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# Lipid-Protein Interactions as Determinants of Activation or Inhibition by Cytochrome $b_5$ of Cytochrome P-450-mediated Oxidations\*

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Activation or inhibition by cytochrome  $b_5$  of benzphetamine *N*-demethylation was studied in micelle-reconstituted systems containing cytochrome P-450 LM<sub>2</sub>, NADPH-cytochrome P-450 reductase, and dilauroylphosphatidylcholine. The effects of cytochrome  $b_5$  were critically dependent on both protein:protein and lipid:protein ratios. A 200% stimulation of *N*-demethylation by cytochrome  $b_5$  was obtained at cytochrome P-450 reductase:cytochrome P-450 ratios similar to those in microsomes, compared to only a 20% stimulation at a ratio of 1:1. At lipid:protein ratios less than 50:1, the addition of cytochrome  $b_5$  caused significant inhibition of benzphetamine *N*-demethylation. Such an inhibition could be partially reversed by increasing phospholipid content of micelles and was not seen in vesicle-reconstituted systems at cytochrome  $b_5$ :cytochrome P-450 ratios of 1:1 or lower. At high cytochrome P-450 reductase:cytochrome P-450 ratios, addition of cytochrome  $b_5$  did not alter the efficiency (80%) with which NADPH was utilized; however, at ratios similar to those in microsomes, an increase in efficiency from 42% to 80% was observed.

The function of cytochrome  $b_5$  was interpreted in terms of a model in which inhibition of cytochrome P-450-mediated reactions results from changes in phospholipid-protein interactions and activation occurs via facilitation of electron transfer between NADPH-cytochrome P-450 reductase and cytochrome P-450 in the membrane.

Liver microsomes are known to contain two possibly linked electron transfer systems. NADPH-cytochrome P-450 reductase-cytochrome P-450 systems are involved in the metabolism of endogenous steroids and fatty acids as well as numerous xenobiotics. NADH-cytochrome  $b_5$  reductase and cytochrome  $b_5$  are components of the stearyl coenzyme A desaturase system (1). The two systems do not necessarily function in total independence. For example, a facilitatory role for cytochrome  $b_5$  in certain cytochrome P-450-mediated reactions has been proposed involving transfer of the second electron for drug oxidations (2-7).

Studies aimed at elucidation of the mechanism of cytochrome  $b_5$  effects on cytochrome P-450-mediated oxidations using micelle-reconstituted systems of purified proteins have revealed a complex pattern of interactions. Cytochrome  $b_5$  has been reported to have no significant effect on cytochrome P-

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450-catalyzed oxidation of some substrates, but can either inhibit or stimulate electron transfer in the metabolism of other compounds (5-10). This variability in effects of cytochrome  $b_5$  has been suggested to be due in part to differences in the substrate metabolized or the type of cytochrome P-450 used in reconstitution experiments (7, 11). However, it may also be reflective of differences in the molar ratios of NADPH cytochrome P-450 reductase, cytochrome  $b_5$ , and cytochrome P-450 and/or the ratios of these proteins to phospholipid molecules in the micelle. The molar ratios of NADPH-cytochrome P-450 reductase to cytochrome  $b_5$  to cytochrome P-450 found in liver microsomes of phenobarbital-induced rabbits is on the order of 1:4:10 (12). In contrast, most studies with micelle-reconstituted systems have involved the addition of 1 to 5 mol of cytochrome  $b_5$  to a 1:1 molar complex of NADPH-cytochrome P-450 reductase and cytochrome P-450. It might have appeared reasonable to attempt magnification of the effects of cytochrome  $b_5$  on the metabolic activity of cytochrome P-450 by its addition to reconstituted systems in molar excess if its effects were both uniform and linear as a function of concentration. However, this does not appear to be the case.

In the present investigation, we have used reconstituted cytochrome P-450 systems including both dilauroylphosphatidylcholine micelles and phospholipid vesicles to study the effects of cytochrome  $b_5$  on the oxidation of a single substrate, benzphetamine. Variations in the molar ratio of NADPH-cytochrome P-450 reductase to cytochrome  $b_5$  to cytochrome P-450 and the ratios of proteins to phospholipids resulted in significant changes of metabolic activity. Depending on the system, cytochrome  $b_5$  could be shown to exert no effect on benzphetamine *N*-demethylation, to cause almost complete inhibition of substrate oxidation, or to cause a 3-fold higher activity. These results are discussed in terms of a proposed model of lipid-protein interactions relating to electron transfer processes in liver microsomal membranes.

