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Interaction of Constitutive and Phenobarbital-Induced Cytochrome P-450 Isozymes during the Sequential Oxidation of Benzphetamine

Explanation for the Difference in Benzphetamine-Induced Hydrogen Peroxide Production and 455-nm Complex Formation in Microsomes from Untreated and Phenobarbital-Treated Rats

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SUMMARY

The following pathway for benzphetamine (Bz) metabolism in rat hepatic microsomes was established: Bz → norbenzphetamine (NorBz) → *N*-hydroxynorbenzphetamine → *N*-benzylethyl-α-phenylnitrone → 2-nitroso-1-phenylpropane. The last product forms a complex with cytochrome P-450 with an absorbance maximum at 455 nm. Steps 1, 2, and 4 are cytochrome P-450-dependent; Step 3 appears to involve the flavoprotein, mixed-function amine oxidase. Step 2 is partially uncoupled, producing H_2O_2 at approximately 3 times the rate of *N*-hydroxylation. Bz is oxidized to NorBz in microsomes from both untreated rats (U-microsomes) and phenobarbital (PB)-treated rats (PB-microsomes), but the 455-nm peak does not appear in U-microsomes until almost all of the Bz has been converted to NorBz; i.e., Bz inhibits the oxidation of NorBz in U- but not in PB-microsomes. The inhibition is competitive. Bz inhibits the oxidation of the nitrone to 2-nitroso-1-phenylpropane in both U- and PB-microsomes; NorBz inhibits this reaction in U-microsomes only. These results can be explained as follows. The substrate affinities of the cytochrome P-450 primarily responsible for the *N*-demethylation of Bz in U- and PB-microsomes differ markedly. The constitutive cytochrome(s) in U-microsomes has a high affinity for Bz; PB induces both this form and a cytochrome(s) with a lower affinity for Bz. The substrate affinities of these two cytochromes P-450 for NorBz do not differ appreciably. Thus, although both forms of cytochrome P-450 can oxidize Bz and NorBz in PB-microsomes, Bz is primarily oxidized by the constitutive form, whereas NorBz is oxidized primarily by the induced form, thereby relieving competition and increasing the over-all sequential oxidation of Bz. The nitrone appears to be oxidized exclusively by the constitutive form in both U- and PB-microsomes. The current study shows that PB induction of monooxygenase activity need not be due entirely to an increase in the amount of cytochrome P-450 or the substrate selectivity of cytochrome P-450 isozyme(s) responsible for that activity, but that, in at least one case, the metabolism of Bz, PB-induced activity can be due, at least in part, to the induction of a cytochrome P-450 isozyme that relieves substrate inhibition.

INTRODUCTION

The cytochrome P-450-dependent monooxygenase system of the liver is responsible for the metabolism of many drugs. This membrane-bound oxygenase also produces hydrogen peroxide (1). Many commonly employed substrates, e.g., ethylmorphine (2), codeine (3), and aminopyrine (4), have no effect on the basal production of hydrogen peroxide. However, Estabrook and Werringloer (5) showed that Bz[1] greatly stimulates the production of hydrogen peroxide in rat hepatic microsomal preparations. The over-all reaction for the *N*-demethylation of Bz can be written as follows:

$$\text{P-450} + \text{NADPH}^+ + \text{H}^+ + \text{O}_2 + \text{Bz} \rightarrow \text{P-450} + \text{NADP}^+ + \text{NorBz} + \text{HCHO} + \text{H}_2\text{O} \quad (1)$$

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[1] The abbreviations used are: Bz, benzphetamine; NorBz, norbenzphetamine; PB, phenobarbital; PB-microsomes, microsomes from phenobarbital-treated rats; U-microsomes, microsomes from untreated rats; HPLC, high-pressure liquid chromatography; DOC, deoxycholate.

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Estabrook and Werringloer visualized the formation of H_2O_2 as an "un-coupling" of Reaction 1 as follows:

$$\text{P-450} + \text{NADPH}^+ + \text{H}^+ + \text{O}_2 + \text{Bz} \rightarrow \text{P-450} + \text{NADP}^+ + \text{Bz} + \text{H}_2\text{O}_2 \quad (2)$$

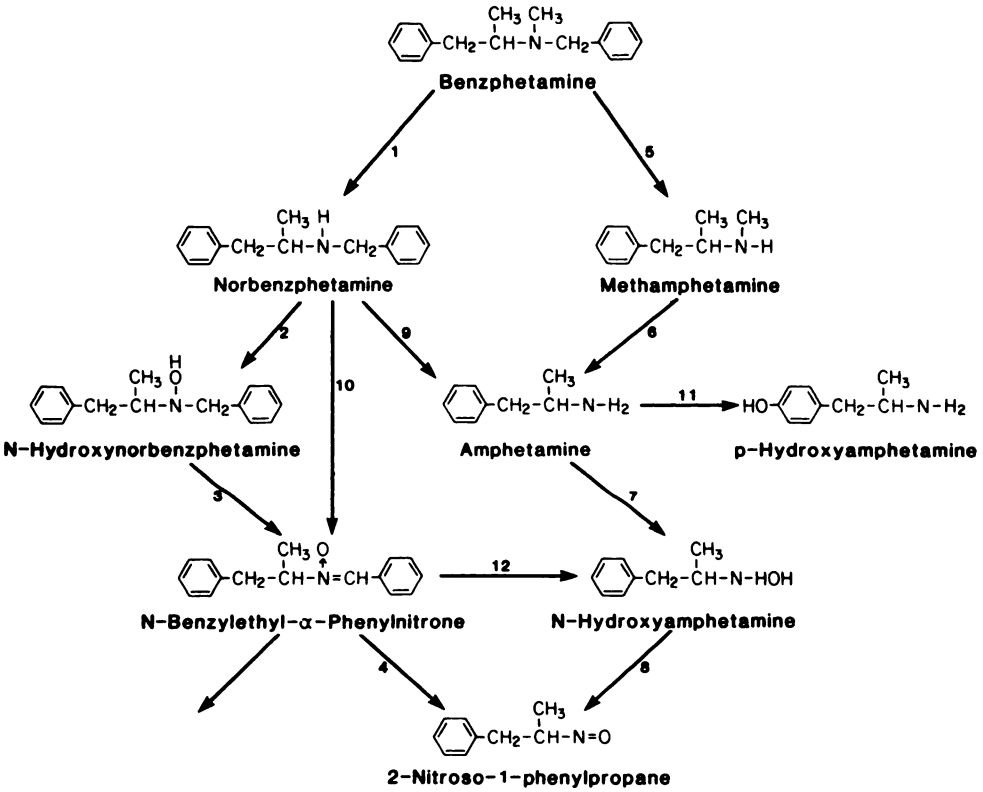
While studying the stoichiometry of NADPH utilization during the oxidation of Bz by hepatic microsomes we found, in disagreement with Estabrook and Werringloer (5), that in the concentration employed by these investigators, Bz did not stimulate hydrogen peroxide generation. This apparent discrepancy was resolved when in preliminary studies we determined that Bz induced hydrogen peroxide formation in microsomes from PB-treated rats (PB-microsomes), the animals used routinely in the laboratory of Estabrook and Werringloer, but not in microsomes from untreated rats (U-microsomes), the animals used routinely in our laboratory. This observation prompted a detailed study of the difference between Bz metabolism by microsomes from untreated and PB-treated rats.

