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INHIBITION OF HEPATIC OXIDATIVE XENOBIOTIC METABOLISM BY PIPERONYL BUTOXIDE

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Abstract—The oxidative metabolism of piperonyl butoxide to a metabolite which forms a complex with reduced cytochrome P-450 is not inhibited by cyanide, but is prevented by the mercurial mersalyl. The apparent K_m for this oxidation of piperonyl butoxide is low, being 65 μM. The inhibition of ethylmorphine *N*-demethylation by piperonyl butoxide *in vitro* is proportional to the amount of cytochrome P-450 present as the cytochrome P-450–piperonyl butoxide metabolite complex. Piperonyl butoxide shows mixed inhibition kinetics when present during ethylmorphine *N*-demethylation, i.e. competitive during the formation of the piperonyl butoxide metabolite, and non-competitive once the metabolite is complexed with cytochrome P-450. Differences in the rate of formation of the piperonyl butoxide metabolite–cytochrome P-450 complex in liver microsomes from rats and mice can account for the greater sensitivity of mice to piperonyl butoxide inhibition of drug metabolism. Enhancement of absorption spectra in turbid solutions, such as piperonyl butoxide dispersions, is examined and discussed.

PIPERONYL butoxide is used extensively as an insecticide synergist. With the isolation of metabolites of piperonyl butoxide[1,2] from mammals and insects, the synergistic action has been attributed to it acting as an alternative substrate for the xenobiotic mixed function oxidase system, thus preventing the metabolism of the insecticide itself. Investigation of the inhibition of drug metabolism *in vitro* has shown that piperonyl butoxide competitively inhibited ethylmorphine *N*-demethylation[3] but that the amount of inhibition decreased with long time periods of incubation. The inability to decrease the inhibitory effect, under anaerobic conditions, suggested oxidative metabolism of the inhibitor to a non-inhibitory product. In the situation *in vivo* this would require the continued replenishment of the synergist to maintain a sufficient concentration to be effective. Recent investigations[4] have shown that aerobic incubation of piperonyl butoxide with hepatic microsomes and NADPH produced a metabolite which complexed with reduced cytochrome P-450 and exhibited an isocyanide-like difference spectrum, although the formation of this complex in the absence of oxygen and presence of NADH seen by Philpot and Hodgson[5] would repudiate a metabolite formed by microsomal mixed function oxidation. The apparent stability of this metabolite–cytochrome P-450 complex produced *in vitro*, i.e. the slow removal of the complex by continued incubation under aerobic conditions (unpublished observations), is also indicated by studies *in vivo* where the pretreatment of animals with methylenedioxyphenyl compounds yields microsomes which can exhibit the same spectral characteristics in the reduced minus oxidized difference spectrum.[6,7] The

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possible role of a stable metabolite–cytochrome P-450 complex in the synergistic action of piperonyl butoxide led us to more closely examine the formation of the metabolite–cytochrome P-450 complex and its effect upon other drug hydroxylation reactions. In addition, the metabolism of piperonyl butoxide in a number of species, and with various pretreatments of the animals within the species, was investigated, in an attempt to relate the formation of the metabolite–cytochrome P-450 complex with the reported species and pretreatment differences in the inhibitory effect *in vivo* of piperonyl butoxide on drug metabolism.[8,9]

METHODS

Liver microsomes from phenobarbital-pretreated male rats were prepared as described previously[10] and were used for spectral studies and enzyme incubations at concentrations up to 2·7 mg microsomal protein/ml. Rats pretreated with 3-methylcholanthrene were given daily i.p. injections (in corn oil vehicle) at a dose of 12 mg/kg. Male rabbits and mice were pretreated with phenobarbital at a dosage of 40 and 120 mg/kg (i.p.) respectively. Protein concentrations were determined by the biuret method[11] and ethylmorphine *N*-demethylation was determined by measuring the formaldehyde produced.[12] NADPH-cytochrome *c* reductase activity was determined using the assay of Masters *et al.*[13] Other spectrophotometric determinations were performed with an Aminco–Chance dual wavelength/split beam recording spectrophotometer.

Piperonyl butoxide (96 per cent pure) was a gift from FMC Corporation (Niagara Chemical Division), ethylmorphine hydrochloride was obtained from Merck & Co., Inc., and NADPH was obtained from P. L. Biochemicals, Inc. Isocitric acid, nicotinamide and isocitrate dehydrogenase (IDH) were obtained from Sigma Chemical Co.

