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Inhibition of microsomal drug metabolism by methylenedioxybenzenes

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METHYLENEDIOXYBENZENE compounds represent a widely employed class of chemicals whose most outstanding property is the synergism of insecticides, namely pyrethrins and carbamates. Furthermore, available evidence indicates that the synergistic effect is due to inhibition of the inactivation of the pesticide.[1] Evidence has also been presented showing that methylenedioxybenzenes inhibit *in vitro* the microsomal metabolism of parathion,[2, 3] carbamates,[4] naphthalene[5] and chlorinated hydrocarbon-type insecticides[6–8] as well as prolong hexobarbital sleeping time in mice.[9]

The experiments reported in this paper represent the results of a systematic study of the inhibitory actions, both *in vitro* and *in vivo*, of three methylenedioxybenzenes, namely piperonyl butoxide, sesamex and tropital (piperonal *bis* (2[2′-*n*-butoxyethoxy] ethyl) acetal). SKF 525-A (2-diethylamino-ethyl-2,2-diphenylvalerate HCl) was included in many of the experiments for purposes of comparison, since it is a known inhibitor of microsomal drug metabolism. In addition, the effect of enzyme induction of the inhibitory action of piperonyl butoxide has been studied.

EXPERIMENTAL

Male Holtzman rats weighing 75–125 g were employed as experimental animals. Microsomal fractions were prepared as described previously.[10]

Incubation mixtures contained varying quantities of substrates and inhibitors, 3 μmole nicotinamide, 15 μmole magnesium chloride, 50 μmole phosphate buffer (pH 7·4), an NADPH-generating system consisting of either 20 μmole glucose 6-phosphate, 1 μmole NADP and 1 Enzyme Units glucose 6-phosphate dehydrogenase or 10 μmole DL-isocitrate, 1 μmole NADP and 1 Enzyme Units isocitrate dehydrogenase and 0·5 ml of the microsomal enzyme preparation in a total volume of 3·0 ml. When formaldehyde was sought as the product, the incubation mixtures contained 3 μmole of neutralized semicarbazide Methylenedioxybenzenes were added to the incubation mixtures in ethanol or acetone when ethylmorphine or *p*-nitroanisole, respectively, served as the substrates. The total amount of solvent added was always 10 μl. Because of inhibition of aniline hydroxylation by most solvents and activation by acetone,[10] the inhibitor was added to the flask and the solvent evaporated before further additions were made. Incubations were carried out at 37° in an atmosphere of air. Incubation times were 30 min when aniline served as the substrate, 6 min in the case of *p*-nitroanis ole and 12 min in the case of ethylmorphine; under these conditions product formation was linear with time.

p-Aminophenol was measured according to the method of Kato and Gillette, [11]formaldehyde produced by the *N*-demethylation of ethylmorphine as described by Nash,[12] and *p*-nitrophenol produced by the *O*-demethylation of *p*-nitroanisole was measured as described by Netter.[13] Hexobarbital blood levels were estimated by gas chromatography by Method A of the procedure described by Anders.[14]

Enzyme kinetic constants were determined by the method described by Cleland.[15] Inhibition was interpreted as being competitive when the 1/v intercepts (no inhibitor versus inhibitor) were not significantly different ($P > 0.05$). Other statistical analyses employed have been described by Steel and Torrie.[16]

RESULTS AND DISCUSSION

As can be seen in Table 1, piperonyl butoxide, sesamex, tropital and SKF 525-A inhibited the metabolism of all the substrates studied. When ethylmorphine served as the substrate, the inhibition produced by piperonly butoxide, tropital and SKF 525-A was approximately equal; sesamex was found to be about an order of magnitude less potent than the other agents. Similar results were obtained with *p*-nitroanisole as the substrate, except that in this case SKF 525-A proved to be more

potent than the other agents. Of the compounds tested, sesamex proved to be the most potent inhibitor of aniline metabolism. The inhibition produced by sesamex was 5 to 10 times greater than that obtained with piperonyl butoxide, tropital or SKF 525-A.

TABLE 1. INHIBITION *in vitro* OF MICROSOMAL DRUG METABOLISM BY METHYLENEDIOXYBENZENES

Inhibitor	I_{50} (M)		
	Substrate		
	Ethylmorphine*	*p*-Nitroanisole†	Aniline‡
Piperonyl butoxide	$2{\cdot}0 \pm 0{\cdot}3 \times 10^{-5}$ (4)§	$4{\cdot}2 \pm 0{\cdot}7 \times 10^{-5}$ (3)	$1{\cdot}3 \pm 0{\cdot}8 \times 10^{-3}$ (3)
Sexamex	$1{\cdot}7 \pm 0{\cdot}3 \times 10^{-4}$ (3)	$1{\cdot}5 \pm 0{\cdot}2 \times 10^{-4}$ (3)	$1{\cdot}1 \pm 0{\cdot}2 \times 10^{-4}$ (3)
Tropital	$2{\cdot}3 \pm 1{\cdot}3 \times 10^{-5}$ (4)	$6{\cdot}7 \pm 0{\cdot}6 \times 10^{-5}$ (3)	$1{\cdot}0 \pm 0{\cdot}5 \times 10^{-3}$ (4)
SKF 525-A	$0{\cdot}8 \pm 0{\cdot}2 \times 10^{-5}$ (3)	$0{\cdot}3 \pm 0{\cdot}1 \times 10^{-5}$ (3)	$2{\cdot}0 \pm 0{\cdot}4 \times 10^{-3}$ (3)

* Substrate concn, $8{\cdot}0 \times 10^{-4}$ M.

