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In Vitro Studies of the Metabolism of Atrazine, Simazine, and Terbutryn in Several Vertebrate Species

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The metabolism of three triazine herbicides (atrazine, simazine, and terbutryn) was studied in rat (Sprague-Dawley and Fischer strains), mouse, goat, sheep, pig, rabbit, and chicken by using in vitro hepatic 10000*g* supernatant or microsomal systems. Principal phase I metabolites were 4- or 6-monodealkylated-*s*-triazines; several observations, including studies with purified enzymes, demonstrated that phase I reactions were cytochrome P-450 mediated. There were species-related variations in rates of metabolism and in ratios of primary metabolites, although no strain- or sex-related differences were noted. Phase II products were glutathione conjugates of the parent compound and of the two monodealkylated products. Experiments with Fenton's reagent, which generates hydroxyl radicals, gave dealkylated 2-chloro-*s*-triazine, supporting the possible role of active oxygen radicals in the cytochrome P-450 mediated reactions.

Atrazine, simazine, and terbutryn are chemically similar, symmetrical or *s*-triazine herbicides. The dominant phase I metabolic reaction (reaction adding a reactive polar group) for triazine herbicides in animals is N-dealkylation (Bohme and Bar, 1967; Crayford and Hutson, 1972; Dauterman and Muecke, 1974; Khan et al., 1979; Erickson et al., 1979; Bradway and Moseman, 1982), and the primary phase II reactions (biological conjugations yielding products that are more water soluble than the unconjugated material) are conjugations with glutathione and with glucuronides (Guddewar and Dauterman, 1979; Dauterman and Muecke, 1974; Crayford and Hutson, 1972; Larsen and Bakke, 1978). 2-(Methylthio)-*s*-triazines, such as terbutryn, have also been shown to form the 2-hydroxyl and the 4-(aminobutyl) alcohol or 4-(aminobutyl) carboxylic acid (Larsen and Bakke, 1978; and Larsen et al., 1978). Because of their persistence in the environment (Parmeggiani, 1983) and their wide-

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spread use on essential food crops (Worthing and Phil, 1987), the metabolic pathways of these compounds merit further study.

In this study, the in vitro metabolism of the three triazine herbicides is characterized, with special emphasis on species likely to be exposed and on a comparative study of in vitro metabolism in two strains of rats, one of which developed mammary tumors when fed atrazine (Ciba-Geigy, 1988).

EXPERIMENTAL PROCEDURES

Materials. ^{14}C-Radiolabeled reference standards [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (atrazine), 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine, 2-chloro-4-(ethylamino)-6-amino-*s*-triazine, 2-chloro-4,6-bisamino-*s*-triazine, 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine), 2-(methylthio)-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine (terbutryn), 2-(methylthio)-4-amino-6-(*tert*-butylamino)-*s*-triazine, and 2-hydroxy-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine] for the phase I metabolism studies were supplied by the Ciba-Geigy Corp., Greensboro, NC. Some phase II glutathione conjugate standards (2-glutathione conjugates of atrazine and of 4,6-bisamino-*s*-triazine) were from Ciba-Geigy, and others [the 2-glutathione conjugates of 4-amino-6-(isopropylamino)-*s*-triazine and of 4-(ethylamino)-6-amino-*s*-triazine] were synthesized from the corresponding ^{14}C-labeled 2-chloro metabolites according to an adaption of the method of Crayford and Hutson (1972): 500 mg of the 2-chloro-*s*-triazine was dissolved in a minimal volume of acetone, and then anhydrous trimethylamine gas was bubbled into this solution, at 20 °C and with stirring, until no more precipitate (the trimethylammonium chloride salt) was formed. The precipitate was collected by filtration, dried, and dissolved in 1000 μL of water containing 99 mg of glutathione and 19 mg of sodium carbonate. This reaction mixture was stirred for 2 h at 50 °C, and then the water was evaporated to yield the [4-(alkylamino)-6-amino-*s*-triazinyl]-2-glutathione.

