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INTERACTION BETWEEN NADPH-CYTOCHROME *P*-450 REDUCTASE AND CYTOCHROME *P*-450 IN THE MEMBRANE OF PHOSPHATIDYLCHOLINE VESICLES

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Summary

Cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, both purified from liver microsomes of phenobarbital-pretreated rabbits, have been incorporated into the membrane of phosphatidylcholine vesicles by the cholate dialysis method. The reduction of cytochrome *P*-450 by NADPH in this system is biphasic, consisting of two first-order reactions. The rate constant of the fast phase, in which 80—90% of the total cytochrome is reduced, increases as the molar ratio of the reductase to the cytochrome is increased at a fixed ratio of the cytochrome to phosphatidylcholine, suggesting that the rate-limiting step of the fast phase is the interaction between the reductase and the cytochrome. The rate constant of the fast phase also increases when the amount of phosphatidylcholine, relative to those of the two proteins, is decreased. This latter observation suggests that the interaction between the two proteins is effected by their random collision caused by their lateral mobilities on the plane of the membrane of phosphatidylcholine vesicles. The rate constant of the slow phase as well as the fraction of cytochrome *P*-450 reducible in the slow phase, on the other hand, remains essentially constant even upon alteration in the ratio of the reductase to the cytochrome or in that of the two proteins to phosphatidylcholine. No satisfactory explanation is as yet available for the cause of the slow-phase reduction of cytochrome *P*-450. The overall activity of benzphetamine N-demethylation catalyzed by the reconstituted vesicles responds to changes in the composition of the system in a similar way to the fast-phase reduction of cytochrome *P*-450, though the latter is not the rate-limiting step of the overall reaction.

Introduction

Two possible mechanisms are conceivable for functional interactions among component proteins of membrane-bound multienzyme systems. Thus, it is possible that the interactions are effected by the lateral motion and subsequent collision of the proteins on the plane of membranes, whereas another possibility is that the proteins exist in membranes as functional clusters in which the interactions take place directly. The hepatic microsomal monooxygenase system consisting of NADPH-cytochrome *P*-450 (cytochrome *c*) reductase and cytochrome *P*-450 is one of such membrane-bound systems and the mode of interaction between the reductase and the cytochrome in microsomal membranes has been extensively studied [1—8]. However, no consensus has yet been reached concerning the mechanism of this interaction. Thus, the results obtained by Estabrook and coworkers [1—5] and by Stier and Sackman [2] seem to support the cluster model. whereas Duppel and Ullrich [5] and Yang and coworkers [3,7] have reported evidence in favor of the view that the interaction is achieved by lateral diffusion and collision of the two proteins. One reason for such disagreement appears to be the fact that most of the experiments so far reported were conducted with intact or variously modified liver microsomes, which are rather complex in both composition and structure.

A promising approach to studies of membrane-bound enzyme systems is to isolate their individual protein components, characterize them, and to reconstitute the systems in artificial phospholipid membranes. Since NADPH-cytochrome *P*-450 reductase [9—12] and multiple forms of cytochrome *P*-450 [13—16] have recently been purified from liver microsomes, it is now feasible to undertake such reconstitution studies on the hepatic microsomal monooxygenase system. Actually, successful incorporation of purified preparations of the reductase and cytochrome *P*-450 into the membranes of microsomes and phospholipid vesicles has already been reported [17—23].

In the present study we incorporated homogeneous preparations of NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450, both purified from liver microsomes of phenobarbital-pretreated rabbits, into egg-yolk phosphatidylcholine vesicles by the cholate dialysis method [24], and studied the kinetics of NADPH-dependent reduction of cytochrome *P*-450 in the monooxygenase system thus reconstituted. The results obtained indicate that the reduction is a biphasic process, consisting of two concurrent first-order reactions, and suggest that the fast-phase reduction, in which 80—90% of the total cytochrome is reduced, takes place by random collision of the reductase with the cytochrome caused by their lateral mobilities in the vesicular membrane.

Materials and Methods

Materials

Cholic acid, purchased from Nissui Yakuhin Co., was recrystallized and converted to sodium salt as described previously [25]. Emulgen 913, a poly-

oxyethylene nonylphenyl ether, was supplied by Kao-Atlas Co., Tokyo. Egg-yolk phosphatidylcholine was prepared by the method of Singleton et al. [26] and its purity was confirmed by thin-layer chromatography on Silica gel G plates with chloroform/methanol/water (65 : 25 : 4, v/v/v) as solvent. *N*-[*Me*-^{14}C]-phosphatidylcholine was obtained from Radiochemical Centre, Amersham, England. Benzphetamine was a generous gift from Dr. T. Kamataki of Keio University School of Medicine, and NADPH was the product of Oriental Yeast Co., Tokyo. Horseheart cytochrome *c* (Type III), catalase, and glucose oxidase (Type IV) were purchased from Sigma Chemical Co., and 2′,5′-ADP-Sepharose 4B from Pharmacia Fine Chemicals, Inc. Other chemicals used were of the highest quality available commercially.

Purification of cytochrome P-450

Cytochrome *P*-450 was purified to a gel-electrophoretically homogeneous state from liver microsomes of phenobarbital-induced rabbits by the method of Imai and Sato [13]. The purified preparation contained 17—18 nmol cytochrome *P*-450 chromophore per mg protein and was free from detergents. It was stored at −70° in 200 mM potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol.

Purification of NADPH-cytochrome P-450 reductase

NADPH-cytochrome *P*-450 reductase was also purified from phenobarbital-induced rabbit liver microsomes by a modification of the method of Yasukochi and Masters [11]. Microsomes (1.5—2.0 g protein) were suspended (to 8 mg protein per ml) in 0.1 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, and 1.5% (w/v) Emulgen 913. The suspension was centrifuged at 77 000 × *g* for 90 min and the clear supernatant fluid was applied to a DEAE-Sephadex A-50 column (3.7 × 18 cm) which had been equilibrated with 0.1 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol, and 0.5% Emulgen 913. The column was washed with 200 ml equilibration buffer and then with 500 ml of the same buffer containing 0.12 M KCl. NADPH-cytochrome *P*-450 reductase was then eluted with 500 ml of a linear KCl concentration gradient from 0.12 to 0.4 M in the equilibration buffer. The fractions containing more than 5 units reductase activity per mg protein were combined and directly applied to a 2′,5′-ADP-Sepharose 4B column (1.7 × 10 cm) equilibrated with 0.1 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 0.5 mM EDTA, 0.1 mM dithiothreitol and 0.1% (w/v) sodium cholate. After washing the column with 500 ml equilibration buffer, the reductase was eluted with the same buffer containing 0.5 mM $NADP^+$. The fractions containing NADPH-cytochrome *c* reductase activity were pooled and dialyzed at 4°C for 24 h against a large volume of 0.1 M Tris-HCl buffer (pH 7.7) containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol to remove $NADP^+$ and most of the detergent. The dialyzed solution was concentrated by means of a collodion bag and stored at −70°. The final preparation had a specific NADPH-cytochrome *c* reductase activity of 40—45 units per mg protein and was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The recovery of the reductase from microsomes was about 40%.

