Microsomal Metabolism of the 1,3-Benzodioxole Ring and Its Possible Significance in Synergistic Action

C. F. Wilkinson and L. J. Hicks

Enzymatic metabolism of 4,5,6,7-tetrachloro-1,3-benzodioxole to the corresponding 3,4,5,6-tetrachlorocatechol has been demonstrated in microsomal preparations from both mammalian liver and insect tissues. In vitro studies have characterized the enzyme responsible as one of the typical mixed-function oxidases associated with this fraction. The investigation strongly supports the view that the 1,3-benzodioxoles are effective in vitro inhibitors of microsomal enzymes, as well as in vivo synergists for a number of drugs and insecticides, as a direct result of being alternative substrates for the mixed-function oxidases. The effectiveness of the 1,3-benzodioxoles results chiefly from a high affinity for the active surface of the microsomal complex.

Derivatives of 1,3-benzodioxole (methylenedioxybenzene) are now well established as inhibitors of the microsomal mixed-function oxidases which are responsible for the metabolism of drugs and other foreign compounds in both vertebrates and invertebrates (Lewis *et al.*, 1967; Philleo *et al.*, 1965). As a result of their inhibitory properties, the 1,3-benzodioxoles have attained commercial significance as insecticide synergists which, when applied in combination with insecticidal compounds, enhance the toxicity of the latter by preventing their metabolic degradation by the insect (Metcalf, 1967, 1968; Wilkinson, 1965, 1968).

Although it has been recognized for some time that compounds containing the 1,3-benzodioxole ring are susceptible to metabolic degradation (Schmidt and Dahm, 1956; Williams, 1959), few comprehensive studies have yet been undertaken to determine fully the nature of the metabolites produced. The metabolites unequivocally identified are largely those resulting from attack by conjugative enzymes on the side chain moiety of the phenyl ring. More recent studies have established the in vivo metabolism of several 1,3-benzodioxoles in houseflies (Wilkinson, 1967), and other investigations (Fishbein *et al.*, 1967a, b) have shown that a number of commercial synergists are chemically altered and excreted largely in the bile following intravenous injection into rats. The most comprehensive investigation to date is that of Casida *et al.* (1966). Employing six 1,3-benzodioxoles labeled with C^{14} at the methylene group, these workers demonstrated cleavage of the dioxymethylene ring by observing the in vivo liberation of $C^{14}O_2$ by mice and houseflies and the production of C^{14}-formate in vitro in suitably fortified homogenates and microsomes from housefly abdomens and mouse liver. In consequence of these data, Casida *et al.* (1966) concluded that the 1,3-benzodioxoles inhibit the microsomal enzymes by virtue of being alternative substrates for the mixed-function oxidase complex.

In attempting to establish the mechanism by which the 1,3-benzodioxoles interfere with mixed-function oxidation, two other theories have been proposed. Hennessy (1965) has suggested that the highly electrophilic benzodioxolium ion resulting from hydride ion transfer from the methylene group of the ring could readily attack some essential moiety of the microsomal system. Molecular orbital calculations of a number of benzodiheterolium ions have recently been reported to support this mechanism (Cloney and Scherr, 1968). Hansch (1968), however, as a result of his analyses of structure activity relationships in a series of 1,3-benzodioxoles, proposed that the synergists are able to interact with free radicals involved in the mechanism of mixed-function oxidation.

Department of Entomology and Limnology, Cornell University, Ithaca, N. Y. 14850

Since synergistic mode of action may be related to metabolism, this investigation was undertaken to study the nature and properties of the microsomal enzyme responsible for cleavage of the 1,3-benzodioxole ring and to compare it with those effecting aldrin epoxidation and aniline hydroxylation.

The fully chlorinated phenyl ring of 4,5,6,7-tetrachloro-1,3-benzodioxole makes this an ideal model substrate for studies of 1,3-benzodioxole cleavage. The compound is readily assayed by electron-capture gas chromatography and the metabolic stability of the halogenated phenyl moiety tends to focus metabolic attack on the dioxymethylene ring itself.

For clarity, the enzyme effecting cleavage of the 1,3-benzodioxole ring is referred to throughout this paper as benzodioxolase.

EXPERIMENTAL

Chemicals. Analytical grade samples of aldrin (1,2,3,4,10,-10-hexachloro-1,4,4*a*,5,8,8*a*-hexahydro-1,4-*endo*-*exo*-5,8-dimethanonaphthalene) and its 6,7-epoxide, dieldrin, were kindly supplied by the Shell Development Co., Modesto, Calif. The hydrochloride salts of aniline and *p*-hydroxyaniline were purchased from Eastman Organic Chemicals and 3,4,5,6-tetrachlorocatechol (TCC) was obtained from the Aldrich Chemical Co. The 4,5,6,7-tetrachloro-1,3-benzodioxole (TCBD) (m.p. 193°–95°C.) was synthesized from TCC by a method identical to that previously reported (Wilkinson, 1967). Samples of SKF 525-A (β-diethylaminoethyl diphenylpropyl acetate hydrochloride) and naphtho-(2,3-*d*)-1,3-dioxole were kindly supplied by Smith Kline and French Laboratories, Philadelphia, and Julius Hyman of the Fundamental Research Co., Berkeley, Calif., respectively. Silylation grade hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were purchased from the Pierce Chemical Co., Rockford, Ill.

Reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), its oxidized form (NADP), reduced nicotinamide adenine dinucleotide ($NADH_2$), flavin adenine dinucleotide (FAD), riboflavin-5-phosphate (FMN), glucose-6-phosphate (G-6-P), G-6-P dehydrogenase, and nicotinamide were purchased from Calbiochem, Los Angeles. All other chemicals and solvents were analytical reagent grade.

Source and Preparation of Microsomes. Mice of the Swiss-

Webster strain were purchased from the Dan Rolfsmeyer Co., Madison, Wis., and other animal species evaluated were kindly supplied by several departments at Cornell University. Larvae of the southern armyworm (*Prodenia eridania*) were obtained from cultures maintained in this laboratory, and those of the Cecropia moth (*Hyalophora cecropia*) were supplied by E. F. Taschenberg, New York State Agricultural Experimental Station, Fredonia, N. Y.

