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Regulation of bile-acid synthesis

Role of sterol carrier protein$_2$ in the biosynthesis of 7α-hydroxycholesterol

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Sterol carrier protein$_2$ (SCP$_2$) is known to stimulate utilization of cholesterol in enzymic reactions in which cholesterol is the substrate. Substantial recent experimental evidence indicates that SCP$_2$: (i) activates enzymic conversion of intermediates between lanosterol and cholesterol; (ii) stimulates the microsomal conversion of cholesterol into cholesterol ester in rat liver; and (iii) enhances mitochondrial utilization of cholesterol for pregnenolone formation in the adrenals. The conversion of cholesterol into 7α-hydroxycholesterol is the rate-limiting step in bile-acid synthesis. We therefore investigated the effect of SCP$_2$ on this physiologically critical reaction by using a gas-chromatography-mass-spectrometry procedure that measures the mass of 7α-hydroxycholesterol formed. The results show that SCP$_2$ enhances 7α-hydroxycholesterol formation by rat liver microsomes (microsomal fractions), utilizing either endogenous membrane cholesterol, cholesterol supplied exogenously in serum or in the form of cholesterol/phospholipid liposomes. Microsomes immunotitrated with anti-SCP$_2$ antibody exhibited considerably less capacity to synthesize 7α-hydroxycholesterol, which was restored to control levels on addition of purified SCP$_2$. These data are consistent with the suggestion that SCP$_2$ may be of physiological significance in the overall metabolism of cholesterol.

Soluble cytosolic proteins have been described that affect cholesterol hydroxylation, although the physiological significance of their role is obscure. Thus Mitton *et al.* (1971) have reported a stimulating effect of 100000*g* rat liver supernatant on microsomal 7α-hydroxycholesterol biosynthesis. On the other hand, Shefer *et al.* (1968) have found this effect to be negative. Spence & Gaylor (1977) have observed that a partially fractionated cytosolic material had the ability to stimulate cholesterol hydroxylation.

Scallen *et al.* (1974) and Johnson & Shah (1974) demonstrated the presence of two proteins in the rat liver soluble fraction that were required for the microsomal conversion of squalene into cholesterol. In later studies, Srikantaiah *et al.* (1976) purified the first of these two proteins, sterol carrier protein$_1$ (SCP$_1$), and showed its requirement in the enzymic conversion of squalene into lanosterol by rat liver microsomal membranes. SCP$_1$ is identical with the supernatant protein factor purified by Ferguson & Bloch (1977). The second of the two proteins, termed 'sterol carrier protein$_2$', was recently purified by Noland *et al.* (1980), and it was shown to activate enzymic conversions of intermediates between lanosterol and cholesterol by liver microsomal membranes. Further studies on the role of SCP$_2$ in reactions that utilize cholesterol as substrate have revealed that: (i) it stimulates utilization of cholesterol, both endogenous and exogenous, in the microsomal conversion of cholesterol into cholesterol ester catalysed by acyl-CoA: cholesterol acyltransferase (Gavey *et al.*, 1981); and (ii) it stimulates transfer of cholesterol from adrenal lipid droplets to mitochondria and mitochondrial utilization of cholesterol for pregnenolone formation (Chanderbhan *et al.*, 1982).

Abbreviation used: SCP, sterol carrier protein.

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The most recent findings of experiments with SCP_2, using either a reconstituted adrenal or rat liver system, show that all cholesterol transport and activation of the conversion of 7-dehydrocholesterol into cholesterol is abolished by immunotitration of the respective cytosols with anti-SCP_2 IgG (Vahouny *et al.*, 1983).

In the present study we provide evidence (i) that a pure, homogeneous preparation of SCP_2 stimulates microsomal 7α-hydroxycholesterol biosynthesis, and (ii) that SCP_2 is effective in promoting cholesterol hydroxylation from both endogenous microsomal cholesterol and cholesterol supplied exogenously in the form of whole serum or as cholesterol/phospholipid liposomes.

Experimental procedures

Materials

Antisera to SCP_2 were prepared in rabbits and characterized by immunoelectrophoresis (Vahouny *et al.*, 1983). Homogenous SCP_2 was prepared as described previously (Noland *et al.*, 1980). Bovine serum albumin (fraction IV, essentially fatty-acid-free) was from Sigma. Asolectin was purchased from Associated Concentrates, Woodside, NY, U.S.A. Sprague–Dawley male rats (150–220g) used in these studies were kept in windowless quarters and under a strict 12h reversed light/dark cycle (15.00–03.00h light). Purina Rat Chow and water were allowed *ad libitum*. All chemicals were of highest purity grade available and were obtained from Fisher.

