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ALTERATIONS IN MOUSE LIVER MONOOXYGENASES BY BENZOTHIADIAZOLES

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Abstract—Administration of 1,2,3-benzothiadiazoles to mice had a biphasic effect on liver microsomal monooxygenases. During the first 15 hr of treatment, an inhibition of the *in vivo* metabolism of hexobarbital, as well as of the *in vitro* hydroxylation of naphthalene and *N*-demethylation of aminopyrine, was observed. An apparent decrease in cytochrome P-450 and in the activity of the NADPH–cytochrome *c* reductase also occurred. The levels of cytochrome b_5 and NADH–cytochrome *c* reductase activity were only slightly affected. A shift to 452 nm in the carbon monoxide difference spectrum was obtained with dithionite-reduced microsomes and this was not modified by ferricyanide. After the initial inhibitory phase, an enhancement of drug-metabolizing activities *in vivo* and *in vitro* and in the levels of some components of the mixed function oxidase system was observed. The carbon monoxide difference spectra of dithionite-reduced microsomes returned to a maximal absorption at 450 nm. The stimulatory effect on monooxygenase activity, elicited by benzothiadiazoles, was prevented completely by actinomycin D and was accompanied by increases in liver weight, microsomal protein, and incorporation of labeled amino acids into microsomal protein, as well as by proliferation of smooth and rough endoplasmic reticulum. Acrylamide gel analysis of liver microsomes from mice, given a single dose of 6-chloro-1,2,3-benzothiadiazole 48 hr prior to being killed, showed preferential induction of cytochrome P-450 apoproteins of 50,000, 52,000 and 53,000 molecular weight.

The administration of certain compounds to mammals has a biphasic effect in both the activity and the content of the mixed function oxidase system. This is usually characterized by a fairly rapid inhibition of monooxygenase activity followed by a slower induction process [1,–4]. The changes in activity, however, are not necessarily related to the degree of induction of cytochrome(s) P-450. The results of this biphasic effect *in vivo* are reflected in altered metabolism and action of drugs, insecticides, carcinogens, and various normal body constituents, such as steroid hormones and bilirubin. This phenomenon is dependent on the nature of the chemical agents, dose, time, route of administration, and the particular substrate assayed.

Several types of chemical are presently used as insecticide synergists [5,6]. The 1,2,3-benzothiadiazoles are a group of insecticide synergists that greatly enhance the toxicity of pyrethrin, organophosphate, and carbamate insecticides [7]. The synthesis of a large series of 1,2,3-benzothiadiazoles and their evaluation as carbaryl synergists against the housefly have been reported [8]. Many of these compounds were found to be potent *in vitro* inhibitors of mammalian and insect microsomal oxidations [9]. Benzothiadiazoles have been described as excellent inhibitors of aminopyrine metabolism in the rat perfused liver [10]. They have also been reported to be inhibitors of irreversible microsomal protein binding of labeled trichloroethylene [11]. In addition to these interactions which occur at the endoplasmic reticulum, benzothiadiazoles inhibit the mitochondrial electron transport chain at the level of coupling site I [12]. Environmental contamination by chemicals with synergistic activity is a potential hazard for mankind; the consequences to humans and other species exposed to them are as yet unknown [13]. The present report describes the effects of 1,2,3-benzothiadiazole administration on the *in vivo* and *in vitro* activities of the mouse hepatic mixed function oxidase system.

MATERIALS AND METHODS

Animal treatment. Six-week-old female mice of the strain Balb/c (H-2^d), weighing 18–24 g, were used. They were housed in wire cages in a humidity and temperature-controlled room with a 12-hr day–night lighting schedule. Animals were randomized and subdivided into groups of four mice each. Groups and treatment are described in the figures and tables. Synergists were injected i.p. in 0.2 ml of corn oil. Hexobarbital (80 mg/kg) was injected i.p. in 0.2 ml of 0.9% isotonic NaCl. Actinomycin D (250 μg/kg) was administered i.p. in 0.2 ml of 0.9% isotonic NaCl.

Preparation of microsomes. Animals were stunned, decapitated, and exsanguinated; livers were removed, pooled by group, weighed, cut out, and rinsed with 0.1 M sodium phosphate buffer, pH 7.5. They were then homogenized in the same buffer using a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle to give a 20% (w/v) preparation. The homogenates were centrifuged at 9000 *g* for 15 min in an International Centrifuge model B-20. Microsomes were obtained by centrifugation of the 9000 *g* supernatant fraction at 100,000 *g* for 60 min in an L2-65B Beckman Ultracentrifuge. The

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microsomal pellet was suspended in 0.1 M potassium phosphate buffer, pH 7.5, for hepatic monooxygenase enzyme assays and in 50 mM–150 mM Tris–KCl buffer, pH 7.5, for determination of cytochrome content.

Preparation of the S-30 fraction. Liver homogenates were prepared as indicated for microsomes except that a 30% (w/v) homogenate was obtained. The S-30 fraction was obtained from the supernatant fraction after centrifugation of the homogenates at 30,000 *g* for 10 min.

Assays for monooxygenase activity in vitro. The monooxygenase activity of microsomes toward naphthalene and aminopyrine was measured in a reaction mixture containing, in a final volume of 5 ml the following: 100 μmoles potassium phosphate buffer (pH 7.5), 10 μmoles $MgCl_2$, 10 μmoles glucose-6-phosphate, 5.0 units of glucose-6-phosphate dehydrogenase, 1 μmole $NADP^+$ microsomes equivalent to 2–6 mg of microsomal protein, dissolved in water, 86 μmoles aminopyrine or 7.8 μmoles naphthalene dissolved in 20 μl ethanol. The reaction was started by the addition of the microsomes. Incubations were carried out at 30°. At zero time and after 15 min of incubation, 1.0-ml aliquots were pipetted into test tubes containing 0.1 ml of concentrated HCl for naphthalene and 0.5 ml of 10% trichloroacetic acid (TCA) for aminopyrine. The formation of 1,2-dihydro-1,2-dihydroxynaphthalene and 1-naphthol was measured by the procedure of Booth and Boyland [14]. Formaldehyde from aminopyrine was determined by the method of Nash [15].

Assays for reductases and cytochromes. Cytochrome b_5 and cytochrome(s) P-450 were determined by the method of Omura and Sato [16]. A molar extinction coefficient of 91 mM^{-1} cm^{-1} was used for cytochrome P-450 and 185 mM^{-1} for cytochrome b_5.

NADPH–cytochrome c *reductase.* NADPH–cytochrome *c* reductase was determined by following the reduction of cytochrome *c* at 550 nm, essentially according to Masters *et al.* [17]. The reaction mixture contained, in a final volume of 1.2 ml, the following: 125 nmoles NADPH, 50 nmoles cytochrome *c* (horse heart), 40 μmoles potassium phosphate buffer (pH 7.5), and 0.31 μmole KCN. The reaction was started by the addition of 120 μg microsomal protein. A change in absorbance of 1.0 corresponded to the reduction of 0.0467 μmole cytochrome *c*.

NADH–cytochrome c *reductase.* NADH–cytochrome *c* reductase was measured with a similar technique using 125 nmoles NADH instead of NADPH and 12 μg microsomal protein. All spectrophotometric determinations were carried out at 20° in a double beam Beckman Spectrophotometer model Acta III on in a Perkin Elmer model 402, using cuvettes of 1.0 ml capacity and 1.0 cm light path.