Liver microsomes from mice and other species employed were prepared in 1.15% KCl at 0° to 4° C. by methods previously reported (Lewis *et al.*, 1967). Centrifugation was accomplished with an International Equipment Co. (IEC) B-60 ultracentrifuge following initial homogenization in a Waring blender. The microsomal pellet was washed by resuspension in 1.15% KCl, followed by recentrifugation at 100,000 G for 1 hour, and the washed pellet was suspended and stored at 0° C. in additional KCl. Microsomes from the gut tissues of the southern armyworm (*Prodenia eridania*) and the Cecropia moth (*Hyalophora cecropia*) were prepared as described by Krieger and Wilkinson (1969).

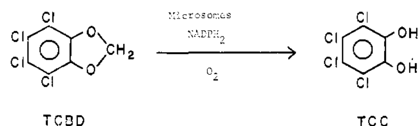
The protein concentration of each preparation was determined by a modified biuret method (Fincham, 1954), employing bovine serum albumin as a standard.

Standard Incubation Procedure. A standard 5.0-ml. incubation mixture, used for each of the three types of assay employed, consisted of 0.5 ml. of microsomal suspension and the following additional components (final concentration): Tris-HCl buffer ($5 \times 10^{-2}M$); G-6-P ($2.4 \times 10^{-3}M$); nicotinamide ($2.45 \times 10^{-3}M$); KCl ($1.23 \times 10^{-2}M$); NADP ($5.2 \times 10^{-5}M$); and G-6-P dehydrogenase (1.6 units). The Tris-HCl was buffered at pH 7.4 for assays of aldrin epoxidation and aniline hydroxylation and at pH 7.8 for investigations of 1,3-benzodioxole cleavage. In all cases, the reaction was initiated by addition of the appropriate substrate in ethanol (aldrin and TCBD) or water (aniline hydrochloride) and incubations were carried out with shaking in open Erlenmeyer flasks at 30° C.

Determination of Enzyme Activity. Aldrin epoxidation was determined by a method identical with that previously reported (Lewis *et al.*, 1967). Gas chromatographic (GC) determination of dieldrin was effected using a Research Specialities instrument provided with a Sr^{90} ionization detector operated at low voltage for electron capture and the 2-foot all-glass column was packed with 5% SE 30 on Gas-chrom Q and maintained at 180° C. Nitrogen was the carrier gas.

The hydroxylation of aniline was determined by the procedure described by Kato and Gillette (1965) and absorbance measurements were made by means of a Norelco Unicam SP 800 spectrophotometer using the scale expansion accessory.

From preliminary investigations, it was established that TCC was the only significant product following incubation of TCBD with suitably fortified microsomal suspensions. The following procedure, involving extraction, trimethylsilylation, and subsequent GC analysis, was adopted for assay of this catechol.



A 4.0-ml. aliquot of the standard incubation medium was transferred to a 40-ml. glass-stoppered tube containing 1.3 ml. of 1*N* trichloroacetic acid. One minute later, 0.7 gram of NaCl was added and the mixture was agitated on a vortex mixer. Following the addition of 1 ml. of acetone, the mixture was extracted into 4 ml. of hexane by means of thorough (1-minute) agitation on the vortex mixer and the organic phase was subsequently transferred to a 10-ml. glass-stoppered tube by means of a Pasteur pipet. The extraction procedure was repeated with additional 4- and 2-ml. aliquots of hexane, the combined extracts were made up to 10 ml., and the solution was dried over anhydrous Na_2SO_4. An 8-ml. aliquot of the dry solution was then transferred to a clean, dry 10-ml. glass-stoppered tube and allowed to react for 30 minutes with 0.4 ml. of trimethylsilylation mixture (5 ml. of pyridine, 1.5 ml. of HMDS, 1.0 ml. of TMCS). The mixture was washed with 2 ml. of saturated NaCl solution and the tube vigorously shaken by hand for 30 seconds. On clearing, the upper hexane phase was rapidly decanted into a clean glass-stoppered tube containing Na_2SO_4 and aliquots of this solution were subsequently assayed on the gas chromatograph. Column packing and conditions were those previously described for dieldrin determination. With this procedure only one GC peak, corresponding to the trimethylsilyl ether of TCC, was obtained in addition to that resulting from the substrate. As a result of protein-dependent variations in catechol recovery, standard curves were always obtained by adding known amounts of catechol to the 5-ml. standard incubation mixture and taking these through the entire procedure described.

Thin-Layer Chromatography (TLC). To obtain sufficient material for TLC investigations, several large scale incubations were carried out at 30° C. for 1 hour in open 250-ml. Erlenmeyer flasks. The contents of each flask comprised the equivalent of 10 standard incubations and contained 800 μg. of TCBD. Following acidification with 2 ml. of 12*N* H_2SO_4, each incubation was extracted into a hexane-acetone mixture, which after drying over anhydrous Na_2SO_4 was evaporated under a stream of nitrogen. The extracts of several incubations were combined to yield a clear yellow solution which was spotted on Brinkman (Polygram) plastic sheets precoated with silica gel N-HR.

Extracts were similarly prepared from incubations to which TCC had been added directly and from those acidified immediately prior to addition of TCBD. In addition, a blank extract was obtained from an incubation mixture to which no addition had been made.

A number of different solvent mixtures were employed to resolve the several components of the incubation extracts. Three which were found to be most useful were hexane-ether (1 to 1), hexane-ether (3 to 1), and chloroform-methanol (100 to 1).

All chlorinated compounds were detected as dark brown or black spots, following ultraviolet exposure of plates sprayed with Mitchell (1957) silver nitrate reagent. Catechols and other hydroxy compounds were visualized as blue spots after spraying with 1% $FeCl_3$ followed by 1% $K_3Fe(CN)_6$. Derivatives of 1,3-benzodioxole appeared as violet spots with the chromotropic acid reagent (Beroza, 1963).

