Biochemical Pharmacology, Vol. 34, No. 11, pp. 1907–1912, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00
Pergamon Press Ltd.

OBLIGATORY ROLE OF CYTOCHROME b_5 IN THE MICROSOMAL METABOLISM OF METHOXYFLURANE*

ELEANOR CANOVA-DAVIS†‡, JOHN Y. L. CHIANG§ and LUCY WASKELL†‡‖

† Department of Anesthesia, Veteran's Administration Medical Center, San Francisco, CA 94121; ‡ Liver Center, University of California, San Francisco, CA 94143; and § Department of Molecular Pathology, Northeastern Ohio Universities College of Medicine, Rootstown, OH 44272, U.S.A.

(*Received* 3 *February* 1984; *accepted* 30 *September* 1984)

Abstract—Cytochrome b_5 has recently been shown to be required in the reconstituted cytochrome P-450 system for the metabolism of the volatile anesthetic methoxyflurane [E. Canova-Davis and L. A. Waskell, *J. biol. Chem.* **259**, 2541 (1984)]. To determine whether this observation in the reconstituted system was merely dependent on the particular ratios of the various components or some other fortuitous, unknown factor, or whether cytochrome b_5 plays a role in the liver microsomal metabolism of methoxyflurane, the following studies were undertaken. Antibody to rabbit holocytochrome b_5 was raised in guinea pigs. The antibody to cytochrome b_5 was able to inhibit 75% of the microsomal metabolism of methoxyflurane. This same antibody also inhibited methoxyflurane metabolism in the reconstituted system. When the antibody to cytochrome b_5 was treated with purified cytochrome b_5 before addition to the microsomes, it did not inhibit methoxyflurane metabolism. Furthermore, the antibody to cytochrome b_5 did not inhibit the microsomal metabolism of benzphetamine. This suggests that cytochrome b_5 was required for the microsomal metabolism of methoxyflurane. It is possible that cytochrome b_5 functioned in the metabolism of methoxyflurane by retaining a specific conformation of cytochrome P-450 and not by transferring the second electron to cytochrome P-450. To explore this possibility, cytochrome b_5 was reconstituted with Mn^{3+}-protoporphyrin IX. The Mn^{3+}-protoporphyrin IX derivative retained the conformation of cytochrome b_5 but not its electron transfer properties. This manganese derivative of cytochrome b_5 was unable to stimulate the metabolism of methoxyflurane. The study demonstrated that cytochrome b_5 was obligatory for the microsomal metabolism of methoxyflurane, whereas it was not required for the microsomal N-demethylation of benzphetamine. Moreover, the heme moiety of cytochrome b_5 functioned to transfer electrons in this reaction.

It is now generally accepted that, under a variety of conditions, cytochrome b_5 is able to transfer electrons to cytochrome P-450 [1–5]. Hildebrandt and Estabrook [1] were the first to propose the currently held view that cytochrome b_5 stimulates cytochrome P-450 activity by providing the second of the two electrons required for NADPH-dependent oxidations. However, the postulate that cytochrome b_5 plays an essential role in the oxidation of all substrates by cytochrome P-450 is not universally accepted for two reasons. First, the addition of cytochrome b_5 to a particular reconstituted system in different laboratories has no effect or results in either inhibition or stimulation of the reaction [6]. Recent experiments by Bösterling *et al.* [6] have begun to unravel some of the reasons for these contradictory results. They report that the effects of cytochrome b_5 in a reconstituted system are dependent on both protein:protein and lipid:protein ratios [6]. The second reason is that few substrates tested in the reconstituted system seem to have an absolute requirement for cytochrome b_5 [7–10]. For example, an absolute requirement for cytochrome b_5 in the O-deethylation of *p*-nitrophenetole in a reconstituted system was observed by Kuwahara and Omura [7]. Shortly afterwards, Vatsis *et al.* [8] demonstrated that cytochrome b_5 is essential for prostaglandin A_1, E_1 and E_2 metabolism by cytochrome P-450_{LM_2}¶, purified from phenobarbital-induced rabbit liver. The same cytochrome P-450 isozyme was later shown to require cytochrome b_5 [9] for the O-demethylation of the volatile anesthetic methoxyflurane ($CHCl_2CF_2OCH_3$).

