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# BINDING OF HOMOGENEOUS CYTOCHROME $b_5$ TO RAT LIVER MICROSOMES
# EFFECT ON N-DEMETHYLATION REACTIONS

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## Summary

Incubation of rat homogeneous detergent-solubilized cytochrome $b_5$ with rat liver microsomes resulted in specific binding of the hemoprotein which was rapidly reduced by NADH. The NADH cytochrome *c* reductase activity in these preparations increased in proportion to the amount of cytochrome bound. However, the extra-bound detergent-solubilized cytochrome $b_5$ did inhibit NADPH-dependent N-demethylations, the NADH synergism and NADPH cytochrome *P*-450 reductase activity. Manganese protoporphyrin-apocytochrome complex when bound to microsomes in amounts equivalent to detergent-solubilised cytochrome $b_5$ showed no effect on N-demethylation activity. Furthermore, the binding of cytochrome $b_5$ preparations reconstituted from heme and apocytochrome $b_5$ had no effect on either the NADPH-dependent N-demethylation of aminopyrene or ethylmorphine or the NADH synergism observed with rat liver microsomes. In addition, homogeneous cytochrome $b_5$ eluted from three additional Sephadex G-100 columns showed no inhibitory effects when bound to liver microsomes. Spectral analyses of the acid-acetone extract of the hemoprotein showed an absorption peak at 278 nm suggesting that the homogeneous $b_5$ contains contaminating amounts of tightly bound detergent which is responsible for the observed inhibition of mixed function oxidase activity and which is removed during extraction of the heme from the apocytochrome and during further gel filtration applications.

## Introduction

While the function of cytochrome *P*-450 in liver microsomes has been well-documented, the physiological role of cytochrome $b_5$, until recently, has remained obscure. Oshino et al. [1] reported that rat liver microsomal cyto-

chrome $b_5$ is an electron carrier in the desaturation reaction, transferring reducing equivalents from NADH and/or NADPH to a cyanide-sensitive factor, presumably the terminal component of the electron-transport chain [2—4]. Using liver microsomes containing exogenously bound cytochrome $b_5$ and an antibody to cytochrome $b_5$, respectively, Strittmatter et al. [5] and Oshino and Omura [6] confirmed cytochrome $b_5$ involvement in fatty acid desaturation. Of paramount interest and still unsettled is the role of cytochrome $b_5$ in the hepatic microsomal mixed function oxidase system, since cytochrome $b_5$ and its reductase have the potential to donate electrons to this system. Based on the synergistic effect of NADH on NADPH-dependent drug hydroxylation reactions [7—10] and on the evidence that partial reoxidation of reduced cytochrome $b_5$ occurs in the presence of excess NADH, NADPH, and ethylmorphine, Hildebrandt and Estabrook [11] postulated that the second reducing equivalent is transferred from NADH to the oxygenated cytochrome *P*-450 substrate complex via cytochrome $b_5$. This hypothesis has created a good deal of controversy; for example, Ichikawa and Loehr [12] reported the reduction of cytochrome *P*-450 by NADH in submicrosomal particles which contained NADH ferricyanide (cytochrome $b_5$) reductase but were devoid of cytochrome $b_5$. However, Sasame et al. [13] showed that in the presence of both NADH and NADPH and anti-cytochrome $b_5$, NADH oxidase activity decreased 58% and rat liver microsomal ethylmorphine N-demethylase activity was reduced to the values obtained prior to addition of NADH. Using cyanide to inhibit the fatty acyl-CoA desaturase pathway, Correia and Mannering [14,15] observed a significant increase in the NADH synergism of ethylmorphine N-demethylation; furthermore, in the presence of stearyl-CoA, the rate of N-demethylation declined in the presence of either NADPH alone or NADPH and NADH. These results strongly suggest that the second electron reaches the cytochrome *P*-450 system via cytochrome $b_5$. Jansson and Schenkman [16] in a recent communication, reported an inhibition of aminopyrine dealkylation following addition of detergent-solubilized cytochrome $b_5$ to liver microsomes, and the addition of NADH to this NADPH-dependent dealkylation did not reverse the inhibition. Yet the findings of Sasame et al. [17] using antibody to cytochrome $b_5$, suggest that cytochrome $b_5$ plays a role in NADPH- and NADH-dependent hydroxylation of lauric acid in rat liver and kidney. More recently, Mannering et al. [18] reported that an antibody to trypsin-solubilized cytochrome $b_5$ inhibited the NADH-stimulated ethylmorphine N-demethylation, but not the NADPH-dependent demethylation, suggesting that the second electron can arise from NADH via cytochrome $b_5$. West et al. [19] reported that in the reconstituted system cytochrome $b_5$ is an obligatory component for the NADH-dependent hydroxylation of benzopyrene. Hrycay and Estabrook [20] showed that extra-bound detergent purified cytochrome $b_5$ to rabbit liver microsomes inhibited both NADPH-dependent N-demethylation and NADPH cytochrome *P*-450 reductase activity but enhanced NADH-cytochrome *P*-450 reductase activity.

