the microsomes which was not removed during the washing procedure.

The components required for the utilization of microsome-bound squalene were investigated. SCP, NADPH, and FAD were required for full activity. Phosphatidylserine was not required, although it did slightly increase sterol formation in some experiments.

**Microsomal Binding of Sterol Product with Exogenous [14C]Squalene as Substrate**—The precise mechanism by which SCP, activates the enzymatic transformation of squalene to lanosterol is still not known. One possibility that has been suggested is that SCP, binds the product of the reaction (lanosterol) and by removing it from the microsomal membrane renews a putative product inhibition.

To test this possibility, microsomes were separated from the incubation media after routine incubations had been performed using exogenously added [14C]squalene as substrate. Flasks were placed in a bath of ice water and chilled for several minutes, and centrifuged at 314,000 \times g for 30 min. The contents of two identical incubations were combined so that microsomal protein equal to that of the controls could be recovered. After centrifugation, supernatant (1.5 ml) and rehomogenized microsomes (2.0 mg) were assayed for radioactive squalene and sterol content.

The results shown in Table II demonstrate that the sterol products remained bound to the microsomes during this procedure. Essentially no sterol was recovered in the supernatant, and recovery from the microsomal pellet was 88\% or more of the total amount of sterol formed. Thus sterol product remains bound to the microsomes in the presence of SCP, and little or no sterol is released from the membranes in the presence of SCP,.

**Microsomal Binding of Squalene and Sterol Biosynthesized from Farnesyl Pyrophosphate**—Microsome-bound squalene was generated from [1,5,9-14C]farnesyl pyrophosphate (0.57 mCi/mmol) as described under "Experimental Procedures" and in the text. Subsequent incubations contained microsomes (0.54 mg of protein, 0.71 nmol of [14C]squalene), NADPH (1.2 mM), FAD (0.05 mM), reduced glutathione (1 mM), and potassium phosphate buffer (20 mM, pH 7.2) containing EDTA (0.1 mM) in a total volume of 1.5 ml. Partially purified SCP, (0.36 mg) was added where indicated. A, disappearance of squalene; B, appearance of sterol; 

![Fig. 2. Conversion of microsome-bound [14C]squalene to sterol as a function of time in the presence or absence of partially purified SCP.](image)

![Fig. 3. A, conversion of microsome-bound [14C]squalene to sterol as a function of partially purified SCP concentration.](chart)

This was designed to be an anaerobic incubation, and no sterol formation was anticipated. However, it is apparent from the data recorded in Table III that oxygen depletion was not completely achieved. A small amount of squalene was metabolized to sterols in each case. The recovery of squalene and sterols after centrifugation appears to be slightly greater than...
100%, this can probably be attributed to losses from the incubation flask during the pipetting.

The amount of squalene and sterol appearing in the supernatant is the same in all cases. Addition of partially purified SCP, or of phosphatidylserine did not affect this result.

The evidence strongly suggests that neither squalene nor sterol is released from the microsomes in the form of a soluble, SCP-bound complex in substantial amounts.

**Effect of Time on Sterol Biosynthesis Using Exogenously Added [3H]squalene** — Sterol biosynthesis over an extended period of time was investigated using exogenously added [3H]squalene as substrate. Incubation flasks and microsomes were prewarmed separately (37°C, 5 min); the incubations were begun with the addition of microsomes. Sterols were determined by co-precipitation with unlabeled cholesterol as the digitonide. The substrate control contained 80 cpm (equivalent to 0.08 nmol of sterol). This amount was subtracted from the results shown in Fig. 4A.

Biosynthesis of digitonin-precipitable sterols showed a distinct lag during the first 30 min but was linear thereafter for the period of time studied (180 min).

Increasing the amount of [3H]squalene substrate added to the incubation media (from 30 nmol/3 ml to 100 nmol/3 ml) increased the amount of sterol formed by only 1 nmol (approximately 11%) in 3 h.