Incorporation of L-[^{14}C]*phenylalanine by S-30 fraction.* Each incubation flask contained the following: 12 μmoles Tris–HCl (pH 7.5), 2 μmoles dithiothreitol, 5.0 μmoles ATP, 1.26 μmoles GTP, 25 μmoles phosphocreatine, 50 μg creatine phosphokinase (EC 2.7.3.2) 5 μmoles $MgCl_2$, 25 μmoles of a mixture of 19 amino acids, 6.25 nmoles of L-[^{14}C]phenylalanine uniformly labeled (sp. act. 20 μCi/μmole) and 25 mg of S-30 protein in a final volume of 0.3 ml. The reaction was started by the addition of S-30 protein. All flasks were prepared in duplicate. Incubations were carried out at 37° for 30 min. The reaction was stopped with 5 ml of 5% TCA, and the flasks were heated at 90° for 15 min. After cooling, the suspension was filtered through Whatman No. 3 paper disks, and washed with 0.1 mM phenylalanine in 5% TCA. The disks were dried, placed in scintillation vials with 10 ml of Bray's solution, and counted in a Nuclear Chicago model Mark I Scintillation Counter.

Gel electrophoresis. Polyacrylamide slab gel electrophoresis was carried out at room temperature in the presence of sodium dodecylsulfate (SDS) using an LKB apparatus (model 3771 D) with a discontinuous buffer system as described by Laemmli [18]. Microsomes were suspended in 0.06 M Tris–HCl buffer (pH 6.8) to a concentration of 0.7 mg protein/ml. Before application to the stacking gel, samples were heated for 2 min at 100° in 0.06 M Tris–HCl (pH 6.8) containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue. The separating gel (14 × 15 × 0.1 cm) contained 9% acrylamide. Electrophoresis was carried out at 10 mA/gel during stacking and at 30 mA/gel during separation. The gels were fixed for 1 hr in water–isopropyl alcohol–acetic acid (65:25:10), stained for 1 hr in 0.05% Coomassie blue with the same solvent and destained overnight at room temperature in water–isopropyl alcohol–acetic acid (80:10:10). The standards with their subunit molecular weights included: bovine serum albumin, 68,000; catalase, 58,000; glutamic dehydrogenase, 53,000; ovalbumin, 43,000; aldolase, 40,000; and lactic dehydrogenase, 36,000.

Electron microscopy. Liver slices were fixed in 2.5 M glutaraldehyde in 0.1 M phosphate buffer at pH 7.3, followed by post-fixation in 1% OsO_4 in the same buffer; dehydration was carried out in ethanol and embedded in Epon 814. Sections were stained with uranyl acetate followed by lead citrate. The grids were examined in a Siemens electron microscope model A at 80 kV. Protein was determined by the method of Lowry *et al.* [19].

Chemicals. Substituted 1,2,3-benzothiadiazoles were synthesized as described elsewhere [8]. Aminopyrine was purchased from Merck & Co. 2,4-Pentanedione was obtained from Carlo, Erba, Milano, Italy and $NADP^+$, NADH, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and cytochrome *c* from the Sigma Chemical Co., St. Louis, MO, U.S.A. Other chemicals used were of the highest purity commercially available.

RESULTS

Effect of a single injection of substituted 1,2,3-*benzothiadiazoles on the mixed function oxidase system.* The effects of a single injection of the synergist 6-chloro-1,2,3-benzothiadiazole on the *in vitro* *N*-demethylation of aminopyrine and naphthalene hydroxylation are shown in Table 1. The biphasic effect observed for both reactions was characterized by an inhibition period during the first 15 hr followed by an enhancement of the microsomal activity after

Table 1. Effect of a single injection of 6-chloro-1,2,3-benzothiadiazole on the hepatic mixed function oxidase systems*

Time after injection (hr)	Aminopyrine *N*-demethylation (%)	Naphthalene hydroxylation (%)	NADH–cytochrome *c* reductase (%)	NADPH–cytochrome *c* reductase (%)	Cytochrome P-450 (%)	Cytochrome *b5* (%)
3	49 ± 6†	51 ± 3†	89 ± 10 (NS)‡	65 ± 10†	58 ± 6†	100 ± 5 (NS)‡
15	55 ± 9†	82 ± 7§			63 ± 8‖	88 ± 10 (NS)‡
24	118 ± 12‖	167 ± 21§			94 ± 10 (NS)‡	100 ± 12 (NS)‡
48	159 ± 20§	215 ± 36¶	88 ± 10¶	120 ± 11¶	215 ± 10†	216 ± 27‖
72	96 ± 14 (NS)‡	154 ± 28 (NS)‡				

* Mice weighing 20–22 g were divided in two groups. The control group was injected with corn oil and the other group was injected with 200 mg/kg of 6-chloro-1,2,3-benzothiadiazole. At the times indicated, both groups were killed. The oxidative activities and cytochrome content were estimated as described in Materials and Methods. Each result is the average ± S.D. of four independent experiments, expressed as treated/control × 100 (%). Control values were: aminopyrine *N*-demethylase, 5.17 ± 0.35 nmoles $HCHO \cdot min^{-1} \cdot (mg\ protein)^{-1}$; naphthalene hydroxylase; 1.70 ± 0.18 nmoles α-naphthol$\cdot min^{-1} \cdot (mg\ protein)^{-1}$; NADH–cytochrome *c* reductase, 431.7 ± 14.3 nmoles cytochrome *c* reduced$\cdot min^{-1} \cdot (mg\ protein)^{-1}$; NADPH-cytochrome *c* reductase, 39.6 ± 1.9 nmoles cytochrome *c* reduced$\cdot min^{-1} \cdot (mg\ protein)^{-1}$; cytochrome P-450, 0.65 ± 0.10 nmole/mg protein; and cytochrome *b5* 0.26 ± 0.03 nmole/mg $protein^{-1}$.

† $P < 0.001$.

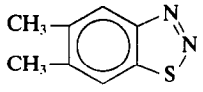
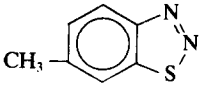
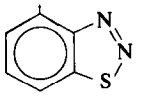
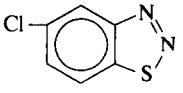
‡ Not significant ($P > 0.05$).

§ $P < 0.02$.

‖ $P < 0.01$.

¶ $P < 0.05$.

Table 2. Effect of a single injection of substituted 1,2,3-benzothiadiazole on *in vitro* aminopyrine *N*-demethylation and naphthalene hydroxylation*

Compound	Time after injection (hr)	Aminopyrine *N*-demethylation (%)	Naphthalene hydroxylation (%)
	3	40 ± 8†	31 ± 3†
	48	157 ± 20‡	187 ± 28‡
	3	29 ± 5†	32 ± 4†
	48	70 ± 9†	79 ± 11‡
	96	100 ± 5 (NS)§	105 ± 8 (NS)§
	3	85 ± 11‖	52 ± 8†
	48	145 ± 15‡	105 ± 10 (NS)§
	3	39 ± 6†	45 ± 8†
	48	185 ± 16†	215 ± 25‡

* Mice weighing 20–22 g were divided into two groups. The control group was injected with corn oil; the other group was injected with 200 mg/kg of substituted 1,2,3-benzothiadiazole. At the times indicated, both groups were killed. The oxidative activities were estimated as described in Materials and Methods. Each result is the average ± S.D. of five independent experiments, expressed as treated/control × 100%.