Analytical and assay methods

Protein was determined by the method of Lowry et al. [27] using bovine serum albumin as a standard. Phospholipid phosphorus was determined by the method of Bartlett [22] after sulfuric acid digestion. ^{14}C radioactivity was measured in a Beckman LS-250 liquid scintillation spectrometer in a medium consisting of 4 g 2,5-diphenyloxazole, 1 l toluene, and 500 ml Triton X-100. Cytochrome *P*-450 was estimated from the CO difference spectrum as described by Omura and Sato [29]; purified cytochrome *P*-450, however, was determined from the absorbance difference between 418 and 500 nm, using a millimolar extinction coefficient increment of 101 (Imai, Y., Hashimoto-Yutsudo, C. and Sato, R., unpublished). NADPH-cytochrome *P*-450 reductase was determined by measuring its NADPH-cytochrome *c* reductase activity in 0.3 M potassium phosphate buffer (pH 7.5) at 25°C as described by Imai [30]. 1 unit of the reductase was defined as the amount catalyzing the reduction of 1 μmol cytochrome *c* per min. Highly purified NADPH-cytochrome *P*-450 reductase was also determined from the intensity of flavin absorption at 455 nm, assuming a millimolar extinction coefficient of 10.7 [31]. Benzphetamine N-demethylation by the reconstituted system was assayed by measuring the formation of formaldehyde by the method of Nash [32]. The reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.25), 1 mM benzphetamine, 0.75 mM NADPH, and the reconstituted system (usually about 0.8 μM in terms of cytochrome *P*-450) in a final volume of 1.0 ml, and the reaction was carried out at 25°C for 5 min.

Reconstitution of monooxygenase system on phosphatidylcholine vesicles

A desired amount of egg-yolk phosphatidylcholine (containing, if necessary, a trace amount of [^{14}C]phosphatidylcholine) dissolved in chloroform was evaporated under a stream of nitrogen gas, and the residual solvent was further removed in vacuo for 1 h. The dried lipid was dispersed in 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, and 1% (w/w) sodium cholate to give a concentration of 2 mg phosphatidylcholine per ml, and the suspension was briefly sonicated to clarify it. An amount (usually 15 nmol) of cytochrome *P*-450 and a desired amount of NADPH-cytochrome *P*-450 reductase were added to 2.0 ml phosphatidylcholine dispersion, and the volume of the mixture was adjusted to 4.0 ml with 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA and 0.1 mM dithiothreitol ("dialysis buffer"). The mixture was then dialyzed at 4°C for 36–48 h against 3 l dialysis buffer; the buffer was renewed every 12 h. The slightly turbid solution thus obtained was briefly sonicated and used as the reconstituted system.

Stopped flow experiments

The reduction of cytochrome *P*-450 by NADPH in the reconstituted system was studied in a Union Giken RA 401 stopped flow spectrophotometer equipped with a kinetic data processor (system 71 or RA 108T) by following the formation of the CO complex of ferrous cytochrome *P*-450 at 450 nm, essentially as described by Imai et al. [33]. The reconstituted system was suspended in 0.1 M potassium phosphate buffer (pH 7.25) containing

0.8 mM benzphetamine, 30 mM glucose, and catalase (1500 units/ml), and the suspension was gently bubbled with CO for 3 min. Glucose oxidase (0.125 mg) dissolved in 50 μl of the same buffer was then added to 2.45 ml suspension and bubbling with CO was continued for 0.5 min. This suspension was mixed in the stopped flow apparatus with 2.5 ml 0.1 M potassium phosphate buffer (pH 7.25) containing 0.6 mM NADPH, 30 mM glucose, catalase (1500 units/ml), and 0.125 mg glucose oxidase, which had been saturated with CO as above. The final concentration of the reconstituted system in the mixing chamber of the stopped flow apparatus was about 0.2 μM cytochrome *P*-450, unless otherwise indicated. The reduction of cytochrome *P*-450 in a reconstituted system consisting of the reductase, the cytochrome, and Emulgen 913 was determined in the same way as described previously [33], except that the Emulgen concentration was 0.005% and the reaction mixture contained glucose, glucose oxidase, and catalase. All measurements were carried out at 25°C.

Electron microscopy

The reconstituted system suspended in 50 mM potassium phosphate buffer (pH 7.25) was placed on a specimen mesh coated with a collodion film. The sample was then stained with 1% phosphotungstic acid (pH adjusted to 7.0 with KOH) and observed in a Hitachi HU-12 electron microscope.

Sucrose density gradient centrifugation

The reconstituted system prepared in the presence of a trace amount of [^{14}C]phosphatidylcholine was suspended in 0.3 ml 50 mM potassium phosphate buffer (pH 7.25) containing 0.1 mM EDTA and 0.1 mM dithiothreitol, and the suspension was layered over 5 ml linear sucrose gradient from 5 to 42% (w/w) containing 50 mM potassium phosphate buffer (pH 7.25), 0.1 mM EDTA, and 0.1 mM dithiothreitol. The sample was centrifuged in an RPS-50 II rotor of the Hitachi 55 P centrifuge at 235 000 × *g* for 31 h. After centrifugation, 4-drop fractions were collected from the bottom of the tube and analyzed for cytochrome *P*-450, NADPH-cytochrome *c* reductase activity, and ^{14}C radioactivity.