Liposomes

Cholesterol/phospholipid liposomes were prepared as described by Feo *et al.* (1978) and Radominska-Pyrek *et al.* (1978). A 9:1 (w/w) mixture of asolectin/cholesterol was prepared in chloroform. The solvent was evaporated under N_2 and 1.0ml of water per 30mg of lipid was added. The mixture was vortex-mixed at 42°C for 15min under N_2, sonicated (Sonifer; setting 6) for 30min in ice and was centrifuged at 134000*g* to remove unincorporated components, which sedimented in the form of a pellet. For some experiments, liposomes were further purified by column chromatography on Sepharose 4B, from which they were eluted as a single peak. Lipid phosphorus of asolectin and of liposomes was measured by the method of Bartlett (1959).

7α-Hydroxycholesterol

Rat liver microsomes (microsomal fractions) were prepared as described previously (Sanghvi *et al.*, 1981). Microsomes with and without SCP_2 were preincubated in phosphate buffer in the absence of cofactors, as described in legends to the Figures and Tables. Incubation, after the addition of cofactors, was for various periods as noted in Tables and Figures. 7α-Hydroxycholesterol production was measured by a g.c.–m.s. method (Hewlett–Packard 5986A mass spectrometer) under conditions described previously (Sanghvi *et al.*, 1978, 1981). Enzyme activity was determined during the period when 7α-hydroxycholesterol production is a linear function of both the time of incubation, as well as the microsomal protein concentration. In some experiments, only the final amount of 7α-hydroxycholesterol formed at the end of a 60 or 90min incubation was measured when the reaction is well past the linear phase. The acetone-dried powder of microsomes was prepared as described by Scallen *et al.* (1968). Microsomal protein was determined by the method of Bradford (1976). The microsomal protein in different assays varied between 0.3 and 0.5mg. The total volume of each incubation flask was 700μl.

Each experiment was performed utilizing combined microsomes obtained from the livers of at least four rats. All assays were performed at least in duplicate. The analytical variation in the measurement of 7α-hydroxycholesterol was less than 5%.

Results and discussion

Previous reports (Mitton *et al.*, 1971; Spence & Gaylor, 1977) have demonstrated the ability of 100000*g* rat liver supernatant, either intact or fractionated, to stimulate microsomal hydroxylation of cholesterol. SCP_2, isolated from rat liver cytosol and purified to homogeneity (Noland *et al.*, 1980), is shown to stimulate cholesterol transport from adrenal lipid droplets to mitochondria and to stimulate the utilization of cholesterol in enzymic reactions in which cholesterol is the substrate (Gavey *et al.*, 1981). This information provided the basis for the present work on the role of SCP_2 in microsomal hydroxylation of cholesterol. In preliminary experiments, conducted in collaboration with the laboratory of Dr. G. S. Boyd (now deceased), University of Edinburgh Medical School, a concentration-dependent increase in the microsomal conversion of [4-^{14}C]cholesterol to labelled 7α-hydroxycholesterol by SCP_2 was observed (B. J. Noland, I. F. Craig, M. E. Lawson, T. J. Scallen & G. S. Boyd, unpublished work). Confirmation of these previous observations by more detailed experiments is reported here.

In order to observe an SCP_2 effect on 7α-hydroxylase, we found that it was necessary to preincubate SCP_2 with microsomes in the absence of cofactors. The greatest increase in SCP_2-stimulated 7α-hydroxycholesterol production occurs in the first 30min of preincubation, with little further increase up to 180min (Fig. 1). Therefore

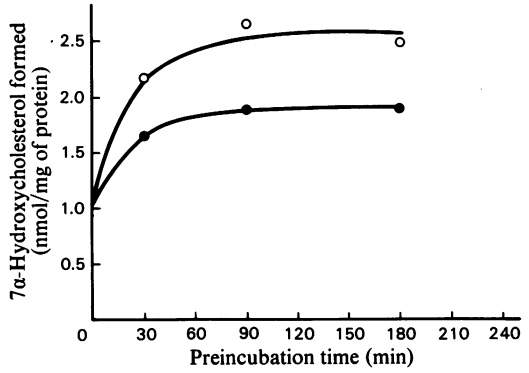

Fig. 1. *Effect of preincubation of SCP_2 and microsomes on 7α-hydroxycholesterol formation*
Microsomes with (○) and without (●) SCP_2 were preincubated at 37°C in the absence of cofactors for various times. The final amount of 7α-hydroxycholesterol formed at the end of a 60min incubation with cofactors was then measured.