Another difference noted in these preliminary studies was that during the metabolism of 1 mM Bz an absorbance maximum at 455 nm developed in PB-microsomes, as first described by Werringloer and Estabrook (6), but not in U-microsomes. This peak is due to the formation of a complex of cytochrome P-450 with 2-nitroso-1-phenylpropane, a metabolite of Bz (7, 8). This suggested the possibility that U- and PB-microsomes differ not only in the production of Bz-induced hydrogen peroxide, but in the over-all metabolic pathway for the metabolism of Bz.

Bz is a substituted amphetamine, with a benzyl and a methyl group α to the nitrogen atom. The predominant pathways for metabolism of substituted amphetamines are oxidation of the nitrogen atom, oxidation at any carbon atom α to the nitrogen atom, and (in the rat) ring-hydroxylation (9). Rat hepatic microsomes *N*-demethylate Bz to form formaldehyde (Scheme 1, Reaction *1*). Formaldehyde could also be released if Bz were dealkylated to methamphetamine (Reaction *5*) and then demethylated to amphetamine (Reaction *6*). It is known that 2-nitroso-1-phenylpropane is formed during the metabolism of Bz, but the exact sequence of reactions leading to its formation has not been determined. Several possible routes for 2-nitroso-1-phenylpropane formation are illustrated in Scheme 1. Franklin (10) has provided evidence that *N*-hydroxyamphetamine is readily oxidized to 2-nitroso-1-phenylpropane (Reaction *8*) to form a 455-nm complex with cytochrome P-450. This precursor of 2-nitroso-1-phenylpropane, *N*-hydroxyamphetamine, is known to be formed from amphetamine (Reaction *7*) in the rabbit, but the occurrence of this reaction in the rat has been questioned (11). The current study examines the several routes shown in Scheme 1 which could conceivably lead to the formation of 2-nitroso-1-phenylpropane from Bz in the rat and attempts to identify the enzymes involved in these reactions.

MATERIALS AND METHODS

Chemicals. Bz and NorBz were gifts from The Upjohn Company (Kalamazoo, Mich.). p-Hydroxy-, D- and L-amphetamine were purchased from Smith Kline & French Laboratories (Philadelphia, Pa.), Sigma Chemical Company (St. Louis, Mo.), and K and K Laboratories (Plainview, N. Y.), respectively. *N*-Hydroxyamphetamine was a gift from Michael Franklin (University of Utah, Salt Lake City, Utah), methamphetamine was a gift from Sheldon Sparber (University of Minnesota, Minneapolis, Minn.), and *N*-hydroxy-NorBz was a gift from



SCHEME 1

Björn Lindeke (Uppsala University, Uppsala, Sweden). Catalase and glucose-6-phosphate dehydrogenase were purchased from Boeringer Mannheim Biochemicals (Indianapolis, Ind.).

Animals and tissue preparation. Male Sprague-Dawley rats (180–230 g) were given food and water ad libitum and were killed between 7 and 9 a.m. Hepatic microsomes were prepared and washed as described previously (3) and used within 8 hr. Where indicated, rats received PB (40 mg/kg, i.p.) in isotonic saline daily for 3 days; they were killed about 24 hr after the last injection.

Metabolism of Bz and metabolites of Bz. Substrates were incubated at 37° with microsomal protein (1 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.4) containing Mg^{2+} (3 mM), 5′-AMP (1 mM), NADPH (0.4 mM), glucose 6-phosphate (4 mM), and glucose-6-phosphate dehydrogenase (0.5 unit/ml). Methanol (0.1 mM) and catalase (5.0 units/ml) were present when hydrogen peroxide production was monitored. Formation of 2-nitroso-1-phenylpropane was determined by monitoring the absorbance of the cytochrome-2-nitroso-1-phenylpropane complex at 455 nm at 37° using an Aminco DW2 spectrophotometer as described by Franklin (10). The same medium as that described above was used in both cuvettes except that the reference cuvette contained no NADPH. NADPH oxidation was estimated in the same manner except that the rate of disappearance of absorbance was recorded at 340 nm ($\epsilon = 6.22$ mM^{-1} cm^{-1}) in the presence and absence of drug. The difference between these rates was used to calculate drug-dependent NADPH oxidation.

Identification and estimation of metabolites of Bz. The incubation medium (3 ml) was alkalinized with 0.2 ml of 10 N NaOH and extracted into 4 ml of chloroform. The chloroform fraction was removed and the extraction was repeated with an additional 4 ml of chloroform. The combined chloroform fractions were evaporated to dryness (water bath) and the sample was resuspended in approximately 30 μl of HPLC solvent. A reverse-phase HPLC column connected to a 254-nm UV monitor was used to separate the metabolites (Fig. 1). Only a single peak on the chromatogram was observed for each metabolite. Bz, NorBz, and methamphetamine were injected onto the column in quantities ranging from 50 to 200 nmoles in a total volume of 30 μl of HPLC solvent either alone, together, or together with a microsomal extract. The peak height for methamphetamine gave a value of 0.23 ± 0.01 *A*/μmole under each of these conditions. The area under the curve was used to quantify the Bz and NorBz content. No methamphetamine was found when microsomes were incubated with Bz. This being the case, 50 nmoles of methamphetamine were added as an internal standard to the chloroform-incubation medium mixture. Bz was also used as a standard (0.18 μmole) in some experiments.

Hydrogen peroxide generation was determined by the method of Keilen and Hartree (12), which measures the formaldehyde formed by the peroxidative action of catalase on methanol. The procedure of Nash (13) was used for the determination of the formaldehyde produced either from hydrogen peroxide or by the *N*-demethylation of Bz. When *N*-demethylation of Bz was measured, catalase and methanol were not included in the medium. The basal formation of hydrogen peroxide was determined in the presence of catalase and methanol and the absence of Bz. The Bz-induced generation of hydrogen peroxide was calculated as the formaldehyde formed in the presence of Bz, catalase, and methanol minus the formaldehyde formed from basal hydrogen peroxide generation and that produced by the *N*-demethylation of Bz.

Extraction and identification of N-benzylethyl-α-phenylnitrone. The eluate from the HPLC column obtained during the 54th min was brought to pH 8 with sodium carbonate and extracted with an equal volume of chloroform. The extraction was repeated, the combined chloroform fractions were evaporated to dryness, and the residue was dissolved in methanol. This residue was used both as a substrate for microsomal metabolism and for identification of the nitrone by UV and gas chromatography/mass spectrometry, which was performed by using a Hewlett Packard Model 5992B gas chromatograph/mass spectrometer [column specifications: 3 feet × 2 mm (inner diameter) glass column, packed with 3 OV-17 on 100/120 GasChrom; helium carrier gas flow rate 15 ml/min; oven temperature programmed from 180° to 280° at a rate of 16°/min; injector port temperature maintained at 220°].

