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STIMULATION OF THE CONJUGATION OF LIPID DIENES IN HEPATIC MICROSOMES BY 3,3′-DICHLOROBENZIDINE

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Abstract—Pretreatment of male rats with 3,3′-dichlorobenzidine (DCB) resulted in the accumulation of conjugated dienes in lipids from hepatic microsomes. *In vitro*, these microsomes had 2-fold the NADPH-dependent malondialdehyde (MDA)-forming capacity of microsomes from untreated rats. To determine the mechanisms of the DCB-induced accumulation of diene conjugation, the effects of added DCB on NADPH- or iron + ascorbic acid- (Fe^{2+}-ascorbate-) dependent diene conjugation, oxygen uptake and MDA formation were examined in microsomes from untreated rats *in vitro*. In the presence of NADPH, added DCB stimulated diene conjugation in microsomal lipids as did *in vivo* DCB pretreatment but inhibited the uptake of oxygen and the formation of MDA. When Fe^{2+}-ascorbate was substituted for NADPH, the formation of diene conjugation, oxygen uptake, and MDA formation were inhibited by added DCB. The DCB-induced stimulation of diene conjugation, in addition to being strictly NADPH dependent, was carbon monoxide sensitive and was concomitant with the binding of added DCB to microsomal lipids. It is postulated that a metabolite of DCB generated by cytochrome P-450 reacts with membrane lipids both *in vivo* and *in vitro* in a manner analogous to the initiation of lipid peroxidation but at the same time prevents the autocatalytic decomposition of the lipids. The DCB-induced diene conjugation is interpreted as predisposing to deleterious changes in microsomes.

3,3′-Dichlorobenzidine (DCB)† is a precursor of industrial dyes and pigments and a curing agent in rubber and polyurethane manufacture [1]. The compound is mutagenic in the Ames test [1–3], binds covalently to nucleic acids [4, 5], is carcinogenic at multiple sites in several animal species, and is a cancer suspect agent in humans [1]. Treatment of rats with DCB induces liver microsomal cytochrome P-450 [6] and also enhances *in vitro* microsomal lipid peroxidation as measured by malondialdehyde (MDA) formation [7]. Toxic xenobiotics, including aromatic amines, interact with membrane lipids, and such interactions may contribute to the deleterious effects of the chemicals. As an example, a correlation exists between the carcinogenicity of an aryl amine and its ability to bind covalently with unsaturated fatty acids of liver microsomes [8]. Furthermore, many xenobiotics stimulate the peroxidation of unsaturated lipids of membranes, a process which has been associated with a variety of pathologies including carcinogenesis [9].

Because DCB pretreatment stimulates *in vitro* lipid peroxidation and because of the deleterious nature of the process, it was of interest in the present studies to verify whether the compound also stimulates lipid peroxidation *in vivo* and, if so, to determine the mechanisms involved. The results show that DCB stimulated the diene conjugation of hepatic microsomal lipids, which is usually attributed to lipid peroxidation [10–13]. The findings in *in vitro* studies suggest that this effect of DCB is NADPH- and cytochrome P-450-dependent and that the resulting diene conjugated lipids do not undergo oxygenation and decomposition in the presence of DCB.

METHODS

Materials. Male Sprague–Dawley rats were obtained from Taconic Farms, Germantown, NY, and were fed a standard laboratory diet and tap water *ad lib.* for 1 week prior to use (at about 175 g body weight). DCB dihydrochloride, benzidine dihydrochloride, 3-methylcholanthrene (3-MC), NADPH, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, ascorbic acid and adenosine diphosphate (ADP) were purchased from the Sigma Chemical Co., St. Louis, MO. Linoleic acid was purchased from Nuchek Prep, Elysian, MN. Cumene hydroperoxide was purchased from ICN Chemicals, Plainview, NY. [^{14}C]DCB (sp. act. 35 mCi/mmol) was purchased from Pathfinders Laboratory, St. Louis, MO. Precoated thin-layer chromatography (TLC) plates (silica gel GF, 250 μm thick) were obtained from Analtech, Newark, DE. Nitrogen (oxygen-free) and carbon monoxide (CO, Research Grade) were purchased from the Matheson Gas Co., East Rutherford, NJ. All solvents were of spectral grade and were obtained from the following

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† Abbreviations: Diene conjugation is used to refer to the isomerization of double bonds from the isolated to the conjugated configuration in microsomal polyunsaturated fatty acids. DCB, 3,3′-dichlorobenzidine; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances (used interchangeably with MDA); Fe^{2+}-ascorbate, iron plus ascorbic acid; 3-MC (MC), 3-methylcholanthrene; ADP, adenosine diphosphate; CO, carbon monoxide; R_f, mobility of a chromatographic spot relative to the solvent; P, microsomes. Cytochrome P-450 is used generically without reference to specific isozymes.

sources: methanol (Burdick & Jackson, Muskegon, MI); *n*-hexane, chloroform and corn oil (Fisher Scientific Co., Fair Lawn, NJ). Hydrofluor was obtained from National Diagnostics, Somerville, NJ. All other reagents were of the highest grade of purity. DCB dihydrochloride was recrystallized from methanol prior to use. The radiochemical was purified to >99% radioactive purity by C_{18}-reverse phase high performance liquid chromatography (HPLC), using phosphate buffer (10 mM, pH 6.0)–methanol (2:3, v/v) as the mobile phase in the isocratic mode. Chloroform was purified by $CaCl_2$ filtration as described previously [14]. All other chemicals and reagents were judged to be free of contamination and were used without further purification.

Preparation of microsomes and isolation of microsomal lipids. Rats were treated by i.p. injection (1 ml/kg) of a 20 mg/ml suspension of either DCB, benzidine, or MC in corn oil, once daily for 2 days, and then killed 48 hr after the last injection. Control rats received corn oil only (1 ml/kg). The rats were killed by decapitation followed immediately by immersing the heads in liquid nitrogen. Washed liver microsomes were prepared by differential centrifugation as described previously [15] and then resuspended in 0.1 M phosphate buffer so that 1 ml contained the equivalent of 1 g of wet liver. Protein was determined by the method of Lowry *et al.* [16]. Lipids from freshly isolated microsomes or from a microsomal incubation mixture were isolated by the procedure of Folch *et al.* [17] and quantitated gravimetrically.