## EXPERIMENTAL PROCEDURES

**Purification of Components**—Cytochrome P-450 LM<sub>2</sub> was purified from livers of phenobarbital-pretreated rabbits by DEAE-cellulose and hydroxylapatite chromatography (13) to a purity of 17 nmol/mg of protein. NADPH-cytochrome P-450 reductase was purified from the same livers by affinity chromatography on 2',5'-ADP-Sepharose (14) to yield a specific activity toward cytochrome *c* of 32 to 40  $\mu$ mol/min/mg of protein. Cytochrome  $b_5$  was purified as previously described (15) to a purity of over 90% as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (16). All protein preparations were concentrated and dialyzed against 0.3 M potassium phosphate buffer, pH 7.5, with 20% glycerol and 0.5% sodium cholate, then stored in liquid nitrogen until used. Egg phosphatidylcholine and egg phosphatidylethanolamine prepared by the method of Singleton (17) were each repurified by preparative high pressure liquid chromatography on a Si-100 column (25  $\times$  1 cm) using mixtures of hexane, isopropyl alcohol, and water. Dilauroylphosphatidylcholine was pur-

chased from Sigma.

**Preparation of Micelle-reconstituted Systems**—Micelle-reconstituted systems were prepared by adding aliquots of a stock solution of 5 mg of dilauroylphosphatidylcholine/ml in 0.3 M potassium phosphate buffer, pH 7.5, with 20% glycerol and 0.5% sodium cholate that was dissolved by sonication in a water bath to a solution of purified microsomal proteins. To prepare a micelle-reconstituted system at a molar ratio of 0.1 NADPH-cytochrome P-450 reductase to 1.0 cytochrome P-450 to 1.0 cytochrome *b*<sub>5</sub> with 50 mol of dilauroylphosphatidylcholine mole per mol of total protein, 13  $\mu$ l of the dilauroylphosphatidylcholine solution were added to a solution containing 0.1 nmol of cytochrome P-450 reductase, 1.0 nmol of cytochrome P-450, and 1.0 nmol of cytochrome *b*<sub>5</sub>. Final concentrations in all assay mixtures were 30 mM potassium phosphate buffer, pH 7.5, 20% glycerol, and 0.025% sodium cholate in 1.0 ml final volume. For different micelle-reconstituted systems, molar ratios of the three microsomal proteins were varied and total protein concentrations ranged from 0.2 to 4.4  $\mu$ M. Dilauroylphosphatidylcholine concentrations were varied from 20 to 250 mol/mol of total protein. The exact concentrations of proteins and lipid for each experiment are given in the figure legends. The micellar preparations were incubated at 4 °C for 2 h prior to assaying for enzymatic activity.

**Preparation of Vesicle-reconstituted Systems**—Reconstitution of the purified proteins into vesicles (monolamellar liposomes) was achieved by a modification of the slow cholate dialysis method previously described (18, 19). An outline for the preparation of a complete system follows. A stock solution of phospholipids was prepared by adding 750  $\mu$ l of a solution of 20% sodium cholate in water and 1.65 ml of 0.3 M potassium phosphate buffer, pH 7.5, to 20 mg of egg phosphatidylcholine and 10 mg of egg phosphatidylethanolamine. The lipids were dissolved by sonication in a bath for 1 min. For a lipid:protein ratio of 5:1 (w/w), 2 ml from this phospholipid stock solution were added to 8 ml of a solution of 2.5 mg of cytochrome P-450, 2.0 mg of NADPH-cytochrome P-450 reductase, and 0.42 mg of cytochrome *b*<sub>5</sub> (1:0.5:0.5 mol/mol/mol) in 0.3 M potassium phosphate buffer, pH 7.5, containing 20% glycerol. The mixture was allowed to equilibrate overnight at 4 °C under argon to assure complete formation of mixed micelles. Vesicles were prepared by dialysis against 10 changes of 700 ml of 20 mM potassium phosphate buffer, pH 7.5, with 20% glycerol for 4 days at 4 °C under a nitrogen atmosphere. Over 90% of the cytochrome P-450 was recovered and no cytochrome P-420 was detectable. Additional vesicle-reconstituted systems containing different ratios of the three proteins were prepared in the same manner as described above. The vesicles which formed were demonstrated to be homogeneous by density gradient centrifugation in a 10 to 50% linear glycerol gradient at 10° *x* g for 18 h.

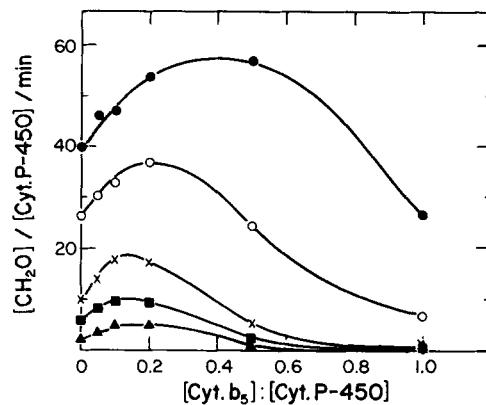
**Incorporation of Cytochrome *b*<sub>5</sub> into Vesicles**—Cytochrome *b*<sub>5</sub> was incorporated into preformed vesicles containing NADPH-cytochrome P-450 reductase and cytochrome P-450. For this procedure, sodium cholate was removed from cytochrome *b*<sub>5</sub> by passage through a Sephadex G-25 column equilibrated in 20 mM potassium phosphate buffer, pH 7.5, with 20% glycerol (15). The incorporation of cytochrome *b*<sub>5</sub> into these vesicles was confirmed by density gradient centrifugation in a 10 to 50% linear glycerol gradient containing 20 mM potassium phosphate buffer, pH 7.5. After 22 h of centrifugation at 10° *x* g at 4 °C, a homogeneous band of opalescent vesicles was observed between the densities 1.032 and 1.046. The proteins in this band were determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis to be NADPH-cytochrome P-450 reductase, cytochrome P-450, and cytochrome *b*<sub>5</sub>. When centrifuged alone, cytochrome *b*<sub>5</sub> was found at densities between 1.062 and 1.083.

