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GENERATION OF CARBON MONOXIDE DURING THE MICROSOMAL METABOLISM OF METHYLENEDIOXYPHENYL COMPOUNDS

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Abstract— Optical difference spectra of several methylenedioxybenzenes in NADPH-reduced rat liver microsomes exhibited a peak at 451.5 nm when conditions became anerobic or when dithionite was added. Subsequent addition of hemoglobin showed formation of a peak at 419 nm, suggesting the presence of carbon monoxide. Metabolic production of carbon monoxide was confirmed by gas chromatographic assay, and regression analysis of data with nine compounds showed a high correlation with the Hammett constants of the aromatic substituents. The enzymes catalyzing carbon monoxide production were localized in rat hepatic microsomal fractions and required NADPH. Microsomal fractions from phenobarbital-treated, but not from 3-methylcholanthrene-treated, rats showed increased production of carbon monoxide and from cobaltous chloride-treated rats showed decreased carbon monoxide formation. The reaction was inhibited by 2-diethylaminoethyl 2,2-diphenylvalerate HCl (SKF 525-A) and substituted imidazoles. The studies show that the carbon monoxide production was mediated by a cytochrome P-450-dependent enzyme system; this is discussed in relation to the inhibitory action of methylenedioxybenzenes toward oxidative drug metabolism.

Methylenedioxyphenyl (1,3-benzodioxole) compounds, typified by piperonyl butoxide, have long been recognized as effective *in vitro* inhibitors of microsomal oxidations [1–8] as well as for their ability to synergize the activity of many drugs and insecticides *in vivo* in both mammals and insects [2–4,7,8].

Following the early discovery that methylenedioxyphenyl compounds were metabolized to the corresponding catechols by mixed-function oxidases [9,10], it was suggested that their inhibitory action might occur largely through competitive alternative substrate interactions. Evidence for a more specific inhibitory mechanism was obtained with the establishment of an apparent decrease in cytochrome P-450 levels in microsomal preparations from mammals [11] and insects [12,13] treated *in vivo* with piperonyl butoxide. A similar decrease in cytochrome P-450 was observed *in vitro* in NADPH-fortified mouse liver microsomal fractions incubated aerobically with piperonyl butoxide [14] and was found to coincide with the development of a characteristic optical difference spectrum (termed 'Type III') with duel Soret peaks at 455 and 427 nm existing in a pH-dependent equilibrium. More detailed studies have confirmed that, in the presence of either NADPH and O_2[4,15–17] or cumene hydroperoxide [18,19], several methylenedioxyphenyl compounds are metabolized by mammalian liver microsomes to intermediates capable of binding to reduced cytochrome P-450 to give relatively stable inhibitory complexes exhibiting Type III difference spectra. The nature of these active intermediates remains unknown [2]. The most recent suggestions favor the formation of a carbene species [20], although homolytic radicals [21], benzodioxolium ions [22] and carbanions [23] have at different times been implicated in the inhibitory mechanism.

Since formation of the complex is correlated with the noncompetitive inhibition of drug metabolism [24] and since it can be demonstrated in microsomal fractions from animals treated *in vivo* with various methylenedioxyphenyl compounds [25,26], it would appear to play a major role in the biological activity of many of these compounds.

However, studies on the interactions of a variety of other derivatives of methylenedioxybenzene with microsomal oxidases and cytochrome P-450 [2] have shown that, although most are inhibitors of drug oxidation, many do not generate a typical Type III difference spectrum in NADPH-reduced microsomal suspensions. Of particular interest is the reported formation with several compounds of a peak at 450 nm which resembles the CO difference spectrum [2,27,28].

Carbon monoxide has been identified only recently as a metabolite of xenobiotics. Kubic *et al.* [29,30] have shown that dihalomethanes are converted to carbon monoxide both *in vivo* and *in vitro*. This reaction is catalyzed by hepatic cytochrome P-450-dependent mixed-function oxidases, and a formyl halide appears to be formed as an intermediate [31]. Other studies have shown that haloforms are also

metabolized to carbon monoxide both *in vivo* and *in vitro* [32,33]. This reaction is also catalyzed by cytochrome P-450-dependent mixed-function oxidases, and reaction mechanism studies indicate that dihalocarbonyl compounds are intermediates in the conversion of haloforms to carbon monoxide [34].

The studies reported in this communication were designed to investigate further the spectral interactions of several methylenedioxyphenyl compounds with hepatic microsomal enzymes and to characterize the enzyme systems involved in the biotransformation of these compounds to carbon monoxide.

MATERIALS AND METHODS

Chemicals. 1,2-Methylenedioxybenzene (MDB) and its 4-bromo-, 4-chloro- and 4-methyl-derivatives were purchased from Frinton Laboratories, Vineland, NJ, and piperonyl nitrile (4-cyano-MDB), sesamol (4-hydroxy-MDB) and piperonyl alcohol (4-hydroxymethyl-MDB) were from the Aldrich Chemical Co., Milwaukee, WI. Piperonyl butoxide was provided by the FMC Corp. (Niagara Chemical Division), Middleport, NY. All of the other MDB derivatives employed in this investigation were synthesized by established procedures [35].

Hemoglobin was purchased from the Pentex Research Corp., Kankakee, IL, and cumene hydroperoxide from Matheson, Coleman & Bell, Norwood, OH. Biochemicals were purchased from CalBiochem, La Jolla, CA, or from the Sigma Chemical Co., St. Louis, MO. All other chemicals and solvents employed were of analytical reagent grade.

Animals. Male Sprague–Dawley derived rats (200–300g) were purchased from either Blue Spruce Farms, Altamont, NY, or from Bio-Labs, White Bear Lake, MN.

Hepatic microsomal fractions for use in the spectral studies were prepared from animals starved for 12 hr before being killed. Livers were perfused *in situ* with 5–10 ml of cold 0.15 M KCl prior to homogenization [36]. The metabolism studies were conducted with microsomal fractions isolated as described previously [37]. Protein was determined by the method of Lowry *et al.* [38].

Phenobarbital sodium was given i.p. at a dose of 50 mg/kg once daily for 4 days; control animals received 0.9% saline solution. 3-Methylcholanthrene, dissolved in corn oil, was given i.p. at a dose of 40 mg/kg once daily for 2 days; control animals were given corn oil alone. Cobaltous chloride hexahydrate was dissolved in water and given by subcutaneous injection once daily for 2 days at a dose of 40 mg/kg; control animals were given a 0.9% saline solution.

