Biochemical Pharmacology, Vol. 16, pp. 1195–1210. Pergamon Press Ltd. 1967. Printed in Great Britain

THE RELATIONSHIP BETWEEN MICROSOMAL EPOXIDATION AND LIPID PEROXIDATION IN HOUSEFLIES AND PIG LIVER AND THE INHIBITORY EFFECT OF DERIVATIVES OF 1,3-BENZODIOXOLE (METHYLENEDIOXYBENZENE)

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(*Received* 7 *November* 1966; *accepted* 2 *February* 1967)

Abstract—The epoxidation of aldrin by microsomal preparations from houseflies and pig liver was markedly inhibited by a number of derivatives of 1,3-benzodioxole. The epoxidation system from the housefly appeared substantially more susceptible to inhibition by these compounds. An active $NADPH_2$-requiring microsomal lipid peroxidation system was found to compete with aldrin epoxidation and inhibitors of lipid peroxidation such as EDTA, Mn^{2+}, Co^{2+}, α,α'-dipyridyl and BHT resulted in a stimulation of dieldrin formation. Housefly preparations contained an endogenous inhibitor of lipid peroxidation.

THE *in vivo* epoxidation of a number of unsaturated cyclodiene insecticides such as aldrin, isodrin and heptachlor has been reported in several vertebrate[1] and invertebrate species[2] as well as in some microorganisms.[3] In mammals these reactions are catalysed by liver microsomal oxidases[4,5] of the type which metabolize a variety of drugs. Similarly in the housefly, epoxidation has been shown to be associated with a microsomal oxidase.[6,7]

1,3-Benzodioxole (methylenedioxyphenyl) derivatives, such as the pyrethrin synergists sesamex and piperonyl butoxide, may potentiate or antagonise the action of a variety of insecticides.[8] These effects appear to be associated with their ability to inhibit microsomal oxidases such as those responsible for the hydroxylation of naphthalene,[9] the oxidation of phosphorothionates,[8,10] carbamates[11–13] and the epoxidation of aldrin, isodrin and heptachlor.[4,5,7] The present study provides additional information on the comparative effects of some simple 1,3-benzodioxole derivatives on a microsomal oxidase from pig liver and from whole houseflies, using the epoxidation of aldrin as a measure of oxidase activity. The simplified planar structure of aldrin and its epoxide, dieldrin, is shown below.

Studies involving the use of tissue homogenates and isolated particles such as mitochrondria and microsomes are often complicated by reactions involving the peroxidation of endogenous lipids. It has been shown by Hockstein, Nordenbrand and Ernster[14] that under aerobic conditions such reactions can be initiated by $NADPH_2$

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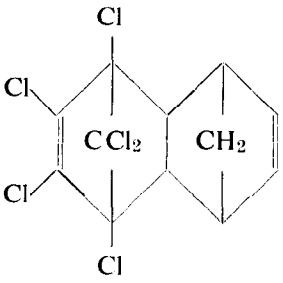
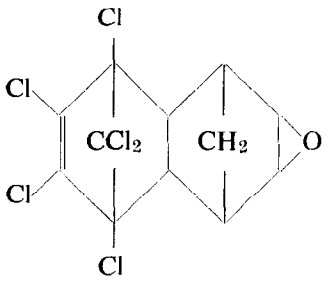
in rat liver microsomes. Since many of the oxidative reactions involved in the metabolism of drugs and insecticides exhibit a similar requirement the relationship between epoxidation and lipid peroxidation has been examined.



Aldrin



Dieldrin

MATERIALS AND METHODS

Materials

Aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-1,4-endo-exo-5,8-di-methanonaphthalene) was obtained as a technical sample from Shell and was recrystallized, m.p. 103–106°.

The 1,3-benzodioxole derivatives (I to XVII) were synthesized and details of the several synthetic procedures employed are described elsewhere.[15] Sesamex [the 2-(2-ethoxyethoxy)ethyl 3,4-(methylenedioxy) phenyl acetal of acetaldehyde] was obtained from Schulton Fine Chemicals Inc., N.Y. and β-diethylaminoethyl diphenylpropylacetate hydrochloride (SKF-525-A) was provided by Smith Kline & French Laboratories, England. A sample of naphtho-(2,3-d)-1,3-dioxole was kindly provided by Dr. Julius Hyman of the Fundamental Research Company, Berkeley, California. Butylated hydroxytoluene (BHT) was obtained from Koch Light Laboratories, and α, α′-dipyridyl from Hopkin & Williams. Nicotinamide-adenine-dinucleotide phosphate (NADP), Glucose-6-phosphate (G-6-P) and G-6-P dehydrogenase were purchased from Boehringer, London, and all other chemicals used were A.R. grade.

Sources and preparation of microsomes

The insect microsomes were prepared in 1·15% KCl from a dieldrin-resistant strain of housefly (*Musca domestica vicina*) as previously described by Ray.[7] The pellet was finally suspended in sucrose (0·25 M), Tris phosphate buffer (10^{-2} M, pH 8·3), NADP (5×10^{-4} M), nicotinamide (10^{-2} M), G-6-P (10^{-2} M) and 2% w/v bovine plasma albumen, fraction V.

Freshly killed pig's liver was obtained from the local slaughterhouse and was homogenized in 1·15% KCl (1:4 w/v) in a Waring–Blendor. The microsomal pellet was obtained by centrifugation at 90,000 *g* max for 1 hr after an initial spin at 20,000 *g* max for ½ hr to remove larger cellular fractions. The pellet was resuspended, washed once in 1·15% KCl and was recentrifuged at 90,000 *g* max for 1 hr before being finally resuspended in KCl by a hand-operated PTFE pestle. All operations were carried out at 0–4°. The final microsomal suspension usually contained 20–25 mg microsomal protein per ml as measured by the Biuret method.[16]

Incubation procedure

The usual incubation mixture contained 0·5 ml of the appropriate microsomal suspension and other components were as follows (final concentration) making up a total volume of 5 ml; Tris phosphate (5×10^{-2} M), G-6-P ($2{\cdot}4 \times 10^{-3}$ M), nicotinamide ($2{\cdot}45 \times 10^{-3}$ M), KCl ($1{\cdot}23 \times 10^{-2}$ M), NADP ($5{\cdot}2 \times 10^{-5}$ M), G-6-P dehydrogenase (1·4 units). The Tris phosphate was buffered at pH 7·3 in incubations with the pig liver microsomes and at 8·3 in those from the houseflies, these being the respective pH optima for the two systems. In the incubation mixtures employing housefly microsomes, the final concentrations of NADP, nicotinamide and G-6-P given above are modified as a result of the inclusion of these materials in the microsome suspension medium. In most cases EDTA (10^{-3} M) was included in the incubation mixtures with pig liver microsomes, and cyanide (2×10^{-4} M) with those from houseflies.