**Effect of Premixing of Incubation Components Using Exogenously Added [14C]squalene** — Attempts to follow the conversion of squalene to sterols over a period of time had

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Squalene</th>
<th>Sterol</th>
<th>Recovery of sterol</th>
<th>Product formed</th>
<th>Product recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.10</td>
<td>0.19</td>
<td>0.10</td>
<td>0.10</td>
<td>0.01</td>
</tr>
<tr>
<td>5</td>
<td>0.10</td>
<td>0.17</td>
<td>0.12</td>
<td>0.12</td>
<td>0.01</td>
</tr>
<tr>
<td>10</td>
<td>0.09</td>
<td>0.15</td>
<td>0.10</td>
<td>0.10</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* Calculated on the basis of radioactivity remaining in the incubation flask.

Calculations performed on the basis of radioactivity in the rehomogenized microsomes and in the supernatant after centrifugation at high speed (314,000 × g, 30 min).

**TABLE II**

**Microsomal binding of sterols biosynthesized from [14C]squalene**

Incubations contained microsomes (2 mg of protein), NADPH (1.2 mM), FAD (0.05 mM), phosphatidylethanolamine (100 μg/ml), potassium phosphate buffer (20 mM, pH 7.2) containing EDTA (0.1 mM), partially purified SCP, (0.36 mg of protein) where used, and [14C]squalene (15 nmol, 1000 cpm/nmol) in a total volume of 1.5 ml. Incubations were conducted in an atmosphere of oxygen for 2 h at 37°C. At the conclusion of the incubation microsomes were sedimented by centrifugation at high speed (314,000 × g, 30 min) as described under "Experimental Procedures" and in the text.

**TABLE III**

**Microsomal binding of squalene and sterols biosynthesized from [1,3,9-14C]farnesyl pyrophosphate**

Microsomal-bound [14C]squalene was prepared from [14C]farnesyl pyrophosphate (specific activity 0.57 mCi/mmol) as described under "Experimental Procedures" and in the text. Partially purified SCP, (0.11 mg) was added to Flasks 2 and 3; PS (150 μg/ml) was also added to Flask 3.

**Fig. 4.** A, effect of time on the biosynthesis of sterol from exogenously added [3H]squalene. Incubations contained microsomes (3 mg of protein), SSEC (30 mg of protein), NADPH (1.2 mM), FAD (0.05 mM), phosphatidylethanolamine (50 μg/ml), potassium phosphate buffer (20 mM, pH 7.4), and [3H]squalene (1000 cpm/nmol). The incubation volume was 3 ml. Incubations were conducted in an atmosphere of oxygen at 37°C for the periods of time shown; [●●●●], 30 nmol of [3H]squalene/incubation; ○○○○, 100 nmol of [3H]squalene/incubation. B and C, effect of premixing of components required for sterol biosynthesis. Incubations contained microsomes (2 mg of protein), NADPH (1.2 mM), FAD (0.05 mM), reduced glutathione (1 mM), phosphatidylethanolamine (100 μg/ml), potassium phosphate buffer (20 mM, pH 7.2) containing EDTA (0.1 mM), partially purified SCP, (0.26 mg), and [14C]squalene (15 nmol, 1000 cpm/nmol) in a total volume of 1.5 ml. All incubations were prewarmed for 30 min in an atmosphere of nitrogen at 37°C before the addition of the final component. [●●●●], begun with the addition of microsomes; [○○○○], begun with the addition of SCP; [□□□□], begun with the addition of NADPH; [●●●●], begun with the addition of [14C]squalene substrate. After the addition of the final component incubations were conducted in an atmosphere of oxygen at 37°C for the times indicated. Sterol formation was measured by silicic acid chromatography (6).
indicated that with exogenously added squalene as the substrate, the rate of the reaction increased rapidly during the first 30 min of the incubation (Fig. 4A).