Spectral studies. Optical difference spectra were measured at 37° with an Aminco DW-2 spectrophotometer using 1 cm cuvettes and 1 ml aliquots of microsomal suspensions (1.8 to 2.5 mg protein/ml) in Tris–HCl buffer (50 μmoles Tris, pH 7.4) containing 150 μmoles KCl and 0.2 μmoles EDTA. NADPH (1 μmole) was added to each cuvette as required and, after base line adjustment, the reactions were initiated by addition to the sample cuvette of appropriate concentrations of MDB derivatives in 10 μl ethanol; the reference cuvette received an equal volume of ethanol. Unless otherwise indicated, repetitive scans (5 nm/sec) between 400 and 500 nm were made at times dictated by the nature of the experiment.

Carbon monoxide (CO) formed during the spectral studies was measured from the CO–hemoglobin difference spectra obtained by addition of a few grains of sodium dithionite and 2 μmoles hemoglobin to each cuvette. The difference in optical density (O.D.) between 419 and 425 nm (the isobestic point) was quantified by comparison with standard curves of the $O.D._{419-425}$ resulting from addition of known microliter amounts of a saturated aqueous solution of CO (1 mM at room temp) to dithionite-reduced microsomal suspensions containing 2 μmoles hemoglobin. Standard curves were prepared for each microsomal suspension.

Simultaneous recordings of oxygen uptake and changes in optical difference spectra were made by means of the Aminco vibrating platinum electrode accessory for the DW-2 spectrophotometer and were conducted in 1 cm cuvettes containing 2.5 ml of microsomal suspension.

Metabolism studies. Incubation mixtures for studying the conversion of methylenedioxyphenyls to CO contained, unless otherwise indicated, Tris–EDTA buffer (150 μmoles Tris and 0.6 μmoles EDTA, pH 7.4), 15 μmoles $MgCl_2$, an NADPH-generating system (10 μmoles DL-isocitric acid, 1 μmole NADP† and 1 unit of pig heart isocitric dehydrogenase), 4.8 μmoles of 4-cyano-MDB added in 32 μl ethanol and 2.70 to 2.88 mg of microsomal protein in a total volume of 3.0 ml.

The 15 min (37°) reactions were initiated by addition of protein and terminated by placing on ice. The incubation mixtures were prepared in 25 ml Erlenmeyer flasks capped with sleeve-type serum stoppers. Carbon monoxide was measured as described previously [30].

Enzyme kinetic constants were calculated by the method of Wilkinson [39] using BASIC programs written in this laboratory.

Regression analysis. Structure–activity correlations were examined by regression analysis [40] using the IBM 360/65 computer at Cornell University. The program employed (ECON) provides estimates of the parameters of single equation models by the method of least squares.

RESULTS

Spectral studies. When MDB (1 mM) was added to a suspension of rat liver microsomes, a Type I difference spectrum appeared with a trough at 420 nm and a peak at about 385 nm. On addition of NADPH to each cuvette, the Type I spectrum was immediately replaced by a Type III difference spectrum with dual Soret peaks at 459–460 nm and 429–430 nm which slowly increased in intensity during the first few minutes of incubation (Fig. 1). A slight increase in absorbance occurred after about 7–9 min and was associated with a bathochromic peak shift from 459 to 455 nm. Only relatively small absorbance changes occurred at the 429 nm peak during this period, and no change in wavelength was observed. On further incubation and depletion of the NADPH

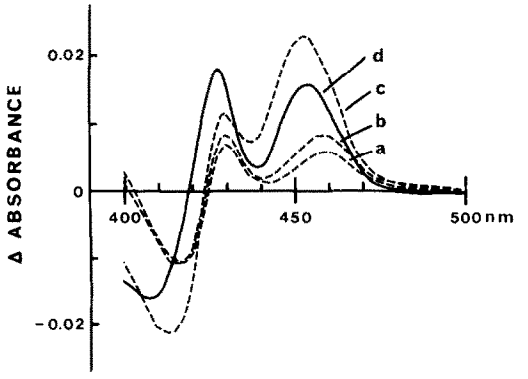


Fig. 1. Optical difference spectra formed on incubation of MDB with NADPH-reduced rat liver microsomes. Microsomal suspension contained 2.53 mg protein/ml; and incubation conditions and reaction mixture were as described in Materials and Methods. Spectra a, b and c were obtained at 5, 9 and 11 min, respectively, after addition of MDB (1 μmole) to the sample cuvette. Spectrum d was obtained at 16 min after addition of sodium dithionite to both cuvettes.

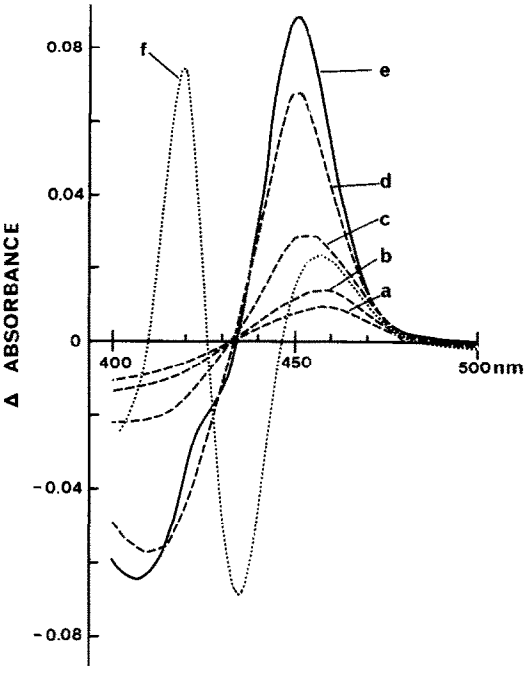


Fig. 2. Optical difference spectra formed on incubation of 4-cyano-MDB with NADPH-reduced rat liver microsomes. Microsomal suspensions contained 2.63 mg protein/ml; incubation conditions and reaction mixture were as described in Materials and Methods. Spectra a, b, c and d were obtained at 2, 4, 6 and 8 min respectively, after addition of 4-cyano-MDB (1 μmole) to the sample cuvette. Spectra e and f, respectively, are those obtained after the addition (in order) of sodium dithionite and hemoglobin (2 μmoles) to each cuvette.

in the reaction mixture, the dual Soret peaks decreased in intensity and were replaced by a single broad peak at 438 nm corresponding to the oxidized form of the Type III difference spectrum. The subsequent addition of sodium dithionite to both cuvettes, either before or after depletion of NADPH, fixed the absorbance maxima at 455 and 427 nm and the spectrum thus formed remained quite stable over a period of at least 3 hr. Spectral characteristics similar to those described for MDB were exhibited by piperonyl butoxide, and the 4-methyl-, 4-methoxy- and 4-hydroxymethyl-derivatives of MDB. In all cases the absorbance maxima of 459–463 nm and 429–432 nm initially observed in difference spectra of NADPH-reduced microsomes were stabilized at 427 nm and 455 nm following incubation and dithionite reduction. Incubation of these compounds with cumene hydroperoxide (0.5 μmole) instead of NADPH first caused formation of the oxidized Type III difference spectrum (438 nm peak) which was replaced immediately by the reduced spectrum (455 and 427 nm peaks) on addition of dithionite. Irrespective of whether the initial incubations were carried out in the presence of NADPH or cumene hydroperoxide, the addition of hemoglobin to both cuvettes after reduction with dithionite caused no additional spectral changes.