Sprague-Dawley and Fischer rats (7–8 weeks of age) were obtained from Charles River Laboratories, Raleigh, NC. ICR mice (25–30 g) were from Dominion Laboratories. New Zealand White rabbits were from a local commercial source. Pig livers were obtained from Jesse Jones abattoir, Raleigh, NC. Sheep and goats were from the North Carolina State University (NCSU) College of Veterinary Medicine. Chickens (3-week-old Arbor Acres X Arbor Acres chicks) were obtained from the NCSU Poultry Science Department.

Methods. Hepatic supernatant fraction (S-10) and microsomes were prepared according to standard methods (Levi and Hodgson, 1983). The cytochrome P-450 (P-450) content of these preparations was determined by measuring the absorption of the dithionite-reduced CO-bound complex of P-450 (Omura and Sato, 1964). Protein content was measured by the method of Lowry et al. (1952). The spectral binding constant, K_s, for a substrate binding with P-450 was determined from the amplitude of the deflections in the characteristic optical difference spectrum at 390 and 420 nm when known concentrations of substrate were added to a dilute microsomal suspension.

For determination of phase I metabolites, incubations contained microsomes (1.0 mg/mL protein), ^{14}C-radiolabeled substrates, an NADPH generating system (0.25 mM NADP, 2.50 mM glucose 6-phosphate, and 1.0 unit of glucose-6-phosphate dehydrogenase in 0.5 mL of buffer), and 50 mM potassium phosphate buffer at pH 7.5 with a total volume of 1.0 mL. Heat-treated microsomes (100 °C for 45 s) were used to assess nonenzymatic oxidations. All reactions were conducted at 37 °C in a shaking water bath. At the end of the reaction period, parent compound and metabolites were extracted into chloroform, concentrated under dry nitrogen gas, and applied to thin-layer chromatographic (TLC) plates (Brinkman Polygram SIL G/UV 254). TLC plates were developed in hexane/isoamyl alcohol (8:2) for atrazine and simazine or in benzene/ethanol (9:1) for terbutryn and quantitated by using a Berthold linear analyzer. Alternate solvent systems used to confirm metabolite identification were benzene/ethanol (9:1) for atrazine and simazine and hexane/isoamyl alcohol (8:2) for terbutryn. It should be noted that some terbutryn metabolites (compounds 5–8 in Figure 3) comigrate in this alternate solvent system. These comigrating terbutryn metabolites were identified by comparison with standards using two-dimensional TLC with hexane/isoamyl alcohol (8:2) for both dimensions, but concentrations were below the level of reliable quantitation.

Phase II metabolism was studied by using a system similar to the phase I system, with the following changes: (1) the supernatant after 10000*g* centrifugation (S-10) was used rather than microsomes; (2) the reaction mixture was supplemented with glutathione; (3) S-10 preparations were used at a higher concentration (approximately 5.0 mg of protein/mL); and (4) the reaction was incubated for 2 h. The parent compound and nonpolar metabolites were extracted into chloroform, and the aqueous layer containing the water-soluble metabolites was freeze-dried. The freeze-dried residue was extracted with aliquots of methanol until no more radioactivity could be detected in the extract. The pooled methanol extracts were concentrated, applied to TLC plates, developed with *n*-butanol/acetic acid/water (11:5:4), and quantitated by using a Berthold linear analyzer. Controls for phase II studies included (1) heat-treated S-10, (2) omission of NADPH, and (3) omission of glutathione. All phase I and phase II incubations were performed in triplicate for all conditions tested.

For the phenobarbital induction experiments, rodents were given 1.0 mg/mL phenobarbital ad libitum in their drinking water for 3 days. Animals were sacrificed, and S-10 and microsomes were prepared on the fourth day.

