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Interaction of steroids with adrenal cytochrome *P*-450 (*P*-450$_{17\alpha,\text{lyase}}$) in liposome membranes

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Purified cytochrome *P*-450$_{17\alpha,\text{lyase}}$ from guinea-pig adrenal microsomes, which catalyzes progesterone 17α-hydroxylation and sequentially C17–C20 bond cleavage of the 17α-hydroxyprogesterone, was successfully incorporated into liposomal membranes composed of only phosphatidylcholine or of a phospholipid mixture of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine at a molar ratio of 5:3:1. Although the purified *P*-450$_{17\alpha,\text{lyase}}$ was readily converted into *P*-420 in the detergent-solubilized system without substrates, the *P*-450 embedded in the liposomal membranes was found to be quite stable without the substrates. Using the *P*-450$_{17\alpha,\text{lyase}}$-proteoliposomes, the interaction of steroids with *P*-450$_{17\alpha,\text{lyase}}$ was studied for progesterone, 17α-hydroxyprogesterone and androstenedione in the liposomal system by optical difference spectroscopy and by equilibrium dialysis. The partition coefficients of steroids between the aqueous phase and the liposomal membranes were determined by the equilibrium dialysis. They were about 1.4–1.6-times higher in phosphatidylcholine liposomes than in the liposomes of the lipid mixture. The dissociation constants of the *P*-450-steroid complexes were calculated from the apparent dissociation constants using the partition coefficients for the situation where the substrate-binding site faces the lipid phase of the membranes or where it faces the aqueous phase. The dissociation constant in the former case was not affected by the lipid composition. These results suggest that *P*-450$_{17\alpha,\text{lyase}}$ might interact only with the substrates in the lipid phase of the liposomal membranes.

Introduction

Several species of cytochrome *P*-450 are located both in mitochondria and endoplasmic reticulum of adrenal cortex cells and are functioning in the biosynthesis of steroid hormones [1]. Cholesterol, the starting material for the steroidogenesis, is converted into pregnenolone by the action of *P*-450$_{scc}$ in the mitochondria and is subsequently transferred to the endoplasmic reticulum, where the pregnenolone is metabolized into progesterone by the aid of 3β-hydroxysteroid dehydrogenase-isomerase and is further converted into de-

Abbreviations: *P*-450$_{C21}$, cytochrome *P*-450 having steroid 21-hydroxylase activity; *P*-450$_{17\alpha,\text{lyase}}$, cytochrome *P*-450 having steroid 17α-hydroxylase and C17,20-lyase activities; *P*-450$_{scc}$, cytochrome *P*-450 having cholesterol desmolase activity; *P*-450$_{11\beta}$, cytochrome *P*-450 having steroid 11β-hydroxylase activity; *P*-450$_{cam}$, cytochrome *P*-450 having camphor methylene hydroxylase activity; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phospathidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

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oxycorticosterone, 17α-hydroxyprogesterone, deoxycortisol and androstenedione by $P\text{-}450_{C21}$ and/or $P\text{-}450_{17\alpha,lyase}$. Most of the metabolites are transferred back to the mitochondria and are metabolized into corticosterone, cortisol or aldosterone by $P\text{-}450_{11\beta}$. In this metabolic pathway $P\text{-}450_{17\alpha,lyase}$, which catalyzes the 17α-hydroxylation of progesterone and also the C17–C20 bond cleavage of 17α-hydroxyprogesterone, is located at a branching point leading to glucocorticoids or to androgens. Most of the 17α-hydroxyprogesterone is released from $P\text{-}450_{17\alpha,lyase}$ to be metabolized into glucocorticoids and the rest is metabolized successively into androgens without leaving $P\text{-}450_{17\alpha,lyase}$ [2,3]. In order to understand the sequential reaction catalyzed by $P\text{-}450_{17\alpha,lyase}$, it is necessary to investigate the interactions of the steroid intermediates with $P\text{-}450_{17\alpha,lyase}$.

