Intermembrane transfer of squalene promoted by supernatant protein factor

Diana Fuks-Holmberg[1] and Konrad Bloch

The James Bryant Conant Laboratories, Harvard University, Cambridge, MA 02138

Abstract Supernatant protein factor (SPF), a protein that stimulates squalene epoxidation, mediates the transfer of squalene between two separable microsomal populations (Kojima, Y., E. J. Friedlander, and K. Bloch, 1981. *J. Biol Chem.* **256:** 7235–7239). We now show that SPF also promotes the transfer of squalene associated with mitochondria or with plasma membranes to total microsomes or rough or smooth microsomal subfractions. Both rough and smooth microsomes have squalene epoxidase activity that is stimulated by SPF.—**Fuks-Holmberg, D., and K. Bloch.** Intermembrane transfer of squalene promoted by supernatant protein factor. *J. Lipid Res.* 1983. **24:** 402–408.

Supplementary key words squalene epoxidation • smooth and rough microsomes • plasma membrane • mitochondria

The conversion of squalene to 2,3-oxido-squalene (epoxidation) by rat liver microsomes is stimulated several fold by supernatant protein factor (SPF), a soluble, presumably cytosolic protein. In previous studies designed to clarify the mode of action of SPF, we have taken advantage of the fact that trypsin treatment abolishes microsomal epoxidase activity. When trypsinized squalene-containing microsomes are mixed with normal microsomes, no epoxidase activity is detected until SPF is added to the system. This finding led to the conclusion that SPF promotes intermembrane squalene transfer (1). This phenomenon was demonstrated more directly by density gradient centrifugation which separates the lighter, trypsinized microsomes from normal microsomes (2). In this report we show that mitochondria and plasma membranes, organelles which do not contain squalene epoxidase, can serve as squalene donors for microsomal epoxidase as well. These transfer processes also have an absolute requirement for SPF. Both rough and smooth microsomes catalyze squalene epoxidation and can serve as acceptors of squalene associated with mitochondria or plasma membranes.

EXPERIMENTAL PROCEDURES

Materials

Female rats (strain CD) weighing 200–220 g, were obtained from Charles River Breeding Laboratories. Tris, NADPH, FAD, and soybean trypsin inhibitor were supplied by Sigma Chemical Co. [^{3}H]Squalene, sp act 3.9 Ci/mmol, was purchased from New England Nuclear Corp. The squalene epoxide cyclase inhibitor AMO-1618 was supplied by Calbiochem and trypsin was supplied by Boehringer, Mannheim. 2,3-Oxidosqualene was synthesized according to the method of Nadeau and Hanzlik (3) by Dr. A. K. Lala in this laboratory. The SPF (acetone fraction) was prepared as described (4); pure protein obtained by isoelectric focusing (4) was supplied by I. W. Caras and E. J. Friedlander of this laboratory.

Preparation of squalene donor and acceptor particles

Animals used for preparation of liver microsomes were starved 20 hr before being killed. Liver microsomes, obtained from a 0.25 M sucrose homogenate as described by Ernster, Siekevitz, and Palade (5), were centrifuged at 105,000 *g* for 60 min and resuspended in 0.25 M sucrose. Mitochondria were prepared according to Guerra (6) with certain modifications. The isolation medium was 0.025 mM Na_2EDTA in 0.44 M sucrose, 45 mM Tris-HCl, pH 7.5; the washing medium was the same, except that EDTA was omitted. Centrifugation (800 *g*) was done twice for 20 min and after each centrifugation the supernatant was carefully decanted. In order to avoid substantial contamination of mitochondria with lysosomes, the 800 *g* supernatant was centrifuged at low speed (4000 *g*) for 20 min. The pellet was resuspended in washing medium and centrifuged again at 4000 *g* for 20 min. This procedure was repeated until glucose-6-phosphatase activity was no longer detectable. In the final step, 1 ml of mitochondrial suspension (3.6 mg/ml) was layered on 4 ml of 1.17 M sucrose, 45 mM Tris-HCl, pH 7.5, and centrifuged in

Abbreviation: SPF, supernatant protein factor.

