Toxic Effects of a Fungicide, 5-Ethoxy-3-(Trichloromethyl)-1,2,4-Thiadiazole (Terrazole), on the Hepatic Drug Metabolizing Enzyme System in Mice

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Widespread presence of a large number of pesticides in the environment has become a potential public health hazard (DALVI and SALUNKHE 1975). Specifically, these man-made chemicals may be identified as insecticides, herbicides, fungicides, acaricides and so on. With regard to toxicologic data, the first two groups of the pesticides have been receiving greater attention than fungicides which are also equally important environmental contaminants. Terrzole is one of the environmental contaminants belonging to the group of fungicides. This chemical has been reported to be useful as a soil fungicide for control of root and stem diseases caused by pythium and phytophthora. It is used alone or in combination with other pesticides for better fungicidal activity. Although acute toxicity studies on terrazole in laboratory animals have been done (ANONYMOUS 1974) no data dealing with the effects of terrazole on the vitally important hepatic drug metabolizing enzyme system have been yet reported. This prompted us to study the hepatic mixed function oxidase catalyzed metabolism of this fungicide in mice.

MATERIALS AND METHODS

Terrazole (5-ethoxy-3-trichloromethyl-1,2,4-thiadiazole) was a generous gift from Olin Corporation, Little Rock, Arkansas. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and $NADP^+$ were purchased from Boehringer Mannheim Corporation, New York. Benzphetamine hydrochloride was obtained as a gift from Upjohn Co., Kalamazoo, Michigan, through the courtesy of Dr. Paul O'Connell. All other chemicals used in the experimental work were of reagent grade purity.

Male albino Swiss mice, weighing 25-40 gm, were purchased from Southern Animal Farms, Prattville, Alabama. All animals were housed in plastic cages on hardwood chips and were maintained on purina chow diet with access to water at all times.

In the experiments dealing with sleeping time test, the animals received a single dose of terrazole in corn oil (100 mg/kg, ip); control animals were given corn oil alone. Twenty-four hr later, the mice were administered intraperitoneally a hypnotic dose of pentobarbital sodium (50 mg/kg). The sleeping time was taken equivalent

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to the time elapsed between the loss and the gain of the righting reflex. The same animals were used for the sleeping tests at 24, 48, and 96 hr after the administration of terrazole.

The in vivo effect of terrazole on hepatic drug metabolizing enzyme (DME) system was studied by dividing the animals into 3 groups each consisting of at least 3 animals. One of the groups received an ip dose of 0.25 mmole/kg terrazole in oil while another group was given 2.0 mmoles/kg terrazole in oil by the same route; the remaining group was taken as a control group which received an equivalent amount of oil alone. The animals were sacrificed by decapitation 24 hr after the treatment. The livers were rapidly removed, weighed, and perfused with ice-cold 1.15% KCl solution. The perfused liver of each animal was homogenized with 3 volumes of ice-cold 0.25 M sucrose solution containing 0.05 mM EDTA first in a small waring blender and then in a Potter Elvehjem type homogenizer. These operations were carried out at 4° C. The homogenates were centrifuged at 9000 x g for 20 min in a refrigerated Sorvall centrifuge. The microsomes from the supernatant fraction were isolated by the procedure described by CINTI et al. (1973). The microsomal pellets were washed twice with 1.15% KCl solution containing 0.05 mM EDTA and were stored at -20° C under nitrogen atmosphere until used for enzyme activity determinations. Liver microsomes from phenobarbital pretreated mice were isolated following the same procedure. In this case mice were administered a daily ip dose of 50 mg/kg phenobarbital sodium in saline for 4 consecutive days. Twenty-four hr after the last injection the animals were sacrificed to obtain the hepatic microsomes.

