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EFFECTS *IN VITRO* AND *IN VIVO* OF METHYLENEDIOXYPHENYL COMPOUNDS ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

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Abstract—The methylenedioxyphenyl compounds, piperonyl butoxide (P.B.), tropital, myristicin and piperonal were tested for their action *in vitro* on oxidative phosphorylation using rat liver mitochondria. These compounds inhibited state 3 respiration and uncoupled oxidative phosphorylation in the decreasing order: piperonyl butoxide, tropital and myristicin. Piperonal had little, if any, effect on mitochondrial function indicating that the side-chain, not the methylenedioxyphenyl moiety, conferred uncoupling activity. The I_{50} of P.B. on state 3 respiration was approximately 50 mμmoles per mg protein with glutamate and β-hydroxybutyrate, and 100 mμmoles/mg with succinate as the substrate. The I_{50} of P.B. on NADH-oxidase and succinoxidase of electron transport particles was 500 and 2500 mμmoles per mg protein, respectively, indicating a lesser effect on the respiratory chain than on phosphorylation.

Studies were also conducted to determine if P.B. selectively inhibited mitochondrial function *in vivo*. Up to 880 mg P.B./kg failed to inhibit either mitochondrial function, or aniline hydroxylase and aminopyrine demethylase activities in mitochondria and microsomes isolated from treated rats and assayed *in vitro*. Similar results were obtained using rats pretreated with phenobarbital for 3 days. The significance of these findings relative to the *in vivo* site of P.B. action is discussed.

METHYLENEDIOXYPHENYL compounds synergize the toxicity of pesticides[1] and other environmental agents.[2–4] The mechanism of synergistic action is believed to be due to the ability of these compounds to competitively inhibit microsomal enzymes involved in hydroxylation reactions.[5,6] Inhibition of microsomal enzymes by methylenedioxyphenyl compounds has been demonstrated both by direct measurement *in vitro*,[3,5,7] and indirectly *in vivo* by reduced elimination rates of drugs[3,8] and increased hexabarbital sleeping times.[3]

Though the action of methylenedioxyphenyl compounds on microsomal enzymes has been well documented, little attention has been given to the possibility that these compounds may have additional sites of action in the cell. In spite of the low mammalian toxicity of most methylenedioxyphenyls, i.e. high LD_{50} values,[4] certain naturally occurring compounds produce cellular pathologies such as fatty livers, cirrhosis and necrosis.[9] These are all indicative of metabolic disturbances which could result from the interaction of synergists with enzyme systems not associated with the microsomal fraction. In view of this, the present study was undertaken to investigate the effects of several methylenedioxyphenol compounds on oxidative phosphorylation both *in vitro* and *in vivo*.

METHODS

Mitochondria were isolated from livers of male Sprague–Dawley rats. Liver tissue was homogenized (33% w/v) for 30 sec in 0·25 sucrose using a Potter–Elvehjem homogenizer. The homogenate was then diluted to 10% (w/v) with 0·25 M sucrose, and mitochondria were removed by centrifugation for 10 min at 8500 *g* after first removing cellular debris by centrifugation at 600 *g* for 10 min. Microsomes used for assaying microsomal enzyme activities were prepared by centrifuging the postmitochondrial supertatant for 1 hr at 100,000 *g*. Microsomes used in the determination of cytochrome P-450 and cytochrome b_5 were prepared by homogenizing liver tissue (10% w/v) in 0·1 M phosphate buffer, pH 7·4, and then centrifuging at 10,000 *g* for 15 min to remove debris. Microsomes were obtained by centrifuging the remaining supernatant at 100,000 *g* for 1 hr. This procedure eliminated a large absorbance peak