Since piperonyl butoxide is very insoluble, it was normally suspended by sonication for 10 sec (at a 3-amp setting) using a Branson Sonifier (with microtip) in the presence of microsomes at a protein concentration of 30 mg/ml. The mixture was maintained cold in an ice bath during sonication. The microsome–piperonyl butoxide suspension which was stable for at least 12 hr was subsequently diluted for determinations of enzyme activity.

Precautionary note. Negligible carbon monoxide was evolved when the piperonyl butoxide was dispersed as described above. However, prolonged sonication of piperonyl butoxide and a variety of other compounds (e.g. safrole) in dilute solutions caused the formation of carbon monoxide, possibly due to local overheating and oxidation of the compounds. This occurred in the absence of microsomes so was not an enzymic formation. Sonication of piperonyl butoxide at a concentration of 2 μl/ml of buffer for 40 sec produced enough carbon monoxide to saturate 5 μM dithionite-reduced cytochrome P-450. The amount of CO formed was dependent on the time of sonication.

RESULTS AND DISCUSSION

The complex of piperonyl butoxide metabolite and reduced cytochrome P-450 differs from an ethyl isocyanide-reduced cytochrome P-450 complex of the same microsomes by the relative intensities of the 455 and 430 nm absorbance maxima. The 455 nm absorbance maximum of the metabolite complex is much more intense than the 430 nm absorbance maximum (at pH 7·4), whereas the converse applies for

the ethyl isocyanide complex. Thus the 455 nm absorbance maximum was used as a measure of the metabolite produced.

Piperonyl butoxide interaction with cytochrome P-450. The apparent K_m (65 μM) for piperonyl butoxide in the oxidation reaction, responsible for the production of the detectable metabolite (see Table 4), was some 5–10 times lower than the apparent K_m for ethylmorphine demethylation (see Tables 2, 3 and 4). The apparent K_m for piperonyl butoxide is higher than the concentration required to half-saturate the type I substrate binding sites of cytochrome P-450 with piperonyl butoxide at this protein concentration. The apparent K_s for type I substrate binding using piperonyl butoxide depends upon the protein concentration employed (Fig. 1). At 2 mg microsomal protein/ml, the apparent K_s is approximately 10 μM, and at lower protein concentrations the apparent K_s is also lowered, indicating that the binding of piperonyl

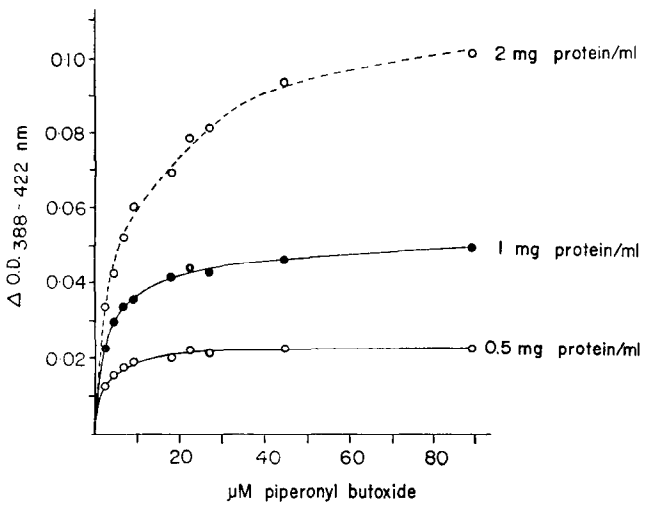

FIG. 1. Titration of the type I binding sites of hepatic microsomes with piperonyl butoxide. Liver microsomes from phenobarbital-pretreated rats were suspended at the three concentrations indicated, in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl and 10 mM $MgCl_2$, and divided into two cuvettes. A baseline of equal light absorbance was established. The difference spectra were then determined after additions (up to 10 μl) of piperonyl butoxide, appropriately diluted in ethanol, to the sample cuvette. Corrections for spectral changes due to the ethanol alone were determined by performing similar experiments in the absence of piperonyl butoxide. The cytochrome P-450 concentration of the microsomes was 2·0 nmoles/mg protein.

butoxide to microsomes does not conform to the Michaelis–Menten concept of substrate–enzyme interaction at these concentrations of enzyme and substrate.

