MOLECULAR PHARMACOLOGY, 8, 204–214

The Effect of Piperonyl Butoxide Concentration on the Formation of Cytochrome P-450 Difference Spectra in Hepatic Microsomes From Mice

RICHARD M. PHILPOT AND ERNEST HODGSON

Department of Entomology, North Carolina State University, Raleigh, North Carolina 27607

(Received September 14, 1971)

SUMMARY

A direct relationship is observed between the formation of the piperonyl butoxide–reduced cytochrome P-450 double Soret difference spectrum (type III) and inhibition of the formation of the carbon monoxide–cytochrome P-450 difference spectrum. This relationship appears to involve a single binding site.

The type III piperonyl butoxide–cytochrome P-450 interaction exerts a similar effect on the formation of the carbon monoxide–, pyridine–, *n*-octylamine–, and ethyl isocyanide–cytochrome P-450 difference spectra. Inhibition of the formation of the spectrum produced by (+)-benzphetamine is greater than that observed with the other ligands.

The relationship between the change in absorbance and the change in absorbance divided by substrate concentration is linear for the formation of the type III spectrum produced by piperonyl butoxide, inhibition by the latter of the formation of the spectrum produced by carbon monoxide, and formation of the pyridine type II substrate spectrum at low pyridine concentrations. At high pyridine concentrations and with (+)-benzphetamine this relationship is curvilinear.

The piperonyl butoxide type III interaction does not inhibit the formation of the pyridine type II substrate spectrum at low pyridine concentrations. At high pyridine concentrations the inhibition observed is similar to the inhibition of the (+)-benzphetamine type I substrate spectrum.

Our observations suggest that piperonyl butoxide binds to one form of cytochrome P-450 at a single site and that an additional form of the cytochrome is present in noninduced hepatic microsomes from mice, which does not bind type I substrates or form a type III interaction with piperonyl butoxide.

INTRODUCTION

Methylenedioxyphenyl compounds are employed as synergists for a number of pesticides. It is now generally accepted that their ability to augment pesticide effectiveness is based on an interaction with the microsomal mixed-function oxidase system (1). There is evidence that MDP[1] compounds are metabolized in the mixed-function oxidase system and that they derive their inhibitory properties by acting as alternate substrates (2–5). This contention is sup-

This is paper 3557 of the Journal Series of the North Carolina State University Agricultural Experiment Station, Raleigh, N. C. The work was supported in part by Grants ES-00044 and ES-00083 from the United States Public Health Service.

[1] The abbreviations used are: MDP, methylenedioxyphenyl; PBO, piperonyl butoxide.

ported by reports that the metabolism of various compounds is competitively inhibited by MDP compounds (6–11). However, other forms of inhibition by MDP compounds—"partly competitive," "partly progressive," "curvilinear," and noncompetitive—have also been reported (7, 11, 9). These results support the conclusion reached by Kuwatsuka (12) that mixed-function oxidase inhibition by MDP compounds involves factors in addition to competitive reactions.

Evidence that MDP compounds interact with cytochrome P-450, the terminal component of the mixed-function oxidase system, in a manner not consistent with a typical enzyme-substrate relationship was first provided by Perry and Buckner (13) from housefly preparations and by Matthews *et al.* (14) from mouse liver preparations. Both studies demonstrated that administration of MDP compounds *in vivo* reduces the apparent level of cytochrome P-450 found in microsomes prepared from treated animals.

Recently we have reported evidence that the attenuation of cytochrome P-450 in mice by piperonyl butoxide, an MDP compound, results from an interaction which prevents CO binding to a portion of the cytochrome (15). In addition, partial inhibition of the formation of the pyridine (type II) substrate difference spectrum and complete inhibition of the hexobarbital (type I) substrate difference spectrum were noted with treatment of microsomes *in vitro* with PBO in the presence of NADPH (15). The inhibition of formation of the type I and II substrate spectra by treatment with PBO *in vivo* (16) was found to correspond to the inhibition of aniline (type II) and hexobarbital hydroxylase activity reported by Škrinjorić-Špoljar *et al.* (17).

The PBO–cytochrome P-450 interaction results in the production of a double Soret difference spectrum similar to that formed by ethyl isocyanide. This spectrum has been demonstrated in mice by treatment with PBO both *in vivo* (16) and *in vitro* (15).

The apparent inability of added ligands to displace PBO bound in the type III[2] configuration makes it possible to study PBO inhibition of the formation of cytochrome P-450 difference spectra (15). In the present communication we have investigated the effect of PBO concentration *in vitro* on the formation of the PBO type III spectrum and the inhibition of several other cytochrome P-450 difference spectra in an attempt to gain further understanding of the mode of action of MDP compounds and the nature of cytochrome P-450 difference spectra and binding sites.

METHODS AND MATERIALS

Six-week-old, male mice from the North Carolina Department of Health strain, an inbred colony maintained since 1910, were used for all experiments. The mice were given free access to Purina laboratory chow and water.