**Assay of Activity**—The rate of benzphetamine *N*-demethylation was determined by measuring formaldehyde formation (20). The reaction mixture contained the reconstituted systems, 1 mM benzphetamine, and 2 mM NADPH. After preincubation for 10 min at 30 °C, NADPH was added. The reaction was stopped after 10 min by the addition of a solution of ZnSO<sub>4</sub>.

The rate of NADPH consumption was measured in an aliquot of the same reaction mixture as was used for benzphetamine *N*-demethylation assays by monitoring the decrease in absorbance at 340 nm. The reaction was performed in a 1-mm path length cuvette thermostated at 30 °C in a Cary 219 spectrophotometer. The NADPH consumed in a 10-min period was calculated using a molar extinction coefficient for NADPH of 5682 M<sup>-1</sup> cm<sup>-1</sup> at 340 nm.

## RESULTS

In Fig. 1, the activity of benzphetamine *N*-demethylation



**FIG. 1. Activity changes by cytochrome *b*<sub>5</sub> in micelle-reconstituted system.** Rates of *N*-demethylation of benzphetamine by micelle-reconstituted systems with cytochrome P-450 reductase and cytochrome P-450 LM<sub>2</sub> containing varying amounts of cytochrome *b*<sub>5</sub> (Cyt. *b*<sub>5</sub>) added to a fixed amount of cytochrome P-450 (Cyt. P-450). Each of the systems contain the following amounts of NADPH-cytochrome P-450 reductase and cytochrome P-450 LM<sub>2</sub>: (●) 0.5 + 0.5  $\mu$ M; (○) 0.5 + 1.0  $\mu$ M; (×) 0.2 + 2.0  $\mu$ M; (■) 0.1 + 2.0  $\mu$ M; (▲) 0.05 + 2.0  $\mu$ M. All systems contain 30  $\mu$ g of dilauroylphosphatidylcholine/ml. The standard error for each point is approximately the size of the symbol.

by micelle-reconstituted systems is shown by the production of formaldehyde at various cytochrome *b*<sub>5</sub>:cytochrome P-450 LM<sub>2</sub> ratios. A fixed concentration of dilauroylphosphatidylcholine was chosen to correspond closely to that used in other studies (5, 21). For every point on each curve, the amounts of cytochrome P-450 and cytochrome P-450 reductase are constant and only the amounts of cytochrome *b*<sub>5</sub> are increased, thereby increasing the ratio. The effect of cytochrome *b*<sub>5</sub> on formaldehyde production is shown at various NADPH-cytochrome P-450 reductase:cytochrome P-450 ratios. The highest rate of formation of formaldehyde is observed in those systems containing the greatest amount of cytochrome P-450 reductase in that this is the rate-limiting component (22). However, the greatest percentage increase in activity due to addition of cytochrome *b*<sub>5</sub> is observed in the system with the lowest ratio of cytochrome P-450 reductase to cytochrome P-450. In every case, strong inhibition of metabolic activity was observed as the cytochrome *b*<sub>5</sub>:cytochrome P-450 ratio approached one.

When similar experiments were reperformed with vesicle-reconstituted systems by the incorporation of detergent-free cytochrome *b*<sub>5</sub> in the membrane of cytochrome P-450- and cytochrome P-450 reductase-containing phospholipid vesicles, a different activation and inhibition profile was observed (Fig. 2). This figure shows the effect of increasing amounts of cytochrome *b*<sub>5</sub> on benzphetamine *N*-demethylation for three cytochrome P-450 reductase:cytochrome P-450 ratios. As in Fig. 1, a stimulation of metabolic activity was observed as a function of addition of cytochrome *b*<sub>5</sub> to the vesicles. Again, activity was highest for the vesicles containing the greatest amount of cytochrome P-450 reductase, and the percentage increase in activity was greatest in those vesicles containing the lowest cytochrome P-450 reductase:cytochrome P-450 ratio. In contrast to micelle-reconstituted systems, the activity in the vesicle-reconstituted systems slowly reached a plateau value at a cytochrome *b*<sub>5</sub>:cytochrome P-450 ratio of 1, and then declined to a lower value as this ratio was increased to 2.

