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Quantitation of Two Forms of Pulmonary Cytochrome *P*-450 in Microsomes, Using Substrate Specificities

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SUMMARY

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The metabolism of *N*-hydroxyamphetamine by the rabbit pulmonary microsomal fraction results in the formation of a metabolic intermediate complex with almost all of the cytochrome *P*-450. However, only half of the cytochrome *P*-450 forms a complex during the metabolism of norbenzphetamine, benzphetamine, or piperonyl butoxide. Immunological studies in which antibodies against each of the two known forms of pulmonary cytochrome *P*-450 were added to microsomes and monooxygenase systems containing purified enzymes demonstrate that one form of cytochrome *P*-450 complexes with both norbenzphetamine and *N*-hydroxyamphetamine, whereas a second form complexes only with *N*-hydroxyamphetamine. The cytochrome *P*-450–metabolic intermediate complexes can be solubilized from the microsomal membrane and separated from each other by chromatography on DEAE-cellulose. The complexes isolated in this manner are the same as those formed in purified systems. As determined by metabolic intermediate complex formation, pulmonary microsomal fractions contain equal amounts of the two forms of cytochrome *P*-450.

INTRODUCTION

The lung is a "portal of entry tissue" (1) and has the ability to concentrate many xenobiotics relative to their concentrations in the blood (2). Thus, the xenobiotic-metabolizing capability and specificity of the lung have been investigated (3–9). Because xenobiotics may be oxidatively metabolized to potentially harmful compounds (10–12), many of these investigations have been directed at the pulmonary cytochrome *P*-450 monooxygenase system.

It has previously been demonstrated that the oxidative metabolism of amphetamine derivatives by microsomes can result in the formation of stable cytochrome *P*-450–metabolic intermediate (MI) complexes (13, 14). Such complexes, which evidence suggests are the result of a ligand interaction between an unstable nitroso intermediate and ferrous cytochrome *P*-450 (15), have an absorbance maximum at 455 nm. However, not all forms of cytochrome *P*-450 are equally capable of forming MI complexes, and in the hepatic microsomes, it appears to be mainly the property of the form(s) of cytochrome *P*-450 induced by phenobarbital (16). The ability of pulmonary microsomes isolated from noninduced animals to form MI complexes from amphetamines is similar to that of phenobarbital-induced hepatic microsomes (17), which suggests that similarities exist between the constituent form(s) of cytochrome *P*-450 in the lung and the phenobarbital-induced form in the liver. However, recent investigations have identified two forms of rabbit pulmonary cytochrome *P*-450 that have different substrate specificities (18). For example, only one form of the cytochrome metabolizes benzo[*a*]pyrene to products which covalently bind to DNA *in vitro* (19). The present investigation demonstrates selective MI complex formation by the two forms of rabbit pulmonary cytochrome *P*-450 and suggests the usefulness of MI complex formation for rapid and easy quantitation of the two forms in microsomes.

MATERIALS AND METHODS

Microsomes were prepared from the lungs of adult male Dutch Belt or New Zealand White rabbits as described previously (20). Protein was determined by the method of Lowry *et al.* (21) using bovine serum albumin as the standard. Microsomal cytochrome *P*-450 was determined from the dithionite difference spectrum of carbon monoxide-saturated microsomes and quantitated using $E = 100\ \text{mM}^{-1}\ \text{cm}^{-1}$ (22). Cytochrome *P*-450–MI complex formation was determined at room temperature

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in 100 mM phosphate buffer (pH 7.6) by dual wavelength spectroscopy, in which the difference in absorption between 455 and 490 nm was monitored as a function of time and quantitated using $E = 65\ mM^{-1}\ cm^{-1}$ (23). After maximal MI complex formation, residual cytochrome *P*-450 (i.e., noncomplexed) was determined from the additional absorbance observed at 450 vs 490 nm upon carbon monoxide gasing of the dithionite-reduced cuvette contents. This technique is possible since carbon monoxide does not displace the metabolic intermediate from the cytochrome. Spectroscopic investigations were usually performed with microsomal suspensions containing 4 mg protein/ml, using an Aminco DW 2 spectrophotometer.