Piperonyl butoxide metabolism by cytochrome P-450. Microsomal oxidative demethylation of ethylmorphine in rats was found to be inhibited in a non-linear fashion by organic mercurials.[10] Both the rate of formation of the 455 nm absorbance maximum associated with the complex of the piperonyl butoxide metabolite with reduced cytochrome P-450 and the final concentration [Δ O.D. $_{455-490\ nm}$ after 10 min] were likewise inhibited in a non-linear fashion, with 50 per cent inhibition occurring at essentially the same mersalyl concentration, 27 nmoles/mg microsomal protein (Fig. 2). This concentration for 50 per cent inhibition would be predicted for any compound being metabolized by the microsomal electron transport system.[10] However, since we are

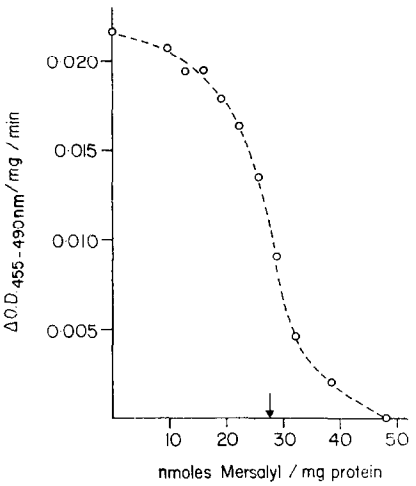

FIG. 2. Effect of mersalyl on the metabolism of piperonyl butoxide. The rate of formation of piperonyl butoxide metabolite-reduced cytochrome P-450 complex (Δ O.D. $_{455-490\ nm}$) upon the addition of 440 μM NADPH to hepatic microsomes (2 mg protein/ml) suspended in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl, 10 mM $MgCl_2$ and 6·28 mM piperonyl butoxide, was determined in the presence of various concentrations of mersalyl.

TABLE 1. EFFECTS OF CYANIDE ON THE OXIDATIVE METABOLISM OF PIPERONYL BUTOXIDE AND OF PIPERONYL BUTOXIDE ON THE MICROSOMAL NADPH-CYTOCHROME *c* REDUCTASE*

Piperonyl butoxide (mM)	KCN (mM)	NADPH-cytochrome *c* reductase (nmoles/mg protein/min)	Δ O.D.$_{455-490}$/ g protein/min
0	0·45	160	
0·07	0·45	150	
0·16	0·45	142	
0·28	0·45	144	
0·44	0·45	145	
0·63	0·00		35·1
0·63	0·17		34·0
0·63	0·33		33·0
0·63	0·45	139	29·4
0·63	0·66		26·6
0·63	1·00		26·2
0·63	1·50		24·4
0·63	2·00		20·8

* Hepatic microsomes from phenobarbital-pretreated rats were used. For the cyanide experiments they were suspended (2 mg protein/ml) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl, 10 mM $MgCl_2$, 0·63 mM piperonyl butoxide and various concentrations of KCN. The rate of change in absorbance at 455 nm, relative to 490 nm, was monitored by dual wavelength spectroscopy after the addition of 0·44 mM NADPH. For the cytochrome *c* reductase experiments, the microsomes were suspended at 0·136 mg protein/ml in 0·05 M potassium phosphate buffer (pH 7·7) containing 0·45 mM KCN, 0·036 mM cytochrome *c* and various concentrations of piperonyl butoxide. The changes in absorbance at 440 mM were monitored after the addition of 0·09 mM NADPH.

detecting a reduced cytochrome P-450–metabolite complex, this inhibition may only reflect the extent of enzymic reduction of cytochrome P-450 in the presence of mersalyl. Piperonyl butoxide itself does not prevent the reduction of cytochrome P-450 by NADPH, and in concentrations of up to 0·6 mM has no significant effect on microsomal NADPH-cytochrome *c* reductase activity (Table 1). Further evidence that the metabolism of piperonyl butoxide proceeds via the cytochrome P-450 system besides the reduced pyridine nucleotide and oxygen requirement, and the inhibition by hexobarbital,[4] is that the formation of the metabolite was only slightly inhibited by cyanide, even at high (mM) concentrations (Table 1).

Piperonyl butoxide inhibition of cytochrome P-450-dependent drug metabolism. With evidence suggesting that piperonyl butoxide is oxidatively metabolized to a compound forming a measurable complex with reduced cytochrome P-450, the effect of this substrate on the metabolism of another mixed function oxidation reaction, ethylmorphine *N*-demethylation, was investigated. The inhibition of *N*-demethylation depended upon both the piperonyl butoxide concentration and the time after the initiation of oxidative metabolism by NADPH (Fig. 3) with the amount of inhibition