The observed difference in the effect of cytochrome *b*<sub>5</sub> appears to reflect a difference between the reconstituted systems used. Since all protein:protein ratios were comparable, it seemed possible that cytochrome *b*<sub>5</sub> has a particularly large molar requirement for phospholipids. Thus, the occurrence of

activation or inactivation could be a consequence of the lipid:protein ratio found in the particular reconstituted system. For this reason, activity was studied in micelle-reconstituted systems as a function of phospholipid present. Fig. 3 shows the N-demethylation of benzphetamine by cytochrome P-450 in two sets of micelle-reconstituted systems as a function of the molar ratio of dilaurylphosphatidylcholine to total protein: one system contains cytochrome b<sub>5</sub>:cytochrome P-450:cytochrome P-450 reductase at a 1:1:1 ratio, whereas the second system lacks cytochrome b<sub>5</sub>. These protein:protein ratios were chosen because they are commonly used in micelle-reconstituted systems. It is seen that micelles containing cytochrome b<sub>5</sub> exhibit either inhibition, activation, or no effect on activity as a function of the molar ratio of phospholipid to total protein. Micelle-reconstituted systems at a molar ratio of cytochrome P-450 reductase to cytochrome P-450 of 0.2:1 gave similar results.

In Fig. 4, the data in Figs. 1 and 2 are combined and

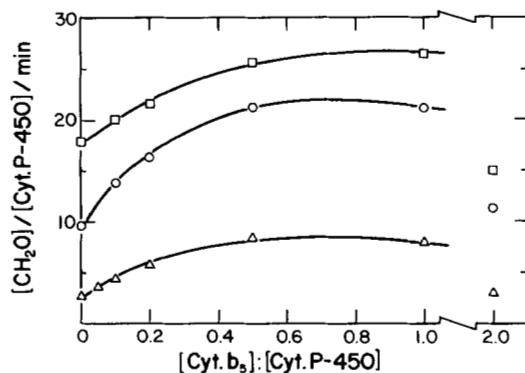


FIG. 2. **Activity changes by cytochrome b<sub>5</sub> in vesicle-reconstituted systems.** Rates of N-demethylation of benzphetamine by NADPH-cytochrome P-450 reductase and cytochrome P-450 (Cyt. P-450) reconstituted in phospholipid vesicles with varying amounts of cytochrome b<sub>5</sub> (Cyt. b<sub>5</sub>) added to a fixed concentration of cytochrome P-450. Each of the vesicle-reconstituted systems contain NADPH-cytochrome P-450 reductase and cytochrome P-450 LM<sub>2</sub> in the following amounts: (□) 0.5 + 1.0  $\mu$ M; (○) 0.25 + 1.0  $\mu$ M; (△) 0.05 + 1.0  $\mu$ M. All vesicles were reconstituted at a lipid:protein ratio of 5:1 by weight. The standard error for each point is approximately the size of the symbol. No decrease of activity is observed at the ratio of cytochrome b<sub>5</sub> to cytochrome P-450 of 1:1.

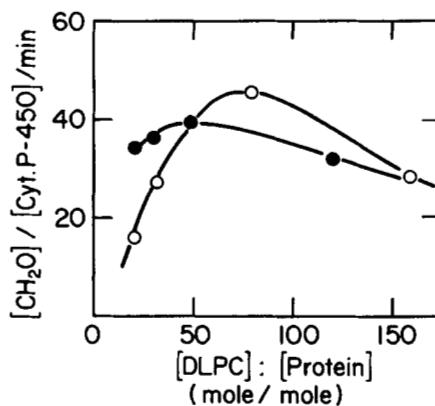


FIG. 3. **Dependence of activity changes by cytochrome b<sub>5</sub> on the lipid:protein ratio.** Rate of N-demethylation of benzphetamine as a function of molar ratio of dilaurylphosphatidylcholine (DLPC) to total protein in micelles containing: NADPH-cytochrome P-450 reductase:cytochrome P-450 (Cyt. P-450):cytochrome b<sub>5</sub> in the following molar ratios: (●) 1:1:0 and (○) 1:1:1. The standard error for each point is approximately the size of the symbol. It is seen that cytochrome b<sub>5</sub> may decrease, increase, or have no effect on activity as a function of phospholipid:protein molar ratio.

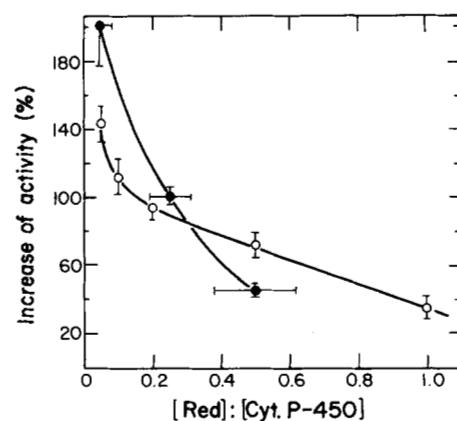
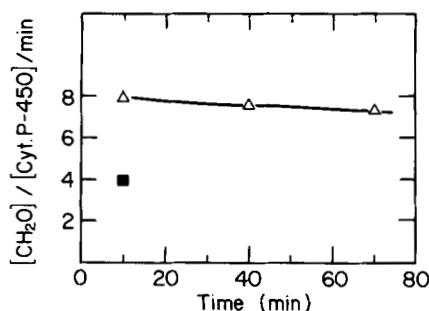


