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The Formation of a 455 nm Complex during Cytochrome P-450-Dependent N-Hydroxyamphetamine Metabolism

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SUMMARY

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The formation of an absorbance maximum at 455 nm during the hepatic microsomal oxidative metabolism of *N*-hydroxyamphetamine requires NADPH and oxygen and is not inhibited by exogenous catalase. This 455 nm complex, which is thought to be a complex of a metabolic intermediate formed during *N*-hydroxyamphetamine metabolism by cytochrome P-450, has an extinction coefficient of 65 mM^{-1} cm^{-1}. It can be formed at a rate of 8 nmoles/mg of protein per minute, which is 10 times the rate of formation of a similar complex from norbenzphetamine, 20 times that from benzphetamine, and 40 times that from *d*-amphetamine and SKF 525-A. Prior treatment of rats with phenobarbital enhances the rate of complex formation, whereas 3-methylcholanthrene administration reduces the rate to less than that observed for untreated animals. Formation of the complex is inhibited by other substrates capable of undergoing mixed-function oxidation. The complex is stable in the presence of dithionite, but is destroyed by ferricyanide oxidation and subsequent further reduction. The 455 nm absorbance maximum is converted to one at 423 nm upon conversion of cytochrome P-450 to P-420 by deoxycholate.

INTRODUCTION

The combination of reduced cytochrome P-450 with ethyl isocyanide to produce absorption maxima at 430 and 455 nm was one of the observations in the original paper by Omura and Sato (1) characterizing this cytochrome. Several years ago it was found that during the microsomal oxidative metabolism of methylenedioxybenzene derivatives an absorbance maximum at 455 nm also occurred, though not if the compounds were added directly to reduced cytochrome P-450 (2–5). The ability of microsomal mixed-function oxidation to produce products or intermediates which act as ligands and bind to cytochrome P-450 (6) was not limited to methylenedioxybenzene derivatives. Similar 455 nm absorption maxima were shown for SKF 525-A, SKF 26754-A (7) and benzphetamine (8). Subsequent investigations, using many compounds related to amphetamine (9, 10) and SKF 525-A (11, 12), have suggested the importance of reactions on the nitrogen atom or on adjacent carbon atoms of these molecules. The work reported here concerns the most active substrate yet, *N*-hydroxyamphetamine. The rate of formation of the 455 nm complex with this substrate is comparable to the rate for many other mixed-function oxidation reactions, and is at least 20 and 40 times faster than the rates of complex formation from

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benzphetamine and SKF 525-A, respectively. A preliminary report of some of these investigations has been presented (13).

MATERIALS AND METHODS

Hepatic microsomes were prepared from 250–300-g male Sprague-Dawley rats as described previously (14). The rats either were untreated or received intraperitoneal injections of phenobarbital in aqueous medium or of 3-methylcholanthrene dissolved in corn oil. Routine phenobarbital treatment consisted of four daily injections, three of 80 mg/kg, and 40 mg/kg on the fourth day. When the phenobarbital treatment regimen differed from this, or when 3-methylcholanthrene was used as the inducing agent, the injection schedule is given in the appropriate legends to tables and figures.

Spectrophotometric observations were generally performed with the microsomes suspended at 2 mg of protein per milliliter in 50 mM Tris-chloride buffer (pH 7.4) containing 150 mM KCl and 10 mM $MgCl_2$. Cytochrome P-450 concentrations were determined by the reduced-CO complex, assuming in all cases an extinction coefficient of 91 mM^{-1} cm^{-1}. The spectral characteristics of the 455 nm complex were determined by difference spectroscopy, and the rates of formation of 455 nm complexes were determined using the dual-wavelength mode (455 vs., 490 nm). All enzymatic reactions were carried out at 25°, using equilibrated buffer and a temperature-regulated Aminco DW-2 spectrophotometer. Oxygen utilization was determined with a membrane-coated Clark oxygen electrode (15). NADPH-cytochrome *c* reductase activity was assayed according to Masters *et al.* (16), and protein concentration was determined by the biuret reaction (17).

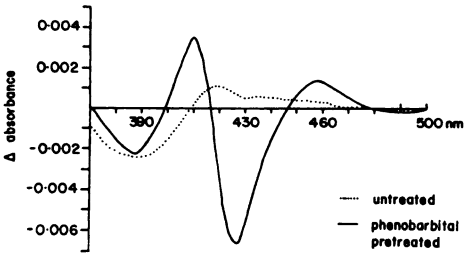

FIG. 1. *Binding spectrum of N-hydroxyamphetamine*

N-Hydroxyamphetamine (33 μM) was added to a suspension of rat hepatic microsomes from phenobarbital-treated rats (cytochrome P-450 concentration, 4.80 μM) and untreated rats (cytochrome P-450 concentration, 2.48 μM), and the difference spectrum was recorded 30 sec later.

