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Methylenedioxyphenyl Insecticide Synergists as Potential Human Health Hazards

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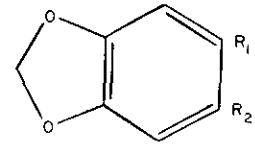
The effects of methylenedioxyphenyl insecticide synergists on human health arise, not from their intrinsic toxicity which is relatively low, but from their dual effect on the oxidative detoxication or intoxication processes of the body. They are able to inhibit these reactions by two mechanisms. They are substrates for mixed function oxidation and, thus, compete with other xenobiotics for available enzyme, and an intermediate in their metabolism is able to bind with cytochrome P-450 to form an inactive complex which absorbs maximally at 455 nm. In addition, they are able to activate xenobiotic metabolism by induction (the increased synthesis and retention) of microsomal oxidative enzymes. Evidence for these effects is presented, including spectroscopic and enzyme kinetic data. Correlations with existing *in vivo* observations are outlined. The inhibition and induction of cytochrome P-450, the central enzyme in the metabolism of xenobiotics, thus provides the focus for consideration of methylenedioxyphenyl synergists as potential hazards.

During the last twenty years, the use of compounds to promote the toxicity and, thus, the desired effect of many insecticides has considerably reduced the concentrations of the insecticides that need to be applied. While this has reduced the environmental load and human exposure to the toxic insecticide, the possibility of untoward effects from the synergists themselves ought to be considered. This brief account illustrates the general mechanisms which could give cause for concern, or at least consideration, when assessing the impact of insecticide synergists on the human population.

An insecticide synergist could be any compound that potentiates insecticide action and, consequently, any compound inhibiting cytochrome P-450-dependent oxidative metabolism could qualify. In actual fact, very few inhibitors have found practical use as synergists. The most widely encountered compounds are those having the general structure I, of which the most extensively investigated and used is piperonyl butoxide. Many methylenedioxybenzene derivatives occur naturally, as illustrated here by safrole and isosafrole, which occur in oil of sassafras and from which most of the synthetic synergists were originally developed. The four other most commonly encountered synthetic synergists are also shown: sesamex, tropital, sulfoxide, and propyl isome. All of these are characterized by a long oxygen-containing substituent (R_1). The nature of the side chains (R_1 and R_2) determines the spectrum of synergistic activity, certain derivatives only being effective synergists for a limited number of insecticide classes. However, the common feature necessary for synergistic activity appears to be the



R_1	R_2	Compound
-H	-H	Methylenedioxybenzene
-H	$-CH_2CH=CH_2$	Safrole
-H	$-CH=CHCH_3$	Isosafrole
$-CH_2(OCH_2CH_2)_3C_2H_5$	$-CH_2CH_2CH_3$	Piperonyl butoxide
$-OCH(CH_3)(OCH_2CH_2)_3H$	-H	Sesamex
$-CH[(OCH_2CH_2)_3C_2H_5]_2$	H	Tropital
$-CH_2CH(CH_3)SOC_8H_{17}$	H	Sulfoxide
$C_3H_7)CH(COOC_3H_7)CH(CH_3)CH_2-$		Propyl isome

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methylenedioxy grouping. The metabolism of these methylenedioxybenzene derivatives involves two principal routes: modification of the side groups and cleavage of the methylenedioxy ring (*1–5*). The relative proportions of each of these reactions depends upon the animal species and the compound studied. If the side groups are small, they are often oxidized to the carboxylic acid group and excreted as conjugated derivatives. The methylenedioxy grouping is metabolized by oxidation (the exact mechanism of which still remains to be resolved), which yields the methylene carbon ultimately as CO_2. This leaves a catechol derivative as the other metabolite.

From the studies of their metabolism, the rational basis for the use of methylenedioxybenzene compounds as insecticide synergists was derived. The enzyme responsible for the oxidation of the methylene carbon was similar to that involved in the oxidative metabolism and detoxication of the insecticides. Thus, their sparing effect was due to their mutual competition at cytochrome P-450, the ubiquitous enzyme responsible for oxidative metabolism of a vast number of foreign compounds (*6*). Thus, it could be seen that synergism would only occur for those compounds which were extensively inactivated by oxidation. Compounds which require oxidation to activate them into more potent insecticides would have their insecticide action decreased.

