14. J. M. Bertoni, G. M. Alexander and R. J. Schwartzman, *Trans. Am. neurol. Ass.* (abstr.) (1980).
15. R. P. Harpur and J. H. Quastel, *Nature, Lond.* **164**, 693 (1949).
16. F. Maley and H. A. Lardy, *J. biol. Chem.* **214**, 765 (1955).
17. T. G. Bidder, *J. Neurochem.* **15**, 867 (1968).
18. H. S. Bachelard, A. G. Clark and M. F. Thompson, *Biochem. J.* **123**, 707 (1971).
19. O. H. Oldendorf, *Am. J. Physiol.* **221**, 1629 (1971).
20. J. Brown, *Metabolism* **11**, 1098 (1962).
21. A. Sols and R. K. Crane, *J. biol. Chem.* **210**, 581 (1954).
22. R. W. Horton, B. S. Meldrum and H. S. Bachelard, *J. Neurochem.* **21**, 507 (1973).

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Inactivation of hepatic cytochrome P-450 by a 1,2,3-benzothiadiazole insecticide synergist*†

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Substituted 1,2,3-benzothiadiazoles synergize the activities of insecticides [1–3], prolong hexobarbital sleeping times in mice [2], inhibit both aldrin epoxidation and dihydroisodrin hydroxylation by rat liver and armyworm microsomes [4], and inhibit the metabolism and accompanying irreversible protein binding of halogenated ethylenes [5, 6]. These biological effects, which are similar to those mediated by the commercially employed methylenedioxyphenyl insecticide synergists [7], presumably reflect inhibition by the heterocycle of the oxidative drug-metabolizing apparatus. Support for this contention is provided by the observation that 5,6-dichloro-1,2,3-benzothiadiazole gives a typical type II difference spectrum on binding to oxidized hepatic cytochrome P-450 [8, 9], and by the finding that the levels of hepatic cytochrome P-450 in mice decrease precipitously after *in vivo* administration of the agent [2]. Despite an extensive correlation of structure with biological activity [3, 4, 8], however, no information is available on the actual mechanism by which 1,2,3-benzothiadiazoles impair the function of cytochrome P-450. We report here a study of 5,6-dichloro-1,2,3-benzothiadiazole which demonstrates that this agent inactivates cytochrome P-450 as the result of a highly specific catalytic interaction.

Materials and Methods

The synergist 5,6-dichloro-1,2,3-benzothiadiazole (DCBT), which was initially provided by Dr. J. H. Davies of Shell Biosciences Laboratory (Sittingbourne, Kent, England), was subsequently synthesized as described in the literature [10]. NADPH, reduced glutathione, tricaprylin, and a serum transaminase determination kit (catalogue No. 505) were purchased from the Sigma Chemical Co. (St. Louis, MO). All other reagents and solvents were of the highest grade commercially available.

Microsomal incubations. Male Sprague–Dawley rats, injected daily with a dose (80 mg/kg, i.p.) of sodium phenobarbital (4 days), were decapitated, and their livers were quickly perfused with cold 1.15% (w/v) KCl solution. The livers were then excised, and a 33–50% (w/v) homogenate in 0.1 M phosphate buffer (pH 7.4) was prepared using a large clearance Dounce tissue grinder. After centrifugation at 10,000 *g* for 20 min to remove cell debris, the supernatant fraction was centrifuged at 96,000 *g* for 60 min. The microsomal pellet thus obtained was washed by resuspension in isotonic KCl solution and recentrifugation at 96,000 *g* for 30 min. The washed microsomes were resuspended in 0.1 M Na/K phosphate buffer (pH 7.4) containing 1.5 mM EDTA and 150 mM KCl at an approximate 10 mg/ml protein concentration. Protein concentrations were measured by the method of Lowry *et al.* [11], using human serum albumin as a standard.