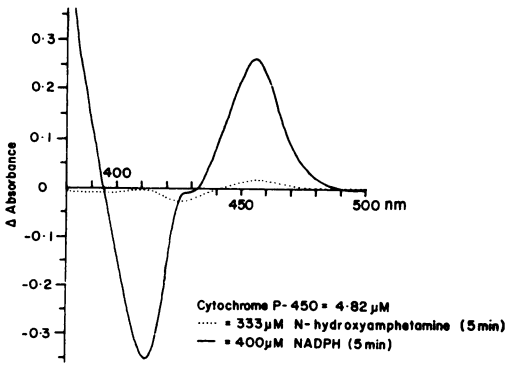

FIG. 2. *Difference spectrum produced during microsomal mixed-function oxidation of N-hydroxyamphetamine*

N-Hydroxyamphetamine was added to a suspension of phenobarbital-treated rat hepatic microsomes, and the difference spectrum was recorded after 5 min (····). NADPH was then added to these microsomes, and the difference spectrum was recorded 5 min later (——).

NADPH, NADH, sodium deoxycholate, 3-methylcholanthrene, catalase (bovine liver), and cytochrome *c* (type IV) were obtained from Sigma Chemical Company. *N*-Hydroxyamphetamine, *d*-amphetamine, and SKF 525-A were gifts from Smith Kline & French, and benzphetamine and norbenzphetamine (*N*-benzyl-α-methylphenylethylamine) were gifts from the Upjohn Company.

RESULTS

The addition of *N*-hydroxyamphetamine to hepatic microsomal suspensions prepared from phenobarbital-treated rats produces the spectrum shown in Fig. 1. It is characterized by absorbance maxima at 410 and 458 nm and minima at 388 and 427 nm. As the *N*-hydroxyamphetamine concentration is increased, the absorbance maximum around 458 nm increases and shifts to the blue slightly, whereas the maxima and minima at 410 and 388 remain essentially

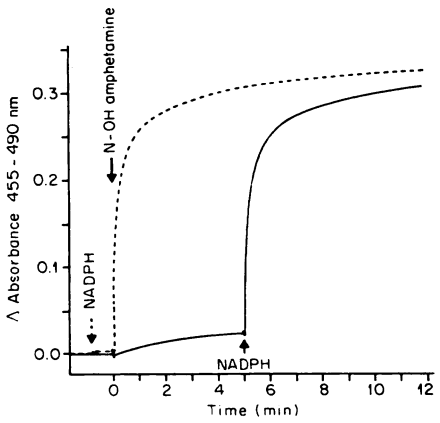

FIG. 3. *Formation of 455 nm absorbance in absence and presence of NADPH*

Under the conditions described in Fig. 2, the spectral changes at 455 nm relative to 490 nm were observed continuously by dual-wavelength spectroscopy (——). The experiment was also performed by adding the NADPH prior to *N*-hydroxyamphetamine (- - -).

unchanged. When *N*-hydroxyamphetamine is added to microsomes from untreated rats, a spectrum is obtained with a minimum at 390 nm and a maximum at 420 nm. With phenobarbital-treated rat microsomes the maximum at 455 nm in the absence of NADPH remains very small in comparison with the amount of 455 nm absorbance measured after the addition of NADPH (Fig. 2). Thus for equal time periods, in this case 5 min, there is at least 10 times greater absorbance at 455 nm under conditions suitable for mixed-function oxidation. The formation of the 455 nm complex is not linear with time in the absence of NADPH, despite an excess of oxygen, cytochrome P-450, and *N*-hydroxyamphetamine (Fig. 3). There is also a rapid increase of the 455 nm absorbance upon the addition of NADPH. This rapid absorbance change is not due to the binding to reduced cytochrome P-450 of an intermediate formed during the absence