With this knowledge we must consider where the possible human health effects might arise. The acute toxicity of methylenedioxybenzene derivatives in mammals is relatively low (Table 1) (*7–9*). For rats, the LD_{50} for piperonyl butoxide is about 10 g/kg and about 15 g/kg for propyl isome (*7*). Other commonly used methylenedioxyphenyl synergists are similarly nontoxic. The smaller the side chains, such as in safrole and isosafrole, the more toxic the compound. The parent compound, methylenedioxybenzene, is quite toxic, having an LD_{50} of about 0.5 g/kg. The compounds also seem to be relatively innocuous on a chronic basis. Toxicity is only seen at relatively high dose levels over extended periods of time. In line with the acute toxicities, the methylenedioxyphenyl compounds with short side chains seem to be the most detrimental. Tumors have been reported from safrole and dihydrosafrole administration (*8–10*), whereas for piperonyl butoxide, only liver cell changes and possibly kidney damage seem to occur (*11,12*). Safrole carcinogenesis probably arises from the formation of 1-hydroxysafrole which is formed during the metabolism of this compound (*13*). No reports of teratogenic or mutagenic effects of methylenedioxyphenyl insecticide synergists have appeared.

Thus, it would appear that for the commonly used methylenedioxyphenyl synergists, both on acute or chronic exposure, there seems to be little need for concern. In one study (*14*), a single 50-mg dose of piperonyl butoxide to humans (equivalent

Table 1. Acute oral toxicity (LD_{50}).[a]

	LD_{50}, mg/kg	
	Rat	Mouse
Propyl isome	15,000	—
Piperonyl butoxide	10,000	—
Piperonal	2,700	—
Dihydrosafrole	2,260	3,700
Safrole	1,950	2,350
Isosafrole	1,340	2,470
Methylenedioxybenzene	580	1,220

[a]Data of Sarles (*7*) and Hagen (*8*).

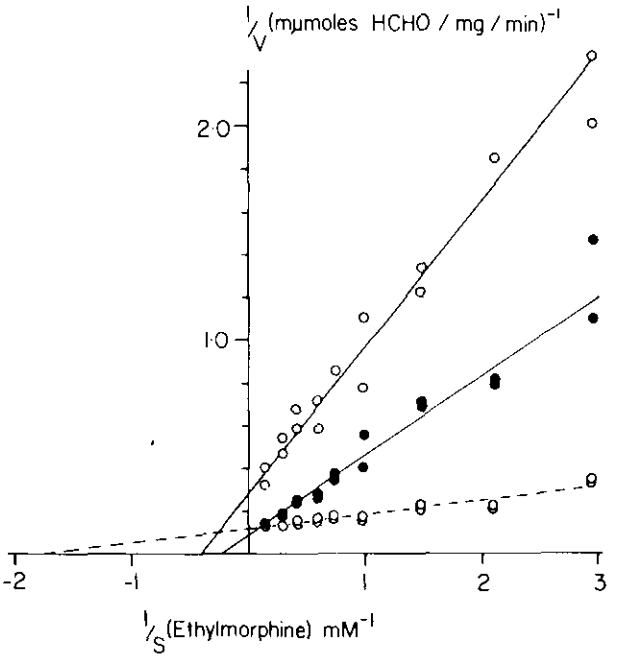

FIGURE 1. Alteration of the inhibitory characteristics of piperonyl butoxide on ethylmorphine *N*-demethylation by preincubation of piperonyl butoxide with microsomes and NADPH. Hepatic microsomes from phenobarbital-pretreated rats were suspended in 50*mM* Tris–chloride buffer (pH 7.4) containing 150*mM* KCl, 10*mM* $MgCl_2$, 10*mM* nicotinamide, 7*mM* isocitrate and 0.27 units of IDH per ml. Lineweaver-Burk plots were constructed for ethylmorphine *N*-demethylation for incubations performed in the absence (○ - -) and presence (● ——) of piperonyl butoxide and also (○—), in the presence of piperonyl butoxide which had been preincubated for 5 min with the microsomes and NADPH.

to 50 times the heaviest likely daily exposure) produced no apparent harmful effects. What does become a matter of concern is the effect that these compounds have on the metabolism of other compounds in the body. A change in the metabolism of many other drugs can, depending on the drug, increase or decrease its toxicity. Thus, a normally nontoxic dose of a drug may then produce toxic symptoms. Before we examine the mechanism of such drug interactions, it is as well to consider the reported *in vivo* effects of methylenedioxyphenyl synergists and their interactions with other drugs and foreign compounds.

