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CHARACTERIZATION OF CYTOCHROME *P*-450$_{scc}$-CONTAINING LIPOSOMES

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Purified cytochrome P_{450scc} from bovine adrenocortical mitochondria was incorporated into liposomes by the cholate-dilution method utilizing either dialysis or Sephadex gel filtration. Among synthetic phospholipids tested, dioleoylglycerophosphocholine showed the best stability during the incorporation of P_{450scc} into liposomes. A maximum amount of heme was incorporated into liposomes at a molar ratio of phospholipid to the cytochrome of approx. 200. When P_{450scc} was incorporated into the dioleoylglycerophosphocholine liposomes by the cholate-filtration method, the P_{450scc}-containing liposomes showed two major populations on the elution pattern of the Sepharose 4B gel filtration, and were seen at a diameter of 200–600 Å and its aggregated forms. When the cytochrome was incorporated into dioleoylglycerophosphocholine liposomes or cholesterol-free adrenocortical mitochondrial liposomes, P_{450scc} was less stable than P_{450scc} in aqueous solution. Cholesterol or adrenodoxin markedly stabilized the liposomal P_{450scc}. Liposomal P_{450scc} required cholesterol for its optimum reduction with adrenodoxin, adrenodoxin reductase, and NADPH in the presence of CO. About 70% of the total heme in the dioleoylglycerophosphocholine liposomes was reduced by the enzymatic reduction in the presence of cholesterol, indicating that 70% of the total molecules are exposed to the surface of the outer monolayer. In order to see the location of the heme in membrane, the dioleoylglycerophosphocholine-liposomal P_{450scc} was subjected to *p*-chloromercuriphenyl sulfonic acid treatment. This reagent destroyed the liposomal P_{450scc}. These results suggest that the heme is located in the proximity of the *p*-chloromercuriphenyl sulfonic acid reacting sites which are exposed to the surface, or located on the vincinity of polar heads of the membrane.

Introduction

The activity of cholesterol side chain cleavage reactions of adrenocortical mitochondria is regulated under the influence of ACTH [1–3]. The topological studies of the enzyme system in the mitochondria revealed that P_{450scc} is located in the matrix side of

Abbreviations and trivial names used ACTH, adrenocorticotropic hormone, cholesterol, cholest-5-en-3β-ol, Emulgen 913, polyoxyethylenenonylphenolether, deoxycorticosterone, 20-hydroxy-4-pregnen-3,20-dione; DLPC, dilauroyl-L-α-glycerophosphocholine, DLPE, dilauroyl-L-α-glycerophosphoethanolamine, DMPC, dimyristoyl-L-α-glycerophosphocholine, DOPC, dioleoyl-L-α-glycerophosphocholine, DPPC, dipalmitoyl-L-α-glycerophosphocholine, DPPE, dipalmitoyl-L-α-glycerophosphoethanolamine, DSPC, distearoyl-L-α-glycerophosphocholine, Hepes, 4-(2-hydroxyethyl)-1-piperazine-2-ethanesulfonic acid, 20*S*-hydroxycholesterol, cholest-5-en-3β, 20*S*-diol, 22*R*-hydroxycholesterol, cholest-5-en-3β, 22*R*-diol, P_{450}, cytochrome *P*-450, P_{450scc}, adrenal mitochondrial cytochrome *P*-450 which functions in the cholesterol side chain cleavage reaction, P_{420}, cytochrome *P*-420, PCMS, *p*-chloromercuriphenyl sulfonic acid, pregnenolone, pregn-5-en-3β-ol-20-one; progesterone, pregn-5-en-3,20-diol, SDS, sodium dodecyl sulfate.

the inner membrane [4–6]. The substrate cholesterol for this NADPH-dependent hydroxylation reaction is believed to be supplied to P_{450scc} through the cholesterol-containing inner membrane. The current hypothesis of the ACTH action on adrenocortical cells strongly suggests that through the sequential activation of adenyl cyclase [7], cyclic AMP-dependent protein kinase [8], and ribosomal labile protein synthesis [9], the availability of cholesterol to P_{450scc} from an as yet undefined cholesterol pool has a final crucial part in the regulatory mechanisms of the overall steroidogenic reactions [1–3].

In this context, the study of the interaction between cholesterol and P_{450scc} in membranes will provide a much better understanding of the molecular function of ACTH stimulus on the subcellular organelles. Previous attempts to incorporate P_{450scc} into artificial vesicles have been reported in recent years [10–13]. However, the topology of P_{450scc} in the reconstituted membranes is poorly understood at the present time. In this study, we have prepared the P_{450scc}-containing liposomes from purified P_{450scc} and various phospholipids First, we examined the stability of P_{450scc} in the phospholipid environments, and secondly the best conditions for the incorporation of P_{450scc} into membranes. Then, we investigated the reducibility of membrane-imbedded P_{450scc} and the topology of the cytochrome molecule in membranes. From these studies, we wish to gain more insight into cholesterol-P_{450scc} interactions in membranes. Preliminary accounts of this study were published elsewhere [14,15]. Related lines of our work in this area have been published previously [16–19]

Materials and Methods

Materials. Cholesterol, PCMS, DEAE-cellulose, dithiothreitol, DLPC, DLPE, DMPC, DOPC, DPPC, DSPC, phosphatidylcholine (type V, egg yolk), digitonin, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV, yeast), Hepes, $NADP^+$, NADH, Sepharose 4B-200, sodium cholate, trypsin inhibitor (type 1-S, soybean), peroxidase (type VI, horseradish), cholesterol oxidase (*Nocardia erythropolis*), cholesterol esterase (bovine pancreas) and 3β-hydroxysteroid dehydrogenase (grade III, *Pseudomonas testerosteroni*) were purchased from Sigma. EDTA was from Aldrich; trypsin from Worthington. Emulgen 913 was a kind gift from Kao-Atlas Other reagents were obtained from the best commercial sources.