Thus the precise role of native cytochrome $b_5$ in microsomal hydroxylation reactions has not been established. Since spectrally and biologically active cytochrome $b_5$ preparations often contain contaminating amounts of tightly bound membranous proteins, lipids and detergents [21], which may introduce

ambiguities in determining the precise role of cytochrome $b_5$ in the above reactions, we undertook the isolation from rat liver microsomes of homogeneous cytochrome $b_5$ with the membranous segment intact. Moreover, we have investigated the binding of this hemoprotein and its manganese derivative to the microsomes, as well as characterized its effect on NADPH-dependent microsomal N-demethylations. Our studies suggest that cytochrome $b_5$ contains tightly bound detergent which appears responsible for the inhibition of NADPH-dependent microsomal oxidation.

A preliminary report of this work was given at the Second Philadelphia Conference on Heme Protein *P*-450 on April 5-6, 1974, and the Federation of American Societies for Experimental Biology in Atlantic City, Vol. 33, 587, 1974.

## Methods

*Reagents and chemicals*

NADH, $NADP^+$, NADPH, isocitric acid (trisodium salt), and isocitric dehydrogenase (Type IV) were obtained from Sigma Chemical Company; ethylmorphine HCl from Mallinckrodt Chemical Works and 4-dimethylaminoantipyrine from Aldrich Chemical Company. The nonionic detergents, Triton X-100 and N-101 were purchased from Rohm and Haas. Sephadex gels were obtained from Pharmacia and diethylaminoethyl cellulose (DEAE) type DE52 was a product of Whatman. Protoporphyrins and hemin were obtained from Calbiochem Inc. The water used in all experiments was distilled in a Corning all-glass apparatus from deionized water. The various gases (CO, $N_2$ and $O_2$) were obtained from Matheson Company; both nitrogen and carbon monoxide were purified further by passing through a deoxygenating system previously described [22]. All other chemicals and reagents were standard commercial products of analytical grade and were not further purified.

*Analytical methods*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was carried out as described by Neville [23] with an 11% acrylamide gel and using the 0.4 Tris · HCl (pH 9.2) system. Prior to preparation of the gels, SDS was recrystallized from ethanol. Staining and destaining were done as described by Weber and Osborn [24] using Coomassie Blue. Protein was measured according to Lowry et al. [25], using bovine serum albumin as the standard.

*Enzyme assays*

NADPH- and NADH-cytochrome *c* reductase activities were estimated as described by Dallner [26]. NADPH-cytochrome *P*-450 reductase activity was determined as described previously [22]. NADH cytochrome $b_5$ reductase activity, using ferricyanide as the electron acceptor, was measured by the procedure of Strittmatter [27]. Cytochromes *P*-450 and $b_5$ were determined by the method of Omura and Sato [28], using an Aminco DW-2 spectrophotometer. Aminopyrine and ethylmorphine *N*-demethylase activities were determined by measuring formaldehyde produced with the pH 6.0 Nash Reagent [29,30].

*Isolation of microsomes*

Normal male Sprague-Dawley rats (225—275 g) were fed and watered ad libitum until sacrifice. One hundred animals were sacrificed by decapitation. Livers were removed and perfused as described previously [31]. 1 kg of liver was homogenized with 9 vol. of 0.25 M sucrose containing 10 mM Tris-acetate (pH 8.1) and 1 mM EDTA for 1 min in a Waring blender. The suspension was then filtered through cheese cloth and centrifuged for 15 min at 600 × *g* and 16 000 × *g*, the pellet being discarded in each case. The microsomal fraction was obtained by centrifugation (25 000 × *g*) of the post-mitochondrial supernatant after addition of $CaCl_2$, according to the procedure developed by Kamath et al. [32] and modified by Cinti et al. [33]. Microsomes used for assaying drug methabolizing enzymes were prepared by the $Ca^{2+}$-dependent sedimentation procedure or by the conventional differential ultracentrifugation method as previously described [34]. Either method of isolation gave microsomal preparations with identical drug metabolizing activities.

*Purification of cytochrome $b_5$*

$Ca^{2+}$ sedimented microsomes were washed once with 0.15 M KCl and suspended in 25% glycerol containing 0.25 M sucrose, 10 mM Tris-acetate (pH 8.1) and 1 mM EDTA. The solubilization of microsomes and the subsequent isolation of cytochrome $b_5$ were accomplished as described by Ozols [21]. In certain experiments purified cytochrome $b_5$ preparation was passed through a Sephadex G-100 column three additional times in attempt to remove contaminating detergent.

