Cytochrome P-450, from bovine adrenal cortex mitochondria was purified and reconstituted into phosphatidylcholine vesicles which varied in both cholesterol content and in the fatty acyl composition of the phospholipid. Under conditions of optimal ionic strength, pH, and excess adrenodoxin and adrenodoxin reductase, it was found that at a constant cholesterol: phospholipid ratio, the membrane composition had large effects on the rate of hemoprotein-catalyzed side chain cleavage of cholesterol. Rate effects were due to phospholipid-induced changes in the enzyme's $K_m$ for cholesterol, and not due to $V_{max}$ effects. Binding of cholesterol to cytochrome P-450 could also be monitored optically by measuring the fraction of enzyme in the high spin form. Dissociation constants determined in this manner for cholesterol binding in phospholipid of differing fatty acyl composition showed an excellent inverse correlation with the rates of pregnenolone formation in the same lipids (at constant cholesterol concentration) (see Fig. 6); thus, phospholipid exerts its rate effects by modulating the binding of cholesterol to the cytochrome. The membrane-mediated effects on spin state and activity mimic closely the effects seen in mitochondria isolated from adrenocorticotropin hormone-treated versus control adrenal cells. This behavior suggests to us that acute steroidogenic action of adrenocorticotropin hormone may be mediated through changes in the composition of the inner mitochondrial membrane in which cytochrome P-450 is embedded.

Cytochrome P-450 from bovine adrenal cortex mitochondria is an inner membrane-associated hemoprotein which can function, together with its soluble electron transport components adrenodoxin (a ferredoxin-type protein) and NADPH-adrenodoxin reductase (a flavoprotein), in the enzymatic side chain cleavage of cholesterol to yield pregnenolone, the common precursor of the adrenal steroid hormones (1, 2). Cleavage of the 20, 22 carbon-carbon bond of cholesterol occurs in three steps utilizing three molecules of oxygen and a total of six electrons (1, 3). We have studied the mechanism of electron transfer in this system and find that adrenodoxin acts as a mobile one-electron shuttle between adrenodoxin reductase and cytochrome P-450 (4-6); the adrenodoxin must interact with an aqueous-exposed site on the cytochrome once during each of its electron shuttles. Our recent successful reconstitution of cytochrome P-450 into phosphatidylcholine vesicles (5) has permitted us to begin studies on the interaction of cholesterol and phospholipid with cytochrome P-450. We find that the cholesterol binding site of the cytochrome is in communication with the hydrophobic milieu of the phospholipid membrane (5). When cholesterol is incorporated into the phospholipid membrane, binding of cholesterol to the cytochrome occurs rapidly and is accompanied by a conversion of the optical spectrum from a low spin to L-type spectrum to a high spin or H-type spectrum (9). The fraction of hemoprotein in the high spin form is a function of the cholesterol:phospholipid molar ratio rather than the total cholesterol concentration expressed in terms of the aqueous volume; we have therefore adopted the convention of expressing cholesterol concentration as a cholesterol: phospholipid molar ratio. The present studies have used the reconstitution technique (5) to investigate further the interactions among cholesterol, phospholipid, and cytochrome P-450 activity and spectral properties of the cytochrome were studied in reconstituted systems which varied in cholesterol content and in the fatty acyl side chain composition of phosphatidylcholine.

Results of the present studies may be useful in interpreting the mechanism by which adrenocorticotropin hormone controls the production of glucocorticoid hormones in the adrenal cortex. The rate of cholesterol side chain cleavage has been shown to be limiting in steroid hormone production (10), and is stimulated by ACTH2 by some as yet poorly understood mechanism. Thus, data pertaining to the rate-limiting step in the catalytic cycle of adrenodoxin reductase: adrenodoxin: cytochrome P-450-catalyzed side chain cleavage is of particular interest. In a recent study of the effects of ions on the rates of adrenodoxin reduction, 11β-hydroxylase, and cholesterol side chain cleavage (using purified lipid-reconstituted complex)

1 Recent work by Handukoglu and Jefcoate (7) using an independent approach has confirmed that adrenodoxin functions as an electron shuttle. We have found no evidence to support a recent proposal (8) that the system operates as a ternary complex among the three protein components.

2 The abbreviations used are: ACTH, adrenocorticotropin hormone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

3 The early steps in the control mechanism are relatively well understood. ACTH binds to a cell surface receptor and activates adenylylate cyclase to form cyclic AMP (11, 12). This, in turn, stimulates the cytosolic translation of a "labile protein" factor, which then acts by some unknown mechanism to stimulate the side chain cleavage reaction (13, 14).
Phosphatidylcholine Vesicle Reconstituted Cytochrome P-450...