† $P < 0.001$.

‡ $P < 0.01$.

§ Not significant ($P > 0.05$).

‖ $P < 0.05$.

24 hr. The effects of the same synergist on the levels of some components of the microsomal electron transport system are also shown in Table 1. The bisphasic effect was similarly observed with respect to the content of cytochrome P-450 and to the activity of the NADPH–cytochrome *c* reductase. Cytochrome b_5 content remained unchanged during the first hours of treatment but was enhanced after 48 hr; NADH–cytochrome *c* reductase activity was essentially unaffected.

The effects of injection of various 1,2,3-benzothiadiazoles substituted with chlorine or methyl in different positions of the phenyl portion of the heterocyclic ring on some monooxygenase activities are

Table 3. Effect of 6-chloro-1,2,3-benzothiadiazole administration on the spectral properties of cytochrome P-450*

Pretreatment with the synergist (hr)	P-450 (nmoles/mg protein)	CO spectra (max nm)	After *in vitro* addition of potassium ferricyanide: P-450 (nmoles/mg protein)	After *in vitro* addition of potassium ferricyanide: Dithionite CO spectra (max nm)
	0.65 ± 0.12†	450		
3	0.38 ± 0.10	452	0.37	452
15	0.43 ± 0.09	452	0.43	452
24	0.61 ± 0.10	450		
48	1.40 ± 0.25	450	1.38	450

* Mice given a single dose of 6-chloro-1,2,3-benzothiadiazole (200 mg/kg) were killed at the times indicated. Microsomes were suspended to give a microsomal protein concentration of 1.0 mg/ml. Potassium ferricyanide was added to a final concentration of 100 μM, 3 min later the suspension was reducted with $Na_2S_2O_4$ and divided into two cuvettes, carbon monoxide was gassed in the sample cuvette, and the difference spectrum was recorded. The carbon monoxide difference spectrum was also obtained with microsomal suspensions to which no ferricyanide was added before reduction with $Na_2S_2O_4$.

† Each result is the mean ± S.D. of 5 experiments.

shown in Table 2. All compounds tested inhibited both reactions assayed, with the most potent being 6-methyl-1,2,3-benzothiadiazole. Some of them, such as 5-chloro-1,2,3-benzothiadiazole and 5,6-dimethyl-1,2,3-benzothiadiazole, enhanced oxidative activity 48 hr after injection. 6-Methyl-1,2,3-benzothiadiazole, however, was unable to increase drug-metabolizing activity even 96 hr after injection.

Effect of 6-chloro-1,2,3-benzothiadiazole administration on the spectral properties of the carbon monoxide binding to cytochrome P-450. Cytochrome P-450, determined by carbon monoxide binding in the dithionite-reduced difference spectrum, appeared to decrease during the first 15 hr in microsomes of mice treated *in vivo* with the synergist and to increase after 48 hr (Table 3). The maximum wavelength absorption for the difference spectrum was 452 nm in microsomes of mice treated for 3 and 15 hr and 450 nm in microsomes of mice treated for 24 and 48 hr. The synergist added directly to microsomal suspensions of untreated mice at 10^{-4} M did not interfere with the determination of cytochrome P-450. It has been reported [20] that the addition of potassium ferricyanide destroyed the complex of cytochrome P-450 with some drugs and made the total cytochrome P-450 available for carbon monoxide binding. Incubation of microsomal preparations of mice treated with 6-chloro-1,2,3-benzothiadiazole for different periods of time with 100 μM ferricyanide changed neither the amount of cytochrome P-450 available for carbon monoxide binding nor the maximum absorption wavelength of the difference spectrum (Table 3).

Hexobarbital sleeping time after in vivo *treatment with 6-chloro-1,2,3-benzothiadiazole.* As shown in Table 4, when hexobarbital was injected 3 hr after the administration of the synergist, the hexobarbital sleeping time was considerably prolonged compared to the controls. But, if the barbiturate was injected 48 hr after the synergist, the duration of narcosis was greatly shortened. Thus, the differential effect on the sleeping time might reflect an inhibition during the first hours immediately following the injection and an increase in the *in vivo* metabolism of the barbiturate.

Table 4. Effect of 6-chloro-1,2,3-benzothiadiazole on hexobarbital sleeping time

Treatment*	Sleeping time (min ± S.D.)	P
Control (corn oil, i.p., then hexobarbital)	78.8 ± 9.7	< 0.001
6-Cl-BTD, i.p., + hexobarbital 3 hr later	335.9 ± 27.3	< 0.002
6-Cl-BTD, i.p., + hexobarbital 48 hr later	13.0 ± 0.99	< 0.001

* Each group contained ten mice. 6-Chloro-1,2,3-benzothiadiazole was injected, i.p., at a dose of 200 mg/kg. At the time indicated, hexobarbital (80 mg/kg) in 0.2 ml of 0.9% isotonic NaCl was injected i.p.

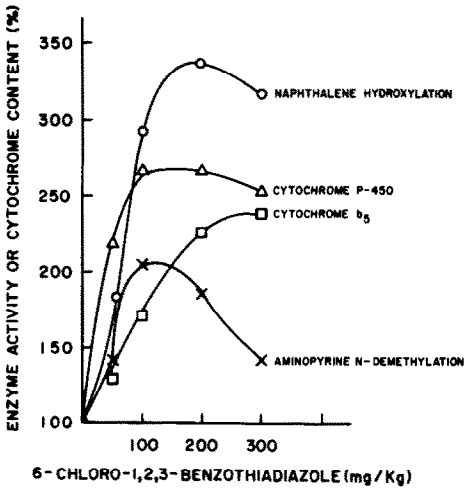


Fig. 1. Effects of various doses of 6-chloro-1,2,3-benzothiadiazoles on the enhancement of microsomal activity and cytochrome content. The control group was injected i.p. with corn oil; the other groups were injected with various doses of synergist. The animals were killed 48 hr later. The oxidative activities and cytochrome content were estimated as described in Materials and Methods. Results are the average of three independent experiments, expressed as treated/control × 100(%).

Dose effect of 6-chloro-1,2,3-benzothiadiazole on the enhancement of some monooxygenase activities and cytochrome content. The dose effect of a 6-chloro-1,2,3-benzothiadiazole injection on aminopyrine *N*-demethylase and naphthalene hydroxylase activities, as well as on the content of cytochrome P-450 and b_5, is shown in Fig. 1. Both activities were increased by treatment with the synergist, the hydroxylase being higher than *N*-demethylase activity at a fixed dose. This effect suggests a different substrate specificity for the oxidizing system. The maximum enhancement was obtained at a dose of 100 mg/kg for the *N*-demethylation and at 200 mg/kg for the hydroxylation. The contents of cytochrome P-450 and b_5 were also increased by injection with the synergist, but no correlation was observed between the magnitude of the total increase in cytochrome P-450 and oxidative activities, suggesting that different species of cytochrome P-450 might be involved in the oxidation of naphthalene and aminopyrene.