Appropriate solutions of the inhibitors were added in either alcohol or acetone (10 μl) and the reaction was initiated by addition of aldrin (100 μg) in alcohol (100 μl). Incubations were carried out in 25 ml conical flasks shaken for 10 min at 30° and the reaction was stopped by addition of 3 ml acetone. The subsequent extraction procedure was identical to that previously described.[7] Aliquots of the light petroleum spirit (40–60° b.p. fraction) extract were assayed for dieldrin by the peak-height method on a Pye Panchromatograph provided with a 100-mc tritium foil electron capture detector. Column conditions were those described by Ray.[7]

The I_{50} values were obtained by plotting the per cent inhibition against the molar concentration of the inhibitor on semi-logarithmic paper. Three or four concentrations of each inhibitor were used and the lines in each case were fitted by eye.

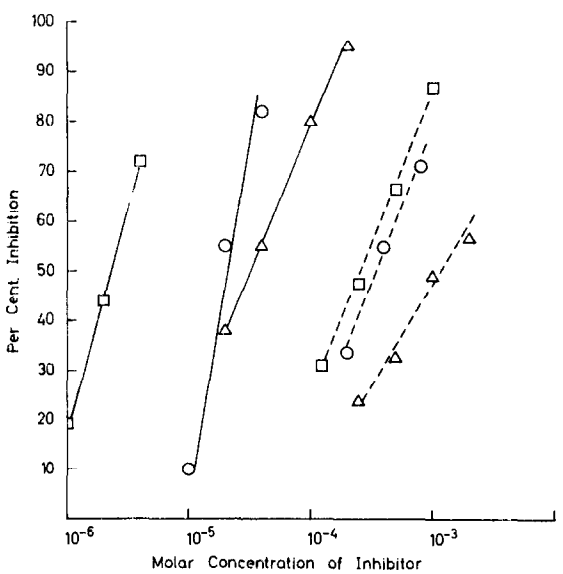


FIG. 1. The inhibition of aldrin epoxidation in housefly and pig liver microsomes. Incubation mixtures and conditions as described in Table 1: – – – – Pig liver microsomes; ——— Housefly microsomes. □ = 5,6-Dibromo-1,3-benzodioxole (X); ○ = 5-dimethylamino-6-nitro-1,3-benzodioxole (XVII); △ = 5-nitro-1,3-benzodioxole (VI).

Lipid peroxidation incubation and assay

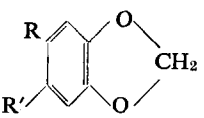
The estimation of lipid peroxidation as measured by the formation of the sodium thiobarbiturate (TBA) chromogen was based on the method described by Hunter *et al.*[17] Incubations were carried out at 30° for a period of 5 min, unless otherwise stated, using 0·2 ml of the microsome suspension in a total volume of 3 ml. The other constituents of the incubation mixture were as follows (final concentration): Tris phosphate, pH 7·3 ($5·2 \times 10^{-2}$ M), G-6-P ($2·5 \times 10^{-3}$ M), NADP ($4·3 \times 10^{-5}$ M), nicotinamide ($2·53 \times 10^{-3}$ M), KCl ($1·28 \times 10^{-2}$ M), and G-6-P dehydrogenase (1·4 units). The reaction was terminated by the addition of 1·63 ml trichloroacetic acid (TCA) (15·4% in 1·15 N HCl); 2·5 ml TBA (0·5% solution) was added and the mixture heated for 10 min in a boiling-water bath. The suspension was centrifuged and the absorption of the supernatant measured at 534 mμ against a reference solution obtained by addition of the TCA to the reaction mixture before the microsomal suspension.

RESULTS

Inhibition of aldrin epoxidation by 1,3-*benzodioxoles*

The results of the inhibition experiments are shown in Table 1. In spite of the considerable variation in the substituent groups of the inhibitors employed, the I_{50} values

TABLE 1. THE INHIBITION BY 1,3-BENZODIOXOLES OF ALDRIN EPOXIDATION IN MICROSOMES PREPARED FROM HOUSEFLIES AND PIG LIVER



Compound	R	R′	I_{50} (Molar concentration) Pig liver microsomes	Housefly microsomes
I	H	H	$2·5 \times 10^{-3}$	$4·8 \times 10^{-4}$
II	H	OH	$2·2 \times 10^{-4}$	$7·2 \times 10^{-5}$
III	H	CH_3	$3·6 \times 10^{-4}$	$1·5 \times 10^{-5}$
IV	H	Cl	$5·0 \times 10^{-4}$	$6·2 \times 10^{-5}$
V	H	Br	$3·6 \times 10^{-4}$	$4·7 \times 10^{-6}$
VI	H	NO_2	$1·3 \times 10^{-3}$	$3·2 \times 10^{-5}$
VII	H	CH_3O	$1·2 \times 10^{-3}$	$2·8 \times 10^{-5}$
VIII	H	NH_2	$4·8 \times 10^{-4}$	6% at $4·0 \times 10^{-4}$
IX	Cl	Cl	$2·5 \times 10^{-4}$	$4·8 \times 10^{-6}$
X	Br	Br	$2·7 \times 10^{-4}$	$2·3 \times 10^{-6}$
XI	Cl	CH_3O	$3·1 \times 10^{-4}$	$4·3 \times 10^{-6}$
XII	Br	CH_3O	$3·1 \times 10^{-4}$	$4·6 \times 10^{-6}$
XIII	NO_2	CH_3O	$6·4 \times 10^{-4}$	$7·0 \times 10^{-6}$
XIV	NO_2	Cl	$7·0 \times 10^{-4}$	$1·9 \times 10^{-5}$
XV	NO_2	NO_2	*	$3·3 \times 10^{-5}$
XVI	NO_2	$NHCH_3$	*	$1·8 \times 10^{-5}$
XVII	NO_2	$N(CH_3)_2$	$3·6 \times 10^{-4}$	$2·0 \times 10^{-5}$
	Naphtho-(2,3-d)-1,3-dioxole		$1·5 \times 10^{-3}$	$9·0 \times 10^{-6}$
	Sesamex		$7·0 \times 10^{-4}$	$4·7 \times 10^{-5}$
	SKF 525 A		$5·0 \times 10^{-4}$	—