Standard 20 ml incubation mixtures contained the following: microsomal protein (1 mg/ml), KCl (150 mM), EDTA (1.5 mM) and NADPH (1.0 mM) in 0.1 M Na/K phosphate buffer (pH 7.4). A 10 mM nominal concentration of DCBT was also present unless otherwise specified. Incubations were initiated by NADPH addition. After the indicated incubation times (20 min unless otherwise specified), 3 ml aliquots were withdrawn and the reaction was stopped by cooling to 0°. Parallel incubations without NADPH or without DCBT were carried out as controls for thermal or lipid peroxidative losses respectively. Alterations in these normal incubation conditions are given in the text. The oxygen tension was reduced when required by sealing the reaction vessel and flushing the reaction mixture with argon prior to initiating the incubation by NADPH addition.

The concentration of cytochrome P-450 was calculated from the 450–520 nm absorbance difference between a dithionite-reduced, carbon monoxide saturated sample and an unreduced, carbon monoxide saturated reference sample, using an Aminco DW-2 spectrophotometer and a value of 100 cm^{-1} mM^{-1} for the molar extinction coefficient [12]. Microsomal heme was measured as the pyridine hemochromogen [13], using a molar extinction coefficient of 34.4 cm^{-1} mM^{-1} for the 557–600 nm absorbance difference

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Table 1. Destruction of hepatic microsomal cytochrome P-450 from phenobarbital-pretreated rats by 5,6-dichloro-1,2,3-benzothiadiazole (DCBT)*

Alterations in standard incubation	Per cent loss of cytochrome P-450	
	20 min†	30 min†
Experiment 1		
None	55 ± 2	56 ± 1
− DCBT	0	0
− NADPH	0	0
Reduced oxygen tension	14 ± 1	
+ Glutathione (1 mM)		56 ± 2
Experiment 2		
None		65 ± 0
+ Glutathione (10 mM)		41 ± 0

* Incubation and assay conditions are given in Materials and Methods. All values, except that obtained under reduced oxygen tension (two determinations only), are the average of three measurements and are given ± S.D. Experiments 1 and 2 were carried out with different groups of rats.

† Incubation time.

[14]. Malondialdehyde levels were measured as described by Asakawa and Matsushita [15]. The formation of malondialdehyde in an incubation containing CCl_4 rather than DCBT was measured as a positive control of the analytical methodology.

The livers of phenobarbital-pretreated rats killed 4 hr after intraperitoneal injection of DCBT (100 mg/kg in tricaprylin) were perfused, removed, homogenized, and extracted as described previously for the isolation of "green pigments" caused by other agents [16, 17]. Thin-layer chromatographic analyses of the extracts provided no evidence for the presence of green red-fluorescing pigments analogous to those isolated in other instances [16–18]. Three separate attempts were made to detect such pigments, including, in one experiment, the parallel examination of untreated control livers.

Serum from phenobarbital-pretreated rats decapitated 24 hr after intraperitoneal injection of DCBT (100 mg/kg) was analyzed for glutamic-oxalacetic (SGOT) and glutamic-pyruvic (SGPT) transaminase activities using reagent kits based on the method of Reitman and Frankel [19]. Similar measurements were made on the serum of phenobarbital-pretreated rats 24 hr after administration of carbon tetrachloride (1 ml/kg) to provide a positive control.

The mutagenicity of DCBT was evaluated by Dr. R. E. McMahon, Lilly Research Laboratories, Indianapolis, IN, using slant cultures of ten tester bacterial strains according to the protocol reported by that laboratory [20]. The tests were carried out with and without the addition of a liver homogenate activating system [20]. The nominal concentration range tested extended from 0.1 μg/ml to 1 mg/ml.

Results and Discussion

Incubation of hepatic microsomes from phenobarbital-pretreated rats with 5,6-dichloro-1,2,3-benzothiadiazole (DCBT) resulted in rapid, spectroscopically measurable loss of cytochrome P-450 (Table 1). This decrease occurred only in the presence of both NADPH and molecular oxygen, the two cofactors required for oxidative turnover of the enzyme.* The previously reported decrease in the levels of cytochrome P-450 in mice injected with DCBT

* Catalytic involvement of the enzyme in its own destruction has been confirmed by studies with a purified, reconstituted cytochrome P-450 (P. R. Ortiz de Montellano, B. A. Mico, J. M. Matthews, K. L. Kunze, G. T. Miwa and A. Y. H. Lu, unpublished).