Evidence of enzyme induction by administration of 6-chloro-1,2,3-benzothiadiazoles. An enhancement in liver weight, microsomal protein, and incorporation of phenylalanine by the S-30 fraction was observed 48 hr after a single injection of the synergist (Table 5). Additional evidence of enzyme induction was shown by the blocking effect of actinomycin D on the 6-chloro-1,2,3-benzothiadiazole-induced stimulation of the mixed function oxidase system. Thus, Fig. 2 shows the *in vitro* rates of *N*-demethylation of aminopyrine, naphthalene hydroxylation, and the levels of cytochrome P-450 and b_5 in mice treated with the synergist, with actinomycin D, and with the synergist plus actinomycin D. At the dose used, the inhibitor of RNA synthesis by itself had

Table 5. Effect of 6-chloro-1,2,3-benzothiadiazole on L-phenylalanine incorporation to S-30 fraction, liver weight, and content of microsomal protein*

	L-[^{14}C]Phenylalanine incorporated (pmoles/mg protein)	Liver wt (g/100 g body wt)	Microsomal protein (mg/g liver)
Control	14.3 ± 2,5	5.0 ± 0.12	15.96 ± 1.89
Treated	24.2 ± 1.2†	6.20 ± 0.26†	19.14 ± 0.57‡

* The control group was injected with corn oil, and the treated group was injected with 200 mg/kg of 6-chloro-1,2,3-benzothiadiazole. The animals were killed 48 hr later. L-[^{14}C]Phenylalanine incorporation was estimated as indicated in Materials and Methods. Each results is the average ± S.D. of four experiments.

† $P < 0.001$.

‡ $P < 0.02$.

very little effect on the oxidative activities and cytochrome content. But, when actinomycin D was administered in combination with the synergist, it prevented completely the stimulation of microsomal oxidative activity but blocked only partially the stimulation of cytochrome content (results not shown). Furthermore, electron microscopy of the livers of mice treated with the synergist showed proliferation of the smooth and rough endoplasmic reticulum 48 hr after the injection (Fig. 3).

Studies on phenylalanine incorporation by S-30 fraction of liver from mice treated with 6-chloro-1,2,3-benzothiadiazole. Incorporation of L-[^{14}C]phenylalanine by the S-30 fraction of liver in synergist-treated mice was notably stimulated. Three hours after treatment, a 31 per cent increase in the incorporation of the amino acid was observed in treated, compared to control, animals. This suggests that enzyme induction started when oxidative activity was still inhibited. Forty-eight hours after treatment with the synergist, a 74 per cent enhancement was observed. In all preparations addition of polyuridylic acid increased incorporation, but it was higher in microsomes from synergist-treated mice. Figure 4 describes the effect of preincubation on the activity of the polypeptide synthetic system. Such preincubated microsomes rapidly lost their ability to incorporate phenylalanine, probably because of degradation of endogenous mRNA. If polyuridylic acid was added, the capacity to incorporate the amino acid was restored.

SDS–polyacrylamide gel electrophoresis. Figure 5 shows the SDS–polyacrylamide gel profiles of microsomes of mice treated with 6-chloro-1,2,3-benzothiadiazole, phenobarbital, and corn oil. Well A contained protein standards of known molecular weights. Wells B and E contained microsomes of mice treated with corn oil for 3 and 48 hr respectively. Wells C and D contained microsomes of mice treated with 6-chloro-1,2,3-benzothiadiazole for 3 and 48 hr respectively. Well F contained microsomes of mice induced by phenobarbital. Protein-staining bands in the range of 48,000 to 60,000 molecular weight have been numbered arbitrarily according to mobility and molecular weight as follows: band 1, mol. wt 50,000; band 2, 52,000; band 3, 53,000; band 4, 56,000; band 5, 58,000; and band 6, 60,000. All these bands stained for peroxidase activity, although some could have

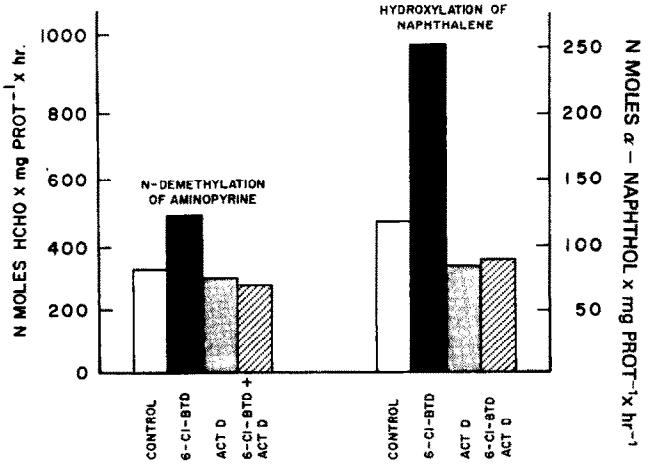


Fig. 2. Effect of actinomycin D on the 6-chloro-1,2,3-benzothiadiazole induction of mixed function oxidase activity. 6-Chloro-1,2,3-benzothiadiazole (200 mg/kg) was administered by a single i.p. injection. Actinomycin D (250 μg/kg) was administered, i.p., at zero time and 24 hr after the synergist or corn oil. Animals were killed 48 hr after synergist or corn oil administration. Monooxygenase activities were determined as described in Materials and Methods. Each value is the mean of four experiments.