Piperonyl butoxide and many other methylenedioxyphenyl compounds were found to prolong the sleeping time of barbiturates and paralysis time of zoxazolamine (*15–20*). Both of these compounds are oxidatively metabolized to inactive products, and the effects seen suggest the inhibitory effect of piperonyl butoxide on their metabolism. Also, as might be expected from their ability to increase the toxicity of insecticides in insects, they can have the same effect with respect to insecticides in mammals. The acute toxicity of freons, griseofulvin and benzopyrene in infant mice has been shown to be increased by piperonyl butoxide (*21,22*). In the realm of carcinogenesis, several methylenedioxyphenyl compounds have been implicated as being cocarcinogens. This will depend upon whether the carcinogenic compound under consideration or its metabolite is the proximate carcinogen. If the compound *per se* is the carcinogen, then an inhibition of its oxidative metabolism to noncarcinogenic metabolites would increase the chances of a cellular interaction necessary for initiation of the tumor. If an oxidative metabolite is the active species responsible for the cellular interaction, then an inhibition of oxidation would slow the formation of reactive metabolites, allowing possible clearance of the

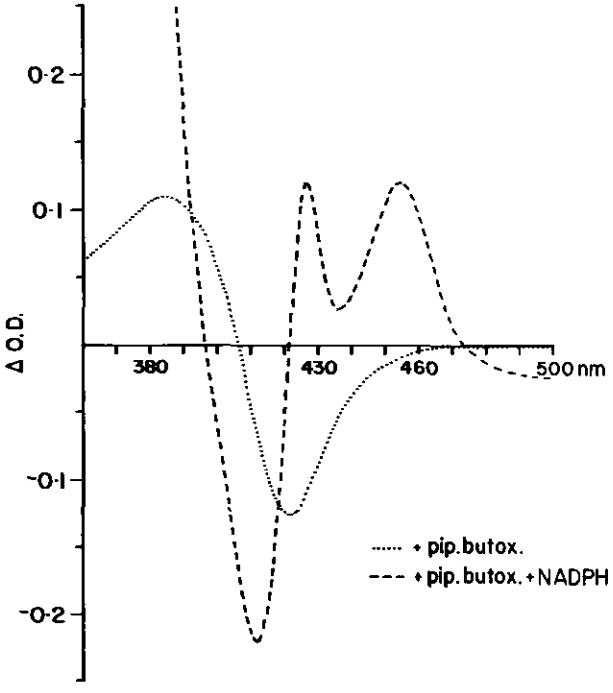

FIGURE 2. Spectral changes produced after addition of piperonyl butoxide and NADPH to an aerobic suspension of liver microsomes. Rat liver microsomes (2.8 nmole cytochrome P-450/mg protein) were suspended in 50*mM* Tris-chloride buffer (pH 7.4) containing 150 *mM* KCl and 10*mM* $MgCl_2$. The suspension was divided equally between two cuvettes and a baseline of equal light absorbance established. Piperonyl butoxide was then added to the contents of the sample curvette to a concentration of 1*mM* and the suspension sonicated to disperse the piperonyl butoxide. The difference spectrum was then determined (· · ·). 500 μM NADPH was then added to the sample cuvette and the difference spectrum again determined 5 min after NADPH addition (- -).

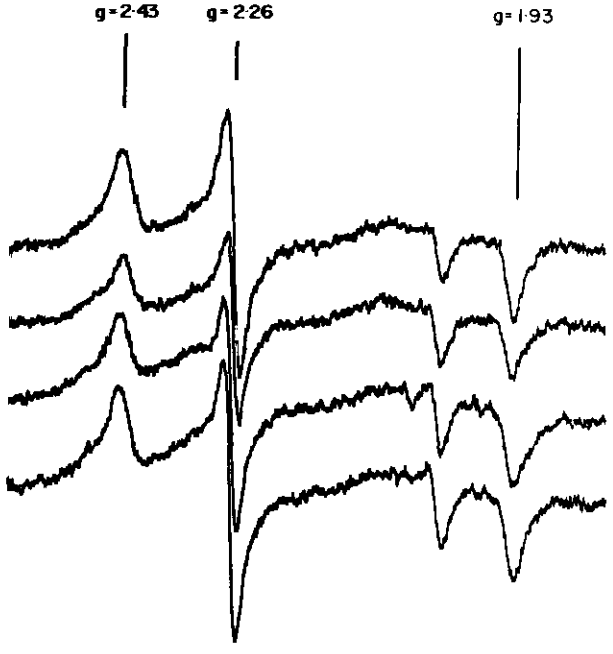

FIGURE 3. The EPR low spin signal of microsomes incubated with piperonyl butoxide and NADPH. The EPR first derivative spectra were obtained from microsomal samples (10 mg protein/ml) which were incubated with piperonyl butoxide (3.15*mM*) and NADPH at 25°C for varying periods of time. The EPR spectra were monitored at −172°C. The uppermost trace was obtained before the addition of NADPH, the next two after 7.2 and 14.0 min of incubations with 2*mM* NADPH. The lower trace was recorded 11.3 min after the addition of 1*mM* NADPH to an identical incubation. The Varian E4 EPR spectrometer settings were 12.5 gauss modulation amplitude and a microwave power setting of 50 mW.