For the 3-methylcholanthrene (3-MC) studies, rodents were injected ip each day with 20 mg/kg of body weight 3-MC in corn oil. A corn oil control of the highest volume of corn oil used in an injection series was also run for each strain studied. Animals were treated for 3 consecutive days. Animals were sacrificed on the fourth day, and microsomes or S-10 was prepared from the liver.

RESULTS AND DISCUSSION

Initial Metabolism Studies in Rodents. Initial studies to determine optimal incubation conditions for phase I in vitro metabolism were conducted with male Sprague-Dawley (SD) rat and male ICR mouse hepatic microsomes and atrazine. Suitable experimental conditions for measurable metabolite production were 50–100 μM substrate, 1.0 mg/mL microsomal protein, and 30 min of incubation time. Positive controls for incubations were SD rat microsomes at 1 mg/mL protein. These conditions and controls were used in the subsequent experiments, except where noted, so that valid comparisons could be made among species and substrates.

There was no product found when NADPH was omitted, suggesting that P-450, which requires NADPH as a cofactor, might be the metabolizing enzyme. Addition of NADH to the complete in vitro system did not increase the rate of metabolism, suggesting that cytochrome b_5, which requires NADH, does not contribute to the microsomal metabolism of atrazine.

Scintillation counts on aliquots from the extracted aqueous layer and the pooled chloroform extracts from the in vitro metabolism experiments showed an extraction efficiency of 95–98% for phase I metabolites into chloroform. Because of this high extraction efficiency of labeled substrate and metabolites, it can be inferred that very little, if any, of the three triazines tested binds covalently to cellular proteins under these experimental conditions. Essentially all of the radioactivity could be accounted for as the sum of aqueous and organic layers.

The principal metabolites of atrazine in the SD rat and the mouse were the two dealkylation products produced by the loss of either the ethyl group [2-chloro-4-amino-6-(isopropylamino)-*s*-triazine] or the isopropyl group [2-chloro-4-amino-6-(ethylamino)-*s*-triazine]. The metabolite with neither ethyl nor isopropyl group (2-chloro-4,6-bisamino-*s*-triazine) was detected at low levels in a few

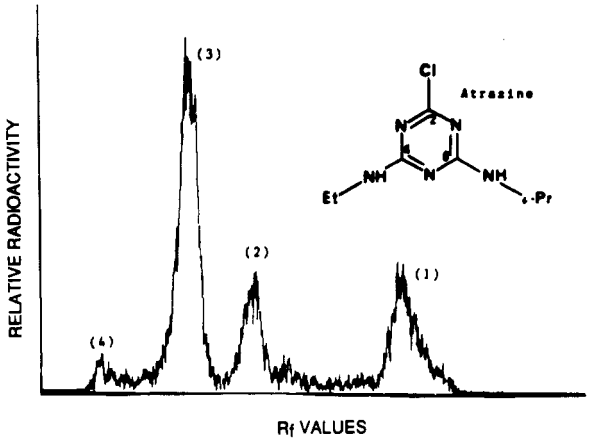

Figure 1. Typical TLC scan of metabolites produced by incubation of atrazine with hepatic microsomes. The numbered peaks are (1) 2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine (atrazine), R_f = 0.74, (2) 2-chloro-4-amino-6-(isopropylamino)-*s*-triazine, R_f = 0.32, (3) 2-chloro-4-(ethylamino)-6-amino-*s*-triazine, R_f = 0.17, and (4) 2-chloro-4,6-bisamino-*s*-triazine, R_f = 0.02.