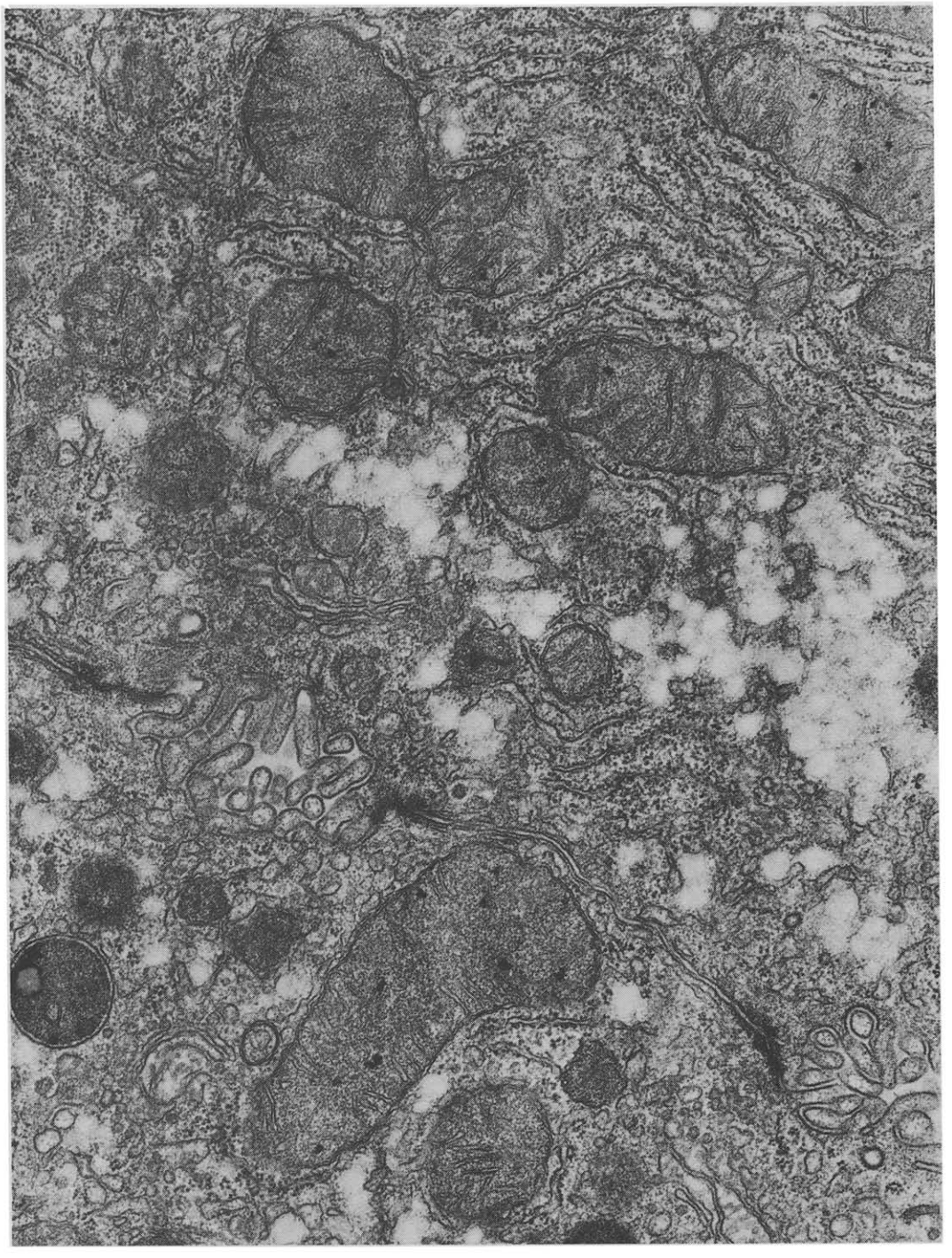


Fig. 3(A).

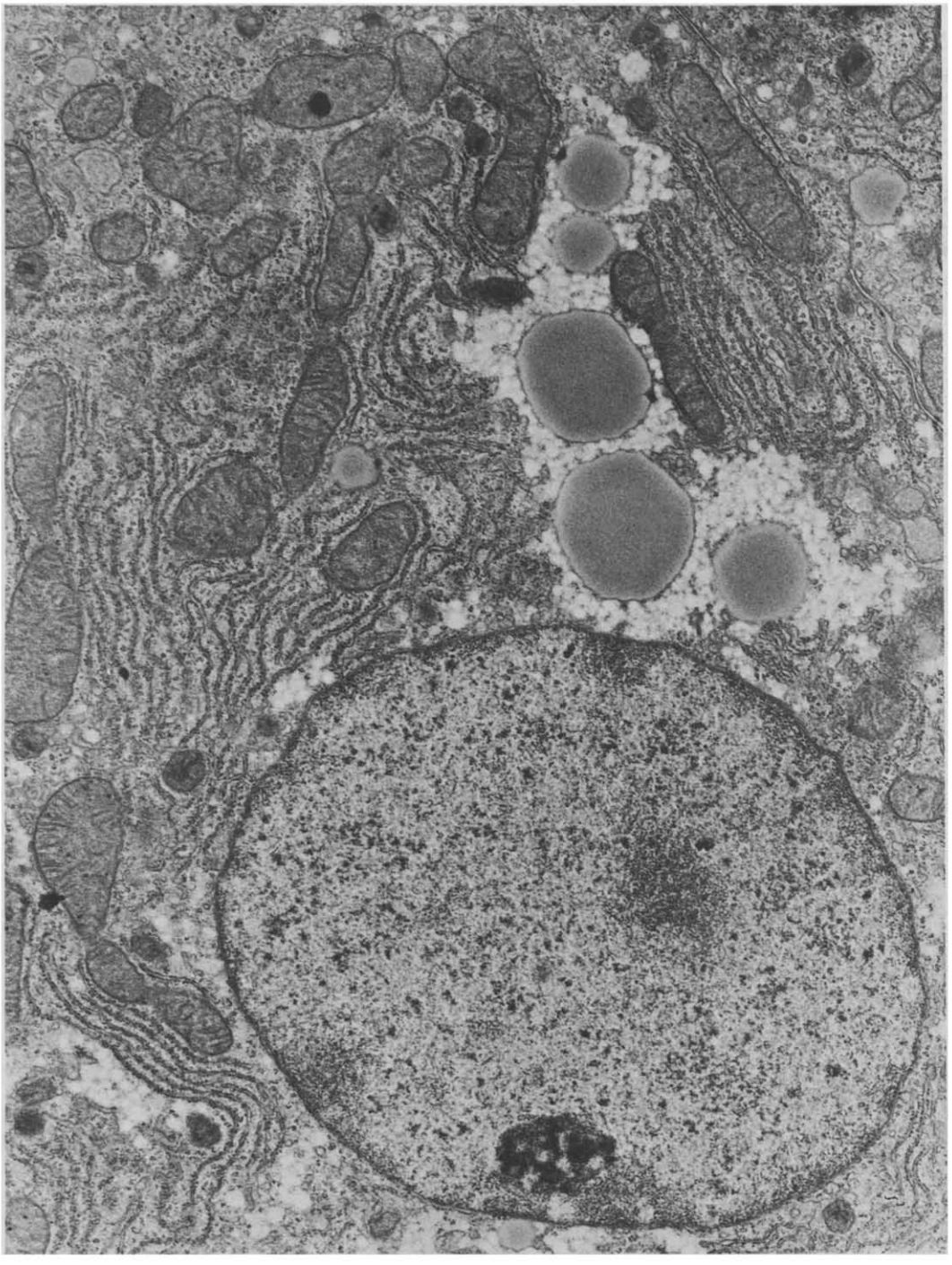


Fig. 3(B).