[1] Present address: National Food Administration, Toxicology Laboratory, Box 622, 75126 Uppsala, Sweden.

15-ml Corex tubes in a Sorval centrifuge at 2000 *g* for 30 min. Each preparation of mitochondria was also tested for squalene epoxidase activity after incorporation of [^{3}H]squalene as described below, in the presence and absence of SPF. No such activity was found. This confirms that the mitochondria were not contaminated by microsomes. Plasma membranes were prepared from the nuclear fraction of liver cells according to Aronson and Touster (7) and checked for squalene epoxidase and glucose-6-phosphatase activities. Rough and smooth microsomes were prepared according to Rothschild (8).

Incorporation of [^{3}H]squalene into mitochondria and plasma membranes

One hundred μl of [^{3}H]squalene (5 nmol/ml, sp act 3.9 Ci/nmol after dilution with nonradioactive squalene) was dispersed with the aid of 100 μl of 0.3% Tween-80 in a stream of argon. A mitochondrial suspension (0.5 ml, 1.8 mg of protein) was added and the mixture was incubated for 30 min at 37°C under argon. Thereafter the suspension was diluted to 25 ml with 0.1 M sucrose, 45 mM Tris-HCl, pH 7.5, and immediately centrifuged (4000 *g*, 20 min). The sedimented pellet was resuspended in 0.1 M sucrose, 45 mM Tris-HCl, pH 7.5, to the original volume. Squalene-containing mitochondria prepared as described provided the substrate for epoxidase assay after addition of microsomes. For assay of squalene transfer to total microsomes prior to sucrose density gradient separation (**Table 1**), the amount of radioactive squalene was doubled (Table 1, Exp. 1a) and quadrupled for experiment 3a (Table 1). For Exp. 2a (Table 1) and the experiments shown in

TABLE 1. Effect of SPF on transfer of squalene from donor (mitochondria or trypsinized microsomes) to acceptor membranes (untreated microsomes or mitochondria)

Exp.	Squalene		Radioactivity (cpm) after Separation on Sucrose Gradient					Efficiency of Squalene Transfer[a]
	Donor	Acceptor	Microsomal Phase	Mitochondrial Phase	2,3-Oxido Squalene in Microsomal Phase		Total cpm Recovered	
					cpm	*nmol*		%
	Mitochondria	Total microsomes						
1a	6000 cpm (5×10^{-3} nmol)	+SPF[b]	1923	2781			5241	22
		−SPF	837	4091			5608	
1b	8200 cpm (1 nmol)	+SPF[c]	3649	2095	428	0.05	6735	30
		−SPF	1925	4238	78	<0.01	7944	
	Mitochondria	Smooth microsomes						
2a	1500 cpm (1.25×10^{-3} nmol)	+SPF[b]	630	400	180	0.15×10^{-3}	1500	33
		−SPF	130	610	35	$<0.02 \times 10^{-3}$	1460	
2b	8200 cpm (1 nmol)	+SPF[c]	3538	2325	510	0.06	6295	24
		−SPF	2088	3875	95	<0.01	6547	
	Mitochondria	Rough microsomes						
3a	12000 cpm (10^{-2} nmol)	+SPF[c]	4115	5143	826	0.68×10^{-3}	10573	26
		−SPF	1462	8391	166	$<0.1 \times 10^{-3}$	11399	
3b	8200 cpm (1 nmol)	+SPF[c]	3105	2170	450	0.06	5962	24
		−SPF	1863	3959	109	<0.01	6718	
	Trypsin-treated total microsomes	Mitochondria						
4	3200 cpm (2.6×10^{-3} nmol)	+SPF[c]	2060	560			2920	11
		−SPF	2500	230			3060	

[a] The efficiency of SPF in squalene transfer (last column) is calculated as difference between $\frac{\text{cpm associated with acceptor}}{\text{total cpm recovered}}$ in the presence and absence of SPF.