FIG. 4. **Dependence of activity increases by cytochrome b<sub>5</sub> on the ratio of cytochrome P-450 reductase to cytochrome P-450.** Percentage increase of activity of N-demethylation of benzphetamine as compared to equivalent control reconstitutions without cytochrome b<sub>5</sub> as a function of molar ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 LM<sub>2</sub> in: (●), vesicle-reconstituted systems containing a molar ratio of cytochrome b<sub>5</sub> to cytochrome P-450 of 0.5:1 reconstituted with a 5:1 weight ratio of phospholipid to total protein; and (○), micelle-reconstituted systems containing a molar ratio of cytochrome b<sub>5</sub> to cytochrome P-450 of 0.5:1 and 50 mol of dilaurylphosphatidylcholine/mol of total protein. The molar ratios in the micelle-reconstituted systems are more accurate because of their simpler preparation. The highest increase of activity is observed at the lower ratios of cytochrome P-450 reductase (Red) to cytochrome P-450 (Cyt. P-450) which mimic the ratios in microsomes.

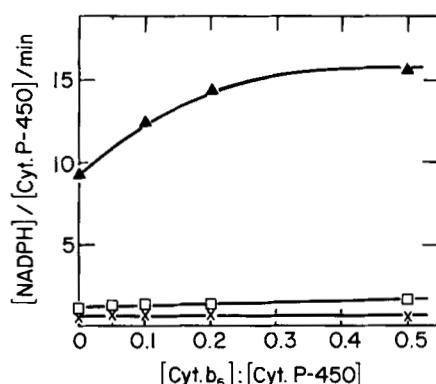
replotted to reveal the striking dependence of the increase in activity caused by cytochrome b<sub>5</sub> on the cytochrome P-450 reductase:cytochrome P-450 ratio. The ratio of cytochrome b<sub>5</sub> to cytochrome P-450 was 0.5:1, similar to that found in rabbit liver microsomes. The stimulatory ability of cytochrome b<sub>5</sub> is greatest at the low cytochrome P-450 reductase:cytochrome P-450 ratios found in microsomes and is lowest at high reductase:cytochrome P-450 ratios employed in other studies on the effect of cytochrome b<sub>5</sub>. This dependence was observed in both vesicle- and micelle-reconstituted systems.

It has been shown that cytochrome b<sub>5</sub> will incorporate rapidly into phospholipid vesicles (23) and into microsomes (24). However, it was necessary to establish that the rate of incorporation of cytochrome b<sub>5</sub> into the reconstituted phospholipid vesicles was sufficiently fast not to be a factor influencing N-demethylation. In Fig. 5, it is seen that within the 10-min time period from addition of cholate-free cytochrome b<sub>5</sub> to reconstituted vesicles held at 4 °C to the 10-min time of the first measurement of activity, the system reaches its maximal activity and then is stable at that level for more than 1 h. A repetition of the time course of incorporation of cytochrome b<sub>5</sub> at 30 °C did not result in further activation. This timespan of constant activity encompasses all of the measurements reported in this study. It should be noted that a slightly higher increase in formaldehyde production was observed when cytochrome b<sub>5</sub> was added in a cholate-containing buffer. Therefore, all studies reported here were performed with cholate-free preparations in order to measure only the effect of cytochrome b<sub>5</sub>.

The increase of N-demethylation activity by cytochrome b<sub>5</sub> described so far has been expressed only as increase in product formation. The reason for this increase could be a higher rate of use of NADPH, a more efficient utilization of NADPH by better coupling of cytochrome P-450 reductase to cytochrome P-450 or a combination of both. In order to differentiate between the two mechanisms, a study of the rate of NADPH utilization by different micelle-reconstituted systems was un-



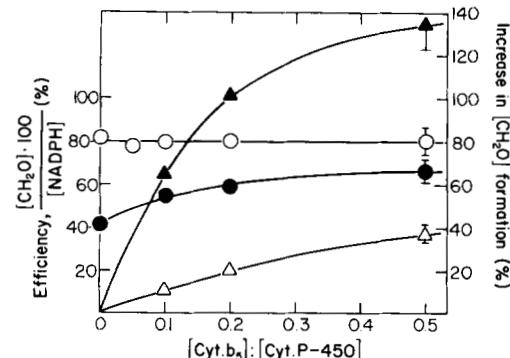
**Fig. 5. Incorporation of cytochrome  $b_5$  in vesicles.** The rate of incorporation of cholate-free cytochrome  $b_5$  into a vesicle-reconstituted system of NADPH-cytochrome P-450 reductase and cytochrome P-450 (Cyt. P-450) was measured by determining the *N*-demethylation rate of benzphetamine at various times after addition of cytochrome  $b_5$  at 4 °C. Aliquots were withdrawn at 10, 30, and 60 min; their activities were measured by warming to 30 °C over a 10-min period, adding NADPH, then measuring formaldehyde production over the following 10-min period. It is seen ( $\Delta$ ) that full activity was reached after a 10-min incubation of cytochrome  $b_5$  with the vesicles at 4 °C and that this activity was maintained after a 60-min incubation at 4 °C. In an experiment not shown here, longer incubation at 30 °C did not result in a higher increase in activity. A control incubation (■) was treated similarly, except that cytochrome  $b_5$  was not added to the initial suspension.