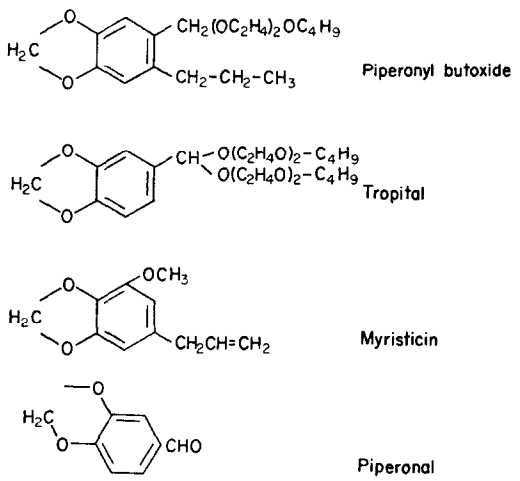

FIG. 1. Structures of methylenedioxyphenyl compounds used in present study.

at 420 mμ which appeared in the reduced difference spectra of microsomes isolated from tissue homogenized in sucrose. The 420 mμ absorbance peak did not appear to be related to degradation of cytochrome P-450[10] since the levels of P-450 were the same in tissues homogenized in either sucrose or phosphate. Electron transport particles (ETP) were prepared from beef heart as described by Green and Ziegler.[11]

Oxidative phosphorylation was measured manometrically in a Warburg apparatus or polarographically with a Clark oxygen electrode. The reaction mix for polarographic determinations contained 120 mM KCl, 12 mM substrate, 8 mM $MgCl_2$, 5 mM K_2HPO_4, and 20 mM glycylglycine buffer, pH 7·4. The total volume was 1·6 ml and the temperature was maintained at 30°. The reaction mix used for manometric determination contained: 30 μmoles of glucose, 1 μmole EDTA, 2·5 μmoles ATP, 10 μmoles $MgCl_2$, 50 μmoles of substrate, 30 μmoles K_2HPO_4, 0·5 mg of hexokinase and 4–5 mg of mitochondrial protein in a total volume of 2·0 ml. Respiration was measured over 30 min at 30°. Mitochondrial ATPase was measured in a medium containing 20 μmoles glycylglycine buffer (pH 7·4), 5 μmoles $MgCl_2$ and 3 μmoles

ATP in a total volume of 1·5 ml. The reaction was incubated for 15 min at 25°. Inorganic phosphate was measured by the method of Fiske and Subbarow.[12]

Aniline hydroxylase activity was measured in microsomes following the methods of Schenkman *et al.*[13] Aminopyrine demethylase was determined using the assay described by Orrenius[14] and the formaldehyde released was measured by the reaction of Nash.[15] Cytochrome P-450 was determined by its carbon monoxide difference spectrum after reduction with dithionite,[10] and cytochrome b_5 was determined by its difference spectra after reducing microsomes in the sample cuvette with NADH.[10]

Piperonyl butoxide, tropital, myristicin and piperonal (Fig. 1) were generously supplied by Dr. L. Fishbein of this Institute. Stock solutions were prepared fresh daily as 15 mM solutions in dimethylsulfoxide (DMSO). Small aliquots of the stock (1–10 μl) were added to the reactions to give the desired final concentration of the inhibitor.

RESULTS

Piperonyl butoxide lowered respiratory control (RC) by inhibiting state 3 (ADP controlled) respiration and enhancing state 4 (minus phosphate acceptor) respiration (Table 1). Respiratory control was partially abolished by 33 mμmoles of piperonyl butoxide/mg of mitochondrial protein regardless of the substrate. It was completely abolished at 65 mμmoles PB/mg protein with β-hydroxybutyrate and glutamate as substrates and at 98 mμmoles PB/mg with succinate as the substrate. The ADP:O ratio could not be measured when the RC ratio reached 1; however, partial uncoupling was observed at concentration of piperonyl butoxide which produced partially reduced RC values. In all cases piperonyl butoxide had a greater effect on RC than on ADP:O ratios.

TABLE 1. EFFECT OF PIPERONYL BUTOXIDE ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	P.B. (mμmoles/mg protein)	mμg Atom O/min/mg protein		R.C.	ADP:O
		State 3	State 4		
Succinate	0	183	25	7·31	2·01
	33	167	25	6·75	1·63
	65	128	30	4·27	1·70
	98	85	85	1·00	
	130	71	71	1·00	
Glutamate	0	72	11	6·43	2·95
	33	49	14	3·50	2·74
	45	38	17	2·23	2·54
	65	22	17	1·30	2·23
β-Hydroxybutyrate	0	52	11	4·71	2·95
	33	40	14	2·91	2·62
	51	23	14	1·64	1·84
	65	26	26	1·00	

* Respiration was determined polarographically. The reaction mix contained 120 mM KCl, 8 mM $MgCl_2$, 5 mM K_2HPO_4, 20 mM glycylglycine (pH 7·4), 12·5 mM substrate, 0·5 μmoles ADP (state 3 respiration), and 2·35 mg mitochondrial protein. The total volume was 1·6 ml.