Mice were killed by decapitation. The livers were removed, rinsed in distilled water, and diced. After thorough washing in 1.15% KCl, the liver tissue was homogenized in 0.05 M Tris–1.15% KCl, pH 7.5 (4.5 ml/liver). The homogenate was centrifuged at 10,000 × *g* for 15 min, and the resulting supernatant fluid was filtered through glass wool and centrifuged at 105,000 × *g* for 1 hr to sediment the microsomal fraction. Microsomes were resuspended in Tris-KCl to give a protein concentration of approximately 20 mg/ml. All operations were carried out at 4°.

All experiments were conducted with microsomal suspensions incubated at room temperature for 15 min. The PBO concentrations indicated were obtained by evaporation of acetone solutions of PBO in the incubation vessels. For determinations involving the PBO type III interaction, NADPH (0.5 mM) was included in both sample and control incubations. In all experiments involving the use of NADPH, the concentration present in both cuvettes was sufficient to reduce cytochrome b_5 fully. NADPH was omitted from the suspensions used for the measurements of the PBO type I spectrum and from untreated controls.

The PBO type I difference spectrum was

[2] We have previously suggested that cytochrome P-450 difference spectra that exhibit two Soret peaks in pH-dependent equilibrium be termed type III (15, 16). This nomenclature is used in the present text.

obtained by comparing microsomal suspensions containing PBO with untreated suspensions. Baselines were obtained by the addition of sodium dithionite to the sample and reference cuvettes after the difference spectrum had been recorded. Recordings of equal light absorbance obtained in this manner agreed with those recorded prior to incubation. The magnitude of the PBO type I difference spectrum was ascertained by the difference in absorbance between the peak formed at 385 nm and the trough at 420 nm.

To obtain the PBO type III difference spectrum, NADPH was added to the incubation mixtures and PBO-treated microsomal suspensions were compared with untreated suspensions. Difference spectra were recorded after reduction with dithionite, using baselines recorded prior to incubation. The differences in absorbance between either 427 and 490 nm or 455 and 490 nm were used to determine the magnitude of the PBO type III spectrum.

Carbon monoxide–cytochrome P-450 difference spectra were obtained from dithionite-reduced microsomes by the method of Omura and Sato (18). The difference in absorbance between 450 and 490 nm was recorded from microsomal suspensions incubated with various concentrations of PBO in the presence of NADPH (0.5 mM). Inhibition of CO binding was calculated from the difference between the above values and those obtained from untreated microsomes.

For the measurement of type I and II substrate difference spectra, incubated microsomal suspensions were divided evenly between sample and reference cuvettes and placed in the spectrophotometer. After a baseline had been recorded for (+)-benzphetamine (type I) or pyridine (type II), solutions were added to the sample cuvette in volumes of 1–2 μl, and an equal amount of buffer was added to the reference cuvette. After each addition the difference spectrum was recorded. In no case did the total volume added exceed 10 μl. The magnitude of the pyridine spectrum was calculated from the difference in absorbance between the peak formed at 424 nm and the trough at 390–405 nm. The degree of (+)-benzphetamine binding was determined by the difference in absorbance between 490 nm and the trough formed at 420 nm.

Ethyl isocyanide and *n*-octylamine difference spectra were obtained by the methods of Sladek and Mannering (19) and Jefcoate *et al.* (20), respectively.

For measurements requiring the maintenance of a specific pH (type III, CO inhibition, and ethyl isocyanide), 0.5 M phosphate buffer was used. For all other experiments microsomes were suspended in 0.05 M Tris–1.15% KCl buffer (pH 7.5).

All spectra were recorded on a Beckman Acta V spectrophotometer equipped with a turbid sample accessory. Difference spectra data are reported using the kinetic plots of Hofstee (21). This was accomplished by substitution of change in absorbance (ΔA) for v and of $\Delta A/S$ for v/S, where S is the concentration of the compound responsible for formation of the cytochrome P-450 difference spectrum in question. This method was used for similar data by Jefcoate *et al.* (20) and Hewick and Fouts (22). K_s values, determined from the slopes of the Hofstee plots, refer to the concentration of substrate required to produce half-maximal spectral change. This constant has been described as a "spectral dissociation constant" by Schenkman *et al.* (23). The term K_i is used in an analogous manner and indicates the substrate concentration required for half-maximal inhibition of spectrum formation. Straight-line expressions shown for the PBO type III and CO inhibition data were obtained by the method of least squares. All experiments were repeated two to six times, with similar results each time.

Ethyl isocyanide was synthesized by the method of Jackson and McKussick (24). (+)-Benzphetamine was a generous gift from Dr. P. W. O'Connell of the Upjohn company. All other chemicals were purchased from commerical sources.