compound from the body by other mechanisms, leading to a decreased incidence of tumor initiation. Examination of the effects of methylenedioxyphenyl synergists, particularly piperonyl butoxide *in vitro*, was then needed to account for observations seen *in vivo*. It was found that they inhibited many mixed-function oxidases (*5, 23, 24*). Although the inhibition was mainly competitive, indicating competition between the drug substrates and methylenedioxyphenyl compounds for the oxidative enzyme, cytochrome P-450, some investigations yielded results which showed other than competitive inhibition (*16, 25, 26*). Closely controlled studies (*27*) revealed that piperonyl butoxide did produce competitive inhibition, but the degree of inhibition at any single concentration increased with time. This suggested the formation of an even more inhibitory product from piperonyl butoxide. Examination of the kinetics of this inhibition (Fig. 1) showed competitive inhibition initially (i.e., increase in K_m with no change in V_{max}), but after conditions conducive to the metabolism of piperonyl butoxide, the kinetics became noncompetitive (i.e., a decrease in V_{max}). Thus, piperonyl butoxide was initially a competitive inhibitor and, subsequently (after metabolism), noncompetitive. What was the product that was capable of inhibiting the cytochrome P-450 noncompetitively?

Spectral examination of the cytochrome P-450 enzyme system (or microsomal suspension) revealed two relevant perturbations (Fig. 2). The addition of piperonyl butoxide to microsomes produced a Type I spectrum (*28*) similar to most of the compounds which are metabolized by cytochrome P-450. After incubation of this suspension with NADPH in the presence of oxygen (i.e., conditions suitable for piperonyl butoxide metabolism), an absorbance appeared particularly noticeable in the 455 nm region (*29, 30*). This was attributed to a product or intermediate formed during the metabolism of piperonyl butoxide, combining with the enzyme, cytochrome P-450, that formed it (*30*).

Evidence that the 455 nm complex involves cytochrome P-450 is shown in Figure 3, where electron paramagnetic resonance spectra of microsomes are presented. Ferric cytochrome P-450 shows a low spin signal of heme iron with *g* tensor values of 2.43, 2.26, and 1.93. Upon addition of NADPH to normal microsomal suspensions, very little change in the low spin signal is observed because the steady state lies in the direction of oxidized cytochrome P-450. However, in the presence of piperonyl butoxide, the formation of the 455 nm complex traps the cytochrome P-450 in the ferrous or reduced state, thus causing a decrease in the low