Results

Characterization of reconstituted system

In this study, the hepatic microsomal monooxygenase system was reconstituted from cytochrome *P*-450, NADPH-cytochrome *P*-450 reductase, and egg-yolk phosphatidylcholine by the cholate dialysis method [24]. Fig. 1 shows a negative-staining electron micrograph of the system reconstituted from cytochrome *P*-450, the reductase, and phosphatidylcholine at a molar ratio of 1 : 0.5 : 350. As can be seen, the preparation consisted of numerous vesicles, the diameter of which ranged from 300 to 2000 Å, and most of the vesicles were single-walled, though multilamellar structures were also seen occasionally. The relatively large size and heterogeneity of the diameter have also been reported for protein-free phospholipid vesicles prepared by the cholate dialysis method [34]. To examine if the cytochrome and the reductase added had actually been incorporated into the vesicles, a reconstituted system consisting

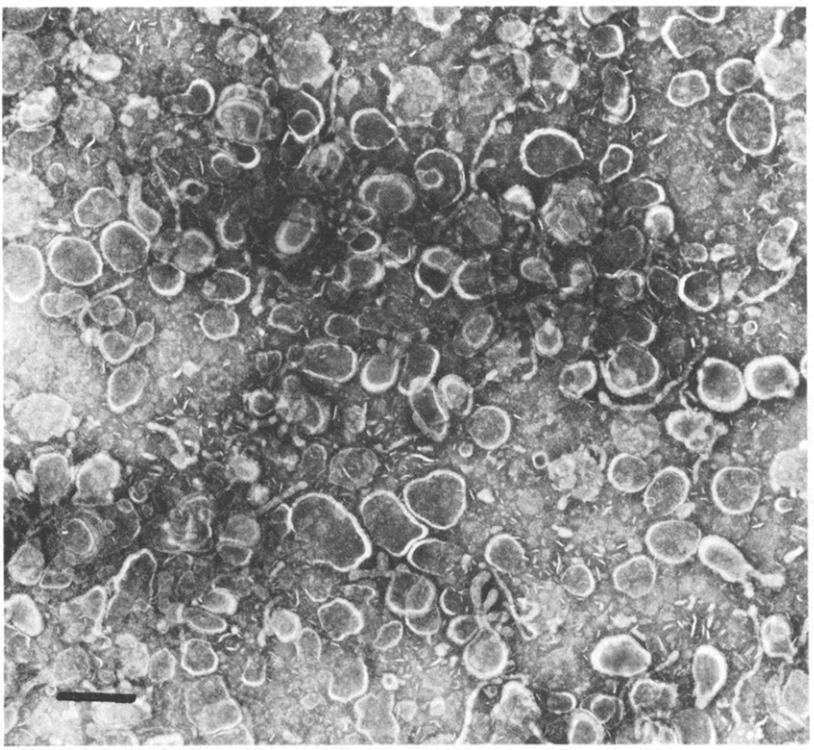


Fig. 1. Negative-staining electron micrograph of a reconstituted monooxygenase system. The system was prepared as described in Materials and Methods and consisted of NADPH-cytochrome *P*-450 reductase, cytochrome *P*-450, and egg-yolk phosphatidylcholine at a molar ratio of 0.5 : 1 : 350. The bar represents 2000 Å.

of the cytochrome, the reductase, and egg phosphatidylcholine (containing a trace amount of [^{14}C]phosphatidylcholine) at the same molar ratio as in Fig. 1 was subjected to sucrose density gradient centrifugation, and the sedimentation behavior of the three components was monitored by measuring the absorbance increment between 418 and 500 nm (for cytochrome *P*-450), NADPH-cytochrome *c* reductase activity (for the reductase), and ^{14}C radioactivity (for phosphatidylcholine). As shown in Fig. 2, all the three components exhibited almost the same sedimentation profiles having sharp peaks at the same position. When the two proteins were separately subjected to centrifugation in the absence of phosphatidylcholine under the same conditions as above, they formed peaks near the bottom of the gradient (data not shown). It could be concluded that all the molecules of the two proteins and phosphatidylcholine had actually formed vesicular complexes, which were rather homogeneous with respect to sedimentation behavior.

To obtain information concerning the topological location of the reductase in the reconstituted system, the NADPH-cytochrome *c* reductase activity of the system was assayed before and after disruption of the vesicular membrane with 1% Emulgen 913. It was thus found that a slight degree of activation (less than 25%) was caused by the detergent treatment, suggesting that only a small fraction of the reductase in the vesicles was not accessible to

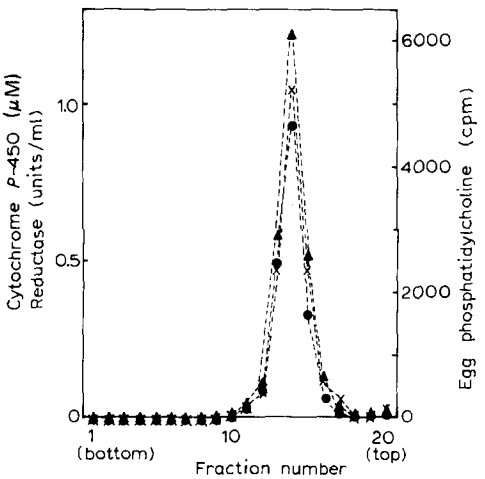


Fig. 2. Sucrose density gradient centrifugation of the reconstituted system. The composition of the system was the same as in Fig. 1, except that it contained a trace amount of [^{14}C]phosphatidylcholine. Centrifugation was conducted as described in Materials and Methods. Each fraction was analyzed for the absorbance increment between 418 and 500 nm (for cytochrome *P*-450, ●), NADPH-cytochrome *c* reductase activity (for NADPH-cytochrome *P*-450 reductase, ▲), and [^{14}C]radioactivity (for phosphatidylcholine, X).

either NADPH, cytochrome *c*, or both. Thus, at least 80% of the reductase seemed to be located at the outer surface of the vesicular membrane*. Despite several attempts, the topology of cytochrome *P*-450, on the other hand, could not be determined. However. the cytochrome in the vesicles was almost completely (more than 95%) reducible by NADPH under anaerobic conditions, indicating the almost all the cytochrome *P*-450 molecules could functionally interact with the reductase that had been reduced by exogenously added NADPH. As will be described below, the reconstituted system could catalyze the NADPH-dependent demethylation of benzphetamine at rates comparable to those reported for other types of reconstituted system [33,35].

Kinetics of cytochrome P-450 reduction in reconstituted vesicles

The reduction of cytochrome *P*-450 by NADPH in the reconstituted vesicles was studied in the stopped flow spectrophotometer by following the formation of the CO complex of ferrous cytochrome *P*-450 at 450 nm in a CO-saturated medium. This method should give the true rate of cytochrome *P*-450 reduction, since the binding of CO to the reduced cytochrome is a much faster reaction than the reduction [36]. To obtain information concerning the kinetics of cytochrome *P*-450 reduction during drug metabolism, all the measurements were carried out in the presence of 0.8 mM benzphetamine. Fig. 3 shows typical time courses of NADPH-dependent reduction of cytochrome *P*-450 measured at different concentrations of the vesicles. The absorbance change at 450 nm after completion of the reduction was proportional to the concentration of the vesicles and, therefore, to that of cytochrome *P*-450 in the reaction mixture. As mentioned above, the final level corresponded to almost complete (more than 95%) reduction of the cytochrome.