RESULTS AND DISCUSSION

Characterization of Metabolites. Gas chromatographic analyses of trimethylsilylated hexane extracts of microsomal incubations with TCBD indicate the presence of only one metabolite of any significance. This cochromatographs with the trimethylsilyl ether of authentic TCC and has a retention time of 1.86 relative to that of TCBD. No GC peaks other than that of the parent compound were observed in extracts not subjected to trimethylsilylation.

Table I. R_f Values for TLC Investigation

Compound	R_f Value Hexane: ether (1:1)	Hexane: ether (3:1)	Chloroform: methanol (100:1)
4,5,6,7-Tetrachloro-1,3-benzodioxole	0.58	0.53	0.64
3,4,5,6-Tetrachloro-catechol	0.19	0.06	0.08

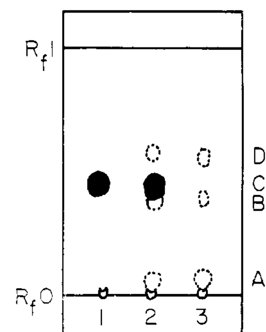


Figure 1. Thin-layer chromatographic behavior in hexane-ether (3 to 1) of extracts of microsomal suspensions

Added
1. TCBD (acidified microsomes)
2. TCBD ($NADPH_2$ fortified)
3. TCC

A. TCC
C. TCBD
B,D. Unknown

Investigations employing TLC on silica gel substantiate the fact that only one major metabolite is produced, and this can readily be resolved from the parent 1,3-benzodioxole in all solvent mixtures employed. This metabolite has the same R_f value as authentic TCC in a number of solvent mixtures (Table I) and reacts in a positive manner with the $AgNO_3$ spray (dark brown) and the $FeCl_3$–$K_3Fe(CN)_6$ reagent (intense blue), indicating, in its structure, the presence of both chlorine and hydroxyl moieties. The absence of the intact 1,3-benzodioxole ring is indicated by a negative response towards the chromotropic acid reagent. Elution of the spots from the thin-layer plate followed by trimethysilylation and GC analysis of the eluent further substantiates the identity of this catechol. Attempts to effect TLC on the trimethylsilyl ether derivative of TCC were unsuccessful, as the derivative reverted immediately to the catechol on application to the plates. This undoubtedly results from hydrolysis of the derivative on the sorbent material of the sheet and indicates the instability of aromatic trimethylsilyl ethers compared with those derived from hydroxylated alicyclic compounds where no breakdown is reported (Brooks and Harrison, 1966).

Although the catechol is the major metabolic product, some evidence of at least two other minor metabolites was initially suggested by TLC. These are indicated by faint spots *B* and *D* in Figure 1, which is a diagrammatic representation of the TLC pattern obtained in hexane-ether (3 to 1). There is, however, a real possibility that these spots do not represent primary oxidative metabolites of TCBD but originate directly from the TCC, as extracts of microsomal incubations to which the catechol had been added directly also gave two faint spots with approximately the same R_f values [0.38 (*B*) and 0.57 (*D*)] in hexane-ether (3 to 1). The actual picture is usually not quite so clear, as suggested by Figure 1; the spots are very faint and the R_f values are somewhat variable, depending on the amount of extract initially applied to the plate. This probably results from materials present in the extracts which tend to retain components of the mixture at the point of application. Spots *B* and *D* gave a positive reaction with both the $AgNO_3$ and the $FeCl_3$–$K_3Fe(CN)_6$ sprays, as did the apparent metabolites from incubations with TCBD, although the blue color resulting from the $FeCl_3$–$K_3Fe(CN)_6$ reagent did not develop immediately. The spots were not apparent when TCC standards were spotted directly on the plates. This suggests that they may be either secondary products resulting from enzymic modification of the catechol in the incubation mixture or artifacts formed during the subsequent extraction and concentration procedure. They may represent oxidation products of TCC.

Although not the major purpose of this investigation, some attempts were made to obtain more information on the compounds represented by spots *B* and *D* in Figure 1. Elution from the plates in hexane-acetone followed by GC analysis showed no detectable peaks. However, after trimethylsilylation a small peak was observed with the eluent from spot *B*, suggesting that this may be a chlorinated hydroxy compound. A GC peak with the same retention value (2.63 relative to TCBD) was detected in concentrated extracts from the large scale incubations following trimethylsilylation but was either absent or of such slight consequence that it was not observed in any of the standard incubations.

The precise mechanism by which cleavage of the 1,3-benzodioxole ring occurs has not been clearly established. It is probably similar to that proposed by Casida *et al.* (1966), in which the process is initiated by microsomal hydroxylation at the methylene group of the five-membered ring. The resulting 2-hydroxy-1,3-benzodioxole intermediate is likely to be highly unstable and in aqueous solution will probably hydrolyze spontaneously to yield the catechol and a one-carbon compound. The demonstration by Casida *et al.* (1966) that the methylene carbon atom is released as formate in vitro and carbon dioxide in vivo tends to support this proposal. Although unlikely, the possibility of direct enzyme involvement in cleavage of the hydroxylated intermediate cannot be entirely discounted.

Optimal Conditions for Catechol Extraction and Assay. Initial studies with a number of derivatives indicated that TCC could be suitably determined by GC analysis of its trimethylsilyl ether. Optimal conditions were therefore established for this assay.

The conversion of the catechol to its trimethylsilyl ether was an extremely fast reaction. Figure 2 (curve *A*, lower abscissa) indicates that the reaction goes essentially to completion in only 15 to 20 minutes at room temperature, 50% of the catechol reacting within the first minute. No further increase in GC peak height was obtained by allowing the reaction to proceed for additional periods of up to 24 hours.