Metabolic intermediates in the biosynthetic pathway of steroid hormones are very lipophilic and favor partitioning into the lipid phase of the membranes [4,5]. Parry et al. [6] pointed out the meaning of substrate partitioning into the membranes and the importance of distinguishing whether the substrate binding site of a membrane-bound enzyme faces the aqueous or the lipid phase. Several studies have provided some evidence suggesting the substrate binding sites of hepatic cytochrome *P*-450's might face the lipid phase of the membranes [7–9]. In our previous study, the other adrenal cytochrome *P*-450, $P\text{-}450_{C21}$, has been shown from the kinetic analysis to have its substrate-binding site in the membranes [10].

In this study, the purified $P\text{-}450_{17\alpha,lyase}$ from the adrenal microsomes of guinea pig was successfully incorporated into phospholipid vesicles. The interaction of steroids with $P\text{-}450_{17\alpha,lyase}$ was studied for progesterone, 17α-hydroxyprogesterone and androstenedione in the liposomal membranes composed of only PC or of a lipid mixture of PC, PE and PS and the effects of the lipid composition on the interaction are discussed.

Materials and Methods

$P\text{-}450_{17\alpha,lyase}$ was purified from guinea-pig adrenal microsomes according to the method previously described, in which progesterone was present in the buffer throughout the purification as the stabilizer of the *P*-450 [2]. Proteoliposome containing $P\text{-}450_{17\alpha,lyase}$ were prepared from only PC or a phospholipid mixture consisting of PC, PE and PS at a molar ratio of 5:3:1 which was comparable with the lipid composition of adrenal microsomes [11]. The phospholipids in the chloroform were dried under a stream of N_2 gas and evacuated for 4 h to remove the residual organic solvent. The dried lipids were mixed with [^{14}C]DPPC in 1.5% (w/v) sodium cholate and the suspension was ultrasonicated at 4°C for a few minutes by a sonicator (Model W-225, Heat System-Ultrasonic Inc.) with a cup horn probe in an atmosphere of Ar until the suspension became clear. $P\text{-}450_{17\alpha,lyase}$ was incubated at 0°C for 1 h in a lipid solution containing 10 mg/ml phospholipids, 6 μM $P\text{-}450_{17\alpha,lyase}$, 12 μM progesterone, 500 mM potassium phosphate buffer (pH 7.2), 1% (w/v) sodium cholate, 0.1 mM EDTA and 20% (w/v) glycerol at the final concentration. The mixture was dialyzed at 4°C for 12 h against 50 mM Tris-HCl buffer (pH 7.2) containing 200 mM NaCl and 0.1 mM EDTA and subsequently applied to a Toyo Soda HPLC system (CCMP and UV8000) with a column of Toyo Soda TSK PWH (0.75 × 7.5 cm), previously equilibrated with the dialysis buffer. The liposome fractions were detected by the ultraviolet scattering at 300 nm and pooled. The liposomes composed of only PC or of a mixture of PC, PE and PS will be referred to as PC liposomes or PCES liposomes, respectively, hereafter. $P\text{-}450_{17\alpha,lyase}$ concentration in the liposomes was determined from the CO-dithionite reduced difference spectrum using $\Delta\epsilon(450\text{ nm} - 490\text{ nm}) = 91\ \text{mM}^{-1} \cdot \text{cm}^{-1}$ [12]. The lipid concentrations of the liposome preparations were estimated from the radioactivity of [^{14}C]DPPC and also from the inorganic phosphate concentration released from the phospholipids [13]. In order to confirm the incorporation of the *P*-450 into the liposome membranes, the prepared liposomes were centrifuged on Ficoll density gradient (3–10% (w/v)) at 190 000 × *g* for 12 h with Hitachi 70P72 centrifuge using RPS 50-2 rotor. Electron microscopic observation of the liposome was carried out as described previously [10].