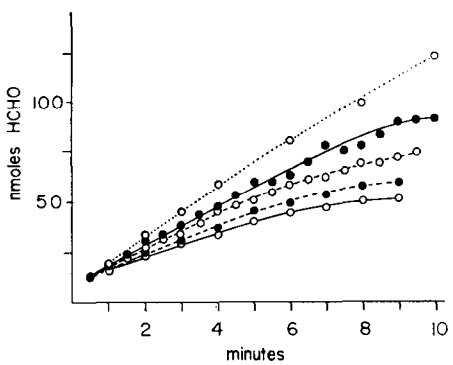

FIG. 3. Effect of piperonyl butoxide concentration on the inhibition of ethylmorphine demethylation. Hepatic microsomes from phenobarbital-pretreated rats were suspended (2·5 mg protein/ml) in 50 mM Tris–chloride buffer (pH 7·4), containing 150 mM KCl, 10 mM $MgCl_2$, 10 mM nicotinamide, 7 mM isocitrate, 0·27 units of IDH/ml, 6·85 mM ethylmorphine and various concentrations of piperonyl butoxide; ○ · · · · ○ = no piperonyl butoxide; ●——● = 0·13 mM; ○– – –○ = 0·25 mM; ●– – –● = 0·38 mM and ○——○ = 0·5 mM. The *N*-dimethylation of ethylmorphine was determined after the addition of 550 μM NADPH.

increasing with time. This is also shown again (solid points) in a subsequent experiment (Fig. 4). However, in this experiment the rate of *N*-demethylation of ethylmorphine in the presence of piperonyl butoxide was compared with that observed upon the addition of ethylmorphine to microsomes which had been preincubated in the presence of NADPH and piperonyl butoxide. The *N*-demethylation rate in microsomes which had been preincubated with NADPH and piperonyl butoxide was very similar to the rate seen after 5 min without preincubation. This suggests that the increased inhibition with time, observed when the reaction was initiated with NADPH in the presence of both substrates, ethylmorphine and piperonyl butoxide, was due to the formation of a non-competitive inhibitor with time. Preincubation with piperonyl butoxide and NADPH forms this non-competitive inhibitor, and the rate then observed is the residual piperonyl butoxide insensitive ethylmorphine *N*-demethylation rate (about

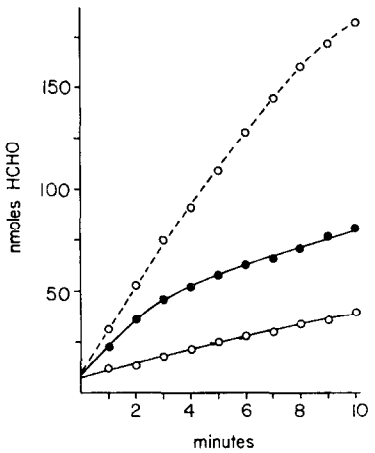

FIG. 4. Similarity of the initial ethylmorphine demethylation rate of microsomes preincubated with NADPH and piperonyl butoxide and the rate observed after 5 min preincubation with ethylmorphine as well as piperonyl butoxide and NADPH. Hepatic microsomes were suspended in the medium described in Fig. 3. The ethylmorphine demethylation rate observed upon the addition of 550 μM NADPH to the suspension was determined in the presence (●——●) and absence (○- - -○) of 0·4 mM piperonyl butoxide. Another sample was preincubated for 5 min with only piperonyl butoxide and NADPH present. The demethylation rate was then determined upon the addition of ethylmorphine (○——○).

TABLE 2. INHIBITORY EFFECT OF PIPERONYL BUTOXIDE ON ETHYLMORPHINE *N*-DEMETHYLATION

Piperonyl butoxide (mM)	K_m (Control %)	V_{max} (Control %)
0[4]	100	100
0·125[5]	890	94
0·16[3]	950	136
0·23[1]	1550	110
0·39[3]	1165	111
0·47[1]	1525	89
0·62[1]	1525	84
0·79[1]	785	64

* Liver microsomes from phenobarbital-pretreated rats were incubated (2 mg protein/ml) for 2 min at 25° in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl, 10 mM $MgCl_2$, 10 mM nicotinamide, 7 mM isocitrate, 0·27 units of IDH/ml and various concentrations of ethylmorphine and piperonyl butoxide. The kinetic parameters of ethylmorphine demethylation were then determined after the addition of 0·137 mM NADPH to the incubation flask. Superscripts in the table denote the number of microsomal preparations examined. The mean K_m for ethylmorphine demethylation in these experiments was 0·35 mM and the V_{max} was 8·0 nmoles HCHO/mg protein/min.