The above discussion reveals that in the reconstituted system, depending on the particular cytochrome P-450 isozyme and substrate, cytochrome b_5 has no effect or acts in either a facilitory, inhibitory or obligatory role. In view of the studies by Bösterling and co-workers [6] which suggest that the

* This research was supported in part by Grants GM-27791 (L. W.) and GM 31584 (J. Y. L. C.) from the National Institutes of Health.

‖ Send correspondence to: Dr. Lucy Waskell, Department of Anesthesia (129), Room 3C-14, Building 203, Veteran's Administration Medical Center, 4150 Clement St., San Francisco, CA 94121.

¶ Abbreviations: anti cytochrome b_5, guinea pig antibody against cytochrome b_5; CM cellulose, carboxymethyl cellulose; DE cellulose, diethylaminoethyl cellulose; dilauroyl-GPC, dilauroyl glycerol-3-phosphoryl choline; HA, hydroxyapatite; IgG, immunoglobulin G; NADP, β-nicotinamide adenine dinucleotide phosphate; P-450_{LM}, liver microsomal cytochrome P-450; PB, phenobarbital; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; and Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol. The isozymes of rabbit liver microsomal cytochrome P-450 are numbered according to their relative electrophoretic mobilities, in accordance with the general recommendation of the Committee on Biochemical Nomenclature of the International Union of Biochemistry.

role of cytochrome b_5 (inhibitory or facilitory) varies depending on the ratio of the lipids and proteins, it was of interest to investigate the role of cytochrome b_5 in the microsomal metabolism of methoxyflurane. In particular, these studies were undertaken to determine whether the results of our reconstituted studies were simply dependent on the proportion of the various components of the reconstituted system or whether cytochrome b_5 actually was required for the microsomal metabolism of a drug. If this latter possibility were true, the results might possibly be of significance *in vivo*. The current study establishes that cytochrome b_5 is obligatory for the microsomal cytochrome P-450-catalyzed metabolism of methoxyflurane and that the heme moiety of cytochrome b_5 is functioning in electron transfer in this reaction.

EXPERIMENTAL PROCEDURES

Purification of microsomal enzymes. Liver microsomes were prepared from phenobarbital-treated white New Zealand male rabbits as described by Haugen and Coon [11]. The procedure for obtaining the heat-stable factor from microsomes has been published previously [12]. The method of Dignam and Strobel [13] as modified by Vermilion and Coon [14] was followed for the isolation of NADPH-cytochrome P-450 reductase from pyrophosphate-extracted PB-induced rabbit liver microsomes. The preparation had a specific activity of 43 μmoles of cytochrome *c* (Sigma Type VI from horse heart) reduced per min per mg of protein in 0.3 M potassium phosphate buffer, pH 7.7, at 30°. The purification to electrophoretic homogeneity of cytochrome P-450_{LM_2} from PB-treated rabbits was performed according to Johnson *et al.* [15] as modified by Canova-Davis and Waskell [9]. The concentration of cytochrome P-450 was determined by the method of Omura and Sato [16]. The method of Chiang [17] was used for the purification of cytochrome b_5 from detergent-solubilized microsomes of PB-treated rabbits as modified by Canova-Davis and Waskell [9]. The concentration of purified cytochrome b_5 was ascertained from the absolute spectrum of the ferric protein using an absorption coefficient of 117 $mM^{-1}\,cm^{-1}$ at 413 nm [18]. In the unpurified preparations, the amount of cytochrome b_5 was estimated from the reduced versus oxidized spectra [19, 20].

Reconstitution of apocytochrome b_5 *with manganese-protoporphyrin IX.* Apocytochrome b_5 was prepared by an acid-acetone treatment as described by Cinti and Ozols [21] as modified by Vatsis *et al.* [8]. The reconstitution of cytochrome b_5 with manganese-protoporphyrin IX (Porphyrin Products, Logan, UT) was effected according to Vatsis *et al.* [8] as modified by Canova-Davis and Waskell [9]. The manganese cytochrome b_5 complex was measured spectrally by the method of Rogers and Strittmatter [22].