*Binding of homogeneous cytochrome $b_5$ to rat liver microsomes*

Unless indicated otherwise in the figure legends, a 40-fold excess of cytochrome $b_5$ was incubated with a liver microsomal suspension containing 0.25 M sucrose and 0.1 M Tris-Cl buffer (pH 8.0) for 15 min at room temperature (20°) essentially as described by Strittmatter et al. [5]. The reaction was stopped by diluting the solution with 10 ml cold Tris buffer followed by centrifugation at 104 000 × *g* for 30 min (at 25 000 × *g* for 15 min in Sorval-RC2-B using $Ca^{2+}$-sedimented microsomes). In the initial experiments, the washing procedure was repeated twice to insure the removal of non-specifically adsorbed cytochrome $b_5$; however, it was later found that one washing was sufficient to remove any non-specifically bound hemoprotein. The microsomes were then resuspended to the original incubation volume with 0.25 M sucrose containing 0.1 M Tris-Cl (pH 7.4) and used for enzymatic assays and drug metabolism. Bound cytochrome $b_5$ was measured by reduced minus oxidized difference spectrum at 424—500 nm using 1.0 μM NADH and an extinction coefficient of 112 $mM^{-1} \cdot cm^{-1}$ [35], rather than the absorbance difference between the peak at 424 nm and the trough at 409 nm [28].

*Preparation of manganese protoporphyrin IX and its apocytochrome $b_5$ complex*

Manganese derivatives of porphyrins were obtained as described by Ozols and Strittmatter [36]. This method involved reacting a 1% solution of the protoporphyrin derivative in 1 : 1 chloroform/pyridine solution with an equal

volume of 3% manganese acetate in glacial acetic acid. The resulting solution was then heated to 90°C for 20 min and the produce was isolated as described previously [36]. A manganese mesoporphyrin preparation with properties identical to the synthetic compound and bovine cytochrome $b_5$ obtained by trypsin treatment of microsomes were generously supplied by Dr Strittmatter's laboratory.

Mn · apocytochrome complex was prepared by a modification of the procedure described by Rogers and Strittmatter [37]. Briefly, heme-free apocytochrome was obtained by adding 20 volumes of acetone containing 0.2% HCl (v/v) to one volume of rat liver cytochrome $b_5$ at 4°C. The resulting suspension was centrifuged and the white precipitate (apocytochrome $b_5$) was redissolved in 0.2—0.4 ml 0.1 M Tris/acetate buffer pH 8.3 containing 0.5% deoxycholate and incubated at 4°C for 30 min with approximately two-fold molar excess of heme or Mn derivative dissolved in a mixture of 50% ethanol/0.05 M Tris-acetate. Following incubation, about 0.5 ml was placed on a Sephadex G-25 column which had been equilibrated with 0.1 M Tris-acetate buffer (pH 8.1) and 1.0 mM EDTA. The heme- or Mn prophyrin-apocytochrome $b_5$ complex and free apocytochrome were eluted while any free metalloporphyrin was retained. The binding of Mn cytochrome $b_5$ to rat liver microsomes employed the procedure described above for binding homogeneous cytochrome $b_5$ to microsomes. Both bound Mn cytochrome $b_5$ and apocytochrome $b_5$ were measured spectrally by the method recently described by Rogers and Strittmatter [37].

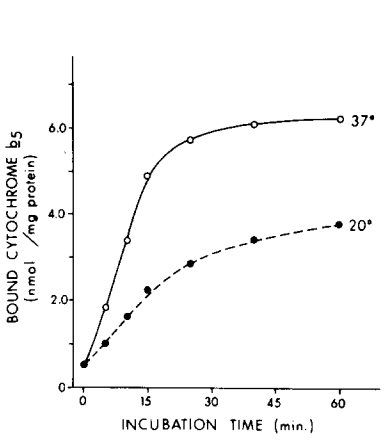
## Results

*Isolation of cytochrome $b_5$ from rat liver microsomes*

The cytochrome $b_5$ preparations migrated as a single protein band when subjected to acrylamide gel electrophoresis in sodium dodecyl sulfate. The spectral properties of rat liver cytochrome were essentially identical to the human, bovine or porcine preparations [21]. No changes in absorbance occurred when either oxidized or reduced hemoprotein preparations were treated with carbon monoxide indicating the complete absence of cytochrome *P*-450 and its denatured form (*P*-420). The cytochrome $b_5$ was not reduced by NADH (1 mM), indicating that the preparation was free of the flavoprotein $b_5$ reductase. In addition, there was no demonstrable NADH or NADPH cytochrome *c* reductase activity.