Incubations. These were at either 37 or 22° and under the additional conditions specified in the legends to figures and tables. All results represent a minimum of two experiments or a typical experiment.

Oxygen uptake was monitored with a Clark-type electrode equipped with a pO_2 amplifier (University of Pennsylvania Bioinstrumentation Shop, Philadelphia, PA). The electrode system was calibrated with gas mixtures containing various concentrations of oxygen and nitrogen. The reaction chamber (0.6 ml total capacity) was closed to ambient air and was equipped with an air-tight inlet for adding reagents and a magnetic system for constant stirring of the reaction mixture.

For the measurement of diene conjugation, isolated lipids were weighed and dissolved in *n*-hexane, and the UV spectra of the hexane solution were recorded. To obtain the 233 nm peak of experimental samples, the difference spectra of lipids from (i) control versus pretreated rats in the case of *in vivo* experiments and (ii) microsomal incubations containing DCB and NADPH versus those from which DCB and NADPH were omitted in the case of *in vitro* experiments, were recorded as described in the legends to the respective figures. The content of diene conjugated lipids was estimated from the 233 nm absorbance, using a millimolar extinction coefficient of 25.25 cm^{-1} [18].

TBA-reactive substances (or MDA) were measured colorimetrically as described by Buege and Aust [11]. Hydroperoxides were measured colorimetrically by the ferrithiocyanate assay [19], using authentic linoleic acid hydroperoxide prepared as described previously [18] or cumene hydroperoxide as the standard; 0.3 μM hydroperoxides were detectable by the assay.

To determine the distribution of [^{14}C]DCB in microsomes, a microsomal reaction containing [^{14}C]DCB was extracted twice, each time with 2 vol. of ethyl acetate to remove unreacted DCB and its polar derivatives. The aqueous fraction was treated with 20% trichloroacetic acid (TCA) (0.2 ml/ml reaction mixture) to precipitate the protein, followed by centrifugation (3000 g × 10 min). The resulting supernatant fraction was added to the combined ethyl acetate extracts, and the total volume was minimized by evaporation and prepared for scintillation spectrometry for the determination of "total extractible" DCB. The protein pellet was extracted twice, each time with a volume of chloroform–methanol (2:1, v/v) equal to the volume of the original reaction mixture; the combined extracts containing "DCB-associated" lipids were dried under nitrogen and prepared for scintillation spectrometry. The residual protein precipitate was solubilized by digestion with 1 N NaOH at 60° and neutralized with 1 N HCl prior to scintillation spectrometry.

For the TLC separation of total organic extracts of microsomal incubations, a microsomal reaction mixture was extracted twice, each time with a volume of chloroform–methanol (2:1, v/v) twice the volume of the original reaction mixture. The combined organic extracts were dried under nitrogen and resuspended in chloroform, and an aliquot was spotted on a silica gel GF plate (preactivated at 110° for 90 min). The plate was developed in chloroform–methanol–water (65:25:4, by vol.), and visualized under UV light followed by exposure to iodine vapor. All spots on the plate that quenched fluorescence and retained iodine (longer than 4 hr) were scraped and prepared for scintillation spectrometry.

For scintillation spectrometry, Hydrofluor was used as the fluor. Gels were prepared from aqueous samples by a 1:1 (v/v) sample–fluor mixture, whereas all dry samples including the TLC scrapings contained only the scintillation fluor. Scintillation spectrometry was carried out on a Tracor Mark III Analytical liquid scintillation spectrometer equipped with an internal standard. The minimum counting efficiency was 80%. All absorption spectra were recorded on an Hitachi 220A spectrophotometer.

RESULTS

Effect of DCB pretreatment on the content of diene conjugated lipids in hepatic microsomes. Diene conjugated lipids are usually considered to be one of several products formed during the peroxidation of polyunsaturated fatty acids. They are thought to be resonance-stabilized products of the abstraction of an allylic hydrogen of a polyunsaturated lipid by an initiator of lipid peroxidation; they have absorption maxima around 233 nm, thus presenting a highly sensitive measure of both *in vivo* and *in vitro* lipid peroxidation [11–13]. Examination of the UV spectra of hepatic microsomal lipids from control and DCB-pretreated rats revealed that the 233 nm absorbance was increased by DCB pretreatment (Fig. 1). The increase was estimated to represent 1.9 nmol/mg

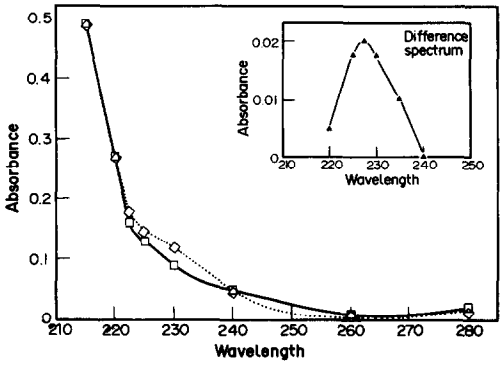

Fig. 1. Absorption spectra of total microsomal lipids from control (———) and DCB-pretreated (. . . . . .) rats. The lipids were dissolved in *n*-hexane, at a final concentration of 435 μg/ml. Insert: difference spectrum between (———) and (. . . . . .). The assay was repeated at least three times, with preparations from some DCB-pretreated rats having up to 2-fold but never less than the 233 nm absorbance shown here.