Experimental Procedures

Materials
Cholic acid, glucose 6-phosphate, pure egg phosphatidylcholine, and glucose-6-phosphate dehydrogenase were obtained from Sigma; NADPH was purchased from P. L. Biochemicals; and [5-3H]pregnenolone (5-pregnen-3β-ol-20-one), [1,2-3H]cholesterol, and [carboxyl-14C]tropolamin were products of New England Nuclear. Hexyl agarose was from Miles-Yeda and antipregnenolone antibody was from Radioassay Systems Laboratories. Cholesterol was purchased from Applied Science Laboratories, and contained less than 1% contamination by gas chromatography and thin layer chromatography. Dimyristoylphosphatidylcholine was from Calbiochem, and all other phospholipids including hydrogenated egg phosphatidylcholine were purchased from Avanti Biochemicals. Cholic acid and was purified prior to use by charcoal treatment and recrystallization from hot 95% ethanol.

Methods
Protein Purification—Adrenodoxin reductase and adrenodoxin were purified as described previously (6, 20). Concentrations were established using extinction coefficients of 10.9 mM-1 cm-1 at 450 nm for adrenodoxin reductase (21) and 11 mM-1 cm-1 at 414 nm for adrenodoxin (22). Purification of cytochrome P-450c, was carried out as described previously (5) with minor modifications as follows. The cytochrome was extracted from sonicated mitochondria (15 mg/ml) with 1% cholate. Following centrifugation, the supernatant was precipitated between 32% and 45% saturated ammonium sulfate. The pellet was redissolved in 50 mM potassium phosphate, pH 7, KDTGA (0.1 mM), dithiothreitol (0.1 mM), dialyzed versus the same buffer, and applied to a hexyl agarose column. Cytochrome was eluted with the same buffer containing 1.0 mM dithiothreitol (0.1 mM NaCl, 0.1 mM dithiothreitol). The concentration of cytochrome P-450c, was determined from the reduced CO absorption spectrum using a Hitachi U1200 spectrophotometer.

Preparation of Phospholipid Vesicles—Small unilamellar phosphatidylcholine vesicles were prepared as described previously (5, 6). Phospholipid in chloroform or ether was then mixed with cholesterol in a test tube, (13 x 100 mm) and solvent was removed under vacuum. Typical phospholipid content was 0.15 to 0.8 mg/ml and cholesterol was as indicated by the cholesterol to phospholipid ratios in the figures. Assay buffer was added to each tube and tubes were then sealed under N2 and sonicated using a Buehler Ultraturm III bath-type sonicator for 10 min. For dimyristoyl- and dipalmitoylphosphatidylcholine, vesicles were prepared at 30°C and 50°C, respectively, temperatures above the phase transitions for these lipids. Resulting vesicles had a diameter of 400 to 500 Å as determined by electron microscopy.1 Cytochrome P-450c, was reconstituted into vesicles as described previously (6, 7) by addition of cytochrome to vesicles and incubation for 12 h. We have previously shown that upon incubation at 30°C, cytochrome becomes associated quantitatively with phospholipid (5).

Assay of Side Chain Cleavage—Side chain cleavage activity was measured by pregnenolone formation using a radioimmunoassay procedure described previously (24). The assay mixture consisted of 0.3 to 1 μM cytochrome P-450c, 0.5 μM adrenodoxin reductase, 10 μM adrenodoxin, 2 mM glucose 6-phosphate, and 2 units/ml of glucose-6-phosphate dehydrogenase, all in a volume of 200 to 300 μl. At time zero NADPH was added to 50 μM final concentration and 25-μl aliquots were taken at 0, 1, 2, and 4 min and pipetted into 1 ml of hexane. Tightly capped vials of hexane were then vortexed for 15 s vacuum dried to stand overnight at 4°C or stored at -35°C for several weeks. Aliquots of the hexane solution were then placed in test tubes (10 x 75 mm) and dried under vacuum. Potassium phosphate buffer (0.6 ml of 0.1 M, pH 7.0), containing 0.1% gelatin and approximately 4000 cpm of 3H progesterone, was then added to each tube and vortexed vigorously for 1 min. NaCl (0.25 M) and ethanol (0.70) was then added and the solution mixed gently for 2 s. Then 0.2 ml of a suspension of activated charcoal plus dextran (0.025 g of charcoal plus 62.5 mg of dextran/100 ml) was added to each tube, mixed gently, and then centrifuged at 3000 rpm for 15 min to remove the charcoal. The supernatant containing antibody plus bound steroid was then pipetted into a scintillation vial and counted. Counts were then compared with those from a standard pregnenolone curve. The assay was sensitive to 0.1 ng of pregnenolone, and each sample was assayed between two and six times to assure accuracy.