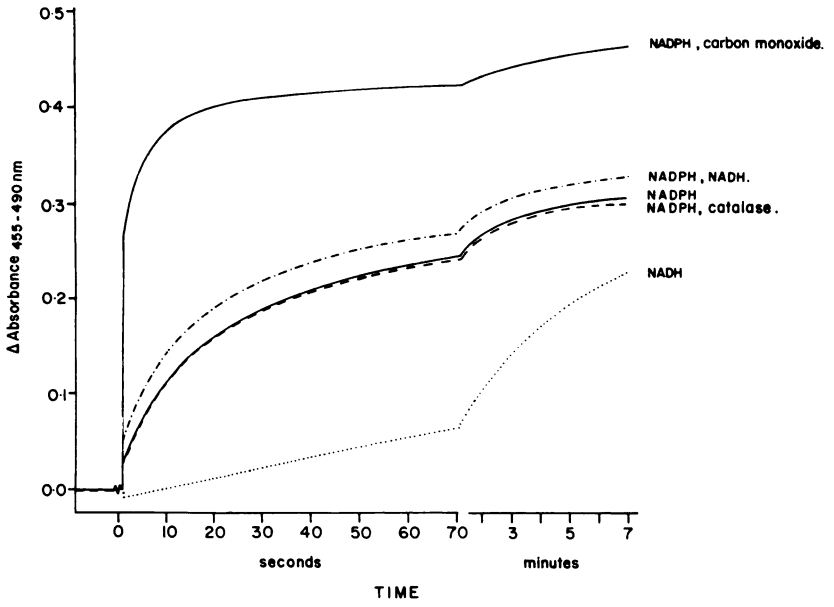

FIG. 4. *Rate of formation of 455 nm absorbance from N-hydroxyamphetamine in the presence of NADPH, NADH, catalase, and carbon monoxide*

Suspensions of hepatic microsomes from phenobarbital-treated rats (cytochrome P-450 concentration, 5.44 μM), containing 330 μM *N*-hydroxyamphetamine, were monitored at 455 nm relative to 490 nm by dual-wavelength spectroscopy. Absorbance changes were determined after the addition of 200 μM NADH (····), 200 μM NADPH (lower continuous line), and both nucleotides together (-·-·). Changes upon the addition of NADPH to suspensions containing catalase (1000 units/ml) (- - -) or that had been gassed for 5 min with carbon monoxide (upper continuous line) were also investigated.

of NADPH. When the order of addition of *N*-hydroxyamphetamine and NADPH is reversed (Fig. 3, dashed line), the rapid change in absorbance occurs immediately upon the addition of *N*-hydroxyamphetamine.

The requirement for conditions of mixed-function oxidation for the formation of the 455 nm complex is reinforced by evidence shown in Fig. 4. The upper continuous line shows the rapid absorbance change upon the addition of NADPH to microsomal suspensions containing *N*-hydroxyamphetamine which had been extensively gassed with carbon monoxide. The removal of most of the oxygen by carbon monoxide gassing and the inhibition of any oxidative reactions by the large $CO:O_2$ ratio suggest that this absorbance change is due solely to the reduction of cytochrome P-450 with the subsequent binding of carbon monoxide. It is much faster than the rate of formation of the 455 nm absorbance maximum upon the addition of NADPH to microsomes in which oxygen and *N*-hydroxyamphetamine are present (lower continuous line). The reaction leading to increased 455 nm absorbance proceeded preferentially with NADPH, and the rate with NADH was at least 10-fold lower (dotted line in Fig. 4). The two cofactors together (dash-dot line) appear to produce a greater absorbance change than does NADPH alone, but the absorbance is less than the sum for the two cofactors added separately. The rate and extent of absorbance change in the presence of NADPH are not affected by large amounts of exogenous catalase (dashed line).

The addition of *N*-hydroxyamphetamine to microsomes containing NADPH produces a rapid utilization of oxygen (Fig. 5, dashed line). This rapid removal of oxygen lasts less than 1 min, a period which corresponds to a rapid rate of formation of the 455 nm absorbance maximum. Thereafter the NADPH-supported endogenous oxygen utilization rate (continuous line) is inhibited.

The conversion of cytochrome P-450 to P-420 by deoxycholate after the formation of the 455 nm complex results in the removal of the 455 nm absorbance and the appearance of an absorbance maximum at 423 nm (Fig. 6). That this was not due to the binding of carbon monoxide (generated by heme oxidation during the NADPH-supported

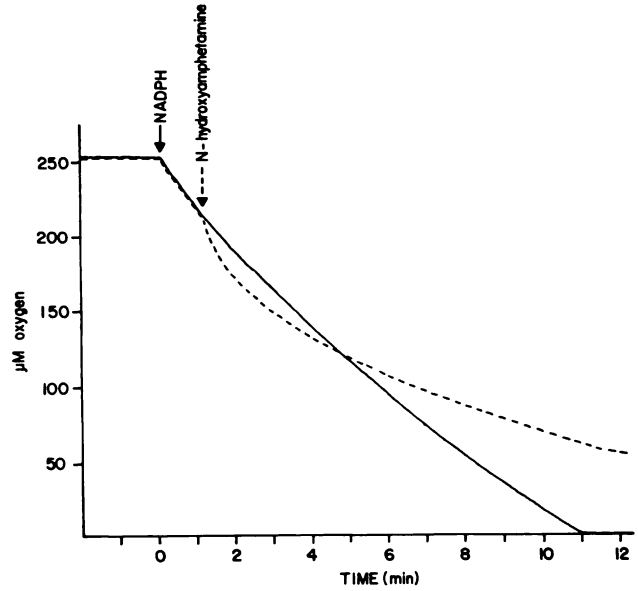

FIG. 5. *Utilization of oxygen during microsomal metabolism of N-hydroxyamphetamine*

The oxygen consumption of suspensions (2 mg of protein per milliliter) of microsomes from phenobarbital-treated rats (cytochrome P-450 concentration, 4.50 μM) was determined upon the addition of 600 μM NADPH with (- - -) and without (——) the subsequent addition of 350 μM *N*-hydroxyamphetamine.

mixed-function oxidation of N-hydroxyamphetamine) to cytochrome P-420 is shown in Table 1. The A_{455}:A_{423} ratio is constant for various starting concentrations of the 455 nm complex, and the 423 nm absorbance is large, even at short (30-sec) time intervals, when one would not expect much carbon monoxide to have been generated.