[b] SPF, acetone fraction, 2 mg of protein.

[c] Pure SPF, 18 μg of protein.

In experiments 1a, 2a, 3a, 0.8 mg of total microsomes, 0.24 mg of smooth, 0.26 mg of rough microsomes, as well as 0.18 mg of mitochondrial protein were used. For experiments 1b, 2b, 3b, 0.6 mg of microsomal and 0.4 mg of mitochondrial protein were used. In experiment 4, 0.24 mg of total microsomal and 0.4 mg of mitochondrial protein were used. The time of incubation prior to centrifugation was 30 min.

Fig. 4, 50 μl of squalene (1500 cpm) was used. In experiments 1b, 2b, and 3b (Table 1) and in the experiment shown in Fig. 2, the concentration of squalene incorporated into mitochondria was 2.5 nmol/mg of protein. Squalene was incorporated into plasma membranes as described for mitochondria. After squalene incorporation, the plasma membranes were washed twice by dilution to 12 ml with 0.1 M sucrose, 45 mM Tris-HCl, pH 7.5, and centrifugation at 100,000 *g* for 1 hr at 4°C. The concentration of incorporated squalene was 8 nmol/mg of plasma membranes.

Preparation of microsome-associated squalene

Squalene was incorporated into microsomes (0.5 ml, 8 mg of protein) as previously described (1) except that phosphatidylglycerol and $CaCl_2$ were omitted. The centrifugation of microsomes (washing) was performed at 105,000 *g* for 60 min. The concentration of incorporated squalene was 2.5 nmol/mg of protein (Fig. 1).

Trypsin treatment of microsomes

The preparation of trypsin-treated microsomes followed the procedure previously described (1).

Separation of microsomes and plasma membranes

For separation of plasma membranes from microsomes that had previously been incubated either in the presence or absence of SPF, the incubated mixture was layered on the top of 12.5 ml of linear sucrose density gradient (0.8 M to 1.6 M) and centrifuged at 38,000 rpm for 20 hr at 4°C in a Beckman SW 40 Rotor. Twenty-drop fractions were collected.

Separation of microsomes and mitochondria

Immediately after the squalene epoxidase assay, the mixture (0.5 ml) was layered on top of 4 ml of 1.17 M sucrose gradient, containing 45 mM Tris-HCl, pH 7.5, and centrifuged in 15-ml Corex tubes at 20,000 *g* for 30 min at 4°C. The upper phase (microsomes), pellet (mitochondria), and the particle-free intermediate layer were analyzed for radioactivity.

Squalene epoxidase assay

Unless otherwise indicated, the amounts of mitochondria and microsomes used for measuring squalene transfer and squalene epoxidase assay (1) were 0.4 mg and 0.6 mg of protein, respectively, and for plasma membranes and microsomes, 0.57 mg and 0.65 mg of protein, respectively.

Radioactivity determination

Radioactivity was measured as described previously (9). Protein estimations were done according to Lowry et al. (10) with bovine serum albumin as standard.

RESULTS

In **Fig. 1** are shown epoxidase activities of squalene-loaded total microsomes and of separated rough and smooth microsomal fractions. Both subfractions contain epoxidase and this activity is markedly stimulated by SPF as are total microsomes. The SPF stimulation observed for total loaded microsomes confirms previous results (1).