TABLE 2. EFFECTS OF TROPITAL ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	Tropital (mμmoles/mg protein)	mμg Atoms O/min/mg protein		R.C.	ADP:O
		State 3	State 4		
Succinate	0	142	33	4·30	1·64
	50	146	38	3·85	1·44
	100	127	44	2·99	1·34
	150	94	61	1·55	1·09
Glutamate	0	67	17	3·94	2·61
	8	59	21	2·81	1·87
	20	55	21	2·62	1·87
	40	47	21	2·24	1·84
	50	47	19	2·48	1·90
	100	40	27	1·49	1·64

* Conditions of the assay are described in Table 1. Each assay contained 1·5 mg mitochondrial protein. The total volume was 1·6 ml.

Tropital (Table 2) also inhibited state 3 respiration and lowered the ADP:O ratio. Inhibition by tropital was less than with piperonyl butoxide until tropital was increased to 100 mμmoles/mg protein. Some reduction in state 3 respiration, RC and the ADP:O ratio was observed at 50 mμmoles/mg protein.

Myristicin at 167 mμmoles/mg protein partially uncoupled succinate-supported oxidative phosphorylation and reduced the RC value but did not completely eliminate either (Table 3). With glutamate as the substrate, little effect of myristicin was observed at 82 mμmoles/mg, whereas 167 mμmoles/mg significantly reduced the RC.

In contrast to the above methylenedioxyphenyl compounds, piperonal had little or no effect on state 3 respiration and RC at 300 and 450 mμmoles/mg protein for succinate and glutamate respectively (Table 4). These results show that the effects of

TABLE 3. EFFECTS OF MYRISTICIN ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	Myristicin (mμmoles/mg protein)	mμg Atoms O/min/mg protein		R.C.	ADP:O
		State 3	State 4		
Succinate	0	167	37	4·50	1·67
	82	155	46	3·37	1·66
	125	142	53	2·68	1·57
	167	126	63	2·00	1·45
Glutamate	0	67	18	3·73	2·65
	82	55	25	2·19	2·17
	167	67	43	1·57	2·00

* Conditions of the assay are described in Table 1. Each assay contained 1·6 mg mitochondrial protein. The total volume was 1·6 ml.

Piperonyl butoxide is related more to the structure of the side-chain than to the methylenedioxyphenyl moiety. This is supported by preliminary experiments in which piperonyl alcohol, sesamol, safrole, isosafrole and dihydrosafrole were tested and found to have no effect on mitochondrial function *in vitro*.

TABLE 4. EFFECTS OF PIPERONAL ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA*

Substrate	Piperonal (mμmoles/mg protein)	mμg Atoms O/min/mg mitochondrial suspension		R.C.	ADP:O
		State 3	State 4		
Succinate	0	150	43	3·49	1·87
	75	148	42	3·55	1·72
	150	140	40	3·50	1·72
	225	145	44	3·25	1·74
	300	139	42	3·34	1·72
Glutamate	0	74	21	3·55	2·43
	75	63	24	2·60	2·12
	150	61	24	2·54	2·04
	300	67	26	2·58	2·12
	450	67	27	2·48	2·06

* Conditions of the assay are described in Table 1. Each assay contained 1 mg mitochondrial protein. The total volume was 1·6 ml.