For calculation of turnover numbers for cytochrome P-450... it was important to measure the concentration of the cytochrome in an aliquot of the vesicle reconstituted system as described below. As much as 50% of the total absorbance was lost in some samples reconstituted into polyunsaturated phospholipids, possibly because of the presence of lipid peroxides. Thus it was necessary to correct for the nonenzymatic contribution of cholesterol to the lipid phase. This correction was made by addition of cholesterol to the vesicles-cytochrome P-450c, also contains approximately 1 mol of cholesterol/mol of cytochrome, and correction was made at lower cholesterol/phospholipid ratios for the contribution of this endogenous cholesterol.

Spectrophotometric Measurements—All optical spectra were recorded using a Varian 219 spectrophotometer. The fraction of cytochrome reduced at 414 nm was determined (418 nm-absorbing species) was calculated as described previously (6); the maximum possible low spin or 418 nm-absorbing form (L-type spectrum) was calculated as described previously (6); the maximum possible high spin form was obtained by adding adrenodoxin to a sample of high cholesterol/phospholipid ratio. In the presence of excess cholesterol, adrenodoxin binding induces a maximal conversion to high spin cytochrome P-450c, (6). In addition, the reduced carbonyl versus reduced difference spectrum revealed the content of cytochrome P-420 present (always under 1%), and spectra were corrected for this 418 nm-absorbing species.

Relative Affinity of Phospholipids for Cholesterol—Relative affinities of phosphatidylcholines for cholesterol were measured by the method of Nakagawa et al. (26). "Donor" and "acceptor" phospholipid vesicles were prepared as described above (12.5 mM KPi, pH 7.0, plus 110 μg/ml of albumin and containing the following molar ratios of components: phospholipid; cholesterol, 0.5 mol/mol, butyrylhydroxylutanol, 0.1 mol/mol, and phosphatic acid, 0.105 mol/mol for acceptor vesicles and 0.024 mol/mol for donor vesicles. In addition, donor vesicles contained ~90 000 cpm each of [1,2-3H]tropolamin. Donor and acceptor vesicles were incubated together (1.83 mg of each...
phospholipid in a total of 0.5 ml of buffer, 37°C, 24 h) and separated on DEAE-cellulose columns (0.8 x 1 cm) with 2.5 mM increments of KPi buffer. Donor vesicles were eluted while acceptor vesicles were retained on the column. Exchange of cholesterol was measured from the ^1^C to H ratio before and after separation of vesicles.

**RESULTS**

**Effect of Saturation of Fatty Acyl Chains of Egg Phosphatidylcholine on Side Chain Cleavage Activity**—We have reported previously that cytochrome P-450, with or without cholesterol, is relatively inactive when reconstituted into purified egg phosphatidylcholine or synthetic dioleoylphosphatidylcholine, but is more active in synthetic dimeristoyl- or dipalmitoylphosphatidylcholine (5). Purified egg phosphatidylcholine has been demonstrated to contain 57% unsaturated fatty acyl chains, primarily C18 (35%) monounsaturated chains. Fig. 1 confirms that the side chain cleavage enzyme is relatively active in native egg phosphatidylcholine, and demonstrates that hydrogenation of this phospholipid results in a reconstituted system with less than 8% of the original activity. Thus, the saturation state of the fatty acyl chains of phosphatidylcholine can exert a major effect on the activity of the side chain cleavage system. The mechanism by which this activity is controlled is considered below.