Table 2. Heme loss, cytochrome P-450 loss, and malondialdehyde formation due to incubation of 5,6-dichloro-1,2,3-benzothiadiazole (DCBT) with hepatic microsomes from phenobarbital-pretreated rats*

Substrate	Cytochrome P-450 content (nmoles/mg protein)	Heme content (nmoles/mg protein)	Malondialdehyde formation (μM)
Experiment 1			
None	2.9 ± 0.1	3.2 ± 0	
DCBT (10 mM)†	1.3 ± 0	1.5 ± 0.1	
(Loss)	1.6	1.7	
Experiment 2			
None			ND
DCBT (10 mM)†			ND
CCl_4‡ (10 mM)			4.9 ± 0.2

* Incubation and assay conditions are given in Materials and Methods. All values (± S.D.) are averages of at least three determinations, non-detectable levels being indicated as ND.

† Nominal concentration (substrate is highly insoluble in aqueous media).

‡ CCl_4 was used as a positive control.

[2] thus reflects a direct interaction of the agent with the enzyme rather than an alteration in the rate of synthesis or degradation of the enzyme.

No cytochrome P-450 was lost when otherwise complete incubations were carried out in the absence of DCBT, a result which shows that lipid peroxidation was efficiently suppressed by the EDTA in the mixture (Table 1). The possibility existed, however, that lipid peroxidation was stimulated by the presence of DCBT, and that the cytochrome P-450 was destroyed by this enhanced peroxidative process. Accelerated hydrogen peroxide formation on binding of certain substrates by cytochrome P-450 has, for example, been documented [21]. The formation of malondialdehyde on incubation of DCBT with hepatic microsomes in the presence of both NADPH and oxygen was therefore investigated, since malondialdehyde formation is a measure of lipid peroxidation [22]. As shown in Table 2, no malondialdehyde was detected either in the presence or the absence of DCBT even though malondialdehyde formation was observed in similar incubations with carbon tetrachloride, an agent known to promote lipid peroxidation [23]. Stimulation of lipid peroxidation by DCBT does not, therefore, appear to be a viable explanation for its destruction of cytochrome P-450.

Addition of 1 mM glutathione to the incubation mixture did not detectably attenuate DCBT-mediated destruction of cytochrome P-450, although some protection of the enzyme was observed when the glutathione concentration was raised to 10 mM (Table 1). This protective effect could result from trapping of a diffusible reactive species by glutathione, although other explanations, such as direct reaction with DCBT prior to interaction with cytochrome P-450, cannot now be excluded. Two experiments were undertaken to determine if generally toxic metabolites were produced enzymatically from DCBT. In one, the hepatotoxic effect of the agent was evaluated by measuring the change in serum transaminase levels 24 hr after injection of DCBT into phenobarbital-pretreated rats (100 mg/kg dose). As shown in Table 3, however, DCBT was not found to have any effect on the levels of serum glutamic-oxalacetic or glutamic-pyruvic transaminases, in marked contrast to the elevated activity of these two enzymes produced by the known hepatotoxin carbon tetrachloride. In a second experiment, the mutagenicity of DCBT was evaluated by a broad screening procedure developed at the Lilly Laboratories [20], both in the presence and in the absence of a liver homogenate activating system. Despite the use of ten different tester strains, and the use of a 10,000-fold concentration range, no evidence was found for a mutagenic or toxic effect of DCBT. Thus, these results are consistent with the argument that the inactivation of cytochrome P-450 by DCBT was not due to a general hepatotoxic effect.