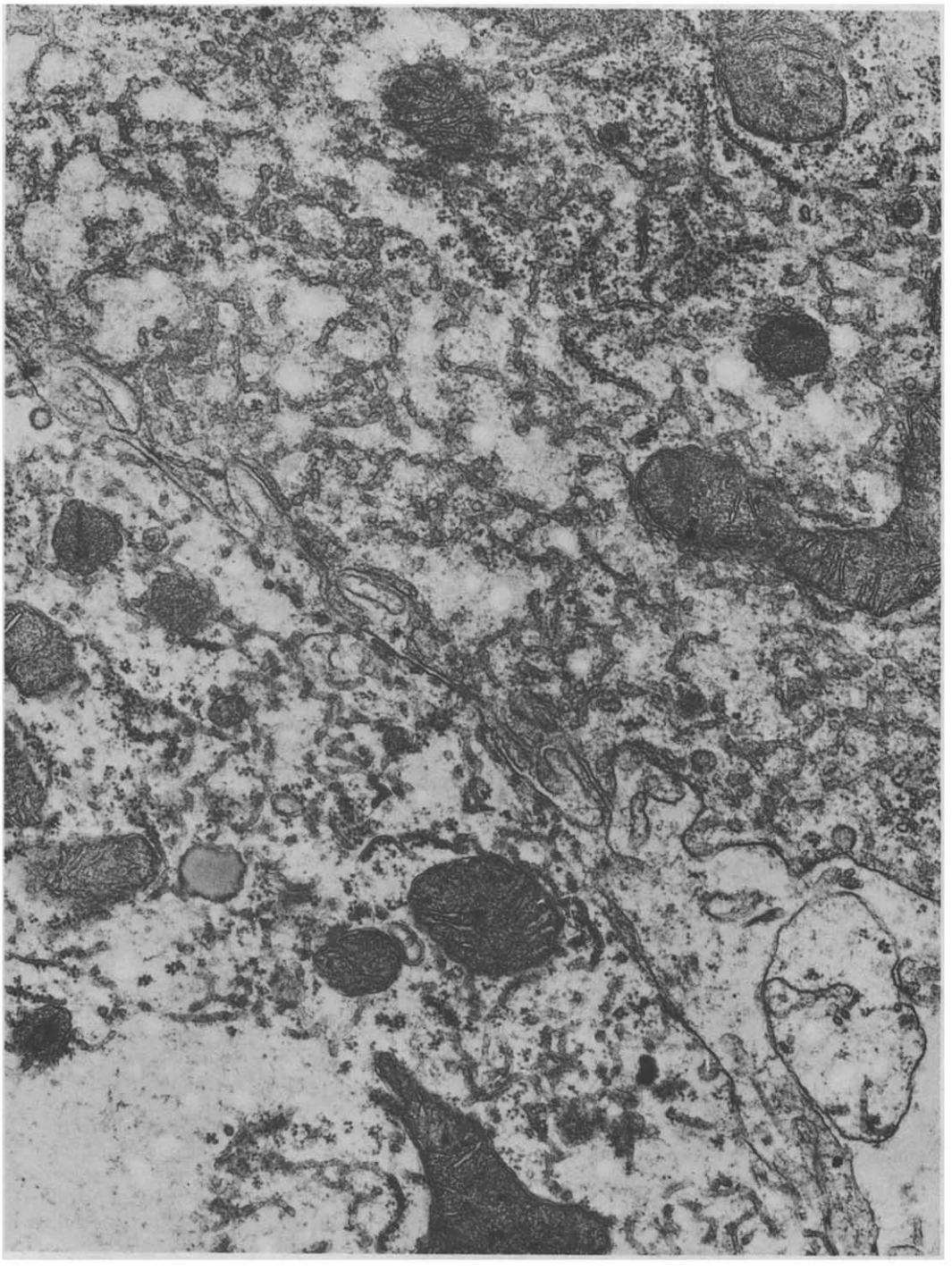


Fig. 3. (A) Electronmicrograph of a liver slice of a mouse injected with corn oil. Border zone between two hepatocytes. Organelles of normal structure and distribution × 55,000. (B) and (C) Electronmicrographs of a liver slice of a mouse injected with 6-chloro-1,2,3-benzothiadiazole 48 hr prior to being killed. Panel B: proliferation of rough endoplasmic reticulum × 22,000. Panel C: Hypertrophy of smooth endoplasmic reticulum × 40,000.

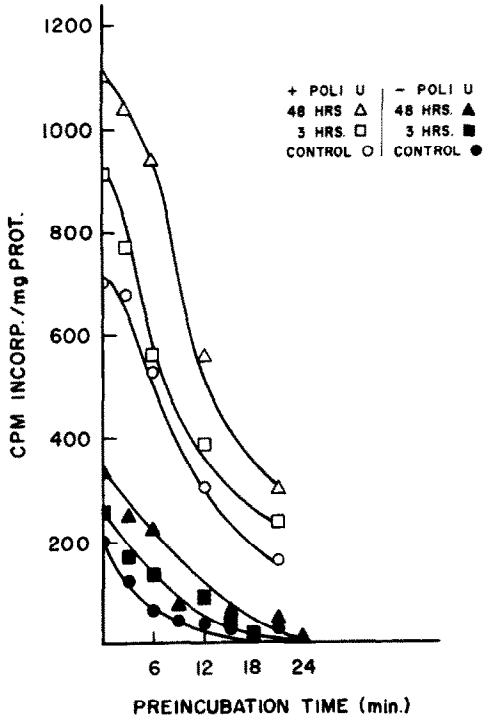


Fig. 4. Rate of loss of mRNA activity in normal and 6-chloro-1,2,3-benzothiadiazole-treated rats. Two groups of four mice each were injected with 6-chloro-1,2,3-benzothiadiazole (200 mg/kg) and were killed 3 or 48 hr later. The control group received corn oil. The preparation of S-30 fraction and incorporation of L-[^{14}C]phenylalanine in the presence or absence of 100 μg of Poly U were was described in Materials and Methods. Preincubations at the times indicated were done with and without Poly U at 37° in the absence of labeled phenylalanine and in the presence of 25 μmoles phosphocreatine and 50 μg creatine phosphokinase.

been proteins that were not cytochrome P-450. In phenobarbital-treated microsomes (Well F), the predominant bands were 1, 2, 5 and 6. In microsomes of mice treated for 48 hr with the synergist (Well D), the predominant bands were 1, 2 and 3; band 5 is less marked, and band 4 is faint. In microsomes of mice treated for 3 hr (Well C), bands 1, 2 and 3 were less marked than microsomes of mice treated with corn oil (Well B).

DISCUSSION

A single intraperitoneal dose of any one of several benzothiadiazoles to mice produced a decrease of certain microsomal activities *in vitro* and a lower hexobarbital metabolism *in vivo* during the first 15 hr after injection. Some of the components of the electron transport system were also affected since an apparent decrease in cytochrome P-450 content and in NADPH–cytochrome *c* reductase was observed. The level of cytochrome b_5 remained almost unchanged, and NADH–cytochrome *c* reductase was affected very little.

An initial apparent decrease in cytochrome P-450 has also been observed for mice treated with piperonyl butoxide and propyl isome [21], as well as with other types of synergists such as NIA 16284, MGK 264, RO 5-8019, and WL 19255 [22,23].

The apparent decrease in cytochrome P-450 after benzothiadiazole administration for 3 hr could result from the binding of the synergist or a metabolite at a site that interferes with the binding of carbon monoxide during the assay. Buening and Franklin [20] and Franklin [24] reported that, during metabolism of SKF 525-A and other drugs, some stable complexes with cytochrome P-450 are formed which interfere with carbon monoxide binding to the terminal oxidase. These complexes were destroyed by *in vitro* addition of potassium ferricyanide, thus making the total cytochrome P-450 available for carbon monoxide binding and restoring some monooxygenase activities to normal levels. The *in vitro* addition of potassium ferricyanide to microsomes of mice treated for a few hours with 6-chloro-1,2,3-benzothiadiazoles neither modified the maximum wavelength absorbance of the dithionite monoxide spectrum which remained at the 452 nm wavelength of cytochrome nor restored it to the original levels of cytochrome P-450. Preliminary results in our laboratory on the mechanism of action of benzothiadiazoles, which will be reported soon, have shown that radiolabeled 6-chloro-1,2,3-benzothiadiazole remains bound very tightly to cytochrome P-450 even after several washings and after treatment with various oxidant agents. The formation of such complexes is related to the apparent decrease in cytochrome P-450 and requires the presence of NADPH and molecular oxygen, thus suggesting a metabolite effect.

The apparent decrease in cytochrome P-450 could also result from destruction of the hemoprotein by the synergist or a metabolite. Although we did not observe the appearance of cytochrome P-420, a decrease in the staining bands of mol. wt 50,000, 52,000 and 53,000 (Fig. 4) was observed when microsomes of mice treated for 3 hr with the synergists were compared with the controls.