Antibodies to the two purified forms of pulmonary cytochrome *P*-450 were raised in goats as described elsewhere (24). The IgG fraction from goat serum was isolated by ammonium sulfate precipitation and anion-exchange chromatography (25).

The separation of two forms of pulmonary cytochrome *P*-450, either uncomplexed or as MI complexes, from solubilized microsomes using DEAE-cellulose was as described previously (18) except for the following: The DEAE-cellulose columns were 1.5 × 6 cm, and the elution of the cytochrome *P*-450-II from the DEAE-cellulose column was accomplished using a 75 mM KCl buffer, rather than a 0–500 mM KCl gradient.

The ratio of the two forms of cytochrome *P*-450 varied among batches of microsomes (for example, see Table 2). The data were therefore difficult to express as mean values ± SE, so where possible the results in each table or figure are those obtained using a single microsomal preparation. However, the experimental results were verified using different microsomal preparations.

RESULTS

The metabolism of a number of compounds by pulmonary microsomes results in the formation of cytochrome *P*-450–MI complexes, and the extent of MI complex formation is shown in Table 1. The substrate concentrations used were those which produced maximal rates of MI complex formation, and the reactions were allowed to proceed until no further increases in complex formation were apparent. Fifty-six, fifty-two, and thirty-nine percent of the cytochrome *P*-450, respectively, complexed with benzphetamine, norbenzphetamine, and piperonyl butoxide (a nonnitrogenous compound). Significantly more cytochrome *P*-450 (85%) was complexed when *N*-hydroxyamphetamine was used as the substrate. No destruction of cytochrome *P*-450 occurred during the incubation; all of the cytochrome could be accounted for by summing the amount as a MI complex and the remaining amount which formed a complex with carbon monoxide.

TABLE 1

The formation of cytochrome P-450–MI complexes from various substrates in rabbit pulmonary microsomes

Microsomes were incubated with 500 μM NADPH and the substrates indicated. The time is that which was required for maximal MI complex formation.

Substrate	Concentration	Time	Cytochrome *P*-450	
			MI complex	Uncomplexed
	μM	min	μM	μM
None	—	—	0	0.81
Benzphetamine	66	60	0.45	0.36
Norbenzphetamine	100	13	0.42	—
N-Hydroxyamphetamine	333	25	0.69	0.11
Piperonyl butoxide	1035	30	0.32	0.52

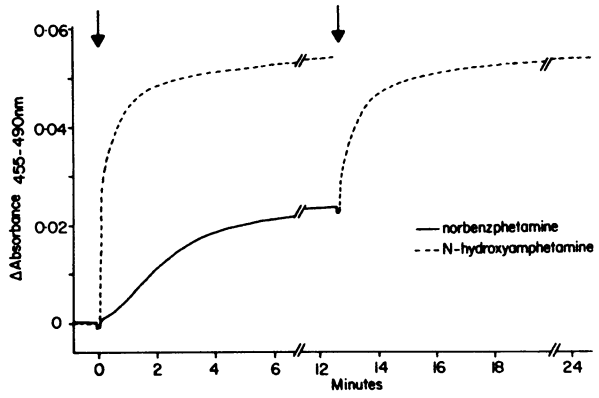

FIG. 1. *The formation of cytochrome P-450–MI complexes from norbenzphetamine and N-hydroxyamphetamine in lung microsomes*

Microsomes were preincubated with NADPH (800 μM), and either norbenzphetamine (100 μM) (solid line) or *N*-hydroxyamphetamine (666 μM) (dashed line) was added at zero time. When the absorbance changes from norbenzphetamine were complete (approximately 12 min), *N*-hydroxyamphetamine was also added and the subsequent absorbance changes were monitored for 12 min.

The addition of *N*-hydroxyamphetamine to microsomes, which had already formed maximal amounts of MI complex from norbenzphetamine, increased the total extent of complex formation to equal the amount seen from *N*-hydroxyamphetamine alone (Fig. 1). The addition of norbenzphetamine to microsomes already containing maximal amounts of MI complex from *N*-hydroxyamphetamine produced no further increase in complex formation (not shown).