Incubations at 30° for 10 min. Incubation medium (final concentration): Tris phosphate ($5·0 \times 10^{-2}$ M), pH 8·3 and 7·3 for housefly and pig liver microsomes respectively, nicotinamide ($2·45 \times 10^{-3}$ M), NADP ($5·2 \times 10^{-5}$ M), G-6-P ($2·4 \times 10^{-3}$ M), KCl ($1·23 \times 10^{-2}$ M), G-6-P dehydrogenase (1·4 units), 0·5 ml of appropriate microsomal suspension containing approximately 12 mg protein. Aldrin (100 μg) added in 100 μl alcohol and inhibitors added in 10 μl alcohol or acetone, total volume 5 ml. EDTA (10^{-3} M) added to pig liver microsomes and cyanide (2×10^{-4} M) to those from houseflies.

* Not measurable due to interference with dieldrin estimation.

obtained with pig-liver microsomes are remarkably constant, 14 of the 17 compounds evaluated having values between $2{\cdot}2 \times 10^{-4}$ and $7{\cdot}0 \times 10^{-4}$ M.

The aldrin epoxidation system in housefly microsomes appears to be more susceptible to inhibition by derivatives of 1,3-benzodioxole and moreover there appears to be more variation between the I_{50} values for different inhibitors. Apart from 5-amino-1, 3-benzodioxole (VIII), which is notable for its lack of inhibition of the housefly system, I_{50} values for the housefly are approximately 10–100 times lower than those obtained with pig liver microsomes, ranging from $2{\cdot}3 \times 10^{-6}$ M for 5, 6-dibromo-1,3-benzodioxole (X) to $4{\cdot}8 \times 10^{-4}$ M for I.

SKF 525-A was found to have an I_{50} similar to those of the 1,3-benzodioxoles in the pig liver system.

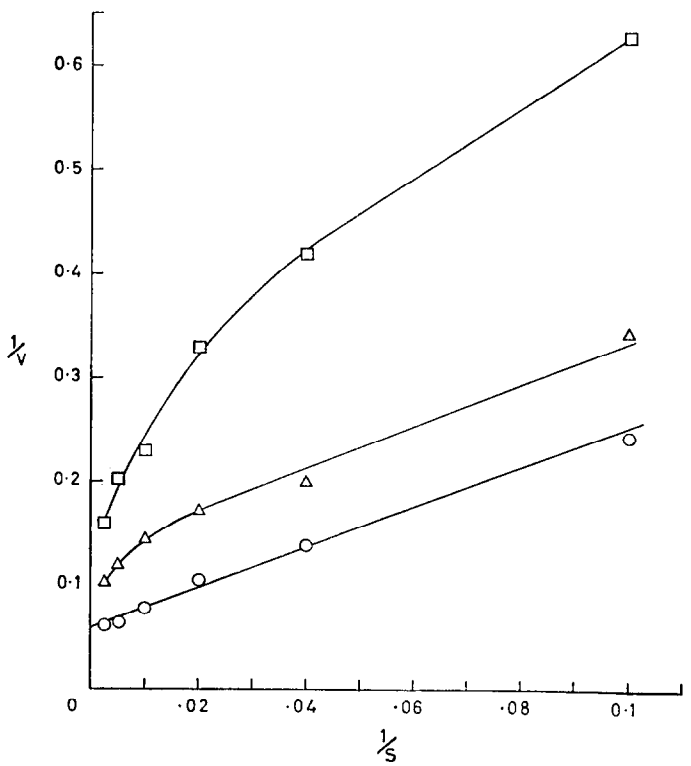


FIG. 2. Reciprocal plots of aldrin epoxidation by pig liver microsomes and the effect of 1,3-benzodioxole inhibitors. Incubation mixture as described in Table 1; incubation time 5 min; aldrin added in 100 μl ethanol; inhibitors added in 10 μl ethanol. S = aldrin added (μg); V = dieldrin produced (μg/5 min). ○ = No inhibitor; △ = 5-methoxy-1,3-benzodioxole (VII) 10^{-3} M; □ = sesamex 2×10^{-4} M.

Attempts to determine whether the inhibition of aldrin epoxidation by the 1,3-benzodioxoles is competitive or non-competitive have not been entirely satisfactory. Fig. 2 is typical of the Lineweaver–Burk plots which have been obtained with pig liver microsomes. It can be seen that in the presence of an inhibitor the lines show a curvilinear trend similar to that previously described with housefly microsomes.[7]

Effect of aldrin solubility on epoxidation

In view of the low solubility of aldrin in aqueous media (< 0·1 ppm) it was surprising to find that there appears to be a normal relationship between the concentration of aldrin in the reaction medium and the rate of dieldrin formation. This question was examined in experiments in which the concentration of aldrin was varied by changing the volume of the reaction medium to which it was added. The results are given in Table 2 together with the results of a parallel experiment with the same

TABLE 2. THE EFFECT OF ALDRIN SOLUBILITY ON EPOXIDATION

Incubation medium						
Reaction mixture (ml)	Microsomal suspension (ml)	Total volume (ml)	Aldrin added (μg)	Conc. in incub. med. (μg/ml)	Dieldrin produced (μg)	Aldrin per 0·2 ml microsomes (μg)
0·8	0·2	1	50	50	9·2	50
1·8	0·2	2	50	25	9·3	50
3·8	0·2	4	50	12·5	9·4	50
4·8	0·2	5	50	10	10·2	50
4·8	0·2	5	200	40	15·0	200
4·8	0·2	5	100	20	12·2	100
4·8	0·2	5	50	10	9·1	50
4·8	0·2	5	20	4	7·3	20