Spectral studies with other MDB compounds including the 4-bromo-, 4,5-dibromo-, 4,5-dichloro-, 4-cyano- and 4-bromo-5-methoxy-derivatives, as well as 2,3-methylenedioxynaphthalene, showed that, although all exhibited Type I difference spectra in oxidized microsomal suspension, their interactions in NADPH-reduced microsomes were quite distinct from those described previously. Thus, the addition of 4-cyano-MDB (1 mM) to an NADPH-reduced microsomal suspension caused the rapid appearance of a difference spectrum with a single, rather broad absorbance maximum at 458–459 nm (Fig. 2). On continued incubation at 37°, the absorbance increased steadily and its maximum shifted progressively to a shorter wavelength (2 min, 456 nm; 4 min, 454 nm, etc.). After about 6 min, a rapid and dramatic increase in absorbance occurred (2- to 4-fold) and the peak stabilized at 451.5–452 nm. The maximum absorbance achieved declined only slowly over the subsequent 10–15 min. Addition of sodium dithionite to both cuvettes after full absorbance development caused a further increase in absorbance at 451.5 nm and the appearance of a shoulder between 420 and 430 nm which was not visible previously. This latter was qualitatively similar to the CO-reduced cytochrome P-450 spectrum; this similarity was strengthened by calculations based on differences between the CO-reduced cytochrome P-450 difference spectra in microsomes incubated with NADPH in the presence and the absence of 1 mM 4-cyano-MDB which showed the extinction coefficient of the 451.5 nm peak to be 93.9 ± 15.3 (N = 6). That the 451.5 nm peak was indeed the CO-reduced cytochrome P-450 spectrum was demonstrated clearly by the fact that addition of hemoglobin to both cuvettes after dithionite reduction caused formation of a peak for carboxyhemoglobin at 419 nm and resulted in a concomitant disappearance of the 451.5 nm peak (Fig. 2f); in all cases, however,

Table 1. Spectral changes occurring in incubations of various methylenedioxybenzene (MDB) compounds with NADPH-reduced rat liver microsomes*

Compound	ΔO.D.$_{451.5-490\ nm}$ NADPH alone (A)	Dithionite (B)	CO to sample (C)	A/B	B/C
Control (none)			0.107†		
4-Cyano-MDB	0.065	0.090	0.101	0.72	0.89
4-Bromo-MDB	0.054	0.084	0.106	0.64	0.79
4,5-Dibromo-MDB	0.060	0.088	0.108	0.68	0.82
4,5-Dichloro-MDB	0.063	0.076	0.101	0.83	0.75
4-Bromo-5-methoxy-MDB	0.040	0.055	0.104	0.72	0.53
2,3-Methylenedioxynaphthalene	0.057	0.070	0.107	0.81	0.65
4-Hydroxy-MDB	0.066	0.902	0.109	0.72	0.84

* Data are means of duplicates obtained with a single microsomal preparation. Incubations were conducted at 37° in 1 cm cuvettes in an Aminco DW-2 spectrophotometer. Reaction mixtures (1 ml) contained microsomal suspension (2.4 mg protein) in 0.05 M Tris–HCl buffer (pH 7.4) containing 0.15 M KCl, 0.2 mM EDTA and 1 mM NADPH. Reactions were initiated by addition of the test compound (1 μmole in 10 μl ethanol) to the sample cuvette (reference cuvette received 10 μl ethanol). Absorbance values at 451.5–490 nm were measured after full development of the NADPH spectrum (6–10 min) (A) and subsequently after addition of sodium dithionite to both cuvettes (B) and after addition of CO to sample (C).

† From CO-difference spectrum in untreated, dithionite-reduced, microsomes.

a rather asymmetric absorbance peak at 455–458 nm remained after addition of hemoglobin.

The same general sequence of spectral changes was observed during incubation with NADPH-fortified microsomes (and subsequent addition of dithionite) of 2,3-methylenedioxynaphthalene and 4-hydroxy-, 4-bromo-, 4,5-dibromo-, 4,5 dichloro- and 4-bromo-5-methoxy-MDB; 4-hydroxy-MDB (sesamol) exhibited a somewhat atypical pattern of spectral changes in that the 451.5 nm peak appeared almost immediately from the start of the reaction and did not entail prior formation of an absorbance peak at a higher wavelength. Similar spectral studies with 4-nitro-MDB also indicated the formation of the 451.5 nm peak but intense absorbance of the compound itself precluded accurate spectral measurements in the 400–450 nm region.

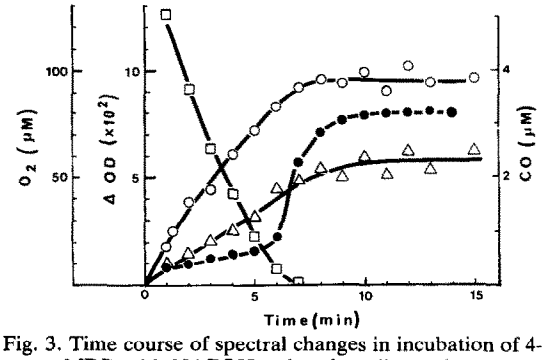


Fig. 3. Time course of spectral changes in incubation of 4-cyano-MDB with NADPH-reduced rat liver microsomes. Reaction mixture and incubation conditions were as described in Materials and Methods. Plots shown are for ΔO.D. observed in NADPH-reduced microsomes (●——●), ΔO.D.$_{451.5-490\ nm}$ after addition of dithionite to both cuvettes (○——○), ΔO.D.$_{419-425\ nm}$ after addition of hemoglobin (2 μmoles) to both cuvettes following dithionite reduction (△——△) and concentration of oxygen in the reaction mixture (□——□). See text for full explanation.

Incubation of these same compounds with cumene hydroperoxide (0.5 μmole to each cuvette) instead of NADPH caused the appearance of a broad ill-defined peak around 400 nm. On reduction with dithionite, the 451.5 nm peak was formed immediately and subsequent addition of hemoglobin to both cuvettes caused the appearance of the typical carboxyhemoglobin spectrum (419 nm) with a small additional peak at 455–456 nm. Quantitative data on CO production in the presence of cumene hydroperoxide were not obtained due to the significant destruction of cytochrome P-450 by this reagent.