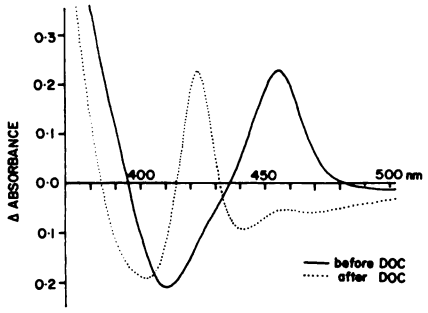

FIG. 6. *Effect of deoxycholate (DOC) on 455 nm absorbance maximum formed during microsomal metabolism of N-hydroxyamphetamine*

Microsomal suspensions (cytochrome P-450 concentration, 4.40 μM) were incubated with 333 μM N-hydroxyamphetamine and 200 μM NADPH for 3 min. A few crystals of sodium dithionite were then added, and the difference spectrum was recorded (——). Sodium deoxycholate (6.7 mM) was then added, and the difference spectrum was recorded after 6 min (····).

The rapid formation of a complex which absorbs maximally at 455 nm during oxidative metabolism of N-hydroxyamphetamine is compared, under identical conditions, with the formation of complexes from some of the other compounds known to exhibit such a property (Fig. 7). Assuming the same

TABLE 1

Relationship between absorbance maximum at 455 nm and that formed at 423 nm after deoxycholate treatment

Microsomes from phenobarbital-treated rats (cytochrome P-450 concentration, 4.40 μM) were incubated with 333 μM N-hydroxyamphetamine and 200 μM NADPH for limited periods of time to form various amounts of the 455 nm complex. The reaction was terminated by the addition of dithionite, and the difference spectrum was recorded. Sodium deoxycholate (6.7 mM) was then added, and the difference spectrum was recorded until the cessation of absorbance changes.

Incubation time with NADPH	$\Delta A_{455\text{-}500}$ upon dithionite addition	$\Delta A_{423\text{-}500}$ after deoxycholate	$A_{455\text{-}500}$: $A_{423\text{-}500}$
min.			
0.5	0.181	0.161	1.12
1.0	0.212	0.181	1.17
3.0	0.239	0.213	1.12

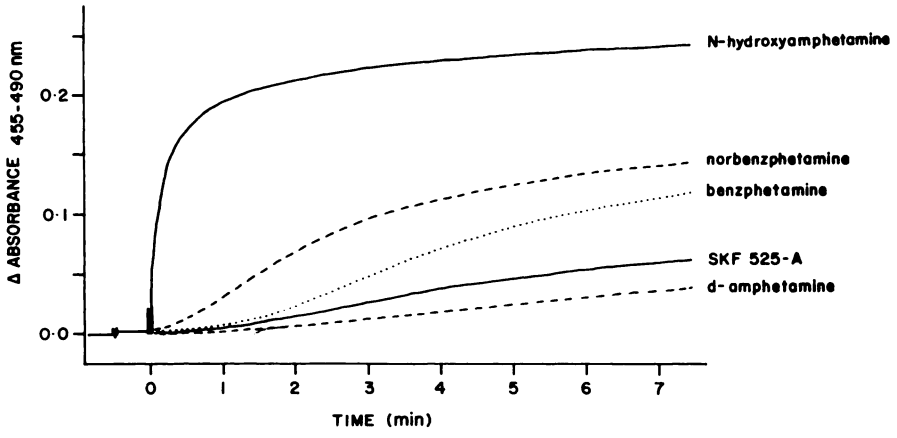

FIG. 7. *Rate of formation of 455 nm complex during microsomal metabolism of N-hydroxyamphetamine compared with that from other substrates*

The formation of an absorbance maximum at 455 nm relative to 490 nm was monitored by dual-wavelength spectroscopy upon the addition of 400 μM NADPH to microsomal suspensions from phenobarbital-treated rats (cytochrome P-450 concentration, 4.82 μM) containing 330 μM N-hydroxyamphetamine, 100 μM norbenzphetamine, 50 μM benzphetamine, 33 μM SKF 525-A, or 1 mM d-amphetamine.