Figure 2 demonstrates the relationship between P.B. inhibition of mitochondrial function and protein concentration. This plot shows inhibition of β-hydroxybutyrate-supported state 3 respiration, reduction of the RC index, and activation of state 4 at two levels of protein concentrations. The I_{50} for state 3 respiration calculated from this plot was 55 and 45 mμmoles/mg protein for 3 and 6 mg protein respectively. Reduction in the RC index and activation of state 4 respiration show a similar dependency upon protein concentration. Inhibitory activity of additional methylenedioxyphenyl compounds was obtained using plots similar to that in Fig. 2. Results showing the I_{50} for state 3 respiration are summarized in Table 5. The I_{50} for piperonyl butoxide is half that for tropital when succinate and glutamate-supported respiration are compared. The I_{50} for piperonyl butoxide is lower with NADH-linked substrates than with succinate, suggesting a greater sensitivity in the NADH-dehydrogenase region. Piperonyl butoxide interferes more with oxidative phosphorylation than with electron transport as shown by the high I_{50} values obtained for succinoxidase and NADH oxidase using electron transport particles. Myristicin and piperonal did not produce 50 per cent inhibition of succinate-supported state 3 at 200 and 300 mμmoles/mg protein, respectively, or glutamate-supported state 3 at 100 or 450 mμmoles/mg. The I_{50} values are, therefore, somewhere above these figures.

Since loss of RC occurred before maximum uncoupling, the effects of certain methylenedioxyphenyl compounds on oxidative phosphorylation could not always be evaluated using polarographic methods. Therefore, oxidative phosphorylation was re-investigated using manometric methods (Table 6). The results show that piperonyl

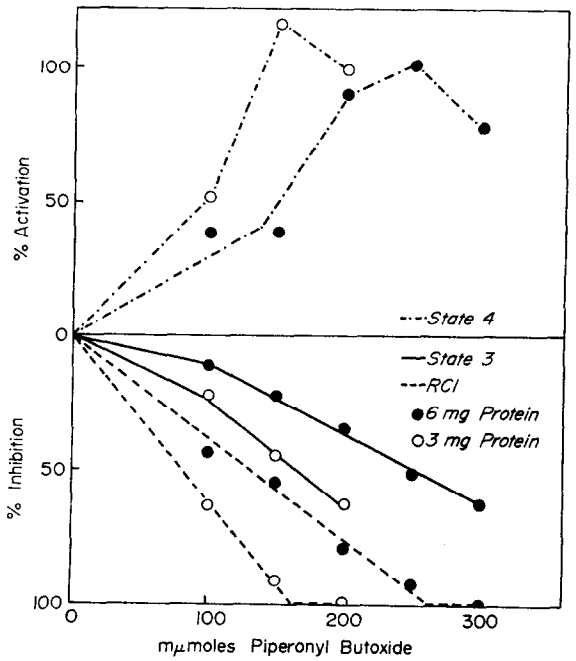

FIG. 2. Effects of protein concentration on piperonyl butoxide inhibition of mitochondrial function. Conditions of the assay are described in Methods.

TABLE 5. I_{50} VALUES FOR SEVERAL METHYLENEDIOXYPHENYL COMPOUNDS ON ELECTRON TRANSPORT AND STATE 3 RESPIRATION*

Inhibitor	I_{50} (mμmoles/mg protein) State 3 respiration Succinate	Glutamate	β-Hydroxybutyrate	Succin-oxidase	NADH-oxidase
Piperonyl butoxide	100	50	60	2500	500
Tropital	200	100			
Myristicin	>200†	>100			
Piperonal	>300	>450			

* I_{50} = mμmoles inhibitor per mg of protein inhibiting respiration by 50 per cent.
† Highest concentration tested.

butoxide uncouples succinate and glutamate-supported oxidative phosphorylation at 44 and 66 mμmoles/mg protein respectively. These figures probably do not represent the minimum levels needed to uncouple. As an uncoupling agent, tropital appears slightly less effective than PB with succinate as the substrate, but nearly equally effective with glutamate. Piperonal on the other hand had little if any effect, even at 115–160 mμmoles/mg protein. These results are in keeping with those from electrode studies. They show that uncoupling decreases in the order piperonyl butoxide, myristicin, piperonal, and that uncoupling activity is a function of the side-chain.