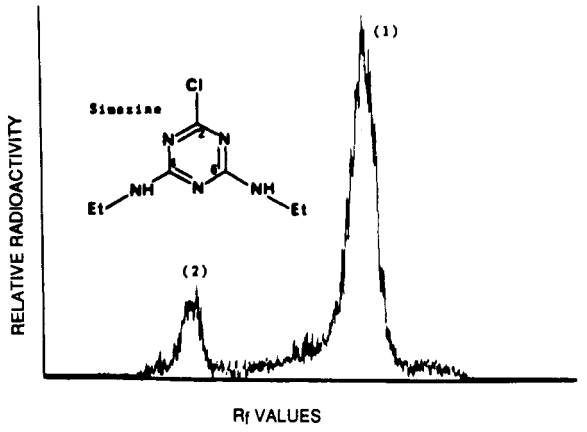

Figure 2. Typical TLC scan of metabolites produced by incubation of simazine with hepatic microsomes. The numbered peaks are (1) 2-chloro-4,6-bis(ethylamino)-*s*-triazine (simazine), R_f = 0.54, and (2) 2-chloro-4-(ethylamino)-6-amino-*s*-triazine, R_f = 0.32.

cases, but the level was too low to quantitate accurately. No dechlorinated or ring cleavage products were noted. The deisopropylated product was formed in greater quantity than the deethylated product in the rat, as had been found by earlier investigators (Dauterman and Muecke, 1974; Bohme and Bar, 1967). Structures, R_f values, and a typical scan of these metabolites after TLC separation with hexane/isoamyl alcohol are shown in Figure 1. Time course experiments showed that production of the two atrazine metabolites was linear through 20 min and essentially complete after 30 min. The ratio of deisopropyl/deethyl metabolites did not vary significantly with time over a 2-h period.

The phase I metabolism of simazine was studied by using the standard incubation system developed with atrazine. As with atrazine, the predominant metabolite was the single dealkylation (deethyl) product. Very small amounts, below the limit of reliable quantitation, of the didealkylated product were found. No products resulting from dechlorination or ring cleavage were found. Figure 2 gives R_f values and a representative scan for simazine and its metabolites.

Phase I metabolism of terbutryn was also studied by using the standard experimental conditions established for atrazine. Terbutryn produced six phase I metabolites, including the two single dealkylation and one double-dealkylation products and their corresponding 2-hydroxy derivatives. R_f values and a typical scan are shown in Figure 3. The prevalent metabolite was the de-*tert*-butyl product, followed by the deethylated and the hydroxyl compounds. In contrast with these in vitro results, in vivo studies in rats and goats produced the deethylated and hydroxyl metabolites but did not yield the de-*tert*-butyl compound. In vivo metabolism also produced the *S*-hydroxyl, *tert*-butyl alcoholic, and *tert*-butyl carboxylic acid metabolites (Larsen and Bakke, 1978; Larsen et al., 1978). Evidently, the 2-methylthio group in terbutryn is more labile than the 2-chloro groups in atrazine and simazine under these experimental conditions, since no 2-hydroxy products were detected from the metabolism experiments with the 2-chloro-*s*-triazines.

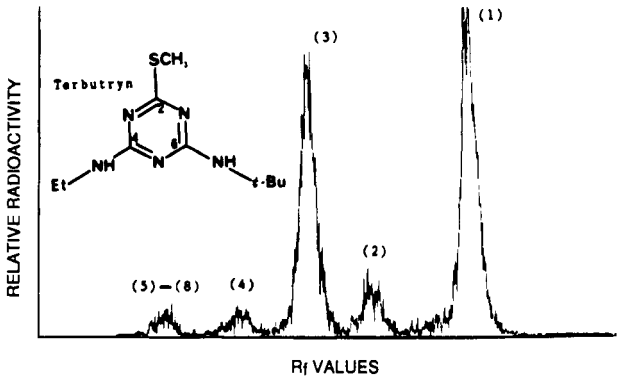

Figure 3. Typical TLC scan of metabolites produced by incubation of terbutryn with hepatic microsomes. The numbered peaks are (1) 2-(methylthio)-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine (terbutryn), R_f = 0.73, (2) 2-(methylthio)-4-amino-6-(*tert*-butylamino)-*s*-triazine, R_f = 0.34, (3) 2-(methylthio)-4-(ethylamino)-6-amino-*s*-triazine, R_f = 0.29, (4) 2-(methylthio)-4,6-bisamino-*s*-triazine, R_f = 0.14, (5) 2-hydroxy-4-(ethylamino)-6-(*tert*-butylamino)-*s*-triazine, R_f = 0.05, (6) 2-hydroxy-4-amino-6-(*tert*-butylamino)-*s*-triazine, R_f = 0.05, (7) 2-hydroxy-4-(ethylamino)-6-amino-*s*-triazine, R_f = 0.05, and (8) 2-hydroxy-4,6-bisamino-*s*-triazine, R_f = 0.05.