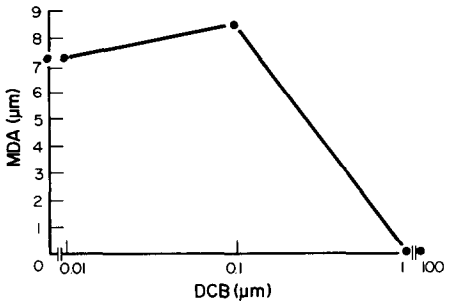

Fig. 2. Effect of added DCB on microsomal NADPH-dependent MDA formation. The incubation mixture (final volume = 1.0 ml phosphate buffer, pH 7.4), in duplicate flasks, contained an NADPH-generating system (150 μM NADP, 1.7 mM glucose-6-phosphate, and 0.5 units glucose-6-phosphate dehydrogenase/ml), 10 μM $FeCl_3$, 2 mM ADP and DCB at the concentrations indicated. Each DCB concentration had a parallel incubation (blank) from which the NADPH-generating system was omitted. The assay was initiated by adding 0.5 mg of microsomal protein from control rats. Following a 15-min incubation at 37°, MDA was determined by the thiobarbituric acid procedure as described in Methods. Each point represents the average of two experiments.

total microsomal lipid. In lipid peroxidation, a major reaction of the resonance-stabilized diene conjugated lipid radical is addition to molecular oxygen, with the eventual formation of lipid hydroperoxides [10–13, 20]. However, hydroperoxides were not detected in either freshly prepared microsomes or in isolated microsomal lipids from DCB-pretreated or untreated rats (Table 1).

Effect of DCB pretreatment on the formation of TBARS/MDA in microsomes. MDA is one of the oxygen-dependent decomposition products of lipid peroxidation and a widely used measure of the reaction [11–13, 20]. The results in Table 1 show that the same amount of MDA was present in microsomes from either control or DCB-pretreated rats. However, these may not have originated *in vivo* as MDA is metabolized extensively in the intact animal [13]. When NADPH was added to the microsomes, the formation of MDA—enzymic lipid peroxidation—was increased 2-fold in microsomes from DCB-pretreated rats, whereas Fe^{2+}-ascorbate-dependent MDA formation—nonenzymic lipid peroxidation—was the same in microsomes from untreated and DCB-pretreated rats (Table 1). Thus, DCB pretreatment enhanced only enzymic lipid peroxidation in microsomes.

Effect of added DCB on in vitro *microsomal MDA formation.* In contrast to the enhancing effect of DCB pretreatment on enzymic microsomal MDA formation (Table 1), added DCB exerted a dose-dependent inhibition of the reaction, with maximum inhibition observed at 1 μM DCB (Fig. 2). In an experiment to determine whether DCB inhibited MDA formation by interfering with the effects of iron, the ratio of iron to DCB was increased 10:1 but the inhibitory effect of the diamine was not overcome (data not presented). DCB also inhibited the nonenzymic reaction but with a potency less than that in the enzymic reaction (Table 2). Hydroperoxides were not detected in either the enzymic or nonenzymic reaction in the presence of DCB (Table 1). The results so far suggested that while DCB may have initiated lipid peroxidation, as evidenced by the formation of diene conjugation *in vivo*, the compound was a potent inhibitor of the reaction *in vitro* as measured by the formation of TBARS.

Effect of added DCB on oxygen uptake during microsomal lipid peroxidation. To verify whether the DCB-induced inhibition of MDA formation resulted

Table 1. Effect of pretreatment with DCB on variables of lipid peroxidation

Microsomes from:	Endogenous MDA* (nmol/mg protein)	Endogenous hydroperoxides*	*In vitro* MDA formation (nmol MDA/mg microsomal protein/10 min)	
			NADPH-dependent	Fe^{2+}-ascorbate-dependent
Control rats	0.38 ± 0.1	ND†	5.2 ± 0.3	21.0 ± 1.2
DCB-pretreated rats	0.43 ± 0.08	ND	10.9 ± 2.1	18.3 ± 1.5

Assay conditions were carried out as described in Fig. 2 except in the Fe^{2+}-ascorbate-dependent reaction where 200 μM ascorbate was substituted for an NADPH-generating system. Hydroperoxides were determined colorimetrically as described in Methods. Each value is the mean ± SD of three experiments.

* In freshly prepared microsomes without prior incubation.

† Not detected.

Table 2. Effect of added DCB on enzymic and nonenzymic microsomal lipid peroxidation

DCB added (μM)	MDA formed [nmol·(mg microsomal protein)$^{-1}$·(15 min)$^{-1}$] Enzymic	Nonenzymic
0	10.4 (100)	20.8 (100)
1	ND*	16.7 (77)
10	ND	6.1 (29)
50	ND	3.6 (17)
100	ND	3.5 (16.5)

The reaction mixtures contained either an NADPH-generating system (enzymic) or Fe^{2+}-ascorbate (nonenzymic) as described in the legends to Fig. 2 and Table 1. Values in parentheses are percentages of the value in the absence of DCB. Each value is the average of two determinations.
* Not detected.

from inhibition of oxygenation, the effect of DCB on oxygen uptake in the peroxidative reaction was assessed. Similar to its effect on MDA formation, added DCB exerted a dose-dependent inhibition of NADPH-dependent microsomal oxygen uptake, with maximum inhibition observed at 1 μM DCB (Fig. 3), the same concentration at which the compound inhibited MDA formation maximally (Fig. 2). Oxygen uptake was also inhibited by the diamine in the Fe^{2+}-ascorbate-dependent reaction (Fig. 4). The concentration of DCB determined in preliminary studies to be maximally inhibitory to either MDA formation or oxygen uptake in either the enzymic or non-enzymic system of lipid peroxidation was 100 μM. When added during the course of the reaction, 100 μM DCB inhibited oxygen uptake instantaneously (Figs. 3 and 5A), whereas 1 μM DCB required a 2-min lag to exert full inhibition (Fig. 3, middle curve). The DCB-induced inhibition of oxygen uptake was not caused by inhibition of NADPH utilization as the oxidation of the reduced nucleotide proceeded unimpeded, as monitored by the loss of absorbance at 340 nm (data not presented).