As a control for the experiments with mitochondria described below, the effect of mitochondria on microsomal squalene epoxidase activity was tested. In the absence of SPF, mitochondria inhibited enzyme activity slightly (14%) at all concentrations tested (up to 0.4 mg of protein). When the system also contained SPF, squalene opoxidase activity declined somewhat more (26% with 0.1 mg and 34% with 0.4 mg of mitochondrial protein). The more marked inhibition by mitochondria

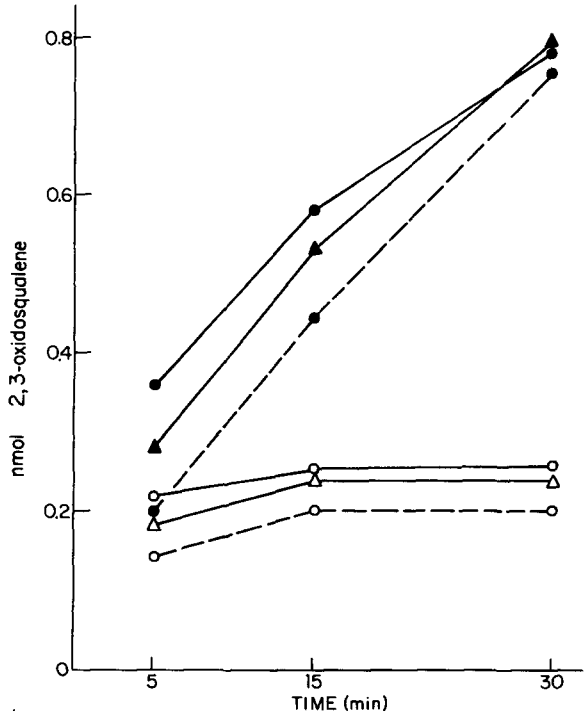

Fig. 1. Squalene epoxide formation by total (● – – – ●, ○ – – – ○), rough (● —— ●, ○ —— ○), and smooth (▲ —— ▲, △ —— △) microsomes. Closed symbols, in the presence of SPF (2 mg of acetone fraction); open symbols, absence of SPF. The microsomes and microsomal fractions (1 mg of protein) contained 2.5 nmol of [^{3}H]squalene incorporated prior to epoxidase assay.

in the presence of SPF may be due to SPF-promoted squalene transfer from squalene-loaded microsomes to squalene-free mitochondria, lowering the amount of squalene available for microsomal epoxidation. An experiment supporting this explanation will be described below.

When mitochondria or plasma membranes serve as squalene donors for microsomal epoxidase (intermembrane substrate transfer, **Fig. 2** and **Fig. 3**), epoxidation rates are more critically SPF-dependent than the rate of epoxidation in squalene-loaded microsomes (Fig. 1, intramembrane substrate transfer). A similar relationship, i.e., greater dependence of mixed membrane systems of SPF, had previously been observed when trypsinized squalene-containing microsomes served as squalene donors for squalene-free epoxidase-active normal microsomes (1, 2). If squalene translocation is the SPF-promoted event, then it is reasonable to assume that a transmembrane process (mitochondria → microsomes, plasma membrane → microsomes, or trypsinized microsomes → normal microsomes) will depend more critically on the translocating agent than an intramembrane process. Plasma membrane-associated squalene is more