This laboratory has provided unequivocal evidence that the autocatalytic inactivation of cytochrome P-450 enzymes, due to turnover of terminal olefinic [17, 18, 24] and acetylenic [16] substrates, results from alkylation of the enzyme prosthetic heme moiety by a reactive species produced within the catalytic site. In agreement with this mechanism, unsaturated substrates have been shown to cause essentially equimolar losses of both hepatic cytochrome P-450 and microsomal heme [25, 26] and also to result in the accumulation of abnormal porphyrins derived from the ravaged prosthetic heme group [27]. Parallel quantitation of cytochrome P-450 and heme losses due to incubation of DCBT with hepatic microsomes (Table 2) revealed a similar 1:1 relationship between the disappearance of the enzyme and of (presumably) prosthetic heme. An intimate, perhaps causal, link between the two processes is thus implied. Despite repeated efforts, however, we have been unable to detect the *in vivo* formation of "abnormal green" porphyrins similar to those obtained with olefins and acetylenes. No characteristic red-fluorescing bands were observed on thin-layer chromatographic analyses of the methylated extracts from livers of phenobarbital-pretreated rats that had been injected with DCBT (100 mg/kg in tricaprylin) 4 hr before they were killed. Inactivation of cytochrome P-450 by DCBT thus resembled the process mediated by allenes [28] and vinyl chloride [29], in which parallel heme loss is also not coupled to detectable pigment formation.

Table 3. Effect of 5,6-dichloro-1,2,3-benzothiadiazole (DCBT) on serum transaminase levels in phenobarbital-pretreated rats*

Agent administered†	SGOT (I.U./l)	SGPT (I.U./l)
None (N = 5)	19 ± 1	60 ± 8
DCBT (N = 4)	17 ± 2	56 ± 5
CCl_4 (N = 2)	>700‡	>800‡

* SGOT (serum glutamic-oxalacetic transaminase) and SGPT (serum glutamic-pyruvic transaminase) levels were measured 24 hr after administration of indicated agents (details in Materials and Methods). Mean values ± S.D. are given.

† DCBT (100 mg/kg) was administered i.p. in tricaprylin. CCl_4 (1 ml/kg) was injected undiluted by the same route. The number of animals in each treatment group is given in parentheses.

‡ Enzyme levels exceeded range which could be accurately measured.

The results reported here clearly demonstrate that oxidative turnover of DCBT by cytochrome P-450 results in irreversible inactivation of the enzyme and in a parallel loss of microsomal heme, even though the nature of the interaction that causes both enzyme and heme loss cannot be defined precisely. Nevertheless, the evidence obtained excludes lipid peroxidative and general hepatotoxic mechanisms and suggests strongly that an oxidative metabolite or transient intermediate derived from DCBT is responsible for the coupled loss of cytochrome P-450 and prosthetic heme. Unfortunately, no data are available on the metabolism of DCBT or of any closely related substrate. Nevertheless, clues to the possible nature of the inactivating interaction can be drawn from the known chemical properties of the 1,2,3-benzothiadiazole ring system. Reaction of this heterocycle with aryl radicals [30, 31], or with oxidative agents such as lead tetra-acetate* and *N*-bromosuccinimide*, yields products the genesis of which can best be explained by the reaction manifold shown in Scheme 1. Although only further work will permit definition of the particular molecular sequence of events that results in enzyme inactivation, it is likely, on chemical grounds, that one of the reactive intermediates shown in Scheme 1 will play a crucial role. Regardless of the detailed mechanism, the present investigation firmly establishes that DCBT inactivates cytochrome P-450 in a catalysis-driven interaction. This demonstration provides a solid rationale for the inhibition of xenobiotic metabolism characteristic of the 1,2,3-benzothiadiazole heterocyclic system and, consequently, for the activity of this family of agents as insecticide synergists.

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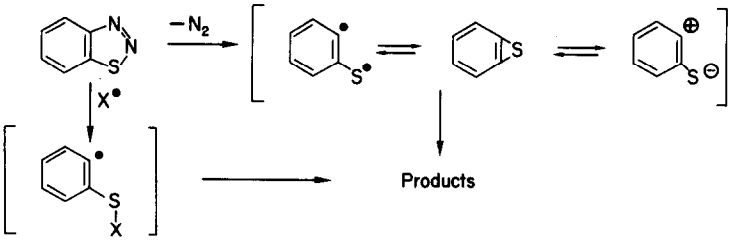
* P. R. Ortiz de Montellano and L. Cuellar, unpublished results.



Scheme 1. Reactive intermediates formed in chemical reactions of the 1,2,3-benzothiadiazole system.