Benzphetamine demethylase activity of the microsomes was assayed as described previously (DALVI et al. 1974). The method involves colorimetric determination of formaldehyde formed from benzphetamine by Nash reagent (NASH 1953). The aniline hydroxylase activity was determined essentially by the method of KATO and GILLETTE (1965). The incubations were carried out in 0.1 M phosphate buffer, pH 7.4, containing an NADPH-generating system ($NADP^+$, 1 mM; glucose-6-phosphate, 12 mM; glucose-6-phosphate dehydrogenase, 1 unit) and 1-1.5 mg microsomal protein per ml of the incubation media in a final volume of 2 ml. Prior to the addition of aniline hydrochloride (1 mM), the reaction mixtures were preincubated for 5 min at 37° C in a gyrotory shaker water-bath. At the end of 20 min incubation period the reaction was stopped by adding 1 ml of 20% TCA and then the incubations were centrifuged at 5000 rpm for 10 min. To a 2 ml supernatant from each centrifuge tube, 1 ml of 10% sodium carbonate reagent and 2 ml of the phenol reagent were added in that order. After incubating the resultant solutions at 37° C for 30 min they were read at 640 nm in a spectrophotometer. The amount of p-aminophenol formed from aniline was calculated from a standard curve plotted for this purpose.

Microsomal cytochrome P-450 was determined by a method of OMURA and SATO (1964) as described previously (DALVI et al. 1975). The absorbance of the complex of cytochrome P-450 and carbon monoxide was recorded in a Beckman Acta CIII dual beam spectrophotometer fitted with a scattered transmission accessory.

Lipid peroxidation was determined by a modification of the method of STOCKS and DORMANDY (1971). The details of the experimental procedure for the measurement of malonaldehyde formed in the incubation mixtures have been reported earlier (DALVI et al. 1975).

Protein concentrations were estimated by the biuret method modified to include 0.1 ml of 1% deoxycholate in each sample.

RESULTS

Effect of a single dose of terrazole (100 mg/kg) on the pentobarbital sleeping time was compared with that of similarly treated control mice. The results of the experiment are shown in Table 1. It is evident that the administration of a single dose of terrazole caused significant prolongation of the sleeping time. The fact that the terrazole-induced effect lasted for at least 4 days suggests that the detoxification of pentobarbital in the liver of poisoned animals occurred at a slower rate than in the normal livers. As can be seen from Table 1, the pentobarbital sleeping time was almost five-times greater in the treated animals than in the control animals.

TABLE 1

Effect of a single dose of terrazole on the pentobarbital "sleeping time" in the mouse

		Time after terrazole (days)		
		1	2	4
Sleeping time (min)[a]	control	9 ± 2	11 ± 4	10 ± 1
	terrazole	47 ± 5†	52 ± 6†	32 ± 1*

[a]Values given are the means ± SEM of four observations.
*P<0.05; †P<0.01, when compared with corresponding control values

As a consequence of the foregoing data the next experiment was designed in which mice were intraperitoneally administered with 0.25 mmole/kg or 2.0 mmoles/kg terrazole. The animals were killed 24 hr after the treatment and the liver microsomes tested in vitro

for their activity of benzphetamine demethylase and aniline hydroxylase. In addition, the concentration of the cytochrome P-450 in the microsomes was measured. The hepatic microsomes were also isolated from the respective control animals for comparison with respect to the aforementioned parameters. In agreement with the results of the in vivo sleeping time experiments, the animals receiving a greater dose of terrazole showed a marked decrease in the activity of both enzymes and a marked loss in the concentration of cytochrome P-450 at 24 hr after the fungicide administration (Table 2). A difference is noted that the activity of aniline hydroxylase was affected considerably more than that of benzphetamine demethylase. It is also evident that a smaller dose of terrazole (0.25 mmole/kg, ip) appears to be almost ineffective against the components of drug metabolizing enzyme system.

TABLE 2

Effect of two different doses of terrazole on the levels of microsomal cytochrome P-450 and on the activity of aniline hydroxylase and benzphetamine demethylase in the mouse.