TABLE 6. EFFECTS OF METHYLENEDIOXYPHENYL COMPOUNDS ON OXIDATIVE PHOSPHORYLATION MEASURED MANOMETRICALLY*

Substrate	Inhibitor (mμmoles/mg protein)	Respiration (μgatoms O)	Phosphorylation (μmole P_i)	P:O
		Piperonyl butoxide		
Succinate	0	5·48	7·3	1·33
	44	6·84	6·2	0·91
	67	6·00	5·3	0·88
Glutamate	0	3·25	9·10	2·80
	66	2·69	3·50	1·30
	70	2·45	0·55	0·22
	79	2·42	1·35	0·56
		Tropital		
Succinate	0	5·21	8·08	1·55
	39	6·30	9·00	1·43
	54	5·90	8·95	1·52
	114	6·34	4·75	0·75
Glutamate	0	3·82	9·77	2·56
	72	3·63	3·60	0·99
	90	2·75	2·18	0·79
		Piperonal		
Succinate	0	5·27	8·20	1·56
	77	5·57	8·00	1·44
	115	5·56	7·45	1·34
Glutamate	0	4·17	11·45	2·75
	120	4·11	9·70	2·36
	160	3·74	10·15	2·72

* Conditions of the assay are described in Materials and Methods. Each assay contained 3·25–4·25 mg of mitochondrial protein. The final volume was 2·0 ml.

The effect of piperonyl butoxide as an uncoupler is reflected in its ability to stimulate mitochondrial ATPase activity (Table 7).

Stimulation of ATPase requires Mg^{2+} and it occurs at approximately 70 mμmoles of piperonyl butoxide/mg mitochondrial protein, a value very close to that found necessary to reduce the P:O ratio (Table 6). Table 8 shows that piperonyl butoxide has no inhibitory effects of DNP-activated ATPases.

TABLE 7. EFFECTS OF PIPERONYL BUTOXIDE ON MITOCHONDRIA ATPASE*

Inhibitor (mμmoles/mg protein)	μmoles P_i/15 min/mg protein $-Mg^{2+}$	$+Mg^{2+}$
0	0·15	0·02
7·3	0·15	0·00
28·5	0·16	0·08
71·5	0·17	0·25
107·0	0·22	0·43
214·0	0·20	0·52

* Conditions of the assay are described in Methods and Materials. Each assay contained 2·1 mg mitochondria protein and, where added, 10 μmoles Mg^{2+}. The total volume was 1·5 ml.

TABLE 8. EFFECTS OF PIPERONYL BUTOXIDE ON DNP-ACTIVATED ATPASE*

	Additions	μmoles P_i/15 min/mg protein	
		$-Mg^{2+}$	$+Mg^{2+}$
Exp. 1	None	0·07	0·12
	DNP	0·32	0·85
	DNP + PB	0·32	0·76
Exp. 2	None	0·03	0·08
	DNP	0·24	0·57
	DNP + PB	0·24	0·54

* DNP $= 2 \times 10^{-5}$ M, PB = 150 mμmoles/mg protein and protein = 2 mg.

Several attempts were made to determine *in vivo* the effect of piperonyl butoxide on oxidative phosphorylation using mitochondria isolated from rats injected with piperonyl butoxide. To determine if piperonyl butoxide selectively inhibited mitochondrial or drug metabolizing enzymes, a microsomal fraction was also isolated and cytochrome P-450, cytochrome b_5, aniline hydroxylase and aminopyrine demethylase were measured. When piperonyl butoxide was suspended in Tween 20 and rats were sacrificed 1 hr after injection (60 mg/kg in 1% v:v Tween as described by Jaffe[16]) either (1) no effects were observed or (2) aniline hydroxylase, aminopyrine demethylase, cytochrome P-450, and cytochrome b_5 were all reduced in treated microsomes. A reduction in cytochrome content suggests that enzyme inhibition could be due to solubilization of membranes rather than a direct effect upon the enzymes. In these experiments RC and ADP:O values were also reduced indicating damage to the mitochondria. In subsequent experiments piperonyl butoxide was dissolved in DMSO and the rat never received more than 0·3 ml/250 g body weight. Under these conditions no inhibition of mitochondrial function or microsomal enzymes was detected in particles isolated from rats receiving a single injection of 160–880 mg piperonyl butoxide/kg over periods of 1–20 hr. In addition, experiments were conducted in which phenobarbital (100 mg/kg) was injected daily for 3 days to induce microsomal enzymes, and piperonyl butoxide (300 mg/kg) was injected 24 hr and 1 hr prior to sacrificing the rats. In these experiments (Table 9), phenobarbital induced a 2- to 3-fold increase in microsomal enzymes and cytochromes. Piperonyl butoxide by itself neither inhibited or induced microsomal enzymes, nor did it alter oxidative phosphorylation. When given with phenobarbital, piperonyl butoxide had no inhibitory effects on induced levels of microsomal enzyme or on mitochondrial function. Preliminary experiments were also conducted in which aniline hydroxylase and aminopyrine demethylase were measured in 9000 *g* supernatants rather than purified microsomes. No effects of piperonyl butoxide could be detected. Inhibition of aniline hydroxylase and aminopyrine demethylase could, however, be demonstrated by adding piperonyl butoxide directly to the enzyme assay system.