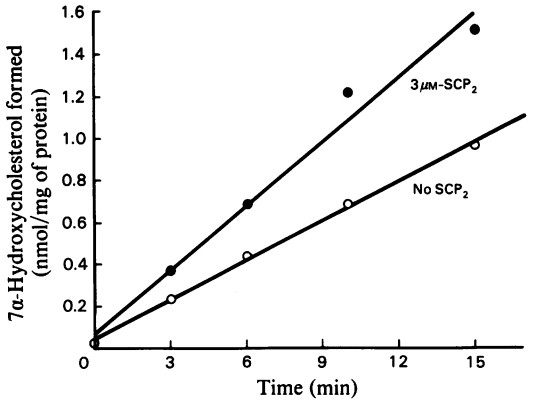

Fig. 2. *Stimulation of cholesterol 7α-hydroxylase activity by SCP_2*
Microsomes were preincubated with (●) and without SCP_2 (○) at 37°C for 60min. Cofactors were then added and enzyme activity was measured during the linear phase.

all subsequent experiments were performed with a 30 or 60min preincubation time. The effects of SCP_2 were studied during both the early, linear phase of the reaction ('enzyme activity') and during the late, stable, phase of the reaction ('final amount formed').

Stimulation of microsomal cholesterol 7α-hydroxylase activity by SCP_2 is shown in Fig. 2, where the presence of SCP_2 in the reaction almost doubles the enzyme activity from 63 to 103pmol/min per mg of protein. When rat liver microsomes were incubated for 90min in the presence of SCP_2, there was a significant increase in the final amount of 7α-hydroxycholesterol produced. If similar incubations were carried out in the presence of increasing amounts of SCP_2, there was, at first, a linear increase in the final amount of 7α-hydroxycholesterol produced. When the SCP_2 concentration was raised above 4μM, this increase was no longer linear and in fact, stabilized at an SCP_2 concentration of 8μM. Thus the data in Fig. 3 give the appearance of a saturation plot. In Fig. 4 the amount of SCP_2 in the incubations is expressed in relation to the amount of microsomal protein and, again, the increase in the production of hydroxylated sterol is proportional to the amount of SCP_2. These data collectively show that SCP_2 was capable of accelerating 7α-hydroxylase activity and increasing the final amount of hydroxycholesterol formed, when endogenous microsomal cholesterol was utilized.

The next series of experiments was carried out to see whether SCP_2 is similarly effective when cholesterol is supplied by an exogenous source, such as cholesterol-containing phospholipid liposomes. As shown in Fig. 5, phospholipid/cholesterol liposomes *alone* bring about an increase in both microsomal 7α-hydroxylase activity and the final amount of hydroxycholesterol formed. The addition of SCP_2 under these conditions has no effect on enzyme activity, but it does markedly increase the final amount of hydroxylated sterol produced at the end of 90min incubation. Moreover, the results in Table 1 show that when phospholipid/cholesterol liposomes serve as an additional source of substrate cholesterol, the increase in the final amount of 7α-hydroxycholesterol formed depends on the amount of SCP_2.

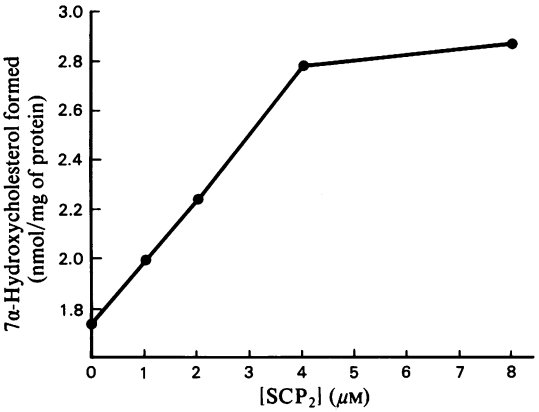

Fig. 3. *Enhanced 7α-hydroxycholesterol production with increasing amounts of SCP_2*
Microsomes were preincubated with SCP_2 (0–8μM) at 37°C for 60min. 7α-Hydroxycholesterol formation was then monitored at the end of 90min incubation with cofactors.