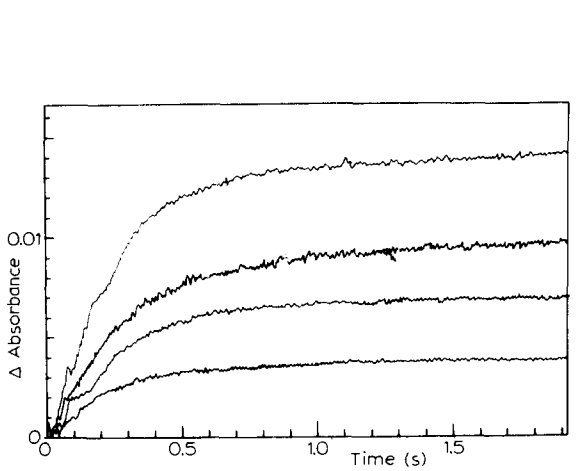
*In the absence of liposomes, the reductase activity was not activated by 1% Emulgen 913.

As is evident from Fig. 4, in which the logarithm of the oxidized cytochrome remaining is plotted against time, the reduction was clearly biphasic, consisting of two concurrent first-order reactions. Approx. 80% of the total cytochrome was reduced in the fast-phase and the apparent first-order rate constant of the fast phase was bout 20-fold as high as that of the slow-phase in the system consisting of cytochrome *P*-450, the reductase, and phosphatidylcholine at a molar ratio of 1 : 0.22 : 400, regardless of the concentration of the vesicles used. These features of kinetics of cytochrome *P*-450 reduction in the reconstituted vesicles are similar to those reported by Peterson et al. [5] for the same reaction in intact liver microsomes.

In Fig. 5 are plotted the fraction of cytochrome *P*-450 reducible in the slow-phase, the rate constant of the fast-phase, and that of the slow-phase against the concentration of the reconstituted vesicles in the reaction mixture. In the concentration range studied, all the three kinetic parameters remained essentially constant even when the concentration of the vesicles was varied 5-fold. It could, therefore, be concluded that the individual vesicles in the mixture acted as discrete, non-interacting units and cytochrome *P*-450 in one vesicle could not be reduced by the reductase residing on another, separate vesicles.

Effect of the cytochrome P-450 to the reductase ratio on kinetics of cytochrome P-450 reduction

To obtain further information concerning the mechanism of cytochrome



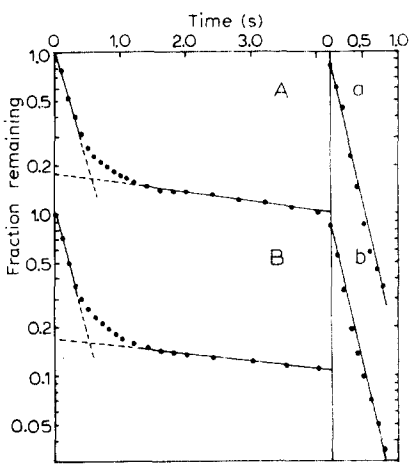


Fig. 3. Time courses of NADPH-dependent reduction of cytochrome *P*-450 in the reconstituted system in the presence of 0.8 mM benzphetamine. Stopped flow spectrophotometric measurements were carried out as described in Materials and Methods. The system used contained NADPH-cytochrome *P*-450 reductase, cytochrome *P*-450, and phosphatidylcholine at a molar ratio of 0.22 : 1 : 400. The final concentrations of the system were 0.04, 0.08, 0.12, and 0.2 μM in terms of cytochrome *P*-450 (from the lowest curve).

Fig. 4. First-order plots of the rate of cytochrome *P*-450 reduction at two different concentrations of the reconstituted system. Logarithm of $(A_t - A_{max})/A_{max}$ was plotted against time after the stop of flow, where A_t and A_{max} are the observed absorbance changes at time t and after the completion of the reduction, respectively. The final concentrations of the system were 0.08 μM (A) and 0.2 μM (B) with respect to cytochrome *P*-450, and the data were taken from Fig. 3. Plots for the fast-phase reduction (a and b) were obtained by subtracting the values for the slow-phase reduction from the uncorrected fast-phase portion of the curves. Rate constants of the fast phase were 4.0 and 4.3 s^{-1} for A and B, respectively, and those of the slow phase were 0.13 and 0.11 s^{-1}, respectively.

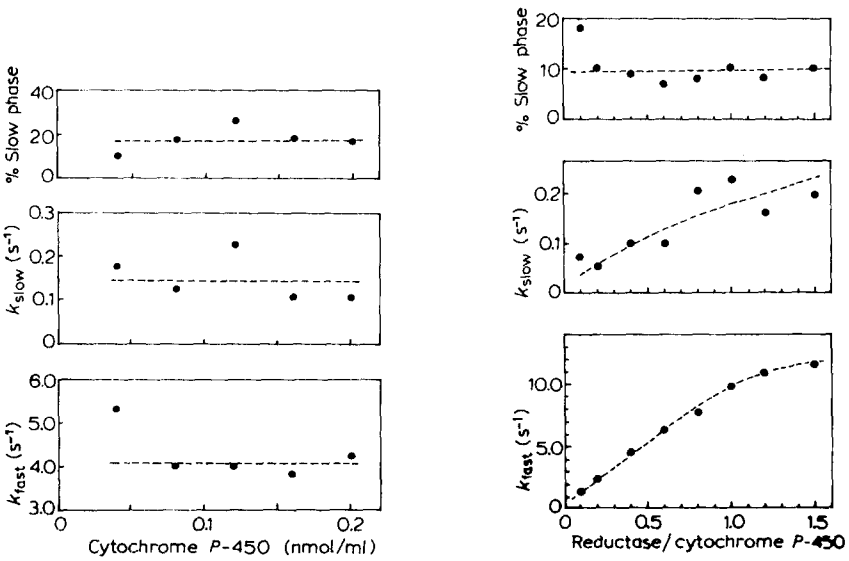


Fig. 5. Dependence of kinetic parameters for cytochrome *P*-450 reduction on the concentration of the reconstituted vesicles in the reaction mixture. The fraction of cytochrome *P*-450 reducible in the slow phase (% slow phase), the rate constant of the fast phase (k_{fast}), and that of the slow phase (k_{slow}) obtained from the data shown in Fig. 3 were plotted against the concentration of the reconstituted system (expressed in terms of the concentration of cytochrome *P*-450).