TABLE 9. EFFECTS OF PIPERONYL BUTOXIDE ON OXIDATIVE PHOSPHORYLATION AND MICROSOMAL ENZYMES IN RATS INJECTED WITH PHENOBARBITAL*

Substrate	Condition	Control	Piperonyl butoxide	Injections Phenobarbital	Phenobarbital + piperonyl butoxide
Succinate	State 3	197 ± 6	210 ± 9	206 ± 13	211 ± 7
Succinate	State 4	43 ± 3	45 ± 3	44 ± 2	45 ± 1
Succinate	R.C.	4·59 ± 0·25	4·68 ± 0·06	4·60 ± 0·15	4·78 ± 0·13
Succinate	ADP:O	1·68 ± 0·04	1·65 ± 0·05	1·64 ± 0·05	1·69 ± 0·05
Hydroxybutyrate	State 3	70 ± 2	72 ± 4	64 ± 2	71 ± 6
Hydroxybutyrate	State 4	16 ± 1	16 ± 1	15 ± 1	16 ± 1
Hydroxybutyrate	R.C.	4·33 ± 0·13	4·74 ± 0·16	4·20 ± 0·21	4·35 ± 0·29
Hydroxybutyrate	ADP:O	2·53 ± 0·04	2·61 ± 0·11	2·65 ± 0·09	2·54 ± 0·11
Cytochrome b_5		0·334 ± 0·046	0·345 ± 0·039	0·405 ± 0·039	0·390 ± 0·037
Cytochrome P-450		0·541 ± 0·066	0·529 ± 0·035	1·030 ± 0·06	1·04 ± 0·12
Aniline hydroxylase		1·36 ± 0·26	1·86 ± 0·4	3·68 ± 0·23	3·52 ± 0·27
Aminopyrine demethylase		1·92 ± 0·32	1·84 ± 0·11	4·09 ± 0·26	4·13 ± 0·15

* State 3 and state 4 respiration are expressed as mμgatoms O per min per mg protein. Aniline hydroxylase and aminopyrine demethylase are expressed as mμmoles of *p*-aminophenol and μg formaldehyde formed, respectively, during 20 min of incubation per mg protein. Cytochrome P-450 and b_5 are expressed as mμmoles per mg microsomal protein. Each value is the $\bar{x} \pm$ S. E. of four rats.

DISCUSSION

The present study demonstrates that methylenedioxyphenyl compounds interfere with oxidative phosphorylation in isolated liver mitochondria. Piperonyl butoxide and tropital were most effective, myristicin was slightly less effective and piperonal had little or no effect. The action of piperonyl butoxide and tropital was primarily on the process of phosphorylation rather than electron transport since the I_{50} for ADP-stimulated respiration (state 3) was ten to twenty-five times lower than for succinoxidase or NADH-oxidase. Uncoupling of oxidation and phosphorylation was also demonstrated both polarographically by the reduction in the ADP:O ratio and manometrically by reduction in the P:O ratio.