15 per cent of the maximum). Examination of the inhibition kinetics of ethylmorphine *N*-demethylation with microsomes showed that piperonyl butoxide was not inhibiting in a simple manner (Table 2). Low concentrations (< 0·13 mM) of piperonyl butoxide only slightly altered the maximum velocity but markedly increased the apparent K_m (about 10-fold), suggesting competitive inhibition. However, with increasing concentrations of piperonyl butoxide, the K_m was further increased and the apparent V_{max} first increased and then decreased.

Comparison of kinetic parameters of three preparations of rat liver microsomes, in which the apparent K_m and V_{max} of ethylmorphine *N*-demethylation were determined (a) in the absence of piperonyl butoxide, (b) in the presence of piperonyl butoxide and (c) after preincubation of the microsomes with NADPH and piperonyl butoxide for 5 min (Table 3), showed that preincubation produced mainly changes in V_{max}, and not

TABLE 3. EFFECT OF THE PREINCUBATION OF MICROSOMES WITH NADPH AND PIPERONYL BUTOXIDE ON THE INHIBITORY ACTION OF PIPERONYL BUTOXIDE ON ETHYLMORPHINE DEMETHYLATION*

	No inhibitor		+ Inhibitor		+ Inhibitor and preincubation	
Expt. No.	K_m (mM)	V_{max} (nmoles HCHO/mg protein/min)	K_m (mM)	V_{max} (nmoles HCHO/mg protein/min)	K_m (mM)	V_{max} (nmoles HCHO/mg protein/min)
1	0·39	9·5	3·3	11·3	2·4	4·1
2	0·42	8·2	2·7	9·2	2·9	3·5
3	0·50	6·9	3·2	7·5	3·3	3·6

* Hepatic microsomes from phenobarbital-pretreated rats were suspended in the medium described in Table 2 except that a piperonyl butoxide concentration of 0·126 mM was used in each experiment. The kinetic parameters of ethylmorphine *N*-demethylation were determined from double reciprocal plots of the initial rates of ethylmorphine *N*-demethylation in the absence and presence of piperonyl butoxide, after the addition of 0·275 mM NADPH, and also after the preincubation of microsomes for 5 min with piperonyl butoxide and NADPH, upon ethylmorphine addition.

K_m, over and above that produced when the piperonyl butoxide was added at the same time as ethylmorphine. These results suggest, therefore, that piperonyl butoxide is a competitive inhibitor of ethylmorphine demethylation, since it is metabolized by the same enzyme system, and that the metabolic product formed is a non-competitive inhibitor. It could be that the non-competitive inhibition is due to the binding of the metabolite to the cytochrome P-450, preventing its participation in further mixed function oxidations. If this were the case then the degree of inhibition of ethylmorphine *N*-demethylation after preincubation of the microsomes with piperonyl butoxide and NADPH would be proportional to the amount of cytochrome P-450 bound to the metabolite.

Piperonyl butoxide metabolite inhibition of cytochrome P-450-dependent drug metabolism. As shown in Fig. 5, the preincubation of NADPH-fortified microsomes with limiting concentrations of piperonyl butoxide produced various amounts of the 455 nm absorbance maximum, representative of the metabolite–reduced cytochrome P-450 complex. The subsequent determination of ethylmorphine *N*-demethylase activity of

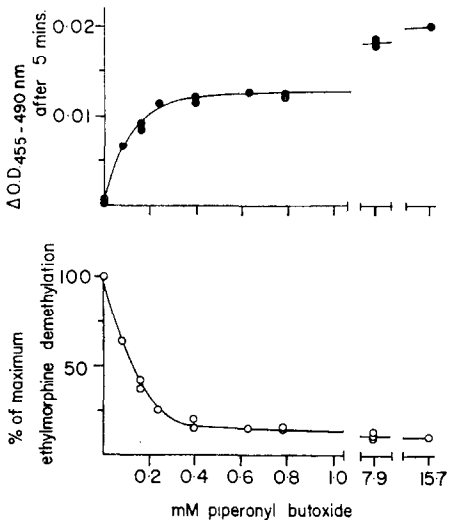

FIG. 5. Correlation between the piperonyl butoxide metabolite–cytochrome P-450 complex and the inhibition of ethylmorphine *N*-demethylation. Hepatic microsomes from phenobarbital-pretreated rats were suspended in the medium described in Fig. 2 with the omission of ethylmorphine, at a concentration of 2·7 mg protein/ml in the presence of various concentrations of piperonyl butoxide. NADPH (550 μM) was then added and after 5 min the absorption spectrum due to the piperonyl butoxide metabolite-reduced cytochrome P-450 complex was determined (Δ O.D.$_{455-490\ nm}$). Ethylmorphine (6·85 mM) was then added and the subsequent initial rate of ethylmorphine *N*-demethylation determined. The ethylmorphine *N*-demethylase activity in the absence of piperonyl butoxide was 6·8 nmoles HCHO/mg protein/min.