TABLE 2

Effect of induction by phenobarbital and 3-methylcholanthrene on rate of formation of 455 nm complexes from N-hydroxyamphetamine and other amphetamines

Rats were given intraperitoneal injections of either phenobarbital, in doses ranging from 10 to 80 mg/kg for 1–4 days, or 3-methylcholanthrene dissolved in warm corn oil, at a dose of 20 mg/kg for 1–4 days. Other animals received no injections at all. Enzyme assays were performed as described in MATERIALS AND METHODS, and 455 nm complex formation was determined using 400 μM NADPH and 333 μM *N*-hydroxyamphetamine, 100 μM norbenzphetamine, or 1 mM *d*-amphetamine.

Treatment	Cytochrome P-450	NADPH–cytochrome *c* reductase	455 nm complex formation from:		
			N-Hydroxyamphetamine	Norbenzphetamine	*d*-Amphetamine
	nmoles/mg protein	*nmoles/mg/min*	$\Delta A_{455\text{-}490}$/*g protein/min*		
None	1.01	76	79	1.50	0.80
	1.17	78	71	1.35	0.55
	1.17	88	85	1.75	
	1.19	92	73	1.10	0.70
	1.28		82	1.76	1.00
	1.29	119	98	2.20	1.30
	1.34	110	97	1.70	0.80
Phenobarbital	1.85	104	186	15.40	2.10
	2.22	142	327	25.20	4.65
	2.34	142	242	20.70	
	2.37	121	236	23.10	2.10
	2.40	156	299	35.70	
	2.95	157	408	40.60	6.00
	3.18	190	466	48.90	8.54
	3.56	198	597	51.30	9.30
3-Methylcholanthrene	1.29	71	51	0.90	0.35
	1.61	68	34	0.50	0.10
	1.63	91	46	1.05	0.30
	2.00	73	35	0.55	0.20
	2.07	78	44	1.05	0.33
	2.45	65	30	0.55	0.20
	2.62	83	51	1.35	0.31

extinction coefficient for the 455 nm complex of each substrate, it can be seen that the complex formation for *N*-hydroxyamphetamine is at least an order of magnitude more rapid than for the next compound, norbenzphetamine. Norbenzphetamine was previously the compound which demonstrated the most rapid formation of a 455 nm complex (18). It was much more rapid than 455 nm complex formation from *d*-amphetamine and SKF 525-A and about twice as fast as complex formation from benzphetamine.

All the preceding observations were made using hepatic microsomes from phenobarbital-treated rats. The effect of phenobarbital induction on the rate of formation of 455 nm complexes is examined in Table 2. Over a range of treatment times and doses, the rate of complex formation increases with increasing cytochrome P-450 concentrations. A 3-fold increase in cytochrome P-450 concentration over that found in untreated rats produces approximately a 6-fold increase in the rate of 455 nm complex formation from *N*-hydroxyamphetamine, as compared to a 30-fold increase from norbenzphetamine and a 10-fold increase from *d*-amphetamine. NADPH–cytochrome *c* reductase activity during these inductions is only increased up to 2-fold. Prior treatment with 3-methylcholanthrene, however, although it increases the total carbon monoxide-binding pigments

(cytochromes P-450 and P_1-450) 2-fold, causes a reduction in the rate of 455 nm complex formation from all three substrates —a reduction to rates below those observed for untreated animals.

In order to convert the rates of 455 nm complex formation from absorbance units to nanomoles per milligram of protein per minute it was necessary to know the extinction coefficient of the 455 nm complex. This was determined by two different methods, and both yielded a value of 65 mM^{-1} cm^{-1}. The first method (Fig. 8) involved the incubation of microsomes with excess *N*-hydroxyamphetamine and NADPH for limited periods of time. The formation of the 455 nm complex was terminated and "fixed" by the addition of excess sodium dithionite (terminated, since dithionite removes the oxygen, and fixed, because it maintains all the cytochrome P-450 in the reduced form). The absorbance at 455 nm relative to 490 nm was then determined, and the contents of the cuvette were gassed with carbon monoxide. By detection of the extra absorbance produced by carbon monoxide, and the known extinction coefficient of the cytochrome P-450–CO complex at 455 nm (73 mM^{-1} cm^{-1}), it was possible to determine the amount of cytochrome P-450 present as the 455 nm complex, assuming no displacement by carbon monoxide. Extrapolation of the values obtained after various times of incubation (and hence various concentrations of the 455 nm complex) to 100% cytochrome P-450 as the complex gives an extinction coefficient of 65 $mM^{-1}cm^{-1}$.