**Effect of Cholesterol to Phospholipid Ratio on Cholesterol Side Chain Cleavage Activity**—As discussed previously the activity of cytochrome P-450, under optimal conditions of ionic strength (4) and pH (25), appears to be governed by the cholesterol to phospholipid ratio (see Fig. 2 and Ref. 5), rather than the absolute cholesterol concentration expressed in terms of the aqueous volume. Such a finding is consistent with our proposal that cholesterol interacts with the cytochrome via the hydrophobic phospholipid milieu rather than through the aqueous environment (5, 6). Using our previous assay system, the double label system of Doering (27), it was difficult to measure rates at high cholesterol to phospholipid ratios since the fractional change in the ^3^H to ^1^C ratio became smaller than the "noise level" of the assay. The use of the radioimmunoassay for pregnenolone allows accurate measurement at high cholesterol to phospholipid ratios, and has permitted us to determine K_m and V_max, values more accurately. Fig. 2 shows the effect of varying the cholesterol to phospholipid ratio upon activity. The V_max value calculated from the y intercept is 21.3 mol of pregnenolone produced/min/mol of cytochrome P-450, under optimal conditions of ionic strength (4) and pH (25). From each pregnenolone produced requires three hydroxylations, this corresponds to a turnover number/hydroxylation of 65 min^{-1}, assuming there is no accumulation of hydrogenation intermediates. This is compared to turnover of 30 min^{-1} obtained by us for 11β-hydroxylation by cytochrome P-450, (4). Thus, when sufficient substrate is supplied to the side chain cleavage enzyme, it can operate at a rate similar to that of the 11β-hydroxylase. Fig. 2 also allows calculation of a K_m for cholesterol of 0.22 (expressed in terms of a cholesterol to phospholipid molar ratio, since phospholipid is the solvent in which cholesterol is "dissolved"). Since the maximum achievable ratio of cholesterol to phospholipid is ~1:1 (28), the relatively high K_m of 0.22 indicates that the system operates at subsaturating levels of cholesterol under all assay conditions. It is likely that this is also the case in vivo; adrenal cortex mitochondrial cholesterol content has been measured under a variety of conditions, and found to be low (19 to 100 nmol/mg of mitochondrial protein) (29, 30).

**Rate of Side Chain Cleavage in Diphyanoylphosphatidylcholine**—The rate of side chain cleavage of cholesterol was measured in diphyanoylphosphatidylcholine at various cholesterol to phospholipid ratios. Comparison of Figs. 1 and 3 reveal that the requirement for unsaturated lipids can be satisfied by the fully saturated diphyanoylphosphatidylcholine which contains a methyl group at every 5th position on its C16 fatty acid chains. Activity (corrected for differences in cytochrome concentration) at the highest cholesterol to phospholipid ratio was 33% higher in the latter lipid than in egg lipid. In experiments (not shown) it was also determined that there was a 30 to 40% loss in hemi absorbance and cytochrome P-450 content (as judged by the CO difference spectrum) when the cytochrome was incubated overnight at 4°C with the egg lipid, whereas there was no detectable loss with diphanyol lipid. Thus, diphyanoylphosphatidylcholine appears to be the lipid of choice in reconstitution studies requiring long incubations with the cytochrome. A double reciprocal plot of activity versus cholesterol to phospholipid ratio as in Fig. 2 (not shown) allowed calculation of a V_max of 22.2, a value identical within experimental error with that for the V_max in egg lipid, and a K_m of 0.20 compared with 0.22 in egg lipid.

**Effects of Various Lipids on Cholesterol Side Chain Cleavage Activity and on the K_m for Cholesterol**—V_max and K_m values determined above, as well as those for synthetic dioleoylphosphatidylcholine determined by the same method, are summarized in Table I. Also shown are turnover numbers (pregnenolone production) in a variety of phosphatidylcholines at a cholesterol to phospholipid ratio of 0.5 mol/mol. As can be seen, there is an almost 20-fold range of turnover numbers depending upon the phospholipid used. For the three lipids in which V_max and K_m could be determined (see Table I), the extrapolated maximal velocities were the same within 15% and the small differences seen could not account for the 2.1-fold difference in rates. The K_m range of 0.02 to 0.36, however, can account for a 20-fold range of rates. Thus, phospholipid appears to exert its effect by changing the enzyme’s K_m for cholesterol, at least for the three phospholipids tested. For phospholipids with lower rates, K_m and V_max values could not be determined. We will show in subsequent sections, however, that the rate in these phospholipids is also determined by cholesterol binding to cytochrome.

Table I also demonstrates that there is no simple correlation of activity with the degree of unsaturation of the phospholipid. The enzyme is least active in fully saturated lipids. A single double bond per acyl chain results in an approximately 8-fold increase in activity, while a second double bond again decreases activity to near that of the fully saturated lipid. Three double bonds per chain again results in an increase in activity (14-fold over saturated phospholipid). Possible explanations for this perplexing series will be discussed below (see "Discussion"). Regardless of the mechanism for these effects, a large range of activities are achievable when the fatty acyl chains are varied while keeping phospholipid head group the same (see Table I).