these preincubated microsomes showed it to be inhibited, and the degree of inhibition at piperonyl butoxide concentrations less than 1 mM was proportional to the amount of the metabolite–cytochrome P-450 complex. These reactions were performed in special vessels containing side-arm cuvettes[14] enabling the 455 nm absorption spectrum and ethylmorphine *N*-demethylation to be determined simultaneously in the same vessel. These results strengthen the hypothesis that piperonyl butoxide is metabolized to a compound which binds to reduced cytochrome P-450 and prevents its enzymic function in the oxidative metabolism of other foreign compounds. The stability of the reduced cytochrome P-450–metabolite complex may be the reason for its effective application *in vivo* as an insecticide synergist.

The apparent discrepancy (Fig. 5) between an increase in the 455 nm absorbance maximum formed with piperonyl butoxide concentrations above 1 mM and no change in the inhibition of ethylmorphine *N*-demethylation is presumably due to the phenomenon of spectral enhancement by very turbid solutions and this will be discussed later. This is due to piperonyl butoxide at these concentrations altering the turbidity characteristics of the suspensions upon sonciation.

Piperonyl butoxide metabolism and inhibition of drug metabolism—variations between species and animal pretreatments. If the hypothesis that the metabolism of piperonyl butoxide to a metabolite, which binds to and inactivates cytochrome P-450, is correct, then we should be able to account for the observations[8] that mice are much more susceptible to piperonyl butoxide inhibition of drug metabolism *in vivo* than rats. The kinetic properties of the oxidative demethylation of ethylmorphine and

TABLE 4. KINETIC PARAMETERS OF PIPERONYL BUTOXIDE AND ETHYLMORPHINE METABOLISM AND THEIR MUTUAL INHIBITION IN VARIOUS PRETREATED ANIMAL SPECIES*

Animal	Pretreatment and no. of preparations†	Cytochrome P-450 (nmoles/mg protein)	Δ O.D.$_{455-490}$ (per g protein/min)	Δ O.D.$_{455-490}$ (per μmole P-450/min)	Δ O.D.$_{455-490}$ K_m (μM)	Ethylmorphine demethylation (nmoles/mg/min)	Ethylmorphine demethylation (nmoles/nmole P-450/min)	Ethylmorphine demethylation K_m (mM)	K_i (μM piperonyl butoxide)
Rat	-(6)	0·78	7	9	63[3]	2·19	2·87	0·32[3]	19[3]
	3 MC (4)	1·49	11	7		2·18	1·47	0·65	62
	Pb (5)	2·10[8]	32	15	65	9·00[6]	4·41	0·26	15
Mouse	- (4)	1·23	16	13	44[2]	4·22	3·47	0·61[3]	6[3]
	Pb (4)	1·92	69	36	53[3]	9·25	4·96	0·50	5
Rabbit	Pb (3)	2·42	11	5	140	2·01	0·81	2·08[3]	137

* Ethylmorphine demethylation by microsomal preparations was determined in a medium described in Table 2, in the presence of 6·85 mM ethylmorphine. Formation of the piperonyl butoxide metabolite-reduced cytochrome P-450 complex was determined at 25° in the presence of 0·63 mM piperonyl butoxide, after the addition of 0·66 mM NADPH to microsomes suspended to 2 mg protein/ml in 50 mM Tris–chloride buffer (pH 7·4) containing 150 mM KCl and 10 mM $MgCl_2$. The K_m and K_i values were determined from double reciprocal plots of ethylmorphine *N*-demethylation reactions performed in various non-saturating concentrations of ethylmorphine, in the presence of between 0·03 and 0·3 mM piperonyl butoxide.