*Binding of cytochrome $b_5$ to rat liver microsomes*

As seen in Fig. 1, incubating rat liver microsomes for 20 min with increasing concentrations of detergent-solubilized homogeneous cytochrome $b_5$ (D-$b_5$) resulted in a slow but significant binding at 20°C, plateauing in the presence of 80 μM D-$b_5$. Incubations at 37°C resulted in a greater than twofold increase in bound D-$b_5$ (7 nmol/mg protein vs 2.8 nmol/mg protein) in the presence of 80 μM D-$b_5$; saturation of binding was not observed at 37°C even in the presence of 100 μM D-$b_5$, since incubations of microsomes with 150 μM D-$b_5$ resulted in further binding. The amount of D-$b_5$ bound to the microsomal membrane at 37°C was 13-fold higher than the control level.



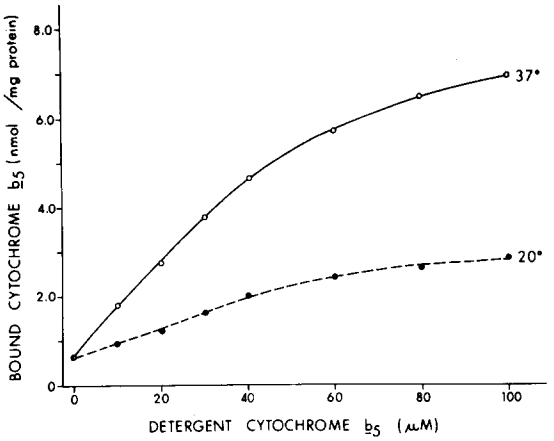




Fig. 1. The effect of temperature on the extent of cytochrome $b_5$ binding to rat liver microsomes. The incubation conditions and the content of bound cytochrome determined as described under Methods. The concentration of endogenous cytochrome $b_5$ was 0.62 nmol/mg of microsomal protein.

Fig. 2. Time course of and temperature effect on cytochrome $b_5$ binding to rat liver microsomes. Microsomes (10 mg/ml) were incubated with 60 μM cytochrome $b_5$ for the indicated time in a 0.1 M Tris-Cl buffer pH 8.0 as described in Methods. The content of endogenous cytochrome $b_5$ in the microsomal preparation used was 0.55 nmol/mg protein.

When microsomes were incubated with 60 μM D-$b_5$ at various time intervals at 37°C, a rapid binding of cytochrome $b_5$ occurred within 15 min and reached a maximum by 22 min (Fig. 2); using the experimental conditions described in Methods, 0.26 nmol of D-$b_5$ were bound per min per mg microsomal protein. At 20°C, the rate of binding was markedly less, 0.1 nmol of D-$b_5$ per min. The binding was not a result of non-specific protein adsorption to the microsomes, since washing with salt solutions (0.1—0.8 M NaCl) and sonication of the preparations failed to solubilize the bound cytochrome. In addition, our trypsin-solubilized cytochrome $b_5$ preparations, in which the membranous segment of the molecule is removed during the proteolytic isolation [5,38], did not bind to rat liver microsomes.

*Enzymatic properties of bound detergent cytochrome $b_5$*

Homogeneous preparations of cytochrome $b_5$ were not reduced by the addition of NADH, whereas upon binding of the hemoprotein to microsomes, complete reduction by NADH was obtained. These results imply that the bound cytochrome $b_5$ has attained a functional orientation in the microsomal membrane. Furthermore, with increasing amounts of bound D-$b_5$, there was a proportional increase in NADH cytochrome *c* reductase activity (Table I), suggesting that the D-$b_5$ molecules can transfer electrons from cytochrome $b_5$ reductase to cytochrome *c*. In this reaction, reducing equivalents from NADH are transferred by the flavoprotein reductase to cytochrome $b_5$, which in turn donates electrons to cytochrome *c* [39]. Although fatty acyl-CoA desaturase activity was not measured in these experiments, Strittmatter et al. [5] reported that bound cytochrome $b_5$ is an effective electron donor to the cyanide-sensi-

TABLE I

ENZYMATIC PROPERTIES OF BOUND DETERGENT-CYTOCHROME $b_5$

60 μM D-$b_5$ was incubated with 7 ml of rat liver microsomes (10 mg protein/ml). At the end of each incubation period, 1 ml of microsomes was removed, centrifuged for 30 min at 104 000 × *g* and resuspended in 1 ml of 0.1 M Tris/Cl buffer (pH 7.5). Bound D-$b_5$ was measured on the Aminco DW-2 Spectrophotometer using 2.0 mM NADH; the reductase activity was measured as described by Dallner [27].

| Incubation time (min) | Bound cytochrome $b_5$ (nmol/mg microsomal protein | NADH-cytochrome *c* reductase (μmol/min/mg microsomal protein) |
|---|---|---|
| 0 | 0.55 | 0.43 |
| 2 | 1.05 | 0.82 |
| 4 | 1.51 | 1.29 |
| 6 | 2.17 | 1.73 |
| 8 | 2.55 | 1.85 |
| 10 | 3.02 | 2.36 |

tive factor during conversion of stearyl-CoA to oleyl-CoA, providing further evidence that exogenously bound cytochrome $b_5$ is functionally active.