RESULTS

Piperonyl butoxide–cytochrome P-450 interactions. A curvilinear relationship was found when change in absorbance is plotted against $\Delta A/S$ for the formation of the PBO type I difference spectrum (Fig. 1). This relationship, obtained with microsomes containing

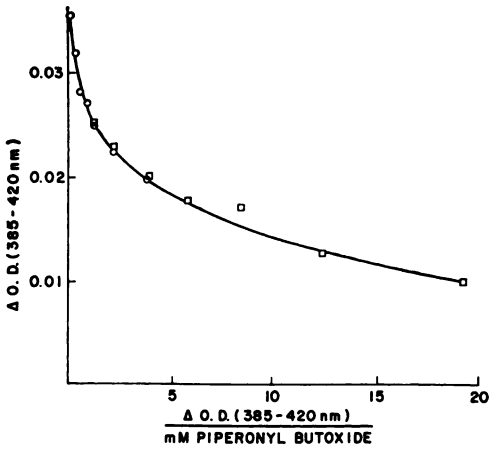

FIG. 1. *Formation of cytochrome P-450–piperonyl butoxide type I substrate difference spectrum*

The different symbols represent two overlapping experiments. The level of cytochrome P-450 in each case was 0.10 ΔA unit as determined by the carbon monoxide difference spectrum (450 − 490 nm).

0.10 ΔA unit of cytochrome P-450 as determined by the CO difference spectrum, was essentially the same for PBO binding with microsomes containing 0.08 and 0.13 ΔA unit of cytochrome P-450. The PBO concentration range employed was 0.5–200 μM.

In contrast, the formation of the PBO type III spectrum at pH 7.0 and 7.5 was linear for both the 427 and 455 nm peaks when ΔA was plotted against $\Delta A/S$ (Figs. 2 and 3). The K_s values of PBO were different for the two peaks (Table 1). However, when the magnitude of the PBO type III spectrum is calculated from the sum of the 427 and 455 nm peaks (see DISCUSSION), both the K_s and maximum ΔA values are nearly identical at each pH (Figs. 2 and 3 and Table 1).

Piperonyl butoxide type III inhibition of CO–cytochrome P-450 binding. Figure 4 shows the linear relationship obtained for the inhibition of CO binding by PBO when ΔA was plotted against $\Delta A/S$. The K_i and maximum ΔA inhibition values are similar at pH 7.0, 7.5, and 8.0. Table 1 shows that the K_i values coincide with the K_s values for the formation of the PBO type III spectrum calculated from the sum of the 427 and 455 nm peaks. CO values were essentially the same with untreated controls and with NADPH-treated or PBO-treated microsomes. Inhibition was observed only with microsomes treated with both PBO and NADPH. At pH 8.0 the type III spectrum was not stable with respect to the baselines established prior to incubation, and quanti-

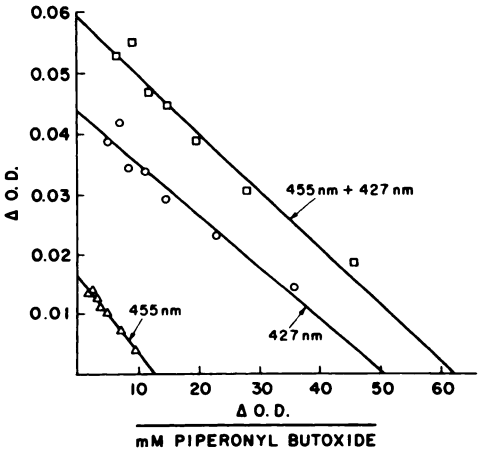

FIG. 2. *Formation of cytochrome P-450–piperonyl butoxide type III difference spectrum at pH 7.0*

The level of cytochrome P-450 was 0.10 ΔA unit as determined by the carbon monoxide difference spectrum (450 − 490 nm).

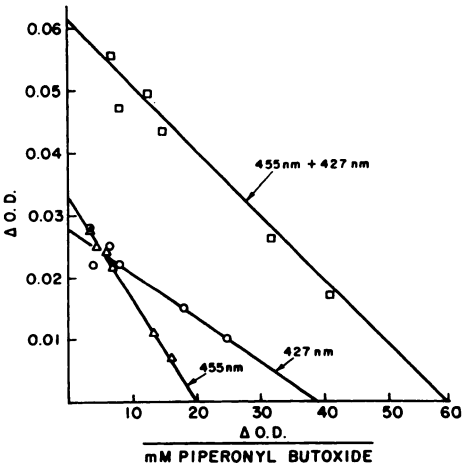

FIG. 3. *Formation of cytochrome P-450–piperonyl butoxide type III difference spectrum at pH 7.5*

The level of cytochrome P-450 was 0.10 ΔA unit as determined by the carbon monoxide difference spectrum (450 − 490 nm).