In order to investigate this phenomenon a series of experiments was conducted in which various components of the reaction were mixed together in the Dubnoff shaker/incubator (30 min, two reciprocations per s) in an atmosphere of nitrogen, before the addition of a final component. FAD and NADPH were added together after the premixing interval, and the reaction was begun with the addition of the final component. Incubation was then conducted in an atmosphere of oxygen. In one case, FAD and NADPH (added together) were the final component.

The results are shown in Fig. 4, B and C. The observation from Fig. 4A was confirmed in the case where the [3H]-squalene substrate was the final component, i.e. the rate of the reaction increased during the 30-min period. An acceleration was also seen when partially purified SCP, was added last, although the reaction did not attain the same velocity in the same interval of time. The reaction was linear (after the first 5 min) when FAD and NADPH were added last.

The reaction also appeared to be linear when microsomes were added last, and was slower than in all of the other cases. This result was different from that of the experiment described in Fig. 4A when the microsomes were added last also; however, in that experiment the components were premixed at 37°C for only 5 min before the reaction was begun.

pH Optimun—Using exogenously supplied [3H]squalene as substrate, the pH optimum range for the conversion of squalene to sterol in phosphate buffer (20 mM) was between 7.1 to 7.3. Activity was substantially lower at pH 7.6.

DISCUSSION

The various techniques used for the presentation of water-insoluble substrates to membrane-bound enzymes sometimes produce effects that are not recognized or carefully evaluated. For example, Fallon et al. (14) have found that when an aqueous dispersion of diglyceride was used as a means of presenting diglyceride as a substrate, no changes in microsomal cholinephosphotransferase or diglyceride acyltransferase activity were seen during high fructose feeding of rats. However, when diglyceride was enzymatically formed in situ from sn-[1,3-14C]glycerol-3-phosphate, increased incorporation of the diglyceride into triglycerides was observed during high fructose feeding, while cholinephosphotransferase activity was unchanged.

Squalene has been presented to microsomal preparations in a variety of ways: in the detergent Tween 80 (3, 15, 16) by sonication (16) and by dioxygen-propylene glycol (1, 6, 17). It is clear that these methods have provided a great deal of information; however, each agent has the potential of affecting the reaction in some way. For example, since Tween 80 is a detergent, it has the potential of artificially activating the conversion of squalene to sterol by solubilizing the substrate squalene. Ono and Bloch (18) have reported that the SPF and phosphatidylserine requirement for squalene epoxidation can be replaced by the detergent Triton X-100. Astruc et al. (19) have shown that contaminants in dioxygen can alter the binding of squalene to proteins present in S100.

Furthermore, Chesterton (20) has shown that most of the squalene and lanosterol derived in vivo from [14C]-mevalonate is bound to the endoplasmic reticulum. He points out that, logically, it can be assumed that squalene which is metabolized to lanosterol is not removed from the endoplasmic reticulum during the process.

It appeared, then, that the study of the metabolism of endogenously generated, microsome-bound squalene could be of advantage. Radioactively labeled microsome-bound squalene, generated from mevalonate, has been successfully used as a substrate by Scallen et al. (17) and by Saat and Bloch (3); however, the conversion of mevalonate to squalene requires enzymes present in S100, as well as microsomes. Farnesyl pyrophosphate is a better precursor to use for this reaction, since only the microsomes are required to convert it to squalene (2). Like mevalonate, farnesyl pyrophosphate is soluble in water. Goodman (21) had shown that this approach was feasible; he did not, however, investigate the effect of any specific protein on the reaction.

Therefore, a technique was devised for the generation of squalene in the microsomal membrane, from its immediate precursor, farnesyl pyrophosphate; the utilization of this membrane-bound squalene as a substrate for sterol biosynthesis is described.

The results indicate that squalene which is newly synthesized in the presence of microsomes, or in the presence of microsomes plus SCP, remains bound to the microsomes (Table III). Squalene generated from farnesyl pyrophosphate adhered to the microsomes through the processes of centrifugation, homogenization, and passage through Sephadex G-25.