The proportion of cytochrome *P*-450 capable of form-

TABLE 2

The effect of antibody to pulmonary cytochrome P-450-I on MI complex formation from norbenzphetamine and N-hydroxyamphetamine in lung microsomes

The maximum amounts of MI complex formed from 100 μM norbenzphetamine (NB) and from the subsequent addition of 667 μM *N*-hydroxyamphetamine (NOHA) were determined in five microsomal preparations (A to E). Maximal changes in MI complex formation were determined after preincubation of the microsomes with the IgG fraction (2 mg/ml for A and B; 4 mg/ml for C, D, and E) containing antibody (Ab) to cytochrome *P*-450-I.

Microsomes	Maximum MI complex formation (μM) from				MI complex ratio, [NB/(NB + NOHA)] × 100%
	NB		(NB + NOHA)		
	−Ab	+Ab	−Ab	+Ab	
A	0.44	0.03	0.79	0.40	56
B	0.42	0.06	0.78	0.38	54
C	0.28	0.04	0.77	0.50	36
D	0.38	0.03	0.73	0.39	52
E	0.31	0.03	0.69	0.37	45

ing a MI complex from norbenzphetamine, relative to the sum of the MI complexes formed with norbenzphetamine and *N*-hydroxyamphetamine, varied from 36 to 56% for the microsomal preparations shown in Table 2. The variation in the ration was not related to the concentration of cytochrome *P*-450 present in the microsomes.

Antibodies to the two known forms of pulmonary cytochrome *P*-450 were used to investigate the relative contributions of these two forms to the substrate specificities stated previously. An IgG fraction containing antibody to cytochrome *P*-450-I (24) inhibited the amount of MI complex formed from norbenzphetamine (83 to 86%) (Table 2). The amount formed from the subsequent addition of *N*-hydroxyamphetamine [i.e., (NB + NOHA) − NB], however, was affected very little (6% increase to 11% decrease) by the presence of the antibody. In companion studies it was found that the total concentration of MI complex formed after *N*-hydroxyamphetamine addition in the presence of the antibody was the same, whether or not the microsomes were first incubated with norbenzphetamine. The inhibition by the antibody to cytochrome *P*-450-I on the rate of MI complex formation from norbenzphetamine was dependent on the antibody concentration and independent of either the time (5 min to 3 h) or the temperature (4 to 25°C) of the incubation of the antibody with microsomes prior to the addition of the substrate and NADPH. Also at the concentrations used, the maximal inhibitory effect of the antibody appeared to be independent of the proportion of cytochrome *P*-450 capable of forming a MI complex from norbenzphetamine relative to the total (Table 2). In contrast to the IgG fraction containing antibody to cytochrome *P*-450-I, the fraction containing antibody to cytochrome *P*-450-II did not inhibit the extent of MI complex formation from norbenzphetamine, but significantly, although incompletely (43 to 57%), inhibited that formed from subsequent *N*-hydroxyamphetamine addition (data not shown). Preimmune IgG did not significantly alter the amount of MI complex formed from either substrate.

The inhibitory effects of antibodies on the extent of MI complex formation were paralleled by their effects on the initial rates of MI complex formation (Fig. 2). MI complex formation from norbenzphetamine was completely inhibited by antibody to cytochrome *P*-450-I but unaffected by antibody to cytochrome *P*-450-II. MI complex formation from *N*-hydroxyamphetamine in the absence of norbenzphetamine was inhibited 50% by antibody to cytochrome *P*-450-I but only 33% by antibody to cytochrome *P*-450-II. Thus, the antibody to cytochrome *P*-450-II inhibits only 66% of the MI complex formation from *N*-hydroxyamphetamine which is not attributable to cytochrome *P*-450-I. Neither antibody significantly interfered with the binding of carbon monoxide to dithionite-reduced cytochrome *P*-450.