Other methods. Benzphetamine N-demethylation and methoxyflurane O-demethylation were assayed as previously published [9]. Inorganic fluoride ion was measured with an Orion ion-specific electrode, and formaldehyde was monitored as described by Nash [23]. Protein concentrations were measured according to Lowry *et al.* [24] after precipitation of the proteins in the presence of trichloroacetic acid and sodium deoxycholate [25]. Synthetic lipids were suspended in water by sonication. Optical spectra were recorded at room temperature with a Cary 219 spectrophotometer operated in the double beam mode. Polyacrylamide slab gel electrophoresis was carried out in the presence of SDS with the discontinuous buffer system described by Laemmli [26].

Preparation of cytochrome b_5 *antibody.* Antibody against rabbit cytochrome b_5 was raised in the guinea pig. Each animal received a total of 1 ml of antigen (1 mg/ml) mixed with an equal volume of complete Freund's adjuvant at several spots on the back and on both foot pads. An additional injection of the same dose was given 4 weeks later. Subsequently, booster injections were given biweekly. Blood was collected by cardiac puncture weekly after the third dose of antigen. The titer of the antisera was tested by Ouchterlony double diffusion [17]. IgG fractions were prepared from high titer antisera by ammonium sulfate fractionation and DEAE-cellulose chromotography [27]. Guinea pig antibody against rabbit cytochrome b_5 formed a precipitin line only with rabbit cytochrome b_5 and rabbit liver microsomes on Ouchterlony double diffusion and rocket immunoelectrophoresis. No cross-reactivity was observed between guinea pig antibody and hamster or rat cytochrome b_5*.

RESULTS

Verification of the cellular location of the protein required for the O-demethylation of methoxyflurane. Previously published data [12] showed that the methoxyflurane-metabolizing activity of partially purified cytochrome P-450_{LM_2} preparations could be stimulated markedly by the addition of a heat-stable factor found in the fractions of solubilized microsomes precipitated by PEG. Subsequently, this factor was identified as cytochrome b_5 [9]. To provide additional supporting evidence that the observed stimulation of the cytochrome P-450_{LM_2}-mediated methoxyflurane metabolism by cytochrome b_5 in the reconstituted system is indeed due to cytochrome b_5, the cellular location of the stimulatory factor was examined. Analysis for various cellular fractions indicated that this factor was found primarily in membranous fractions (Table 1). The amount of cytochrome b_5 in a given cellular fraction was approximately parallel to the activation properties of the individual fractions. The mitochondrial pellet contained a significant amount of cytochrome b_5 because of its marked contamination with microsomes. The cytosol was essentially devoid of activity while the cytosolic lipids were completely inactive (Table 1).

Inability of cytochrome c to activate methoxyflurane metabolism in the reconstituted system. To determine whether the effects of cytochrome b_5 were specific for methoxyflurane metabolism in the reconstituted system or whether other cytochromes would

* John Y. L. Chiang, unpublished results.

Table 1. Activating properties of selected cellular fractions

Fraction	Activation properties* (nmoles F^-/mg/min)	Concentration of cytochrome b_5 (nmoles/mg protein)
10,000 *g* pellet of the whole cell homogenate	1.0	1.9
Cytosol	0.05	ND†
Phosphate-washed microsomes	1.2	1.4
Cytosolic lipids	0	ND

* The assay mixture contained partially purified cytochrome P-450_{LM_2} (1.8 nmoles/ml), cytochrome P-450 reductase (0.5 nmole/ml), 0.1% Nonidet P-40 and the standard NADPH-generating system of 0.1 M Tris acetate buffer, pH 7.4, at 37°, 10 mM glucose-6-phosphate, 1 mM NADP, and 0.3 units of glucose-6-phosphate dehydrogenase in a total volume of 300 μl. The cellular fraction, heated at 75° for 2 min, was used as the source of the activation factor. The reaction mixture was incubated in a shaking water bath for 60 min at 37°. The reaction was started by addition of 1 μl of methoxyflurane.

† ND indicates not detected.

work as well, cytochrome *c* was substituted for cytochrome b_5. When cytochrome *c*, in contrast to cytochrome b_5, was added to the reconstituted system, it was unable to stimulate methoxyflurane metabolism (Table 2). Thus, the effects of cytochrome b_5 appear to be specific with respect to methoxyflurane metabolism.