Although the time at which rapid development of the 451.5 nm peak occurred varied somewhat from one compound to another during the NADPH-mediated reactions, the relative increase in absorbance observed following addition of dithionite (A/B, Table 1) was quite similar with each compound. In all cases, bubbling CO through the sample cuvette after reduction with dithionite caused a further increase in absorbance at 451.5 nm (B/C, Table 1), indicating the presence of a residual amount of cytochrome P-450 not complexed with the CO generated during the reaction; this varied from 11 per cent with 4-cyano-MDB to 47 per cent with the 4-bromo-5-methoxy-derivative. The final absorbance attained at 451.5 nm after bubbling CO through the sample cuvette was identical to that of the CO-reduced cytochrome P-450 measured in untreated control microsomes (Table 1).

In an attempt to explain the rapid development of the large 451.5 nm peak in NADPH-reduced microsomes, simultaneous recordings were made of spectral changes and oxygen concentration. These clearly showed (Figs. 3 and 4) that the 451.5 nm peak became visible only when the oxygen concentration in the sample cuvette had been reduced to about 10–15 μM. The time at which both events occurred could be varied by changing conditions in the cuvette (temperature, etc.), and reintroduction of oxygen after full development of the 451.5 nm

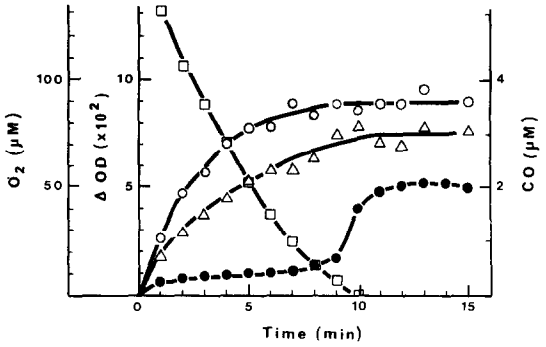


Fig. 4. Time course of spectral changes in incubations of 4,5-dichloro-MDB with NADPH-reduced rat liver microsomes. Reaction mixture and incubation conditions were as described in Materials and Methods. Plots shown are for ΔO.D.observed in NADPH-reduced microsomes (●——●), $\Delta O.D._{451.5-490\ nm}$ after addition of dithionite to both cuvettes (○——○), and $\Delta O.D._{419-425\ nm}$ after addition of hemoglobin (2 μmoles) to both cuvettes following dithionite reduction (△——△) and concentration of oxygen in the reaction mixture (□——□). See text for full explanation.

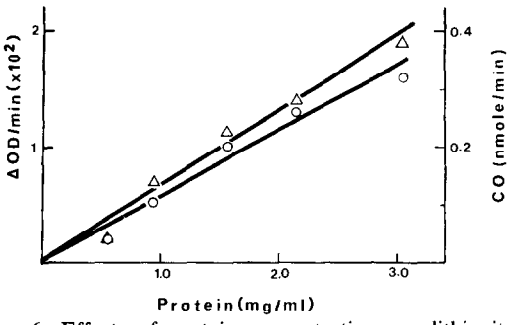


Fig. 6. Effects of protein concentration on dithionite-reduced difference spectra and CO production in incubations of 4-cyano-MDB with NADPH-reduced rat liver microsomes. Incubations were conducted at 37° in 1 cm cuvettes containing a 1 ml reaction mixture, as described in Materials and Methods. The reaction was initiated by addition of 4-cyano-MDB (1 μmole) to the sample cuvette. After 5 min of incubation, dithionite was added to both cuvettes and the $\Delta O.D._{451.5-490\ nm}$ was measured (○——○). CO production was determined from $\Delta O.D._{419-425\ nm}$ after subsequent addition of hemoglobin (2 μmoles) to each cuvette (△——△).

peak caused a rapid decline in its absorbance and reappearance of the smaller 455–457 nm peak.

Addition of sodium dithionite to each cuvette at different times after initiation of the reaction caused the immediate formation of the 451.5 nm peak, and the size of the peak increased with time to reach a plateau level which was always greater that that which developed following incubation with NADPH alone (Figs. 3 and 4, Table 1). The rate of formation of the 451.5 nm peak observed after dithionite addition remained linear for the first few minutes of the reaction and paralleled the rate of production of CO as measured spectrophotometrically ($\Delta O.D._{425-419}$) following addition of hemoglobin (Figs. 3 and 4). Using this method, the rate of formation of CO with 4-hydroxy-, 4-cyano-, 4-bromo-, 4,5-dibromo- and 4,5-dichloro-MDB and 2,3-methylenedioxynaphthalene (all at 1 mM) varied between 0.1 and 0.15 nmole/min/mg microsomal protein. The rate was somewhat less for 4-bromo-5-methoxy-MDB, and no CO production was detected by this method with piperonyl butoxide, piperonyl alcohol, MDB, 4-methyl-MDB or 4-methoxy-MDB.

The size of the 451.5 nm peak, developed after addition of sodium dithionite to microsomal suspensions incubated with NADPH and 4-cyano-MDB (1 mM) for different times, increased with increasing protein concentration (Fig. 5), but its rate of formation remained linear for only about 5 min. Over this latter period, the formation of the 451.5 nm peak correlated well with CO production up to 3 mg protein/ml (Fig. 6), and both parameters exhibited

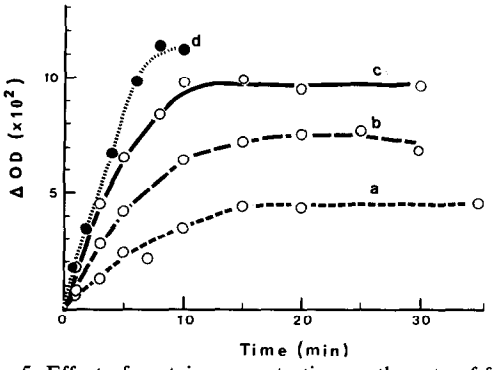


Fig. 5. Effect of protein concentration on the rate of formation of dithionite-reduced difference spectra in incubations of 4-cyano-MDB with NADPH-reduced microsomes. Incubations were conducted at 37° in 1 cm cuvettes containing a 1 ml reaction mixture, as described in Materials and Methods. Reactions were initiated by addition of 4-cyano-MDB (1 μmole) to NADPH-reduced microsomal suspensions containing 1.05, 1.60, 2.50 and 3.30 mg protein/ml. After appropriate incubation times, dithionite was added to both cuvettes and $\Delta O.D._{451.5-490\ nm}$ was determined. Curves a, b, c and d were obtained in order of increasing protein concentration.