Cytochrome *P*-450–MI complex formation and the inhibitory effect of antibodies were investigated in a reconstituted monooxygenase system, containing purified pulmonary cytochrome *P*-450-I or cytochrome *P*-450-II and purified hepatic NADPH–cytochrome *P*-450 reductase (Table 3). Cytochrome *P*-450-I formed MI complexes from both norbenzphetamine and *N*-hydroxyamphetamine, which were inhibited (80%) by antibody to cytochrome *P*-450-I. Antibody to cytochrome *P*-450-II had no effect on these activities. Cytochrome *P*-450-II did not form a MI complex from norbenzphetamine. However, using *N*-hydroxyamphetamine, a MI complex the same as that seen with the purified cytochrome *P*-450-I system was observed. This MI complex formation from *N*-hydroxyamphetamine was inhibited approximately 90% by the antibody to cytochrome *P*-450-II but was unaffected by the antibody to cytochrome *P*-450-I. In both the reconstituted system and the microsomes (Fig. 2), preimmune IgG had a slight activating effect on MI complex formation from *N*-hydroxyamphetamine, but the reason for this selective effect is unclear.

With the considerable evidence that cytochrome *P*-450-I could form MI complexes from both norbenzphetamine and *N*-hydroxyamphetamine, and that cytochrome *P*-450-II forms a MI complex only from *N*-hydroxyamphetamine, the separation of the two cytochromes from microsomes, as their MI complexes, was

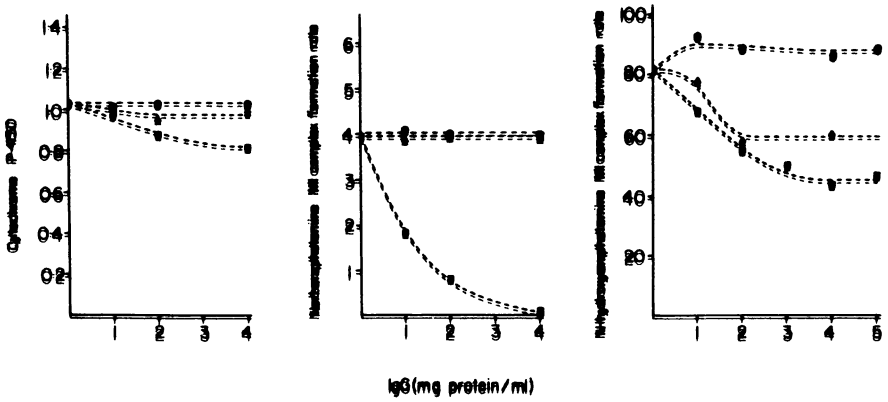

FIG. 2. *The effect of antibodies to cytochromes P-450-I and -II on microsomal cytochrome P-450 detectability and rates of MI complex formation*

The IgG fractions used were either preimmune (●) or contained antibody to cytochrome *P*-450-I (■) or cytochrome *P*-450-II (▲). Microsomes were incubated with IgG fractions for 5 min prior to assay. MI complex formation rates (Δ absorbance 455–490 nm × 10^4/min) were determined with either 100 μM norbenzphetamine or 333 μM *N*-hydroxyamphetamine and 800 μM NADPH. The cytochrome *P*-450 concentration (μM) was determined from the carbon monoxide spectrum 5 min after the addition of dithionite, as described in Materials and Methods.

TABLE 3

The inhibition of purified rabbit pulmonary cytochromes P-450-I and P-450-II activities by antibodies

Cytochrome *P*-450 concentrations in the reconstituted systems were 0.55 μM for cytochrome *P*-450-I and 0.43 μM for cytochrome *P*-450-II. The flavoprotein concentration was 6250 units of cytochrome *c* reductase activity (nmol/min) per ml. With cytochrome *P*-450-I, phospholipid (dilauroyl lecithin) was added (0.7 mg/ml) by vortexing the purified components with the phospholipid which had been dried to the glass wall by evaporation of a chloroform solution. With cytochrome *P*-450-II, the components were preincubated together for 10 min at 37°C prior to the IgG addition. IgG was added to the reconstituted systems and incubated for 5 min before MI complex formation rates were determined. The concentrations of *N*-hydroxyamphetamine (NOHA), norbenzphetamine (NB), and NADPH were 800 μM, 100 μM, and 1.5 mM, respectively.