Fig. 6. Dependence of kinetic parameters for cytochrome *P*-450 reduction on the molar ratio of NADPH-cytochrome *P*-450 reductase to cytochrome *P*-450 in the reconstituted system. All the reconstituted preparations contained the cytochrome and phosphatidylcholine at a fixed molar ratio of 1 : 400, but the molar ratio of the reductase to the cytochrome were varied as indicated.

P-450 reduction in the reconstituted vesicles, the kinetics of the reduction were studied in preparations which contained fixed amounts of cytochrome *P*-450 and phosphatidylcholine, at a molar ratio of 1 : 400, and different amounts of the reductase. As shown in Fig. 6, the first-order rate constant of the fast-phase reduction increased clearly as the ratio of the reductase to cytochrome in the vesicles was increased. Thus, at a reductase to cytochrome ratio of 0.1 the rate constant of the fast-phase was 1.4 s^{-1}, whereas it became as high as 11.5 s^{-1} when the ratio was increased to 1.5. The rate constant of the slow phase also showed a tendency of increase as the reductase to cytochrome ratio was increased. However, the determination of the rate constant of the slow phase, in contrast to that of the fast phase, is difficult and usually accompanied by considerable errors. Therefore, the significance of the increase observed is not yet established. The amount of cytochrome *P*-450 reducible in the slow phase was essentially unchanged by the alteration in the reductase to the cytochrome ratio. Since it is expected that an increase in the concentration of the reductase at a fixed level of the cytochrome, will enhance the opportunity of interaction between the two component proteins, these results suggested that at least the fast-phase reduction of cytochrome *P*-450 involved such a protein-protein interaction in the vesicles as the rate-limiting step.

Effect of dilution of the protein with phosphatidylcholine

The results shown in Fig. 6, however, do not allow us to conclude

whether the protein-protein interaction was caused by lateral diffusion of the two proteins on the plane of the vesicular membrane or effected by their direct interaction within each functional cluster. To distinguish these two alternative possibilities, experiments were then carried out in which the molar ratio of the reductase to the cytochrome was kept constant but the amount of phosphatidylcholine, relative to the two proteins, was varied. As can be seen in Fig. 7, the rate constant of the fast phase decreased from 11.0 to 2.9 s^{-1} when the molar ratio of phosphatidylcholine to cytochrome *P*-450 was increased from 200 to 2000 at a fixed molar ratio of the reductase to the cytochrome of 1 : 1. In contrast to the rate constant of the fast phase, that of the slow phase as well as the fraction of cytochrome *P*-450 reducible in the slow phase were again essentially unchanged by the alteration in the phosphatidylcholine content. Table I shows further that the same tendency was also observable when the molar ratio of the reductase to the cytochrome was kept to 0.1 : 1 and the ratio of phosphatidylcholine to the cytochrome was varied from 150 to 450. These findings could be readily accounted for by assuming that

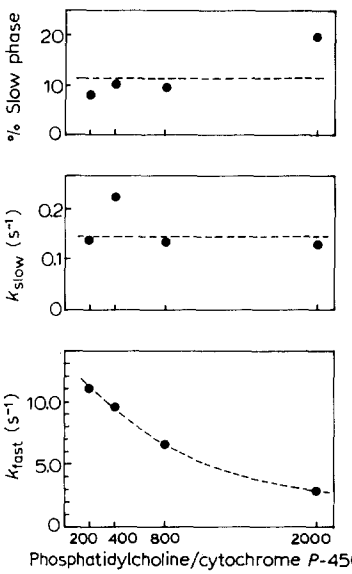


Fig. 7. Effect of dilution of the two protein components in the reconstituted vesicles with phosphatidylcholine on kinetic parameters of cytochrome *P*-450 reduction. All the reconstituted preparations contained NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 at a fixed molar ratio of 1 : 1, but the molar ratio of phosphatidylcholine to the cytochrome was varied as indicated.

TABLE I

EFFECT OF THE MOLAR RATIO OF PHOSPHATIDYLCHOLINE TO THE TWO COMPONENT PROTEINS ON THE REDUCTION KINETICS OF CYTOCHROME *P*-450 AND BENZPHETAMINE N-DEMETHYLATION ACTIVITY OF THE RECONSTITUTED VESICLES

The molar ratio of the reductase to cytochrome *P*-450 was fixed to 0.1 : 1, and that of phosphatidylcholine to cytochrome *P*-450 was varied as indicated. The N-demethylation activity is expressed in terms of nmol formaldehyde formed per s per nmol cytochrome *P*-450. PC = phosphatidylcholine.

PC/*P*-450	k_{fast} (s^{-1})	k_{slow} (s^{-1})	slow phase (%)	Formaldehyde formation (nmol/s per nmol *P*-450)
150	2.3	0.12	14	0.065
450	1.5	0.12	18	0.035

the rate of the fast-phase reduction of cytochrome *P*-450 was limited by lateral mobilities of the two proteins, because a decrease in the two-dimensional concentrations of the reductase and the cytochrome on the plane of phosphatidylcholine membrane should decrease the frequency of their collision caused by their lateral diffusion. It should be emphasized that these results cannot be explained by the cluster model, because the rate of direct interaction between the two proteins within each cluster should not be affected by the amount of phosphatidylcholine present in the vesicles.

Benzphetamine N-demethylation by reconstituted vesicles

As mentioned above, the reconstituted vesicles could catalyze the NADPH-dependent N-demethylation of benzphetamine in the presence of molecular oxygen. It was, therefore, of interest to examine the effect of the composition of the vesicles on the rate of this overall hydroxylation reaction. It was thus found that this rate responded to changes in the composition of the vesicles in a way similar to that of the fast-phase reduction of cytochrome *P*-450. For example, Fig. 8 shows that the overall benzphetamine demethylation activity increased as the molar ratio of the reductase to cytochrome *P*-450 in the vesicles was increased at a fixed ratio of the cytochrome *P*-450 to phosphatidylcholine of 1 : 400, and the extent of increase was comparable to that in the rate of fast-phase reduction of cytochrome *P*-450 (cf. Fig. 6). Furthermore, as shown in Fig. 9, the demethylation activity decreased significantly when the molar ratio of phosphatidylcholine to cytochrome *P*-450 was increased at a fixed ratio of the reductase to the cytochrome of 1 : 1. When compared with the data shown in Fig. 7, it will be seen that the decrease in the benzphetamine N-demethylation activity was almost in parallel to that in the rate of fast-phase reduction of cytochrome *P*-450 as a function of the phosphatidylcholine to cytochrome *P*-450 ratio. Similar parallel decreases in the two activities were also observed when the phosphatidylcholine to cytochrome *P*-450 ratio was increased at a fixed molar ratio of the reductase to the cytochrome of 0.1 : 1 (Table I).