The partition coefficients of steroids between aqueous and lipid (liposome membranes) phases

were determined with an equilibrium dialysis method at 25 °C, using an equilibrium dialyzer (Spectrum Inc.). One cell was divided into two half cells of 1 ml by a cellulose membrane of 4.5 cm^2 (Spectrapor 2, Spectrum Inc.). A ^{3}H-labeled steroid solution of a known concentration in one half cell was equilibrated with the liposome solution in the other half cell. The buffer used in the dialysis experiments was 50 mM Tris-HCl (pH 7.2) containing 50 mM NaCl and 0.1 mM EDTA, which was used as the basal buffer in this experiment. The dialysis was continued until no more change in the concentration of the steroid was observed, which took about 2 h. The partition coefficient was defined as the ratio of the steroid concentration in the lipid phase to that in the aqueous phase, where 1 g of lipid was assumed to occupy 1 cm^3 in liposome membranes [14].

The dissociation constant of the P-$450_{17\alpha,lyase}$-steroid complex was estimated from the substrate-induced difference spectra and also from the equilibrium dialysis method. The details of the optical measurements have been described previously [10].

[1,2-^{3}H]Progesterone, 17α-[1,2-^{3}H]hydroxyprogesterone, [1,2-^{3}H]androstenedione and L-α-dipalmitoyl[*2-palmitoyl*-1-^{14}C]phosphatidylcholine were purchased from New England Nuclear Corp., Boston, MA. Androstenedione, sodium cholate, L-α-phosphatidylcholine from egg york (Type III E), L-α-phosphatidylethanolamine from egg yolk (Type III) and L-α-phosphatidylserine from bovine brain were obtained from Sigma Chemical Co., St. Louis, MO. 17α-Hydroxyprogesterone was from Fluka AG, Buch, and progesterone was from Nakarai Chemical Co., Kyoto.

Results

In order to investigate the interaction of steroids with P-$450_{17\alpha,lyase}$, it is required to prepare the P-450 in the steroid-free form, but the presence of the substrate was necessary in the detergent-solubilized state to protect the P-450 from the inactivation [2]. The incubation of P-$450_{17\alpha,lyase}$ in the phospholipid solution was performed in the presence of progesterone and glycerol, but the following dialysis for the preparation of the proteoliposomes was carried out without progesterone and glycerol. Progesterone concentration in the dialyzed solution was reduced to 0.2 μM from 12 μM in the incubation medium, which was still a quite high concentration, and almost all P-$450_{17\alpha,lyase}$ was in the high-spin form. The HPLC gel filtration with a TSK PWH column decreased the concentration of progesterone to less than 5 nM and that of cholic acid to less than 0.001% (w/v), where P-$450_{17\alpha,lyase}$ was all in the low-spin form. The incorporation of P-$450_{17\alpha,lyase}$ into the liposomal membranes was ascertained by the Ficoll density gradient centrifugation, as shown in Fig. 1. The purified P-$450_{17\alpha,lyase}$ on the density gradient was found from fraction 3 (approx. density 1.046) to fraction 11 (approx. density 1.023), whereas the P-450 dialyzed with cholate-solubilized lipids was located around fraction 15 (approx. density 1.012) which was the same position as phospholipids. Moreover, the molar ratio of P-$450_{17\alpha,lyase}$ to the phospholipids of the liposome fraction was about the same as that of the sample before the density gradient centrifugation, suggesting that almost all the P-450 in the prepared sample was present in the liposome membranes. Electron microscopic observations showed that the prepared proteoliposomes were unilamellar vesicles of about 50 nm in