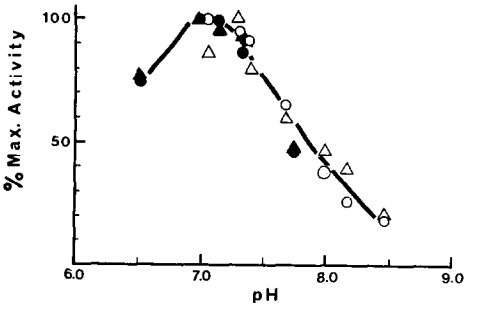


Fig. 7. Effects of pH on formation of dithionite-reduced difference spectra and CO production in incubations of 4-cyano-MDB with NADPH-reduced rat liver microsomes. Incubations were conducted at 37° in 1 cm cuvettes in 1 ml reaction mixtures at appropriate pH. Reactions were initiated by addition of 4-cyano-MDB (1 μmole) to the sample cuvette and incubations were for 5 min. $\Delta O.D._{451.5-490\ nm}$ was measured after addition of dithionite to both cuvettes containing either 0.1 M phosphate buffer 0.2 mM EDTA (●——●) or 0.05 M Tris–HCl, 0.15 M KCl and 0.2 mM EDTA (○——○). CO production was measured from $\Delta O.D._{419-425\ nm}$ following subsequent addition of hemoglobin (2 μmoles) to the respective reaction mixtures (▲——▲) and (△——△).

Table 2. Cofactor requirements for the metabolism of 4-cyano-methylenedioxybenzene to carbon monoxide*

Cofactor	CO (nmoles/min)
NADPH	1.63 ± 0.23
NADPH (heat-inactivated enzyme)	0
NADH	0.18 ± 0.12
NADPH +NADH	1.06 ± 0.23
GSH	0.01 ± 0.01
NADPH + GSH	1.57 ± 0.38
Cumene hydroperoxide	1.78 ± 0.15
NADPH − air	0.48 ± 0.26

* Incubations and analyses were conducted as described in Materials and Methods except that the NADPH-generating system was omitted. The final concentrations of both NADPH and NADH were 1 mM; GSH, solution adjusted to pH 7.4, was added to a final concentration of 10 mM. The cumene hydroperoxide concentration was 0.5 mM; this experiment and that in which air was omitted were carried out under anerobic conditions produced by evacuating and refilling the capped incubation flasks with nitrogen gas five times. Data represent the means ± S.D., N = 3, except in the experiment where air was omitted where N = 4.

a similar dependence on pH (Fig. 7), the optimum occurring between 6.9 and 7.5.

More complete characterization of the biotransformation of MDB compounds to CO was effected through metabolic studies utilizing a direct and sensitive measure of CO production.

Metabolic studies. Initial metabolic experiments clearly confirmed that CO was generated during the microsomal metabolism of MDB compounds, and 4-cyano-MDB was selected as a model substrate for further characterization studies.

The reaction was localized in hepatic microsomal fractions (data not shown) and its enzymatic nature indicated by the fact that insignificant metabolic rates were observed in heat-inactivated (5 min at 95°) preparations.

Cofactor studies showed that NADPH was required for maximal activity (Table 2). NADH was much less effective as a cofactor and the addition of NADH to incubation mixtures containing NADPH resulted in a lowered rate of CO production. GSH alone did serve as a cofactor and did not alter CO production from 4-cyano-MDB in the presence of NADPH. In agreement with the data from the spectral studies, cumene hydroperoxide, which is known to support the cytochrome P-450-dependent metabolism of MDB [18,19] and of many other compounds [41], was also effective in supporting the conversion of 4-cyano-MDB to CO (Table 2).

The enzyme exhibited a rather broad pH optimum extending from pH 7.0 to 8.0 (Fig. 8). The reaction was linear with time for 15 min and showed a non-linear dependence on protein concentration (Fig. 8). On the basis of these studies, a reaction time of 15 min was used and the protein concentration was held constant at 0.90 to 0.96 mg protein/ml; reaction rates are expressed as nmoles CO formed/min.

The metabolism of 4-cyano-MDB to CO was also studied in rats treated with agents known to alter

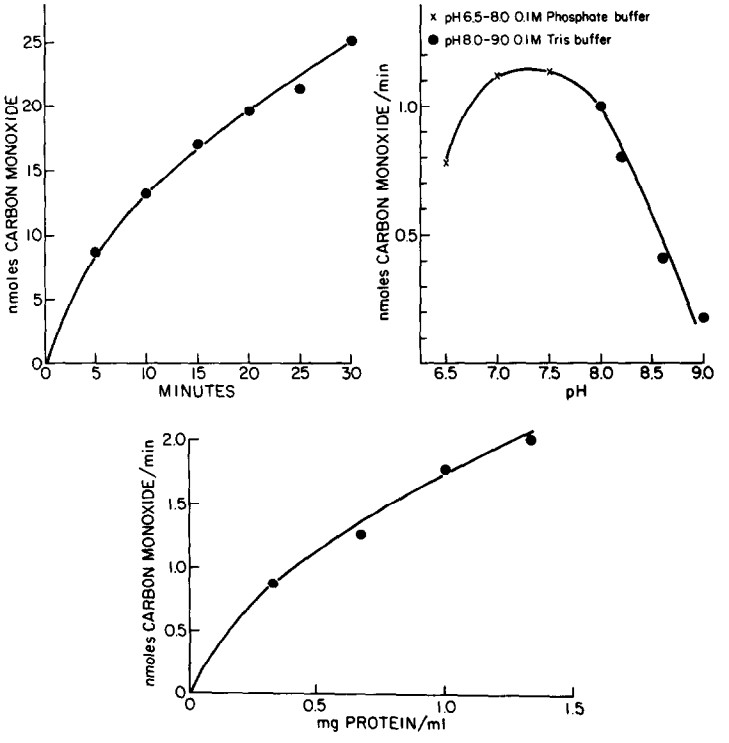


Fig. 8. Dependence of the metabolism of 4-cyano-MDB to carbon monoxide on time (upper left), pH (upper right) and protein concentration (lower). The incubation conditions and analyses were carried out as described in Materials and Methods except that the parameters were varied as indicated on the abscissa of each panel.