Role of the heme prosthetic group of cytochrome b_5 *in the cytochrome P-*450_{LM_2}*-catalyzed methoxyflurane O-demethylation.* Previously reported studies [9] have demonstrated that apocytochrome b_5 is unable to replace holocytochrome b_5 in the metabolism of methoxyflurane by purified cytochrome P-450_{LM_2}. To illustrate that the heme is actually needed for electron transfer and not merely to retain a specific conformation of the cytochrome, apocytochrome b_5 was reconstituted with the hexacoordinate porphyrin Mn^{3+}-protoporphyrin IX. The Mn^{3+}-protophorphyrin IX-cytochrome b_5 derivative was unable to duplicate the stimulatory properties seen with reconstituted Fe^{3+}-protoporphyrin IX-cytochrome b_5 (Fig. 1).

NADH synergism of NADPH-dependent O-demethylation of methoxyflurane in phenobarbital-induced rabbit liver microsomes. As shown in Table 3, NADH catalyzed the O-demethylation of methoxyflurane at 19% of the rate which was observed when NADPH was the sole source of electrons. The addition of NADH to incubation mixtures containing an NADPH-generating system increased the rate of anesthetic metabolism to a greater extent than could be accounted for by the metabolism in the presence of NADH alone. This synergistic effect implicates cytochrome b_5 in the transfer of electrons to cytochrome P-450 in microsomes.

Inhibition of the O-demethylation of methoxyflurane by antibody directed against cytochrome b_5. The metabolism of methoxyflurane by PB-induced rabbit liver microsomes was inhibited by 75% with guinea pig anti cytochrome b_5 IgG (Fig. 2). Control immunoglobulin from guinea pig sera had no effect. Additional evidence that the inhibition was due to

Table 2. Effects of cytochrome b_5 and cytochrome *c* on the metabolism of methoxyflurane in the reconstituted system*

Sample	F^- (nmoles/60 min)
Compete system − b_5	1.1
Complete system + b_5	20.0
Complete system + Cyt *c*	1.4

* The complete system contained purified cytochrome P-450_{LM_2} (17.2 nmoles P-450/mg), NADPH-cytochrome P-450 reductase (10.81 nmoles/mg), cytochrome b_5 (32.39 nmoles/mg) and cytochrome *c* as indicated at a final concentration of 1.15 nmoles/ml, dilauroyl phosphatidyl choline (final concentration 100 μg/ml), 0.1 M Tris acetate, 1 mM NADP, glucose-6-phosphate dehydrogenase (final concentration 1 unit/ml) and 10 mM glucose-6-phosphate in a total volume of 300 μl.

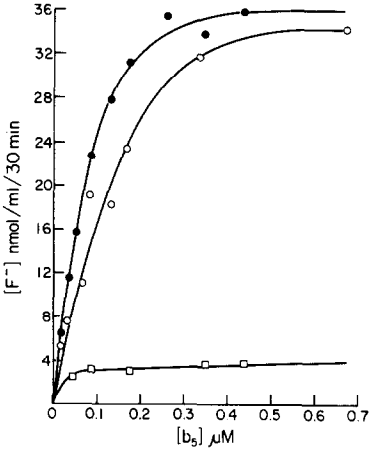

Fig. 1. Role of Mn^{3+}-protoporphyrin IX and heme reconstituted cytochrome b_5 in the metabolism of methoxyflurane. The assay mixture contained the standard NADPH-generating system as described in Table 1, partially purified cytochrome P-450_{LM_2} (0.9 nmole/ml), cytochrome P-450 reductase (0.27 nmole/ml), and the cytochrome b_5 analog as indicated: (●——●) native cytochrome b_5, (○——○) heme reconstituted cytochrome b_5, and (□——□) Mn^{3+}-protoporphyrin IX reconstituted cytochrome b_5. The reaction was conducted for 30 min at 37°.

Table 3. Synergistic effect of NADH on NADPH-dependent O-demethylation of methoxyflurane in PB-induced rabbit liver microsomes*

NADPH (μM)	% Activity	
	− NADH	+ 1 mM NADH
0	0	19
11	32	59
22	64	97
44	77	117
89	94	142
179	100	153
531	99	142

* The reaction mixture contained an NADPH-generating system as described in Table 1 at the concentrations indicated and 0.37 mg/ml of PB-induced microsomes. The specific activity at 100% activity in the presence of NADPH alone was 5.2 nmoles of F^- produced per mg of microsomal protein per min.

the interaction of anti b_5 IgG with microsomal cytochrome b_5 is recorded in Table 4. Preincubation of anti b_5 IgG with purified cytochrome b_5 neutralized the inhibitory effect of anti b_5 IgG on the microsomal metabolism of methoxyflurane. The anti cytochrome b_5 preparation was also capable of inhibiting the O-demethylation of methoxyflurane in a reconstituted system (Fig. 3).