Purification of cytochrome P_{450scc}, adrenodoxin, and adrenodoxin reductase. P_{450scc} was purified from bovine adrenocortical mitochondria with some modifications of previous procedures [17]. All procedures were carried out at 4°C. P_{450scc} was extracted from sonicated mitochondria with a cholate solution (cholate/protein ratio of 1.0, w/w). After centrifugation at $105\,000 \times g$ for 60 min, the supernatant solution was subject to ammonium sulfate fractionation, and the precipitate at 32–45% ammonium sulfate saturation was dissolved in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/20% (v/v) glycerol, and dialyzed against the same buffer overnight. The dialyzed solution was applied to an aniline-Sepharose 4B column, which was equilibrated previously with the same buffer without glycerol. After washing the column with the buffer and then with the buffer containing 0.2 m KCl, P_{450scc} was eluted with the buffer containing 0.45 M KCl/0.3% sodium cholate. The fractions, which had a high A_{393}, were pooled and subject to the ammonium sulfate fractionation (32–45% saturation). After dialysis against 10 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA/0 1 mM dithiothreitol/20% glycerol, the solution was applied to a cholesterol-Sepharose 4B column, which was equilibrated previously with the 10 mM potassium phosphate buffer, pH 7.4. After washing the column with the buffer containing 0.4 M KCl, the cytochrome was eluted with the buffer containing 0 04% Emulgen 913. The eluted fraction was subject to the poly(ethylene) glycol precipitation (13%, w/v) to remove the detergent, and the pellet was dissolved in the 10 mM potassium phosphate buffer, pH 7 4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/20% glycerol. The dialyzed solution was then applied to a DEAE-cellulose column, which was equilibrated previously with 2 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA/ 0.1 mM dithiothreitol/20% glycerol After washing the column extensively with the buffer to remove residual Emulgen 913, P_{450scc} was eluted with the 50 mM potassium phosphate buffer containing 0.3 mM KCl. The eluted solution was dialyzed against 50

mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/20% glycerol. Final preparation showed a single band by SDS-polyacrylamide gel electrophoresis. The sample contained 1–2 nmol free cholesterol/nmol P_{450scc} and 1–2 nmol esterified cholesterol/nmol P_{450scc} and contained 10–15 nmol heme/mg protein. The heme content in sample was determined by the pyridine hemochromogen method [20]. Steroid-free P_{450scc} was made by the addition of adrenodoxin, adrenodoxin reductase, NADPH, 3β-hydroxysteroid dehydrogenase, and NAD^+ to the cholesterol-bound P_{450scc} as described before [18].

Adrenodoxin and adrenodoxin reductase were purified according to the method described before [21–23]. Aniline-Sepharose 4B and cholesterol-Sepharose 4B were prepared as described previously [17,24].

Preparation of cytochrome P_{450scc}-containing liposomes. The standard Hepes buffer used in this study consisted of 50 mM Hepes buffer, pH 7.4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/0.1 M NaCl. We have used two different methods for the preparation of liposomes. In order to know basic conditions for the incorporation of P_{450scc} into liposomes, we used the cholate-dialysis method. A chloroform solution of the phospholipid (20–700 nmol) was dried by a continuous stream of nitrogen at 50°C. The dried sample was dissolved by diethyl ether and then the solution was dried again. To the dried materials, appropriate amounts of the standard Hepes buffer were added, and the suspension was subject to a vortex mixing for 10 min. Sodium cholate (9.3 nmol/nmol phospholipid) was then added to make a clear solution. P_{450scc} was then added and the mixture was gently shaken. Final volume of the mixture was adjusted to 1.0 ml. The mixture was dialyzed against the same buffer for 36 h at 4°C with two changes of the outside buffer. To measure the amount of P_{450scc} after dialysis, the content in the solution was determined by the reduced CO-bound minus reduced difference spectrum.

For a large preparation of P_{450scc}-containing liposomes, we used the cholate-filtration method. Phospholipid (40 μmol)/sodium cholate (200 mg) mixture was made as described under the cholate-dialysis method. When cholesterol-containing liposomes were prepared, appropriate amounts of cholesterol were added to the chloroform solution of phospholipid. Final volume was adjusted to 1.2 ml. To this solution, 0.33 ml P_{450scc} (72 μM) was added and the mixture was kept on ice for 10 min. The mixture was applied to a Sephadex G-50 column (1.6 × 25 cm) and eluted with the standard Hepes buffer. The cytochrome-rich fractions were combined and centrifuged at 85 000 × g for 15 min to remove multilamellar liposomes. Only a small portion (less than 10%) of the P_{450scc} was precipitated by this centrifugation. The P_{450scc}-containing liposomes were concentrated before centrifugation, if necessary, by ultrafiltration using an Amicon cell with YM-30 membrane

Preparation of mitochondrial lipid. Adrenal cortex mitochondria were prepared as described previously [16]. Mitochondrial lipids were extracted from the mitochondria according to the Folch procedure [25]. The extract was evaporated to dryness and dissolved with chloroform and kept at −18°C under argon atmosphere. Cholesterol content of the extract varied by preparation, being a cholesterol mol% of 7.4–14 Cholesterol-free adrenal mitochondrial lipids were prepared as follows: the lipid extract was mixed at a molar ratio of cholesterol to digitonin of 0.91 at room temperature for 10 min. Then, the precipitate was removed by centrifugation and filtration. The resulting lipid preparation contained cholesterol of less than 0.1 mol%.

Electron microscopy. The ultrastructural analysis of the P_{450scc}-containing liposomes were performed by the negative staining method. 1% phosphotungstic acid was used as a staining reagent. Electron microscopy was performed using a Philips 201 microscope operated at 80 kV.