**Effects of Phospholipid Acyl Chains on the Cholesterol-Induced High Spin Conversion of Cytochrome P-450**.—Our preparation of purified cytochrome P-450, contains between 0.5 and 1 mol of cholesterol/mol of cytochrome (4, 5) and exists at pH 7 as a mixture of H- and L-type hemoprotein (always greater than 70% H-type spectrum). In previous studies (4, 5), we showed that reconstitution of cytochrome P-
Phosphatidylcholine Vesicle Reconstituted Cytochrome P-450<sub>acc</sub>

**Fig. 1 (left).** Effect of fatty acyl chain saturation of egg phosphatidylcholine on the rate of side chain cleavage of cholesterol. Cytochrome P-450<sub>acc</sub> (1 mM final) was reconstituted into pure egg phosphatidylcholine (PC) vesicles (0.15 mg in 1 ml of 10 mM Hepes, pH 7.2, 100 mM NaCl) as described under "Experimental Procedures." Cholesterol was present in a ratio of 0.5 mol of cholesterol/mol of phospholipid. Adrenodoxin reductase (0.5 mM), adrenodoxin (10 μM), glucose 6-phosphate (2 mM), and glucose-6-phosphate dehydrogenase (2 units/ml) were included, and the reaction was initiated using 50 μM (final) NADPH. Pregnenolone formed was assayed as described under "Experimental Procedures." Effects of various phospholipids on cholesterol side chain cleavage activity are shown in Fig. 2 (center). Effect of cholesterol/ phospholipid ratio on cholesterol side chain cleavage activity. Conditions were as in Fig. 1 using pure egg phosphatidylcholine (PC) except that the cholesterol/phospholipid (CHOL/PL) molar ratio was varied as indicated on the x-axis. The reciprocal of the turnover number (moles of pregnenolone formed/mol of cytochrome P-450/min) is plotted versus the reciprocal of cholesterol to phospholipid ratio.

**Fig. 3 (right).** Rate of cholesterol side chain cleavage in diphytanoylphosphatidylcholine. The rate of pregnenolone formation using pure diphytanoylphosphatidylcholine is shown at the indicated cholesterol:phospholipid (CHOL/PL) molar ratios. Conditions were as in Fig. 1.

**TABLE 1**

| Phospholipid          | V<sub>max</sub> (mol/mol) | K<sub>d</sub> (CHOL/PL) molar ratio | Turnover (0.9) |
|-----------------------|---------------------------|------------------------------------|----------------|
| Diphytanoyl-PC<sup>a</sup> | 22                        | 0.20                               | 20             |
| Egg PC                | 21                        | 0.22                               | 15.5           |
| Dimyristoyl-PC (18:3) | 19                        | 0.36                               | 9.5            |
| Dioleoyl-PC (18:1)    | 19                        | 0.36                               | 9.5            |
| Dilinoleoyl-PC (18:2) | 19                        | 0.36                               | 9.5            |
| Hydrogenated egg PC   | 19                        | 0.36                               | 9.5            |

<sup>a</sup> Turnover (moles of pregnenolone formed/mol of cytochrome P-450/min) at a cholesterol to phospholipid molar ratio of 0.5.

<sup>4</sup> PC, phosphatidylcholine.

450<sub>acc</sub> into cholesterol-free phospholipid vesicles resulted in conversion to the L-type spectrum (see Ref. 5, Figs. 1 and 3 for examples of H- and L-type spectra). When the cytochrome was reconstituted into cholesterol-containing vesicles, however, the extent of conversion was diminished, and the amount of H-type remaining was proportional to the membrane cholesterol content. Thus, the H-type spectrum can be used as a measure of cholesterol bound to the hemoprotein’s active site, and we have used this property to determine a K<sub>d</sub> for cholesterol binding in egg phosphatidylcholine in terms of a cholesterol/phospholipid molar ratio.

Fig. 4 shows the dependence of the hemoprotein spectrum on cholesterol/phospholipid ratio for a variety of saturated phospholipids, where f represents the fraction of cytochrome with the H-type spectrum calculated as described under "Experimental Procedures." Only dimyristoylphosphatidylcholine demonstrated ideal behavior. In addition, side chain cleavage activity was observed only at the highest cholesterol to phospholipid ratio for dipalmitoyl- and hydrogenated egg phosphatidylcholine. Although all vesicles were prepared above their phase transition temperatures, reconstitution and activity measurements were carried out at 4°C and 37°C, respectively, well below the transition temperatures for dipalmitoyl- and hydrogenated egg phosphatidylcholine, respectively. Thus, we suggest that the nonideal behavior of the latter lipids may result either from poor reconstitution of the cytochrome or from unavailability of substrate for binding to cytochrome in gel state lipids.