Table 3. Effects of phenobarbital, 3-methylcholanthrene or cobaltous chloride treatment on the metabolism of 4-cyano-methylenedioxybenzene to carbon monoxide*

Treatment	CO (nmoles/min)	% Control
Saline control	1.16 ± 0.34	100
Phenobarbital	1.92 ± 0.06	166
Corn oil control	1.21 ± 0.07	100
3-Methylcholanthrene	1.13 ± 0.08	93
Saline control	1.53 ± 0.23	100
Cobaltous chloride	0.81 ± 0.20	53

* Animal treatments, incubations and analyses were conducted as described in Materials and Methods. Data are shown as means ± S.D., N = 3.

hepatic cytochrome P-450 levels. Microsomal fractions isolated from phenobarbital-treated rats showed a significant increase in rates of metabolism, while those isolated from 3-methylcholanthrene-treated rats were unchanged (Table 3). Treatment of rats with cobaltous chloride resulted in markedly lower rates of conversion of 4-cyano-MDB to CO (Table 3).

Three known inhibitors of cytochrome P-450-mediated reactions, SKF 525-A (2-diethylaminoethyl 2,2-diphenylvalerate HCl), 1-phenylimidazole and 1-(2-isopropylphenyl)imidazole, all inhibited the metabolism of 4-cyano-MDB to CO (Table 4); the latter inhibitor was by far the most potent, causing 95 per cent inhibition at a concentration of 10 μM.

The rates of CO formation from a total of fifteen substituted MDB compounds were determined (Table 5), and data for twelve of these were measured at a comparable substrate concentration of 2.0 mM; as a result of their insolubility in the reaction mixture, data for 4-nitro- and 4-methoxy-5-nitro-MDB and for 2,3-methylenedioxynaphthalene could only be obtained at the lower concentrations indicated. The CO production data for nine of the twelve compounds were subjected to regression analysis using various forms of equation,

$$\text{Log CO} = a\pi^2 + b\pi + \rho\sigma + k \qquad (1)$$

Table 4. Effects of inhibitors on the metabolism of 4-cyano-methylenedioxybenzene to carbon monoxide*

Inhibitor (μM)	CO (nmoles/min)	% Control
None	1.34 ± 0.24	100
SKF 525-A (1)	1.20 ± 0.19	90
SKF 525-A (10)	0.73 ± 0.23	54
SKF 525-A (100)	0.42 ± 0.12	31
1-Phenylimidazole (1)	1.13 ± 0.15	84
1-Phenylimidazole (10)	0.64 ± 0.09	48
1-Phenylimidazole (100)	0.13 ± 0.04	10
1-(2-Isopropylphenyl)imidazole (0.1)	1.21 ± 0.18	90
1-(2-Isopropylphenyl)imidazole (1)	0.46 ± 0.06	34
1-(2-Isopropylphenyl)imidazole (10)	0.07 ± 0.04	5

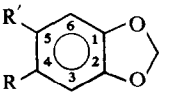
* Incubations and analyses were conducted as described in Materials and Methods; the concentration of 4-cyano-MDB was 1.6 mM. Data are shown as the means ± S.D., N = 3.

Table 5. Metabolism of substituted methylenedioxybenzenes to carbon monoxide*

Substrate	Concn. (mM)	CO (nmoles/min)
4-Cyano-MDB	2.0	2.06 ± 0.13
4-Nitro-MDB	0.2	0.58 ± 0.07
4-Hydroxy-MDB	2.0	3.19 ± 0.38
4-Hydroxymethyl-MDB	2.0	0.28 ± 0.11
4-Methoxy-MDB	2.0	0.21 ± 0.10
4-Methyl-MDB	2.0	0.21 ± 0.10
4-Bromo-MDB	2.0	0.86 ± 0.18
4-Allyl-MDB (Safrole)	2.0	0.26 ± 0.13
4-Methoxy-5-nitro-MDB	0.13	0.44 ± 0.02
4-Bromo-5-methoxy-MDB	2.0	0.58 ± 0.10
4,5-Dibromo-MDB	2.0	1.40 ± 0.15
4,5-Dichloro-MDB	2.0	2.24 ± 0.38
Methylenedioxybenzene	2.0	0.22 ± 0.11
2,3-Methylenedioxynaphthalene	1.0	1.12 ± 0.18
Piperonyl butoxide	2.0	0.17 ± 0.12

* Incubations and analyses were conducted as described in Materials and Methods except that the substrates indicated (added in alcohol) were used. Data represent the means ± S.D., N = 3.

Table 6. Physicochemical parameters and observed and calculated values for CO production from MDB derivatives

General structure: 

Compound R	R′	Physicochemical parameters* $\Sigma\sigma$	$\Sigma\pi$	Log [nmoles CO/min ($\times 10^2$)]† Observed	Calculated
H—	H—	0.00	0.00	1.34	1.53
CH_3—	H—	−0.17	0.52	1.32	1.34
CH_3O—	H—	−0.27	−0.04	1.32	1.23
Br—	H—	0.23	1.02	1.93	1.80
CN—	H—	0.63	−0.32	2.31	2.25
CH_2=CH—CH_2—	H—	−0.15‡	1.20	1.42	1.36
Br—	Br—	0.62§	1.96§	2.15	2.24
Cl—	Cl—	0.60§	1.46§	2.35	2.22
Br—	CH_3O—	0.35§	1.14§	1.76	1.93

* Values are *para* substituent constants [44] unless otherwise indicated. All relate to the effect of the substituents at carbon-1.

‡ Since no σ constant was available for this group, the value for ethyl was used.

§ Obtained by summation of *para* and *meta* substituent constants for R and R′, respectively.

† Observed values were from Table 5 and were multiplied by 100 for computational convenience; calculated values from equation 2.

where log CO is the log of CO production (nmoles/min), π is the hydrophobic bonding constant [42], σ the Hammett constant [43], and a, b, ρ and k are constants obtained from the analysis. Since Hammett sigma constants for 4-hydroxymethyl-MDB and piperonyl butoxide are not available, these compounds were omitted from the analysis. Sesamol (4-hydroxy-MDB) was also omitted since the spectral data obtained with this compound clearly indicate a reaction different from the other compounds.

As a result of the symmetrical nature of the methylenedioxyphenyl ring (see general structure, Table 6), substituents (R) in the 4-position are *para* with respect to carbon-1 and *meta* with respect to carbon-2; the opposite is true for substituents (R′) in the 5-position of the ring. To investigate the substituent effects at carbon-1, the *para* substituent constants for π and σ were used for the 4-monosubstituted MDB derivatives. In the case of the three disubstituted compounds, advantage was taken of the additive character of π and σ, and the net effect at carbon-1 was calculated as the sum of the appropriate *para* and *meta* substituent constants for the 4- and 5-substituents, respectively. Equations 2–4 were obtained using the data in Table 6. Equation 2 clearly establishes that, with the nine compounds examined, the rate of CO production

	N	r	S.D.	
$\text{Log CO} = 1.139\sigma + 1.534$	9	0.951	0.135	(2)
$\text{Log CO} = 1.141\sigma - 0.018\pi + 1.535$	9	0.942	0.145	(3)
$\text{Log CO} = 1.182\sigma + 0.603\pi - 0.046\pi^2 + 1.530$	9	0.927	0.158	(4)

is highly correlated ($r = 0.951$) with the Hammett constant and is favored by electron withdrawing substituents which create an electron deficient locus at carbon-1. The correlation coefficient was not improved by inclusion in equations 3 and 4 of the hydrophobic terms π and/or π^2, clearly indicating that hydrophobicity of the compounds plays little or no role in their interaction with cytochrome P-450.