Microsome-bound squalene thus biosynthesized was metabolically active and was converted to sterol (Figs. 1, 2, and 3). The components required for the utilization of membrane-bound squalene in vivo were investigated. SCP, NADPH, and FAD were required for full activity.

A series of experiments using endogenously generated, microsome-bound squalene as substrate, and using S100 and increasingly purified preparations of SCP, as the source of supernatant protein, showed that SCP, was responsible for activation of the conversion of squalene to sterol (lanosterol), even when squalene was biosynthesized in situ from farnesyl pyrophosphate (Fig. 2). Kinetic studies (Fig. 3) confirmed the observations of Srikanthiah et al. (1) that SCP, behaves kinetically as a noncatalytic carrier in that a hyperbolic relationship is seen between SCP, concentration and sterol formation (Fig. 3A). The Edlin-Heftree plot (Fig. 3B) is biphasic; the velocity at low concentrations of partially purified SCP, is greater than is accounted for by the amount of SCP, added.

There are a number of significant differences seen between the reactions involving microsome-bound squalene and exogenously added squalene. When exogenously added squalene is the substrate, a distinct lag phase is seen in some circumstances before a constant velocity is achieved (Fig. 4). No evidence of an equivalent lag phase was seen for the metabolism of endogenously generated squalene, nor was any lag observed when exogenously supplied farnesyl pyrophosphate was the substrate (2).

In addition, it is clear that the microsomes without added S100 are more active when microsome-bound squalene is the substrate than when exogenously added squalene is the substrate, although microsomes alone are capable of low levels of activity with exogenously added squalene (1).

The picture is complicated by another factor, i.e. the presence of endogenous unlabeled squalene in the microsomes that has been synthesized in vivo. Assay of buffer-washed microsomes by the method of Rothblat et al. (11) showed that there may be as much as 0.8 nmol of endogenous squalene/mg of microsomal protein present in these preparations. (Microsomes are capable of binding more squalene than this, as shown in experiments reported here (Fig. 1).)

The sterol carrier protein hypothesis (1, 17, 22) implies the presence of SCP, in (or on) the endoplasmic reticulum during the conversion of squalene to lanosterol, and this process is a continuous one. An explanation that appears to fit the ob-
served experimental results is as follows.

Squalene that has been biosynthesized in vivo is bound to microsomes that are prepared as described here. A fraction of this squalene, but not all, is present as an SCP₁-squalene-microsome (enzyme) complex. When incubated with the necessary cofactors in the presence of radioactively labeled, exogenously added squalene, initially only this endogenous squalene fraction is converted to sterols. No evidence of this sterol formation is seen, since no radioactively labeled sterol is formed.

If SCP₁ (in the form of 10₀ or purified preparations) is added to the incubation, or if the microsomes have not been carefully washed, more SCP₁ is available, and exogenously added, radioactively labeled squalene is also bound to the microsomes and transformed into an SCP₁-squalene-microsome complex and subsequently converted into sterols.

When squalene is biosynthesized in vitro from farnesyl pyrophosphate, however, the newly synthesized, radioactively labeled squalene equilibrates with the unlabeled squalene of both pools that was biosynthesized in vivo. When these microsomes are subsequently incubated with cofactors, the equilibration of the radioactively labeled squalene with the unlabeled squalene-SCP₁ complex ensures that a certain amount of it will be converted to sterols. Again the limiting factor is the amount of SCP₁ present, and the addition of more SCP₁ increases the amount of squalene that is metabolized (Figs. 1 and 3).

Saat and Bloch (3) found that microsomes which were preincubated with [³H]squalene and 10₀, then centrifuged at high speed and washed, were just as active in converting the bound [³H]squalene to 2,3-oxidosqualene as were microsomes that had been incubated with [³H]squalene and 10₀ in a conventional manner. Their interpretation was that a supernatant protein factor (SFP) performed a transport function and that once this function had been accomplished, SFP was washed from the membranes, leaving the transported squalene in the metabolically active site. The results can also be interpreted to mean, however, that the supernatant protein was bound to the membranes as an SCP₁-squalene-microsome complex and was not removed by the washing procedure.