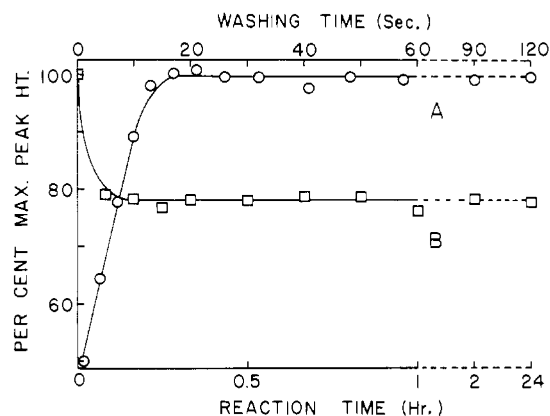


Figure 2. Conversion of catechol

A. Rate of trimethylsilylation of TCC in hexane
B. Effect of washing on recovery of trimethylsilyl ether of TCC

Table II. Subcellular Distribution of the Enzyme Responsibile for 1,3-Benzodioxole Cleavage

Subcellular Fraction	Specific Activity, Mμmoles/Min./Mg. Protein
Mitochondria plus larger cellular organelles and debris	0.015
Mitochondrial supernatant	0.12
Microsomes	0.55
Microsomal supernatant	Trace

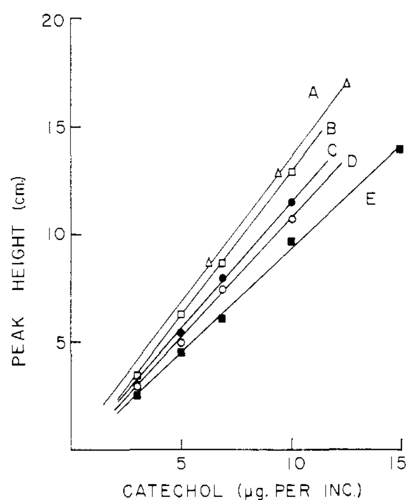


Figure 3. Effect of microsomal protein concentration on recovery of TCC

Mg. of protein per incubation
$A = 0$
$B = 2.5$
$C = 4.9$
$D = 9.8$
$E = 19.6$

Following trimethylsilylation of standard solutions of TCC in hexane, aliquots of these solutions could be applied directly to the GC, as with the highly purified reagents employed no interference was observed from excess trimethylsilylation mixture in the solution. However, in attempting the similar direct assay of trimethylsilylated solutions of catechol extracted from the standard microsomal incubations, additional peaks plus a very broad solvent front were obtained. Although many trimethylsilyl ether derivatives are highly unstable in aqueous solution, washing the hexane solution with saturated NaCl was investigated in the course of attempts to improve the GC analysis. The results shown in Figure 2 (curve *B*, upper abscissa) indicate that washing for 10 seconds causes an initial rapid decrease in the peak height as observed on GC. However, additional washing for periods of up to at least 2 minutes results in no further decrease in peak height and the 30-second wash which was finally adopted for the assay procedure greatly improved the GC analysis. Experiments using 5, 10, and 20 μg. of catechol established that the decrease in peak height resulting from the washing procedure (approximately 22%) was apparently independent of catechol concentration over the range normally encountered in the incubation extracts.

The extraction procedure described earlier was finally chosen only after considerable attention had been given to ascertaining optimal conditions, as even apparently minor variations can result in marked changes in the final recovery of the catechol.

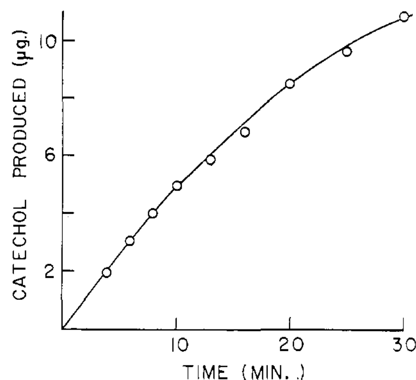


Figure 4. Rate of enzymatic formation of TCC by mouse liver microsomes

Although the absolute amount of trichloroacetic acid used to stop the reaction was not found to be too critical, allowing the acidified incubations to stand longer than a few minutes usually decreased catechol recovery.

However, the amount of NaCl and acetone subsequently added to the mixture had marked effects on extraction efficiency. Extractions made directly into hexane without addition of acetone gave only about 20% of the recovery obtained under optimal conditions. The best results were obtained by addition of 0.5 to 1.5 ml. of acetone per incubation, and any further increase decreased extraction efficiency. Furthermore, the optimal amount of NaCl per incubation was between 0.5 and 1.0 gram.

These extraction conditions are valid only for TCC and modifications will be required before the method can be applied to other catechols with different solubility characteristics.

Total recovery was found to be largely dependent on the protein concentration of the microsomal suspensions employed (Figure 3), ranging from about 95% in the case of incubations containing 2 to 3 mg. of protein to about 70% with those containing approximately 20 mg. As a result, all standard curves were obtained by direct addition of known amounts of catechol to the standard incubation mixtures, followed by the usual procedure for extraction and assay.

In Vitro Properties of Benzodioxolase. To establish the subcellular distribution of benzodioxolase, the enzymatic activity of several centrifugal fractions of liver homogenates was determined. Table II clearly shows that maximum activity is associated with the microsomal fraction of the cell. Only very low activity was observed in the 20,000 G particulate fraction comprising the mitochondria plus larger cell organelles and debris and no measurable catechol production was found with the microsomal supernatant.

Under the in vitro incubation conditions employed, formation of TCC increased normally with time over a 30-minute period (Figure 4). However, a 15-minute incubation period was selected for the routine procedure, as true linearity was not maintained for much longer than 12 to 15 minutes following initiation of the reaction with TCBD. The relationship between benzodioxolase activity and protein concentration (Figure 5) similarly indicates a deviation from linearity when microsomal protein exceeds 8 to 10 mg. per incubation.