The incubation medium consisted of the reaction mixture, to which was added 0·2 ml microsomal suspension containing approximately 5 mg protein. Incubations were at 30° for 10 min. The reaction mixture contained Tris phosphate pH 7·3 ($5{\cdot}8 \times 10^{-2}$ M), G-6-P ($2{\cdot}8 \times 10^{-3}$ M), nicotinamide ($2{\cdot}85 \times 10^{-3}$ M), KCl ($1{\cdot}43 \times 10^{-2}$ M), NADP ($6{\cdot}0 \times 10^{-5}$ M), G-6-P dehydrogenase (1·4 units), EDTA ($1{\cdot}16 \times 10^{-3}$ M).

preparation in which the concentration was varied in the more usual manner, i.e. by the addition of different amounts of aldrin to a constant volume of reaction medium. In all cases the amount of microsomal material was kept constant. These results show that the rate of epoxidation is related not to the concentration of aldrin in the reaction medium but to the concentration in or on the microsomal material in suspension.

Endogenous lipid peroxidation in pig liver microsomes

Preliminary experiments to establish a relationship between the oxygen uptake by pig liver microsomes and the disappearance of $NADPH_2$ were unsuccessful. The amount of oxygen consumed was variable and always in excess of that required to oxidize the $NADPH_2$ added.

A similar phenomenon has been reported by Hochstein *et al.*[14] for rat liver microsomes where the excess oxygen uptake was associated with the formation of the TBA chromogen which is known to be produced as a result of lipid peroxidation. Similar experiments with pig liver microsomes, incubated with $NADPH_2$ or NADP plus the $NADPH_2$-generating system, though without addition of Fe^{++} and/or pyrophosphate, showed a rapid production of the TBA chromogen reaching a maximum in about 12 min (Fig. 3).

Factors influencing lipid peroxidation and their effect on the epoxidation of aldrin

In view of the reported suppression of lipid peroxidation in rat liver microsomes catalysing the oxidative demethylation of codeine and aminopyrine,[18] it became of

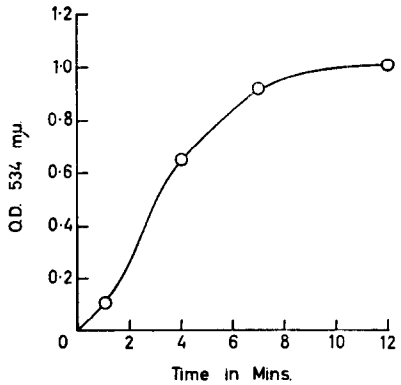


FIG. 3. Rate of $NADPH_2$ stimulated lipid peroxidation in pig liver microsomes. Incubation mixture and conditions as described in Table 4.

interest to examine the effect of aldrin and dieldrin on lipid peroxidation. Table 3 shows that neither of these compounds produced any effect on the formation of the TBA chromogen during a 5-min incubation period, each substance being added (500 μg in 100 μl alcohol) immediately before the microsome suspension.

TABLE 3. THE EFFECT OF ALDRIN AND DIELDRIN ON LIPID PEROXIDATION IN PIG LIVER MICROSOMES

Additions	Lipid peroxidation (o.d. 534 mμ)
100 μl alcohol	1·2
500 μg aldrin in 100 μl alcohol	1·1
500 μg dieldrin in 100 μl alcohol	1·1

Incubations were for 5 min at 30°. Incubation medium (final concentrations): Tris phosphate pH 7·3 ($5{\cdot}2 \times 10^{-2}$ M), G-6-P ($2{\cdot}5 \times 10^{-3}$ M), NADP ($4{\cdot}3 \times 10^{-5}$ M), nicotinamide ($2{\cdot}53 \times 10^{-3}$ M), KCl ($1{\cdot}28 \times 10^{-2}$ M), G-6-P dehydrogenase (1·4 units), microsomal suspension (0·2 ml) containing approximately 5 mg protein, total vol. 3 ml.

A number of metallicions (Mn^{++} and Co^{++})[19] and other substances [α,α'-dipyridyl, EDTA, butylated hydroxytoluene (BHT)] known to inhibit lipid peroxidation were also examined for their effect on TBA chromogen formation. At the same time parallel experiments were conducted to investigate the effect of these same materials on aldrin epoxidation. In all cases appropriate amounts of each compound were added to the reaction mixture in 10 μl of water or alcohol immediately before the microsomes. Incubations were carried out for 5 min for lipid peroxidation and for 10 min in the estimation of aldrin epoxidation. Checks were made with each substance to examine the possibility of any direct effect on the development of the TBA chromogen itself; no such effect was observed.

The results are summarized in Table 4. In all cases there appears to be a direct relationship between the inhibition of lipid peroxidation and the stimulation of epoxidation. This is particularly well illustrated in the experiments with Mn^{++}. When

BHT was added to microsomes incubated in the presence of EDTA, no further stimulation of epoxidation was observed, indicating that the effect of at least two of the substances is not additive.

TABLE 4. THE EFFECT OF ANTIOXIDANTS ON LIPID PEROXIDATION AND DIELDRIN FORMATION IN PIG LIVER MICROSOMES

Addition	Lipid peroxidation (o.d. 534 mμ)	Dieldrin produced (μg)
None	0·85	5·0
2×10^{-5} M Mn^{++}	0·47	6·6
5×10^{-5} M Mn^{++}	0·30	8·7
1×10^{-4} M Mn^{++}	0·11	10·7
2×10^{-4} M Mn^{++}	0·05	10·9
5×10^{-4} M Mn^{++}	0	11·7
None	0·42	4·3
10^{-3} M α,α′-dipyridyl	0·02	—
10^{-4} M α,α′-dipyridyl	0·02	13·4
None	0·70	4·2
8×10^{-4} M BHT	0·04	—
5×10^{-4} M BHT	—	22·4
1×10^{-3} M EDTA	—	29·8
None	—	1·0
1×10^{-3} M EDTA	—	4·5
5×10^{-4} M Mn^{++}	—	5·0
5×10^{-4} M Co^{++}	—	5·0
$2{\cdot}5 \times 10^{-4}$ M Co^{++}	—	2·5
None	0·25	5·5
5×10^{-4} M DOPA	0·02	7·7

Incubation conditions for epoxidation as described in Table 1 with the exclusion of EDTA and for lipid peroxidation as in Table 3. Other additions as indicated.