**Fig. 6. Increase of NADPH consumption by cytochrome  $b_5$ .** Rate of consumption of NADPH in the presence of benzphetamine as a function of adding increasing amounts of cytochrome  $b_5$  (Cyt.  $b_5$ ) to a fixed amount of cytochrome P-450 (Cyt. P-450) in micelles containing 50 mol of dilaurylphosphatidylcholine/mol of total protein and a ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 of 0.1:1 ( $\Delta$ ). Control systems contain no cytochrome P-450 (□) and no benzphetamine (x). Total NADPH content was decreased by less than 10% at the end of the experiment.

dertaken. In Fig. 6, the consumption of NADPH by micelle-reconstituted cytochrome P-450 reductase and cytochrome P-450 is shown as a function of cytochrome  $b_5$ :cytochrome P-450 molar ratio at a constant ratio of 50 mol of dilaurylphosphatidylcholine/mol of total protein. Consumption of NADPH increases from 9.3 nmol/nmol of P-450/min to 15.3 when 0.5 molar equivalents of cytochrome  $b_5$  are added to the micelle-reconstituted system. Very low amounts of NADPH were utilized and no stimulation of activity with increasing amounts of cytochrome  $b_5$  was observed in control micelle-reconstituted systems if either the substrate benzphetamine or cytochrome P-450 were omitted.

Since NADPH consumption as well as formaldehyde formation increase simultaneously with increasing cytochrome  $b_5$ , the efficiency was determined for two sets of micelle-reconstituted systems. Of particular interest were the systems at a cytochrome P-450 reductase:cytochrome P-450 ratio of 1:1 because of its frequent use and the one at 0.1:1 because of



**Fig. 7. Comparison of efficiency of electron transfer to increase of product formation.** The points plotted to the left axis measure the efficiency of electron transfer between NADPH-cytochrome P-450 reductase and cytochrome P-450 (Cyt. P-450) measured by production of formaldehyde from *N*-demethylation of benzphetamine divided by consumption of NADPH in micelle-reconstituted systems containing 50 mol of dilaurylphosphatidylcholine/mol of total protein. NADPH-cytochrome P-450 reductase:cytochrome P-450 molar ratios are: (○) 1:1 and (●) 0.1:1. The points plotted to the right side of the figure show the percentage increase in formaldehyde formation compared to the corresponding control systems without cytochrome  $b_5$  at the following molar ratios of NADPH-cytochrome P-450 reductase to cytochrome P-450: (▲) 0.1:1 and (Δ) 1:1. In a point not plotted in this figure, the efficiency increases to 80% with a cytochrome  $b_5$ :cytochrome P-450 molar ratio of 1. The standard error is less than indicated for one ratio. It is seen that, at high ratios of cytochrome P-450 reductase to cytochrome P-450, the high efficiency does not change and the observed activity increase by cytochrome  $b_5$  (Cyt.  $b_5$ ) is due to increased NADPH consumption, whereas at low ratios, efficiency as well as consumption is affected.

its similarity to the natural ratio in microsomes. The data points plotted to the left axis of Fig. 7 show that the efficiency of electron transport from cytochrome P-450 reductase to cytochrome P-450 is constant as a function of adding increasing amounts of cytochrome  $b_5$  to a fixed amount of cytochrome P-450, when the ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 is 1. However, when the ratio of cytochrome P-450 reductase to cytochrome P-450 is 0.1:1, the addition of cytochrome  $b_5$  increases efficiency from 40% to 67% at a molar ratio of cytochrome  $b_5$  to cytochrome P-450 (0.5:1), and, in a point not plotted on the graph, to 80% at a ratio of cytochrome  $b_5$  to cytochrome P-450 of 1. In order to make a comparison possible between the increase of formaldehyde production and the increase in efficiency, they are both shown on the same scale. The points plotted to the right axis of Fig. 7 show the increase in formaldehyde production by cytochrome P-450 and cytochrome P-450 reductase as a function of adding cytochrome  $b_5$  to a fixed concentration of cytochrome P-450. The small increase in formaldehyde production observed in the case of the micelles containing cytochrome P-450 reductase and cytochrome P-450 in a 1:1 ratio is entirely attributable to an increased consumption of NADPH due to the presence of cytochrome  $b_5$  and the efficiency of the system does not change. However, in the case of the system containing cytochrome P-450 reductase and cytochrome P-450 at a 0.1:1 ratio, it is seen that the large percentage increase in formaldehyde production must represent both increased electron transfer efficiency and increased consumption of NADPH.