Treatment (mmoles/kg)	Benzphetamine demethylase activity (nmoles formaldehyde formed/min/mg protein)[a]	Aniline hydroxylase activity (nmoles p-aminophenol formed/min/mg protein)[a]	Cytochrome P-450 (nmoles/mg protein)[a]
none	1.50 ± 0.08	0.32 ± 0.04	0.22 ± 0.05
0.25	1.17 ± 0.16	0.31 ± 0.02	0.18 ± 0.01
2.00	0.40 ± 0.15*	0.04 ± 0.01*	0.08 ± 0.02*

[a]Results given are the means ± SEM of four observations.
*P<0.05, when compared with corresponding control values.

Next, an experiment was carried out in which microsomes isolated from phenobarbital pretreated mice were incubated with terrazole in the absence and presence of NADPH-generating system in order to examine whether terrazole requires metabolism for its liver toxicity. After the preincubation, the microsomes were sedimented, resuspended in Hepes buffer and their ability to catalyze the demethylation of benzphetamine and hydroxylation of aniline examined. In addition, cytochrome P-450 content of these microsomes was also determined. An examination of these data (Table 3) indicates that there was no significant loss of cytochrome P-450 concentration or of the activity of benzphetamine demethylase and aniline hydroxylase in the microsomes preincubated with NADPH alone as compared to those

TABLE 3

Effect of terrazole on the concentration of cytochrome P-450 and the activity of the mixed function oxidase enzyme system in hepatic microsomes from phenobarbital-pretreated mice[a].

Incubation conditions		Benzphetamine demethylase activity (nmoles formaldehyde formed/min/mg protein)[b]	Aniline hydroxylase activity (nmoles p-aminophenol formed/min/mg protein)[b]	Cytochrome P-450 (nmoles/mg protein)[b]
Terrazole	NADPH			
-	-	5.54 ± 1.19	0.88 ± 0.01	0.67 ± 0.13
-	+	4.75 ± 0.18	0.66 ± 0.02	0.64 ± 0.10
+	-	1.34 ± 0.03*	0.42 ± 0.02*	0.62 ± 0.08
+	+	0.92 ± 0.05*	0.22 ± 0.02†	0.38 ± 0.09*

[a]Hepatic microsomes and, where indicated, an NADPH-generating system in a final volume of 5 ml (20-25 mg protein) were preincubated in 30 ml beakers. Terrazole in DMSO (final concentration, 1 mM) was added to the appropriate incubations. An equal volume of DMSO (10 µl) was added to those beakers containing no terrazole. After 15 min incubation period, the reactions were terminated by placing the beakers in water-ice bath and then the microsomes sedimented by centrifugation, resuspended in Hepes buffer and used for the determination of the components of microsomal enzyme system.

[b]Results are expressed as the means ± SEM of four observations.

*$P<0.05$; †$P<0.01$, when compared with corresponding control values

preincubated in the absence of both NADPH and terrazole. On the other hand, the microsomes preincubated with terrazole in the presence of an NADPH-generating system metabolized benzphetamine and aniline at a significantly slower rate than did the microsomes preincubated with terrazole in the absence of NADPH. Similarly, significantly more loss of cytochrome P-450 occurred in the microsomes preincubated with terrazole and NADPH than in those with terrazole alone. Microsomes preincubated with terrazole minus NADPH metabolized benzphetamine and aniline at a considerably slower rate than those not preincubated with terrazole. The probable explanation for the difference in the ability of these microsomes to metabolize benzphetamine and aniline could be the incomplete removal of terrazole bound to the microsomes preincubated with this toxicant. This residual terrazole could act as a competitive inhibitor of the metabolism of benzphetamine and aniline.