Test for integrity of liposomes. The P_{450scc}-containing liposomes were prepared as described above except that the mixture contained 50 mM Hepes buffer (7.4)/0.1 mM EDTA/0.1 M NaCl/0.4 M potassium ferricyanide. A Sephadex G-50 gel filtration was applied for the removal of the excess ferricyanide. The internal and external ferricyanide concentration of the ferricyanide-loaded liposomes were determined by the method of Holloway and Katz [26]. A molar extinction coefficient of 1000 $M^{-1} \cdot cm^{-1}$ at 420 nm was employed for calculation of the ferricyanide concentration.

Other procedures. Protein concentration was

determined by the method of Lowry et al. [27] or by the biuret method [28]. Spectrophotometric determination was carried out with a Cary 118 spectrometer. Phospholipid was measured as inorganic phosphate by the method of Bartlett [29] or by the method of Stewart [30]. Heme content of the P_{450scc} was determined by the pyridine hemochromogen method [20]. Cholesterol was determined by the cholesterol oxidase-peroxidase method [18]. The content of cholesterol ester was estimated after hydrolysis by cholesterol esterase. Light scattering was measured with a Perkin-Elmer spectrofluorometer (model 203). PCMS titration was carried out spectrophotometrically.

Results

Selection of phospholipid for the preparation of stable P_{450scc} liposomes.

We have surveyed phospholipids which serve as the membrane component of a stable liposome-reconstituted P_{450scc}. As shown in Table I, cholesterol-DOPC liposome is the best membrane among phospholipids tested for our present purpose These results clearly show that P_{450scc} in membranes with saturated phospholipids is more unstable than that in membranes with unsaturated phospholipids. DPPC, DPPC-DPPE (1.1, mol/mol), DMPC, and egg phosphatidylcholine, which were used previously by other groups [10–13], were all found to be unsatisfactory We have tested the effects of steroids on the P_{450scc} recovery Cholesterol, 20*S*-hydroxycholesterol and 22*R*-hydroxycholesterol were found to stabilize P_{450scc}, whereas pregnenolone, progesterone, and deoxycorticosterone had no effect.

Based on these results, we have decided to use DOPC with cholesterol in most of the following experiments Detailed stability examinations of the cholesterol-containing liposome-reconstituted P_{450scc} are presented later in this paper.

TABLE I

SELECTION OF PHOSPHOLIPID FOR THE PREPARATION OF STABLE P_{450scc} LIPOSOMES

The mixture of P_{450scc} and phospholipid in the presence of 0.5% cholate was placed in a dialysis bag and dialyzed against the standard buffer for 36 h at 4°C. The experiments were carried out more than three times and the values shown are the mean Standard derivations were approx. 20% of the mean value

Phospholipids	Recovery (%) [a]	
	Cholesterol-free P_{450scc}	Cholesterol-bound P_{450scc}
DLPC	13	15
DMPC	23	33
DPPC	17	24
DSPC	14	29
DOPC	33	62
Egg phosphatidylcholine	31	37
Adrenocortical mitochondrial lipids [b]	–	56

[a] Before the separation of liposomes from the P_{450scc}/phospholipid mixture.

[b] 15 mol% cholesterol-containing lipids.

Effect of molar ratio of phospholipid to P_{450scc} on the incorporation of the cytochrome into liposomes

When DOPC, DOPC-DLPC (1:1, mol/mol), DOPC-DLPE (1 1, mol/mol), and DOPC-DLPE-cholesterol (1:1:1, mol/mol/mol) were used as liposomal phospholipids, a maximum heme content in the reisolated liposomes by centrifugation was consistently observed at a molar ratio of phospholipid to cytochrome of approx. 200 (Fig. 1). The membrane chemistry tells us that the DOPC-DLPC vesicles have asymmetric distribution of phospholipids, DOPC tends to locate preferentially in the outer monolayer [30] The DOPC-DLPE membrane has a phase separation; phosphatidylcholine and phosphatidylethanolamine prefer bilayer and hexagonal structures, respectively [30]. The cholesterol-containing DOPC membrane has a different fluidity from the cholesterol-free membrane; the cholesterol-containing vesicle has a more rigid structure of the fatty acyl chains than the cholesterol-free vesicle at our experimental temperature [31]. From these facts, together with our results, we came to the following conclusion when DOPC was used as one of the membrane components, the chemical nature of membranes did not seriously affect the extent of the P_{450scc} incorporation into liposomes. In rat adrenocortical mitochondria, the

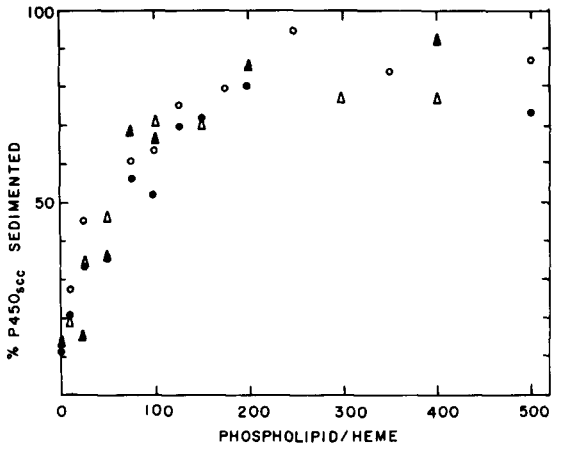

Fig 1. Effect of molar ratio of phospholipid to P_{450scc} on the incorporation into liposomes. Liposomes consisting of various phospholipids were obtained by the cholate-dialysis method as described in Materials and Methods. The dialyzed solutions were centrifuged at 105 000 × *g* for 60 min. The precipitates were suspended by 25 mM Hepes buffer, pH 7.4, containing 0.1 mM EDTA/0.1 M NaCl, and subjected to the heme determination. Percentage sedimented was calculated as

$$\frac{\text{amount of heme in pellet fraction}}{\text{total amount of heme in supernatant + pellet fractions}} \times 100 .$$

○, DOPC; ▲, DOPC + DLPC (1 1); ●, DOPC + DLPE (1 1), △, DOPC + DLPE + cholesterol (1 1 1).