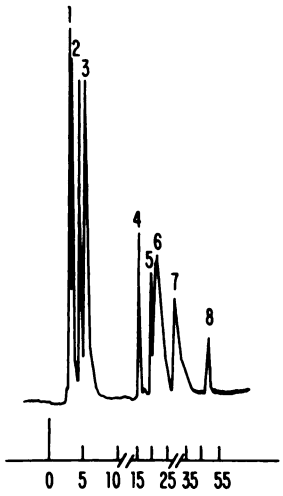


FIG. 1. *Separation of Bz and possible metabolites of Bz*

Authentic compounds were separated by HPLC, by using a reverse-phase column (C-18 Ultrasphere ODS; Altex) and a liquid phase consisting of 30:70 acetonitrile/0.17 M acetic acid containing 10 mM triethylamine. Individual compounds or mixtures of compounds were applied to the column as described under Materials and Methods and eluted at a flow rate of 1 ml/min. The elution pattern, as detected by absorbance at 254 nm, is as follows: *1*, *p*-hydroxyamphetamine; *2*, amphetamine; *3*, methamphetamine; *4*, benzylamine; *5*, phenylacetone; *6*, NorBz; *7*, Bz. Retention times for the substrate and metabolites were as follows: Bz, 27 min; NorBz, 22 min; amphetamine, 6 min; methamphetamine, 3 min; and *p*-hydroxyamphetamine, 2 min, respectively. The metabolite represented by Peak *8*, obtained after incubation of 1 mM Bz with PB-microsomes, was subsequently identified as *N*-benzylethyl-α-phenylnitrone (Fig. 5). The peak between *2* and *3* is of microsomal origin and was not altered by substrate or the duration of incubation period.

RESULTS AND DISCUSSION

Stoichiometry of Bz metabolism and H_2O_2 formation in U- and PB-microsomes. When U-microsomes were incubated with 1 mM Bz, formaldehyde and NorBz were produced at equal rates (Table 1). Drug-induced NADPH disappearance and Bz disappearance were stoichiometric with HCHO and NorBz formation. This is interpreted to mean that the demethylation of Bz to NorBz is the only dealkylation reaction that occurs in U-microsomes when the initial Bz concentration is 1 mM. This conclusion was supported by HPLC chromatography, which revealed no

TABLE 1

Bz metabolism and H_2O_2 formation in hepatic microsomes from untreated and PB-treated rats

U-microsomes (1.0 mg/ml) or PB-microsomes (0.25 mg/ml) were incubated as described under Materials and Methods with 1 mM Bz for 10 min. Values are means and standard error of three separate experiments.

	Untreated	PB-treated
	nmoles/ml/10 min	
Bz disappearance[a]	75 ± 15	79 ± 12
NorBz accumulation[a]	80 ± 10	18 ± 2
Formaldehyde formation	70 ± 1	87 ± 2
Bz-induced H_2O_2 formation	0	71 ± 7
Bz-induced NADPH oxidation[b]	73 ± 1	347 ± 8

[a] Estimated from 254-nm absorbance of HPLC peak.
[b] Estimated from disappearance of absorbance at 340 nm.

metabolites other than NorBz. When PB-microsomes were used, the stoichiometry was very different from that seen with U-microsomes. Bz disappearance was approximately stoichiometric with HCHO formation, but not with NorBz accumulation. This could mean that NorBz had been formed in the amount indicated by HCHO formation and then metabolized further, or it could mean that Bz was largely dealkylated to methamphetamine, which was then demethylated to amphetamine. If the latter situation prevailed, methamphetamine, amphetamine, or hydroxyamphetamine should have appeared in the incubation medium; HPLC analysis revealed none of these products. Instead, an unidentified product leaving the column at 54 min was observed. As is shown later, this product was determined to be *N*-benzylethyl-α-phenylnitrone, which could result from the metabolism of NorBz but not from amphetamine. Of the 347 nmoles of NADPH utilized by PB-microsomes, 158 nmoles can be accounted for by HCHO and H_2O_2 formation. This leaves 189 nmoles of NADPH unaccounted for, which is not excessive when one considers that each of the three NADPH-dependent steps involved in the oxidation of NorBz to 2-nitroso-1-phenyl-propane would require 60 nmoles of NADPH.

Effect of the concentration of Bz on H_2O_2 generation and the formation of 2-nitroso-1-phenylpropane. Preliminary studies showed that the metabolism of Bz (1 mM) led to the formation of a difference absorption spectrum with a maximum at 455 nm with PB-microsomes but not with U-microsomes. Because Bz-induced H_2O_2 formation occurs in PB-microsomes and not in U-microsomes, the question was raised as to whether there was a direct or reciprocal correlation between H_2O_2 formation and 455-nm complex formation. Our experimental approach to the question was to determine whether changes in Bz concentration caused parallel changes in H_2O_2 formation and the formation of the 455-nm complex. U-microsomes were used as controls. This led to the observation that, when low concentrations of Bz (0.05 and 0.1 mM) were used, both H_2O_2 and the 455-nm complex were formed by both U- and PB-microsomes. This unexpected finding led to the studies summarized in Fig. 2. A comparison of Fig. 2A, B, and C shows that, when the concentration of Bz is 1 mM, both H_2O_2 formation and 455-nm complex formation are induced in PB-microsomes but that neither is induced by Bz in U-microsomes. It can be seen in Fig. 2D, E, and F that, after a lag period, H_2O_2 and 455-nm complex formation occurred in both U- and PB-microsomes when the concentration of Bz was lowered to 0.025 nM. The lag period was sufficiently long to allow for almost complete Bz disappearance (determined by HCHO formation; Fig. 2D) from U-microsomes before H_2O_2 and 455-nm complex formation commenced (Fig. 2E and F, respectively). This suggested that a product of Bz metabolism induces H_2O_2 production in U-microsomes, but only in the absence of Bz. When NorBz replaced Bz as the substrate (Fig. 2H and I), H_2O_2 and a 455-nm complex were formed with no visible lag phase. It therefore seems likely that NorBz is the metabolite responsible for H_2O_2 production, not Bz, as was implied by Estabrook and Werringloer (5).