Effect of antibody directed against cytochrome b_5 *on benzphetamine N-demethylase activity.* In contrast to the inhibition of methoxyflurane metabolism by PB-induced liver microsomes seen with cytochrome b_5 antibody, there was no effect upon the metabolism of benzphetamine (Table 5). Neither control

Table 4. Neutralization of antibody against cytochrome b_5 by purified cytochrome b_5*

Addition	F^- (nmoles/ml/20 min)	% Activity
None	23.7	100
Anti b_5 (mg/ml)		
2.0	17.7	75
2.4	16.5	70
Anti b_5 + b_5 (nmoles b_5/mg anti b_5)		
0.09	17.5	74
0.17	17.4	73
0.21	20.0	84
0.35	23.7	100
0.43	22.0	93
0.65	22.2	94
0.86	22.8	96

* Assay mixtures contained a standard NADPH-generating system described in Table 1, 0.22 mg/ml of PB-induced microsomes, and additions as indicated in a final volume of 300 μl. Anti cytochrome b_5 and cytochrome b_5 were preincubated at room temperature for 5 min before other components were added. Then, the complete reaction mixture was preincubated at 4° for 15 min before the reaction was started with 1 μl of methoxyflurane. The reaction mixture was incubated for 20 min at 37° in a shaking water bath.

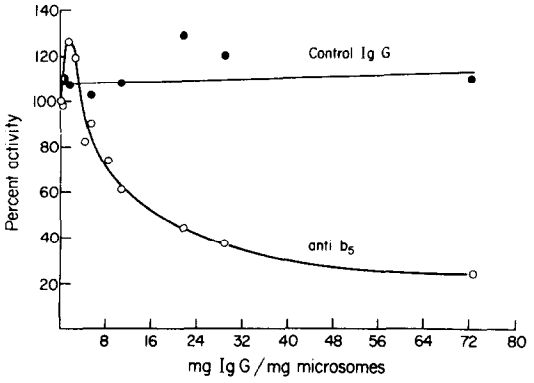

Fig. 2. Inhibition of the metabolism of methoxyflurane in PB-induced microsomes by antibody directed against cytochrome b_5. The reaction mixture contained the standard NADPH-generating system as described in Table 1, 0.22 mg/ml of PB-induced microsomes, and antibody as indicated. The complete mixture was preincubated at 4° for 15 min before the reaction was begun with 1 μl of methoxyflurane. The incubation was conducted for 20 min at 37°. In the absence of immunoglobulin, the specific activity was 4.8 nmoles of F^- formed per mg of microsomal protein per min.

immunoglobulin from guinea pig sera nor cytochrome b_5 antibody caused a significant inhibition of the N-demethylation of benzphetamine.

DISCUSSION

It has been reported that liver microsomes of PB-treated rabbits metabolize methoxyflurane at an enhanced rate [28]. However, this activity is lost upon solubilization and purification of the PB-induced P-450 from microsomes [12]. Addition of

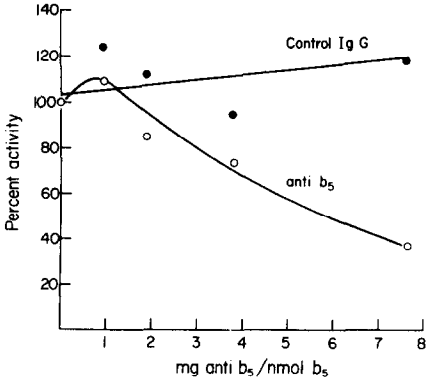

Fig. 3. Inhibition of methoxyflurane metabolism in a reconstituted enzyme system by antibody directed against cytochrome b_5. The reaction mixture contained the standard NADPH-generating system as described in Table 1, 0.42 nmole/ml of cytochrome $P\text{-}450_{LM_2}$ and 0.41 nmole/ml of cytochrome P-450 reductase, 0.42 nmole/ml of cytochrome b_5, 43 nmoles/ml of dilauroyl-GPC, and antibody as indicated. The complete assay mixture was preincubated at 4° for 15 min before the reaction was begun with 1 μl of methoxyflurane. The incubation was conducted for 60 min at 37°. In the absence of immunoglobin, the specific activity was 0.7 nmole of F^- produced per nmole of cytochrome P-450 per min.