† Superscripts in main table when the number of preparations examined differs from that stated.

the metabolism of piperonyl butoxide (to a cytochrome P-450–metabolite complex) in these species, and with various pretreatments of these animals, was examined (Table 4). These show that the K_i for piperonyl butoxide on ethylmorphine *N*-demethylation in mice is much lower than in rats. When examining the metabolism of piperonyl butoxide in these two species, mice showed both a greater affinity for the substrate and, more importantly, up to a 2-fold increase in the rate of metabolism as compared to rats, suggesting that the metabolism of piperonyl butoxide is of prime importance in its inhibitory effect. The differences between rats and mice apply both to normal and phenobarbital-pretreated animals. Phenobarbital pretreatment does not significantly alter the K_m for either ethylmorphine or piperonyl butoxide in these animals, but markedly increases the rate of metabolism of both these substrates, either expressed per milligram of protein or as a turnover number. 3-Methylcholanthrene pretreatment of rats, however, increases the cytochrome P-450 concentration in the microsomes but decreases the rate of piperonyl butoxide and ethylmorphine metabolism when expressed as a turnover number, adding further to the wealth of evidence in the literature that the cytochrome P-450 induced by 3-methylcholanthrene has different substrate specificity, especially since the apparent K_m for ethylmorphine demethylation in these experiments is also markedly increased. The turnover numbers in microsomes from 3-methylcholanthrene-pretreated rats, were calculated, for convenience (pending general agreement on a true value for the total population of cytochrome P-450 in these microsomes) using an extinction coefficient of 91 mM^{-1} cm^{-1}. The higher K_i for piperonyl butoxide inhibition of ethylmorphine metabolism in 3-methylcholanthrene-pretreated rats can explain the results shown by Anders[9] where drug metabolism in microsomes from these animals was less susceptible to piperonyl butoxide inhibition. That drug metabolism in phenobarbital-pretreated rats was also less inhibited by piperonyl butoxide is at variance with the similarities in the K_i for normal and phenobarbital-pretreated animals reported here. The results obtained here with phenobarbital-pretreated rabbits (Table 4) demonstrate both a much higher K_m and slower rate of metabolism of both substrates, ethylmorphine and piperonyl butoxide, and a correspondingly high K_i for piperonyl butoxide inhibition of ethylmorphine *N*-demethylation in comparison to similarly treated rats. From the similarity in K_i for control and phenobarbital-pretreated rats, one could predict that rabbits would be very resistant to inhibitory effects *in vivo* of piperonyl butoxide on drug metabolism.

Enhancement of chromophore absorbance by turbid solutions. The spectral enhancement by turbid solutions, alluded to earlier, will be discussed here, since much spectrophotometric work in drug metabolism is performed on turbid suspensions, and with relatively insoluble substrates. The phenomenon discussed here may limit or at least caution the use of sonication in the dispersion of immiscible liquid substrates. As shown in Fig. 6, the 455 nm absorbance maximum formed from piperonyl butoxide, at piperonyl butoxide concentrations between 30 and 150 times the apparent K_m (0·67 μl piperonyl butoxide/ml is equivalent to 2·07 mM), apparently increases with increasing piperonyl butoxide concentration. That this is probably not due to increased formation of the piperonyl butoxide metabolite–cytochrome P-450 complex is seen in Fig. 7. Here the magnitude of the dithionite–reduced cytochrome P-450–CO complex (absorbance maximum 450 nm) was determined at various protein and piperonyl butoxide concentrations. The absorbance maximum at any given protein concentration is increased with increasing concentrations of piperonyl butoxide. However, the

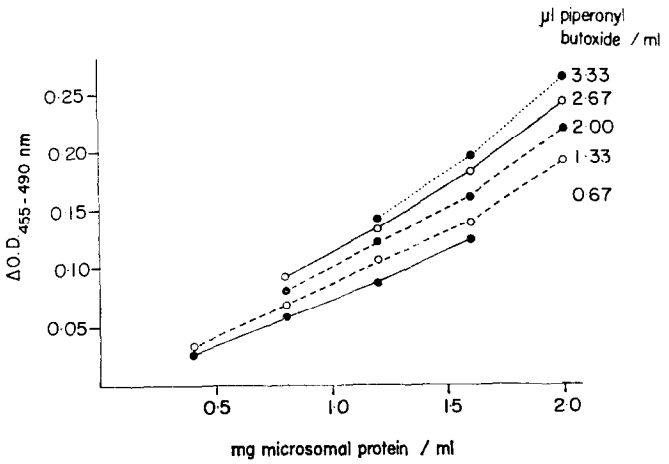

FIG. 6. Enhancement of the piperonyl butoxide metabolite–cytochrome P-450 absorption spectrum by turbid solutions. Liver microsomes from phenobarbital-pretreated rats were suspended at the protein concentrations shown (0·4–2·0 mg/ml) in 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl, 10 mM $MgCl_2$ and various concentrations of piperonyl butoxide. The mixture was divided into two cuvettes and a baseline of equal light absorbance determined. NADPH (940 µM) was then added and 10 min later the absorption spectra due to the oxidative metabolism of piperonyl butoxide were determined.