When unsaturated or branched chain (saturated) phosphatidylcholines were used, however, linear plots were obtained (see Fig. 5). Dissociation constants obtained from these plots indicate that cholesterol binding to cytochrome P-450<sub>acc</sub> is highly dependent upon the fatty acyl portion of the phospholipid used, with the affinity in the following order: diphytanoyl > egg > dioleoyl > dilinoleoyl > dimyristoylphosphatidylcholine. This order is identical with that seen for activity at a cholesterol to phospholipid ratio of 0.5 (see Table 1). This correlation is demonstrated quantitatively in Fig. 6. Activity in the various lipids (at a constant cholesterol to phospholipid ratio) is plotted versus the inverse of the dissociation constant values (K<sub>d</sub>) as determined in Figs. 4 and 5. An excellent correlation is seen (correlation coefficient = 0.975), demonstrating that the activity in various phospholipids is determined by fatty acyl chain effects on the binding of cholesterol to cytochrome P-450.<sup>11</sup>

Equilibrium Distribution of Cholesterol among Phospholipid Vesicles of Various Compositions—One can consider at least three general classes of mechanisms by which fatty acyl chains of phospholipids might affect the binding of cholesterol to cytochrome P-450<sub>acc</sub>. First, some physical property of the lipid itself (as reflected for example, in the fluidity or the transition temperature of the lipid) might influence activity.

Cholesterol, at high concentrations, appears to have a "fluidizing" effect on phospholipids below their phase transitions (see, for example, Ref. 31) and may account for the more ideal binding and activity mechanisms at very high cholesterol concentration in dipalmitoyl- and hydrogenated egg phosphatidylcholine.

A cholesterol binding constant in dilinoleoylphosphatidylcholine could not be determined due to extensive development of turbidity during the reconstitution.

A fatty acid alone, although a high spin inducer, acts as an inhibitor of enzyme activity. In contrast to the acylated form, its effects are independent of unsaturation. The mechanism of these effects is under study (Lambeth, unpublished).
Phosphatidylcholine Vesicle Reconstituted Cytochrome P-450<sub>sec</sub>

Fig. 4 (left). Effects of some saturated phosphatidylcholines on the cholesterol-induced high spin conversion of cytochrome P-450<sub>sec</sub>. Cytochrome P-450<sub>sec</sub> was reconstituted into saturated phospholipids (DMPC, synthetic dimyristoylphosphatidylcholine, ●); hydrogenated egg phosphatidylcholine (PC), ○; and synthetic dipalmitoylphosphatidylcholine, □ containing the indicated concentration of cholesterol (CHOL). Sonication to form vesicles was carried out above the phase transition temperature for each lipid. Reconstitution was carried out in 20 mM Hepes buffer, pH 7.2, 100 mM NaCl, 1 mM dithiothreitol. The fraction of cytochrome P-450<sub>sec</sub> in the high spin form (f) was determined optically as described under ”Experimental Procedures.” PL, phospholipid.

Fig. 5 (center). Effects of unsaturated and branched chain phosphatidylcholines on the cholesterol-induced high spin conversion of cytochrome P-450<sub>sec</sub>. As in Fig. 4, DLPC, synthetic dilinoleoylphosphatidylcholine, ○; DOPC, synthetic dioleoylphosphatidylcholine, ●; egg phosphatidylcholine, □; and dipalmitoylphosphatidylcholine, □ PC, phospholipid; CHOL, cholesterol; PL, phospholipid.

Fig. 6 (right). Correlation of cholesterol binding to cytochrome P-450<sub>sec</sub> with cholesterol side chain cleavage activity. The turnover of cytochrome P-450<sub>sec</sub> (pregnenolone formation) was determined at a fixed cholesterol:phospholipid ratio (see Table I) and plotted as a function of the reciprocal of the K<sub>D</sub> for cholesterol binding, as determined from the cholesterol-induced high spin conversion of cytochrome P-450<sub>sec</sub> (see Figs. 4 and 5). PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

Second, each lipid might have a given affinity for cholesterol, and might compete with the protein for binding of cholesterol, as illustrated by Equation 1.

\[
L - C + P-450 = L + P-450 - C
\]

where L = lipid and C = cholesterol. A third possibility and the one which we prefer, is that the phospholipid affects cholesterol binding by interacting with the cytochrome itself. The first and third possibilities are considered in the “Discussion,” while the second is explored below.

If the mechanism represented by Equation 1 were operating, then the order of affinity of various phospholipids for cholesterol should be inversely correlated with the affinity of cytochrome P-450 for cholesterol in the respective phospholipid. Nakagawa et al. (26) have measured the relative affinity of various phospholipids for cholesterol by preparing two populations of egg phosphatidylcholine vesicles and attributing this to the different contents of phosphatidic acid (see ”Experimental Procedures”).