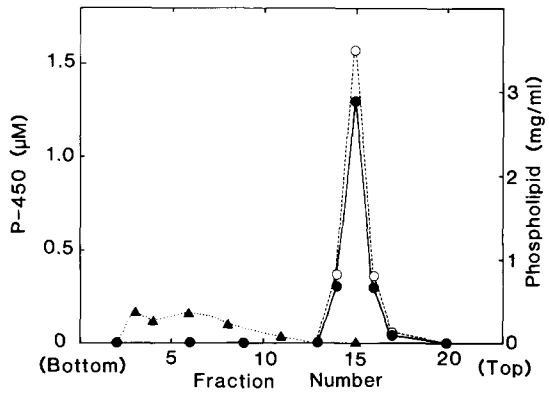

Fig. 1. Sedimentation patterns of P-$450_{17\alpha,lyase}$ and P-$450_{17\alpha,lyase}$-proteoliposomes on the Ficoll density gradient (3–10%, w/v). The dotted line with closed triangles shows the distribution of the P-450 after the centrifugation of 0.5 nmol of purified P-$450_{17\alpha,lyase}$. The full line with closed circles shows concentration of P-$450_{17\alpha,lyase}$ and the broken line with the open circles shows the concentration of phospholipids after the centrifugation of 0.5 nmol of P-$450_{17\alpha,lyase}$ incorporated into 1.2 mg of PCES liposomes by the cholate-dialysis method.

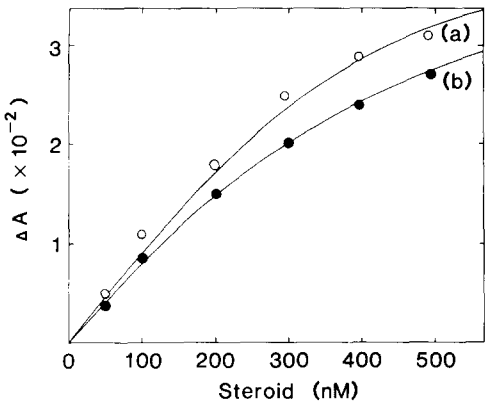

Fig. 2. Effects of lipid concentration on the substrate induced-difference spectra of $P\text{-}450_{17\alpha,\text{lyase}}$ embedded in PC liposome membranes. The horizontal axis represents the total concentration of 17α-hydroxyprogesterone in the system. The closed and open circles show the magnitudes of the difference spectra, ΔA(389 nm − 421 nm), observed at 25 °C in the system containing 380 nM $P\text{-}450_{17\alpha,\text{lyase}}$ in 1 mg/ml and 3 mg/ml of phospholipid membranes in the basal buffer, respectively. The curves (a) and (b) were generated using apparent dissociation constants of 40 nM and 95 nM, respectively, which were obtained by the least squares method for the observed points.

the average diameter. $P\text{-}450_{17\alpha,\text{lyase}}$ incorporated into liposomal membranes was quite stable without progesterone and glycerol, and only about 20% of the incorporated $P\text{-}450_{17\alpha,\text{lyase}}$ was converted into the inactive form during the storage at 25 °C for 24 h, while 80% of the detergent-solubilized $P\text{-}450_{17\alpha,\text{lyase}}$ was inactivated under the same condition.

Upon the addition of 17α-hydroxyprogesterone to $P\text{-}450_{17\alpha,\text{lyase}}$ proteoliposomes, the optical absorption spectrum in the oxidized form was converted from the low-spin to the high-spin form and the difference between those two showed a maximum at 389 nm and a trough at 421 nm with an isosbestic point at 407 nm. The magnitude of the difference spectrum was considered to be proportional to the concentration of the *P*-450-substrate complex. The magnitudes of the difference spectra were plotted against the apparent concentrations of the added 17α-hydroxyprogesterone in Fig. 2. Curve (a) was obtained for the $P\text{-}450_{17\alpha,\text{lyase}}$-proteoliposomes prepared from only PC in which the lipid concentration was 1 mg/ml. The curve (b) was measured for the sample where $P\text{-}450_{17\alpha,\text{lyase}}$ concentration was the same as (a), but the lipid concentration was three times higher than that in (a). At the higher PC concentration, the magnitude of the difference spectrum at a certain steroid concentration was apparently smaller, which might be reflecting the lower concentration of the steroid around $P\text{-}450_{17\alpha,\text{lyase}}$. The magnitude of the 17α-hydroxyprogesterone-induced difference spectrum of $P\text{-}450_{17\alpha,\text{lyase}}$ incorporated into PCES liposomes was a little smaller than that observed for PC liposomes under the same conditions except for the lipid composition. In order to assess the *P*-450-steroid complex concentration, the absorption coefficient differences, $\Delta\epsilon$(389 nm − 421 nm), in the difference spectra were required, which were determined from the magnitudes of the difference spectra induced by the excess addition of the steroids to the system. As in Table I, the absorption coefficient difference is not much different for each steroid between those in PC and in PCES liposomes. The values for 17α-hydroxyprogesterone were similar to those for progesterone either in PC or in PCES liposomes, but the values for androstenedione were quite smaller than those of other steroids. The similarity of the absorption coefficient difference between two liposomal systems suggests that the conformation of the *P*-450-steroid complex in PC liposomes might be similar to that in PCES liposomes. The apparent dissociation constants of the $P\text{-}450_{17\alpha,\text{lyase}}$-steroid complexes could be determined using the absorption coefficient differences under an assumption that $P\text{-}450_{17\alpha,\text{lyase}}$