Studies of Metabolites of Atrazine as Substrates for in Vitro Microsomal Metabolism. The initial metabolism studies had shown a consistent difference between mouse and rat with regard to the ratios of the main metabolites of atrazine (deisopropyl/deethyl). The mouse hepatic microsomal preparations gave ratios of approximately 1, while Sprague-Dawley rat microsomes gave ratios of 3–4. A series of experiments were performed to determine if there were differences between mouse and rat when either the deisopropyl metabolite or the deethyl metabolite was used as the starting material (substrate) in the in vitro system. When the deethyl product was the substrate, only 3–4% was metabolized by either species, with the product being 2-chloro-4,6-bisamino-*s*-triazine. When the deisopropyl metabolite was used as the substrate, there was a complete conversion to 2-chloro-4,6-bisamino-*s*-triazine in both species. These same values, 3–4% metabolism of the deethyl compound and 100% metabolism of the deisopropyl compound, held for severalfold variations in the concentration of NADPH and of microsomes in the reaction mixture. One possible explanation for this observation is steric inhibition of enzyme–substrate binding by the deethyl compound. This is a subject for further investigation.

Species-Related Differences in Phase I Metabolism. A series of incubations were carried out by using atrazine, simazine, or terbutryn as the substrate with hepatic microsomal preparations from various species of animals. The standard experimental conditions (1.0 mg

Table I. Species Differences in in Vitro Phase I Atrazine Metabolism

species	N[a]	nmol 30 min^{-1} (mg of protein)$^{-1}$		nmol 30 min^{-1} (nmol of P450)$^{-1}$	
		-iPr[b]	-Et[c]	-iPr[b]	-Et[c]
mouse, ICR male	3	10	10	*d*	*d*
mouse, ICR female	3	6	8	*d*	*d*
pig	3	3	1	11	3
rabbit, male	2	5	3	4	2
rat, SD[e] male	3	10	4	24	8
rat, SD male	2	20	5	49	12
rat, SD female	2	24	6	58	14
rat, F[f] male	2	23	6	28	7
rat, F female	2	25	6	30	7
rat, SD male	2	21	5	35	8
rat, SD female	2	21	5	28	7
rat, F male	2	22	6	42	11
rat, F female	2	20	5	34	8
sheep, female	1	8	5	20	12
chicken, female	1	5	3	53	33
chicken, female	1	8	5	49	29
chicken, male	1	7	4	53	29
chicken, male	1	12	6	59	30
goat, female	1	6	2	5	2
goat, female	1	12	6	15	7
goat, female	1	12	8	22	16

[a] Number of livers from different animals combined in one microsomal preparation. [b] The deisopropyl product [2-chloro-4-amino-6-(ethylamino)-*s*-triazine]. [c] The deethyl product [2-chloro-4-(isopropylamino)-6-amino-*s*-triazine. [d] Not tested. [e] SD, Sprague-Dawley. [f] F, Fischer.