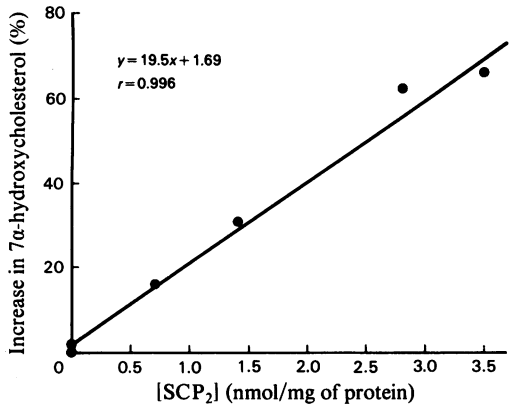

Fig. 4. *Enhanced 7α-hydroxycholesterol production as a function of the SCP_2/protein ratio*
Microsomes were preincubated with SCP_2 (0–8 μM) at 37°C for 60min. 7α-Hydroxycholesterol formation was then monitored at the end of 90min incubation with cofactors. The percentage increase compared with the control is presented.

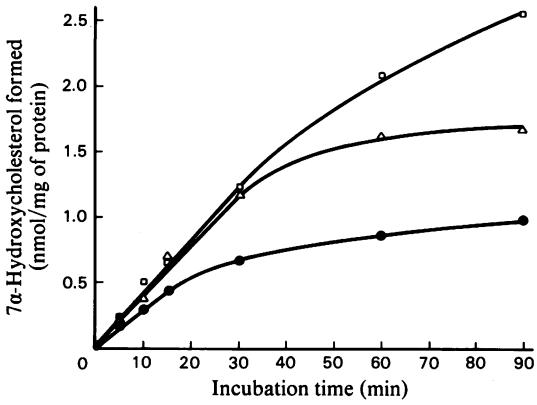

Fig. 5. *Effect of SCP_2 and liposomes on 7α-hydroxycholesterol formation*
After a 60min preincubation of microsomes (●) at 37°C with SCP_2 (1.5 μM) and asolectin/cholesterol liposomes (□) or with liposomes alone (△), the 7α-hydroxycholesterol formation at indicated times was monitored. The basal levels of 7α-hydroxycholesterol in boiled controls of respective preparations are subtracted from all values.

Recently we have demonstrated (Sanghvi *et al.*, 1984) that an acetone-dried-powder preparation of rat liver microsomes, which is completely devoid of non-esterified cholesterol, exhibits no 7α-hydroxylase activity unless an exogenous source of substrate cholesterol is provided. Results in Table 2 indicate that SCP_2 brings about an increase in acetone-dried-powder 7α-hydroxylase activity, when either liposomes or serum is the donor of substrate cholesterol. Again, the change in enzyme activity is proportional to the amount of SCP_2 added. Thus SCP_2 behaves similarly towards both the acetone-dried powder and intact microsomes.

Table 1. *Effect of different concentrations of SCP_2 on 7α-hydroxycholesterol production in microsomes with and without liposomes*
Microsomes with and without liposomes and/or SCP_2 were preincubated at 37°C for 30min before the addition of cofactors. 7α-Hydroxycholesterol formed at the end of 90min of incubation with cofactors was measured. The cholesterol content of the liposomes was 40 μg.

	7α-Hydroxycholesterol (pmol/mg of protein)	
	−Liposomes	+Liposomes
Microsomes	1733	2400
+SCP_2 (2 μM)	2244	2800
+SCP_2 (4 μM)	2800	3180

Table 2. *Effect of SCP_2 on cholesterol 7α-hydroxylase activity in microsomal acetone-dried powder*
Microsomal acetone-dried powder was preincubated at 37°C with liposomes or serum without cofactors for 30min. After the addition of cofactors, cholesterol 7α-hydroxylase activity was measured over a period of 10min during the linear phase. Serum and liposomes contained 15 and 12 μg respectively of non-esterified cholesterol.