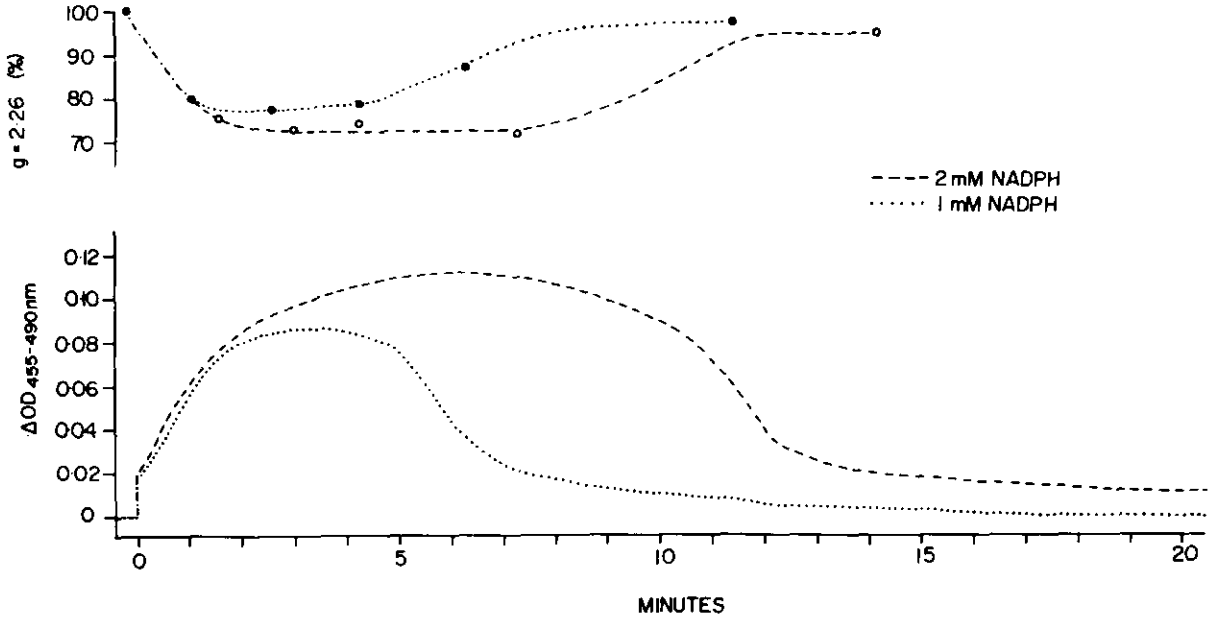

FIGURE 4. The correlation of the low spin EPR signal (g = 2.26) with the amount of cytochrome P-450 existing as the 455 nm complex. Microsomes from phenobarbital-pretreated rats were incubated (10 mg protein/ml) in 50*mM* Tris-chloride buffer (pH 7.4) containing 150*mM* KCl and 10*mM* $MgCl_2$, 3.15*mM* piperonyl butoxide (sonicated into a fine suspension) and NADPH as indicated. After various time intervals samples were removed and frozen in tubes suitable for EPR determinations. Optical determinations of the 455 nm complex were performed continuously in a 2 mm light path cuvette (attached to the incubation flask). The EPR low spin signal (g = 2.26) was calculated as the peak—trough height of the first derivative spectrum, since no signal broadening was detected, and presented as % of the low spin signal present in the absence of NADPH.

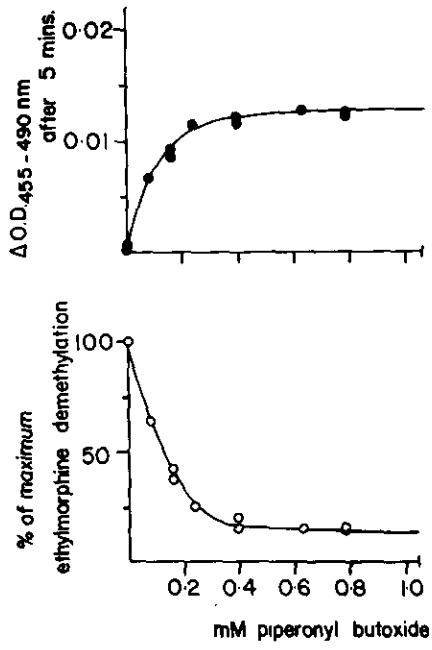

FIGURE 5. Correlation between the piperonyl butoxide metabolite-cytochrome P-450 complex and the inhibition of ethylmorphine *N*-demethylation. Hepatic microsomes from phenobarbital-pretreated rats were suspended at a concentration of 2.7 mg protein/ml in 50*mM* Tris-chloride buffer, pH 7.4, containing 150*mM* KCl, 10*mM* $MgCl_2$ and various concentrations of piperonyl butoxide. NADPH (50μM) was then added, and after 5 min the absorption spectrum due to the piperonyl butoxide metabolite-reduced cytochrome P-450 complex was determined (Δ OD 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 nmole HCHO/mg protein/min.

spin signal, shown here as a decrease in the amplitude of the first derivative spectrum. Examination of the $g = 2.26$, or predominant low-spin signal, of microsomal samples removed at specific times during the metabolism of piperonyl butoxide (Fig. 4) revealed that the decrease observed in the signal amplitude (since there was no signal broadening) correlated with the amount of 455 nm complex which was detectable by optical spectroscopy. This agreement held when the experiment was performed with two concentrations of NADPH. If the 455 nm-absorbing complex was responsible for the noncompetitive inhibition, then the extent of piperonyl butoxide inhibition of mixed-function oxidation after preincubation should be dependent on the amount of the 455 nm complex formed. To determine this, the amount of the 455 nm complex formed was measured after 5 min preincubation of microsomes with NADPH and limited quantities of piperonyl butoxide (Fig. 5). The ethylmorphine demethylation reaction was then initiated and the degree of inhibition determined. The two parameters, 455 nm complex formation and degree of inhibition, were found to correlate very well (*27*). Thus, it now seems possible to explain the very effective insecticide synergistic action of piperonyl butoxide and probably other methylenedioxybenzene derivaties, not only on an alternative substrate hypothesis but also on the theory that metabolism of the compound results in an intermediate which complexes with the cytochrome P-450 in a ligandlike manner to form a very stable complex. This prevents the participation of cytochrome P-450 in insecticide metabolism.