*Effect of bound cytochrome $b_5$ on N-demethylation activities*

The N-demethylation of ethylmorphine by normal rat liver microsomes prior to incubation with D-$b_5$ was approximately 7.0 nmol/min/mg microsomal protein (Table II). In the presence of both pyridine nucleotides, the synergistic effect of NADH was observed, resulting in a 60% stimulation of ethylmorphine demethylase activity. However, when microsomes were preincubated with 60 μM D-$b_5$ at various time periods (from 5 min to 60 min) and subsequently used for determining demethylase activity, an unexpected decrease in the demethylation of ethylmorphine occurred with increasing amounts of bound D-$b_5$

TABLE II

EFFECT OF INCREASING AMOUNTS OF BOUND CYTOCHROME $b_5$ ON THE N-DEMETHYLATION OF ETHYLMORPHINE IN THE PRESENCE AND ABSENCE OF NADH

Microsomes were pre-incubated with 60 μM cytochrome $b_5$ from 5 to 60 min. At the end of each time period, the microsomes were diluted with cold Tris/Cl buffer (pH 8.0), centrifuged at 104 000 × *g* for 30 min and then resuspended to the original incubation volume with 0.25 M sucrose containing 0.1 M Tris/Cl (pH 7.4). The N-demethylation reaction was initiated with the pre-incubated microsomes and run for 8 min. Control N-demethylation activities (6.95 and 11.36 in absence and presence of NADH, respectively) were essentially the same throughout the various pre-incubation time periods. Equimolar concentrations (0.5 mM) of NADPH and NADH were used.

| Pre-incubation time (min) | Microsomal content of cytochrome $b_5$ (nmol/mg protein) | Ethylmorphine N-demethylation (nmol HCHO/min/mg protein) | |
|---|---|---|---|
| | | —NADH | +NADH |
| 0 | 0.51 | 6.95 | 11.36 |
| 5 | 1.04 | 5.61 | 6.55 |
| 10 | 1.65 | 4.11 | 4.73 |
| 15 | 2.33 | 3.93 | 3.75 |
| 20 | 2.64 | 3.45 | 3.89 |
| 30 | 3.01 | 3.56 | 4.12 |
| 40 | 3.35 | 3.33 | 4.18 |
| 60 | 3.74 | 3.31 | 4.15 |

TABLE III

EFFECT OF BOUND NATIVE CYTOCHROME $b_5$, RECONSTITUTED CYTOCHROME $b_5$ AND MANGANESE DERIVATIVE ON MICROSOMAL NADPH CYTOCHROMES *c* AND *P*-450 REDUCTASE ACTIVITIES

Prep. I consists of microsomes containing native cytochrome $b_5$ in which N-demethylations were inhibited. Prep. II consists of microsomes which had been incubated with the reconstituted cytochrome $b_5$. Prep. III is the Mn · $b_5$ derivative bound to microsomes. *represents the amount of Mn · $b_5$ bound to rat liver microsomes (nmol/mg protein).

| Treatment | Cytochrome $b_5$ (nmol/mg protein) | NADPH cytochrome *c* reductase | NADPH cytochrome *P*-450 reductase |
|---|---|---|---|
| | | (nmol/min/mg protein) | |
| Microsomes | 0.55 | 65 | 6.4 |
| Microsomes | | | |
| +Prep. $I_a$ | 0.96 | 38 | 2.3 |
| +Prep. $I_b$ | 2.40 | 21 | 1.7 |
| Microsomes | | | |
| +Prep. $II_a$ | 1.13 | 59 | 6.9 |
| +Prep. $II_b$ | 3.08 | 70 | 6.0 |
| Microsomes | | | |
| +Prep. $III_a$ | 0.82* | 62 | 5.9 |
| +Prep. $III_b$ | 2.15* | 66 | 6.7 |

(Table II); the stimulatory effect of NADH was also abolished by exogenously bound D-$b_5$. Identical results were obtained when aminopyrine replaced ethylmorphine. Furthermore, both NADPH cytochrome *c* reductase and NADPH cytochrome *P*-450 reductase activities were markedly inhibited (45% and 65%, respectively) when the microsomal cytochrome $b_5$ concentration was almost doubled (Table III, Prep. Ia). An explanation of the data could be found in one or more of several possibilities: (1) that bound D-$b_5$ is acting as an electron sink channelling reducing equivalents away from the mixed function oxidase system, (2) that bound D-$b_5$ by its very presence in large amounts (relative to endogenous cytochrome $b_5$) on the membrane is disrupting the normal sequential flow of electrons through the mixed function oxidase pathway, (3) that incorporation of cytochrome $b_5$ into the microsomes results in an increase in the membrane protein-lipid ratio (such alteration of the membrane components could affect the architecture or fluidity of the membrane, which in turn may have an adverse effect on the mixed function oxidase system), (4) that our cytochrome $b_5$ preparation, although homogeneous, contains trace amounts of tightly bound detergent and (5) the inhibitory effect observed could be attributed to any combination of these possibilities.