A similar regression analysis using data from the same nine compounds but employing substituent constants relating to the effect of the substituents on carbon-2 of the ring yielded equation 5 which again indicates the importance of the Hammett constant and provides a

	N	r	S.D.	
$\text{Log CO} = 1.277\sigma + 1.439$	9	0.930	0.156	(5)

correlation coefficient only slightly lower ($r = 0.930$) then equation 2.

DISCUSSION

The metabolism of methylenedioxyphenyl compounds in insects and mammals has been discussed in some detail in several reviews [2,3,45]. The metabolic pathways reported usually involve either cleavage of the methylenedioxy ring to the corresponding catechol [9,10,46] or a variety of oxidations and subsequent conjugations of substituents on the aromatic ring. Ring cleavage and at least some of the reactions resulting in side chain modifications are catalyzed by microsomal cytochrome P-450-mediated enzymes requiring NADPH and O_2[2,3]. During ring cleavage, the methylenic carbon of the methylenedioxy ring is reportedly oxidized to formate *in vitro* [46] and ultimately to carbon dioxide *in vivo* [9,46]. Little is known concerning the mechanism through which ring cleavage occurs, although it has been suggested that it might be initiated by hydroxylation of the methylenic carbon to an unstable hydroxymethylene intermediate [3].

The studies reported here show that a variety of substituted methylenedioxybenzenes are metabolized to carbon monoxide by enzymes located in hepatic microsomal fractions requiring NADPH for maximal activity. That the carbon monoxide originates from the methylenic carbon and is not a product of heme breakdown has been clearly demonstrated in preliminary gas chromatographic/mass spectroscopic studies with [^{13}C-methylene]-4,5-dichloro-MDB.* In contrast to the NADH synergism of certain NADPH-dependent drug-metabolizing pathways [47, 48], the addition of NADH was found to decrease the rate of CO production from 4-cyano-MDB. The mechanism for this decrease is not understood but may be due to diversion of an intermediate to a different product.

A strict requirement for oxygen could not be established unequivocally since, even after evacuating and refilling the capped reaction vessels with nitrogen five times, the rate of CO production remained at about 30 per cent of the control level. A somewhat similar problem was encountered in establishing a requirement for oxygen in the formation of the Type III spectrum from piperonyl butoxide until it was found that the spectrum could be formed anerobically in the presence of NADPH if an opportunity was first provided for aerobic interaction between the microsomes and piperonyl butoxide [27]. It is possible that, in the present investigation, the microsomal suspension with which the reaction was initiated (after making all other reaction components anerobic) contained sufficient oxygen to support limited metabolism.

Treatment of animals with phenobarbital, but not 3-methylcholanthrene, markedly increased the rate of conversion of 4-cyano-MDB to CO. In contrast, treatment with cobaltous chloride, which is known to decrease cytochrome P-450 levels [49], led to decreased rates of metabolism of 4-cyano-MDB to CO.

Finally, both SKF 525-A and substituted imidazoles, which are known to inhibit mixed function oxidases [36,50], inhibited the biotransformation of 4-cyano-MDB to CO. These observations, along with the subcellular location and cofactor requirements, indicate the participation of cytochrome P-450 in the reaction.

Regression analysis of data for nine substituted methylenedioxybenzenes show an excellent correlation ($r = 0.951$) between the production of CO and the Hammett sigma constants of the aromatic substituents. This demonstrates that CO production is favored by electron withdrawing substituents which cause an electron deficiency at the two aromatic carbon atoms attached to the methylenedioxy ring. Although the mechanism of the reaction awaits clarification, the CO is certainly formed from the methylene carbon and may arise from an intermediate similar to that proposed in the pathway for ring cleavage and the generation of formate. The production of CO from sesamol (4-hydroxy-MDB) apparently proceeds by a mechanism different from the other MDB derivatives since results obtained with this compound do not fit the regression equation 2 and it exhibits atypical spectral interactions. This may result from ionization of the phenolic hydroxyl moiety in the reaction medium.

Speculation concerning the relevance, if any, of the metabolic production of carbon monoxide to the mechanism by which methylenedioxybenzenes inhibit oxidative drug metabolism is of considerable interest. Inhibition resulting from direct ligand interaction of the generated CO with reduced cytochrome P-450 is not possible under most aerobic *in vitro* assay conditions and, although oxygen levels in intact liver cells *in vivo* can vary and are difficult to define [51], it is in most circumstances highly unlikely that sufficiently anoxic conditions can occur to allow any more than transient formation of CO-reduced cytochrome P-450 complexes.

It has been suggested that the primary mechanism of inhibition occurs through metabolic formation of an active intermediate which forms a stable complex with cytochrome P-450 [2,4,15–17,25,52]. It is further suggested that the 455 nm absorbance maximum in the Type III difference spectrum represents the reduced (ferrous) form of the complex [2,4,52] and that under oxidizing conditions the ferric form appears as a peak at 438 nm. Formation of the 455 nm peak has been suggested to involve ligand binding of a hypothesized carbanion [23] or carbene [20] intermediate to the ferrous heme iron of cytochrome P-450. Although this may provide a satisfactory explanation for the inhibitory activity of many methylenedioxybenzene compounds, the fact remains that several derivatives which are excellent inhibitors of oxidative drug metabolism and CO binding to cytochrome P-450 do not cause formation of typical Type III spectra in NADPH-reduced microsomal suspensions [2,27,28]. Thus *cis*- and *trans*-methylenedioxycyclohexane exhibit spectra with a single peak at 427 nm [27,28] and other methylenedioxybenzenes, including those reported here to generate CO, do not produce an obvious 455 nm spectral complex [27,28]. It appears, therefore, that little or no relationship exists between formation of the 455 nm complex and the inhibitory potency of methylenedioxybenzene derivatives toward drug metabolism. It is of interest to note, however, that following development of the carboxyhemoglobin spectrum in incubations with the CO-producing compounds, a small residual absorbance at 455–457 nm is always visible (Fig. 1) and may represent the 455 nm complex observed with other methylenedioxybenzenes. Indeed, qualitatively at least, there seems to be an inverse relationship between 455 nm complex formation and CO production; compounds which produce a relatively large 455 nm complex generate little CO and those generating CO do not exhibit 455 nm complex formation. It is not yet clear whether this indicates the direct formation of CO from a carbene-type intermediate (if such a species is involved) or whether both CO and the carbene are formed by alternative pathways from a common unstable intermediate.