The question that remains at this point is whether this *in vitro* effect is of consequence *in vivo* and so could account for the whole animal observations mentioned earlier. The following observations (Fig. 6–8) show what happens to the liver microsomal system for up to 48 hr after treatment of the rats with a single intraperitoneal injection of piperonyl butoxide at two dose levels (500 and 1000 mg/kg). The microsomal cytochrome b_5 concentration remained unchanged. The carbon monoxide detectable cytochrome P-450 undergoes a biphasic change. First, there was a decrease in concentration which was followed by an increase. These changes appear to be dose-dependent, the higher the dose of piperonyl butoxide, the longer the decrease and the larger the subsequent increase. The type I and type II binding spectra show similar biphasic changes. The NADPH-cytochrome c reductase activity undergoes continuous elevation. The mixed-function oxidase activities (Fig. 7) determined as ethylmorphine *N*-demethylation, *p*-nitroanisole *O*-demethylation, and the rate of 455 nm complex formation from piperonyl butoxide correspond to the changes seen in the carbon monoxide detectable cytochrome P-450; that is, an inhibition below control values followed by an activation.

The second phase, or increase, can be accounted for by the phenomenon of induction. It has been known for many years that animals can respond to exposure to foreign compounds by increasing the liver enzymes responsible for the metabolism of these compounds (*31*). With piperonyl butoxide, as seen here, this is a graded response, the higher dose producing the larger induction. The explanation for the initial decrease can be seen in Figure 8. The animal treated with piperonyl butoxide forms a 455 nm complex which survives the preparation of microsomal fractions and can be measured *in vitro*. The larger the dose of piperonyl butoxide, the

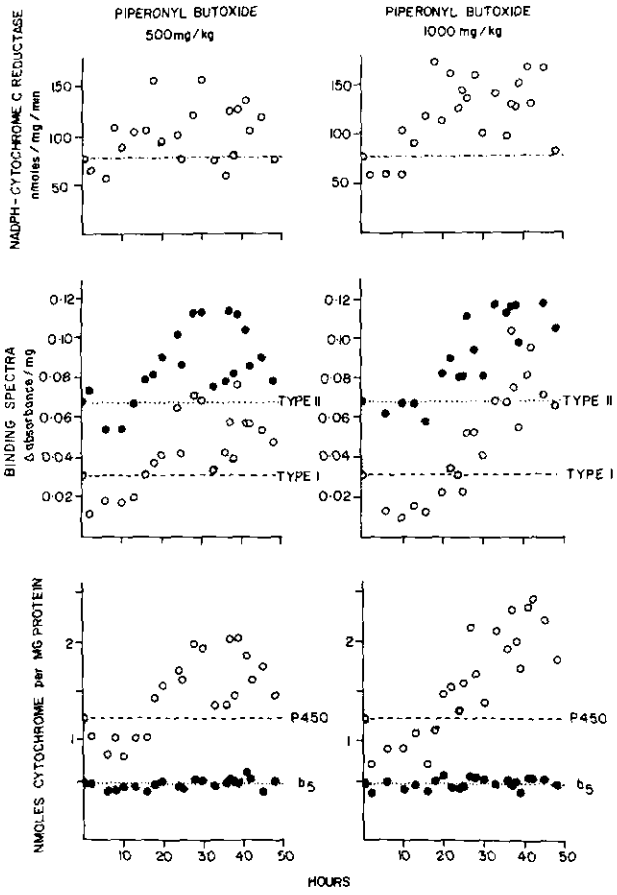

FIGURE 6. Changes in microsomal cytochrome content and enzymic activity following piperonyl butoxide administration: (left) 500 mg/kg; (right) 1000 mg/kg. Hepatic microsomes were prepared from male rats sacrificed at 0–48 hr after a single IP injection of piperonyl butoxide. Cytochrome b_5, cytochrome P-450, type I and type III binding spectra were determined in microsomal suspensions containing 2 mg protein/ml. NADPH-cytochrome c reductase was determined with microsomes at 0.2 mg protein/ml. Cytochrome b_5 was determined with a 200μM NADH microsomal difference spectrum, and calculated by using the wavelength pair 420-409 nm (ϵ = 185/mM-cm). Cytochrome P-450 was determined with a CO difference spectrum of dithionite reduced microsomes, using the wavelength pair 450-490 nm (ϵ = 91/mM-cm). The binding spectra were peak to trough absorbance changes after the addition of 3mM hexobarbital (type I) or saturating concentrations of aniline (type II) to microsomal suspensions. NADPH-cytochrome c reductase was determined using absorbance changes at 550 nm (ϵ = 21/mM-cm). Each point represents one animal; the hatched area shows ± standard deviation for untreated animals.