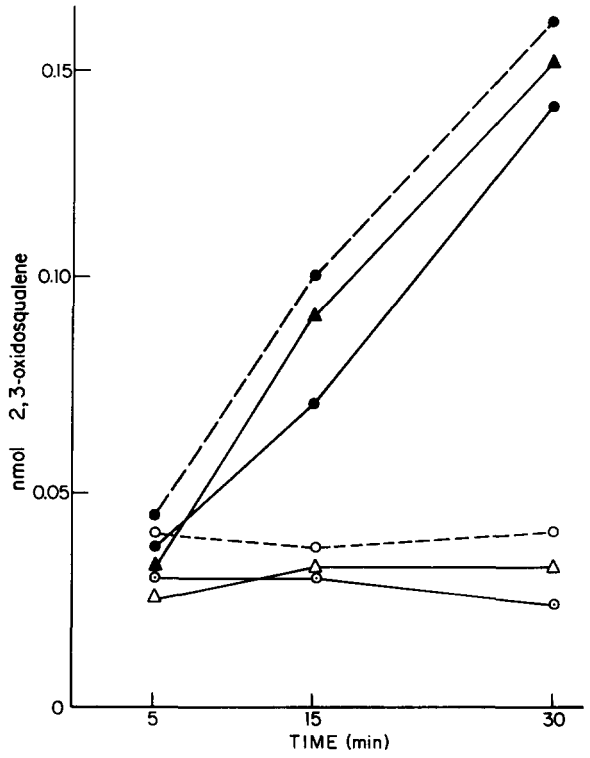

Fig. 2. Squalene transfer from mitochondria (0.4 mg of protein containing initially 1 nmol of [^{3}H]squalene) to *a*) total microsomes (● - - - ●, ○ - - - ○), *b*) rough microsomes (● —— ●, ○ —— ○), and *c*) smooth microsomes (▲ —— ▲, △ —— △). Closed symbols, in the presence of SPF (2 mg of acetone fraction); open symbols, absence of SPF. Microsomal protein in *a*), *b*), and *c*) was 0.6 mg.

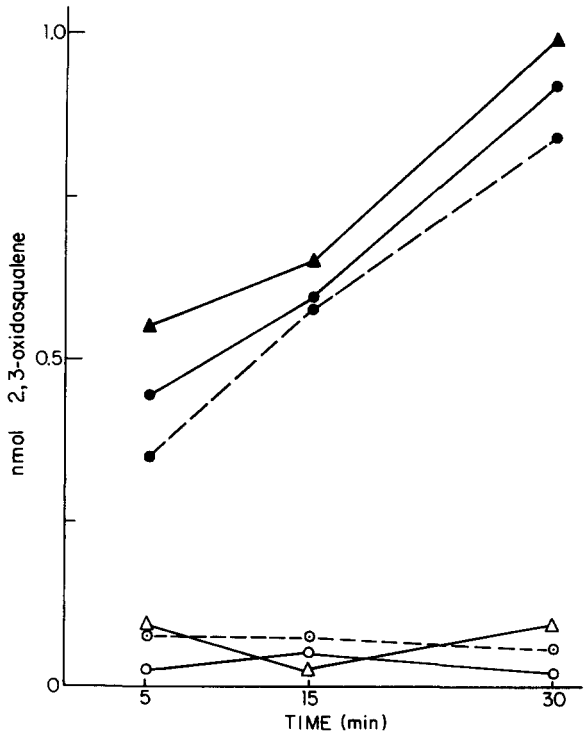

Fig. 3. Squalene transfer from plasma membranes (0.57 mg of protein containing initially 4.6 nmol of [^{3}H]squalene) to *a*) total microsomes (● - - - ●, ○ - - - ○), *b*)rough microsomes (● —— ●, ○ —— ○), and *c*) smooth microsomes (▲ —— ▲, △ —— △). Closed symbols, in the presence of SPF (acetone fraction, 2 mg); open symbols, absence of SPF. Microsomal protein in *a*), *b*), and *c*) was 0.65 mg.

rapidly transferred initially to microsomal epoxidase sites than is mitochondrial squalene (Figs. 2 and 3). It should be noted that neither mitochondria nor plasma membranes by themselves contain epoxidase activity.

Squalene epoxide production in the mixed system consisting of squalene-containing mitochondria or plasma membranes and epoxidase-containing but initially squalene-free microsomes, will depend on two sequential processes, transfer of substrate from the donor to the acceptor membrane followed by substrate translocation within the microsomal membrane, perhaps from a general pool to a pool more accessible to the active site. To test this hypothesis, mitochondria preloaded with squalene and unloaded total microsomes were preincubated for varying lengths of time, separated on a sucrose density gradient, and then analyzed for both total radioactivity and radioactive squalene epoxide. The preincubations were performed both in the presence and absence of SPF. The results are shown in **Fig. 4.** The microsomal phase is seen to contain substantially more unchanged squalene than squalene epoxide at all times. This indicates that the intermembrane squalene transfer is rapid by comparison with the rate of epoxidation.