TABLE I

DIFFERENCE ABSORPTION COEFFICIENTS, $\Delta\epsilon$ (389–421 nm), OF THE STEROID-INDUCED DIFFERENCE SPECTRA OF $P\text{-}450_{17\alpha,\text{lyase}}$ IN LIPOSOMAL MEMBRANES

The difference spectra were measured at 25 °C in the basal buffer upon the addition of 5 μM of progesterone, 5 μM of 17α-hydroxyprogesterone and 30 μM of androstenedione, respectively.

Steroids	$\Delta\epsilon$ ($mM^{-1}\cdot cm^{-1}$)	
	PC liposomes	PCES liposomes
Progesterone	110 ± 4	108 ± 5
17α-Hydroxyprogesterone	105 ± 2	106 ± 5
Androstenedione	19 ± 2	20 ± 2

TABLE II

PARTITION COEFFICIENTS OF STEROIDS BETWEEN AQUEOUS AND LIPID PHASES

The partition coefficients were measured in the basal buffer at 25 °C by equilibrium dialysis using [^{3}H]steroids.

Steroids	K_p	
	PC liposomes	PCES liposomes
Progesterone	3200 ± 200	2300 ± 200
17α-Hydroxyprogesterone	1500 ± 100	920 ± 50
Androstenedione	440 ± 40	320 ± 20

might bind only one steroid molecule. The solid lines in Fig. 2 are the theoretical curves using the apparent dissociation constants calculated by the least-squares method, which agree fairly well with the observed points.

The decrease in the magnitude of the difference spectrum caused by the increment of the amount of the lipid in the system must be attributed to the difference in the concentration of the steroids around the *P*-450 molecules, which might be originated by partitioning of the steroid molecule between the aqueous and the lipid phases. The partition coefficients of the steroids, K_p, were measured by the equilibrium dialysis as described in Materials and Methods. The values of partition coefficient of the steroids in PC and PCES liposomes were constant when the lipid concentration of the system was varied and were not changed by the variation of the steroid concentration, which are listed in Table II.

The Scatchard plot was used for the determination of the number of substrate binding site in

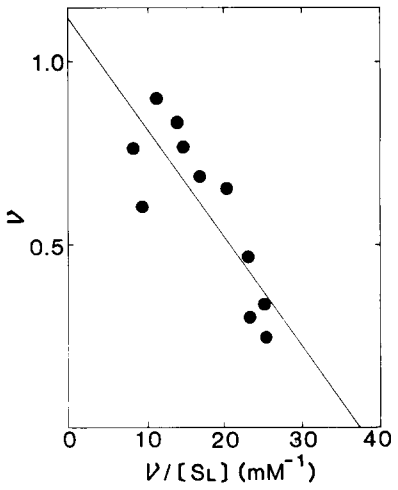

Fig. 3. Scatchard plot for the determination of the number of substrate binding site in $P\text{-}450_{17\alpha,\text{lyase}}$ incorporated into the PCES liposome membranes. ν in the vertical axis represents the average number of 17α-hydroxyprogesterone molecules bound to one $P\text{-}450_{17\alpha,\text{lyase}}$ molecule, which were estimated by the equilibrium dialysis in the basal buffer at 25 °C. [SL] represents the concentration of 17α-hydroxyprogesterone in the lipid phase of the membranes. Details are described in the text.