Effect of Bz and its metabolites on H_2O_2 production. Of seven putative metabolites of Bz, only NorBz induced

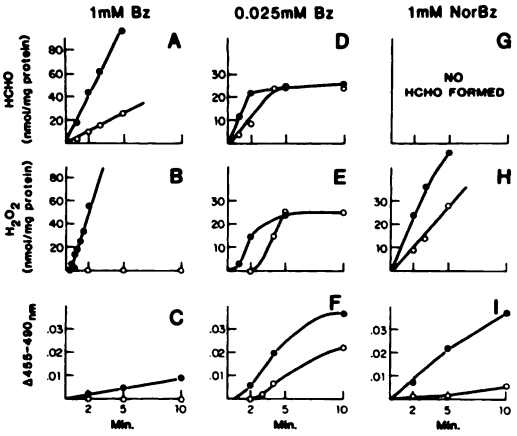


FIG. 2. *Effect of substrate (Bz or NorBz) concentration on the formation of HCHO, H_2O_2, and 455-nm complex*

U-microsomes (○) or PB-microsomes (●) (1 mg/ml) were incubated with Bz or NorBz as described under Materials and Methods. Demethylation, H_2O_2 generation, or 2-nitroso-1-phenylpropane formation (455-nm complex formation) was monitored by using 1 mM Bz (A, B, and C), 0.025 mM Bz (D, E, and F), or 1 mM NorBz (G, H, and I) as substrate. H_2O_2 values represent H_2O_2 formed in the presence of substrate minus H_2O_2 formed in the absence of substrate.

H_2O_2 generation in U-microsomes (Table 2). Both Bz and NorBz induced H_2O_2 generation in PB-microsomes (Table 2). However, an initial 30-sec lag phase was seen with Bz (Fig. 2B), indicating that NorBz formation was required before H_2O_2 generation was induced. Since NorBz is the primary metabolite of Bz, this raised the question of why NorBz-induced H_2O_2 generation was not seen in U-microsomes in the presence of Bz. This might be explained if Bz competes with NorBz as a substrate inhibitor during this early lag phase (Fig. 2E), when both Bz and the NorBz derived from Bz are present. Formaldehyde formation from Bz was therefore estimated in the presence and absence of NorBz (Fig. 3). NorBz was found to inhibit Bz demethylation in U-microsomes, and this inhibition was not relieved by PB induction. In contrast, although Bz inhibited both NorBz-induced H_2O_2 production and NorBz *N*-oxidation (estimated as *N*-benzylethyl-α-phenylnitrone accumulation) in U-microsomes, neither

TABLE 2

Effect of possible products of Bz metabolism on microsomal H_2O_2 production

Microsomes (1 mg/ml) were incubated with each compound (1.0 mM) as described under Materials and Methods. Values are means and standard error of three separate experiments except for *N*-hydroxyamphetamine, which is the mean of two experiments.

Addition	Untreated	PB-treated
	nmoles H_2O_2 produced/min/mg protein	
None	9.6 ± 0.4	12.5 ± 0.8
Bz	11.0 ± 0.4	23.6 ± 1.3
NorBz	15.4 ± 0.5	22.4 ± 1.3
Methamphetamine	9.8 ± 0.3	12.5 ± 0.6
D-Amphetamine	9.9 ± 0.8	13.3 ± 0.4
L-Amphetamine	9.2 ± 0.3	13.3 ± 0.3
N-Hydroxyamphetamine	4.3	3.7
Benzaldehyde	10.0 ± 0.9	10.8 ± 1.4
Benzylamine	5.3 ± 2.4	7.1 ± 2.4

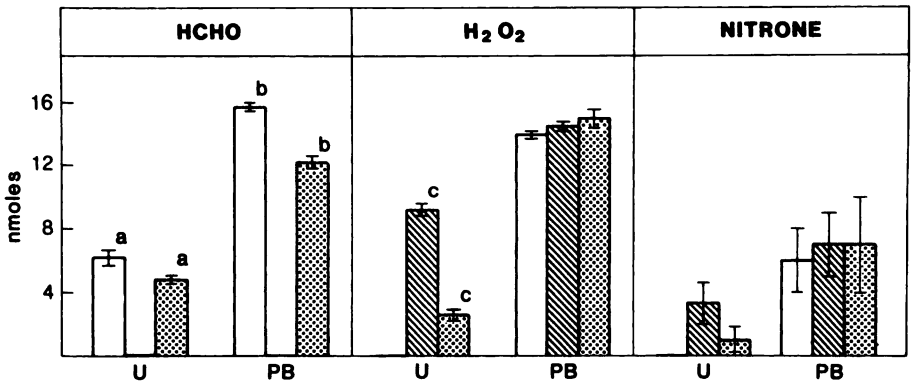


FIG. 3. *Inhibitory effect of Bz and NorBz on HCHO, H_2O_2, and nitrone formation*

Bz (1 mM, *open bars*), NorBz (1 mM, *hatched bars*), or Bz (1 mM) together with NorBz (1 mM, *stippled bars*) was incubated with U- or PB-microsomes as indicated. The microsomal protein content was 1.0 mg except when the formation of HCHO by PB-microsomes was determined, in which case, 0.5 mg was used. The reaction was stopped after 1 min, and HCHO, H_2O_2, and nitrone were estimated. Significant difference ($p < 0.05$) is indicated by *a*, *b*, or *c*.

H_2O_2 nor nitrone production was inhibited by Bz after PB induction (Fig. 3). It is to be noted that in U-microsomes, Bz was a far more potent inhibitor than NorBz. A more extensive study revealed that both compounds acted as competitive inhibitors in U-microsomes, and that the K_i for Bz inhibition of NorBz-induced H_2O_2 production was 2 orders of magnitude smaller than the K_i for NorBz inhibition of Bz demethylation (Table 3). This type of competitive inhibition between Bz and NorBz suggests that a single enzyme is responsible for the metabolism of both substrates. Alternative substrate kinetics were used to test this possibility. Cha (14) has shown that if Substrates A and B interact with a single enzyme under the conditions where the concentration of Substrate B (S_B) is constant while that of Substrate A (S_A) is varied, a nonlinear plot will be obtained when the reciprocal of the combined velocities of the metabolism of Substrates A and B ($1/v_A + 1/v_B$) is plotted against $1/S_A$. If V_A is greater than V_B, this curve will intercept a similarly plotted linear curve obtained when varying concentrations of Substrate A are incubated with the enzyme in the absence of Substrate B. When $K_A = K_{iA}$ and $K_B = K_{iB}$, the intercept will be located on the ordinate by $1/v = 1/V_B$ and on the abscissa by $1/S_A = (V_A - V_B)/V_B \cdot K_A$; the intercept will be independent of S_B (Fig. 4II).