† Substrate concn, $4{\cdot}0 \times 10^{-4}$ M.

‡ Substrate concn, $1{\cdot}0 \times 10^{-3}$ M.

§ Values are shown as mean ± S.E.; numbers in parentheses refer to the number of determination

Experiments were conducted in order to determine whether the inhibition produced by methylenedioxybenzenes was competitive or noncompetitive. When piperonyl butoxide served as the inhibitor, competitive inhibition was consistently obtained with ethylmorphine ($K_i = 3{\cdot}0 \pm 1{\cdot}1 \times 10^{-6}$ M, n = 6) and *p*-nitroanisole ($K_i = 6{\cdot}0 \pm 0{\cdot}9 \times 10^{-7}$ M, n = 5) as the substrates. In the case of sesamex and tropital, the type of inhibition proved to be variable and could not be determined with certainty.

Table 2 shows the effect of varying concentrations of piperonyl butoxide, sesamex, tropital and SKF 525-A on the half-life ($T_{\frac{1}{2}}$) *in vivo* of hexobarbital. At a ratio of inhibitor:hexobarbital of 0·5,

TABLE 2. EFFECT OF METHYLENEDIOXYBENZENES ON THE METABOLISM *in vivo* OF HEXOBARBITAL*

Inhibitor†	Dose (μmoles/kg)	$T_{\frac{1}{2}}$ (min)	P
None		42 ± 4 (8)‡	
Piperonyl butoxide	160	58 ± 4 (7)	< 0.02
Piperonyl butoxide	320	100 ± 14 (7)	< 0·001
Sesamex	160	45 ± 8 (8)	> 0·1
Sesamex	320	96 ± 16 (7)	< 0·01
Tropital	160	66 ± 9 (7)	< 0·05
Tropital	320	89 ± 12 (8)	< 0·01
SKF 525-A	16	56 ± 11 (6)	> 0·1
SKF 525-A	32	170 ± 52 (6)	< 0·02

* Dose, 320 μmole/kg.

† The inhibitors were suspended in 1% (w/v) polysorbate 80 and administered i.p. in a volume of 1 ml/100 g body wt. 45 min before the hexobarbital was given.

‡ Values are shown as the mean ± S.E.; numbers in parentheses refer to the number of determinations.

piperonyl butoxide and tropital produced a significant prolongation of the $T_{\frac{1}{2}}$ of hexobarbital. However, when the inhibitor:hexobarbital ratio was 1·0, piperonyl butoxide, sesamex and tropital all produced a significant increase in the $T_{\frac{1}{2}}$ of hexobarbital. At a ratio of inhibitor:hexobarbital of 0·05, SKF 525-A did not significantly alter the $T_{\frac{1}{2}}$ for hexobarbital; the effect of SKF 525-A was significant when the ratio was 0·1.

Results of experiments designed to test the effect of enzyme induction by phenobarbital and 3-methylcholanthrene on the inhibitory properties of piperonyl butoxide are shown in Table 3. When the test system employed was the *N*-demethylation of ethylmorphine *in vitro*, it can be seen that treatment with both phenobarbital and 3-methylcholanthrene significantly reduced the inhibitory action of piperonyl butoxide. In the case of hexobarbital, sleeping time was taken as a measurement of hexobarbital metabolism *in vivo*. Hexobarbital sleeping time was significantly prolonged in control

TABLE 3. EFFECT OF ENZYME INDUCTION BY PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON THE INHIBITORY PROPERTIES *in vitro* AND *in vivo* OF PIPERONYL BUTOXIDE

Treatment	Metabolism of ethylmorphine *in vitro*		Metabolism of hexobarbital *in vivo*		
			Sleeping time (min)		
	% Inhibition*	P	Hexobarbital†	Hexobarbital + piperonyl butoxide†	P
Vehicle (saline)	57·8 ± 3·3 (4)‡		73 ± 4 (6)‡	142 ± 11 (6)‡	< 0·001
Phenobarbital§	17·6 ± 8·1 (4)	< 0·01	8 ± 1 (6)	11 ± 1 (5)	> 0·2
Vehicle (corn oil)	64·9 ± 11·0 (4)		101 ± 19 (6)	177 ± 31 (6)	> 0·05
3-Methylcholanthrene§	8·6 ± 5·0 (4)	< 0·01	230 ± 20 (6)	216 ± 15 (5)	> 0·5

* Inhibitor concn, 1×10^{-4} M; substrate concn, 8×10^{-4} M. Uninhibited rates of metabolism, expressed as μmoles formaldehyde/g liver/hr, were as follows: saline, 2·54; phenobarbital, 12·00; corn oil, 2·00; 3-methylcholanthrene, 1·29.