The second technique involved the incu-

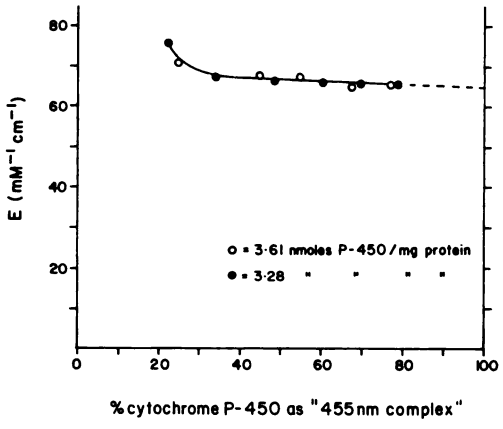

FIG. 8. *Determination of extinction coefficient of 455 nm complex formed during microsomal metabolism of N-hydroxyamphetamine. I*

Microsomal suspensions from phenobarbital-treated rats were incubated with excess NADPH (200 μM) and *N*-hydroxyamphetamine (500 μM) for limited periods of time. A small amount of sodium dithionite was then added, and the difference spectrum was recorded. The contents of the cuvette were then gassed with carbon monoxide and the difference spectrum was again recorded. Comparison of the absorbance at 455 nm before and after carbon monoxide gassing, with the absorbance obtained after carbon monoxide gassing of a dithionite-reduced sample which had not been incubated with NADPH, permitted determination of the amount of cytochrome P-450 bound as the 455 nm complex, and hence the extinction coefficient. The extinction coefficient obtained from reactions terminated at various times up to 200 sec (and hence with various amounts of cytochrome P-450 as the 455 nm complex) were plotted against the percentage of cytochrome P-450 as the 455 nm complex.

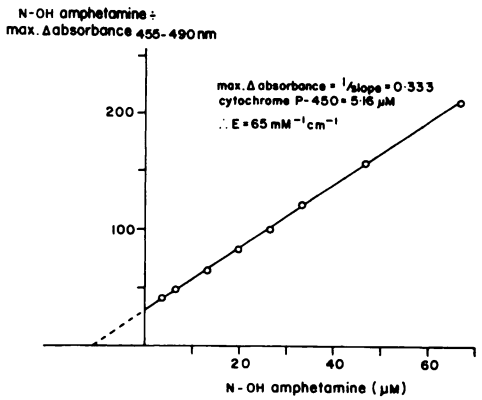

FIG. 9. *Determination of extinction coefficient of 455 nm complex formed during microsomal metabolism of N-hydroxyamphetamine. II*

Microsomes from phenobarbital-treated rats incubated with limiting amounts of *N*-hydroxyamphetamine and excess NADPH (500 μM), and the maximum absorbance (at 455 nm relatve to 490 nm) ever attained was recorded. A plot of *N*-hydroxyamphetamine concentration, divided by maximum absorbance, against *N*-hydroxyamphetamine concentration gives a slope which is the reciprocal of the maximum absorbance possible. Dividing this by the cytochrome P-450 concentration gives the extinction coefficient.

bation of microsomes with limiting amounts of *N*-hydroxyamphetamine and excess NADPH. The maximum absorbance obtained (about 12 min later) at each *N*-hydroxyamphetamine concentration was determined and graphed on what would be a substrate (s)/velocity (v) vs. S plot if it were an enzymatic reaction; in this case v is the maximum absorbance obtained, not a rate (Fig. 9). The slope of such a plot is $1/V_{max}$ or, in this case, 1/maximum absorbance. Dividing this by the known concentration of cytochrome P-450 in the assay medium again gives an extinction coefficient of 65 mM^{-1} cm^{-1}.

The V_{max} and apparent K_m for the reaction responsible for 455 nm complex formation from *N*-hydroxyamphetamine could be calculated using this value. A value of 6.3–6.5 nmoles/mg of protein per minute could be derived for maximum velocity, and an apparent K_m value of 33 μM for reactions performed at several protein concentrations (Fig. 10). The apparent K_m value decreased slightly and the V_{max} increased slightly as the protein concentration of the medium decreased. Linear extrapolation of these values to infinitely small protein concentrations produced an apparent K_m of 30 μM and a V_{max} of 6.6 nmoles/mg of protein per minute.

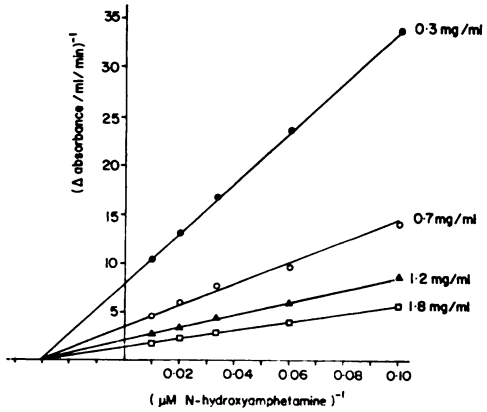

FIG. 10. *Determination of kinetic constants of microsomal formation of 455 nm complex from N-hydroxyamphetamine*

The initial velocity of the formation of the 455 nm absorbance (relative to 490 nm) was derived from the absorbance changes observed upon the addition of various concentrations of *N*-hydroxyamphetamine (10–100 μM) to microsomal suspensions containing 0.3, 0.7, 1.2, or 1.8 mg of protein per milliliter and 200 μM NADPH.