Fig. 4I contains data from an experiment in which Bz (Substrate B) was present in a concentration of either 0.05 mM or 0.1 mM while NorBz (Substrate A) was varied between 0.03 mM and 2 mM. These plots are not consistent with those applicable to classical alternative substrate kinetics (Fig. 4II) for the interaction of two substrates with a single enzyme. However, deviations from the classical example do not exclude the possibility that Bz and NorBz may be interacting with the same enzyme, because the classical example is only applicable when the K_i is equal to the K_m for the metabolism of the inhibiting substrate (15). The V_A remained unchanged in the presence of S_B (Fig. 4I); this could only occur if a single enzyme was reacting with both substrates. That the intercept varied with S_B indicates that K_{iA} was not equal to K_A or that K_{iB} was not equal to K_B (15). Velocity in the presence of a competitive inhibitor can be described by

$$v = \frac{V \cdot S/K}{1 + S/K + I/K_i}$$

and the sum of the velocities of the two reactions is

$$v_A + v_B = \frac{V_A \cdot S_A/K_A}{1 + S_A/K_A + S_B/K_{iB}} + \frac{V_B \cdot S_B/K_B}{1 + S_A/K_{iA} + S_B/K_B}$$

where *A* is NorBz and *B* is Bz. If $K_A = K_{iA}$ and $K_B = K_{iB}$, the denominators are the same, which is the classical alternative substrate case drawn in Fig. 4II. The theoretical plots obtained when K_B is larger than K_{iB} for concentrations of Substrate B of 0.05 and 0.1 mM (*solid lines*, Fig. 4III) resemble those obtained experimentally (Fig. 4I). Conversely, if K_B were much smaller than K_{iB}, the *broken-line* plots shown in Fig. 4III would be obtained. The observation that K_B is much larger than K_{iB} (Table 3) supports the interpretation that Bz and NorBz react with the same enzyme and that the affinity of Bz for the binding site exceeds its capacity for being metabolized; i.e., $K_{iB} < K_B$. Although it would seem less likely, these kinetic observations do not exclude the possibility that

TABLE 3

Apparent kinetic constants for Bz demethylation and NorBz-induced hydrogen peroxide formation

Microsomes were incubated as described under Materials and Methods. Concentrations of each substrate varied between 0.03 and 2.0 mM; the inhibitor concentration was 0.05 or 0.1 mM. Values are the means of three experiments.

Kinetic constant	Untreated	PB-treated
Bz demethylation		
K_m Bz (μM)	30	34
V_{max} (nmoles/min/mg microsomal protein)	5.7	33
K_i NorBz (μM)	120	100
NorBz-induced H_2O_2		
K_m NorBz (μM)	200	100
V_{max} (nmoles/min/mg microsomal protein)	10	22
K_i Bz (μM)	4	—
Bz-induced H_2O_2		
K_m Bz (μM)	—	180
V_{max} (nmoles/min/mg microsomal protein)	—	19

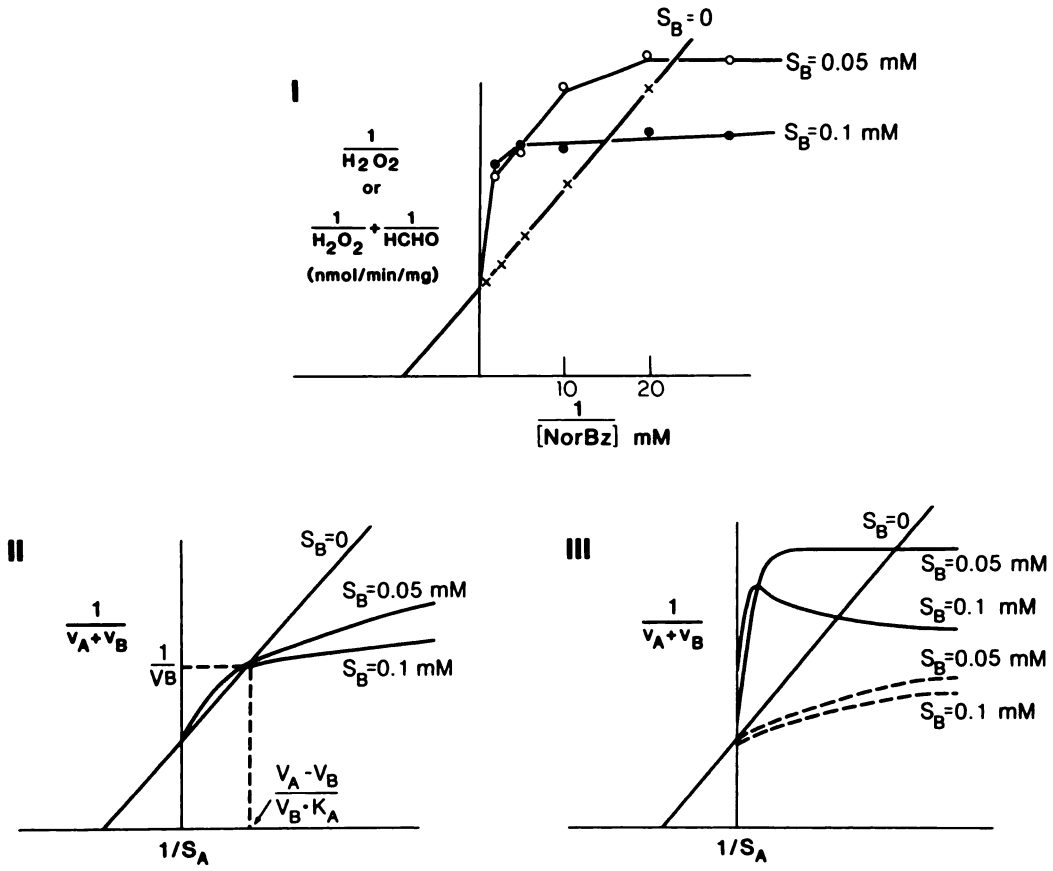


FIG. 4. *Alternative substrate kinetics: effect of a constant amount of Bz (S_B) and varying concentrations of NorBz (S_A)*

I, Double-reciprocal plot of product formation versus NorBz concentration. U-microsomes were incubated as described under Materials and Methods with concentrations of NorBz varying between 0.03 and 2.0 mM in the absence (×) or presence of 0.05 mM (○) or 0.1 mM (●) Bz. II, classical alternative substrate kinetics where $K_A = K_{iA} = 200$ μM and $K_B = K_{iB} = 30$ μM. III, K_A, K_{iA}, and K_B values are the same as in II, but K_{iB} has been given a value of 3 μM (*solid-line* plots) or 300 μM (*broken-line* plots).

each of two enzymes metabolizes only one of the two substrates while interacting with both.

A very different picture is seen in PB-microsomes. Not only does NorBz not accumulate during Bz demethylation, but after an initial 30-sec lag phase (Fig. 2B) H_2O_2 is produced with a K_m (Bz as substrate) of 180 μM (Table 3), a K_m very similar to the K_m (NorBz as substrate) for H_2O_2 production. When alternative substrate kinetic experiments were repeated using PB-microsomes, a pattern like that of the *broken-line* plot in Fig. 4III was obtained (data not shown) which indicates that, although Bz may be metabolized by the enzyme that produces H_2O_2 in the presence of NorBz, it does not successfully compete with NorBz. This lack of competition between Bz and NorBz for H_2O_2 production in PB-microsomes is indicative of the induction of a new cytochrome(s) with a greater affinity for NorBz than for Bz. Theoretically, one should be able to inhibit the metabolism of a low concentration of NorBz by raising the Bz concentration. This is not possible experimentally, because the conversion of Bz to NorBz is so rapid when the concentration of Bz is high that a low concentration of NorBz cannot be maintained long enough to permit a meaningful kinetic evaluation.