*Binding of manganese derivative of cytochrome $b_5$ to rat liver microsomes and their effect on N-demethylation*

In an attempt to determine the mechanism by which exogenously bound D-$b_5$ inhibited drug metabolism, manganese analogue of cytochrome $b_5$ was prepared and its binding to microsomes examined. The reason for using the manganese derivative of cytochrome $b_5$ was two-fold. Firstly, to delineate the

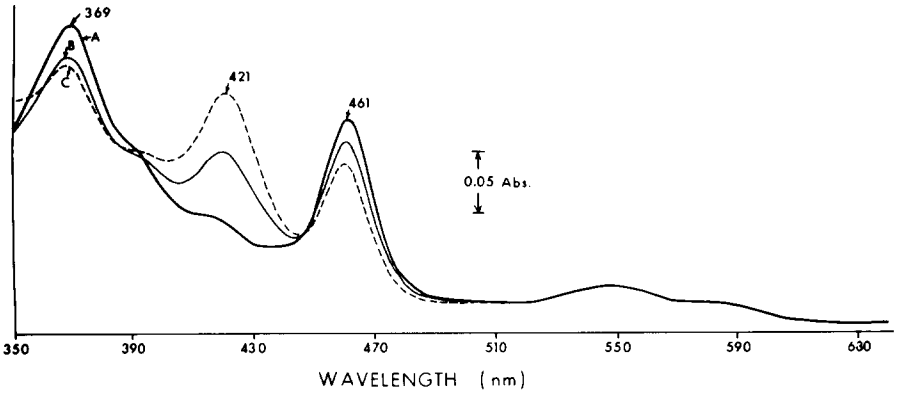



Fig. 3. Absorbance spectra of manganese protoporphyrin in 20 mM Tris/HCl buffer (pH 7.5) containing 50% ethanol. The concentration of metalloporphyrin was 5 $\mu$M. Curve A, oxidized form; Curve B, dithionite-reduced form; Curve C, CO complex of the reduced form. Substitution of Mn mesoporphrin for the protoporphyrin gave identical spectra.

role of heme in the above reaction and secondly, to determine whether the physical presence of the hemoprotein was disrupting sequential flow of electrons.

The absolute absorbance spectrum of the manganese protoporphyrin complex shown in Fig. 3 (Curve A) consisted of 2 major peaks at 369 nm and 461 nm with shoulders at 390 nm and 415 nm (cf. ref 36). The spectrum of the metalloporphyrin upon reduction with dithionite (Curve B) resulted in a decrease in the absorbance maxima and the formation of a new peak at 421 nm. When the oxidized derivative was complexed with apocytochrome $b_5$, a 4 nm spectral shift to 465 nm and a loss of both shoulders was observed.

The rate and extent of binding of the metalloporphyrin-apocytochrome $b_5$ (Mn · $b_5$) complex to rat liver microsomes is shown in Fig. 4. About 1 nmol of Mn · $b_5$ was bound to microsomes when 30 nmol were incubated at 20°C for 30 min; as much as 4—6 nmol/mg microsomal protein were bound when the temperature was raised to 37°C and the concentration increased to 50—60 nmol Mn · $b_5$ (Fig. 4).

When microsomal preparations containing various amounts of bound Mn · $b_5$ were tested for N-demethylation of either ethylmorphine or aminopyrine, no inhibition of drug metabolism was observed (Fig. 5). Moreover, neither NADPH cytochrome *c* reductase nor NADPH cytochrome *P*-450 reductase activity was inhibited in microsomes containing 0.82 nmol and 2.15 nmol of Mn · $b_5$/mg microsomal protein (Table III, Prep. $III_a$ and $III_b$), strongly suggesting that the physical presence of extra bound cytochrome $b_5$ is not the reason for the observed inhibition of demethylation activity. Results obtained using the Mn protoporphyrin derivative were identical to those obtained with Mn mesoporphyrin derivative.