Effect of $NADH_2$

As reported in rabbit liver microsomes,[5] we have observed that not only $NADPH_2$ but also to a lesser extent $NADH_2$, is able to support the epoxidation of aldrin by pig liver microsomes. $NADH_2$ however will not initiate lipid peroxidation in these preparations. The effect of EDTA (10^{-3} M) on lipid peroxidation and on aldrin epoxidation in the presence of either $NADPH_2$ or $NADH_2$ is shown in Table 5. No stimulation of epoxidation is obtained by addition of EDTA to the $NADH_2$-containing system suggesting that $NADH_2$ is itself providing the reducing system necessary for epoxidation rather than acting in combination with trace amounts of NADP and a transdehydrogenase system.

Effect of inhibitors of lipid peroxidation and preincubation on epoxidation

The stimulation of aldrin epoxidation resulting from the inhibition of lipid peroxidation could be due to the prevention of the accumulation of lipid peroxides, since these are well known inhibitors of many enzyme systems.[20] Alternatively there may be competition between the lipid peroxidation and epoxidation systems for a common intermediate of microsomal electron transport between $NADPH_2$ and oxygen.[18]

These possibilities were examined in two ways. Firstly the effect of EDTA was studied on the initial rate of epoxidation and the period of time over which this rate was maintained. Secondly the effect of preincubating the microsomes in the presence

of $NADPH_2$ (before addition of aldrin) was examined, thereby allowing the accumulation of lipid peroxides before the initiation of epoxidation.

The effect of EDTA on the initial rate of epoxidation is shown in Fig. 4. In these experiments 500 μg aldrin was added to twice the normal volume of incubation

TABLE 5. THE EFFECT OF EDTA ON $NADPH_2$ AND $NADH_2$ STIMULATED DIELDRIN FORMATION AND ON LIPID PEROXIDATION

Addition	Lipid peroxidation (o.d. 534 mμ)	Dieldrin produced (μg)
None	0	0·0
$NADPH_2$ ($4{\cdot}3 \times 10^{-5}$ M)	0·6	9·2
$NADPH_2$ ($4{\cdot}3 \times 10^{-5}$ M) plus EDTA (10^{-3} M)	0·03	12·5
$NADH_2$ ($4{\cdot}3 \times 10^{-5}$ M)	0·03	3·6
$NADH_2$ ($4{\cdot}3 \times 10^{-5}$ M) plus EDTA (10^{-3} M)	—	3·1

For lipid peroxidation the incubations were at 30° for 5 min. Incubation medium: Tris phosphate pH 7·3 ($5{\cdot}2 \times 10^{-2}$ M), G-6-P ($2{\cdot}5 \times 10^{-3}$ M), nicotinamide ($2{\cdot}53 \times 10^{-3}$ M), KCl ($1{\cdot}28 \times 10^{-2}$ M), 0·2 ml microsomal suspension containing approximately 5 mg protein, total volume 3 ml. For epoxidation the incubations were for 10 min at 30°. Incubation medium: Tris phosphate pH 7·3 (5×10^{-2} M), G-6-P ($2{\cdot}4 \times 10^{-3}$ M), nicotinamide ($2{\cdot}45 \times 10^{-3}$ M), KCl ($1{\cdot}23 \times 10^{-2}$ M), 0·5 ml microsomal suspension (approx. 12 mg protein), total volume 5 ml. Aldrin (100 μg) added in 100 μl alcohol.

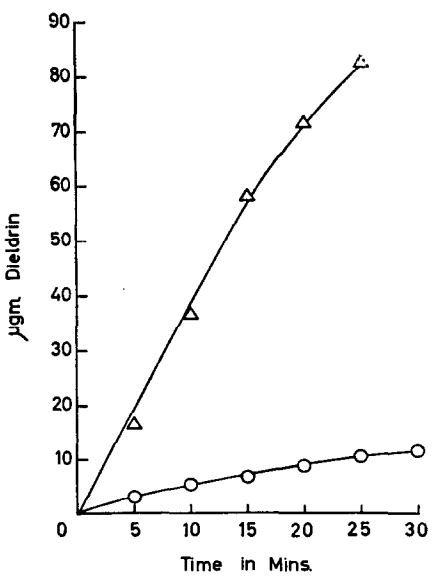


FIG. 4. Effect of EDTA on the rate of dieldrin formation in pig liver microsomes. Incubation at 30°. Incubation mixture: Tris phosphate 5×10^{-2} M (pH 7·3); nicotinamide $2{\cdot}45 \times 10^{-3}$ M; NADP $5{\cdot}2 \times 10^{-5}$ M; KCl $12{\cdot}3 \times 10^{-3}$ M; G-6-P $2{\cdot}4 \times 10^{-3}$ M; G-6-P dehydrogenase 2·8 units; microsomal protein approximately 24 mg; aldrin 500 μg; total vol. 10 ml. Aliquots of 1 ml withdrawn at intervals. △ = with EDTA 10^{-3} M; ○ = without EDTA.

medium, both in the presence and in the absence of EDTA (10^{-3} M). Aliquots of 1 ml were withdrawn at intervals and pipetted into 7 ml acetone. After the addition of 4 ml water the aldrin and dieldrin were extracted and assayed in the usual way. Figure 4 shows quite clearly that EDTA produces a marked stimulation in the initial rate of epoxidation. In addition it can be seen that the rate of epoxidation falls off more rapidly in the absence of EDTA.