Table II

| Lipid                  | Egg cholesterol/total cholesterol |
|------------------------|-----------------------------------|
| Diphytanoyl-PC<sup>a</sup> | 0.60                              |
| Dllinoleoyl-PC (18:3)   | 0.86                              |
| Egg PC                 | 0.54 (0.55)<sup>b</sup>           |
| Dioleoyl-PC (18:1)     | 0.61                              |
| Dillnoleoyl-PC (18:2)  | 0.66                              |
| Dimyristoyl-PC (14:0)  | 0.37 (0.30)<sup>b</sup>           |

<sup>a</sup> PC, phosphatidylcholine.

<sup>b</sup> Numbers in parentheses refer to values obtained from the data of Nakagawa et al. (26). These authors also obtained a slightly unequal distribution of cholesterol between the two populations of egg phosphatidylcholine vesicles, and attributed this to the different contents of phosphatidic acid (see “Experimental Procedures”).

was used as the donor phospholipid, and our results, as well as those of Nakagawa et al. (26), are summarized in Table II. Inspection of the data reveal an affinity order for cholesterol as follows:

DMPC > egg PC > diphytanoyl-PC > dioleoyl-PC > dilinoleoyl-PC

where DMPC is dimyristoylphosphatidylcholine and PC is phosphatidylcholine. A striking feature of this series is the decrease in cholesterol affinity with increased amount of fatty acyl chain unsaturation. Although this order is of interest in its own right, the inverse correlation with cholesterol binding to enzyme is poor, indicating that some other mechanism controls the cholesterol-cytochrome binding equilibrium.

**DISCUSSION**

Phospholipid has been shown to affect the activity of a variety of membrane-associated enzymes and transport proteins (see Ref. 32 for a review). In many cases, a relatively nonspecific requirement for lipid is seen, while a more specific role has been proposed for a few enzymes. Liver microsomal cytochrome P-450 together with its flavoprotein reductase have been shown to require phospholipid for activity in hydroxylation reactions (33). Cytochrome oxidase isolated by a variety of procedures contained 1 mol/mol of tightly bound cardiolipin which appeared to be important for catalytic activity (34, 35). β-Hydroxybutyric dehydrogenase requires phosphatidylcholine, and highest activity was seen when the lipid contained unsaturated fatty acyl chains (36). We have previously demonstrated an effect of phospholipid on the activity of purified cytochrome P-450<sub>sec</sub> from beef adrenal cortex mitochondria (5). An activity difference dependent upon the nature of the fatty acyl side chain was found; little or no activity was seen with fully saturated chains, while activity was high when the phospholipid contained unsaturated chains.

In the present investigation, the role of phospholipid in the cholesterol side chain cleavage system has been investigated further. The cytochrome has been reconstituted into phosphatidylcholine vesicles in which the fatty acyl groups of the
Phosphatidylcholine Vesicle Reconstituted Cytochrome P-450<sub>ac</sub>.

phospholipid were varied. Phosphatidylcholines produce stable lamellar or vesicular structures in the presence of excess water; and use of this head group eliminated possible artifacts due to the nonbilayer arrangements of hydrated lipids seen with some other head groups (37). Large acyl chain-dependent rate effects were seen in the reconstituted system (see Table I); this finding is consistent with our previous proposal of a membrane phase interaction of cholesterol with the cytochrome (5, 6). The effects occur only when the fatty acids were part of a phospholipid molecule, since free fatty acids, whether saturated or unsaturated, were potent inhibitors in the vesicle reconstituted system. " There appears to be no simple relationship between activity and the degree of chain unsaturation; although activity was relatively low in straight chain saturated phosphatidycholines, another saturated lipid, diphytanoylphosphatidylcholine, gave the highest activity. Similarly, among unsaturated lipids, the activity order (dilinolenoylphosphatidylcholine (C18:3) > dioleoylphosphatidylcholine (C18:1) > dilinoleoylphosphatidylcholine (C18:2), see Table II) failed to correlate with unsaturation.

While the molecular explanation for the activity order with various fatty acyl groups is not yet fully understood (see below), it is clear from the present studies that these groups exert their effect by modulating the binding of cholesterol to the active site of cytochrome P-450<sub>ac</sub>; in Fig 6 the dissociation constants for binding of cholesterol to hemoprotein in the various lipids show an excellent correlation with the activities in these lipids (at a constant cholesterol:phospholipid ratio). Table I demonstrates that this lipid effect on cholesterol binding is also seen in the catalytic K<sub>c</sub> for cholesterol, and little or no effect on the V<sub>max</sub> is seen.