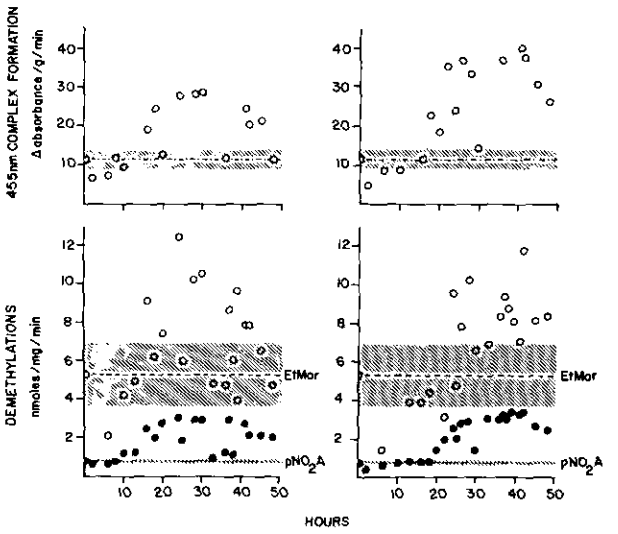

FIGURE 7. Changes in microsomal mixed-function oxidase activities after piperonyl butoxide administration: (left) 500 mg/kg; (right) 1000 mg/kg. Hepatic microsomes were prepared from male rats sacrificed at 0–48 hr after an IP injection of piperonyl butoxide. All determinations were made with microsomal suspensions at 2 mg protein/ml in 50mM Tris buffer containing 50mM KCl and 10mM $MgCl_2$. p-Nitroanisole demethylation was determined from continuous monitoring of p-nitrophenol formation (in pH 7.85 buffer) at 417 nm. Ethylmorphine demethylation activity was determined from the colorimetric determination of the formaldehyde produced. The formation of the 455 nm complex was determined by dual wavelength spectroscopy (455–490 nm) in the presence of 0.63mM piperonyl butoxide after the addition of 400μM NADPH. Each point represents one animal; the hatched area shows ± standard deviation for untreated animals.

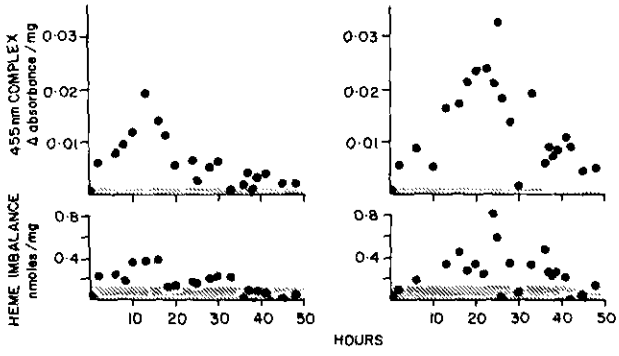

FIGURE 8. Relationship between heme and the 455 nm complex in microsomes after piperonyl butoxide administration: (left) 500 mg/kg; (right) 1000 mg/kg. Hepatic microsomes were prepared from male rats sacrificed at 0–48 hr after a single IP injection of piperonyl butoxide. Total microsomal heme was determined by using alkaline pyridine (ϵ = 32.4/mM-cm for 557-575 nm). Cytochrome b_5 and cytochrome P-450 concentration (Fig. 6) were then subtracted to give the heme imbalance. Microsomal 455 nm complex concentration was determined using absorbance at 455 nm, relative to 490 nm for NADPH reduced versus native microsomal suspensions. Each point represents one animal; the hatched area shows the standard deviation for untreated animals.

larger and of longer duration is the presence of the complex. This complex prevents the measurement of cytochrome P-450 with carbon monoxide, since the carbon monoxide is unable to displace the metabolite or intermediate from the cytochrome P-450 (*32*). This conclusion is reinforced if the total heme in the microsomes is determined and the measurable cytochrome P-450 and cytochrome b_5 subtracted. The resulting discrepancy or imbalance (lower traces) parallels the amount of the 455 nm complex detectable. Thus, the initial decrease in measurable cytochrome P-450 and the inhibition of mixed-function oxidation (*23, 33, 34*) can be accounted for by some of the cytochrome P-450 existing in a complexed and, thus, inactivated form (with a piperonyl butoxide metabolic intermediate).