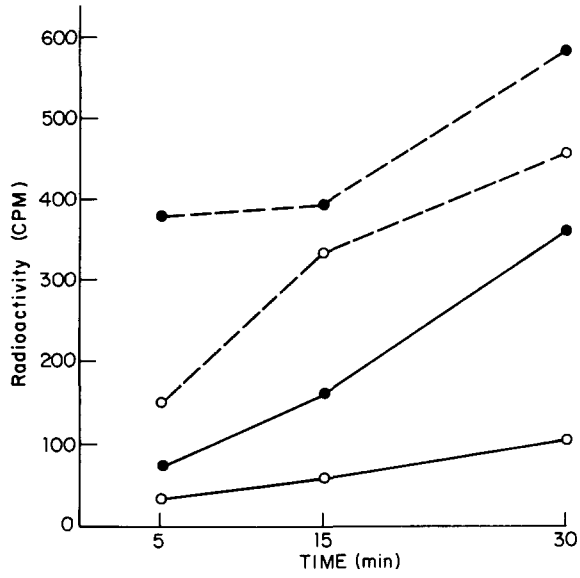

Fig. 4. Radioactivity in normal microsomes (0.8 mg protein) after incubation with [^{3}H]squalene containing mitochondria (0.18 mg of protein, 1.3×10^{-3} nmol of [^{3}H]squalene). ● – – – ●, ○ – – – ○, unchanged squalene in microsomes; squalene epoxide in microsomes, ● —— ●, ○ —— ○. Closed symbols, in the presence of SPF (acetone fraction, 2 mg); open symbols, absence of SPF. After incubation microsomes were separated from mitochondria by sucrose density gradient centrifugation as described in the Experimental Procedures.

Inter- and intramembrane squalene transfer cannot be experimentally distinguished but we believe the rate of the intermembrane substrate translocation to be the faster of the two for the following reason. In microsomes previously loaded with squalene (11) or containing endogenously generated squalene (12), the epoxidation rate is stimulated to the same degree as in assay systems containing exogenous squalene. This is most reasonably explained by an effect of SPF on the intramembrane distribution of squalene between two pools or compartments as stated above. This implies that, in the absence of SPF, the concentration of squalene at the epoxidase site is not saturating and hence that the replenishment of substrate at the active site is slow, SPF controlling this rate-limiting step.

A significant amount of squalene is found associated with the microsomal acceptor membrane even in the absence of SPF. This nonspecifically transferred squalene appears to be less accessible to the microsomal epoxidase site.

Additional data showing squalene transfer from mitochondria are given in Table 1. Squalene in the mitochondrial donor fraction decreases as the squalene content and squalene epoxide production in the microsomal phase increase. This exchange process requires SPF. Not only total microsomes but also the rough and smooth fractions serve as squalene acceptors.

In order to ascertain whether SPF also mediates the reverse process, squalene-loaded trypsin-treated microsomes served as donor and mitochondria as acceptor (Table 1, Exp. 4). Epoxidase activity in microsomes, tested directly after trypsin treatment, could not be detected. The two membranes were separated on a sucrose gradient and the radioactivity in mitochondria, microsomes, and in the intermediate fractions was determined. In this system the extent of squalene transfer was much lower (11%) than that observed for SPF-mediated transfer of squalene from mitochondria to microsomes (more than 22%) (Table 1, Exp. 1 and 5).

Fig. 5 shows the separation of squalene-loaded plasma membranes from total microsomes and microsomal subfractions by sucrose density gradient centrifugation. Total and rough microsomes both sedimented in a visible pellet after centrifugation and separated clearly from plasma membranes (Fig. 5A, B). Smooth microsomes did not sediment and were separated from plasma membranes on the gradient (Fig. 5C). In all three separations plasma membranes were almost completely depleted of squalene, while a significant amount of squalene appeared in microsomes when incubations were carried out in the presence of SPF, as was also observed for squalene transfer from mitochondria. SPF-mediated transfer of squalene from plasma membranes is nearly complete, which is not the case when mitochondria serve as the squalene donors (Table 1).