Experiment	Cholesterol 7α-hydroxylase activity (pmol/min per mg of protein)
(*a*) Microsome powder	
+liposomes	10
+SCP_2 (5 μg)	18
(*b*) Microsome powder	
+serum	12
+SCP_2 (23 μg)	22
+SCP_2 (46 μg)	32

The specificity of SCP_2 in stimulating cholesterol 7α-hydroxylase was assessed in an experiment in which we compared its effects to that of albumin and fatty-acid-binding protein. The results in Fig. 6 show that, whereas SCP_2 increases 7α-hydroxycholesterol production, equimolar albumin and fatty-acid-binding protein are without effect.

Since SCP_2 is derived from rat liver cytosol, we reasoned that treatment of rat liver cytosol with anti-SCP_2 IgG should compromise the 7α-hydroxylase-stimulating ability of cytosol. In a number of experiments carried out to investigate this phenomenon, we observed that immunotitration of cytosol by anti-SCP_2 IgG did not produce a significant decrease in the ability of cytosol to

enhance 7α-hydroxycholesterol synthesis. In an attempt to confirm this finding, we treated rat liver cytosol with anti-SCP_2 IgG and then tested its ability to affect adrenal mitochondrial pregnenolone production. Results in Table 3(*a*) show that cytosol treated with anti-SCP_2 has indeed lost its ability to enhance pregnenolone production by adrenal mitochondria, whereas the native cytosol, or cytosol treated with non-immune IgG, retains this ability. These same fractions were then tested for their effect on 7α-hydroxycholesterol synthesis. Again, consistent with our previous observations, the anti-SCP_2-treated cytosol showed virtually the same ability to stimulate 7α-hydroxycholesterol formation as did the cytosol treated with non-immune IgG (Table 3*b*). These results suggest that rat liver cytosol contains protein(s) or non-protein components besides SCP_2 with the ability to enhance 7α-hydroxylation of cholesterol. This observation is consistent with reports suggesting the presence in rat liver cytosol of such factors (Craig *et al.*, 1979; Danielsson *et al.*, 1980, 1984).

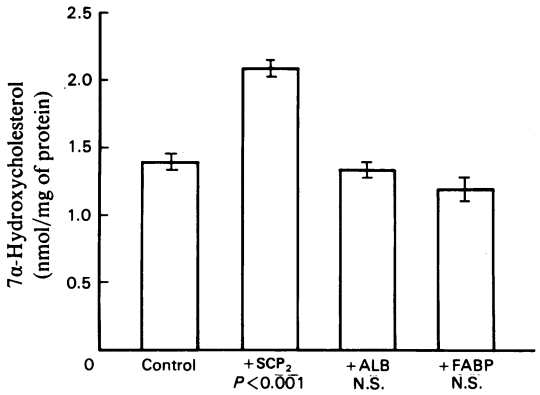

Fig. 6. *Specificity of SCP_2 in stimulating microsmal 7α-hydroxycholesterol synthesis*

Microsomes were preincubated at 37°C for 60 min in the absence of cofactors with SCP_2 (2 μM), albumin (ALB, 4 μM), and fatty-acid-binding protein (FABP, 2 μM). The final amount of 7α-hydroxycholesterol formed at the end of a 90 min incubation with cofactors was then measured. The data are means ± S.D., $n = 4$. The significance values are with respect to control (N.S., not significant).

The presence of endogenous SCP_2 in microsomes was ascertained by direct titration of microsomes with anti-SCP_2 IgG. Fig. 7 shows that immunotitrated microsomes form progressively less 7α-hydroxycholesterol relative to microsomes treated with non-immune IgG or untreated microsomes. Importantly, when SCP_2 is added to immunotitrated microsomes they are able to form 7α-hydroxycholesterol at levels comparable with those in microsomes treated with non-immune IgG (Table 4). This series of experiments would seem to

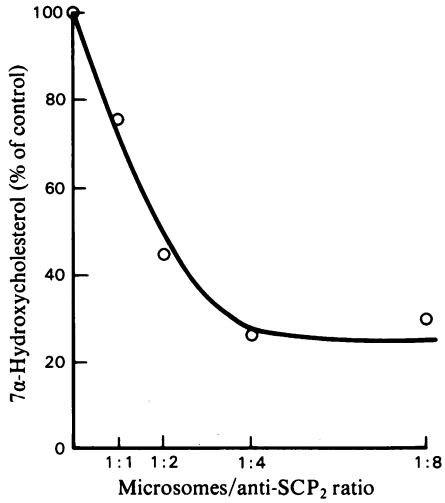

Fig. 7. *Immunotitration of microsomal 7α-hydroxycholesterol synthesis by anti-SCP_2 IgG*