TABLE 1

Spectral dissociation and inhibition constants of piperonyl butoxide for formation of piperonyl butoxide (type III)- and inhibition of carbon monoxide-cytochrome P-450 difference spectra, and maximum ΔA values for formation and inhibition

Determination	pH	K_s	K_i	Maximum ΔA	
				Formation[a]	Inhibition[b]
		M	*M*		
455 nm peak, type III	7.0	1.3×10^{-5}		0.016	
	7.5	1.7×10^{-5}		0.033	
427 nm peak, type III	7.0	8.8×10^{-6}		0.044	
	7.5	7.2×10^{-6}		0.028	
455 nm + 427 nm	7.0	9.7×10^{-6}		0.060	
	7.5	1.0×10^{-5}		0.061	
Inhibition of CO spectrum	7.0		1.0×10^{-5}		0.050
	7.5		1.1×10^{-5}		0.047
	8.0		1.1×10^{-5}		0.047

[a] Cytochrome P-450 control levels equal to 0.10 ΔA unit as determined by the carbon monoxide difference spectrum.

[b] Cytochrome P-450 control levels equal to 0.12 ΔA unit as determined by the carbon monoxide difference spectrum.

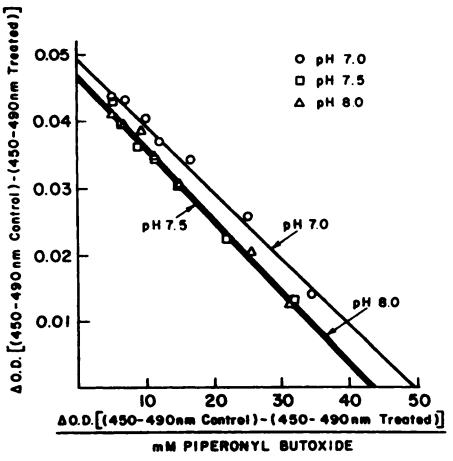

FIG. 4. *Inhibition of cytochrome P-450-carbon monoxide difference spectrum by piperonyl butoxide type III binding at pH 7.0, 7.5, and 8.0*

Inhibition was calculated as the difference in absorbance between the carbon monoxide difference spectrum from untreated and treated microsomes. The level of cytochrome P-450 in the untreated microsomes was 0.12 ΔA unit as determined by the carbon monoxide difference spectrum (450 — 490 nm).

tation of the 427 and 455 nm peaks was not possible.

Piperonyl butoxide inhibition of formation of types I and II substrate difference spectra. The effect of the PBO type III interaction on the pyridine–cytochrome P-450 type II substrate difference spectrum is shown in Figs. 5 and 6. A Hofstee plot of pyridine binding with untreated microsomes was curvilinear over a large concentration range (11.7 μM–11.7 mM), but for concentrations below approximately 5 μM a linear relationship was achieved. When microsomes were incubated with increasing concentrations of PBO in the presence of NADPH, inhibition of pyridine binding occurred only in the nonlinear portion of the curve. The effect of NADPH alone was to lower slightly the upper part of the curve because of a reduction in the magnitude of the trough formed by the pyridine spectrum. This reduction was reflected in a gradual shift of the trough from 390 to 405 nm at pyridine concentrations greater than 10 μM. The shift in the trough increased with increasing pyridine concentration and was accompanied by a shift in the isosbestic point from 413 to 417 nm. No shift in the peak absorbance at 424 nm was noted. In all cases the magnitude of the trough was determined from the point

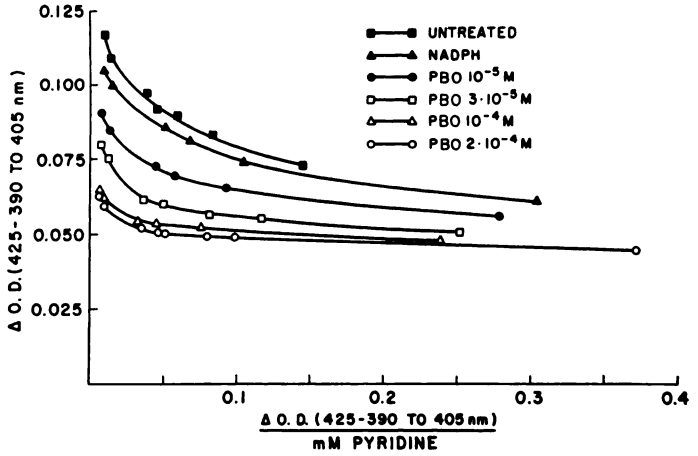

FIG. 5. *Effect of cytochrome P-450–piperonyl butoxide type III interaction on formation of cytochrome P-450–pyridine type II substrate difference spectrum at high pyridine concentrations (0.2–20 mM)*

The level of cytochrome P-450 was 0.20 ΔA unit as determined by the carbon monoxide difference spectrum (450 — 490 nm).