In the second series of experiments microsomes were incubated for 10 min in the presence and absence of $NADPH_2$, both with and without either EDTA or Mn^{++}, prior to the addition of aldrin. The results of these experiments (Table 6) show that

TABLE 6. THE EFFECT OF PREINCUBATION OF PIG LIVER MICROSOMES ON THE EPOXIDATION OF ALDRIN

Preincubation conditions	Additions for incubation	Dieldrin produced (μg)
Not preincubated	None	1·0
Not preincubated	5×10^{-4} M Mn^{++}	6·0
Without $NADPH_2$*	5×10^{-4} M Mn^{++}	6·0
No addition	5×10^{-4} M Mn^{++}	3·0
Plus 5×10^{-4} M Mn^{++}	None	6·0
Without $NADPH_2$ + 5×10^{-4} M Mn^{++}	None	6·0
Not preincubated	None	8·0
Not preincubated	10^{-3} M EDTA	42·0
No addition	None	3·2
Plus 10^{-3} M EDTA	None	38·0
Not preincubated	None	0·9
Not preincubated	10^{-3} M EDTA	6·7
Without $NADPH_2$	10^{-3} M EDTA	6·8
No addition	10^{-3} M EDTA	4·6
Plus 10^{-3} M EDTA	None	7·6
Without $NADPH_2$ + 10^{-3} M EDTA	None	7·6

Preincubations and incubations were carried out for 10 min at 30° in the following medium (fina concentration): Tris phosphate pH 7·3 (5×10^{-2} M), G-6-P ($2{\cdot}4 \times 10^{-3}$ M), nicotinamide ($2{\cdot}45 \times 10^{-3}$ M), KCl ($1{\cdot}23 \times 10^{-2}$ M), NADP ($5{\cdot}2 \times 10^{-5}$ M), G-6-P dehydrogenase (1·4 units), 0·5 ml microsomal suspension (approx. 12 mg protein), total vol. 5 ml. Aldrin (100 μg) in 100 μl alcohol added after preincubation.

* $NADPH_2$ generated in medium [NADP ($5{\cdot}2 \times 10^{-5}$ M), plus G-6-P dehydrogenase (1·4 units)].

preincubation in the absence of $NADPH_2$ made little difference to the subsequent rate of dieldrin formation. Preincubation in the presence of $NADPH_2$ however, resulted in a 50 per cent reduction in epoxidation, although when Mn^{++} (5×10^{-4} M) or EDTA (10^{-3} M) was included during preincubation, this reduction was not observed. Nevertheless, after preincubation with $NADPH_2$ alone, the subsequent rate of dieldrin formation in the presence of Mn^{++} was three times that in microsomes incubated in the absence of Mn^{++} which had not been subjected to preincubation.

The results of both series of experiments indicate that although the inhibition of epoxidation is due in part to preformed lipid peroxide, it is chiefly the result of competition between the two systems.

Effect of fly microsomes on lipid peroxidation

Contrary to the above findings with pig liver microsomes, dieldrin formation by fly microsomes is not stimulated by EDTA;[7] in fact a slight inhibition has consistently been observed. Furthermore the housefly microsomes did not produce the TBA

chromogen on incubation with $NADPH_2$, either alone or together with Fe^{++} and ADP.

Samples of housefly microsomes and of the 100,000 *g* fly supernatant were incubated with pig liver microsomes in the presence of $NADPH_2$ and the TBA chromogen measured. It is clear from the results shown in Table 7 that both the fly microsomes

TABLE 7. THE EFFECT OF MICROSOMES AND OF SUPERNATANT FROM THE HOUSEFLY ON LIPID PEROXIDATION IN PIG LIVER MICROSOMES

Additions	Lipid peroxidation (o.d. 534 mμ)
None	0·62
0·25 ml housefly microsomes	0
0·25 ml housefly microsomes (heat killed)	0·10
0·25 ml housefly microsomes (added after incubation)	0·65
0·25 ml 100,000 *g* housefly supernatant	0

Incubations were for 5 min at 30°. Incubation medium (final concentrations): Tris phosphate pH 7·3 ($4·7 \times 10^{-2}$ M), G-6-P ($2·25 \times 10^{-3}$ M), nicotinamide ($2·28 \times 10^{-3}$ M), KCl ($1·15 \times 10^{-2}$ M), NADP ($4·3 \times 10^{-5}$ M) G-6-P dehydrogenase (1·4 units), microsomal suspension (0·25 ml), containing approximately 5 mg protein, total vol. 3 ml.

and the supernatant contain an inhibitor of lipid peroxidation. Inhibition was still observed with housefly microsomes which had been heated for 1 min at 100° suggesting that the inhibition is not due to competitive enzyme action.

Although no attempts have been made to identify this inhibitor, certain possibilities have been considered.

It has recently been demonstrated that considerable amounts of the enzyme tyrosinase are present in both housefly microsomes[21] and supernatant,[22] in contrast to the absence of activity in microsomes obtained from pig liver.[23]

In order to examine the possibility that the tyrosinase system might be involved in the inhibition of lipid peroxidation, a crude preparation of this enzyme was isolated from the common mushroom (*Psalliota campestris*).[24] The results of these experiments are shown in Table 8. No inhibition of TBA chromogen formation was obtained with tyrosine alone, and only slight inhibition resulted from the addition of tyrosinase to the system. When both tyrosine and tyrosinase were added together however, lipid peroxidation was completely inhibited, although no inhibition was observed when the tyrosinase had been heat killed. That the inhibition of lipid peroxidation results from a product of tyrosinase oxidation (possibly a polyphenol or benzoquinone derivative) is further suggested by the inhibition observed on addition of a solution tyrosine + tyrosinase which had been heat killed after a prior period of incubation. 3,4-Dihydroxyphenylalanine (DOPA) itself inhibits lipid peroxidation (Table 8) with a consequent stimulation of aldrin epoxidation (Table 4).

Inhibition of lipid peroxidation by 1,3-*benzodioxoles*

In view of the marked effect of EDTA and other inhibitors of lipid peroxidation on epoxidation, the effect of the 1,3-benzodioxoles on lipid peroxidation was examined.