Uncoupling of oxidation and phosphorylation and inhibition of state 3 respiration are associated with the side-chain of the methylenedioxyphenyl moiety. This is in contrast to the mechanism of inhibition believed responsible for inhibition of microsomal enzymes.[5–7] Considerable evidence has accumulated that inhibition of drug hydroxylation or epoxidation[5,7] is due to competition of the methylenedioxyphenyl moiety for the substrate binding site on the enzyme.[5,6] Thus, most compounds tested containing this group have been found to be effective inhibitors.[7,8] Though the side-chain is important in conferring uncoupling activity, the possibility that the methylenedioxyphenyl moiety is directly involved can not be neglected. The function of the side-chain could be to allow binding to the membrane, or enzyme, in a manner which facilitates interaction of the methylened ioxyphenyl moiety with the coupling site.

The level of piperonyl butoxide found to be inhibitory to mitochondrial function *in vitro* (I_{50} for state 3 of approximately 50 mμmoles/mg of mitochondrial protein) is similar to that calculated from published values for *in vitro* inhibition of pig liver microsomal enzymes by several methylenedioxyphenyl compounds.[5,7] In fact, the

I_{50} obtained for piperonyl butoxide in the present study represents the lower limits of I_{50} obtained for methylenedioxyphenyls on microsomal enzymes. Converting literature values from molar to mμmoles per mg of microsomal protein, the I_{50} of various methylenedioxyphenyls on aldrin epoxidation is 110–135 mμmoles/mg protein[7] and the I_{50} of sesamex and SKF-525 A for aldrin epoxidation, aniline hydroxylase and 1,3-benzo-dioxide cleavage ranges from 35 to 2000 mμmoles/mg protein,[5] depending on whether the calculation is based upon the use of 2 or 20 mg of protein per assay.[5] From these calculations it seems probable that piperonyl butoxide could be equally effective *in vivo* as an inhibitor of mitochondrial or microsomal enzymes. A number of experiments were conducted to test if, indeed, piperonyl butoxide interfered with mitochondria function *in vivo* and if inhibition was selective for either mitochondrial or microsomal enzymes. Injections of piperonyl butoxide ranging from 160–880 mg/kg produced no inhibition of either mitochondrial or microsomal enzymes when the particles were isolated from treated rats and the enzymes assayed *in vitro*. These results are in contrast to those of Jaffe *et al.*[16,17] Regarding the results of Jaffe *et al.*[16] however, it seems doubtful to us that piperonyl butoxide injected into rats should inhibit microsomal enzymes measured *in vitro* using purified microsomes. Evidence indicates that methylenedioxyphenyl compounds are competitive inhibitors of microsomal enzymes[5,6] and, as such, act as alternate substrates for the enzymes.[5] Wilkinson and Hicks[5] have shown that the benzol-dioxole ring is cleaved by microsomal enzymes and does not form a stable enzyme–inhibitor complex. This being the case, the small amount of synergist bound to isolated microsomes should be rapidly removed from the enzyme in the high concentrations of substrate used for the assay. The findings of Jaffe *et al.*[16,17] might be attributable to the fact that their enzymes measurements were made using a 10,000 *g* supernatant[16] which could contain enough of the injected synergist to inhibit enzyme activity. However, we were not able to demonstrate inhibition of microsomal enzymes in similar preparations of rat livers after injecting piperonyl butoxide i.p.

Though it is still not clear if piperonyl butoxide inhibits mitochondrial function *in vivo*, the available evidence suggests that piperonyl butoxide does selectively inhibit microsomal enzymes *in vivo*. As shown in the present experiments, 880 mg piperonyl butoxide kg had no measurable effect on oxidative phosphorylation. In contrast, PB equivalent to 250 mg/kg delayed biliary excretion of benz(o)pyrene,[8] and 120 mg/kg significantly reduced hexobarbital metabolism and increased hexobarbital-induced sleeping time,[3] presumably because of the action of piperonyl butoxide on microsomal enzymes. Though it is possible that damaged mitochondria are eliminated during isolation, it must be concluded from the present data that P.B. has little or no effect on mitochondria *in vivo* and that microsomal enzymes are selectively inhibited.

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