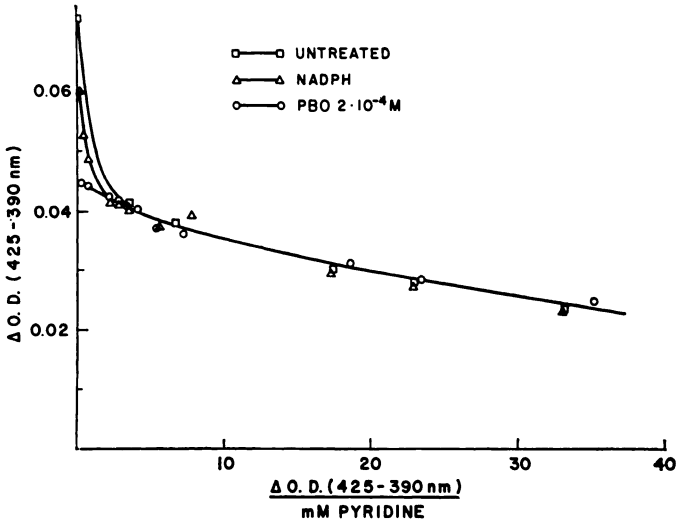

FIG. 6. *Effect of cytochrome P-450–piperonyl butoxide type III interaction on formation of cytochrome P-450–pyridine type II substrate difference spectrum at low pyridine concentrations (0.7–200 μM)*

The level of cytochrome P-450 was 0.20 ΔA unit as determined by the carbon monoxide difference spectrum (450 — 490 nm).

of least absorbance. PBO alone (not shown in the figures) increased the apparent magnitude of the pyridine type II spectrum. This was most probably due to displacement of the PBO by pyridine, resulting in the formation of a type II complex in the sample cuvette while the reference still contained a type I complex. This would produce an additive effect. In fact, shifts in the pyridine peak and trough to lower wavelengths were noted. This is compatible with the addition of peaks occurring at 424 nm (pyridine, sample cuvette) and 420 nm (PBO, reference cuvette) and troughs occurring at 390 nm (pyridine, sample cuvette) and 385 nm (PBO, reference cuvette).

Additions of (+)-benzphetamine to microsomal suspensions containing NADPH led

to an increase in NADPH oxidation in the sample cuvette as compared to the reference, probably because of the metabolism of a small amount of (+)-benzphetamine. This was apparent even in the short period of time necessary to complete each experiment (5–7 min). This difference, which formed a trough at 340 nm, was of sufficient magnitude to interfere with the peak formed at 385 nm by (+)-benzaphetamine. However, the isosbestic point (404 nm) and trough (420 nm) were not affected, and the magnitude of the trough was used as a measure of the (+)-benzphetamine spectrum. The difference in NADPH oxidation between the sample and reference suspensions is detailed in Table 2. Figure 7 shows that (+)-benzphetamine binding in the presence of NADPH was slightly reduced. As noted, this was probably due to oxidative metabolism of a small amount of the benzphetamine. With increasing concentrations of PBO, (+)-benzphetamine binding was greatly inhibited (Fig. 7). This could not have been due to increased metabolism, since increasing concentrations of PBO correspond to decreasing NADPH oxidation (Table 2).

TABLE 2

NADPH oxidized during (+)-benzphetamine binding experiment, sample minus reference

The level of cytochrome P-450 in each case was 0.2 ΔA unit as determined by the carbon monoxide difference spectrum (450 − 490 nm).

Conditions	ΔA (340 nm)	NADPH oxidized
		μmole/5 min
NADPH[a]	−0.192	7.5×10^{-2}
PBO (10 μM) + NADPH	−0.138	5.5×10^{-2}
PBO (50 μM) + NADPH	−0.067	2.8×10^{-2}
PBO (0.2 mM) + NADPH	−0.031	1.2×10^{-2}

[a] NADPH (0.61 μmole) was present in both the sample and reference cuvettes prior to the first addition of (+)-benzphetamine.

Effect of PBO type III binding on ethyl isocyanide and n-octylamine spectra. The effects of NADPH and PBO on the *n*-octylamine–cytochrome P-450 difference spectrum are shown in Fig. 8. The spectrum obtained with untreated microsomes consisted of a peak at 432 nm and a broad trough with minima occurring at about 394 and 410 nm. The addition of NADPH resulted in a shift in the peak from 432 to 427 nm and an increase in the trough

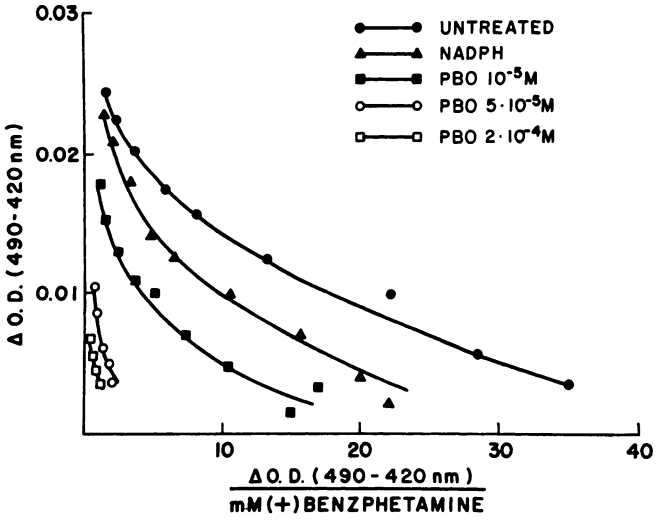

FIG. 7. *Effect of cytochrome P-450–piperonyl butoxide type III interaction on formation of cytochrome P-450–(+)-benzphetamine type I substrate difference spectrum*