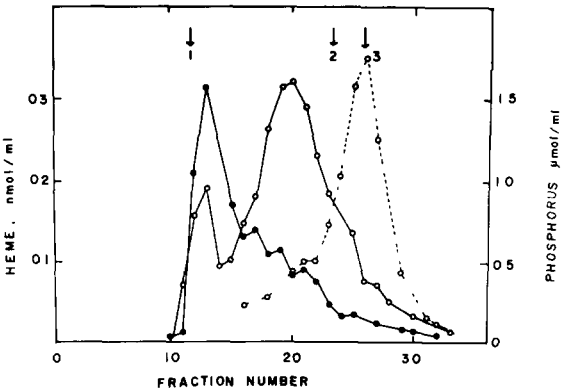

Fig. 2. Separation of P_{450scc}-containing liposomes by Sepharose 4B gel filtration. P_{450scc}-containing DOPC liposomes (6 nmol heme, 15 nmol cholesterol) were prepared from DOPC by the cholate-filtration method described in Materials and Methods. Concentrated solution (0.5 ml) was applied to a column of Sepharose 4B (1.6 × 26 cm) The column was eluted with 50 mM Hepes buffer, pH 7.4, containing 0.1 mM EDTA/0.1 ml dithiothreitol/0 1 M NaCl, and each fraction (1.6 ml) was examined for phospholipid phosphorus and heme content. Purified P_{450scc} (7 nmol) was applied to the column separately. The void volume of the column was determined by blue dextran (arrow 1) and eluted positions of the two marker proteins were indicated. β-galactosidase (M_r 750 000) (arrow 2), catalase (M_r 240 000) (arrow 3). ●———●, phospholipid phosphorus, ○———○, heme of liposomal P_{450scc}, ○- - - - -○, heme of purified P_{450scc} (membrane-free). Lipid was determined by the method of Stewart [30], and heme was estimated by the pyridine hemochromogen method [20].

molar ratio of phospholipid to P_{450} is known to be approx. 250 [32]. Our previous study showed that all P_{450} cytochromes are located in the inner mitochondrial membrane [4–6]. The ratio in the inner membrane must be less than 250. Therefore, the incorporation of P_{450scc} into artificial membranes under our conditions appears to be slightly less efficient than incorporation into the natural membrane.

Separation of P_{450scc}-containing liposomes by gel filtration

The P_{450scc}-containing DOPC liposomes were prepared at a molar ratio of phospholipid to cytochrome of 1670 in the presence of cholesterol. The resulting liposomal mixture was subjected to Sepharose 4B gel filtration. Fig. 2 shows the elution profile of the liposomes and P_{450scc}. The elution fractions containing heme were well coincided with the fractions containing phospholipid phosphorus, and these fractions were separated from those containing soluble liposome-free P_{450scc}. When reisolated liposomes were washed with 0.5 M KCl, P_{450scc} was not liberated from liposomes, indicating the hydrophobic interactions between P_{450scc} molecule and acyl chains. These results strongly indicate that P_{450scc} molecules are associated with the DOPC vesicle. It was also seen that there were two major populations of P_{450scc}-containing liposomes. The large liposomes, which were eluted at the first peak, had a molar ratio of lipid to heme of 8300. The small liposomes, which were eluted at the second peak. had a ratio of 1300. Our estimation of the molecular weights for the large and small vesicles was over $1 \cdot 10^7$ and $3 \cdot 10^6$, respectively.

When P_{450scc} was incorporated into lipids prepared from bovine adrenocortical mitochondria by the cholate-filtration method, the elution profile ob-

tained from a Sepharose 4B column was similar to that shown in Fig. 2. The large vesicles have a molar ratio of phospholipid to heme of 2050, which is significantly smaller than that of the DOPC liposomes. The small vesicles had the ratio of 1130, which is comparable to the corresponding value of the DOPC liposomes.

The absorption spectrum of liposome-reconstituted P_{450scc} displayed a typical low-spin type spectrum with maxima at 362 (broad), 415 (sharp), 530 (broad), and 565 nm (broad). There was no distinct peak at 650 nm, which is a typical maximum of the high-spin form. These absorption peaks are in good agreement with those of purified lipid-free P_{450scc} (355, 416, 535, and 568 nm). Hall et al. [10] reported the absorption spectrum of liposome-reconstituted P_{450scc}, which exhibited a mixed high- and low-spin type spectrum. We have never observed a mixed-spin type spectrum in the environment of phospholipid, a low-spin inducer, unless cholesterol is enriched in membrane; when molar ratios of heme/cholesterol/phospholipid were 1:860:1253, a distinct shoulder of the high-spin form was observed

Electron microscopy

In order to evaluate the size and shape of the P_{450scc}-containing liposomes, an electron microscopic examination was carried out by the negative staining method. Bilayer structures with diameters from 200 to 600 Å were most abundant. Some aggregated liposomes were also seen in the photograph. These two major populations may represent the large and small vesicles separated by the gel filtration procedure. The average diameter for the small vesicles appears to be larger than that of egg yolk phosphatidylcholine vesicles (270 Å) [33], which were prepared by the cholate-filtration method

Leakiness of liposomes

Ferricyanide, a nonpermeable salt, can be trapped into the matrix space of liposomes. In order to examine the leakiness of the P_{450scc}-containing liposomes, we prepared the liposomes in the presence of 0.4 M ferricyanide. After concentrating by ultrafiltration, the liposomes were separated from contaminating ferricyanide by a Sephadex G-50 column. The inside and outside ferricyanide concentrations of liposomes were determined spectrophotometrically. The average internal volume was calculated from the ferricyanide and phospholipid phosphorus concentrations in the suspension. These results are summarized in Table II. Egg yolk phosphatidylcholine liposomes, which were prepared by a cholate-filtration method, had an average internal volume of 0.47 μl/μmol phospholipid phosphorus [33]. Compared with the egg phosphatidylcholine vesicles, the DOPC liposomes without P_{450scc} appear to be substantially larger. When P_{450scc} was present in the DOPC liposomes, the trapped amount of ferricyanide was less than that of the P_{450scc}-free liposomes. This is consistent with the

TABLE II

SIZE AND FERRICYANIDE LEAKINESS OF LIPOSOMES

P refers to the phospholipid phosphorus.