Effects of oxygen scavenger and substrate concentration on kinetics of cytochrome P-450 reduction

Since cytochrome *P*-450 is autoxidizable, its reduction kinetics could be affected by the presence of a small amount of oxygen remaining in the reaction mixture. For this reason, an oxygen scavenging system consisting of glucose, glucose oxidase, and catalase was included in the reaction mixture in the present study. It was, however, found that the reduction kinetics was unaltered even when the scavenging system was omitted, if the system was carefully kept under anaerobic conditions. Previous studies have shown that the reduction of cytochrome *P*-450 in intact liver microsomes [37] and in a reconstituted system containing a detergent [33] is greatly stimulated by the presence of type I substrates. In this study, therefore, all the measurements were carried out in the presence of 0.8 mM benzphetamine. It was, however, found that the reduction in the absence of benzphetamine also proceeded in a biphasic fasion, although the rates of both phases were greatly decreased, as expected. Fig. 10 shows the effect of benzphetamine concentration on the

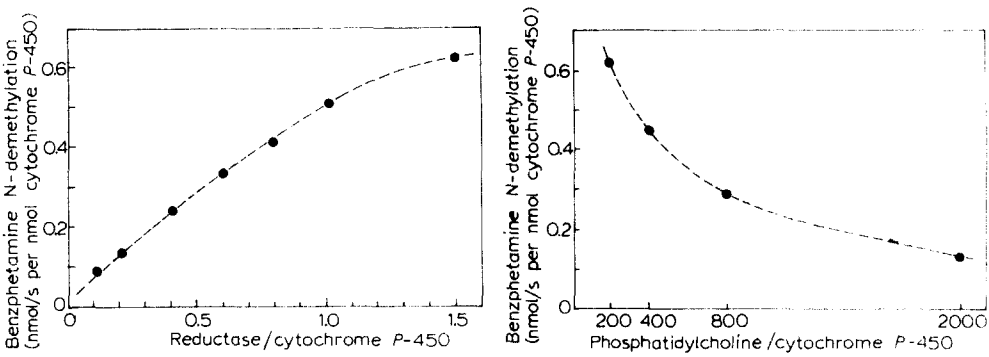


Fig. 8. Dependence of NADPH-dependent benzphetamine N-demethylation activity of the reconstituted vesicles on the molar ratio of NADPH-cytochrome *P*-450 reductase to cytochrome *P*-450. The same reconstituted preparations as used in Fig. 6 were assayed for their benzphetamine demethylation activities as described in Materials and Methods.

Fig. 9. Effect of dilution of the two protein components in the reconstituted vesicles with phosphatidylcholine on NADPH-dependent benzphetamine N-demethylation activity. The same reconstituted preparations as used in Fig. 7 were assayed for their benzphetamine demethylation activities.

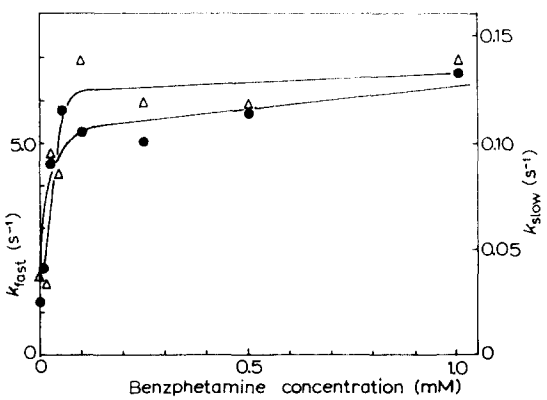


Fig. 10. Effect of benzphetamine concentration on the rates of cytochrome *P*-450 reduction in the reconstituted vesicles. The reconstituted system contained NADPH-cytochrome *P*-450 reductase, cytochrome *P*-450, and phosphatidylcholine at a molar ratio of 0.5 : 1 : 400. The stopped flow measurements were carried out as described in Materials and Methods, except that the concentration of benzphetamine was varied as indicated. The rate constants of the fast phase (●) and of the slow phase (△) were plotted against the benzphetamine concentration.

rate constants of the fast and slow phases. It can be seen that both rate constants became almost maximal at about 0.1 mM benzphetamine, and 0.8 mM benzphetamine was sufficient to ensure the maximal stimulation effect. The fraction of cytochrome *P*-450 reducible in the slow phase again remained constant (about 25%) regardless of the substrate concentration employed.

Reduction of cytochrome P-450 in a system reconstituted with a nonionic detergent

It has been shown that a drug hydroxylase activity can be reconstituted by mixing the reductase, cytochrome *P*-450, and low concentrations of nonionic detergents such as Triton X-100 and Emulgen 913 [33,38]. It is likely that the reconstituted system thus prepared is not vesicular and consisting of

spherical micelles of the detergent to which both the reductase and the cytochrome are inserted. To examine if the biphasic nature of cytochrome *P*-450 reduction resulted from the intrinsic property of the lipid vesicular system, the reduction kinetics was measured in a system reconstituted in the presence of 0.005% Emulgen 913. In confirmation of the finding of Imai et al. [33] with a similar system, the reduction was again biphasic, as shown in Fig. 11. The fraction of cytochrome *P*-450 reducible in the slow phase was about 30%, and an increase in the molar ratio of the reductase to the cytochrome in the Emulgen 913 system did not affect the fraction of cytochrome *P*-450 reducible in the slow phase appreciably. The whole process, therefore, proceeded in a similar way to the phosphatidylcholine-containing vesicular system.

Discussion

In this study we have incorporated homogeneous preparations of cytochrome *P*-450 and NADPH-cytochrome *P*-450 reductase, both purified from liver microsomes of phenobarbital-pretreated rabbits, into egg-yolk phosphatidylcholine vesicles by the cholate dialysis method and used this reconstituted system as a model for studies of the mode of interaction of the two protein components in hepatic microsomal membranes. Besides its simplicity, this system is advantageous in that its composition can be varied as desired. The results thus obtained indicate that the NADPH-dependent reduction of cytochrome *P*-450 in this vesicular system is a biphasic process, consisting of two first-order reactions, and that 80—90% of the total cytochrome is reduced in the fast phase regardless of the composition of the system. In these respects, the reduction kinetics of cytochrome *P*-450 in the present system is similar to that in intact liver microsomes [5,39] and in a non-vesicular reconstituted system containing a nonionic detergent instead of phosphatidylcholine [33].