A concentration range of 1–150 μM was employed for (+)-benzphetamine. The cytochrome P-450 level was 0.20 ΔA unit as determined by the carbon monoxide difference spectrum (450 − 490 nm).

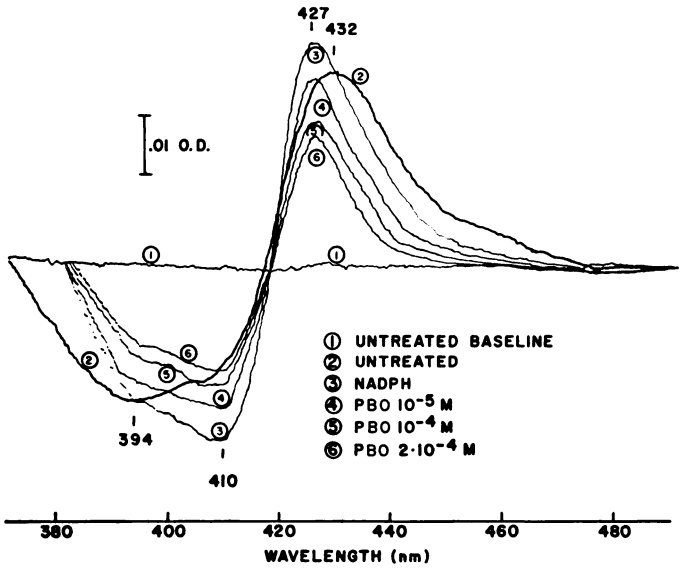

FIG. 8. *Effect of cytochrome P-450–piperonyl butoxide type III interaction on cytochrome P-450–n-octylamine difference spectrum*

The cytochrome P-450 level was 0.15 ΔA unit as determined by the carbon monoxide difference spectrum (450–490 nm). (The spectra shown are taken directly from actual recordings and are representative of all the spectra obtained in this study.) *1*, untreated baseline; oxidized microsomes in sample and reference; *2*, untreated; addition of *n*-octylamine to sample; *3*, NADPH; *n*-octylamine in sample, NADPH in sample and reference; *4–6*, *n*-octylamine in sample, piperonyl butoxide at indicated concentrations in sample, NADPH in sample and reference.

absorbance at 410 nm. When PBO was included with NADPH during incubation of the microsomes, the magnitude of the subsequently formed *n*-octylamine difference spectrum was reduced. The degree of reduction coincides with the concentration of PBO, and the shape of the spectrum appears consistent with the observed effect of NADPH regardless of the PBO concentration.

The magnitude of the ethyl isocyanide–cytochrome P-450 spectrum was also reduced when formed with microsomes incubated with PBO and NADPH. Proportional decreases in the 430 and 455 nm peaks of the dithionite-reduced spectrum occurred with increasing PBO concentrations, resulting in no alteration of the equilibrium point (the pH at which both peaks are of equal magnitude) (Table 3).

TABLE 3

Effect of cytochrome P-450–piperonyl butoxide type III interaction on cytochrome P-450–ethyl isocyanide difference spectrum and pH equilibrium point

PBO concentration[a]	ΔA (430 or 455 nm − 490 nm)				pH equilibrium point
	pH 7.5		pH 7.75		
	455 nm	430 nm	455 nm	430 nm	
M					
0	0.102	0.125	0.121	0.100	7.6
10^{-5}	0.087	0.105	0.106	0.087	7.6
5×10^{-5}	0.076	0.091	0.090	0.074	7.6
2×10^{-4}	0.072	0.082	0.084	0.071	7.6

[a] Microsomal suspensions containing 0.17 ΔA unit of cytochrome P-450 (as measured by the carbon monoxide difference spectrum) were incubated with piperonyl butoxide in the presence of NADPH (0.5 mM).

DISCUSSION

Treatment of microsomes with PBO in the presence of NADPH effects reductions in the magnitudes of all the cytochrome P-450 difference spectra examined. For CO, ethyl isocyanide, *n*-octylamine, and pyridine

(type II) the maximum reductions are similar—about 40%. For (+)-benzphetamine the reduction is about 70%. This differential effect, caused by PBO binding, agrees with evidence supporting the contention that interactions with cytochrome P-450 produced by CO, ethyl isocyanide, and type II substrates—but not type I substrates—are related by a common factor. Aniline (type II) competes with CO for NADPH-reduced microsomes (23) and with ethyl isocyanide for both oxidized (25) and dithionite-reduced (26) microsomes. CO is also known to compete with ethyl isocyanide for dithionite-reduced microsomes (18). In addition, aniline, pyridine, and ethyl isocyanide all give similar spectra with dithionite-reduced microsomes (27). On the other hand, hexobarbital (type I) does not compete with either ethyl isocyanide (26) or CO (23, 26).