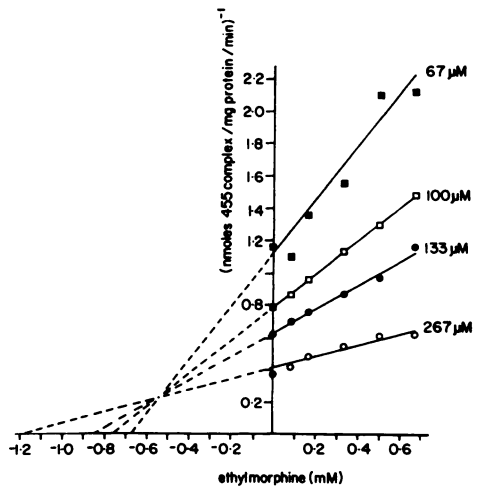

FIG. 11. *Inhibition of 455 nm complex formation from N-hydroxyamphetamine by ethylmorphine*

Absorbance changes at 455 nm relative to 490 nm were monitored upon the addition of 200 μM NADPH to suspensions containing 2 mg of microsomal protein per milliliter, various concentrations of N-hydroxyamphetamine (67 → 267 μM), and ethylmorphine. (The cytochrome P-450 concentration was 4.80 μM, and the K_m for 455 nm complex formation was 25 μM *N*-hydroxyamphetamine.)

Examination of the inhibitory effects of ethylmorphine on 455 nm complex formation from *N*-hydroxyamphetamine (Fig. 11) shows competitive inhibitory characteristics and an inhibitor constant (K_I) for ethylmorphine of 0.54 mM, a value very close to the K_m for the *N*-demethylation of this substrate (14, 19–21).

DISCUSSION

N-Hydroxyamphetamine is not unique in its ability to form a 455 nm complex during microsomal oxidative metabolism. The data presented here do much to confirm the original interpretation of the formation of such complexes (3, 19), i.e., that a mixed-function oxidation reaction is responsible for the formation of an intermediate which then binds to and inactivates the cytochrome P-450 that formed it. Some of the major

complications using other substrates were the slowness of the reaction (7) and the ability to bind less than 40% of the cytochrome P-450 as the 455 nm complex (8). With *N*-hydroxyamphetamine, the speed of the reaction is comparable to many other classical xenobiotic oxidations, and at least 80% of the cytochrome P-450 can be sequestered as the 455 nm complex.

Although the formation of the 455 nm complex requires NADPH, small amounts of the complex are formed in the absence of exogenous NADPH, as shown in Figs. 2 and 3. The reason for this remains unresolved at present, since the possibility of endogenous NADPH being present in the microsomes after their isolation appears extremely unlikely. This small 455 nm complex, with a negative absorbance around 412 nm (Fig. 2), perturbates the rather small binding spectrum obtained upon the addition of *N*-hydroxyamphetamine to aerobic microsomes (Fig. 1). The very small amount of 455 nm complex formed with microsomes from untreated rats gives a clearer picture of the binding spectrum of *N*-hydroxyamphetamine, i.e., reverse type I (22), with an absorption minimum at 390 nm and a maximum at 420 nm. Perturbation of this spectrum by the larger amount of 455 nm complex in microsomes from phenobarbital-treated rats could result in the rather peculiar binding spectrum seen in such microsomes.

The formation of a small amount of the 455 nm complex in the absence of exogenous NADPH raised the possibility that the fast rate of formation of the complex seen upon the addition of NADPH (Fig. 3) could be due to the reduction of cytochrome P-450 coupled with rapid binding to a metabolite previously formed during aerobic incubation of microsomes with *N*-hydroxyamphetamine. This possibility was eliminated by two observations. The reversal of the order of addition of *N*-hydroxyamphetamine and NADPH, thus eliminating the aerobic incubation period referred to above, did not decrease the rapid rate of formation of the 455 nm complex. Furthermore, the rate of reduction of cytochrome P-450, as measured in the presence of carbon monoxide (and *N*-hydroxyamphetamine), was much faster than the rate of formation of the 455 nm complex.