Table 5. Effects of anti-cytochrome b_5 and control immunoglobulin on benzphetamine N-demethylation by microsomes*

Immunoglobulin (mg/mg microsomes)	% activity Control	Anti b_5
0	100	100
1.1	90	102
2.2	99	88
4.3	97	104
8.6	101	106
17.3	98	97
21.6	103	92
32.4	111	92
43.2	114	104

* The reaction was assayed as described in Fig. 2 except that 0.37 mg/ml of PB-induced microsomes was used. The reaction was started by additing benzphetamine to a final concentration of 1 mM. The specific activity in the absence of immunoglobulin was 5.8 nmoles of formaldehyde produced per mg of microsomal protein per min.

cytochrome b_5 to the purified PB-induced cytochrome P-450, LM_2, resulted in a recovery of activity comparable to that observed in microsomes [9]. A systematic search of the various cellular fractions (Table 1) revealed that only membrane containing fractions were able to stimulate the metabolism of methoxyflurane by solubilized cytochrome P-450. These data are consistent with the contention that cytochrome b_5 is required for methoxyflurane metabolism.

It has been postulated that cytochrome b_5 functions in the metabolism of methoxyflurane by donating the second of the two electrons needed for oxidation [9]. This was deduced from previous studies which investigated the reoxidation of cytochrome b_5 [1, 3], the more recent observations that cytochrome b_5 was needed in a 1:1 molar ratio to effect maximal metabolism, and that apocytochrome b_5, lacking the heme prosthetic group, could not stimulate the metabolism of methoxyflurane [9]. An alternative interpretation of the latter observation is that the function of cytochrome b_5 was merely to induce a particular conformation on the cytochrome P-450 system and not to transfer an electron. It is possible that the apocytochrome b_5 obtained by acid-acetone precipitation exists in a different conformation than native cytochrome b_5. This possibility was eliminated by the experiment in which a Mn^{3+}-protoporphyrin IX-cytochrome b_5 preparation, which retains the conformation of holocytochrome b_5 but which cannot transfer electrons, was reconstituted and shown to be inactive ([29], Fig. 1).

Similarly, the demethylation of methoxyflurane in a reconstituted enzyme system may not reflect the metabolism as it proceeds in microsomes. It is known that NADH donates its electrons to cytochrome b_5 via the cytochrome b_5 reductase [1]. Electrons from NADPH can reach cytochrome P-450 either directly through NADPH-cytochrome P-450 reductase or indirectly from the reductase through cytochrome b_5 [1, 3]. If cytochrome b_5 is truly a component in the metabolism of methoxyflurane in the microsomes, adding NADH to the system containing NADPH should produce a rate greater than the additive rate calculated from the velocity in the presence of NADH or NADPH alone [1, 5]. Table 3 illustrates that this synergistic effect can be demonstrated.

Cytochrome b_5 has been implicated in the hydroxylations by cytochrome P-450 with a number of substrates in both a facilitory [2, 6, 17] and an obligatory role [7, 8, 10]. The work with the reconstituted system of P-450-catalyzed methoxyflurane metabolism allocates the involvement of cytochrome b_5 to an obligatory one since cytochrome b_5 must be added to effect metabolism ([9], Table 2). The experiments in which antibody to cytochrome b_5 was used to inhibit the NADPH-dependent methoxyflurane metabolism in microsomes (Fig. 2 and Table 4) suggest that cytochrome b_5 is also obligatory for the microsomal metabolism of methoxyflurane. The fact that anti cytochrome b_5 (Fig. 2) inhibited methoxyflurane metabolism by a maximum of 75% may be explained by the existence of other isozymes capable of metabolizing methoxyflurane (L. Waskell and E. Canova-Davis unpublished results) in the absence of cytochrome b_5. The inhibition of methoxyflurane metabolism in a reconstituted system by anti cytochrome b_5 (Fig. 3) corroborates the specificity of action of the anti cytochrome b_5 preparation. The lack of inhibition of benzphetamine N-demethylation by anti cytochrome b_5 (Table 5) was anticipated since cytochrome b_5 is not an obligatory component in this reaction [2]. The result adds further credence to the assumption that anti cytochrome b_5 is not a general inhibitor of the microsomal mixed-function oxidases.