The molecular mechanism by which phospholipid acyl chains exert their effect is of particular interest. The present studies, while they do not explain the details of the molecular interactions involved, allow us to choose among various general types of mechanisms. First, as has been suggested for several enzymes and transport proteins, the activity might be regulated by the physical properties of the lipid phase. For example, sugar transport in fatty acid auxotrophs of Escherichia coli is apparently dependent upon the fluidity of the phospholipid membrane (38). Such a mechanism is not supported for the side chain cleavage system. Cornwall and Patil (39) have pointed out that the physical properties of phospholipids vary with unsaturation as a step function rather than a continuous function; major changes occur with the introduction of a one double bond, and additional unsaturation results in only minor variations in physical properties. This is in contrast to the activity order seen in Table I for side chain cleavage, where introduction of additional double bonds produces large effects on activity. Similarly, lateral diffusion in lipid bilayers above their transition temperatures is rapid (40), and we have provided evidence that the rate of association of cholesterol with cytochrome P-450<sub>ac</sub> is far more rapid than turnover (9). Thus our data do not support a rate effect due to known physical properties of the lipid phase itself. A second possible mechanism, that lipid effects cholesterol binding to cytochrome competitively due to its own affinity for cholesterol (see Equation 1), is discussed above under "Results." Comparison of Table I and Fig. 6 with Table II reveals no positive or negative correlation of activity of K<sub>c</sub> with the affinity of a given lipid for cholesterol, thus indicating that this simple mechanism alone does not modulate the cholesterol binding equilibrium.

A third possibility, that the binding effects are due to a direct interaction of lipid with the enzyme, is most consistent with the available data. Thus, the structural specificity of a lipid binding site(s) on the enzyme could explain the observed activity order in Table I. The lipid could, for example, stabilize or destabilize a cholesterol-binding confirmation of the enzyme, thus affecting binding by interacting with a site physically distinct from the cholesterol binding site. Adrenodoxin, which forms a 1:1 complex with cytochrome P-450<sub>ac</sub>, by binding at an aqueous phase site, facilitates the binding of cholesterol by such a mechanism (9). Another possible mechanism of this general class is suggested by the observation that the cholesterol binding site on cytochrome P-450<sub>ac</sub> is in communication with the hydrophobic phospholipid milieu (5): the fatty acyl chains themselves might participate in the binding of cholesterol to its enzyme binding site. Thus, a thermodynamically stable complex among phospholipid, cholesterol, and enzyme might be formed. The present data suggest strongly that the lipid exerts its effect by a direct interaction with the enzyme, but do not distinguish between these latter two possibilities.

Finally the present studies are of particular interest regarding the short term response (~15 min) of the adrenal cortex to ACTH. This pituitary peptide hormone binds to a specific cell surface receptor and, via a series of intracellular steps (see Footnote 1 and Ref. 41 for a review), acts ultimately within the mitochondrion by an unknown mechanism to stimulate the rate of side chain cleavage of cholesterol (10). A hallmark of the steroidogenic effect of ACTH is an increase in the fraction of the mitochondrial cytochrome P-450<sub>ac</sub> the high spin form (17) with no short term change in the total content of cytochrome P-450. The spin state change has been interpreted as reflecting cholesterol binding to cytochrome, but no correlation of spin state with total mitochondrial cholesterol content is seen (18); the results are therefore often explained by proposing ACTH control of cholesterol pools within the mitochondrion. From the present studies it is clear that the parallel changes in spin state and activity seen with ACTH treatment are consistent with an ACTH-mediated decrease in the cholesterol K<sub>c</sub> of cytochrome P-450<sub>ac</sub>. Variations in the hydrophobic composition of the phospholipid in the reconstituted system induce spin state and activity changes which mimic those seen in mitochondria isolated following ACTH treatment of cells or tissues, and we suggest the possibility that these ACTH-induced changes could be mediated by some membrane phase component or components. In support of this working hypothesis, ACTH has been demonstrated to exert a variety of effects on adrenal phospholipid metabolism; ACTH causes an increased turnover of both phosphatidic acid and phosphatidyninositol within 2 min (42, 44) and an increased production of polyphosphoinositides within 15 min (43). Although the subcellular location and catalytic significance of these ACTH-controlled changes in lipid metabolism have not been reported, the present studies suggest that changes in the composition of the inner mitochondrial membrane in which cytochrome P-450<sub>ac</sub> is embedded could mediate the steroidogenic effects of ACTH.

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