## DISCUSSION

Studies of cytochrome P-450-mediated reactions in hepatic microsomal preparations have led to proposals that cytochrome  $b_5$  can play a facilitatory role in the transfer of a second

electron in substrate oxidations (2-4). In attempts to understand this action of cytochrome  $b_5$  use has been made of reconstituted systems containing the purified components NADPH-cytochrome P-450 reductase and cytochrome P-450 in phospholipid micelles. The addition of purified cytochrome  $b_5$  to such NADPH-dependent micellar systems has led to reports of variable effects on substrate metabolism. For example, inhibitory effects of cytochrome  $b_5$  on the *N*-demethylation of benzphetamine by systems containing cytochrome P-450 LM<sub>2</sub> (5, 9) have led to suggestions that it exerts regulatory actions on electron transfer processes (5). Cytochrome  $b_5$  is also reported to inhibit the *O*-de-ethylation of various substrates catalyzed by cytochrome P-450 forms purified from hepatic microsomes of methylcholanthrene-treated rats (11). However, in some reconstituted systems, cytochrome  $b_5$  activates rather than inhibits cytochrome P-450-mediated substrate metabolism. Stimulatory effects of cytochrome  $b_5$  have been reported for *N*-demethylation of benzphetamine (6) for the *O*-de-ethylation of *p*-nitrophenetole (11), for the hydroxylation of lauric acid (8), and of acetanilide (9). The involvement of cytochrome  $b_5$  in the *O*-de-ethylation of certain specific substrates is concluded to be obligatory (7, 11), while rates of metabolism of other substrates in the same systems were unaffected by cytochrome  $b_5$ .

The type of cytochrome P-450 and the particular substrate are only two of the determinants of the effects of cytochrome  $b_5$  in reconstituted systems. Microsomal phospholipids, particularly phosphatidylcholines, are important components mediating molecular interactions between NADPH-cytochrome P-450 reductase and cytochrome P-450 (25). It is reasonable to anticipate that the effects of cytochrome  $b_5$  on substrate reaction rates in reconstituted effects of cytochrome  $b_5$  on substrate reaction rates in reconstituted systems could also be dependent on phospholipid-protein interactions. Phospholipid requirements could change radically in systems containing three, rather than two, interacting protein components. Therefore, the present study included examination of the effects of cytochrome  $b_5$  on substrate metabolism plus its dependence on both phospholipid:protein and protein:protein ratios. The *N*-demethylation of benzphetamine by cytochrome P-450 LM<sub>2</sub> was selected as the single reaction for our studies since both inhibitory and stimulatory effects of cytochrome  $b_5$  have been reported in reconstituted micelles (5, 6). In our reconstituted systems, cytochrome  $b_5$  causes both activation and inhibition of the *N*-demethylation reaction, or has no measurable effect, depending upon the ratio of cytochrome  $b_5$  to cytochrome P-450 (Figs. 1 and 2). In vesicles, which contain a higher phospholipid:protein ratio than micelles, the inhibitory effects of cytochromes  $b_5$  are present only when the mole ratio of cytochrome  $b_5$  to cytochrome P-450 exceeds unity. Experiments designed to assess directly the phospholipid dependence of the effects of cytochrome  $b_5$  in reconstituted micelles reveal that inhibition occurs when the phospholipid:protein mole ratio is less than 50:1 in a system containing cytochrome  $b_5$ , cytochrome P-450, and its reductase in a 1:1:1 ratio (Fig. 3). At higher phospholipid:protein molar ratios, the rate of *N*-demethylation of benzphetamine is either stimulated or minimally affected by the addition of cytochrome  $b_5$ .

Considerations of the molecular structure of cytochrome  $b_5$  and its potential for interactions with phospholipids may furnish a possible explanation for the strong lipid dependence of its inhibitory effects in certain reconstituted systems. Cytochrome  $b_5$  consists of a heme-containing globular component that does not interact with detergents and an elongated "tail" structure capable of interactions with detergent micelles and phospholipid vesicles (23, 24, 26). Insertion of this hydro-

phobic "segment" into the membrane is anticipated to cause reorganization of the protein-phospholipid interactions in the bilayer. Due to the amphipathic structure of cytochrome  $b_5$ , an unusually large number of phospholipid molecules would occupy the region under the hydrophilic portion of the protein molecule that is extrinsic to the membrane. Fig. 8 shows such a structural arrangement wherein the hydrophilic portion of the cytochrome  $b_5$  molecule which has clusters of charged groups on its surface (27) is lying over the polar head groups of the phospholipids to form a "cap." Similar changes in a protein-lipid domain, involving heterogeneous lateral distribution of phospholipids, occur in systems containing asymmetric polypeptides such as polymyxin (28). The model is in agreement with EPR studies on vesicles suggesting motional restriction of phospholipid molecules adjacent to the hydrophobic segment of the cytochrome  $b_5$  molecule (23). Distortion of phospholipid structures leading to a change of lipid association with other proteins including cytochrome P-450 is suggested as a plausible explanation for certain inhibitory effects of cytochrome  $b_5$  in reconstituted micelles. Increasing the phospholipid:protein ratio in such systems would predictably reverse such inhibitory effects. Similarly, the absence of inhibitory effects of cytochrome  $b_5$  in reconstituted vesicles at cytochrome  $b_5$ :cytochrome P-450 ratios below unity, is expected given the comparatively high phospholipid:protein molar ratio in the bilayer system compared to the micelle.