Phenobarbital induction increases the rate of Bz demethylation with no significant change in K_m (Table 3), which suggests that the constitutive[2] isozyme(s) responsible for Bz demethylation is induced. This concept is strengthened by the observation that the percentage of inhibition of Bz demethylation by antibody to the purified isozyme P-450$_b$ is the same in U- and PB-microsomes (16). Ryan *et al.* (17, 18) have shown that PB induces P-450$_b$ and at least two other P-450 isozymes. One of these (P-450$_a$) reacts very poorly with Bz (specific activity; 1.9 nmole of Bz demethylated per minute per nanomole of P-450$_a$); the other (P-450$_e$) is only about one-sixth as active as P-450$_b$ (specific activity of P-450$_e$ and P-450$_b$: 20 and 127 nmoles/min/nmole of P-450, respectively). As shown by the failure of Bz to inhibit NorBz in PB microsomes (Table 3; Fig. 3), an isozyme has been induced in these microsomes which has a greater affinity for NorBz than for Bz. Thus, whereas more of the isozyme with a high affinity for Bz and a low affinity for NorBz is present in PB-microsomes than in U-micro-

[2] A *constitutive P-450* is defined arbitrarily as any species of P-450 that exists in the liver of untreated animals. *PB-induced P-450* is arbitrarily defined as any species of P-450 that is increased in the liver after the administration of PB to rats; this may include both "constitutive P-450s" and P-450s that did not exist prior to the administration of PB or that existed in only very small amounts.

somes, PB-microsomes also contain an isozyme which has a greater affinity for NorBz than for Bz.

N-Hydroxylation and hydrogen peroxide formation. Up to this point, the following observations have been made: (a) NorBz is responsible for hydrogen peroxide production (Table 2; Fig. 2H); (b) NorBz is oxidized to a nitrone (Fig. 3) and a nitroso derivative (Fig. 2). Two mechanisms might account for peroxide production during NorBz *N*-oxidation: (a) the reaction might produce a single peroxy intermediate, which could then break down to produce either peroxide or *N*-oxidized product; (b) the reaction could be uncoupled, as suggested by Estabrook and Werringloer (5), so that oxygen is either inserted into the substrate or expelled as peroxide, leaving the substrate unchanged.

In accordance with Mechanism a, the possibility was considered that NorBz is oxidized by a single isozyme to a single *N*-peroxy intermediate, which might break down via any of three competing routes: (i) the release of substrate (NorBz) and H_2O_2; (ii) the formation of a primary amine, a ketone, and H_2O_2 (19); or (iii) the formation of *N*-hydroxy-NorBz and water; *N*-hydroxy-NorBz could then be oxidized further to the nitrone. Any change in flux through the hypothetical peroxy intermediate along one of these three pathways might lead to compensatory changes in the other pathways. To test this concept, rates of nitrone formation were altered with an inhibitor and an activator to determine whether the rate of H_2O_2 formation would change accordingly. Low concentrations of octylamine increase the activity of hydroxylamine oxidase, the enzyme putatively responsible for the conversion of *N*-hydroxy-NorBz to the nitrone (20). DOC inhibits hydroxylamine oxidase (20). Octylamine (10 μM) and DOC (0.3 mg/mg of protein) increased and decreased formation of the nitrone from NorBz by 39% and 43%, respectively (Table 4). When DOC depressed nitrone formation, there was no compensatory increase in H_2O_2 production, which might have been expected if nitrone and H_2O_2 were alternate products of a single intermediate. When nitrone formation was increased by octylamine, there was no compensatory depression in H_2O_2 production (Table 4). Although these compounds lowered NorBz-induced H_2O_2 production slightly, they also lowered basal H_2O_2 production and ethylmorphine *N*-demethylation activity to the same extent. The effects of these compounds on H_2O_2 production and demethylase activity thus appear to be due to some effect not related to their action on hydroxylamine oxidase activity. The results are not consistent with the suggestion that a common peroxy intermediate is broken down via alternative paths to produce H_2O_2 or *N*-hydroxy-NorBz. They are consistent with the suggestion that a single enzyme is partially uncoupled.

The effects of DOC and octylamine on nitrone formation (Table 5) suggest that the formation of nitrone is mediated by the flavin enzyme, mixed-function amine oxidase, rather than by cytochrome P-450. To test this possibility, nitrone formation from NorBz was monitored in the presence of methimidazole (a competitive substrate for mixed-function amine oxidase), SKF-525-A (an inhibitor of some cytochrome P-450 systems), and two concentrations of CO, which competes with oxygen for cytochrome P-450. The results are shown in Table 5. Inhibition of nitrone production occurred under all conditions, whereas inhibition of H_2O_2 production did not occur in the presence of methimidazole. That NorBz-induced peroxide production and *N*-oxidation are cytochrome P-450-dependent activities is shown by the inhibition of both activities by SKF 525-A and CO (Table 5). In the presence of methimidazole, a peak appeared at 16 min on the HPLC chromatogram, which co-eluted with authentic *N*-hydroxy-NorBz. These results are consistent with a cytochrome P-450-mediated oxidation of NorBz to *N*-hydroxy-NorBz, which is further oxidized to nitrone by a flavin enzyme (Scheme 1, Reactions *2* and *3*).

Not only are both NorBz-induced peroxide production and *N*-oxidation cytochrome P-450-dependent, but both activities are inhibited by Bz in U-microsomes and neither activity is inhibited by Bz in PB-microsomes (Fig. 3). These observations suggest that it is unlikely that two separate isozymes of P-450 interact with NorBz, one to produce *N*-hydroxy-NorBz and the other to produce H_2O_2. An uncoupling of *N*-oxidation to produce H_2O_2 could explain these results. The kinetic studies in Table 3 and Fig. 4 describe a linear Lineweaver-Burk

TABLE 4

Effect of octylamine (10 μM) and deoxycholate (0.3 mg/kg of microsomal protein) on nitrone formation and H_2O_2 generation from NorBz, basal H_2O_2 formation, and ethylmorphine N-demethylase activity

Fresh hepatic microsomes from untreated rats were incubated as described under Materials and Methods with 1 mM NorBz or 1 mM ethylmorphine for 10 min. Control values (nanomoles/10 min/mg of protein) were as follows: NorBz disappearance, 42; nitrone formation, 4.3; NorBz-induced and basal H_2O_2 production, 126 and 88, respectively; ethylmorphine *N*-demethylation, 90.

	Deoxycholate	Oxtylamine
	% control	
NorBz disappearance[a]	64	109
Nitrone formation[a]	57	139
NorBz-induced H_2O_2	75	87
Basal H_2O_2	73	79
Ethylmorphine *N*-demethylation	69	87

[a] Estimated from 254-nm absorbance of HPLC peak.