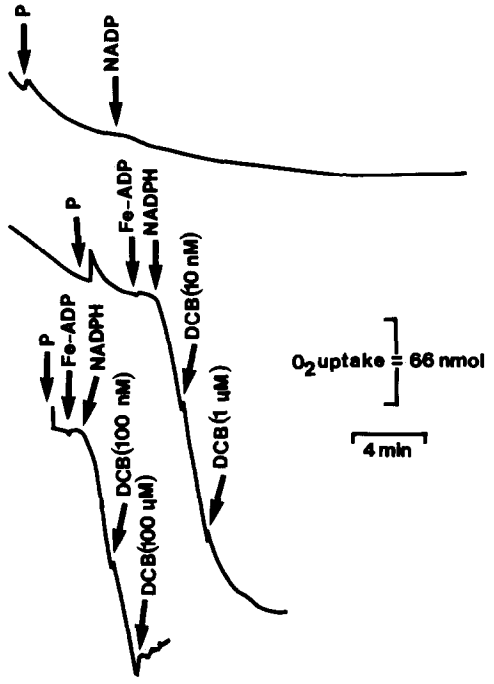

Fig. 3. Dose-dependent effect of DCB on the oxygen uptake of NADPH-dependent microsomal lipid peroxidation. The assay (at 22°) was carried out as described in Methods and contained the following (final concentrations) as shown: microsomes (P) from control rats (1 mg protein/ml), NADPH (300 μM), $FeCl_3$ (10 μM), ADP (2 mM) and DCB (at the concentrations indicated).

Effect of DCB addition on the formation of diene conjugated lipids in microsomes in vitro. To verify whether DCB also inhibited the initial reactions of lipid peroxidation *in vitro*, the effect of the diamine on diene conjugation was examined. The result was contrary to the expected in that the diamine stimulated diene conjugation (Fig. 6). It can be seen in Fig. 6 also that unchanged DCB, which has an absorption maximum at 280 nm, decreased concomitantly with increase in the 233 nm absorbance. This suggested, perhaps, a precursor–product relationship between DCB and diene conjugation. The 233 nm absorbance was not contributed by a product of DCB *per se* because none of the free metabolites of DCB, which are totally extractible from a microsomal reaction mixture [6], had an absorption maximum or shoulder around 233 nm (Lang and Iba, unpublished observations). The data in Table 3 show that almost as much diene conjugation was induced by 1 μM DCB as by 100 μM, in agreement with the similarity in the magnitude of inhibition of oxygen uptake by the two concentrations of the diamine. In the presence of added iron, the content of DCB-induced diene conjugation was reduced to 20% of the content in the absence of Fe^{3+}-ADP (Table 3). We attribute this effect to a possible iron-catalyzed decomposition of the diene conjugated lipids.

Distribution of DCB in microsomes. The stimulation of the conjugation of lipid dienes by DCB on the one hand and the inhibition of oxygenation of the lipids by the diamine on the other suggested, among several possibilities, that DCB or its metabolite was competing with oxygen for reaction with the diene conjugated lipids. To determine whether DCB indeed bound to microsomal lipids, [^{14}C]DCB was incubated with NADPH-supplemented micro-

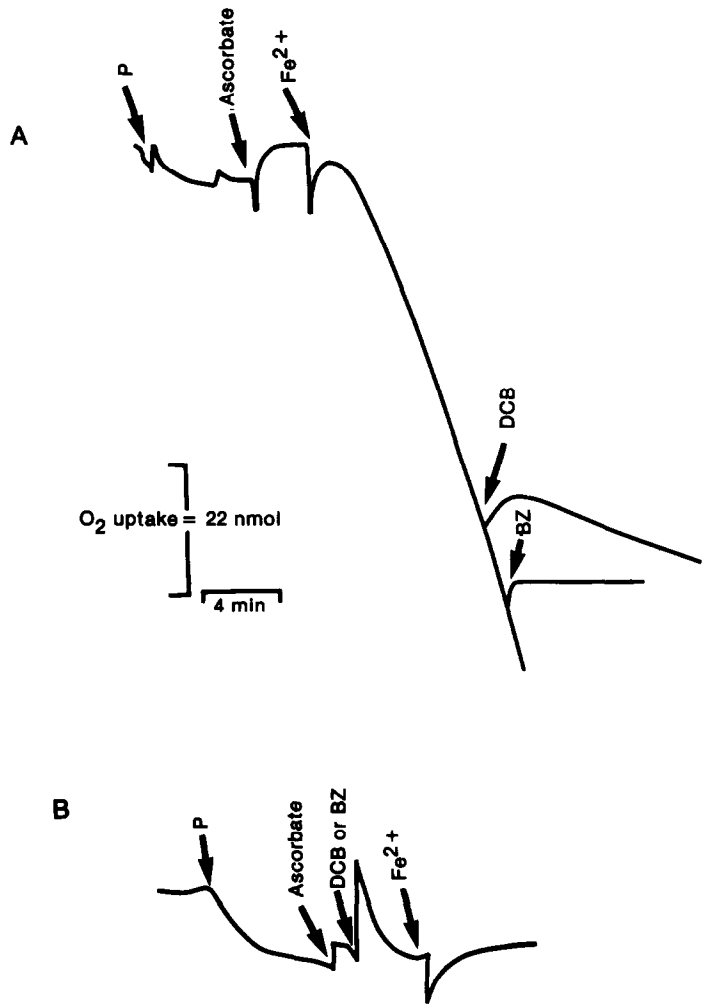

Fig. 4. Effect of DCB or benzidine on the oxygen uptake of nonenzymic microsomal lipid peroxidation. Assay conditions were as in Fig. 3 except that ascorbic acid (200 μM, final concentration) replaced NADPH and only 100 μM DCB or benzidine was added either during the course of the reaction (A) or prior to the initiation of the reaction (B).