Additional evidence for this contention has been obtained by studying the effects of microsome storage on difference spectra. Hewick and Fouts (22) demonstrated that the loss of the ethyl isocyanide, CO, and aniline difference spectra during storage occurs concomitantly with loss of heme, but that the (+)-benzphetamine spectrum disappears at a much faster rate. Shoeman *et al.* (28) noted that the stability of the aniline-induced spectrum during storage is much greater than that of the hexobarbital-induced spectrum (type I).

The differences noted above suggest (23, 29) that type I and type II substrates occupy different binding sites and that type II substrates possibly bind to the same site as CO and ethyl isocyanide.

The effect of PBO on the formation of the pyridine substrate difference spectrum appears significant with respect to the points noted above. At low pyridine concentrations, which produce a linear ΔA vs. $\Delta A/S$ relationship, no inhibition by the PBO type III interaction was observed. Since the linear segment of the curve suggests a single binding site, a maximum ΔA value for this site can be calculated by extrapolation and the effect of PBO on the formation of the pyridine spectrum at high pyridine concentrations can be determined. 0.2 mM PBO this inhibition is about 70%—the same value obtained for inhibition of the formation of the (+)-benzphetamine difference spectrum. This could be explained by the existence of binding sites for pyridine and (+)-benzphetamine that are equally affected by the PBO type III interaction in a noncompetitive manner and by an additional, unaffected binding site for pyridine.

It is suggested that PBO binds to one cytochrome P-450 to exert a similar effect on the formation of all the difference spectra examined and that another cytochrome P-450, which does not bind PBO or (+)-benzphetamine, is present. This hypothesis is supported by the following observations: (a) PBO (in the absence of NADPH) and (+)-benzphetamine are both type I substrates; (b) inhibition by 0.2 mM PBO, in the presence of NADPH, of the formation of the CO–, ethyl isocyanide–, *n*-octylamine–, and pyridine–cytochrome P-450 difference spectra is similar in magnitude; (c) inhibition of the CO spectrum and the formation of the PBO type III spectrum, as determined from the sum of the two peaks, are coincident and appear to involve a single site; (d) the effect of PBO on the formation of the (+)-benzphetamine spectrum is similar to that observed for the pyridine spectrum at high pyridine concentrations; and (e) no inhibition by PBO of the formation of the pyridine spectrum is seen at pyridine concentrations producing a linear ΔA vs. $\Delta A/S$ relationship.

The observation of different K_s values derived from the formation of the 427 and 455 nm peaks of the PBO type III spectrum appears to conflict with the above hypothesis. However, there is a direct correlation between the formation of the PBO type III spectrum and inhibition of the CO spectrum when the 455 and 427 nm peaks are summed. The spectral equilibrium of the PBO type III interaction suggests that two distinct responses to the concentration of PBO occur in reduced microsomes.

If the spectral equilibria observed reflect ligand binding, two possibilities could account for the data. First, two binding sites exist which are spectrally distinguishable only when the cytochrome is reduced. [The oxidized PBO type III spectrum has a single peak (16), as does that produced by ethyl isocyanide (18).] Second, a single bind-

ing site is present in the oxidized state, and conformational changes of this site occur in the reduced "forms" of the cytochrome. The proposed single-site hypothesis is contradicted by the former explanation and can only be true with the latter if the K_i values of PBO for inhibition of the formation of the CO spectrum are the same at each site.

A third explanation exists if the magnitudes of the 427 and 455 nm peaks of the PBO type III spectrum do not directly reflect binding of PBO to the cytochrome, but rather a combination of binding, the effects of pH, and the concentration of PBO on the equilibrium of two forms of the reduced PBO–cytochrome P-450 complex. If this explanation is correct, total PBO binding could only be calculated from the sum of two peaks. Combinations of pH and ligand concentration effects have been observed for another parameter related to cytochrome P-450. Imai and Sato (30) noted that the stimulation of aniline hydroxylase activity by ethyl isocyanide is dependent on pH, ethyl isocyanide concentration, and aniline concentration. Hansen and Fouts (31) showed that the pH optimum for the same reaction in microsomes from benzpyrene-induced rats is dependent on the aniline concentration employed.

The spectral interactions of varous compounds appear to be complex, either as a result of complex binding relationships or because of the presence of undescribed effectors. Our data showing curvilinear relationships between ΔA and $\Delta A/S$ for the formation of type I (benzphetamine and PBO) and type II substrate spectra are in agreement with those published by Jefcoate *et al.* (20) and Hewick and Fouts (22). [Linear Lineweaver-Burk relationships have been reported for type I and II substrate binding (23, 32, 33), but they may be due to the narrow substrate concentration ranges employed.]