Table II. Species Differences in in Vitro Phase I Simazine Metabolism

species	N[a]	deethylated product	
		nmol 30 min^{-1} (mg of protein)$^{-1}$	nmol 30 min^{-1} (nmol of P450)$^{-1}$
rat, SD[b] male	3	8	10
mouse, ICR male	3	11	*c*
pig	3	1	3
rabbit, male	2	5	5
sheep, female	1	4	9
chicken, female	1	8	81
chicken, female	1	8	48
chicken, male	1	8	62
chicken, male	1	8	39
goat, female	1	3	3
goat, female	1	4	6
goat, female	1	4	10

[a] *N*, number of livers from different animals combined in one microsomal preparation. [b] SD, Sprague-Dawley. [c] Not tested.

of microsomal protein, 50–100 μM substrate, 30-min incubation time), established for atrazine metabolism with rodent microsomes, were used in all incubations. Tables I–IV show the results for atrazine, simazine, and terbutryn, calculated as nmol of product 30 min^{-1} (mg of protein)$^{-1}$ and as nmol of product 30 min^{-1} (nmol of P-450)$^{-1}$. From the data, it is apparent that there are considerable differences in the rates of metabolism (quantities of metabolites produced in 30 min, both within and among species), but, when the difficulty of detecting low levels of metabolites (e.g., <3%) is considered, there are striking qualitative similarities in the types of metabolites produced. This suggests a common pathway for the metabolism of the triazine herbicides in these species.

It is interesting to note that normalizing data to total P-450 content does not establish a concurrence among species or within species as to the amount of product produced in 30 min. Walker (1980) also noted a similar lack of agreement between P-450 level and microsomal enzyme activity. This lack of correlation when data are normalized to the total P-450 level could be explained as

Table III. Species Differences in in Vitro Phase I Terbutryn Metabolism (Normalized to Microsomal Protein Content)

species	N[a]	nmol 30 min^{-1} (mg of protein)$^{-1}$				
		-SCH_3[b]	-Et, -tBu[c]	-tBu[d]	-Et[e]	X[f]
mouse, ICR male	3	2	1	18	5	ND[g]
pig	3	ND	ND	2	1	ND
rabbit	2	ND	0.5	5	5	ND
rat, SD[h] male	3	2	3	16	4	ND
rat, SD male	2	0.2	1	12	2	ND
rat, SD female	2	ND	2	12	4	ND
rat, F[i] male	2	ND	3	17	4	ND
rat, F female	2	ND	ND	14	2	ND
chicken, female	1	0.3	2	10	2	1
chicken, male	1	1	2	12	2	2
chicken, male	1	1	4	12	2	2
goat, female	1	ND	2	10	4	ND

[a] *N*, number of livers from different animals combined in one microsomal preparation. [b] The dethiomethyl products (all 2-hydroxy compounds). [c] The double-dealkylated product (2-(methylthio)-4,6-bisamino-*s*-triazine). [d] The de-*tert*-butyl product (2-(methylthio)-4-amino-6-(ethylamino)-*s*-triazine). [e] The deethyl product [2-(methylthio)-4-(*tert*-butylamino)-6-amino-*s*-triazine]. [f] An unidentified metabolite. [g] ND, not detected. [h] SD, Sprague-Dawley. [i] F, Fischer.

Table IV. Species Differences in in Vitro Phase I Terbutryn Metabolism (Normalized to Microsomal P-450 Content)

species	N[a]	nmol 30 min^{-1} (nmol of P-450)$^{-1}$				
		-SCH_3[b]	-Et, -tBu[c]	-tBu[d]	-Et[e]	X[f]
pig	3	ND[g]	ND	6	4	ND
rabbit, male	2	ND	0.5	4	4	32
rat, SD[h] male	3	4	6	38	9	ND
rat, SD male	2	0.2	2	8	2	ND
rat, SD female	2	ND	3	20	6	ND
rat, F[i] Ma	2	ND	9	53	12	ND
rat, F female	2	ND	ND	18	3	ND
chicken, female	1	2	11	60	13	7
chicken, male	1	6	19	94	15	15
chicken, male	1	5	17	54	10	7
goat, female	1	ND	2	13	6	ND

[a] *N*, number of livers from different animals combined in one microsomal preparation. [b] The dethiomethyl products (all 2-hydroxy compounds). [c] The double-dealkylated product [2-(methylthio)-4,6-bisamino-*s*-triazine]. [d] The de-*tert*-butyl product [2-(methylthio)-4-amino-6-(ethylamino)-*s*-triazine]. [e] The deethyl product [2-(methylthio)-4-(*tert*-butylamino)-6-amino-*s*-triazine]. [f] An unidentified metabolite. [g] ND, not detected. [h] SD, Sprague-Dawley. [i] F, Fischer.