Samples	Trapped ferricyanide			Internal volume (μl/μmol P)	Diameter [a] (Å)	Stokes' radius [b] (Å)	Ref.
	0 h (μmol/μmol P)	16 h (μmol/μmol P)	16 h 0 h (%)				
DOPC liposomes	0.225	0.204	91	0.42	–	210	This work
P_{450scc} liposomes [c]	0.082	0.063	77	0.21	200–600	180–400	This work
Egg phosphatidylcholine	0 122	–	–	0.17	–	–	26
	–	–	–	0.47	270	210	33
	–	–	–	0.40	300	–	54

[a] Based on the electron microscopic experiments

[b] Based on the gel filtration experiments.

[c] 0 2 mol% cholesterol containing DOPC.

fact that less retention of ferricyanide was observed for the cytochrome b_5-containing liposomes than for the protein-free liposomes [26]. After storage of the liposomes at 4°C for 16 h, the P_{450scc}-containing liposomes retained 77% of the trapped ferricyanide, whereas the protein-free liposomes retained 91%. Although the P_{450scc}-containing liposomes appears to be leaky, the trapped ferricyanide was not depleted completely. For our present purposes, the P_{450scc}-containing liposomes were satisfactorily utilized

Light-scattering of liposomal P_{450scc}

We examined the size of liposomal P_{450scc} by 90° light scattering measurements. In comparison with the P_{450scc}-free DOPC liposomes, the P_{450scc}-containing liposomes had approximately twice as much scattering intensity at 400 nm, indicating that the cytochrome-containing liposomes are larger in average than the hemoprotein-free liposomes. The effect of cholesterol was then tested. The result indicated that the P_{450scc}-containing liposomes with cholesterol had a comparable size to that without cholesterol. Therefore, the low amount of ferricyanide per phospholipid phosphorus in the cytochrome-containing lipo-

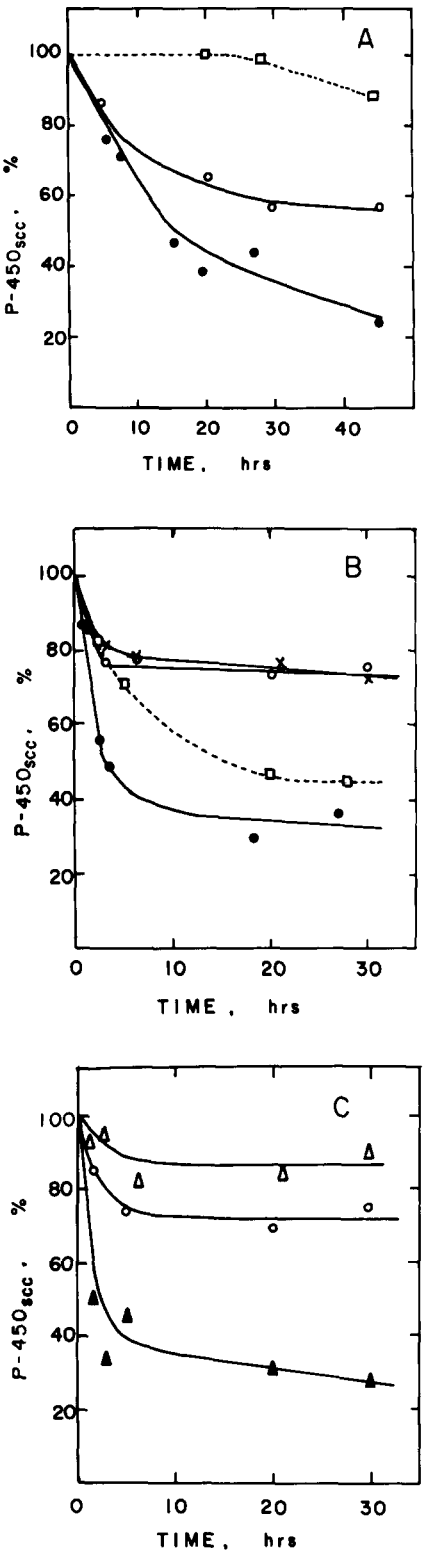

Fig. 3. Stability of liposomal P_{450scc}. P_{450scc}-containing liposomes (0.5 nmol P_{450scc}, 0.8 μmol phospholipids) were prepared by the cholate-filtration method. The cholesterol-bound P_{450scc} was used in these experiments. After incubation of liposomal P_{450scc} in 0.2 ml 50 mM Hepes buffer, pH 7.4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/0.1 M NaCl at 4 (A), 30 (B), and 30°C (C), the remaining heme was measured by the pyridine hemochromogen method. Percent of the remaining P_{450scc} was plotted against the time of incubation. (A): □------□, aqueous P_{450scc} solution (0.7 μM); ●———●, 0.2 mol% cholesterol-containing DOPC liposomal P_{450scc}; ○———○, 44 mol% cholesterol-containing DOPC liposomal P_{450scc}. (B) □------□, aqueous P_{450scc} solution (0.7 μM), ●———●, 0.2 mol% cholesterol-containing DOPC liposomal P_{450scc}; ○———○, 16 mol% cholesterol-containing DOPC liposomal P_{450scc}; X———X, 0.2 mol% cholesterol-containing DOPC liposomal P_{450scc} + adrenodoxin (2 nmol). (C) ▲———▲, 0.2 mol% cholesterol-containing mitochondrial liposomal P_{450scc}; △———△, 10 mol% cholesterol-containing mitochondrial liposomal P_{450scc}, ○———○, 16 mol% cholesterol was added to the 0.2 mol% cholesterol-containing liposomal P_{450scc}. The method for mitochondrial phospholipids is described in Materials and Methods.

somes (Table II) does not indicate that the vesicle with the hemoprotein is smaller than that without the hemoprotein. Rather, it is explained by a small accomodation of ferricyanide in the protein-containing liposomes and partially by a slight leakiness of membrane.