As seen from Table 4, formation of malonaldehyde is stimulated in the incubation mixtures containing NADPH alone. In agreement with the work previously reported by others (KAMATAKI and KITAGAWA 1974; DEMATTEIS and SEAWRIGHT 1973), the NADPH-stimulated lipid peroxidation of microsomal membranes could be almost completely prevented by addition of EDTA to the incubations. In contrast, the formation of malonaldehyde due to terrazole was not prevented by addition of EDTA to the incubation mixtures.

TABLE 4

Effect of terrazole and NADPH on the formation of malonaldehyde in the in vitro incubation of microsomes isolated from phenobarbital-pretreated mice.

Addition			Malonaldehyde formed (nmoles/15 min/mg protein)[a]
EDTA	Terrazole	NADPH	
none	-	-	1.55 ± 0.07
	-	+	3.94 ± 0.34†
	+	-	1.76 ± 0.01
	+	+	5.12 ± 0.09†
1 mM	-	-	1.28 ± 0.01
	-	+	1.80 ± 0.01
	+	-	1.76 ± 0.02
	+	+	3.08 ± 0.01†

[a]Results are expressed as the means ± SEM of two observations.
†$P<0.01$, when compared with corresponding control values.

DISCUSSION

The data presented in Table 1 clearly indicate that like other pesticides (NEAL et al. 1975) terrazole also inhibits the drug metabolizing enzyme system as evidenced by the marked prolongation of pentobarbital sleeping time. In the present study, the inhibition of the activity of hepatic DME system caused by terrazole at a dosage level of 100 mg/kg persisted for about 48 hr after the administration of the fungicide and thereafter the activity appears to come back to normal as seen from the increased metabolism of

pentobarbital at 96 hr after terrazole treatment. These observations suggest that terrazole-induced inhibition of hepatic DME system appears to be dependent on duration and on dose. The results on the activities of benzphetamine demethylase and aniline hydroxylase in the liver microsomes isolated from animals that had received 2 mmoles/kg terrazole (Table 2) demonstrate that these enzymes are markedly inhibited after the terrazole administration with concomitant loss of cytochrome P-450. Thus the decreased activity of benzphetamine demethylase and aniline hydroxylase observed in the present study reflects -at least in part- the lower level of cytochrome P-450 which is involved in the catalytic activity of several oxidative drug metabolizing enzymes. Although these results account for the decreased pentobarbital metabolism and increased sleeping time seen in the terrazole-treated animals the mechanism by which the loss of cytochrome P-450 occurred remains unknown.

Next, an experiment was planned (Table 3) to study whether terrazole by itself or its metabolite(s) are responsible for the decreased activity of microsomal enzymes and the loss of cytochrome P-450 content. The fact that there is no decrease in the concentration of cytochrome P-450 by terrazole in the incubation not containing NADPH as compared to that containing NADPH suggests that terrazole requires metabolism for its liver toxicity. Further, as can be seen from Table 4, terrazole in the presence of NADPH-generating system stimulates microsomal membrane lipid peroxidation which does not appear to be protected by the addition in the incubation of EDTA, an inhibitor of lipid peroxidation. Thus it seems likely that a terrazole metabolite(s) might be causing hepatotoxicity by initiating destructive lipid peroxidation of the microsomal membranes. A similar mechanism of toxicity has been proposed for carbon tetrachloride which is metabolized by DME to $\cdot Cl$ and $\cdot CCl_3$ free radicals responsible for microsomal membrane disruption (GHOSHAL and RECKNAGEL 1965).

It is interesting to note that both terrazole and captan, an extensively used fungicide, contain trichloromethyl moiety in their molecules. In fact fungitoxicity of captan has been attributed to this grouping (LUKENS 1966). Moreover, a well known insecticide DDT also contains a trichloromethyl moiety in its molecule and has been reported to be the site of biotransformation by microsomal enzymes (OBRIEN 1967). In light of these analogous reports it may be postulated that trichloromethyl moiety of terrazole undergoes metabolic alteration by hepatic DME system and the product(s) thus formed are perhaps responsible for the observed liver toxicity.

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