The relationship between enzyme activity and pH is shown in Figure 6, where in addition to the curve for 1,3-benzodioxole cleavage, curves for aldrin epoxidation and aniline hydroxylation have been included for comparative purposes. The curve for benzodioxolase exhibits a pH optimum of about 7.9, and in both this property and general shape it closely resembles the pH profile obtained for aniline hydroxylase. The sharply de-

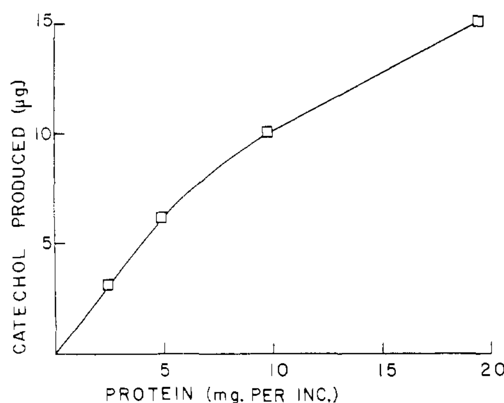


Figure 5. Effect of protein concentration on microsomal benzodioxolase activity

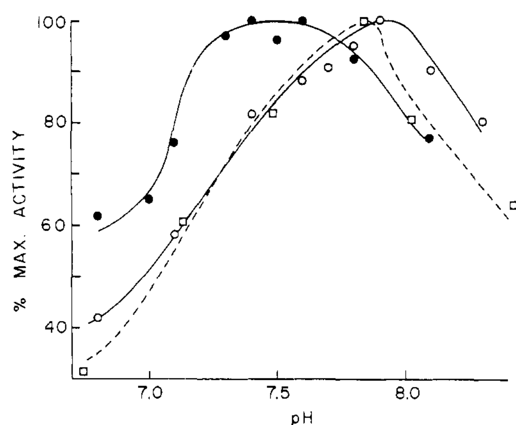


Figure 6. Effect of pH on microsomal enzyme activity

● Aldrin epoxidation
○ 1,3-Benzodioxole cleavage
□ Aniline hydroxylation

fined pH optima for benzodioxolase and aniline hydroxylase are in marked contrast to the generally broader curve exhibited by aldrin epoxidase, which indicates an optimum pH of 7.4 to 7.6. Possibly the decline in activity observed above pH 8.0 with both aniline hydroxylase and benzodioxolase does not truly reflect an effect on the enzymes themselves but may be an artifact resulting from the alkaline instability of the respective enzymatic products.

The effects of a number of cofactors were evaluated on the in vitro metabolism of TCBD and comparative data were obtained with regard to the effect of these materials on aniline hydroxylation and aldrin epoxidation. Table III shows that all three enzymes exhibit a strict requirement for $NADPH_2$. In the case of benzodioxolase, this requirement can be partially satisfied by $NADH_2$, as indicated by the 39% activity observed on substituting this cofactor for $NADPH_2$. The requirement for $NADPH_2$, combined with the fact that incubations carried out under anaerobic conditions gave only trace amounts of catechol (and therefore presumably require oxygen), is strong evidence that benzodioxolase can be classified as a typical mixed-function oxidase.

A considerable decrease in the activity of all three enzymes was observed in the absence of nicotinamide at $10^{-3}M$ and is presumably due to rather high pyridine nucleotidase activity in the microsomal preparation (Schenkman *et al.*, 1967). However, in spite of the high concentration of nicotinamide employed (which should far exceed that of pyridine nucleotidase), the effect of this cofactor varied considerably with protein concentration. Thus in the case of aldrin epoxidation, omission of $10^{-3}M$ nicotinamide from the incubation medium resulted in decreases of 10 and 92%, respectively, at protein concentrations of 5 and 20 mg. per incubation.

Table III. Effect of Cofactors and Metal Ions on Microsomal Enzyme Activity

Incubation Medium	% Control Activity: Aldrin epoxidation	Aniline hydroxylation	1,3-Benzodioxole cleavage
Cofactors			
Complete[a]	100	100	100
Minus $NADPH_2$	Trace	4.4	0
Minus nicotinamide ($10^{-3}M$)	57	21.3	22
Minus $NADPH_2$ plus $NADH_2$ ($10^{-3}M$)	81.3	30.1	39
Plus FAD ($10^{-3}M$)	36.7	55.7	46.5
Plus FMN ($10^{-3}M$)	97	19.9	15
Plus glutathione ($10^{-3}M$)	115	73.6	97
Plus EDTA ($10^{-3}M$)	134	118.6	100
Metal ions			
Plus $NiCl_2$ ($10^{-3}M$)	85	89.4	86.7
Plus $MnCl_2$ ($10^{-3}M$)	120	130	105
Plus $CoCl_2$ ($10^{-3}M$)	101	121	94
Plus $MgCl_2$ ($10^{-3}M$)	116	104	100
Plus $FeCl_2$ ($10^{-5}M$)	83	87.6	94.3
($10^{-4}M$)	75	37.4	41.5
($10^{-3}M$)	20	...	0
Plus $FeCl_3$ ($10^{-5}M$)	94	108.3	97.6
($10^{-4}M$)	87	81.3	60.8
($10^{-3}M$)	70	...	0
Plus CuCl ($10^{-5}M$)	78.8	92.4	96.7
($10^{-4}M$)	9	14.6	29.4
Plus $CuCl_2$ ($10^{-5}M$)	98	69.1	81.1
($10^{-4}M$)	1	9.3	28

[a] Standard incubation medium.

Both FAD and FMN at $10^{-3}M$ resulted in marked inhibition of all three enzyme systems, aniline hydroxylase and benzodioxolase being particularly susceptible to FMN.

The marked stimulation of epoxidation and hydroxylation resulting from inclusion of $10^{-3}M$ EDTA in the incubation medium may indicate the presence in mouse liver microsomes of a lipid peroxidation system (Lewis *et al.*, 1967), though this was not further investigated.

Each of the three enzyme systems reacted in a similar manner to the presence of a number of metal ions (Table III). All were inhibited to a similar degree by inclusion in the incubations of Fe^{2+} or Fe^{3+} and were particularly sensitive to copper in either of its valence forms. Although both aldrin epoxidation and aniline hydroxylation were stimulated to some extent in the presence of Mn^{2-}, Co^{2+}, or Mg^{2+}, these ions had only a slight effect on benzodioxolase activity.