The data shown in Fig. 6 indicate that the rate constant of the fast-phase reduction of cytochrome *P*-450 increases significantly when the molar ratio of the reductase to the cytochrome in the vesicles is increased. This finding can be best explained by assuming that the rate-limiting step of the fast-phase re-

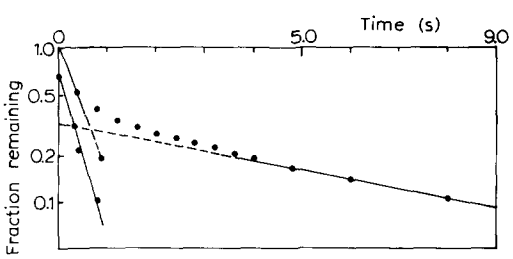


Fig. 11. First-order plot of the rate of cytochrome *P*-450 reduction by NADPH in a system reconstituted with Emulgen 913 (instead of phosphatidylcholine). In one syringe of the stopped flow apparatus a CO-saturated solution was placed containing 0.33 μM cytochrome *P*-450, 0.32 μM NADPH-cytochrome *P*-450 reductase, 0.01% Emulgen 913, 0.8 mM benzphetamine, and the oxygen scavenging system (see Material and Methods) in 0.1 M potassium phosphate buffer (pH 7.25). In another syringe a CO-saturated solution was placed containing 0.3 mM NADPH, 0.8 mM benzphetamine, and the oxygen scavenging system in 0.1 M potassium phosphate buffer (pH 7.25). Stopped flow measurement was carried out as described in Materials and Methods. The rate constant of the fast phase was 2.4 s^{-1}, and of the slow phase 0.145 s^{-1}.

duction is the electron transfer from the reduced reductase to the oxidized cytochrome, since an increase in the concentration of the reductase at a constant level of the cytochrome would be expected to enhance the opportunity of such electron transfer. It is inconceivable that the reduction of the reductase by NADPH is determining the rate of cytochrome *P*-450 reduction*. The results shown in Fig. 7 and Table I indicate further that the rate constant of the fast phase decreases significantly when the two proteins, which are present at a fixed molar ratio, are diluted with phosphatidylcholine in the vesicular membrane. This latter observation cannot be satisfactorily accounted for by assuming that the two proteins exist in the membrane as clusters, because the rate of electron transfer occurring in each cluster should not be affected by the amount of phosphatidylcholine. Instead, it seems that all the observations presented above are consistent with the view that the fast-phase reduction of cytochrome *P*-450 in the reconstituted vesicles involves random collision of the two proteins caused by their lateral diffusion in the membrane and that their collision is the rate-limiting step of the reduction process. If this is so, then the rate of the fast-phase reduction should also be influenced by changes in the fluidity of phospholipid membranes. This possibility is now being studied in this laboratory by examining the temperature effect on the reduction in the systems reconstituted with synthetic phosphatidylcholines.

Although the results reported in this paper appear to provide strong evidence for the lateral diffusion mechanism for the interaction between the reductase and cytochrome *P*-450 in the membrane of phosphatidylcholine vesicles, this conclusion may not be directly applicable to the situation taking place in intact liver microsomes in view of considerable complexity of the latter membranes. However, it does not seem unreasonable to assume that the events occurring in the reconstituted phosphatidylcholine vesicles reflect, at least with respect to their basic features, those taking place in the membranes of intact liver microsomes. Strittmatter and coworkers [40,41] have actually reported evidence suggesting that the electron transfer from NADH-cytochrome b_5 reductase to cytochrome b_5 in liver microsomes is accomplished by their lateral diffusion. The lateral mobilities of NADPH-cytochrome *P*-450 reductase and cytochrome *P*-450 in the microsomal membranes also appear to be necessary for these proteins to interact with other components of the electron transport system such as cytochrome b_5 [35,42] and squalene epoxidase [43].

In contrast to the rate constant of the fast-phase, that of the slow-phase is essentially unaffected by changes in the molar ratio of the reductase to cytochrome *P*-450 in the vesicles and by dilution of the two proteins with phosphatidylcholine. Furthermore, the fraction of the cytochrome reducible in the slow-phase is also virtually constant regardless of the composition of the vesicles and the presence and absence of the substrate (benzphetamine). The

*The turnover rates of NADPH-cytochrome *P*-450 reductase with various artificial electron acceptors are very similar to one another and have values of about 20 nmol/s per nmol enzyme [11,45]. It is, therefore, likely that the same turnover rate can hold with cytochrome *P*-450 as acceptor. This turnover rate is higher than the rate of cytochrome *P*-450 reduction in the reconstituted vesicles, suggesting that the reductase is kept in the reduced form during the catalysis and, therefore, the reduction of the reductase by NADPH cannot be the rate-limiting step of the cytochrome *P*-450 reduction reaction.

results obtained in the present study indicate that the slow-phase is due neither to the inter-vesicular electron transfer (Fig. 5) nor to the slow reduction of the cytochrome not in combination with the substrate (Fig. 10). One possible cause of the biphasicity would be the presence of two topologically different populations of cytochrome *P*-450 in the reconstituted vesicles. It is conceivable that 80—90% of the cytochrome is located on the outer surface of the membrane and thus reducible rapidly and the rest which is bound to the inner surface is reducible only very slowly. Evidence against this possibility is the finding that the biphasicity of cytochrome *P*-450 reduction is also observable in a reconstituted system containing Emulgen 913 instead of phosphatidylcholine (Fig. 11). In such a system, which seems to consist of spherical (rather than vesicular) mixed micelles, it is unlikely that cytochrome *P*-450 exists in two topologically different states. From all these considerations, it may be said that the biphasic nature of cytochrome *P*-450 reduction reflects an intrinsic property or properties of the cytochrome, which are not influenced by the composition of the reconstituted vesicles. Further studies are surely needed to elucidate the cause of the slow-phase reduction.