TABLE 5

Effects of methimidazole, SKF 525-A and CO on H_2O_2 and Nitrone production from NorBz in U-microsomes

Microsomes were incubated as described under Materials and Methods except that Bz was replaced with NorBz (1 mM). Results are means of two separate experiments. The percentage of "none" values is given in parentheses.

Addition	Nitrone production	H_2O_2 Production
	nmoles/min/mg	
None	2.8	7.2
Methimidazole (0.5 mM)	1.3 (47%)	6.9 (95%)
SKF 525-A (0.5 mM)	0.4 (14%)	NA[a]
Air	2.1	8.4
CO/O (1:1)	1.1 (52%)	4.8 (57%)
CO/O (2.5:1)	0.8 (38%)	2.1 (25%)

[a] SKF 525-A caused an increase (5.3 nmoles/min/mg of protein) in H_2O_2 over control levels in both the absence and presence of NorBz.

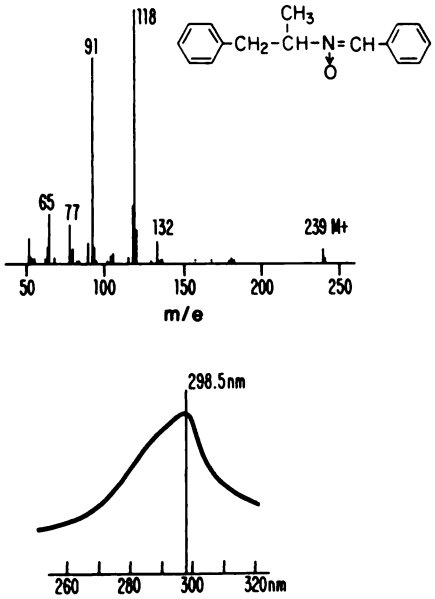


FIG. 5. *UV- and mass spectrographs of N-benzylethyl-α-phenylnitrone*

Bz (1 mM) was incubated with PB-microsomes; an extract containing *N*-benzylethyl-α-phenylnitrone was prepared from the incubation mixture and applied to an HPLC column as described under Materials and Methods. The nitrone was eluted in Fraction *8* (Fig. 1), purified and subjected to UV- and mass spectroscopy.

plot for H_2O_2 production. This linearity demands that, if a single enzyme is uncoupled to produce both *N*-hydroxy-NorBz and H_2O_2, H_2O_2 must be a constant percentage of all products formed at all concentrations of NorBz employed. If this were not the case, the plot would curve with a change in the substrate concentration as the formation of one product was favored over that of the other (21). This constant percentage is consistent with uncoupling rather than with the formation of an intermediate that breaks down to produce alternate products.

Nitrone metabolism. The HPLC chromatograph shown in Fig. 1 includes a peak (Peak *8*) obtained after Bz (1 mM) was incubated with PB-microsomes. The fraction responsible for this peak was subjected to gas chromatography/mass spectrometry. The spectrum (Fig. 5) was identical with that obtained by Beckett and Gibson (22) for *N*-benzylethyl-α-phenylnitrone. The metabolite also exhibited the characteristic UV-absorption peak (Fig. 4) for this nitrone.

When the nitrone obtained from Fraction *8* was incubated with microsomes and an NADPH-generating system, the nitrone disappeared, as was determined by HPLC analysis, and a 455-nm peak appeared, thus indicating the formation of 2-nitroso-1-phenylpropane. The nitrone did not disappear and the 455-nm absorbance peak did not appear when the NADPH-generating system was absent. This finding appears to contradict the hypothesis (23) that the nitrone is hydrolyzed to *N*-hydroxyamphetamine prior to formation of 2-nitroso-1-phenylpropane, at least in microsomal preparations. It was therefore concluded that the nitrone is an intermediate in the metabolism of NorBz to 2-nitroso-1-phenylpropane. This does not exclude the possibility that the nitrone might be hydrolyzed by some non-microsomal enzyme in the hepatocyte to *N*-hydroxyamphetamine, which in turn could be oxidized to 2-nitroso-1-phenylpropane.

Lack of amphetamine formation during Bz metabolism. It might be argued that NorBz could undergo *α-C*-oxidation to amphetamine, followed by *N*-oxidation to 2-nitroso-1-phenylpropane (Steps *7* and *8*, Scheme 1), rather than via *N*-oxidation to the nitrone (Steps *2* and *3*, Scheme 1) followed by a *α-C*-oxidation to 2-nitroso-1-phenylpropane (Step *4*, Scheme 1). When microsomes from untreated rats are incubated with 1 mM amphetamine, less 455-nm complex is produced than when 0.1 mM NorBz is the substrate (Table 6). This implies that amphetamine is not on the pathway between NorBz and 2-nitroso-1-phenylpropane. This implication was strengthened when it was determined that no amphetamine of *p*-hydroxyamphetamine was formed when NorBz was incubated with microsomes for 10 min (HPLC data not shown). *p*-Hydroxyamphetamine did accumulate when amphetamine or methamphetamine was the substrate (HPLC data not shown). H_2O_2 production, nitrone formation, and 2-nitroso-1-phenylpropane formation from 0.1 mM NorBz were determined in the presence and absence of 1 mM amphetamine (Table 6) to determine whether amphetamine is on the metabolic pathway from NorBz to 2-nitroso-1-phenylpropane. The presence of amphetamine had no effect on any of these measurements, and it was therefore concluded that *α-C* oxidation of NorBz to amphetamine does not occur and is not required for 2-nitroso-1-phenylpropane formation from NorBz. Two other points might be used to argue that the pathway of Bz oxidation via amphetamine has not been eliminated by the presented evidence: (a) the failure to detect amphetamine as a product of NorBz could mean that the amphetamine produced from NorBz is so rapidly converted to *N*-hydroxyamphetamine that a detectable concentration is not reached; (b) failure to produce 2-nitroso-1-phenylpropane when microsomes are incubated with a relatively high concentration of amphetamine could be due to inhibition of the conversion

TABLE 6

Effect of excess amphetamine on product formation from NorBz incubated with U-microsomes

Microsomes (1 mg of protein per milliliter) were incubated as described under Materials and Methods except that Bz was excluded and either no drug (basal), amphetamine (1 mM), NorBz (0.1 mM), or both drugs were included. Results are means and standard error of three separate experiments.

Product formed	Basal	Amphetamine (A)	NorBz (B)	A + B
H_2O_2 (nmoles/min/mg)	4.9 ± 0.09	4.8 ± 0.09	7.0 ± 0.2	7.4 ± 0.2
2-Nitroso-1-phenylpropane ($A_{455\ nm}$ at 10 min)	0.00	0.0002 ± 0.0001	0.0018 ± 0.0001	0.0017 ± 0.0001
Nitrone formation (nmoles/min/mg)	0.00	0.00	0.55 ± 0.6	0.56 ± 0.06

of *N*-hydroxyamphetamine to 2-nitroso-1-phenylpropane (Scheme 1, Reaction *8*) by amphetamine. Both arguments were eliminated by the observation that a high concentration of amphetamine (1 mM) did not alter the production of 2-nitroso-1-phenylpropane from NorBz (0.1 mM).