DISCUSSION

It is generally believed that the membrane-associated enzymes catalyzing the various steps in the hepatic conversion of squalene to cholesterol reside exclusively in the microsomal fraction but are absent from other organelles. For the purposes of the present experiments on intermembrane transfer, the specificity of this enzyme localization had to be verified. We find, indeed, that squalene epoxidase activity is not detectable either in mitochondria or plasma membranes. Whether this is true for both of the two components of the squalene epoxidase system (NADPH-cytochrome c reductase and epoxidase proper) (13) has not been investigated. It was also of interest to establish whether the "rough" and "smooth" components of microsomes both catalyze squalene epoxidation. Our experiments show that both microsomal subfractions are enzymatically active with no detectable differences in specific activity. If, as we have postulated, squalene epoxidase is nonrandomly distributed in the membrane space of the endoplasmic reticulum, association with ribosomes cannot be a determining factor.

As shown in this laboratory (1, 11, 14) and by others (12, 15, 16), squalene epoxidation is markedly stimu-

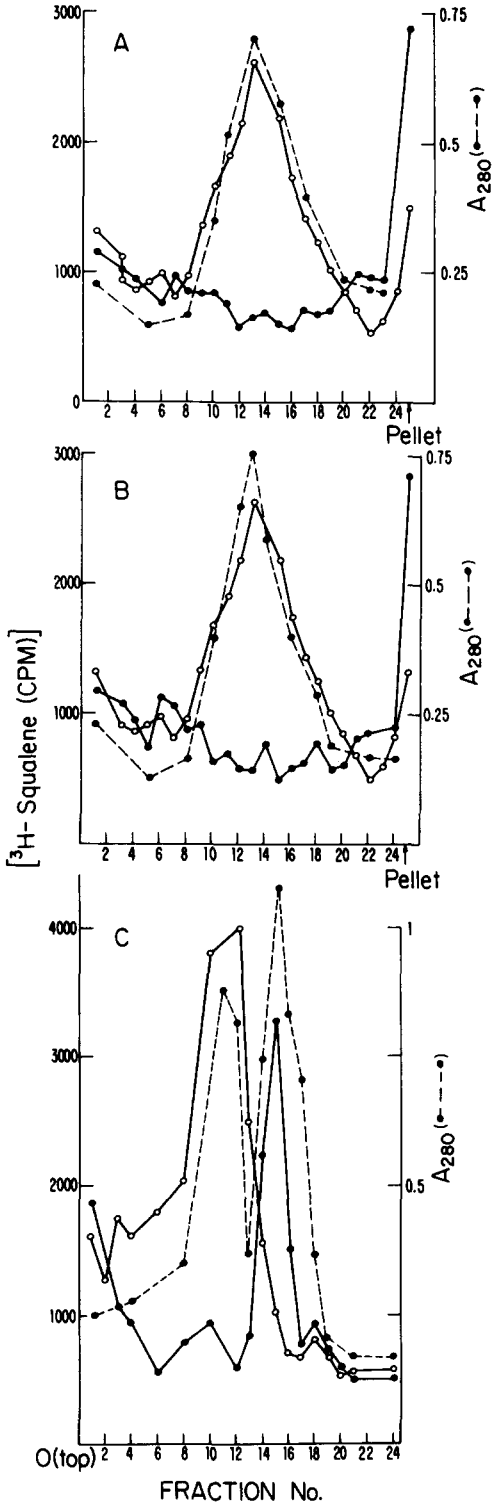

Fig. 5. Transfer of squalene from plasma membranes (0.65 mg of protein, 5.2 nmol of [^{3}H]squalene) to normal microsomes (1.4 mg of protein). The two membrane fractions were incubated either without or in the presence of SPF (10 μg pure protein) and the mixture was applied to a linear sucrose gradient (0.8–1.6 M). After centrifugation at 38,000 rpm for 20 hr at 4°C in a Beckman SW 40 Rotor, fractions were collected (20 drops per fraction) with the aid of Fluorinert. Panel A: ● —— ● [^{3}H]squalene distribution in the presence, and ○ —— ○ in the absence of SPF. Absorbance at 280 nm was monitored in a continuous flow Gilford spectrophotometer. The protein profile of microsomes and plasma membranes centrifuged separately was also checked. Panel B: [^{3}H]squalene distribution after incubation of squalene-containing plasma membranes with rough microsomes (0.84 mg of protein) and centrifugation. Panel C: same as in B with 0.84 mg of smooth microsomes.

lated by SPF whether the substrate is supplied exogenously or already incorporated into microsomes. This led to the working hypothesis that SPF regulates in some unknown manner the distribution of squalene internally in the membrane, e.g., between metabolically active and inactive pools. Recently we have demonstrated more directly that SPF actively promotes squalene transfer across the membranes of two separable microsomal membrane populations (2). The question therefore arose whether organelles other than endoplasmic reticulum can serve as squalene reservoirs for donating squalene to receptor microsomes containing squalene epoxidase. Mitochondria and plasma membranes were chosen for this purpose, especially because by themselves they fail to metabolize squalene to epoxide or by any other route. The results obtained show very clearly that both mitochondria and plasma membrane can serve in this capacity. As in the system consisting of trypsinized squalene-containing microsomes and normal microsomes (1, 2), squalene