Solutions of the appropriate inhibitors were prepared in alcohol and were added to the usual incubation mixture in a 10 μl volume. Parallel checks established that none of these compounds interfered with TBA chromogen development. The results (Table 9) show that, although some inhibition is obtained with all the compounds

TABLE 8. THE EFFECT OF TYROSINASE AND TYROSINE ON LIPID PEROXIDATION IN PIG LIVER MICROSOMES

Addition	Lipid peroxidation (o.d. 534 mμ)
None	0·7
Tyrosine ($1·5 \times 10^{-4}$ M) + tyrosinase	0
Tyrosinase	0·5
Tyrosine ($1·5 \times 10^{-4}$ M)	0·7
None	0·7
Tyrosine ($1·5 \times 10^{-4}$ M) + tyrosinase (Heated after preincubation)*	0·02
Tyrosinase (heat killed) + tyrosine ($1·5 \times 10^{-4}$ M)	0·7
None	0·25
DOPA (5×10^{-4} M)	0·02

Incubation medium and conditions as in Table 7. Tyrosine or tyrosine/tyrosinase mixture added in 0·25 ml.

* Tyrosine and tyrosinase were incubated together and heat killed before being added to the microsomes.

TABLE 9. EFFECT OF 1,3-BENZODIOXOLES ON LIPID PEROXIDATION BY PIG LIVER MICROSOMES

Inhibitor		Lipid peroxidation (o.d. 534 mμ)	Inhibition (%)
	None	0·7	0
I	2×10^{-4} M	0·7	0
VII	8×10^{-4} M	0·4	43
IV	8×10^{-4} M	0·5	29
VIII	4×10^{-5} M	0·6	14
VIII	8×10^{-4} M	0	100
	None	0·8	0
I	$1·7 \times 10^{-3}$ M	0·7	12
II	$1·7 \times 10^{-3}$ M	0	100
IV	$1·7 \times 10^{-3}$ M	0·6	25
V	$1·7 \times 10^{-3}$ M	0·65	19
VII	$1·7 \times 10^{-3}$ M	0·43	46
	None	0·66	0
II	$7·0 \times 10^{-6}$ M	0·56	15
II	$1·7 \times 10^{-4}$ M	0·48	27
II	$3·3 \times 10^{-4}$ M	0·17	74
	None	0·64	0
VIII	1×10^{-5} M	0·63	2
VIII	3×10^{-5} M	0·55	14
VIII	1×10^{-4} M	0·25	61
VIII	5×10^{-4} M	0·01	98

Incubation conditions were the same as those described in Table 3. Inhibitors were added in 10 μl alcohol.

examined, in most cases the concentration required to inhibit lipid peroxidation is considerably higher than that required to inhibit epoxidation. The two obvious exceptions are sesamol (II) and 5-amino-1,3-benzodioxole (VIII), both of which are efficient inhibitors of lipid peroxidation. The inhibition obtained with 5-methoxy-1,3-benzodioxole (VII) could result from its *O*-demethylation to produce II during the incubation. Sesamol (II) has in fact been used as an antioxidant.[25]

DISCUSSION

The remarkable similarity in the degree of inhibition of epoxidation in pig liver microsomes by the several 1,3-benzodioxoles (Table 1) is interesting in view of the considerable variation of ring substituents within the group. This suggests that the substituents themselves have little effect on the inhibitory properties of the 1,3-benzodioxole nucleus and that secondary binding or orientation properties at the site of action are of minor importance *in vitro*. Considerably more variation occurs in the case of the housefly system, although even here most of the I_{50} values lie between fairly narrow limits and no correlations between structure and activity are immediately obvious.

This contrasts with the situation with these same compounds *in vivo*, where considerable variations occur with respect to their synergistic activity with carbaryl (1-naphthyl *N*-methylcarbamate)[15] and lends support to the view that *in vivo* the major role of ring substituents relates to penetration through the insect cuticle.

The fact that many of the substituents incorporated into the 1,3-benzodioxole ring [e.g. CH_3O-, $(CH_3)_2N$-] are themselves susceptible to oxidative attack by the microsomes might suggest additional competitive action with respect to aldrin epoxidation. The results indicate that this does not appear to be the case and that any such additional effects are overridden (or inhibited) by the 1,3-benzodioxole nucleus.

The greater sensitivity of the housefly epoxidation system to inhibition by the 1,3-benzodioxoles (Table 1, Fig. 1) may be an important factor in selectivity which would decrease any deleterious metabolic effects in mammals resulting from exposure to these compounds. Coupled with the more rapid metabolic breakdown of these compounds in mammalian systems compared with those in insects[26] the possible hazards associated with residues of the 1,3-benzodioxole synergists should be minimized. Microsomal epoxidation in insects also seems to be more susceptible to inhibition by carbon monoxide[7] and real differences in the nature of the enzymes involved may be indicated.

The variation in the ability of the 1,3-benzodioxoles to inhibit lipid peroxidation is in marked contrast to their more uniform action on epoxidation. This suggests that in the inhibition of lipid peroxidation it is the ring substituent (particularly OH and NH_2) rather than the 1,3-benzodioxole nucleus, which is playing the major role.

The experiments with pig liver microsomes indicate that the degree of inhibition of epoxidation by the 1,3-benzodioxoles is influenced by the presence of EDTA in the reaction medium (Table 10). In the absence of EDTA a very complex situation exists regarding the inhibition of epoxidation which makes the results difficult to interpret. Under such conditions the activity of the lipid peroxidation system will result in an inhibition of epoxidation additional to that resulting from the 1,3-benzodioxoles. However, the inhibition of lipid peroxidation by the latter would be expected to stimulate dieldrin formation, so that the net result would be dependent on a complex

balance between the inhibition incurred by each of the two systems with any particular synergist. The results in Table 10 indicate variations in I_{50} values of some of the inhibitors in the presence and absence of EDTA. To eliminate the complicating factors attendant on lipid peroxidation all I_{50} values with pig liver microsomes were obtained in the presence of EDTA.

TABLE 10. EFFECT OF EDTA ON INHIBITION BY DERIVATIVES OF 1,3-BENZODIOXOLE ON EPOXIDATION OF ALDRIN BY PIG LIVER MICROSOMES

Inhibitor	I_{50} (Molar concentration)	
	With EDTA (10^{-3} M)	Without EDTA
V	$3{\cdot}6 \times 10^{-4}$	$1{\cdot}6 \times 10^{-3}$
VI	$1{\cdot}3 \times 10^{-3}$	$4{\cdot}4 \times 10^{-4}$
VII	$1{\cdot}2 \times 10^{-3}$	$3{\cdot}1 \times 10^{-4}$
VIII	$4{\cdot}8 \times 10^{-4}$	$1{\cdot}5 \times 10^{-3}$
XII	$3{\cdot}1 \times 10^{-4}$	$3{\cdot}6 \times 10^{-4}$

Incubation conditions as described in Table 1. Inhibitors added in 10 μl alcohol.