In considering the health aspects of the methylenedioxyphenyl compounds we, therefore, have to consider two facets: their ability to inhibit and their ability to induce. The balance of these two at the time when the individual is exposed to a second drug or foreign compound may be critical in determining the consequence. Also, the relative stability, or persistence, of the inactive cytochrome P-450 complex (*33, 35, 37*) raises the possibility of additive inhibitory effects from exposure to the synergists several days apart.

The investigations, together with the omissions, that have been directed at determining the possible health effects of the most commonly encountered methylenedioxyphenyl compound, piperonyl butoxide, are shown in Table 2. The most visible omission is the lack of studies to determine any pharmacological or toxicological effects when compounds are administered with long time intervals between them and the piperonyl butoxide exposure. The majority of investigations have been with compounds which are inactivated by cytochrome P-450-dependent reactions and thus show increased toxicity or pharmacological effect when given shortly after piperonyl butoxide, i.e., during the inhibitory phase. For some compounds, such as bromobenzene and acetaminophen, the toxicity is

Table 2. Alteration of pharmacological or toxicological effect after exposure to piperonyl butoxide.

Action of cytochrome P-450 metabolism	Alteration of pharmacological (P) or toxicological (T) effect at the following time intervals after piperonyl butoxide exposure: Short (P-450 inhibition)	Long (P-450 induction)
Active → Inactive	Increase (Insecticides) Barbiturates (P) Zoxazolamine (P) Antipyrine (P) Griseofulvin (T) Freons (T) Benzopyrene (T) (acute)	Decrease
Inactive → Active	Decrease (Insecticides) Acetaminophen (T) Bromobenzene (T)	Increase

Table 3. Amines capable of forming metabolic 455 nm complexes.

d- and *l*-Amphetamine	Methamphetamine	*d*- and l-Benzphetamine
4-Chloroamphetamine	4-Chloromethamphetamine	
4-Methoxyamphetamine (PMA)	Methoxyphenamine	SKF 525-A
3,4-Methylenedioxyamphetamine (MDA)	Propylhexedrine	Lilly 18947
2,3-,2,5-, and 3,5-Dimethoxyamphetamine	Cyclopentamine	Adiphenine
2,3,4-,3,4,5-,2,4,5-, and 2,4,6-, Trimethoxyamphetamine	Fenfluramine	Benactyzine
2,5-Dimethoxy-4-bromoamphetamine	Phenmetrazine	Propoxyphene
2,5-Dimethoxy-4-methylamphetamine (STP)	Benzylamphetamine	Methadone
2,4-Dimethoxy-5-methylamphetamine	*N*-Hydroxyamphetamine	Diphenhydramine
3,4-Ethylenedioxy-5-methoxyamphetamine	Propylamphetamine	Acetylmethadol
3,4-Methylenedioxy-5-methoxyamphetamine		
SKF 26754-A	SKF 8742-A	
	Desipramine	
	Nortriptyline	

mainly due to metabolites formed during cytochrome P-450-dependent oxidative reactions (*8, 39*). If these compounds are received shortly after methylenedioxyphenyl exposure, their toxicity is reduced since less of the toxic metabolite will be formed. However, if the exposure to methylenedioxyphenyl compounds had been large and long enough to cause induction, then the increased amount of enzyme will increase the amount of toxic metabolite produced and one would, therefore, expect an increase in toxicity.

In conclusion, it is obvious that much of the glamorous work concerning piperonyl butoxide metabolism has been done. However, there are many areas, perhaps less glamorous, which need extensive investigation if we are to judge the potential human health hazards of insecticide synergists, in particular, and inhibitors of drug metabolism in general. The need has become more urgent with the discovery that methylenedioxyphenyl synergists are not the only compounds capable of forming 455 nm complexes with cytochrome P-450 during metabolism (Table 3). A variety of amines, especially amphetamine derivatives (*40–44*), also form such complexes. We may find that the study of insecticide synergists will develop a whole new understanding of clinical drug interactions which will, hopefully, reduce health hazards in drug therapy.

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