somes, and the distribution of radioactivity in the lipid and protein fractions was measured. In the absence of NADPH, 98 nmol (98%) of the 100 nmol of added DCB was extracted by ethyl acetate mostly as the unchanged compound, whereas 2.1 nmol (2.1%) was non-extractible and was distributed equally between microsomal lipids and protein (Table 4). However, in the presence of NADPH, the amount of total extractible DCB was reduced to 95.4 nmol (95.4%), about 30% of which was judged to be metabolites based on their retention times when analyzed by HPLC (data not presented). Of the 4.6 nmol of DCB that resisted extraction, 2.9 nmol was bound to microsomal lipids—a 2.4-fold increase over the amount bound in the absence of NADPH (Table 4). To determine whether the radioactivity in the microsomal lipids represented irreversible lipid–DCB complexes, the total chloroform–methanol extracts of a microsomal incubation containing [^{14}C]DCB were analyzed by TLC. The chromatography resolved seven components including unchanged DCB. Five of these had lower R_f values than unchanged DCB, whereas one had a higher R_f value (Table 5). The fraction that migrated less than unchanged DCB ($R_f = 0.88$) may have represented metabolites of DCB; however, we have observed in preliminary studies that metabolites of the compound do not separate well from unchanged DCB under these chromatographic conditions. Furthermore, the low migrating components stained iodine, suggesting them to be lipid–DCB complexes. The component with $R_f = 0.93$ was more than likely a DCB–lipid complex because of its reactivity with iodine and because no DCB metabolite had a higher R_f value than the unchanged diamine under these chromatographic conditions (Iba and Lang, unpublished observation). When Fe^{3+}-ADP was added at the end of the microsomal incubation but prior to chloroform–methanol extraction, the radioactive content and the iodine-staining intensity of the fraction with $R_f = 0.93$ became substantially reduced (data not presented). This supports the speculation above that the diminution of DCB-induced diene conjugation by Fe^{3+}-ADP (Table 3) resulted most

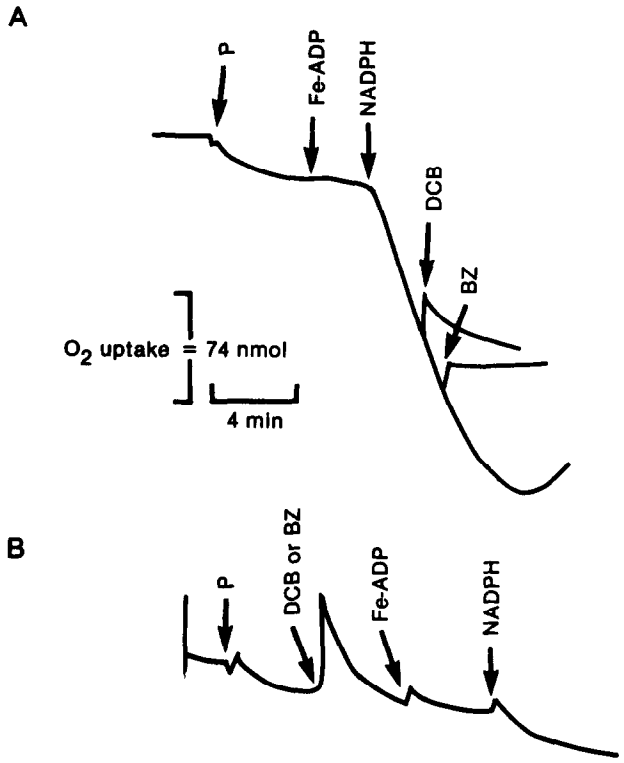

Fig. 5. Comparative effects of DCB and benzidine on the oxygen uptake of enzymic microsomal lipid peroxidation. The assay was as described in the legend of Fig. 4 except that NADPH (300 μM) was substituted for ascorbic acid.

likely from iron-induced decomposition of the diene conjugated lipids.

Effect of DCB on Fe^{2+}*-ascorbate-induced diene conjugation of microsomal lipids.* Fe^{2+} alone or Fe^{2+} plus ascorbic acid also induced the conjugation of microsomal lipid dienes (Fig. 7). However, added DCB, in contrast with its effect in the presence of NADPH, totally prevented diene conjugation in either the Fe^{2+} or Fe^{2+}-ascorbate system (Fig. 7). Microsomal DCB metabolism proceeds in the presence of NADPH [6] but not in the presence of Fe^{2+} or Fe^{2+}-ascorbate (Lang and Iba, unpublished observations). This induction of diene conjugation by DCB in the enzymic but not in the nonenzymic system strongly implicated a metabolite of the diamine in the diene conjugation reaction.

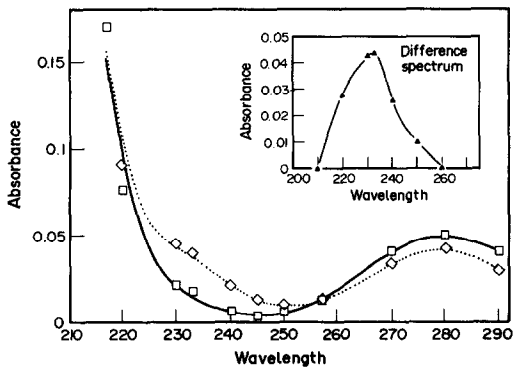

Fig. 6. Effect of added DCB on diene conjugation in microsomal lipids. Microsomes from control rats (1 mg) were incubated with 100 nmol DCB with (. . . . . .) or without (———) an NADPH-generating system in a final volume of 1 ml phosphate buffer (0.1 M, pH 7.4). After a 15-min incubation at 37°, the entire reaction mixture was extracted with chloroform–methanol as described in Methods. Absorption spectra are of the dried lipoid extracts in *n*-hexane (final concentration 128 μg lipid/ml). Insert: difference spectra between (———) and (. . . . . .).