IgG (3.5 mg protein/ml)	Rates of MI complex formation (Δabsorbance 455 vs 490 nm × 10^3/min)			
	MI complex formation with purified cytochrome *P*-450-I from		MI complex formation with purified cytochrome *P*-450-II from	
	NB	NOHA	NB	NOHA
—	2.6	27	0	25
Preimmune	2.1	35	0	30
Anti-cyt. *P*-450-I	0.3	7	0	25
Anti-cyt. *P*-450-II	2.9	27	0	3

attempted (Table 4). The microsomes were incubated under conditions suitable for MI complex formation and then solubilized and chromatographed on DEAE-cellulose. The preincubation conditions did not alter the chromatographic properties of the cytochromes. After incubation of the microsomes with norbenzphetamine, 56% of the cytochrome in the *P*-450-I fraction was found to be complexed, and more important was that none of the cytochrome in the *P*-450-II fraction was complexed. After incubation with *N*-hydroxyamphetamine, about 59% of the cytochrome in the *P*-450-I fraction and 80% of the cytochrome in the *P*-450-II fraction were complexed. The yields of both cytochromes were approximately the same whether they were in the uncomplexed, norbenzphetamine-complexed, or *N*-hydroxyamphetamine-complexed

TABLE 4

Chromatography of microsomal MI complexes on DEAE-cellulose

Microsomes (3.8 mg protein/ml) were incubated for 30 min in the presence of 0.5 mM NADPH, or NADPH and 0.11 mM norbenzphetamine, or for 10 min with NADPH and 0.74 mM *N*-hydroxyamphetamine. The microsomes were then centrifuged (120,000 *g*, 30 min), and the pellet was resuspended in 0.015 mM phosphate buffer (pH 7.7) containing 0.15 mM EDTA and 1.5 mM dithiothreitol. The solubilization and DEAE-cellulose chromatography were performed as described in Materials and Methods. The fractions are those designated previously (18). Values given are total cytochrome *P*-450 (nmol followed by the % of cytochrome existing as MI complex).

Microsomal preincubation with	Cytochrome *P*-450 and MI complex in		
	Fraction I	Fraction II	Fractions I and II
—	2.82 (0)	2.45 (0)	5.27 (0)
Norbenzphetamine	3.05 (56)	2.07 (0)	5.12 (34)
N-Hydroxyamphetamine	3.75 (59)	2.47 (80)	6.22 (67)

state, i.e., 2.82, 3.05, and 3.75 nmol for cytochrome *P*-450-I and 2.45, 2.07, and 2.47 nmol for cytochrome *P*-450-II, respectively. The amount of total eluted cytochrome (Fractions I and II) existing as a MI complex after incubation with *N*-hydroxyamphetamine (67%) was twice that seen after incubation with norbenzphetamine (34%). Although these numbers are lower than the maximal amounts seen in microsomal suspensions, the ratio of 2:1 for the two substrates agrees with that seen in microsomes.

DISCUSSION

The use of MI complex formation to differentiate between specific forms of cytochrome *P*-450 present in the hepatic microsomes has been limited to the changes observed after phenobarbital induction since microsomes from untreated animals do not readily form such complexes. In hepatic microsomes from phenobarbital-induced rats, 90% of the cytochrome *P*-450 formed a MI complex from *N*-hydroxyamphetamine (23, 26), while only 50% formed a MI complex from benzphetamine (26, 27). However, since at least four forms of cytochrome *P*-450 are present in hepatic microsomes from phenobarbital-induced rats (28), the assignment of MI complex formation specificities to specific forms is extremely difficult. Similar problems exist in the rabbit since multiple forms of cytochrome *P*-450 are also present in the livers of this species (29, 30).

Several observations indicated that the rabbit pulmonary forms of cytochrome *P*-450 might be more easily delineated by MI complex formation than those in the liver. First, rabbit pulmonary microsomes are very similar to hepatic microsomes from phenobarbital-treated animals in their ability to form MI complexes from amphetamine derivatives (17). Second, a minimum of 70% of the cytochrome *P*-450 in rabbit pulmonary microsomes can be accounted for by two forms (18) which have significantly different substrate specificities (18, 19).