If these explanations of the experimental results are correct, then it may also be true that squalene bound in the SCP₁-squalene-microsome complex in vivo represents the metabolically more active pool seen by Loud and Bucher (23), and that microsome-bound squalene that is not present in such a complex is that which is metabolically less active.

Finally, a series of observations on the rate of sterol biosynthesis using exogenously added squalene as the substrate have provided information which gives insight into the processes of squalene binding and metabolism (Fig. 4). The effect of preincubation of [³H]squalene and partially purified SCP₁, for 30 min at 37°C was to partially inactivate the protein as far as squalene metabolism is concerned. This act of incubations, where microsomes were added as the final component, was the least active of any.

It will be recalled that Tai and Bloch (16) incubated 10₀ with squalene at 37°C for 30 min and observed large molecular weight protein aggregates containing squalene at the void volume on Sephadex G-100. These aggregates were inactive for the conversion of squalene to 2,3-oxidosqualene. The same aggregation phenomenon was seen by Scallen et al. (17). In that case partially purified SCP₁ was incubated with [³H]squalene at 37°C for 15 min; the aggregate retained partial activity for the conversion of squalene to sterols. A possible explanation for the effect is that incubation of SCP₁ with squalene in the absence of microsomes results in an aggregation of protein and squalene into a metabolically inert complex, while the binding of squalene and SCP₁ to microsomes produces a metabolically active complex.

The addition of partially purified SCP₁, as the last component also results in reduced activity. The reason for this is not entirely clear; it may be that both processes described above (binding of SCP₁ in active and inactive complexes) are taking place during the time interval examined.

Finally, the most striking differences in the rate of the reaction occur when cofactors (NADPH and FAD) are added last or when squalene is added as the final component. When cofactors are the final component, the reaction is linear after the first 5 min, indicating that the necessary association and complex formation steps have already taken place.

When squalene is the final component added (Fig. 4C), a distinct lag phase occurs before any conversion to sterol is seen, and the rate of the reaction increases thereafter for the duration of these incubations (30 min). This order of addition, premixing microsomes, SCP₁, and cofactors prior to the addition of [³H]squalene as the final component, gave the best yield of sterol at 30 min.

Binding of exogenously added squalene to microsomes shows a similar dependency on time, as was observed by Saat and Bloch (3). They also noted that the fraction of radioactively labeled squalene converted to 2,3-oxidosqualene depended upon the length of preincubation of microsomes with squalene.

The results reported here (Fig. 4) and those of Saat and Bloch (3) can be interpreted as representing the equilibration of exogenously added squalene with unlabeled squalene already present in the microsomes, and the conversion of both labeled and unlabeled squalene to 2,3-oxidosqualene, or sterols, or both.

In conclusion, the work described here and in the accompanying article (2) demonstrates that there is no supernatant protein requirement for the microsomal conversion of farnesyl pyrophosphate to squalene. Activation of the microsomal conversion of exogenously added squalene to sterol (lanosterol) by SCP₁ is not an artifact, since 10₀ and SCP₁ activate this reaction even when squalene has been generated from farnesyl pyrophosphate in situ bound to the microsomal membrane. Therefore, a requirement for a specific supernatant protein, e.g., SCP₁, for the activation of microsomal enzymes in cholesterol biosynthesis, is first seen only after the formation of squalene, the first water-insoluble intermediate in the pathway.

In addition, a technique has been devised by which endogenously generated squalene can be prepared for use as a substrate. Results of investigations using this form of substrate and by conventional techniques strongly suggest that the metabolically active form of squalene and SCP₁ is a microsome(enzyme)-bound complex, and supports the hypothesis that SCP₁ facilitates the movement of squalene on or within the microsomal membrane.

These same observations also demonstrate that additional work is necessary to further characterize this interaction.

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Conversion of Microsome-bound Squalene to Sterol

Studies on the conversion of enzymatically generated, microsome-bound squalene to sterol.
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