P-450 isozyme specificity in triazine metabolism, with different species showing different P-450 isozyme composition. However, subsequent induction studies suggest that the rate of metabolism corresponds to total P-450 rather than to specific (induced) isozyme levels.

There was one exception to the general qualitative agreement among species, in the metabolism of terbutryn. Chickens consistently produced an unidentified metabolite that elutes between the parent compound and the deethyl metabolite in the solvent system used. Since the general pattern in this elution system produces polar compounds near the origin and more nonpolar compounds nearer the solvent front, the unidentified metabolite was a relatively nonpolar material. R_f values are shown in Figure 3. This material might have been the carboxylic acid or the alcohol found at the 4- or 6-amino position as reported in earlier studies with goats and rats (Larsen and Bakke, 1978; Larsen et al., 1978).

All species tested generally had comparable rates of terbutryn metabolism when data were calculated on the

Table V. Rates of Phase I Atrazine Metabolism in Sprague-Dawley (SD) and Fischer (F) Rats

	nmol of product 120 min^{-1}			
	deisopropyl		deethyl	
basis of calculations	SD	F	SD	F
per milligram of S-10 protein	6.4	8.9	3.2	4.0
per nanomole of microsomal P-450	64.0	56.0	32.0	25.3
per milligram of wet liver	0.52	2.7	0.25	1.2
per gram of body	0.027	0.13	0.013	0.059

basis of microsomal protein. However, when the data were calculated on the basis of P-450, chickens had much higher rates. If P-450 was responsible for catalyzing this reaction, the forms in the chicken preparations were more active toward terbutryn than the forms in preparations from other species. This might explain the appearance of an additional metabolite, perhaps from a faster reaction rate in the chicken metabolic pathway, permitting a detectable quantity of a minor product to accumulate.

When both sexes of the same species were studied in mouse, rat, and chicken, there were no significant sex-related differences ($p > 0.05$).

Strain-Related Differences in the Phase I and Phase II Metabolism of Atrazine in the Rat. Since earlier in vivo studies with atrazine had shown the development of mammary tumors in the high-dose group of female Sprague-Dawley (SD) rats but not in female Fischer (F) rats or in other rodent species (Ciba-Geigy, 1988), the basic phase I and phase II metabolism of atrazine was studied in both strains of female rats. The metabolism studies were performed by using 7–8 week-old Fischer rats weighing 101–125 g with average liver weights of 5.6 g and 8–9-week-old Sprague-Dawley rats weighing 176–200 g with average liver weights of 9.7 g. This age–weight range was chosen because it is frequently used to prepared the S-9 metabolic activation system for in vitro studies. Fischer rats had an average of 23 mg of microsomal protein and 5.5 nmol of P-450 per gram of liver (4.2 nmol of P-450/mg of protein), and the Sprague-Dawley rats had an average of 8 mg of microsomal protein and 1.7 nmol of P-450 per gram of liver (4.7 nmol of P-450/mg of protein). Both strains of rats were obtained from the same supplier in the same shipment, and both microsomal preparations were prepared on the same day with the same buffers and reagents.

Both strains gave the same metabolites. Time course experiments using 5 mg of S-10 protein/mL over 30 min of incubation showed that both strains gave equivalent amounts of metabolites at all time points sampled (5, 10, 15, 20, and 30 min). If rates of phase I metabolism were compared on the basis of microsomal P-450 content, Fischer rats and Sprague-Dawley rats give roughly equivalent rates. However, if comparisons were made on the basis of liver weight or body weight, rates of metabolism were higher in the Fischer strain. Values are shown in Table V.