Indicated ratios of microsomes and anti-SCP_2 IgG were incubated at 25°C for 1 h. They were then left overnight at 4°C. The tubes were shaken very gently and centrifuged at 2200 rev./min. (Beckman Model TJ-6 refrigerated centrifuge) for 30 min. The supernatant was decanted, leaving behind a pellet. Microsomes, recovered after centrifugation of supernatant for 20 min at 300000*g*, were preincubated for 60 min at 37°C and 7α-hydroxycholesterol formation was measured at the end of a 90 min incubation with cofactors. The control 7α-hydroxycholesterol levels in untreated microsomes, or in microsomes treated with nonimmune IgG, were 2653 and 2651 pmol/mg of protein respectively.

Table 3. *Effect of anti-SCP_2-treated liver cytosol on (b) 7α-hydroxycholesterol and (a) pregnenolone synthesis*

Microsomes with and without additions were preincubated at 37°C for 30 min. 7α-Hydroxycholesterol produced at the end of a 90 min incubation with cofactors was then measured (*b*).

Expt.	(*a*) Pregnenolone (ng/mg of adrenal mitochondrial protein)	(*b*) 7α-Hydroxycholesterol (pmol/mg of microsomal protein)
Control	50.4	548
+Cytosol	153.0	2311
+Cytosol+$IgG_{non\text{-}immune}$	146.0	1932
+Cytosol+IgG_{immune}	53.0	1830

Table 4. *Effect of SCP_2 on 7α-hydroxycholesterol synthesis in microsomes treated with anti-SCP_2 IgG*
Conditions for the experiments were the same as those in Fig. 7.

Expt.	7α-Hydroxycholesterol produced (pmol/mg of protein)
(*a*) Microsomes (M)	1739
+ Non-immune IgG (M-IgG_c)	1499
+ Immune IgG (M-IgG_i)	930
(*b*) M-IgG_i + SCP_2 (8 μM)	1444

confirm the presence of SCP_2 in microsomes and indicate a possible role for it in 7α-hydroxycholesterol biosynthesis.

In all our experiments we have noticed that the magnitude of stimulation of 7α-hydroxylation of cholesterol brought about by SCP_2 is typically a doubling, relative to controls. This is in contrast with changes of much higher magnitude observed by Gavey *et al.* (1981) with respect to the stimulation of cholesterol ester formation and by Chanderbhan *et al.* (1982) with respect to stimulation of transfer of cholesterol from adrenal lipid droplets to mitochondria and mitochondrial utilization of cholesterol for pregnenolone formation by SCP_2. It is not clear, on the basis of available data, why such a difference should exist. It may be that microsomes have greater amounts of SCP_2 than mitochondria and may, therefore, have been prestimulated with respect to 7α-hydroxylase. At present, it is not possible to measure directly the endogeneous levels of SCP_2. Secondly, it is also likely that SCP_2-sensitive enzymes are differentially affected by SCP_2. This possibility may be explored in experiments with highly purified enzyme preparations. Unfortunately, this again is not possible at the present time.

In summary, the results presented here demonstrate that SCP_2 can enhance 7α-hydroxycholesterol formation by rat liver microsomes under various experimental conditions. It is important to note that SCP_2 is able to accomplish this regardless of the source of substrate cholesterol. These observations are compatible with the data of Gavey *et al.* (1981) and Chanderbhan *et al.* (1982), which show that SCP_2 promotes utilization of cholesterol by rat liver microsomes in the formation of cholesterol esters catalysed by acyl-CoA : cholesterol acyltransferase and the formation of pregnenolone by the adrenal mitochondria. In these instances there is evidence suggesting that SCP_2 serves as the specific intracellular transport system that delivers either endogenous or exogenous cholesterol to the respective enzymes that further metabolize it.

We do not as yet know the mechanism by which SCP_2 appears to promote cholesterol utilization in the formation of 7α-hydroxycholesterol. It might function to improve the accessibility, as well as availability, of substrate cholesterol and, in addition, play some role in the removal of the product, 7α-hydroxycholesterol.

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