In the present model, cytochrome  $b_5$  is given a transmembrane orientation. Studies of incorporation of purified cytochrome  $b_5$  into preformed phosphatidylcholine vesicles have led to the suggestion of localization of the charged COOH terminus of the hydrophobic segment on the same side of the bilayer as the catalytic moiety (29). However, such vesicles are known to be relatively impermeable to transmembrane diffusion of charged molecules. Irrespective of its precise orientation, the amphipathic nature of cytochrome  $b_5$  is anticipated to exert greater effects on phospholipid organization than would be expected from its molecular weight.

The inhibitory effects of cytochrome  $b_5$  on the *N*-demethylation of benzphetamine in reconstituted micelles are increased at high substrate concentrations (9), which may even exceed the concentration of the phospholipids present (5). From consideration of the physicochemical properties of the benzphetamine molecule, which contains both hydrophilic and hydrophobic groups, it can be expected to have a high lipid:water partition coefficient. Thus, at high substrate concentrations, such as 1 mM in the aqueous phase, the concentration of benzphetamine in the lipid phase could be sufficient to contribute to the disruption of those phospholipid-protein interactions involved in efficient electron transfer (30, 31).

The magnitude of the stimulatory effects of cytochrome  $b_5$

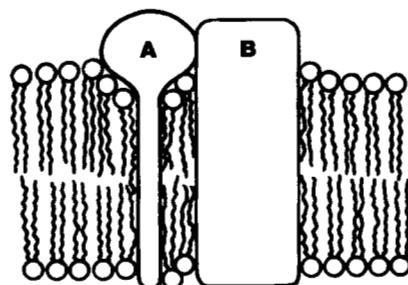


FIG. 8. Diagrammatic representation of phospholipid interactions of cytochrome  $b_5$  (A) and another membrane protein (B) such as cytochrome P-450. The model shows high phospholipid requirement of cytochrome  $b_5$  in its protein-protein interactions for electron transfer.

on *N*-demethylation is highly dependent on the mole ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 in both micelle- and vesicle-reconstituted systems (Fig. 4). Maximal stimulation occurs at lower ratios of reductase to cytochrome P-450 similar to those found in rabbit hepatic microsomes. The addition of cytochrome *b*<sub>5</sub> also increased rates of NADPH utilization (Figs. 6 and 7). When the ratio of NADPH-cytochrome P-450 reductase to cytochrome P-450 was 1:1, the increase in NADPH utilization was stoichiometric with increased formation of CH<sub>2</sub>O, reflecting minimal change in efficiency of coupling. At ratios of the reductase to cytochrome P-450 similar to microsomes, stimulation of the rate of *N*-demethylation by cytochrome *b*<sub>5</sub> exceeded that of NADPH utilization with a resultant increase in efficiency.

There are several possible mechanisms by which cytochrome *b*<sub>5</sub> mediates activation of *N*-demethylation by cytochrome P-450. In the reconstituted systems used in the present study, which lack NADH and NADH-cytochrome *b*<sub>5</sub> reductase, the stimulatory effects of cytochrome *b*<sub>5</sub> are presumed to depend on its interaction with NADPH-cytochrome P-450 reductase, cytochrome P-450, or both protein components. Increases in reaction rate could involve a direct participation of cytochrome *b*<sub>5</sub> in electron transfer or, alternatively, allosteric interactions with the two other protein components to facilitate electron flow between them. Evidence for a direct role in electron transfer includes reduction of cytochrome *b*<sub>5</sub> by NADPH-cytochrome P-450 reductase (32, 33) and demonstration of electron transfer between cytochrome *b*<sub>5</sub> and cytochrome P-450 (34). However, in kinetic studies of the oxidation of reduced cytochrome P-450 in micelles, cytochrome *b*<sub>5</sub> was not oxidized significantly during the time of formation of the oxo-cytochrome P-450 complex (35). In this particular system, cytochrome *b*<sub>5</sub> increased the rate constant for the rate-limiting step in the reaction, thus acting as an effector as well as an intermediate in electron transfer.

The pronounced dependence of metabolic activity on protein:lipid and protein:protein molar ratios demonstrates the importance of full optimization of all parameters of a model system prior to use of its activity or electron-coupling efficiency (10) for characterization of the effects of additional components. In particular, the apparent high requirement of cytochrome *b*<sub>5</sub> for phospholipids over that of other protein compounds in micelle-reconstituted systems demonstrates the need for compensatory changes in the total phospholipid content in such systems as protein:protein ratios are changed. The dependence of activity on molar ratios of components of the endoplasmic reticulum also illustrates the potential for modulation of metabolic activity by changes in the lateral organization with the membrane.

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