Stability of Benzodioxolase. The storage of microsomal suspensions at 0° C. in 1.15% KCl usually results in a steady decrease in mixed-function oxidase activity. It was, therefore, of interest to investigate the stability of benzodioxolase under these conditions and to compare it with that of each of the other two enzymes studied. Figure 7 indicates that during a 9-day storage period under nitrogen, little if any ring cleavage activity was lost, and in fact a slight increase was observed after about 3 days. This pattern of stability proved to be intermediate between that observed for aldrin epoxidase and that for aniline hydroxylase. The former showed a 43% increase in activity during the first 24 hours of storage and this level was essentially maintained over a 9-day

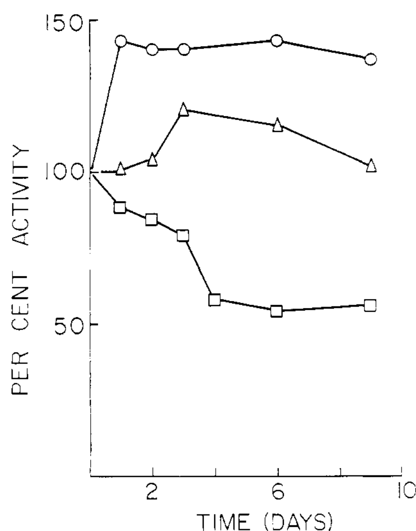


Figure 7. Stability of microsomal enzymes to storage under nitrogen

○ Aldrin epoxidation
△ 1,3-Benzodioxole cleavage
□ Aniline hydroxylation

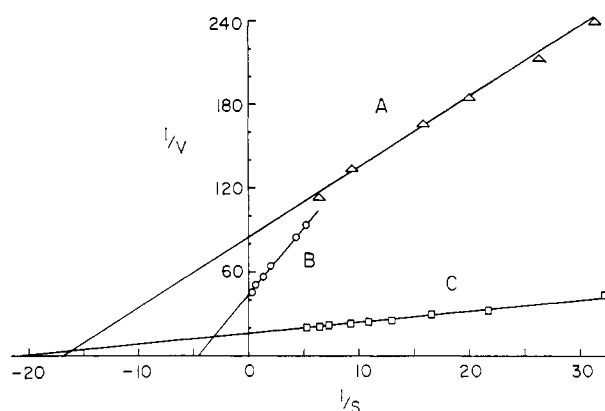


Figure 8. Lineweaver-Burk reciprocal plots

A. Aldrin epoxidation
B. Aniline hydroxylation
C. 1,3-Benzodioxole cleavage
S. μMoles substrate per incubation
V. μMoles product per 15 minutes

period. Aniline hydroxylase activity, however, showed a fairly steady decrease in activity throughout, indicating an enzyme of somewhat lower stability under conditions employed.

These data can be interpreted as indicative of the presence of three distinct enzymes, the activities of which decrease at different rates. Alternatively, it is equally plausible to conclude that a single enzyme of low specificity is involved and that storage results in variations in the ability of the enzyme to metabolize different substrates as a consequence of changes in the structural conformation at the active surface.

Relative Affinity of Microsomes for Different Substrates. Lineweaver-Burk reciprocal plots for aldrin epoxidation, aniline hydroxylation, and 1,3-benzodioxole cleavage are shown in Figure 8; in each case substrate concentration is expressed in terms of micromoles of substrate per incubation. The use of molar concentration based on the total volume of the aqueous incubation mixture is avoided because of problems surrounding the extremely low solubility of aldrin and TCBD. In the case of aldrin, for example, aqueous solubility is approximately 0.01 p.p.m. and 0.05 μg. of aldrin would therefore be expected to saturate a 5-ml. aqueous volume. The concentration of aldrin routinely employed in incubations (100 μg.) exceeds this figure by a factor of 2000 and even the lowest concentrations employed in obtaining plot *A* in Figure 8 (10 μg.) is 200-fold greater than that required for saturation. In view of this rather disturbing fact, it is surprising that additional increases in substrate concentration result in further increases in enzyme activity. As discussed by Lewis *et al.* (1967), this anomaly can be rationalized only if it is assumed that aldrin is solubilized in the lipoprotein material of the microsomal suspension itself and that in this form it remains freely available to the active surface of the enzyme. This assumption is supported by the apparent independence of dieldrin formation on the total volume of the aqueous incubation mixture (Krieger and Wilkinson 1969; Lewis *et al.*, 1967) and renders questionable K_m values expressed in terms of molar concentration of this parameter.

However, apparent K_m values should still give some indication of the relative affinity to the microsomal complex for different substrates, if the same enzyme source is employed throughout. Calculations made from the data shown in Figure 8 establish K_m values of 0.22, 0.06, and 0.046 μmole, respectively, for aniline, aldrin, and TCBD. These figures suggest that the microsomal affinity for TCBD is greater than that for either aldrin or aniline by factors of 1.3 and 4.7, respectively.

The mixed-function oxidase complex of liver microsomes is able to accommodate a remarkable variety of compounds classified as foreign compounds. It is not yet clear whether the complex consists of one, two, or a considerable number of enzymes of minimal or low specificity linked with the microsomal electron-transport chain through cytochrome P 450. Possibly a protein moiety of P 450 itself may comprise a number of binding sites more or less closely associated with one another. Whatever the case, it has been established (Rubin *et al*,, 1964) that a number of drugs which are oxidatively metabolized by microsomal enzymes can inhibit the metabolism of other drugs by this same complex. Thus, the microsomal metabolism of ethylmorphine was found to be inhibited in a competitive manner by such drugs as hexobarbital, chlorpromazine, and zoxazolamine, and in some cases, as with the *N*-demethylation of ethylmorphine and the sulfoxidation of chlorpromazine, inhibition was of a mutual nature, each drug inhibiting the metabolism of the other. Such inhibition, referred to as alternative substrate inhibition, can arise by either competition of the different substrates for a common intermediate, or direct competition for a site on the binding surface of the protein itself. The latter can be envisaged more clearly if it is postulated that the binding portion of the microsomal complex is a single moiety of extremely low specificity, but competitive interaction is not necessarily precluded if a number of distinct but closely associated binding sites are involved.