The data suggest that a given anesthetic or drug may affect the *in vivo* utilization of NADH and NADPH via its preference for either cytochrome P-450 reductase or cytochrome b_5. In addition, anesthetic metabolism may be competing with essential biosynthetic reactions for electrons since the *in vivo* role of cytochrome b_5 is to provide electrons via NADH for a host of important reactions, including prostaglandin, cholesterol and fatty acid synthesis [30–32]. This competition could have significant consequences on the susceptibility of an organism to the cytotoxic effects of different xenobiotics.

Acknowledgements—We thank Dr. H. Loh of the University of California at San Francisco for his gift of benzphetamine.

REFERENCES

1. A. Hildebrandt and R. W. Estabrook, *Archs Biochem. Biophys.* **143**, 66 (1971).
2. A. Y. H. Lu, S. B. West, M. Vore, D. Ryan and W. Levin, *J. biol. Chem.* **249**, 6701 (1974).
3. C. Bonfils, C. Balny and P. Maurel, *J. biol. Chem.* **256**, 9457 (1981).
4. M. Noshiro, N. Harada and T. Omura, *Biochem. biophys. Res. Commun.* **91**, 207 (1979).
5. G. J. Mannering, S. I. Kuwahara and T. Omura, *Biochem. biophys. Res. Commun.* **57**, 476 (1974).
6. B. Bösterling, J. R. Trudell, A. J. Trevor and M. Bendix, *J. biol. Chem.* **257** 4375 (1982).
7. S. Kuwahara and T. Omura, *Biochem. biophys. Res. Commun* **96**, 1562 (1980).
8. K. P. Vatsis, A. D. Theoharides, D. Kupfer and M. J. Coon, *J. biol. Chem.* **257**, 11221 (1982).

9. E. Canova-Davis and L. A. Waskell, *J. biol. Chem.* **259**, 2541 (1984).
10. T. Sugiyama, N. Miki and T. Yamano, *J. Biochem., Tokyo* **87**, 1457 (1980).
11. D. A. Haugen and M. J. Coon, *J. biol. Chem.* **251**, 7929 (1976).
12. E. Canova-Davis and L. A. Waskell, *Biochem. biophys. Res. Commun.* **108**, 1264 (1982).
13. J. D. Dignam and H. W. Strobel, *Biochem. biophys. Res. Commun.* **63**, 845 (1975).
14. J. L. Vermilion and M. J. Coon, *J. biol. Chem.* **253**, 2694 (1978).
15. E. F. Johnson, G. E. Schwab and U. Müller-Eberhard, *Molec. Pharmac.* **15**, 708 (1979).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. J. Y. L. Chiang, *Archs Biochem. Biophys.* **211**, 662 (1981).
18. P. Strittmatter and S. F. Velick, *J. biol. Chem.* **211**, 253 (1956).
19. R. W. Estabrook and J. Werringloer, *Meth. Enzym.* **52**, 212 (1978).
20. J. Ozols, *Biochemistry* **13**, 426 (1974).
21. D. L. Cinti and J. Ozols, *Biochim. biophys. Acta* **410**, 32 (1975).
22. M. J. Rogers and P. Strittmatter, *J. biol. Chem.* **249**, 895 (1974).
23. T. Nash, *Biochem J.* **55**, 416 (1953).
24. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
25. G. L. Peterson, *Analyt. Biochem.* **83**, 346 (1977).
26. U. K. Laemmli, *Nature, Lond.* **227**, 680 (1970).
27. W. L. Dean and M. J. Coon, *J. biol. Chem.* **252**, 3255 (1977).
28. L. R. Greenstein, B. A. Hitt and R. I. Mazze, *Anesthesiology* **42**, 420 (1975).
29. J. Ozols and P. Strittmatter, *J. biol. Chem.* **239**, 1018 (1964).
30. P. Strittmatter, E. T. Machuga and G. J. Roth, *J. biol. Chem.* **257**, 11883 (1982).
31. V. V. R. Reddy, D. Kupfer and E. Caspi, *J. biol. Chem.* **252**, 2797 (1977).
32. N. Oshino, Y. Imai and R. Sato, *J. Biochem. Tokyo* **69**, 155 (1971).