Studies designed to define more clearly the mechanism of the reaction leading to CO are currently in progress.

* M.W. Anders and C.F. Wilkinson, unpublished data.

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REFERENCES

1. M. W. Anders, *Biochem. Pharmac.* **17**, 2367 (1968).
2. E. Hodgson and R. M. Philpot, *Drug Metab. Res.* **3**, 231 (1975).
3. J. E. Casida, *J. agric. Fd Chem.* **18**, 753 (1970).
4. M. R. Franklin, *Envir. Hlth Perspect.* **14**, 29 (1976).
5. C. F. Wilkinson, *Enzymatic Oxidations of Toxicants* (Ed. E. Hodgson), p. 113. North Carolina State University Press, Raleigh (1968).
6. C. F. Wilkinson, *Proceedings of the Second International IUPAC Congress on Pesticide Chemistry* (Ed. A. S. Tahori), Vol. 2, p. 117. Gordon & Breach, New York (1971).
7. C. F. Wilkinson, *Bull. Wld Hlth Org.* **44**, 171 (1971).
8. C. F. Wilkinson, in *The Future for Insecticides: Needs and Prospects* (Eds. R. L. Metcalf and J. McKelvy, Jr.) p. 195. John Wiley, New York (1976).
9. J. E. Casida, J. L. Engel, E. G. Esaac, F. X. Kamienski and S. Kuwatsuka, *Science* **153**, 1130 (1966).
10. C. F. Wilkinson and L. J. Hicks, *J. agric. Fd Chem.* **17**, 829 (1969).
11. H. B. Matthews, M. Skrinjaric-Spoljar and J. E. Casida, *Life Sci.* **9**, 1039 (1970).
12. A. S. Perry and A. J. Bucknor, *Life Sci.* **9**, 335 (1970).
13. H. B. Matthews and J. E. Casida, *Life Sci.* **9**, 989 (1970).
14. R. M. Philpot and E. Hodgson, *Life Sci.* **10**, 503 (1971).
15. M. R. Franklin, *Xenobiotica* **1**, 581 (1971).
16. M. R. Franklin, *Xenobiotica* **2**, 517 (1972).
17. R. M. Philpot and E. Hodgson, *Molec. Pharmac.* **8**, 204 (1972).
18. C. R. Elcombe, J. Bridges, R. H. Nimmo-Smith and J. Werringloer, *Biochem. Soc. Trans.***3**, 967 (1975).
19. A. P. Kulkarni and E. Hodgson, *Pestic. Biochem. Physiol.* **9**, 75 (1978).
20. W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **27**, 387 (1978).
21. C. Hansch, *J. med. Chem.* **11**, 920 (1968).
22. D. J. Hennessy, *J. agric. Fd Chem.* **13**, 218 (1965).
23. V. Ullrich and K. H. Schnabel, *Drug Metab. Dispos.* **1**, 176 (1973).
24. M. R. Franklin, *Biochem. Pharmac.* **21**, 3287 (1972).
25. R. M. Philpot and E. Hodgson, *Chem. Biol. Interact.* **4**, 185 (1971).
26. C. R. Elcombe, J. W. Bridges, T. J. B. Gray, R. H. Nimmo-Smith and K. J. Netter, *Biochem. Pharmac.* **24**, 1427 (1975).
27. E. Hodgson, R. M. Philpot, R. C. Baker and R. B. Mailman, *Drub Metab. Dispos.* **1**, 391 (1973).
28. A. P. Kulkarni and E. Hodgson, *Pestic. Biochem. Physiol.* **6**, 183 (1976).
29. V. L. Kubic, M. W. Anders, R. R. Engel, C. H. Barlow and W. S. Caughey, *Drug Metab. Dispos.* **2**, 53 (1974).
30. V. L. Kubic and M. W. Anders, *Drug Metab. Dispos.* **3**, 104 (1975).
31. V. L. Kubic and M. W. Anders, *Biochem. Pharmac.* **27**, 2349 (1978).
32. A. E. Ahmed, V. L. Kubic and M. W. Anders, *Drug Metab. Dispos.* **5**, 198 (1977).
33. M. W. Anders, J. L. Stevens, R. W. Sprague, Z. Shaath and A. E. Ahmed, *Drug Metab. Dispos.* **6**, 556 (1978).
34. J. L. Stevens and M. W. Anders, *Toxic. appl. Pharmac.* **45**, 297 (1978).
35. C. F. Wilkinson, *J. agric. Fd Chem.* **15**, 139 (1967).
36. C. F. Wilkinson, K. Hetnarski, G. P. Cantwell and F. J. DiCarlo, *Biochem. Pharmac.* **23**, 2377 (1974).
37. M. W. Anders, *Biochem. Pharmac.* **27**, 1098 (1978).
38. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
39. G. N. Wilkinson, *Biochem. J.* **80**, 324 (1961).
40. C. Hansch and T. Fujita, *J. Am. chem. Soc.* **86**, 1616 (1964).
41. P. J. O'Brien, *Pharmac. Ther.* (A) **2**, 517 (1978).
42. T. Fujita, J. Iwasa and C. Hansch, *J. Am. chem. Soc.* **86**, 5175 (1964).
43. H. Jaffe, *Chem. Rev.* **53**, 191 (1953).
44. P. N. Craig, *J. med. Chem.* **14**, 680 (1971).
45. L. Fishbein and H. L. Falk, *Envir. Res.* **2**, 297 (1969).
46. F. X. Kamienski and J. E. Casida, *Biochem. Pharmac.* **19**, 91 (1970).
47. M. A. Correia and C. J. Mannering, *Molec. Pharmac.* **9**, 455 (1973).
48. M. A. Correia and G. J. Mannering, *Molec. Pharmac.* **9**, 470 (1973).
49. T. R. Tephly and P. Hibbeln, *Biochem. biophys. Res. Commun.* **42**, 589 (1971).
50. C. F. Wilkinson, K. Hetnarski and L. J. Hicks, *Pestic. Biochem. Physiol.* **4**, 299 (1974).
51. H. Sies, in *Hypoxia and Ischemia* (Eds. M. Reivich, R. Coburn, S. Lahiri and B. Chance), pp. 51–66. Plenum Press, New York (1977).
52. M. R. Franklin, *Pharmac. Ther.* (A) **2**, 277 (1977).