Inhibition of nitrone metabolism by Bz and NorBz. The 2-nitroso-1-phenylpropane formed from the nitrone (Scheme 1, Step *4*) complexes with the cytochrome P-450 responsible for its formation to give a maximal absorbance at 455 nm (6, 7). The complex occurs when 1 mM Bz is incubated with PB-microsomes but not with U-microsomes (Fig. 2C). However, it is formed in U-microsomes when relatively low concentrations of Bz are used (Figs. 2F and 6A). The effect of Bz concentration on the formation of the complex in U- and PB-microsomes is shown in Fig. 6A. Complex formation was increasingly depressed in both microsomal preparations as the concentration of Bz was increased between 0.1 and 1.0 mM. Werringloer and Estabrook (6) observed a decrease in complex formation in PB-microsomes with increasing concentrations of Bz, but they did not describe the pathway of Bz metabolism or locate the site of inhibition. NorBz was also tested as an inhibitor of complex formation (Fig. 6B). Concentrations of NorBz above 0.1 mM inhibited complex formation in U-microsomes but not in PB-microsomes; therefore, the depressant effect of Bz on complex formation in PB-microsomes (Fig. 6A) must be due to Bz directly, not to NorBz or another metabolite. NorBz has a lesser depressant effect than Bz on complex formation in U-microsomes (Fig. 6A and B). Therefore the inhibition by Bz in U-microsomes cannot be due entirely to NorBz formation; both Bz and NorBz must inhibit 2-nitroso-1-phenylpropane formation. The question was then raised as to how Bz and NorBz produce their inhibitory effects. Steps *1* (3), *2* (24), and *4* (7) are believed to be cytochrome P-450-dependent; Step *3* is not (25). The current studies (Figs. 3 and 4) suggest that Steps *1* and *2* involve the same cytochrome P-450 in U-microsomes. The data in Fig. 6 are consistent with the view that the P-450 isozyme involved in Step *4* is also the isozyme involved in Steps *1* and *2* in U-microsomes. This statement is based on the observation that at high concentration, both Bz and NorBz inhibit complex formation (Fig. 6A and B) and that Bz (1 mM) inhibits complex formation by about 50% when the nitrone (1 μM) is incubated with U-microsomes (data not shown).

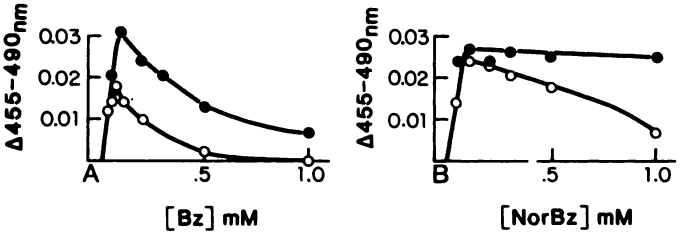


FIG. 6. *Effect of substrate concentration on the extent of 455-nm complex formation*

The absorbance change at 455 nm was monitored by difference spectroscopy during the incubation of U-microsomes (○) or PB-microsomes (●) with Bz (A) or NorBz (B).

Whereas a metabolic pathway involving a single cytochrome P-450 for Steps *1*, *2*, and *4* is compatible with the data obtained for U-microsomes, it is not compatible with the data obtained for PB-microsomes because (a) NorBz inhibits complex formation in U-microsomes but not in PB-microsomes; (b) Bz inhibits 455-nm complex formation but not NorBz metabolism in PB-microsomes; (c) although increasing the concentration of NorBz above 0.1 mM does not increase the 455-nm complex formed, neither does it inhibit complex formation. The results obtained with PB-microsomes can be explained if the cytochrome involved in Step *2* is not the same isozyme as that involved in Steps *1* and *4*. The suggestion that Steps *1* and *2* are mediated by different cytochrome P-450s in PB-microsomes is supported by the observation that in these microsomes Bz does not inhibit NorBz-induced hydrogen peroxide production (Fig. 3B).

In summary, the data presented in this communication support a metabolic pathway of Bz to 2-nitroso-1-phenylpropane in U-microsomes via Steps *1*, *2*, *3*, and *4* rather than Steps *5*, *6*, *7*, and *8*; Steps *1*, *10*, and *4*; Steps *1*, *9*, *7*, and *8*; or Steps *1*, *2*, *3*, *12*, and *8* (Scheme 1). Step *1* is preferred over Step *5* because Bz disappearance and NorBz disappearance are stoichiometric with HCHO formation; this could not be the case if HCHO had been formed via Steps *5* and *6*. Moreover, the rate of HCHO formation was linear (Fig. 2A); if HCHO had been formed via Steps *5* and *6*, an initial lag phase in the rate of HCHO formation would have occurred. Finally, no amphetamine (Steps *5* and *6* or Steps *1* and *9*) or *p*-hydroxyamphetamine (Step *11*) was detected in the incubation medium. Step *2* is preferred over Step *9* because no amphetamine or *p*-hydroxyamphetamine was formed; however, *N*-benzylethyl-α-phenylnitrone, which could not be derived from amphetamine, was formed (Step *3*). Finally, incubation of amphetamine (1 mM) with U-microsomes produced very little 2-nitroso-1-phenylpropane, and the inclusion of amphetamine with NorBz did not increase the amount of 2-nitroso-1-phenylpropane above that produced by NorBz alone (Table 6). The absence of Step *9* precludes the involvement of Step *7* in the oxidation of Bz to 2-nitroso-1-phenylpropane. Steps *2* and *3* are preferred over Step *10* because stimulators and inhibitors of hydroxylamine oxidase alter *N*-benzylethyl-α-phenylnitrone formation predictably. Step *4* is preferred over Steps *12* and *9* because the disappearance of the nitrone required NADPH, which would not be the case for Step *12*, a hydrolytic reaction. In summary, of five pathways that have been considered feasible, only the pathway involving Steps *1*, *2*, *3*, and *4* survives the elaborated processes of elimination. It is of interest that two of the feasible pathways were deficient in only a single step. When methamphetamine was incubated with microsomes, 2-nitroso-1-phenylpropane complex was formed, thus demonstrating that only Step *5* is missing in the pathway involving Steps *5*, *6*, *7*, and *8*. Step *9* is likewise the only step missing in the pathway involving Steps *1*, *9*, *7*, and *8*.

PB induction of a given monooxygenase activity has been attributed to (a) formation of an increased amount

of one or more of the P-450 isozymes responsible for that activity, (b) the formation of P-450 isozyme(s) with greater substrate selectivity, or both a and b. The current study shows that PB can also induce drug metabolism, in this case that of Bz, by inducing a P-450 isozyme(s) that relieves substrate inhibition.

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