On the basis of the observations that the rate of cytochrome *P*-450 reduction is affected in parallel to the overall monooxygenase activity of liver microsomes by the addition of various type I substrates and that selective inactivation of the reductase by chemical modification decreases the overall activity, it has been suggested that the reduction of cytochrome *P*-450 is the rate-limiting step of the microsomal monooxygenase reaction [1,3,37]. However, it has later been shown that the rate of cytochrome *P*-450 reduction in intact liver microsomes [39] and in a detergent-containing reconstituted system [33] is much faster than the overall monooxygenase activity. In the present study, too, it has been shown that the rate of cytochrome *P*-450 reduction in the phosphatidylcholine-containing reconstituted system is about 20-fold as high as that of the overall benzphetamine N-demethylation activity regardless of the composition of the vesicles, implying that the fast-phase reduction cannot be the rate-limiting step. Nevertheless, the present results indicate that the activity of benzphetamine demethylation by the reconstituted vesicles is affected almost in parallel to the rate of the fast-phase reduction of cytochrome *P*-450 by changes in the molar ratio of the reductase to the cytochrome (Fig. 8) and in the amount of phosphatidylcholine in the vesicles (Fig. 9 and Table I). It has further been demonstrated that the rate of cytochrome *P*-450 reduction in the detergent-containing reconstituted system and the overall monooxygenase activity are also similarly influenced by the substrate concentration, pH, and various substrates [33]. These findings suggest that the interaction between the reductase and cytochrome *P*-450 is somehow interrelated to the rate-limiting step of the overall reaction, which seems to be the introduction of the second electron to the oxygenated intermediate or in a later step [33]. It is of interest to note that the introduction of the second electron also involves the interaction of the reductase and cytochrome *P*-450. Guengerich et al. [44] have further suggested that the reductase acts as an effector on the final step of the overall activity, where an unidentified cytochrome *P*-450-substrate complex is decomposed to form the hydroxylated product and regenerate ferric cytochrome *P*-450. However, much remains to be learned

about the interrelation between the rate-limiting step of the overall reaction and the fast-phase reduction of cytochrome *P*-450 (introduction of the first electron).

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References

1 Franklin, M.R. and Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 318—329
2 Stier, A. and Sackmam, E. (1973) Biochim. Biophys. Acta 311, 400—408
3 Yang, C.S. (1975) FEBS Lett. 54, 61—64
4 Stier, A. (1976) Biochem. Pharmacol. 25, 109—113
5 Peterson, J.A., Ebel, R.E., O'Keeffe, D.H., Matsubara, T. and Estabrook, R.W. (1976) J. Biol. Chem. 251, 4010—4016
6 Duppel, W. and Ullrich, V. (1976) Biochim. Biophys. Acta 426, 399—407
7 Yang, C.S., Strickhart, F.S. and Kicha, L.P. (1977) Biochim. Biophys. Acta 465, 362—370
8 Yang, C.S. (1977) Life Sci. 21, 1047—1058
9 Vermillion, J.L. and Coon, M.J. (1974) Biochem. Biophys. Res. Commun. 60, 1315—1322
10 Dignam, J.D. and Strobel, H.W. (1975) Biochem. Biophys. Res. Commun. 63, 845—852
11 Yasukochi, Y. and Masters, B.S.S. (1976) J. Biol. Chem. 251, 5337—5344
12 Dignam, J.D. and Strobel, H.W. (1977) Biochemistry 16, 1116—1123
13 Imai, Y. and Sato, R. (1974) Biochem. Biophys. Res. Commun. 60, 8—14
14 Hashimoto, C. and Imai, Y. (1975) Biochem. Biophys. Res. Commun. 68, 821—827
15 Haugen, D.A. and Coon, M.J. (1976) J. Biol. Chem. 251, 7929—7939
16 Ryan, D., Lu, A.Y.H., Kawalek, L., West, S.B. and Levin, W. (1975) Biochem. Biophys. Res. Commun. 64, 1134—1141
17 Yang, C.S. and Strickhart, F.S. (1975) J. Biol. Chem. 250, 7958—7972
18 Yang, C.S. (1977) J. Biol. Chem. 252, 293—298
19 Pokorovsky, A., Mishin, V., Rivkind, N. and Lyakhovich, V. (1977) Biochem. Biophys. Res. Commun. 77, 912—917
20 Miwa, G.T. and Cho, A.K. (1976) Life Sci. 18, 983—988
21 Miwa, G.T., West, S.B. and Lu, A.Y.H. (1978) J. Biol. Chem. 253, 1921—1929
22 Yang, C.S., Strickhart, F.S. and Kicha, L.P. (1978) Biochim. Biophys. Acta 509, 326—337
23 Ingelman-Sundberg, M. and Glaumann, H. (1977) FEBS Lett. 78, 72—76
24 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477—5487
25 Imai, Y. and Sato, R. (1974) J. Biochem. 75, 689—697
26 Singleton, W.S., Gray, M.S., Brown, M.C. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53—56
27 Lowry, O.H., Rosebrough, A.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
28 Bartlett, G.R. (1959) J. Biol. Chem. 234, 466—468
29 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2370—2378
30 Imai, Y. (1976) J. Biochem. 82, 267—276
31 Iyanagi, T. and Mason, H.S. (1973) Biochemistry 12, 2297—2308
32 Nash, T. (1953) Biochem. J. 55, 416—421
33 Imai, Y., Sato, R. and Iyanagi, T. (1977) J. Biochem. 82, 1237—1246
34 Brunner, J., Slrabal, P. and Hauser, H. (1976) Biochim. Biophys. Acta 455, 322—331
35 Imai, Y. and Sato, R. (1977) Biochem. Biophys. Res. Commun. 75, 420—426
36 Rosen, P. and Stier, A. (1973) Biochem. Biophys. Res. Commun. 51, 601—611
37 Gigon, P.L., Gram, T.E. and Gillette, J.R. (1969) Mol. Pharmacol. 5, 109—122
38 Lu, A.Y.H., Levin, W. and Kuntzman, R. (1974) Biochem. Biophys. Res. Commun. 60, 266—272
39 Matsubara, T., Baron, J., Peterson, L.L. and Peterson, J.A. (1976) Arch. Biochem. Biophys. 172, 463—469
40 Rogers, M.J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 895—900
41 Rogers, M.J. and Strittmatter, P. (1974) J. Biol. Chem. 249, 5565—5569
42 Hildebrandt, A. and Estabrook, R.W. (1971) Arch. Biochem. Biophys. 143, 66—79
43 Ono, T., Ozasa, S., Hasegawa, F. and Imai, Y. (1977) Biochim. Biophys. Acta 486, 401—407
44 Guengerich, F.P., Ballou, D.P. and Coon, M.J. (1976) Biochem. Biophys. Res. Commun. 70, 951—956
45 Vermillion, J.L. and Coon, M.J. (1978) J. Biol. Chem. 253, 2694—2704