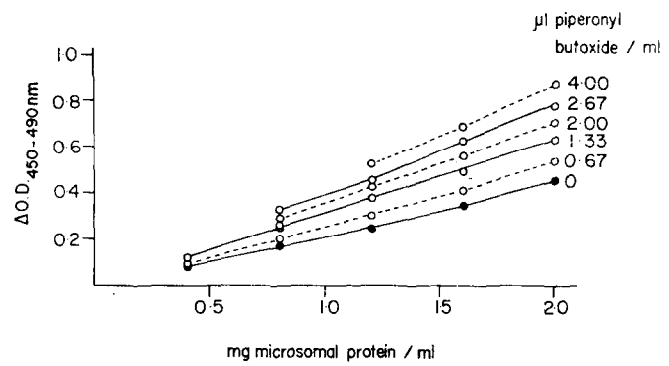

FIG. 7. Enhancement of the reduced cytochrome P-450–CO absorption spectrum by turbid solutions of piperonyl butoxide. Liver microsomes from phenobarbital-pretreated rats were suspended at various protein concentrations (0·4–2·0 mg protein/ml) in 50 mM Tris–chloride buffer (pH 7·4) containing 150 mM KCl, 10 mM $MgCl_2$ and various concentrations of piperonyl butoxide. A few crystals of sodium dithionite were then added and the suspension was divided into two cuvettes. A baseline of equal light absorbance was established. The contents of the sample cuvette were then gassed with CO for 2 min and the subsequent absorption spectrum was determined.

increase in absorbance is not linearly related to the piperonyl butoxide concentration (Fig. 8). Whether this is a true characteristic of the turbidity, or whether it is our inability to fully disperse the higher concentrations of piperonyl butoxide, is not known at present. That this phenomenon of turbidity enhancement is not solely a property of insoluble methylenedioxphenyl compounds is shown in Table 5 where dispersions of other compounds such as corn oil and Antifoam A emulsion were also found to enhance the cytochrome P-450–CO absorbance maximum. Although this

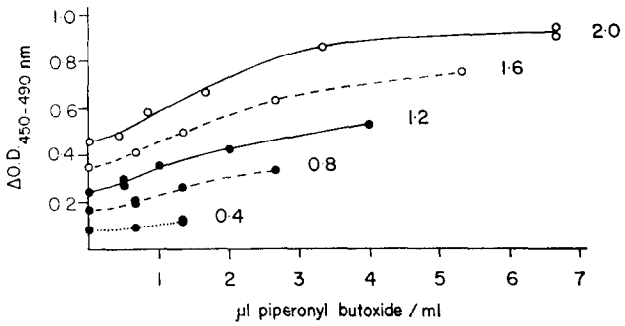

FIG. 8. Dependence of the spectral enhancement of the reduced cytochrome P-450–CO absorption spectrum upon the turbidity of the medium. Hepatic microsomes were treated as in Fig. 7, and the absorption of the reduced cytochrome P-450–CO complex was plotted as a function of the amount of piperonyl butoxide sonicated into the medium.

TABLE 5. ENHANCEMENT OF ABSORPTION SPECTRA BY COMPOUNDS CAPABLE OF FORMING EMULSIONS*

Compound	Concn. (μl/ml)	Cytochrome P-450 (Δ O.D. $_{450-490}$/mg protein)
None		0·177
Safrole	1·12	0·195
Isosafrole	1·12	0·227
Piperonyl butoxide	2·20	0·314
None		0·161
Antifoam A emulsion	1·33	0·190
Antifoam A emulsion	2·67	0·201
Antifoam A emulsion	6·67	0·219
None		0·204
Piperonyl butoxide	13·3	0·287
Trilinolein	13·3	0·287
Corn oil	13·1	0·331

* Hepatic microsomes from phenobarbital-pretreated rats were suspended at 2 mg protein/ml in emulsions of 50 mM Tris–chloride buffer, pH 7·4, containing 150 mM KCl, 10 mM $MgCl_2$ and various immiscible liquids. After the addition of a few grains of sodium dithionite to the suspension, the mixture was divided between two cuvettes, and a baseline of equal light absorbance determined. The contents of the sample cuvette were then gassed with CO and the difference spectrum was recorded.

limits the quantitative measurement of chromophores in turbid solutions, this enhancement can be usefully applied to intensify minor absorbance maxima and aid their identification or even establish their existence.

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