Effect of carbon monoxide or nitrogen on the DCB-induced conjugation of microsomal lipid dienes. Because DCB is metabolized by cytochrome P-450 [6], the involvement of the hemoprotein in producing the putative DCB species responsible for the conjugation reaction was examined. Carbon monoxide, which inhibits the activity of ferrocytochrome P-450 [21], totally prevented the NADPH-dependent DCB-induced conjugation of microsomal lipid dienes, whereas nitrogen inhibited the reaction by 28% (Table 6).

Effect of pretreatment with or addition of benzidine or other compounds on MDA formation and conjugation of microsomal lipid dienes. Benzidine differs from DCB structurally in lacking *o*-chlorination. Both diamines are carcinogenic at multiple sites in several experimental animals, nevertheless with some differential species- and tissue-specificity [1]. It was of interest, therefore, to (1) assess whether induction of microsomal lipid peroxidation is a general effect of the toxic benzidines and (2) obtain some information on the structural features of DCB

Table 3. Effect of DCB pretreatment or addition on the content of diene conjugated lipids in microsomes

	Diene conjugated lipid (nmol/mg total microsomal lipid)
Microsomes from DCB-pretreated rats	1.9*
Microsomes from control rats	ND†
Microsomes from control rats:	
+NADPH‡ + DCB (0.1 μM)	4.2§
+NADPH + DCB (1.0 μM)	12.6§
+NADPH + DCB (100 μM)	14.4‖
+NADPH + DCB (100 μM) + Fe^{3+}-ADP¶	2.4§

* Value is from the data in Fig. 1.
† Not detected.
‡ NADPH was added as a generating system. Other assay conditions were as described in the legend of Fig. 6.
§ Average of two determinations.
‖ Value is from the data in Fig. 6.
¶ $FeCl_3$ and ADP were 5 μM and 10 mM, respectively; diene conjugated lipids were not detected when $FeCl_3$ was increased above 5 μM.

Table 4. Distribution of preincubated [^{14}C]DCB in microsomes

	nmol DCB equivalent in: Protein	Lipid
(A) Microsomes + DCB (100 μM)	0.7 ± 0.2* (0.7)†	2.8 ± 0.3* (1.1)†
(B) Microsomes + DCB (100 μM) + NADPH	1.7 ± 0.3* (1.7)†	8.2 ± 1.4* (3.3)†

The reaction mixture contained 100 nmol [^{14}C]DCB (10 mCi/mmol), 1 mg microsomal protein from control rats, with (B) or without (A) an NADPH-generating system, in a total volume of 1 ml phosphate buffer, pH 7.4. Following a 15-min incubation at 37°, the reaction was terminated with 0.2 ml of 20% TCA. Subsequent separation of protein and lipids and radiometric determination of DCB-bound protein and lipids were carried out as described in Methods. Each value is the average of three determinations.
* nmol Bound/mg tissue.
† nmol Bound/total tissue.

Table 5. TLC separation of total chloroform–methanol extracts of microsomal incubations containing [^{14}C]DCB

Chromatographic spot (R_f)	Reactivity with I_2*	Percent radioactivity added: −NADPH	+NADPH
0	−	0.09	0.17 (+0.08)
0.15	±	0.06	0.20 (+0.14)
0.35	+	0.20	0.20
0.50	+	0.40	0.50 (+0.10)
0.65	+	0.10	0.60 (+0.50)
0.88	−	92.50	87.00 (−5.50)†
0.93	++	6.40	10.80 (+4.40)

Assay conditions were as described in Table 4 except that the reaction was terminated and extracted with chloroform–methanol (2:1, v/v) as described in Methods. TLC and scintillation spectrometry of the chromatographic fractions were as described in Methods. Values in parentheses are the net NADPH-dependent change.
* Spots retaining iodine stain for >4 hr following exposure to iodine vapor.
† Unchanged DCB.

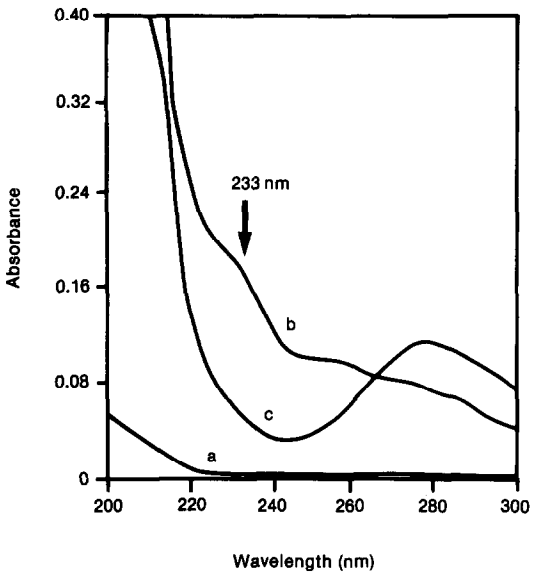

Fig. 7. Effect of DCB on Fe^{2+}-ascorbate-induced diene conjugation in microsomal lipids. Assay conditions were as in Fig. 6 except that either ferrous ammonium sulfate (Fe^{2+}, 10 μM) alone or Fe^{2+} (10 μM) and ascorbic acid (200 μM) were substituted for NADPH. DCB (100 nmol) was either omitted from (b) or added to (c) the reaction mixture. (a): *n*-Hexane only.

that may have contributed to the conjugation of lipid dienes. Benzidine, unlike DCB did not stimulate diene conjugation of microsomal lipids nor the NADPH-dependent microsomal MDA formation following its administration to rats (data not shown). However, similar to DCB, benzidine totally inhibited both MDA formation (data not shown) and oxygen uptake (Figs. 4 and 5) when added to microsomes. Thus, benzidine shares the antioxidant effect but not the diene conjugating effects of DCB.

Because 3-MC and DCB induce qualitatively identical types of hepatic microsomal cytochrome P-450, we investigated if *in vivo* conjugation of microsomal lipid dienes was a common effect of MC-type inducers of cytochrome P-450. However, conjugated lipid dienes did not accumulate in microsomes following MC pretreatment. These findings support the contention that the stimulation of diene conjugation in microsomal lipids by DCB is an effect intrinsic to the DCB molecule and, perhaps, unrelated to cytochrome P-450 induction by the compound.