translocation is markedly stimulated in the presence of SPF. Clearly, the identity of the donor membrane is not an important factor in these transfer processes, at least qualitatively. This suggests that neither the presence of squalene epoxidase nor specific squalene binding sites in the donor membrane system is critical for translocation to the microsomal acceptor. The efficiencies of the various squalene donor systems in this transfer are difficult to quantitate because their capacity to incorporate squalene from aqueous media differs widely. Thus under identical conditions plasma membranes incorporate 16% and mitochondria incorporate 4% of exogenous squalene. It is to be expected that the fraction of squalene transferred to the receptor microsomes will be greater the higher the squalene concentration in the donor membranes. This expectation is borne out by the observation that during the same period, about 6 times as much squalene is transferred from plasma membranes than from mitochondria.

The presence or absence of active squalene epoxidase also influences the efficiency of intermembrane squalene transfer. We have previously shown (2) that the extent of squalene transfer from normal to trypsin-treated microsomes (containing inactive epoxidase) is significantly lower (20%) than in the reverse direction. Similarly, we now find that the fraction of squalene transferred from trypsin-treated microsomes to mitochondria is the least efficient of all transfer processes

studied (Table 1, Exp. 4).[2] First, as previously noted, the capacity of mitochondria to incorporate exogenous squalene is relatively limited. A further explanation for these differences is that in acceptor membranes containing epoxidase the translocated squalene will be metabolized, at least in part, making more squalene storage sites available. The data shown in Fig. 4 are consistent with this interpretation. The acceptor membranes contain nearly twice as much squalene as squalene epoxide. The same data show that squalene epoxidation is slower than squalene transfer.

The present data extend our previous findings by showing that SPF promotes squalene translocation between a variety of separable membrane systems. The transfer process has some specificity but this specificity is not absolute. However, the underlying mechanism, i.e., the manner in which SPF, a cytoplasmic protein, induces the movement of a membrane-associated molecule from one site to another both within and across membranes, remains unknown.

The present experiments were undertaken for further clarification of the mechanism of SPF action. The results obtained with mitochondria and plasma membranes are not meant to imply that these organelles are involved in hepatic squalene transport under physiological conditions.

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[2] We have neglected the potential presence of nonradioactive squalene in membrane fractions which has been estimated by a colorimetric procedure to be as high as 0.8 nmol per mg of microsome protein (15). Therefore our data for efficiencies of squalene transfer (Table 1) calculated from squalene-associated radioactivities are not necessarily equivalent to mass transfer of squalene.