† Piperonyl butoxide, 420 μmole/kg, was suspended in 1% (w/v) polysorbate 80 and administered 45 min before hexobarbital, 420 μmole/kg, was given. In both cases the drugs were administered i.p. in a volume of 1 ml/100 g body wt.

‡ Values are shown as mean ± S.E.; numbers in parentheses refer to the number of determinations.

§ Phenobarbital schedule, 160 μmole/kg of phenobarbital once daily for 5 days; 3-methylcholanthrene schedule, 75 μmole/kg of 3-methylcholanthrene once daily for 5 days.

(saline-treated) animals receiving piperonyl butoxide. However, in rats treated with phenobarbital, hexobarbital sleeping time was markedly reduced and piperonyl butoxide failed to produce any significant prolongation of sleeping time. In the case of the corn oil control rats, piperonyl butoxide produced a prolongation of hexobarbital sleeping time, but the effect was not significant. Treatment with 3-methylcholanthrene resulted in a prolonged hexobarbital sleeping time; piperonyl butoxide did not produce any significant alteration in the 3-methylcholanthrene-treated rats.

The results of the inhibition studies with the methylenedioxybenzenes show that these agents are effective inhibitors, both *in vitro* and *in vivo*, of microsomal drug metabolism. When studied in a system *in vitro* with ethylmorphine as the substrate, piperonyl butoxide and tropital were nearly as potent as SKF 525-A; with *p*-nitroanisole as the substrate, SKF 525-A proved to be considerably more potent than the methylenedioxybenzenes. These findings are difficult to compare directly with those of other workers, since the effectiveness of the inhibitors varies considerably depending on the substrate employed.[5, 6]

When studied as inhibitors of the metabolism of hexobarbital *in vivo*, piperonyl butoxide, sesamex and tropital were found to be approximately equipotent in prolonging the $T_{\frac{1}{2}}$ of hexobarbital (Table 2). SKF 525-A, however, proved to be much more effective as an inhibitor of hexobarbital metabolism than any of the methylenedioxybenzenes tested.

Piperonyl butoxide was also found to prolong significantly the hexobarbital sleeping time (Table 3). This finding is in agreement with the data showing that piperonyl butoxide inhibits the metabolism of hexobarbital *in vivo* (Table 2) and supports the contention of Fine and Molloy[9] that synergists prolong hexobarbital and pentobarbital sleeping time by preventing the metabolism of the barbiturate.

The mechanism by which the methylenedioxybenzenes inhibit microsomal drug metabolism may involve an alternative substrate mechanism, although this cannot be established with certainty on the basis of the available information. Evidence favoring an alternative substrate mechanism has been presented by Wilkinson[17] and by Casida *et al.*,[18] who showed that the compounds can serve as substrates for the microsomal mixed function oxidases, and by Philleo *et al.*,[5] who showed that methylenedioxybenzenes are competitive inhibitors of certain microsomal oxidations. Finally,

before an alternative substrate mechanism can be definitely established, it must be shown that the Michaelis constant of the compound, when employed as a substrate, is not significantly different from the inhibition constant when serving as an inhibitor.[19, 20] Although Hennessy[21] has postulated that methylenedioxybenzenes might interact with enzymes via hydride transfer and formation of benzodioxolium ions, this mechanism would lead to irreversible inhibition and thus not be consistent with an alternative substrate mechanism.

Finally, it was of interest to determine the effect of enzyme induction of the inhibitory action of piperonyl butoxide. The data in Table 3 show that, while piperonyl butoxide is an effective inhibitor of drug metabolism both *in vivo* and *in vitro* in normal rats, its effectiveness is markedly diminished by chronic treatment with either phenobarbital or 3-methylcholanthrene. A possible explanation for these observations is that induction results in a rapid metabolism of piperonyl butoxide to products having minimal inhibitory capacity. In this regard piperonyl butoxide resembles ethyl 2-diethylaminoethyl 2-ethyl-2-butylmalonate (Sch 5712), which has been shown to be less effective as an inhibitor of hexobarbital metabolism in phenobarbital-treated animals.[22] In contrast, SKF 525-A is an effective inhibitor in phenobarbital-treated rats, since its products of metabolism are potent inhibitors.[23] The finding that induction with 3-methylcholanthrene prolongs hexobarbital sleeping time (Table 3) is in agreement with the observations by Gram *et al.*,[24] who found a significantly lower rate of hexobarbital metabolism in smooth liver microsomes derived from 3-methylcholanthrene-treated rabbits.

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