$P\text{-}450_{17\alpha,\text{lyase}}$ in the liposome membranes [15]. The vertical axis in Fig. 3 represents, ν, the average number of 17α-hydroxyprogesterone molecules bound to one *P*-450 molecule which can be obtained by the equilibrium dialysis. The horizontal axis represents ν/[SL], where [SL] is the concentration of 17α-hydroxyprogesterone in the lipid phase. The values of [SL] were estimated as the product of the partition coefficient and the steroid concentrations in the aqueous phase. The observed points in Fig. 3 scatter considerably and

TABLE III

DISSOCIATION CONSTANTS OF $P\text{-}450_{17\alpha,\text{lyase}}$-STEROID COMPLEXES IN THE LIPOSOMAL MEMBRANES

The dissociation constants were calculated using the experimental results obtained by the equilibrium dialysis under the assumption that $P\text{-}450_{17\alpha,\text{lyase}}$ interacts only with the steroids in the aqueous phase (K_{d_A}) or with those in the lipid phase of the membrane (K_{d_L}). The equilibrium dialysis was performed in the basal buffer at 25 °C. The details were in the text.

Steroids	K_{d_L} (μM)		K_{d_A} (nM)	
	PC liposomes	PCES liposomes	PC liposomes	PCES liposomes
Progesterone	35 ± 5	40 ± 8	10 ± 3	18 ± 4
17α-Hydroxyprogesterone	26 ± 6	30 ± 8	20 ± 5	32 ± 8
Androstenedione	600 ± 80	600 ± 90	1100 ± 200	1900 ± 300

the line calculated by the least-squares method crosses over the vertical axis at 1.1, suggesting one substrate binding site in $P\text{-}450_{17\alpha,\text{lyase}}$. The dissociation constant can be obtained as the slope of the line in Fig. 3 under the assumption that the $P\text{-}450_{17\alpha,\text{lyase}}$ interacts only with steroids in the lipid phase of the membranes. The K_d values for other systems were obtained in a similar way, which are listed in Table III. K_{d_L} and K_{d_A} represent dissociation constants of $P\text{-}450_{17\alpha,\text{lyase}}$-steroid complexes in the cases where $P\text{-}450_{17\alpha,\text{lyase}}$ interacts only with the steroids in the lipid phase and only with those in the aqueous phase, respectively.

Discussion

The partition coefficients for the steroids in PC liposomes were apparently larger than those in PCES liposomes as summarized in Table II. The ratio of the K_p value in PC liposomes to that in PCES liposomes ranged around 1.4–1.6 for each steroid, which means that the solubility of the steroid in lipid phase might be affected in a similar way by the head groups of the phospholipids. The partition coefficients of steroids for the microsomal membranes are expected to be different from these values in Table II but might be similar to those in PCES liposomes rather than those in PC liposomes. The partition coefficient of progesterone in DMPC liposomes has been reported by Arrowsmith et al. [16], which is about half of the value obtained in this study, suggesting the partition coefficient might be affected by the fatty acid chains of the phosphatidylcholine.