This investigation demonstrates that approximately half of the rabbit pulmonary cytochrome forms MI complexes from a variety of compounds in a manner similar to the hepatic cytochrome *P*-450 induced by phenobarbital. The majority of the remaining pulmonary cytochrome *P*-450 forms a MI complex only from *N*-hydroxyamphetamine. Results of experiments with the two purified cytochromes and investigations of the inhibitory effects of antibodies on microsomal MI complex formation demonstrate that pulmonary cytochrome *P*-450 designated "I" (18) forms MI complexes that are the same as those formed in phenobarbital-induced hepatic microsomes. In fact, recent catalytic, immunological, and structural evidence demonstrates that this form of pulmonary cytochrome *P*-450 is indistinguishable from the major hepatic form induced by phenobarbital (24; C. R. Wolf and S. Slaughter, unpublished data). Complex formation from *N*-hydroxyamphetamine appears to be a function of both cytochrome *P*-450-I and cytochrome *P*-450-II. The antibody to cytochrome *P*-450-II inhibits 90% of the complex formation from *N*-hydroxyamphetamine in purified systems containing cytochrome *P*-450-II. In microsomes, however, the antibody inhibits cytochrome *P*-450-I-independent MI complex formation by only 66%. Either

of two possibilities probably accounts for this difference: First, all of the cytochrome P-450-II in microsomes may not be accessible to the antibody; or second, pulmonary microsomes may contain a third form of cytochrome P-450 which forms a MI complex from N-hydroxyamphetamine but which is not inhibited by the antibodies to either cytochrome P-450-I or cytochrome P-450-II. Thus, while MI complex formation from norbenzphetamine provides a measure of active cytochrome P-450-I, the possibility exists that additional complex formation from N-hydroxyamphetamine in microsomes is not totally a function of cytochrome P-450-II.

The locus of the inhibition of MI complex formation by antibodies to cytochrome P-450 appears to be at the substrate binding site rather than at the heme because neither antibody prevents carbon monoxide binding to dithionite-reduced cytochrome P-450. An interference with the flow of electrons through the reductase to the cytochrome is also excluded because the amount of NADPH-reducible cytochrome P-450 under anaerobic conditions in the presence of carbon monoxide is also unchanged by the antibodies (data not shown). Thus, antibody inhibition of complex formation appears to reflect a failure to form the metabolic intermediate rather than an interference with its subsequent ligand binding to the heme. The inhibitory effect of the antibody to cytochrome P-450-II on N-hydroxyamphetamine MI complex formation may suggest that the formation of the heme–ligand interaction is restricted to the molecule where the intermediate or ligand was generated. If this were not so, 50% inhibition of MI complex formation from N-hydroxyamphetamine in microsomes would never be seen for the following reason. In the presence of antibody to cytochrome P-450-II, intermediates generated on the uninhibited cytochrome P-450-I could migrate to the cytochrome P-450-II (which, although inhibited with respect to MI complex formation, is still capable of binding CO) and bind to the ferrous heme to form a complex. The net result would be sequestration of both cytochrome P-450-I and cytochrome P-450-II as MI complexes. Alternatively, the 50% inhibition of N-hydroxyamphetamine MI complex formation by cytochrome P-450-II antibody could indicate that the instability of the metabolic intermediate is such that it does not survive migration.

The results also demonstrate the stability of the complexes formed between metabolic intermediates and ferrous cytochrome P-450. It is possible to isolate the complex intact from sodium cholate-solubilized microsomes by chromatography on DEAE-cellulose under aerobic conditions. The stability of the MI complexes should prove advantageous in future studies on the function of the two forms of pulmonary cytochrome P-450 *in situ*.

The results in this paper demonstrate that the formation of MI complexes from two amphetamine derivatives provides a unique, reliable, and rapid means of differentiating two major forms of cytochrome P-450 in rabbit lung, and could be of great importance in investigating the effects of drugs and toxic substances on this organ.

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