In the presence of two compounds, both potential substrates for the microsomal enzymes, that substrate for which the active surface exhibits the highest affinity, or lowest K_m, will be bound in preference to the other. The present investigation has established that of the three substrates studied, the microsomal complex shows the highest affinity for TCBD. This fact alone could explain the marked inhibitory activity of compounds containing the 1,3-benzodioxole ring, as their presence within the microsomal complex could readily preclude the binding and subsequent metabolism of other substrates. However, in considering the inhibitory action of the

1,3-benzodioxoles yet another parameter should be included—the relative metabolic turnover for each of the substrates involved. As discussed elsewhere (Wilkinson, 1968), an ideal competitive inhibitor is one which is strongly bound to the active site of the enzyme and at the same time remains relatively stable to enzymatic degradation. In effect, a good competitive inhibitor should exhibit a high affinity for, but a low rate of metabolism by, the enzyme with which it interferes. If the inhibitor is itself extensively metabolized, its enzymic products must continually diffuse away from the surface, effectively reopening the active site and allowing more opportunity for subsequent interaction with the other substrate(s).

Table IV shows the specific activities for epoxidation, hydroxylation, and 1,3-benzodioxole cleavage in a number of vertebrate and invertebrate species. The relative specific activities of microsomal enzymes for different substrates vary considerably from one species to another. Thus, in the case of mouse liver microsomes, the specific activity for benzodioxolase is essentially the same as, or slightly higher than, that for aldrin epoxidase. This is in marked contrast to the situation observed with microsomes from the gut tissues of *Prodenia eridania* and *Hyalophora cecropia*, where the specific activity for aldrin epoxidation is greater than that observed for 1,3-benzodioxole cleavage by factors of 9 and 5, respectively.

In general agreement with Casida *et al.* (1966), it therefore appears that the 1,3-benzodioxoles are considerably more stable to enzymatic attack by insect microsomes than by those from mammalian liver. If the 1,3-benzodioxoles are acting competitively as alternative substrates for the microsomal enzymes, this greater stability should enhance their inhibitory activity in the insect preparation. That this is the case is indicated by the considerably greater inhibitory action of TCBD on aldrin epoxidation in *Prodenia* gut tissue microsomes (I_{50} 7.7 $\times$ $10^{-7}M$) than that observed in mouse liver microsomes (I_{50} 7.4 $\times$ $10^{-5}M$). The epoxidase in microsomal preparations from whole houseflies was also found to be 10- to 100-fold more susceptible to inhibition by derivatives of 1,3-benzodioxole than that obtained from pig liver (Lewis *et al.*, 1967).

The effects of three inhibitors of microsomal oxidation were studied on epoxidation, hydroxylation, and 1,3-benzodioxole cleavage. Table V shows that the I_{50} values for each inhibitor are similar for each of the enzymic reactions considered, SKF 525-A being the least effective of those evaluated. The marked inhibition of benzodioxolase by naphtho-(2,3-*d*)-1,3-dioxole (I_{50} 1.6 $\times$ $10^{-5}M$) and to a lesser extent sesamex (1.9 $\times$ $10^{-4}M$) is particularly interesting, as both compounds contain the 1,3-benzodioxole ring and are presumably both susceptible to the same type of metabolic attack as the substrate. Such inhibition is additional evidence in favor of the alternative substrate theory of 1,3-benzodioxole activity. The degree of inhibition of benzodioxolase activity by other compounds containing this same group will depend largely on the relative affinity of each compound for the active enzyme surface and on the relative concentration of each in the assay medium. Examination of the I_{50} values of a series of 1,3-benzodioxoles for aldrin epoxidation affords some indication of their relative affinity for the microsomal complex. It shows a relative order as follows for the three compounds under discussion: naphtho-(2,3-*d*)-1,3-dioxole (4.4 $\times$ $10^{-5}M$), TCBD (7.8 $\times$ $10^{-5}M$), sesamex (3.5 $\times$ $10^{-4}M$). It is not entirely surprising, therefore, that naphtho-(2,3-*d*)-1,3-dioxole is an effective inhibitor of the metabolism of TCBD (I_{50} 1.6 $\times$ $10^{-5}M$) while sesamex (I_{50} 1.9 $\times$ $10^{-4}M$) inhibits only at a concentration sufficiently

Table IV. Variation of Microsomal Enzyme Activity in Different Species

Species	Specific Activity, Mμmoles/Min./Mg. Protein × 10^2		
	Aldrin epoxidation	Aniline hydroxylation	1,3-Benzodioxole cleavage
Prodenia eridania[a]	59.3	3.2	6.8
Hyalophora cecropia[a]	21.1	Trace	4.4
Dog			
Male[b]	7.6	5.3	7.2
Female[b]	3.5	5.1	12
Mouse			
Male[b]	36	16	45
Female[b]	22	6.5	23
Rat			
Male[b]	30	3.1	5.4
Female[b]	9	1.7	2.4
Chicken (female)[b]	31	12	11
Mink (male)[b]	1.4	0.12	0

[a] Gut tissue microsomes.
[b] Liver microsomes.

Table V. Effect of Inhibitors on Microsomal Enzyme Activity

Inhibitor	I^{50} (Molar Concentration)		
	Aldrin epoxidation	Aniline hydroxylation	1,3-Benzodioxole cleavage
Sesamex	3.5 × 10^{-4}	1.3 × 10^{-4}	1.9 × 10^{-4}
Naptho-(2,3,-*d*)-1,3-dioxole	4.4 × 10^{-5}	1.4 × 10^{-5}	1.6 × 10^{5}
SKF 525-A	5.5 × 10^{-4}	26% at $10^{-3}M$	8.5 × 10^{-4}

high to counteract its apparently lower microsomal affinity. The I_{50} value for sesamex is approximately 5 times the concentration of TCBD routinely employed in the assay procedure (3.2 $\times$ $10^{-5}M$). Also naphtho-(2,3-*d*)-1,3-dioxole is approximately twice as effective as the TCBD as an in vivo synergist for carbaryl against houseflies (Wilkinson, 1967).

Inhibition by alternative substrates can result from either competition for a common intermediate or competitive binding to the active surface of the enzyme. If the former is correct, a direct relationship should exist between the rate of metabolism of the inhibitor and the degree of inhibition observed, as presumably a greater metabolic turnover of the inhibitor would involve the utilization of more of the common intermediate which consequently becomes rate-limiting on the other substrate. It appears from this investigation, however, that an inverse relationship exists and that more effective inhibition is associated with a lower metabolic turnover of the inhibitor. This situation favors the idea that competitive inhibition by the 1,3-benzodioxoles results chiefly from competitive binding at the active surface of the microsomal complex.