Inhibitors of the microsomal enzymes can block the oxidative process in different ways [25], e.g. by competition with substrates for the microsomal enzymes, by blocking the reduction of cytochrome P-450, by interfering with the transfer of active oxygen from cytochrome P-450 to substrate, or by destruction of the terminal oxidase. The mechanism of inhibition of the mixed function oxidases by insecticide synergist is not completely understood, although several hypotheses have been proposed [26–29]. Our results suggest that inhibition of drug-metabolizing activities by benzothiadiazoles could be the results of one or both of the following phenomena:(a) a decrease in the reduction of cytochrome P-450 as suggested by the low activity of NADPH–cytochrome *c* reductase in mice treated with the synergist, and (b) a destruction of labile form(s) of cytochrome P-450 which will prevent drug oxidation. The active form that interacts with cytochrome P-450 could be a carbanion, a carbonium ion, or a free radical. All such intermediates can be easily formed from benzothiadiazoles. The activities of benzothiadiazoles as carbaryl synergists in *Musca domestica* correlate well with the hydrophobic binding constant

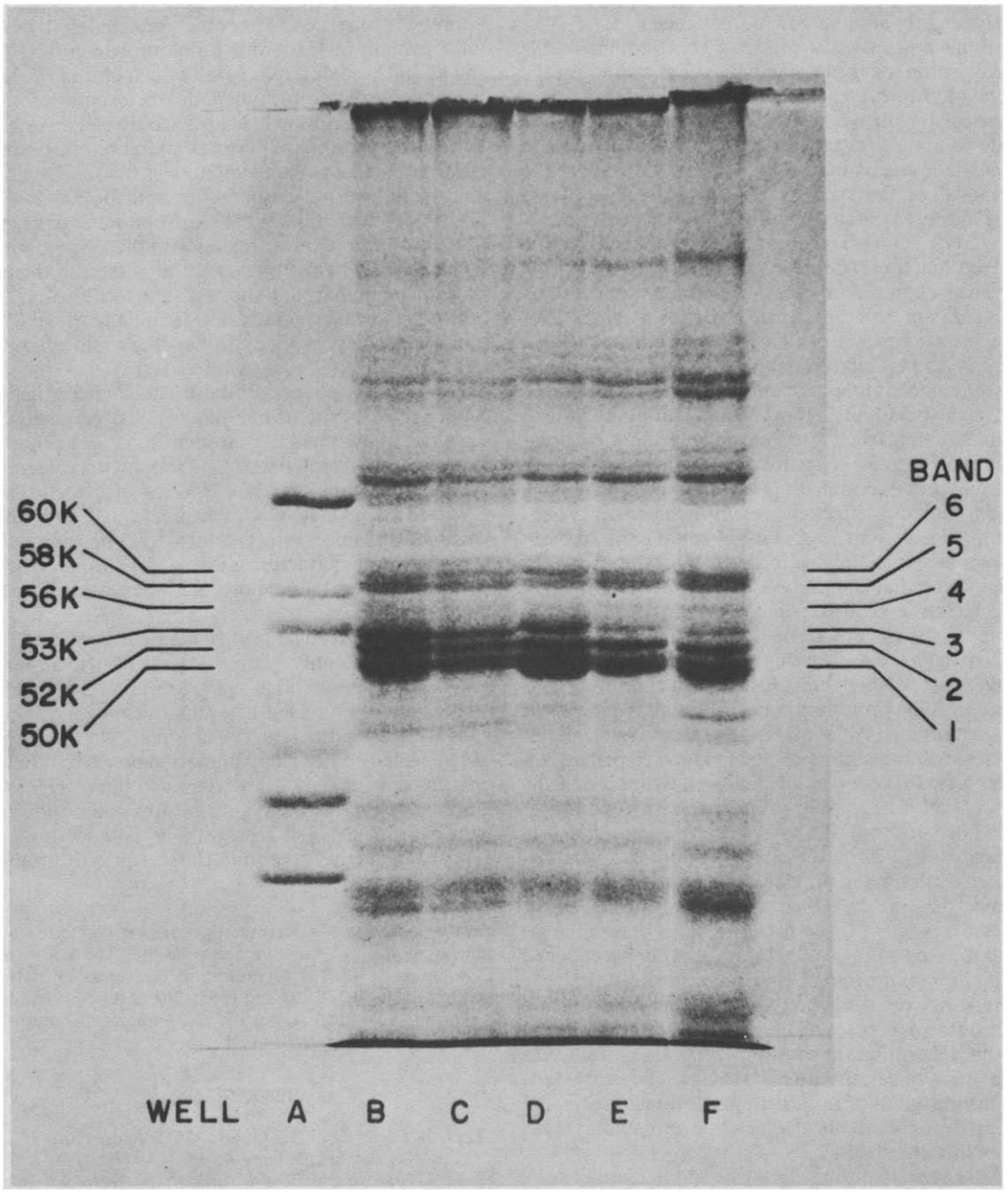


Fig. 5. SDS–polyacrylamide slab gel of mice liver microsomes. Electrophoretic migration is from top to bottom. The samples were analyzed at the microsomal protein and cytochrome P-450 levels indicated. Well A contained proteins standards (3 μg) with molecular weights of 68,000, 58,000, 53,000, 43,000, 40,000 and 36,000. Wells B and E contained, respectively microsomes from mice treated with corn oil 3 and 48 hr prior to the animals being killed plus in well B (70 μg microsomal protein and 0.05 nmole cytochrome P-450), and in well E (70 μg microsomal protein and 0.05 nmole cytochrome P-450). Wells C and D contained, respectively, microsomes of mice injected with 6-chloro-1,2,3-benzothiadiazole 3 and 48 hr prior to the animals being killed plus in well C (70 μg microsomal protein and 0.02 nmole cytochrome P-450), and in well D (70 μg microsomal protein and 0.10 nmole cytochrome P-450). Well F contained microsomes of mice induced by phenobarbital (45 μg protein and 0.11 nmole cytochrome P-450). Arrows indicate electrophoretic bands of approximately 50,000, 52,000, 53,000, 56,000, 58,000 and 60,000 molecular weight.

(π) and the homolytic free radical constant (τ) [8]. This is not a sufficiently strong reason, however, to postulate a free radical as one of the intermediates responsible for the inhibition.

After the initial inhibitory phase which lasted around 15 hr, an enhancement of drug-metabolizing activities *in vivo* and *in vitro* was observed. A similar increase was observed in the content of cytochrome P-450 and b_5. Our results suggest that the enhancement of some monooxygenase activities is due mainly to an increase in cytochrome P-450 content, since the activity of NADPH–cytochrome *c* reductase was

only slightly stimulated; the enhancement of cytochrome b_5 could also make a contribution to the higher monooxygenase activities, but at present the role of this cytochrome in drug metabolism is a matter of controversy. The increase in the oxidation rate of some substrates observed after a few days of a single injection of the synergist may be due to the removal of the synergist in the active site of the enzyme by a catabolic process and/or to the accelerated *de novo* synthesis of some components of the mixed function oxidase system.