DISCUSSION

The major focus of the present study was to verify if DCB induced lipid peroxidation in the liver *in vivo* and to determine the mechanisms involved, using *in vitro* hepatic microsomal systems. The results show that DCB pretreatment caused the accumulation of diene conjugated lipids, which is a widely accepted index of lipid peroxidation, in hepatic microsomes; similarly, added DCB stimulated the conjugation of microsomal lipid dienes *in vitro*. It may be concluded, therefore, that the DCB-induced conjugation of microsomal lipid dienes proceeded by the same mechanism *in vivo* and *in vitro*. However, the resulting diene conjugated lipids were not associated with hydroperoxides, as would be expected for lipid peroxidation, suggesting that the latter process, which forms characteristic end products including MDA, was not an effect of DCB. This could be interpreted to mean that either (1) the DCB-induced diene conjugation was not associated with lipid peroxidation or (2) DCB initiated lipid peroxidation, but prevented the subsequent reactions that are usually associated with lipid peroxidation, e.g. formation of hydroperoxides and their decomposition.

The first of the above two possibilities is feasible because nitroxides, which can be formed oxidatively from aryl amines, can undergo an oxygen-dependent Alder-ene-type addition to double bonds of polyunsaturated fatty acids [22, 23]. The polyunsaturated fatty acid adducts so formed can undergo isomerization from the isolated to the conjugated double bond configuration [24]. DCB would be expected to be N-oxygenated prior to adding to unsaturated lipids to cause the conjugation of their double bonds. However, the inhibition of only 28% of the total DCB-induced diene conjugation by nitrogen and the observation that diene conjugation and oxygen uptake appeared to be mutually exclusive argue against the oxidative pathway as a major mechanism by which DCB stimulated the conjugation of lipid dienes.

The second possibility would involve a peroxidation-type mechanism. Its feasibility is streng-

Table 6. Effects of carbon monoxide and nitrogen on DCB-induced conjugation of lipid dienes in microsomes

		Diene conjugated lipids (nmol formed/mg total lipid)
(A)	Microsomes + NADPH + DCB (100 μM)	14.0 (100)
(B)	+Carbon monoxide : oxygen (80 : 20)	ND (0)
(C)	+Nitrogen	10.1 (72)

The reaction mixture, in a Thurnberg-type test tube, was as described in the legend of Fig. 6 except that the reaction was carried out in either open air (A) or closed under a stream of either a $CO : O_2$ mixture supplied through a gas mixer after purging the reaction mixture (for 5 min) with the gas mixture followed by adding DCB (B) or nitrogen following a 5-min purging of the reaction mixture with the nitrogen and adding DCB (C). Lipid isolation and determinations of diene conjugation were as described in Methods. ND = not detected. Values in parentheses are percentages of diene conjugation formed in open air. Each value is the average of three determinations.

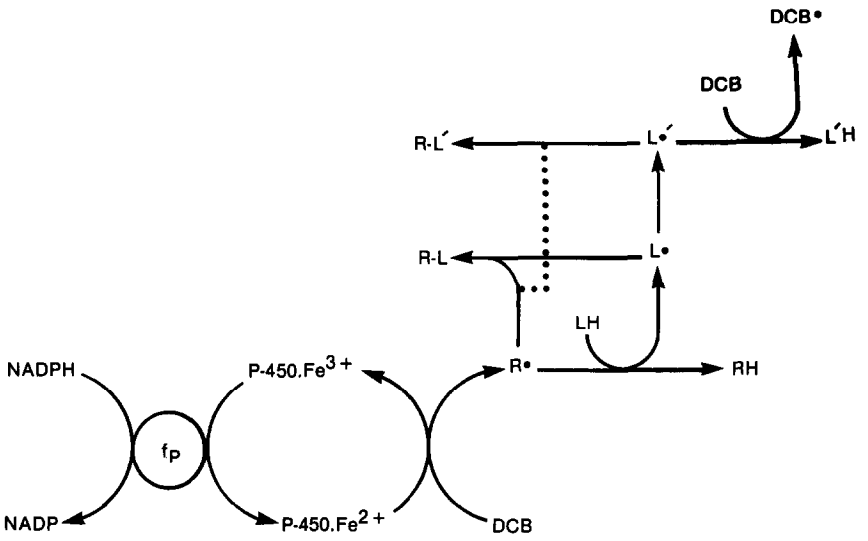

Fig. 8. Proposed scheme of the conjugation of microsomal lipid dienes induced by DCB. The species R˙ represents a proton-abstracting, lipid-binding product of DCB formed by cytochrome P-450. LH = polyunsaturated lipid. L˙ and L˙′ represent the carbon-centered polyunsaturated lipid radical and its resonance-stabilized conjugated diene derivative, respectively. R-L and R-L′ represent complexes of the reactive DCB derivative R˙ with L˙ and L˙′, respectively. f_P = NADPH-dependent cytochrome P-450 reductase. L′H represents other lipid derivatives with conjugated dienes formed from L˙′ and hydrogen donors such as DCB.

thened by the observation that DCB stimulated the formation of diene-conjugated lipids but inhibited the uptake of oxygen and the formation of TBARS. This peroxidation-based mechanism of DCB-induced diene conjugation can be discussed in the context of the reaction steps in the peroxidation of polyunsaturated fatty acids, which include, as shown in the scheme below:

$$LH + R^{\cdot} \longrightarrow L^{\cdot} + XH \quad (1)$$
$$L^{\cdot} \longrightarrow L^{\cdot\prime} \quad (2)$$
$$L^{\cdot\prime} + O_2 \longrightarrow LOO^{\cdot} \quad (3)$$
$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \quad (4)$$
$$LOOH + O_2 \rightarrow \rightarrow \rightarrow \text{Products (MDA, etc.)} \quad (5)$$
$$L^{\cdot\prime} + DCB(H) \longrightarrow L^1H + DCB^{\cdot} \quad (6)$$

(1) formation of lipid radicals (L˙) from a polyunsaturated fatty acid (LH) by an initiating species R˙, (2) isomerization of the isolated double bonds in L˙ to form a lipid radical (L˙′) with conjugated dienes, (3) oxygenation of L˙′ to form a hydroperoxyl radical intermediate (LOO˙), (4) propagation of the radicals, and (5) subsequent decomposition of the oxygenated lipids into several products such as short chain alkanes and alkenes, alcohols, aldehydes and ethers [11–13].