In any discussion relating to the biological activity of the 1,3-benzodioxoles an important fact must be born in mind—the extreme specificity of the 1,3-benzodioxole ring itself. A number of structure-activity investigations have clearly established the essentiality and specificity of the 1,3-benzodioxole nucleus for in vivo synergistic activity with both the pyrethrins (Hewlett, 1960) and the carbamates (Metcalf, 1967; Moorefield and Weiden, 1964; Wilkinson, 1965; Wilkinson *et al.*, 1966). Even slight structural modifications in this central bicyclic system usually result in a marked decrease or complete loss of synergistic activity. Furthermore, until the recent discovery of a number of aromatic propynyl ethers (Kooy, 1966; Sacher *et al.*, 1968), the 1,3-benzodioxoles were

as a group essentially unique in promoting this type of biological activity.

If, as suggested, the 1,3-benzodioxoles are acting as alternative substrates, it is somewhat surprising that synergistic activity is not a more common property associated with a considerable number of compounds which are similarly bound and metabolized by the microsomal enzymes. This is not the case, however, and apparently some special structural feature incorporated into the 1,3-benzodioxole nucleus greatly enhances the ability of compounds containing this moiety to compete successfully with a large number of other compounds for a site on the active surface of the enzyme.

At first sight the alternative substrate theory is in marked opposition to other proposals to explain the mechanism of synergistic action. The very fact that the 1,3-benzodioxoles are metabolized by an enzyme comprising part of the microsomal mixed-function oxidase complex casts immediate doubt on the veracity of Hennessy's (1965) hydride ion transfer concept and Hansch's (1968) suggestion for the involvement of homolytic free radicals. If either of these proposals is correct, the 1,3-benzodioxoles should immediately block all mixed-function oxidase activity and consequently no cleavage of the 1,3-benzodioxole ring would occur. Furthermore, inhibition by either mechanism would almost certainly prove to be of a noncompetitive nature whereas, although not absolutely clear, available information suggests that it more closely resembles a competitive situation (Lewis *et al.*, 1967; Philleo *et al.*, 1965; Wilkinson, 1968).

Acceptance of the alternative substrate theory need not necessarily involve a simultaneous rejection of the principles incorporated in the proposals of Hennessy or Hansch. It is still necessary to elucidate the nature of the essential structural features associated with the 1,3-benzodioxole ring which will allow compounds containing this group to compete so successfully with a large number of other compounds for sites on the microsomal enzymes. Virtually nothing is known regarding the molecular requirements for efficient binding at the active surface and it is probable that electronic interactions as well as steric properties are involved in the process. It is therefore entirely possible that such properties as the ability of the 1,3-benzodioxoles to produce free radicals or their tendency to produce the benzodioxolium ion by hydride ion transfer are in some way related to their superior binding capacity with the microsomal complex.

LITERATURE CITED

Beroza, M., J. Agr. Food Chem. **11**, 51 (1963).
Brooks, G. T., Harrison, A., *Chem. Ind. (London)* **1966,** 1414.
Casida, J. E., Engel, J. L., Essac, E. G., Kamienski, F. X., Kuwatsuka, S., *Science* **153,** 1130 (1966).
Cloney, R. D., Scherr, V. M., J. Agr Food Chem. **16,** 791 (1968).
Fincham, J. R. S., *J. Gen. Microbiol.* **11,** 236 (1954).
Fishbein, L., Fawkes, J., Falk, H. L., Thompson, S., *J. Chromatog.* **27,** 153 (1967a).
Fishbein, L., Fawkes, J., Falk, H. L., Thompson, S., *J. Chromatog.* **29,** 267 (1967b).
Hansch, C., *J. Med. Chem.* **11,** 920 (1968).
Hennessy, D. J., J. Agr. Food Chem. **13,** 218 (1965).
Hewlett, P. S., *Advan. Pest Control Res.* **3,** 27 (1960).
Kato, R., Gillette, J. R., *J. Pharmacol. Exptl. Therap.* **150,** 279 (1965).
Kooy, H. J., Jr. (to Hoffman-La Roche), Dutch Patent **6,601,926** (Aug. 17, 1966).
Krieger, R. I., Wilkinson, C. F., *Biochem. Pharmacol.* **18,** (1969, in press).
Lewis, S. E., Wilkinson, C. F., Ray, J. W., *Biochem. Pharmacol.* **16,** 1195 (1967).
Metcalf, R. L., *Ann. Rev. Entomol.* **12,** 229 (1967).
Metcalf, R. L., in "Enzymatic Oxidations of Toxicants," p. 151, North Carolina State University, Raleigh, 1968.
Mitchell, L. C., *J. Assoc. Offic. Agr. Chemists* **40,** 294 (1957).
Moorefield, H. H., Weiden, M. H. S., *Contrib. Boyce Thompson Inst.* **22,** 425 (1964).
Philleo, W. W., Schonbrod, R. D., Terriere, L. C., J. Agr. Food Chem. **13,** 113 (1965).
Rubin, A., Tephly, T. R., Mannering, G. J., *Biochem. Pharmacol.* **13,** 1007 (1964).
Sacher, R. M., Metcalf, R. L., Fukuto, T. R., J. Agr. Food Chem. **16,** 779 (1968).
Schenkman, J. B., Ball, J. A., Estabrook, R. W., *Biochem. Pharmacol.* **16,** 1071 (1967).
Schmidt, C. H., Dahm, P. A., *J. Econ. Entomol.* **49,** 729 (1956).
Wilkinson, C. F., doctoral dissertation, University of California, Riverside, Calif., 1965.
Wilkinson, C. F., in "Enzymatic Oxidations of Toxicants," p. 113, North Carolina State University, Raleigh, 1968.
Wilkinson, C. F., J. Agr. Food Chem. **15,** 139 (1967).
Wilkinson, C. F., Metcalf, R. L., Fukuto, T. R., J. Agr. Food Chem. **14,** 73 (1966).
Williams, R. T., "Detoxication Mechanisms," 2nd ed., Wiley, New York, 1959.

Received for review November 18, 1968. Accepted February 3, 1969.