It has been suggested that multiple binding sites, bound endogenous substrates, or allosteric effects could account for curvilinear binding kinetics (22). These possibilities all affirm the assumption that spectral magnitude reflects interaction of the substrate with binding site(s). Indirect effects, particularly at high substrate concentrations, resulting in alterations of the cytochrome affecting binding affinities or extinction coefficients, also seem possible.

In summary, PBO appears to combine with cytochrome P-450 to inhibit ligand interaction effectively with both the heme and type I binding sites. In addition, the similar effect of PBO on the pyridine–, CO–, ethyl isocyanide–, and *n*-octylamine–cytochrome P-450 spectra and the observation of an apparently unaffected type II binding site suggest an additional cytochrome P-450 which does not bind PBO or type I substrates.

REFERENCES

1. J. E. Casida, *J. Agr. Food Chem.* **18**, 753 (1970).
2. J. E. Casida, *Science* **153**, 1130 (1966).
3. F. X. Kamienski and J. E. Casida, *Biochem. Pharmacol.* **19**, 91 (1970).
4. C. F. Wilkinson and L. J. Hicks, *J. Agr. Food Chem.* **17**, 539 (1969).
5. E. G. Esaac and J. E. Casida, *J. Agr. Food Chem.* **17**, 829 (1969).
6. G. T. Brooks and A. Harrison, *Biochem. Pharmacol.* **18**, 557 (1969).
7. T. Nakatsugawa and P. A. Dahm, *J. Econ. Entomol.* **58**, 500 (1965).
8. W. W. Philleo, R. D. Schonbrod and L. C. Terriere, *J. Agr. Food Chem.* **13**, 113 (1965).
9. L. G. Hansen, Ph.D. thesis, "Microsomal oxidations of xenobiotics in the housefly, *Musca domestica* (L.)." North Carolina State University at Raleigh, 1970. (University Microfilms, Ann Arbor, Mich.).
10. M. W. Anders, *Biochem. Pharmacol.* **17**, 2367 (1968).
11. S. E. Lewis, C. F. Wilkinson and J. W. Ray, *Biochem. Pharmacol.* **16**, 1195 (1967).
12. S. Kuwatsuka, *in* "Biochemical Toxicology of Insecticides" (R. D. O'Brien and I. Yamamoto, eds.), p. 131. Academic Press, New York, 1970.
13. A. S. Perry and A. J. Buckner, *Life Sci.* **9**, 335 (1970).
14. H. B. Matthews, M. Škrinjarić-Špoljar and J. E. Casida, *Life Sci.* **9**, 1039 (1970).
15. R. M. Philpot and E. Hodgson, *Life Sci.* **10**, 503 (1971).
16. R. M. Philpot and E. Hodgson, *Chem.-Biol. Interactions*, **4**, 185 (1971).
17. M. Škrinjarić-Špoljar, H. B. Matthews, J. L. Engel and J. E. Casida, *Biochem. Pharmacol.* **20**, 1607 (1971).

18. T. Omura and R. Sato, *J. Biol. Chem.* **239**, 2370 (1964).
19. N. E. Sladek and G. J. Mannering, *Biochem. Biophys. Res. Commun.* **24**, 668 (1966).
20. C. R. E. Jefcoate, J. L. Gaylor and R. L. Calabrese, *Biochemistry* **8**, 3455 (1969).
21. B. H. J. Hofstee, *Nature* **184**, 1296 (1959).
22. D. S. Hewick and J. R. Fouts, *Biochem. Pharmacol.* **19**, 457 (1970).
23. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 113 (1967).
24. H. L. Jackson and B. C. McKussick, *Org. Syn.* **35**, 62 (1955).
25. Y. Imai and R. Sato, *Biochem. Biophys. Res. Commun.* **22**, 620 (1966).
26. A. P. Alvares, G. Schilling, W. Levin and R. Kuntzman, *J. Pharmacol. Exp. Ther.* **176**, 1 (1971).
27. Y. Imai and R. Sato, *J. Biochem. (Tokyo)* **62**, 464 (1967).
28. D. W. Shoeman, M. D. Chaplin and G. J. Mannering, *Mol. Pharmacol.* **5**, 412 (1969).
29. J. Schenkman, *Biochemistry* **9**, 2081 (1970).
30. Y. Imai and R. Sato, *J. Biochem. (Tokyo)* **63**, 380 (1968).
31. A. R. Hansen and J. R. Fouts, *Chem.-Biol. Interactions* **3**, 123 (1971).
32. J. B. Schenkman, I. Frey, H. Remmer and R. W. Estabrook, *Mol. Pharmacol.* **3**, 516 (1967).
33. Y. Imai and R. Sato, *J. Biochem. (Tokyo)* **62**, 239 (1967).