Since the Mn derivatives of cytochrome $b_5$ were prepared by removing the iron protoporphyrin from the cytochrome and then reconstituting the apocytochrome with the metalloporphyrin, control experiments using reconstituted cytochrome $b_5$ prepared from heme and apocytochrome were also performed. Contrary to the native bound cytochrome $b_5$, the microsomal preparations

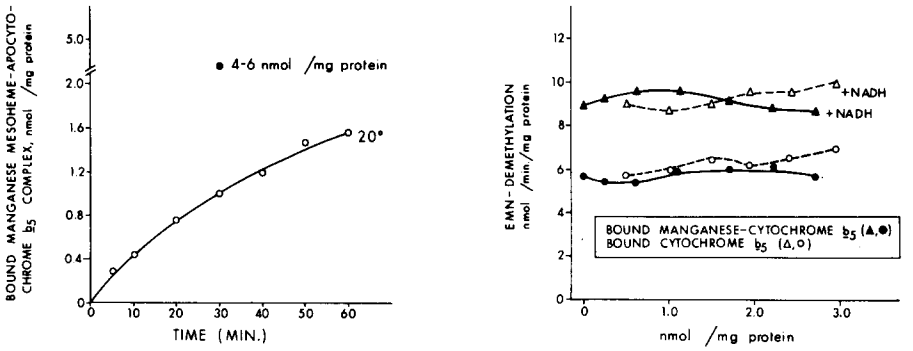



Fig. 4. Time course of binding of manganese porphyrin-apocytochrome $b_5$ complex to rat liver microsomes at 20° C. The metalloporphyrin-apocytochrome complex (60 μM) was incubated with microsomes (10 mg/ml) as described in Methods. The single solid circle represents the amount of the Mn derivative bound to microsomes following incubation at 37° C for 30 min.

Fig. 5. Effect of increasing amounts of bound Mn · $b_5$ (▲, ●) and reconstituted cytochrome $b_5$ (△, ○) on demethylation activity in the presence and absence of NADH. The binding of cytochrome $b_5$ and the manganese derivative to microsomes and the determination of demethylase activity were carried out as in Table II.

containing reconstituted cytochrome $b_5$ did not inhibit ethylmorphine or aminopyrine N-demethylation (Fig. 5) in the absence or presence of NADH even at concentrations of 3 nmol cytochrome $b_5$ /mg microsomal protein; as shown in Table II, native cytochrome $b_5$ inhibited demethylation approximately 50% at this concentration. Furthermore, no inhibition of either NADPH cytochrome *c* reductase or NADPH cytochrome *P*-450 reductase activity was observed in microsomal preparation containing reconstituted cytochrome $b_5$ (Table II, Prep. II).

The fourth possibility, that our cytochrome $b_5$ preparation contained tightly bound detergent responsible for the observed inhibited activities, could explain our data, provided our reconstituted cytochrome $b_5$ was identical to the preparation prior to heme extraction. Strittmatter [40] had previously reported that microsomal cytochrome $b_5$ can be completely resolved into an apoprotein which will recombine with 1 mol of heme to yield the original cytochrome $b_5$ absorbance spectrum and a hemoprotein having the same physical and enzymatic characteristics as the native molecule. As observed with native D-$b_5$, the NADH cytochrome *c* reductase activity increased in proportion to the amount of reconstituted cytochrome $b_5$ bound to microsomes (Fig. 6). The NADH cytochrome $b_5$ reductase activity, determined with ferricyanide as the electron acceptor, did not change with increasing amounts of cytochrome $b_5$, as was expected, since the rate-limiting step in the reduction of microsomal cytochrome $b_5$ is the transfer of electrons from the flavoprotein reductase to the hemoprotein [5]. The reconstituted cytochrome $b_5$ was active in the desaturation of stearyl-CoA, as previously observed by Strittmatter et al. [5].

On the assumption that the homogeneous cytochrome $b_5$ preparations contained detergent, three additional Sephadex G-100 columns were prepared and a sample of our purified hemoprotein was passed through the columns. The cytochrome $b_5$ preparation eluted from the third column was then used for

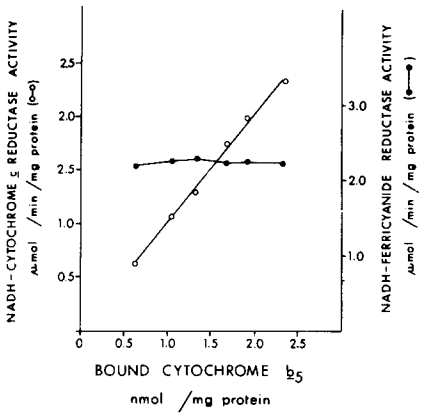



Fig. 6. Effect of reconstituted bound cytochrome $b_5$ on NADH-cytochrome *c* reductase and NADH cytochrome $b_5$ reductase activities. Heme-free apocytochrome was prepared and reconstituted with heme as described in Methods. The preparation was then incubated with 10 mg/ml microsomes in the usual manner. Microsomal preparations used in this experiment contained 0.62 nmol of endogenous cytochrome $b_5$ and the endogenous NADH cytochrome *c* and $b_5$ reductase activities were 0.6 and 2.2 μmol/min/mg protein, respectively.

binding studies. The results obtained were identical to those obtained with the reconstituted cytochrome $b_5$, i.e., there was no inhibition of either ethylmorphine or aminopyrine demethylase activities as well as no effect on NADPH cytochrome *P*-450 reductase activity with increasing amounts of cytochrome $b_5$ bound to microsomes.