Implicit in the peroxidation-based model of DCB-induced conjugation of microsomal lipid dienes is the assumption that the initiating species R˙ in Reaction 1 may have been a metabolite of DCB. That the initiating species was a metabolite of DCB is supported by the following arguments: (i) Diene conjugation in microsomes was enhanced by DCB in the presence of NADPH (Fig. 6), and NADPH supports the microsomal metabolism of DCB [6]; in contrast, the diamine inhibited diene conjugation in the presence of either Fe^{2+} or Fe^{2+}-ascorbate (Fig. 7) and these reagents did not support DCB metabolism (Lang and Iba, unpublished observations). (ii) In microsomes, unsubstituted benzidine does not undergo NADPH-dependent biotransformation [3], and did not induce diene conjugation in the present studies. (iii) DCB is metabolized by cytochrome P-450 in microsomes [6] and carbon monoxide, an inhibitor of the monooxygenase, prevented DCB-induced diene conjugation (Table 6). The apparently mutually exclusive relationship between the diene conjugation reaction and oxygen uptake would rule out R˙ being any of the oxygen species, e.g. perferryl [25], ˙OH [26], $^1O_2$ [27], postulated to initiate lipid peroxidation in other systems. It also strongly suggests that the initiating species may have resulted from the reductive metabolism of DCB, e.g. dechlorination. Although dechlorination of arylchlorides is not considered an energetically favorable reaction [28], recent findings show that these compounds can undergo both oxidative [29] and reductive [30] cytochrome P-450-dependent dechlorination. The stimulation of diene conjugation by DCB but not by benzidine supports the involvement of dechlorination in the reaction. Alternatively, the initiating DCB species may have been an N-oxidized species, as arylamines readily undergo electronic N-oxidation to nitrogen-centered radicals [31, 32]. However, benzidine, which undergoes electronic N-oxidation [33] did not stimulate the conjugation of microsomal lipid dienes, suggesting that the nitrogen-centered radical may not have been the initiating species.

Implicit also in the peroxidation-based model is that Reaction 3 of the peroxidation scheme shown above did not occur to a significant extent. This would explain the inhibition of oxygen uptake by DCB during lipid peroxidation, as the putative init-

iating DCB species rather than molecular oxygen may have bound to the lipid radicals (L˙, L˙˙). Even though the affinity of oxygen for lipid radicals is very high [34], that of the reactive DCB species for the lipid radicals may be higher. Furthermore, the reactive DCB species and the lipid radicals may have been generated in close proximity and concentrated in poorly oxygenated domains of the microsomes. The involvement of a DCB derivative in the inhibition of oxygen uptake is also supported by the time-course of the inhibition in which a 2-min lag preceded complete inhibition of oxygenation when 1 μM DCB was added during the course of microsomal lipid peroxidation (Fig. 3). The lag could be interpreted as the time necessary for microsomes to generate the concentration of the requisite species that was maximally inhibitory to oxygenation. The instantaneous inhibition of oxygen uptake by 100 μM DCB probably indicated the higher rate of formation of the inhibitory species at the high substrate concentration. An additional and perhaps important cause of inhibition of the oxygenation (Reaction 3) may have been a direct antioxidant effect of unchanged DCB via hydrogen donation to the diene conjugated lipid radical (L˙˙) to form the stable diene conjugated lipid (L'H) (Reaction 6). This reaction, which is also in competition with oxygen addition to L˙˙ (Reaction 3), should be expected to predominate, yielding increased levels of diene conjugation. Conditions favoring Reaction 6 over Reaction 3 in the present studies included the availability of the hydrogen donor DCB and decreased levels of metal catalysts capable of decomposing the diene conjugated lipid intermediates and products. The identity and fate of the putative radical DCB˙ formed in Reaction 6 are unknown but it could possibly serve as an initiating DCB derivative similar to R˙ (Reaction 1). It should be pointed out that the formation of L' would neither require nor preclude the covalent binding of DCB to lipids.

The events associated with DCB-induced formation of diene conjugation in microsomal lipids could be interpreted according to the scheme in Fig. 8. In the scheme, R˙ represents a radical or some other proton-abstracting derivative of DCB. R-L and R-L' represent complexes that the putative initiating DCB metabolite forms with either the initial lipid radicals (L˙) or their resonance stabilized derivatives with conjugated double bonds (L˙˙). Evidence that such complexes were formed include the data presented in Table 6. L'H represents an additional stable diene conjugated lipid product via hydrogen donation by DCB to the diene conjugated lipid radical (L˙˙) as discussed above.

The observation that DCB prevented MDA formation *in vitro* does not mean that DCB is an antioxidant. It is possible that initiation reactions of lipid peroxidation, such as those stimulated by DCB, are of more toxicological relevance *in vivo* than the terminal reactions of the process, e.g. MDA formation, which are commonly measured *in vitro*. We have observed previously that microsomes from DCB-pretreated rats have diminished content of the antioxidant vitamin E [7], an effect which may have contributed to the enhanced enzymic TBARS formation therein. Because microsomes from pretreated rats had enhanced enzymic lipid peroxidation, we speculate that DCB exposure predisposes membranes to peroxidative damage. Further studies will be necessary to characterize the DCB species that stimulate the conjugation of double bonds in microsomal lipids as well as details of the lipid–DCB interactions. Further studies will also determine if and how these interactions contribute to the pathological effects of DCB.

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