Stability of the liposomal P_{450scc}

When P_{450scc} was incorporated into the cholesterol-free DOPC liposomes, P_{450scc} was found to be less stable than the soluble cytochrome at 4 and 30°C (Fig. 3A and B). Approx 40% of the heme remained intact after 3 h at 30°C *. When cholesterol was present in liposomes, P_{450scc} was considerably more stable under the same conditions (80%). The hemoprotein with cholesterol was weakly stabilized at 4°C, however. Since the fatty acyl chain melting point of DOPC is reported to be −22°C [30], the fluidity of the membrane may not be largely responsible for the stability between 4 and 30°C. In addition to this, adrenodoxin acted as a stabilizer of the liposomal P_{450scc} (Fig. 3B). When P_{450scc} was incorporated into cholesterol-containing lipids prepared from adrenocortical mitochondria, the cytochrome showed the best stability among the conditions tested; after 30 h at 30°C, about 90% of the heme remained intact No formation of P_{420} was detected under these conditions. When P_{450scc} was incorporated into the cholesterol-free adrenocortical lipids, the hemoprotein was found to be unstable under the same conditions (Fig. 3C). From these results, we concluded that the liposomal P_{450scc} requires cholesterol or adrenodoxin for its maximum stability.

The kinetics of the P_{450scc} degradation reactions clearly showed the fast and slow phases. The half-times of the respective reactions were calculated as summarized in Table III. The fast phase of the reaction is due to the peroxidation reaction of heme by contaminating hydroperoxides of phospholipid used. The distinct protective effects of cholesterol in the fast reaction can be accounted for by the fact that

* In the absence of cholesterol, liposomal P_{450scc} was very unstable upon dithionite reduction. The reduced CO-bound difference spectroscopy for the determination of the cytochrome was found to be unreliable. The stability is referred, therefore, to the heme degradation reaction, but not to the conversion reaction of P_{450} to P_{420}.

TABLE III

HALF-TIMES OF THE DEGRADATION REACTION OF DOPC LIPOSOMAL P_{450scc} AT 30°C

The experimental conditions are described in Fig. 4.

Conditions	Fast reaction	Slow reaction
1.9 μM cholesterol [a] in aqueous medium	10.5 h	–
10 mol% cholesterol [b] in mitochondrial lipids	17.0 h	8 days
0.2 mol% cholesterol [a] in mitochondrial lipids	3.0 h	54 h
0.2 mol% cholesterol [a] in DOPC	2.5 h	34 h
16 mol% cholesterol [b] in DOPC	8.5 h	9 days
0.2 mol% cholesterol [a] in DOPC with 10 μM adrenodoxin	11.0 h	5.5 days

[a] Cholesterol was bound to P_{450scc}.
[b] Cholesterol was incorporated into liposomes.

cholesterol acts as a radical trapping agent, the 7α- and 7β-hydroperoxy derivatives are products of the radical-mediated reaction [34,35].

Reduction of liposomal P_{450scc} by adrenodoxin-adrenodoxin reductase system

We have examined whether or not liposome-incorporated P_{450scc} is reduced by externally added adrenodoxin, adrenodoxin reductase, and NADPH. Since the reducible portion of P_{450scc} in liposomal membranes should represent the molecules located in the outer monolayer of the bilayer, we hoped to gain some clue on the asymmetric distribution of the hemoprotein molecules from these studies. When cholesterol-free P_{450scc} was incorporated into the cholesterol-free DOPC liposomes, no reduced P_{450scc} was detected by the enzymatic system in the presence of CO (Fig. 4A). Approx. 20% of the total P_{450scc} in 0.2 mol% cholesterol-DOPC liposomes were reduced by the same system (Fig. 4B) When 7.3 mol% cholesterol was included into the P_{450scc}-containing DOPC liposomes, approx. 70% of the total heme were observed as the reduced CO-bound form (Fig. 4C). To be noted is the fact that at this concentration of cholesterol, the oxidized spectrum of mem-

brane-bound P_{450scc} displayed a considerable amount of the low-spin form. In the presence of 40 mol% cholesterol in the membrane, 70% of the total cytochrome were reduced under the same conditions (Fig. 4D). For a control experiment, 95% were reduced readily by the addition of dithionite in the presence of 40 mol% cholesterol. These results indicate that P_{450scc} molecules distribute asymmetrically within the DOPC liposomal bilayer, approx. 70% of the total P_{450scc} molecules are located in the outer monolayer, whereas 30% of the total hemoprotein are not accessible to the reducing system.