The curvilinear trend of the reciprocal plots (Fig. 2) is consistently repeatable and renders impossible any definite conclusions regarding the characterization of the inhibition (competitive vs. non- competitive). The shape of these lines in the presence of an inhibitor is characteristic of systems in which a common substrate is attacked by two distinct enzymes.[27] The curvature is not very obvious in the presence of substrate alone which would suggest a close similarity between the K_m values for the two enzymes. With inhibitor present however, the deviation at higher substrate concentrations becomes more pronounced and suggests a difference in susceptibility to inhibition of the enzymes involved. The curvature of the lines in the presence of an inhibitor suggests that the K_m for one of the enzymes is changed and indicates competitive kinetics. However, the situation is too complex for a more detailed interpretation.

Results in this laboratory indicate competitive inhibition by 5,6-dibromo-1,3-benzodioxole (X) of the *O*-demethylation of *para*-nitroanisole, which is in agreement with the reported competitive action of sesamex towards microsomal hydroxylation.[9] More recent work[26] has established the microsomal metabolism of derivatives of 1,3-benzodioxole and suggests that the latter compounds may be acting as alternative substrates for the mixed function oxidase system.

The question of the aqueous insolubility of aldrin in relation to its epoxidation in *in vitro* systems arose during the course of these investigations. As shown in Table 2 the amount of dieldrin produced was found to be independent of the total volume of the reaction mixture and directly related to the absolute amount of aldrin added. This suggests that the true concentration of the aldrin (relating to its availability at the enzyme surface) is not that expressed as a molar concentration in the total incubation volume, but is probably the concentration present in the lipid phase of the microsomal suspension. It is therefore difficult to define concentration when applied to such biphasic systems and K_m values should be accepted with some reservation. However, the 'concentration' in any one preparation will be related to the absolute amount of substrate added. The above considerations will be relevant in all *in vitro* studies in which lipid-soluble substrates are employed.

In our experiments with pig-liver microsomes the addition of either $NADPH_2$, or NADP plus a $NADPH_2$-generating system, resulted in a rapid uptake of oxygen and the formation of the TBA chromogen associated with the formation of lipid peroxides. In the experiments of Hochstein *et al.*[14] with rat liver microsomes, $NADPH_2$-linked lipid peroxidation occurred only on addition of Fe^{++} and pyrophosphate, whereas Gram and Fouts[28] found that the 9000 *g* supernatant supported lipid peroxidation without any such addition. Similarly in our experiments with washed microsomes from pig liver, the addition of Fe^{++} was not required to initiate $NADPH_2$-linked peroxidation. This fact, plus the observation that peroxidation is inhibited by EDTA and α,α'-dipyridyl suggests that traces of Fe^{++} are present within the microsomes or in the reaction medium.

Lipid peroxidation was not influenced by the addition of either aldrin or dieldrin. When lipid peroxidation was inhibited however, either by a chelating agent such as EDTA or by an antioxidant, there was a marked stimulation of aldrin epoxidation, often as much as seven fold. The causal relationship between these two observations is suggested by the experiments with Mn^{++}, where the concentration required for maximum stimulation of epoxidation is similar to that required for the complete inhibition of lipid peroxidation. Furthermore the epoxidation supported by $NADH_2$ is not stimulated by EDTA, and in our preparations, as in those used by Orrenius *et al.*,[18] $NADH_2$ did not stimulate lipid peroxidation. Similarly, the failure of EDTA to stimulate epoxidation in housefly microsomes is presumably associated with the absence of lipid peroxidation in this preparation. In this case however a further factor is involved, since both the microsomes and the 100,000 *g* supernatant from the fly homogenate contain a heat-stable inhibitor of lipid peroxidation. The association of this inhibitor with the presence of tyrosinase in the insect preparation, and the fact that one of the products of tyrosinase action (DOPA) can inhibit lipid peroxidation may be coincidental since the identity of the inhibitor has not been clearly established.

With regard to the mechanism by which lipid peroxidation may inhibit epoxidation two possibilities have been considered; either that the lipid peroxides themselves inhibit the enzymes involved in epoxidation or that lipid peroxidation and epoxidation compete for a common intermediate, as suggested by others for oxidative demethylation.[18,28] Since inhibition of lipid peroxidation by EDTA results in a marked stimulation in the initial rate of epoxidation (Fig. 4) the second mechanism would seem to be more important. However, there does appear to be some inhibition by lipid peroxides or their decomposition products since pre-incubation of the microsomes with NADP plus $NADPH_2$-generating system results in a partial inhibition of the subsequent epoxidation. This is not completely reversed by EDTA or by Mn^{++}, although either substance will protect the epoxidation system if it is present during the pre-incubation period.

Since competition appears to be the major factor involved it is surprising that aldrin fails to have any effect on lipid peroxidation. This is particularly striking in view of the fact that *O*-demethylation can compete so successfully with lipid peroxidation in rat-liver microsomes.[18] It can be inferred from the work of Cooper and Rosenthal,[29,30] that in its response to lipid peroxidation the epoxidation system may show a closer relationship to the C-21 hydroxylation of steroids. These workers showed that ascorbic acid and catecholamines influence C-21 hydroxylation either by stimulating or inhibiting a reaction which competes for electrons from $NADPH_2$. Although this

reaction was not identified the nature of the compounds involved suggests that this too may be the peroxidation of endogenous lipids. Ascorbic acid is in fact an activator[14] and adrenalin a known inhibitor[31] of lipid peroxidation.

Acknowledgements—We wish to thank Miss C. A. Greenfield for very competent technical assistance. One of the authors, C. F. Wilkinson, was the recipient of a U.K. Civil Service Commission Research Fellowship during this investigation.

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