The fact that 48 hr after the administration of some benzothiadiazoles increases are observed in liver weight, microsomal protein, incorporation of labeled amino acid to microsomal protein, and proliferation of the smooth and rough endoplasmic reticulum supports the possibility of enzyme induction. Additional evidence that the observed enhancement in drug-metabolizing activity is due to enzyme induction was obtained in experiments with actinomycin D, since the stimulatory effect elicited by 6-chloro-1,2,3-benzothiadiazole on the activity of some monooxygenases was completely suppressed by actinomycin D, suggesting that the process is dependent on mRNA transcription. The fact that actinomycin D was not able to block the basal rate of monooxygenase activity at a concentration that completely prevented 6-chloro-1,2,3-benzothiadiazole induction indicated that protein synthesis in control animals is dependent on preformed stable mRNA. On the other hand, an increase in the number of microsomal incorporation sites was also observed in microsomes obtained 48 hr after 6-chloro-1,2,3-benzothiadiazole injection (Fig. 3). Furthermore, similar rates of loss of mRNA activity in normal and 6-chloro-1,2,3-benzothiadiazole-treated animals (Fig. 3) support the evidence that the increased amino acid incorporation in microsomes from 6-chloro-1,2,3-benzothiadiazole-treated mice is not due to a synergist-induced inhibition of the loss of endogenous mRNA activity. Thus, enzyme induction by the synergist could be due to an increase in the number of active microsomal incorporation sites and also to an increase in the levels of certain mRNA species. The exact steps in transcription or in post-transcriptional processing of mRNA precursors at which benzothiadiazoles act remain to be defined.

Other mechanisms of enzyme induction by xenobiotics have been proposed. Some compounds, which presumably increase the intracellular concentration of cyclic AMP, lead to an increase in the activity of some hydroxylases [30], suggesting that protein kinase(s) activity and phosphorylation of specific proteins may be important in drug induction. On the other hand, some polycyclic aromatic hydrocarbons bind avidly to cytosolic receptor(s) [31] that are presumably involved in the transport of the inducer to the nucleus; the inducer–receptor complex could become the signal to evoke enzyme induction. These mechanisms could also be a possible target for the activities of benzothiadiazoles as inducers of the mixed function oxidase system.

It is now evident that multiple forms of cytochrome P-450 are present in the liver microsomes of animals untreated or treated with different inducers. These forms differ in their catalytic, spectral, and electrophoretic properties. Phenobarbital-treated hybrid mice contain at least four forms of cytochrome P-450 [32]. Three different forms of cytochrome P-450 have been isolated from hepatic microsomes of rats treated with Arochlor 1254 [33]. Isosafrole, an insecticide synergist that behaves as an inhibitor of cytochrome P-450 mediated monooxygenation, is also able to induce novel species of cytochrome P-450 [34]. An examination of the electrophoretic patterns of microsomes of mice treated with the carrier, with 6-chloro-1,2,3-benzothiadiazole at different times, or with phenobarbital showed that 6-chloro-1,2,3-benzothiadiazole induced preferentially a 50,000 mol. wt species, similar to phenobarbital, but also induced a 53,000 mol. wt band that was barely observable in phenobarbital-treated microsomes. Although this type of one-dimensional gel analysis does not allow unequivocal analysis of the protein levels, it is interpreted as strong supporting evidence for the induction of a new species of cytochrome P-450. This change is clearly associated with the treatment of mice with 6-chloro-1,2,3-benzothiadiazole. Further characterization of cytochrome P-450 species induced by 6-chloro-1,2,3-benzothiadiazole is under progress.

The use of chemical probes has been proposed as a method to identify various classes of cytochrome P-450; thus, metyrapone, SKF 525-A, and amphetamines have been used to evaluate changes associated with alterations in cytochrome P-450 content of liver microsomes from animals exposed to different inducing agents [35]. The fact that 6-chloro-1,2,3-benzothiadiazole is able to form stable adducts with cytochrome P-450 suggests its possible use as a probe to identify various classes of cytochrome P-450.

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REFERENCES

1. H. Jaffe, K. Fujii, H. Guerin, M. Sengupta and S. S. Epstein, *Biochem. Pharmac.* **18**, 1045 (1969).
2. H. L. Falk and P. Kotin, *Ann. N. Y. Acad. Sci.* **160**, 299 (1969).
3. M. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2**, 341 (1966).
4. R. Kato, E. Chiesara and P. Vassanelli, *Medna exp.* **6**, 254 (1962).
5. J. E. Casida, *J. agric. Fd. Chem.* **18**, 753 (1970).
6. C. F. Wilkinson and L. B. Brattsten, *Drug Metab. Rev.* **1**, 153 (1972).
7. J. C. Felton, D. W. Jenner and P. Kirby, *J. agric. Fd. Chem.* **18**, 671 (1970).
8. D. L. Gil and C. F. Wilkinson, *Pestic. Biochem. Physiol.* **6**, 338 (1976).
9. D. L. Gil and C. F. Wilkinson, *Pestic. Biochem. Physiol.* **7**, 183 (1977).
10. H. Schwab and H. Sies, *Hoppe-Seyler's Z. physiol. Chem.* **359**, 385 (1978).
11. H. M. Bolt, A. Buchter, L. Wolowski, D. L. Gil and W. Bolt, *Int. Archs. occup. envir. Hlth.* **39**, 103 (1977).
12. D. L. Gil, J. Ferreira and B. Reynafarje, *Xenobiotica* **10**, 7 (1980).

13. E. E. Hodgson and R. M. Philpot, *Drug Metab. Rev.* **3**, (2), 231 (1974).
14. J. Booth and E. Boyland, *Biochem. J.* **70**, 681 (1958).
15. T. Nash, *Biochem. J.* **55**, 416 (1953).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. B. S. S. Masters, C. H. Williams, Jr. and H. Kamin, *Methods in Enzymology* Vol. 10, p. 565. Academic Press, New York (1967).
18. U. K. Laemmli, *Nature Lond.* **227**, 680 (1970).
19. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
20. M. K. Buening and M. R. Franklin, *Drug. Metab. Dispos.* **4**, 244 (1976).
21. R. M. Philpot and E. Hodgson, *Chem.-Biol. Interact.* **4**, 185 (1971/1972).
22. H. B. Matthews, M. Skrinjaric-Spoljar and J. E. Casida, *Life Sci.* **9**, 1039 (1970).
23. M. Skrinjaric-Spoljar, H. B. Matthews, J. L. Engel and J. E. Casida, *Biochem. Pharmac.* **20**, 1607 (1971).
24. M. R. Franklin, *Chem.-Biol. Interact.* **14**, 337 (1976).
25. J. R. Gillette, *Adv. Pharmac.* **4**, 219 (1966).
26. J. E. Casida, J. L. Engel, E. G. Essac, F. X. Kamienski and S. Kuwatsuka, *Science* **151**, 1130 (1966).
27. D. J. Hennessy, *J. agric. Fd. Chem.* **13**, 218 (1965).
28. C. Hansch, *J. med. Chem.* **11**, 920 (1968).
29. V. Ullrich and K. H. Schnabel, *Drug Metab. Dispos.* **1**, 176 (1973).
30. H. Yamasaki, E. Huberman and L. Sachs, *J. biol. Chem.* **250**, 7766 (1975).
31. T. M. Guenthner and D. W. Nebert, *J. biol. Chem.* **252**, 8981 (1977).
32. M.-T. Huang, S. W. West and A. Y. H. Lu, *J. biol. Chem.* **251**, 4659 (1976).
33. D. W. Ryan, P. E. Thomas, D. Korzemouski and W. Levine, *J. biol. Chem.* **254**, 1365 (1979).
34. M. Dickins, J. Bridges, G. R. Elcombe and K. L. Netter, *Biochem. biophys. Res. Commun.* **80**, 89 (1978).
35. J. Werringloer and R. W. Estabrook, in *The Induction of Drug Metabolism* (Eds. R. W. Estabrook and E. Lindenlaub), p. 267. F. K. Schattauer, Stuttgart (1979).