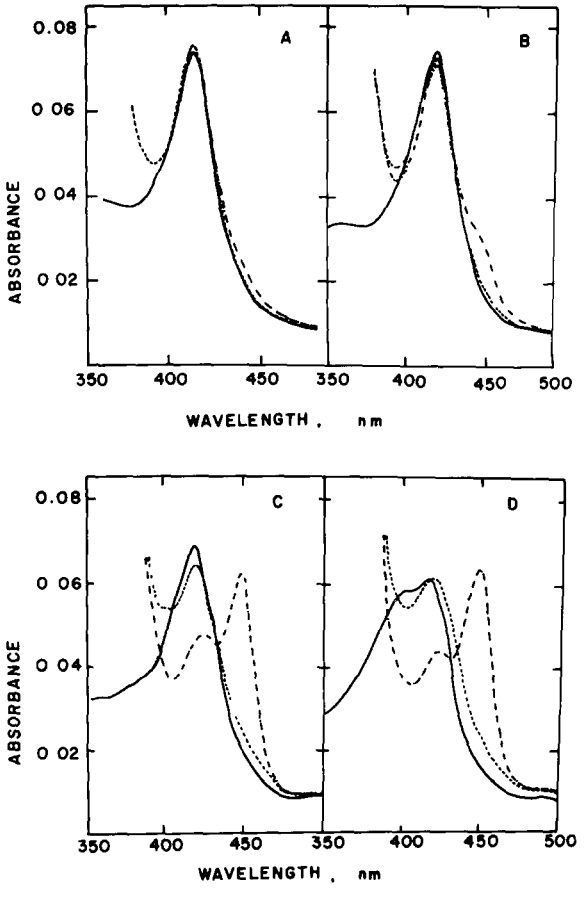

Fig 4. Reduction of the DOPC liposomal P_{450scc} by the adrenodoxin-adrenodoxin reductase system. The liposomal P_{450scc} was prepared by the cholate-filtration method. The liposomal P_{450scc} (0.65 nmol heme) was reduced by adrenodoxin (0.41 nmol), adrenodoxin reductase (0.21 nmol), NADP (30 nmol), glucose 6-phosphate (500 nmol), and glucose-6-phosphate dehydrogenase (0 22 unit) in 1.0 ml 50 mM Hepes buffer, pH 7.4, containing 0.1 mM EDTA/0.1 mM dithiothreitol/0.1 m NaCl After enzymatic reduction, CO was bubbled gently, and kept at 25°C for 10 min. (———), oxidized spectrum, (- - - - -) reduced spectrum; (· — · —) reduced CO-bound spectrum (A) Steroid-free P_{450scc} in DOPC liposomes (1.43 μmol phospholipid), (B) Cholesterol-bound P_{450scc} (2 7 nmol cholesterol/nmol P_{450scc}) in DOPC liposomes (1.17 μmol phospholipid, 0.15 mol% cholesterol), (C) Cholesterol-bound P_{450scc} in cholesterol-supplemented DOPC liposomes (1.27 μmol phospholipid, 7.3 mol% cholesterol), (D) Cholesterol-bound P_{450scc} in cholesterol-supplemented DOPC liposomes (0 83 μmol phospholipid, 39.5 mol% cholesterol).

PCMS tritration of DOPC liposomal P_{450scc}

We have investigated the location of the heme site of P_{450scc} in the bilayer membrane in order to observe in more detail the interaction of cholesterol and the cytochrome. When PCMS, an impermeable sulfhydryl reagent, was added to the DOPC liposomal and detergent-dispersed P_{450scc}, the absorption

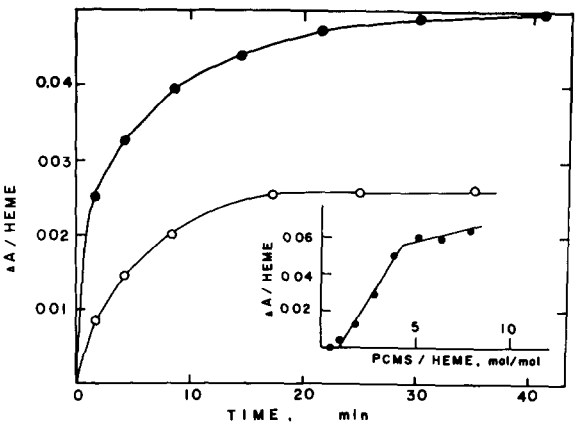

Fig. 5. Reaction of P_{450scc} with *p*-chloromercuriphenyl sulfonic acid. The reference and sample cuvettes contained 0.53 nmol P_{450scc} in 1.0 ml 10 mM potassium phosphate buffer, pH 7.4/0.1 mM EDTA/0.1 M NaCl. PCMS was added to the sample cuvette and the mixtures were incubated at 25°C. The difference spectra were taken with time until the spectrum reached a maximum. ΔA/heme = ΔA_{393nm} (maximum) $-\Delta A_{424nm}$ (minimum) in the DOPC liposomal P_{450scc}/nmol heme, ΔA_{385nm} (maximum) $-\Delta A_{422nm}$ (minimum) in P_{450scc} with 0 02% of Emulgen 913/nmol heme •———•, DOPC liposomal P_{450scc} (0.89 μmol phospholipid) Molar ratio of PCMS/heme was 3.8. ○———○, P_{450scc} with 0.02% Emulgen 913. Molar ratio of PCMS/heme was 3.7. ΔA/heme was calculated from the maximum difference spectrum at each PCMS concentration in the inserted figure.

TABLE IV

COMPARISON OF PROPERTIES AMONG VARIOUS LIPOSOMAL P_{450scc}

Abbreviations A–L stand for DPPC, DPPE, DOPC, soya phosphatidylcholine, egg phosphatidylcholine, DMPC, diphytanoylphosphatidylcholine, DLPC, dilinolenoylphosphatidylcholine, dilinoleoylphosphatidylcholine, adrenal mitochondrial phospholipid, and DSPC, respectively.