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REFERENCES

1. J. C. Felton, D. W. Jenner and P. Kirby, *J. agric. Fd Chem.* **18**, 671 (1970).
2. M. Skrinjaric-Spoljar, H. B. Matthews, J. L. Engel and J. E. Casida, *Biochem. Pharmac.* **20**, 1607 (1971).
3. D. L. Gil and C. F. Wilkinson, *Pestic. Biochem. Physiol.* **6**, 338 (1976).
4. D. L. Gil and C. F. Wilkinson, *Pestic. Biochem. Physiol.* **7**, 183 (1977).
5. H. M. Bolt, H. Kappus, A. Buchter and W. Bolt, *Archs. Toxic.* **7**, 153 (1977).
6. H. M. Bolt, A. Buchter, L. Wolowski, D. L. Gil and W. Bolt, *Int. Archs. occup. envir. Hlth* **39**, 103 (1977).
7. E. Hodgson and R. M. Philpot, *Drug Metab. Rev.* **3**, 231 (1974).
8. D. L. Gil, *Ph.D. Dissertation*, Cornell University, Ithaca, NY (1973); *Dissert. Abstr. Part B Sci. Eng.* **34**, 3143 (1974).
9. H. B. Matthews, M. Skrinjaric-Spoljar and J. E. Casida, *Life Sci.* **9**, 1039 (1970).
10. P. Kirby, S. B. Soloway, J. H. Davies and S. B. Webb, *J. chem. Soc.* (*C*) 2250 (1970).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall. *J. biol. Chem.* **193**, 265 (1951).
12. R. W. Estabrook, J. Peterson, J. Baron and A. Hildebrandt, in *Methods in Pharmacology*, Vol. 2 (Ed. C. F. Chignell), p. 303. Appleton-Century-Crofts, New York (1972).
13. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
14. M. R. Waterman, in *Methods in Enzymology*, Vol. 52 (Eds. S. Fleischer and L. Packer), p. 457. Academic Press, New York (1978).
15. T. Asakawa and S. Matsushita, *Lipids* **15**, 137 (1980).
16. P. R. Ortiz de Montellano and K. L. Kunze, *J. biol. Chem.* **255**, 5578 (1980).
17. P. R. Ortiz de Montellano and B. A. Mico, *Molec. Pharmac.* **18**, 178 (1980).
18. P. R. Ortiz de Montellano, G. S. Yost, B. A. Mico, S. E. Dinizo, M. A. Correia and H. Kambara, *Archs Biochem. Biophys.* **197**, 524 (1979).
19. S. Reitman and S. Frankel, *Am. J. clin. Path.* **28**, 56 (1957).
20. R. E. McMahon, J. C. Cline and C. Z. Thompson, *Cancer Res.* **39**, 682 (1979).
21. G. D. Nordblom and M. J. Coon, *Archs. Biochem. Biophys.* **180**, 343 (1977).
22. J. I. Gray, *J. Am. Oil Chem. Soc.* **55**, 539 (1978).
23. R. D. Recknagel and E. A. Glende, Jr., *CRC Crit. Rev. Toxic.* **2**, 263 (1973).
24. P. R. Ortiz de Montellano, K. L. Kunze and B. A. Mico, *Molec. Pharmac.* **18**, 602 (1980).
25. F. De Matteis, *Biochem. J.* **124**, 767 (1971).
26. J. J. Bradshaw, M. R. Ziman and K. M. Ivanetich, *Biochem. biophys. Res. Commun.* **85**, 859 (1978).
27. F. De Matteis, in *Heme and Hemoproteins, Handbook of Experimental Pharmacology*, Vol. 44 (Eds. F. De Matteis and W. N. Aldridge), p. 95. Springer, New York (1978).
28. P. R. Ortiz de Montellano and K. L. Kunze, *Biochem. biophys. Res. Commun.* **94**, 443 (1980).
29. F. P. Guengerich and T. W. Strickland, *Molec. Pharmac.* **13**, 993 (1977).
30. L. Benati, A. Tundo and G. Zanardi, *J. Chem. Soc., Chem. Commun.* 590 (1972).
31. L. Benati, P. C. Montevecchi, A. Tundo and G. Zanardi, *J. org. Chem.* **41**, 1331 (1976).