*Detection of detergent in acid-acetone extract of D-cytochrome $b_5$*

On the basis of the aforementioned data which suggest the presence of detergent tightly bound to cytochrome $b_5$, acid-acetone was added to a solution of cytochrome $b_5$ as described in the methods section. The acid-acetone extract was then evaporated to 10% of its original volume to concentrate the detergent, if present. Analysis of the concentrated extract as measured by difference spectra showed a broad absorbance band at about 278 nm, with an extinction of 0.159. At this wavelength region heme does not absorb and hence does not interfere, and a protein determination on the extract was negative. Assuming an extinction coefficient of 25 per 1% solution, we found 0.4 μmol of detergent per μmol of cytochrome $b_5$. No detergent was detected in the cytochrome $b_5$ preparations which were eluted from 3 additional Sephadex G-100 columns. The acid-acetone extract obtained from the reconstituted cytochrome $b_5$ also had no measurable absorbance peak at 278 nm.

## Discussion

Estabrook and co-workers [11] put forward the concept that cytochrome $b_5$ participates in the transfer of electrons to mixed function oxidase system during the oxidation of drugs, other xenobiotics and steroids. This postulated mechanism is supported by the elegant studies of Correia and Mannering [14,15] who showed that stearyl-CoA decreased the rate of metabolism of

ethylmorphine by shunting electrons from cytochrome $b_5$ to the microsomal fatty acyl-CoA desaturase system and that low concentrations of cyanide increased the rate of drug metabolism by diverting electrons away from the desaturase system. The findings of Sasame et al. [13] who reported a decrease in both NADH oxidation and N-demethylation in the presence of cytochrome $b_5$ antibody support the view that the transfer of the second electron from NADH to ternary *P*-450 complex is mediated by cytochrome $b_5$. More recently, Mannering et al. [18] reported that anti-cytochrome $b_5$ immunoglobulin inhibited the NADH synergism of NADPH-dependent ethylmorphine N-demethylation implicating cytochrome $b_5$ participation in the transfer of reducing equivalents from NADH to the mixed function oxidase system.

Our data are not necessarily in disagreement with the aforementioned evidence. The inhibition of drug metabolism (N-demethylation of aminopyrine and ethylmorphine) observed with increasing amounts of bound native cytochrome $b_5$ presumably was not due to disruption of electron flow through the mixed function oxidase pathway by the physical presence of D-$b_5$, since the binding of the Mn porphyrin-apocytochrome complex to microsomes failed to show inhibition of N-demethylation. As much as 6 nmol of the Mn derivative per mg protein were bound to rat liver microsomes without affecting either NADPH cytochrome *c* reductase or NADPH cytochrome *P*-450 reductase activities. The Mn derivative was the analogue of choice since it combined stoichiometrically with apocytochrome $b_5$ and is not reduced by the cytochrome $b_5$ reductase system, despite its ability to accept electrons from dithionite.

Contrary to the detergent-solubilized cytochrome $b_5$, binding to liver microsomes of either the reconstituted cytochrome $b_5$ or the preparation which was eluted through three additional columns did not inhibit drug metabolism. The inhibition observed with native cytochrome $b_5$ is most probably attributed to the presence of Triton bound to cytochrome $b_5$ since as little as 0.005% Triton N-101 present in an assay mixture inhibited demethylase activity by 15—20% (Lemelin and Cinti, unpublished observation) and since this detergent was used in the isolation of cytochrome $b_5$. This could explain the apparent discrepancies present in the literature [16,20].

With regard to the detergent effect, it is of interest to indicate that the NADPH-dependent, but not the NADH-dependent reactions were inhibited by added D-cytochrome $b_5$. Both reductases are flavoproteins which, on the basis of their ease of solubilization by detergents and proteolytic enzymes, are believed to be localized to the external surface of the microsomal membrane. Yet the detergent appears to disrupt only the microsomal electron transfer chain which utilized NADPH since NADH ferricyanide reductase and NADH cytochrome *c* reductases activities were not inhibited.

Finally, although increased amounts of bound cytochrome $b_5$ have no stimulatory effect on N-demethylase activity, our data do not exclude the possibility of a common component between the cytochrome $b_5$ and *P*-450 pathways which may be rate-limiting and therefore increasing the amount of cytochrome $b_5$ bound to the membrane would not stimulate drug oxidation. Furthermore, if sufficient quantities of cytochrome $b_5$ are already present on the membrane so that the hemoprotein is not rate-limiting, then increasing the amount of bound cytochrome $b_5$ would also not stimulate drug oxidations.

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