The slow rate of 455 nm complex formation in the presence of NADH, in this case about 10% of the rate observed with NADPH, is consistent with the small amount of oxidative drug metabolism that can be supported by NADH (23). Also, the slight synergism of NADPH and NADH on the rate of 455 nm complex formation is in agreement with similar effects for other mixed-function oxidations (23, 24). The changes of oxygen consumption observed upon the addition of *N*-hydroxyamphetamine to microsomes fortified with NADPH again suggest that an oxidative reaction is necessary for 455 nm complex formation, since there is an initial burst of oxygen utilization upon addition of the substrate. The subsequent inhibition of the endogenous oxygen consumption conforms with the theory that the 455 nm complex is cytochrome P-450 inactivated by the formation of the 455 nm complex, thus inhibiting any endogenous cytochrome P-450-dependent oxidations. That formation of the 455 nm complex proceeds via a mixed-function oxidation rather than a peroxide-dependent reaction, which has been suggested by Kadlubar *et al.* (25) for the oxidation of *N*-hydroxyamphetamine (25), is suggested here by the finding that large amounts of exogenous catalase had no effect on the formation of the 455 nm complex.

The cytochrome P-450 complex formed from *N*-hydroxyamphetamine, like all such complexes so far observed, is stable in the presence of dithionite. It was thus possible to "freeze" the complex with dithionite, since this renders all the cytochrome P-450 in the reduced form and removes the oxygen to prevent further complex formation. When the complex was treated with deoxycholate, at concentrations which converted normal cytochrome P-450 to P-420, it was possible to observe a decrease in the 455 nm absorbance and the formation of absorbance at 423 nm (Fig. 6). This was probably due to complexation of the intermediate responsible for the 455 nm complex with P-420 (denatured cytochrome P-450). The relation-

ship between these two complexes is shown by the constancy of the $A_{455}:A_{423}$ ratio (Table 1), using several starting concentrations of the 455 nm complex. The possibility that the 423 nm absorbance was due to the binding of P-420 to carbon monoxide formed from oxidation reactions taking place during the formation of the 455 nm complex cannot be excluded, although the large absorbance at 423 nm after only very short periods of incubation (30 sec) and the lack of detectable carbon monoxide in similar incubations in the absence of *N*-hydroxyamphetamine make such an explanation extremely unlikely.

The rate of 455 nm complex formation from *N*-hydroxyamphetamine is the highest for any substrate yet observed. This applies both to untreated and phenobarbital-treated rats. It is compared in Table 2 with 455 nm complex formation from norbenzphetamine (the second fastest substrate so far observed) and *d*-amphetamine, the prototype of this group of compounds. Thus complex formation from *N*-hydroxyamphetamine was 50 times that from norbenzphetamine and 100 times that from *d*-amphetamine in untreated rats. After phenobarbital treatment, however, it was only 10 times that from norbenzphetamine and 60 times that from *d*-amphetamine. Thus phenobarbital induces less 455 nm complex formation for *N*-hydroxyamphetamine than for *d*-amphetamine, and much less than for norbenzphetamine. For a 3-fold increase in cytochrome P-450 there is a 2-fold increase in NADPH–cytochrome *c* reductase, and 6-, 30-, and 10-fold increases in 455 nm complex formation from *N*-hydroxyamphetamine, norbenzphetamine, and *d*-amphetamine, respectively. Thus either different enzymes catalyze the formation of the intermediate responsible for the 455 nm complex per se, or else different enzymes are responsible for the metabolism of these compounds into substrates for the 455 nm complex-forming reaction. The ability to bind about 80% of the cytochrome P-450 as the 455 nm complex with *N*-hydroxyamphetamine as substrate, in contrast to about 40% with benzphetamine as substrate (8), lends credence to the latter idea. If several cytochrome P-450-dependent reactions were necessary to form metabolites which are substrates for the 455 nm complex-forming reaction, the sequestering of cytochrome P-450 as the 455 nm complex would severely limit the availability of substrates, and thus the formation of the 455 nm complex. With *N*-hydroxyamphetamine, which appears to be the most favored or key substrate for the 455 nm complex formation reaction, the availability of substrate is not limited, but rather the cytochrome P-450 is, since each molecule turns over only once before inactivating itself as a 455 nm complex. This would also account for the difference in shape of the curves depicting 455 nm complex formation for *N*-hydroxyamphetamine and the other substrates (Fig. 7).

Another explanation for the relative differences in 455 nm complex formation among the three substrates after phenobarbital induction is that these differences could be due to a new type of cytochrome P-450 present in microsomes after phenobarbital treatment. This could manifest itself as differences either in extinction coefficients or in affinity of the cytochrome for the metabolic intermediate, among the three substrates.

Induction by 3-methylcholanthrene, or polycyclic hydrocarbons in general, is known to result in the formation of a different hemoprotein cytochrome P-450 (26) as well as to stimulate only certain enzyme activities (27). In the experiments shown a 2-fold increase in CO-binding hemoprotein gave little or no change in NADPH–cytochrome *c* reductase activity, but caused a decrease in the rate of 455 nm complex formation for all three substrates. This suggests that induction by 3-methylcholanthrene does not cause induction of the enzymes or the type of cytochrome P-450 necessary for the formation of the 455 nm complex.

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