Properties	Hall et al. [10]	Kamin et al. [11–13]	This work
Phospholipid used in most experiments	A/B (1 1, mol/mol)	D [11] E [12,13]	C
Incorporation method	Cholate-dilution	Mixing without detergent	Cholate-dilution
Cholesterol in membrane	None	Variable amounts	Variable amounts up to 50 mol%
Phospholipid/protein in reconstituted membrane (mol/mol)	500	50–100 [11]	200
Vesicle size	305–405 Å	1000 Å [11]	200–600 Å
Stability	No loss (6 weeks, 0°)	Phospholipid stabilizes [11] 50% loss [13]	Phospholipid destabilizes Cholesterol stabilizes
Preference for phospholipid	–	C > D > E > F [11] [a] G > E > C > H [13] [b] G > I > E > C > J > F > A [13] [a]	C ≒ K > F > A > H > L [c]
% of P_{450scc} in the outer monolayer	–	–	70% in C

[a] Steroid hydroxylase activity.
[b] Spectrophotometric determination from the low to high spin conversion.
[c] Stability test.

peak at 418 nm was shifted to 408 and 412 nm in DOPC liposomal and detergent-dispersed P_{450scc}, respectively The PCMS-treated P_{450scc} exhibited a reduced CO-bound maximum at 420 nm. As shown in the inset of Fig 5, one molecule of PCMS/heme was consumed without affecting the spectrum. The maximum difference absorbance was obtained at the molar ratio of PCMS to heme of approx. 4 in the liposomal and the detergent-dispersed protein, respectively The reaction rates of the PCMS reactions with liposomal and detergent-dispersed P_{450scc} are shown in Fig. 5. At a fixed concentration of PCMS, the reaction rate of PCMS with liposomal P_{450scc} was faster than that with detergent-dispersed P_{450scc}. The difference in these reactivities of PCMS is not well understood at the present time. Nevertheless, impermeable PCMS is capable of reacting with the protein in a manner to change its heme structure, suggesting that the sulfhydryl groups at the proximity of the heme site are exposed to the surface of the membrane. In addition, it is reasonable to say that the liposomal P_{450scc} molecules are more dispersed in the membrane than the detergent-dispersed P_{450scc} molecules.

Discussion

We have investigated the properties of liposome-incorporated P_{450scc} in order to mimic the in vitro states of the cytochrome in adrenocortical mitochondrial inner membrane. Earlier studies on this line of work demonstrated the incorporation of the heme-protein into phospholipid vesicles [10–13]. Differences in phospholipids used and in the preparation procedures of liposomes produced some obvious discrepancies among these results. In this sense, they are worthy of being summarized in comparison with our present results (Table IV) For the artificially

reconstituted system, it is essential to know the topographic properties of P_{450scc} molecules in the membrane in order to mimic the native membrane. Otherwise, a conclusion from the reconstituted system may be irrelevant to the native mitochondrial system. In this investigation, we have concentrated our efforts on the characterization of P_{450scc}-containing liposomes.

First of all, we have examined carefully the conditions for the stable incorporation of P_{450scc} into liposomes. Among phospholipids tested, DOPC and lipids extracted from adrenocortical mitochrondria were found to be the best for the purpose. Under our best stabilizing conditions, the half-time was revealed to be 8–9 days at 30°C after the initial rapid degradation. It was observed previously that after hypophysectomy of rats, the half-time of the decay process of P_{450scc} was 3.5 days [39–41]. Reconstituted P_{450scc} appears to be substantially more stable compared with the in vitro environment, which may involve such degradation mechanisms as lipid-peroxidative, proteolytic, and phospholipase-catalyzed reactions.

From our reducibility experiments of liposomal P_{450scc}, 70% of cholesterol-DOPC-reconstituted P_{450scc} was reduced by the external additions of adrenodoxin, adrenodoxin reductase, and NADPH, which are all impermeable across phospholipid membranes. These observations lead us to conclude that 70% of the incorporated cytochromes are located in the outer monolayer of bilayers. When non membrane-bound cholesterol is present, we have never seen any substantial amount of the reduced CO-bound form by the external addition of the enzymatic reducing system regardless of phospholipids used. Seybert et al. [11] found that when P_{450scc} was incorporated into soya phosphatidylcholine vesicles. 100% of liposomal P_{450scc} were reduced by the same enzymatic system in the absence of cholesterol. In their experiments, the time for mixing the cytochrome with liposomes was 10 min at room temperature. Such conditions may not be enough time to accomodate the hemoprotein into the membrane. Indeed, their current conditions for the incorporation were 12 h under N_2 [13].

From our PCMS titration and trypsin-digestion experiments of liposome-reconstituted P_{450scc}, we are able to envisage the topographic picture of P_{450scc} in membrane. Water-soluble and membrane-impermeable PCMS could convert the liposomal cytochrome to the inactive forms. These results indicate that the nonpermeable reagent acts at least in the vicinity of the heme moiety of the protein, implying that the heme region is in close proximity to the polar head of phospholipids. Cholesterol in membrane is known to be complexed with phospholipid. Most likely, the 3β-hydroxyl group of cholesterol interacts with the carbonyl group of the ester linkage of phospholipid At a high concentration of cholesterol, more than 20 mol%, the cluster formation of cholesterol molecules becomes dominant [42]. The cleavaging C-C bond at the 20 and 22 positions of cholesterol by P_{450sc} would be approx. 20 Å * below the plane of polar heads of phospholipids. The ability of cholesterol to convert the heme iron spin state at the substrate-binding site of P_{450scc} reasonably indicates that it must be close to the heme site. This idea is in agreement with the two-dimensional crystallographic ESR measurements, which revealed that the heme site is less than 20 Å from the surface of the membrane [43].

Finally, we wish to say a few words on the function of ACTH on the mitochondrial cholesterol availability to P_{450scc}. From our present results together with our previous work [17–19], the availability of cholesterol to the cytochrome depends on the membrane concentrations of cholesterol. This implication is consistent with works by others on the intact mitochondria under various physiological conditions, including the ACTH action [1–3]. In this connection, the transmembrane movement of cholesterol molecules appears to be the step which is indeed stimulated by the action of ACTH.

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* The sum of lengths of phospholipid polar head and cholesterol nucleus.

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