There are two possible schemes for the interaction of substrates with $P\text{-}450_{17\alpha,\text{lyase}}$. One is that the substrate-binding site of the *P*-450 faces aqueous phase and the *P*-450 interacts only with the substrates in the aqueous phase, which will be referred to as model A. The other is that the binding site faces lipid phase of the membrane and the *P*-450 interacts only with the substrates in the lipid phase, which will be referred to as model L. The dissociation constant of $P\text{-}450_{17\alpha,\text{lyase}}$-17α-hydroxyprogesterone complexes in model L was obtained as the slope of the Scatchard plot in Fig. 3. In order to determine the dissociation constant in model A, K_{d_A}, the horizontal axis should be changed to represent $\nu/[SA]$, where [SA] is the concentration of the steroid in the aqueous phase, which is attained by expansion of the horizontal scale of Fig. 3 by a factor of K_p. The scale expansion in the horizontal axis does not change the intercept at the vertical axis and the number of binding sites can be reasonably assumed to be one for $P\text{-}450_{17\alpha,\text{lyase}}$ in either of the models. There are, however, some reports that *P*-450 might have two substrate-binding sites, one for strong binding and the other for weaker binding [17]. The existence of a weaker substrate-binding site in $P\text{-}450_{17\alpha,\text{lyase}}$ can not be excluded by the Scatchard plot in Fig. 3, because high concentration of the steroid makes it difficult to obtain the correct concentration of *P*-450-steroid complex by the equilibrium dialysis method. The dissociation constant for model A, K_{d_A}, can be calculated as K_{d_L}/K_p, the values of which are listed in Table III. The dissociation constants of $P\text{-}450_{17\alpha,\text{lyase}}$-steroid complexes were also determined by the differences spectra measurements where the apparent dissociation constants, obtained directly from the titration curve as Fig. 1, should be divided by $(V_L/V_T + 1/K_p)$ for the determination of K_{d_L} and by $(K_pV_L/V_T + 1)$ for K_{d_A} in which V_T and V_L represent the volumes of the system and lipid phase, respectively [10]. The K_d values obtained by the difference spectra were fairly in good agreement with those obtained by the equilibrium dialysis in Table III.

The values of K_{d_L} of the complexes for all the three steroids in PC liposomes are equal within the experimental error to those in the PCES liposomes, suggesting that in the model L the difference in the lipid composition of the membranes might exert little effect on the conformation of $P\text{-}450_{17\alpha,\text{lyase}}$. The absorption coefficients of the $P\text{-}450_{17\alpha,\text{lyase}}$-steroid complexes in PC liposomes are almost equal to those in PCES liposomes, which supports the above discussion. The values of K_{d_L} lie in the order of 17α-hydroxyprogesterone, progesterone and androstenedione, which seems physiologically reasonable, because some of 17α-hydroxyprogesterone, the metabolic intermediate, must be further metabolized sequentially into androstenedione [1] and the final product, androstenedione, must be readily released from $P\text{-}450_{17\alpha,\text{lyase}}$. In model A, K_{d_A} values for steroids in PCES liposomes are about 1.7-times

higher than those in PC liposomes and K_{d_A} values for 17α-hydroxyprogesterone are 1.8–2.0-times higher than those for progesterone, which is in conflict with the above discussion for model L. Furthermore, K_{d_A} values for 17α-hydroxyprogesterone and progesterone are much smaller than the value for $P\text{-}450_{cam}$-camphor complex, where $P\text{-}450_{cam}$ is functioning in aqueous phase and the substrate-binding site undoubtedly faces the aqueous phase [18]. The agreement of K_{d_L} values of three different steroids in PC liposomes with those in PCES liposomes within the the experimental error can not be conceived as accidental, but must be a strong evidence in support of model L.

The hydrophobic compounds such as cholesterol can dissolve very slightly in aqueous phase and almost all of them in the lipid phase of the membrane. Cholesterol-$P\text{-}450_{scc}$ complex has been reported to release cholesterol when the complex was incorporated into the membrane, indicating that the cholesterol-binding site of $P\text{-}450_{scc}$ faces the lipid phase [19,20]. Lombardo et al showed a concrete evidence for model L that the 34 kDa hydrophobic fragment of $P\text{-}450_{11\beta}$, produced by tryptic treatment of $P\text{-}450_{11\beta}$ embedded in membranes, had a partial 11β-hydroxylase activity [21]. It has been shown experimentally that $P\text{-}450_{C21}$ has the substrate-binding site in the lipid phase in the membrane [10] and the present study also indicates the same orientation for $P\text{-}450_{17\alpha,lyase}$. Thus, the results of these studies convince us that the substrate-binding sites of all of the major steroidogenic P-450 species in adrenal cortex face the lipid phase of membranes.

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