Rates of conjugation of atrazine and its metabolites with glutathione (phase II metabolism) were the same in both strains of rats ($p > 0.05$), normalized to S-10 protein content. In the in vitro system, the phase I reactions proceeded much more rapidly than the phase II reactions; approximately 96% of the ring-labeled metabolites were recovered in the chloroform-extracted (nonpolar) phase I portion, and only 4% in the polar phase II aqueous portion, after 2 h of incubation of the reaction mixture at 37 °C. Increasing the levels of glutathione and/or NADPH in the reaction mixture did not increase the phase II reaction rate. The predominant phase II conjugation product was with atrazine rather than with the dealkylation products.

Table VI. Effects on Atrazine Metabolism and P-450 Levels of PB and 3MC Induction

		nmol 30 min^{-1} (mg of protein)$^{-1}$		
inducer	strain (P-450, nmol/mg of protein)	-iPr, -Et[a]	-iPr[b]	-Et[c]
3MC	SD (0.27)	4.0 ± 0.9	19.3 ± 3.2	8.6 ± 1.4
	F (0.45)	2.5 ± 2.3	19.3 ± 2.7	9.2 ± 2.2
PB	SD (0.97)	3.4 ± 0.9	29.0 ± 1.1	9.6 ± 0.5
	F (0.93)	2.6 ± 1.5	28.8 ± 0.6	10.6 ± 0.5
corn oil	SD (0.21)	1.7 ± 0.3	12.5 ± 0.9	3.9 ± 0.4
	F (0.24)	2.1 ± 0.7	18.0 ± 0.9	7.8 ± 1.4

[a] The double-dealkylated product. [b] The deisopropylated product. [c] The deethyl product.

Kinetics and Binding Constants for Atrazine Metabolism. The apparent K_m of atrazine using hepatic microsomal preparations from female Sprague-Dawley rat was 27.5 μM, and with female Fischer rats, 27.8 μM. Determinations were made after 15 min of incubation at 37 °C using 20, 40, 60, and 80 μM atrazine. V_{max} values were 0.72 and 1.16 nmol min^{-1} (mg of protein)$^{-1}$ for Sprague-Dawley and Fischer rats, respectively. When expressed in terms of P-450 content, the V_{max} values were equivalent: 3.03 (Sprague-Dawley) and 3.28 (Fischer) nmol min^{-1} (nmol of P450)$^{-1}$. For both strains of rats, no binding spectra (no apparent optical difference spectrum) could be detected for atrazine concentrations from 0.67 nM to 33 μM. Benzphetamine, a positive control, gave the typical type 1 optical difference spectrum with microsomal preparations from both strains of rats. These results suggest either that atrazine does not bind tightly to P-450 or that atrazine binds with P-450 in some atypical fashion that does not produce a characteristic type 1 binding spectrum.

Induction Studies with Phenobarbital and 3-Methylcholanthrene. Phenobarbital (PB) and 3-methylcholanthrene (3-MC) are known to induce specific isozymes of P-450 that have differing substrate affinities (Guengerich, 1987). Female Sprague-Dawley and Fischer rats were treated in vivo with PB or with 3-MC according to the procedures described under Methods. Controls for PB induction were untreated animals from the same shipment/supplier given drinking water without PB. Vehicle (corn oil) treated controls were employed for 3-MC treatment. Hepatic microsomal preparations from induced and from control animals were incubated with atrazine under standard experimental conditions. The results of these experiments are presented in Table VI. PB induction increased P-450 levels, while 3-MC in corn oil produced P-450 levels roughly equivalent to the uninduced corn oil control. The corn oil control was equivalent to the untreated control. The total rates of metabolism roughly corresponded to the total P-450 level, with PB induction producing the most metabolism. The ratios of metabolites were unchanged from those of the uninduced state with all induction regimens. Since PB and 3-MC cause very pronounced in vivo synthesis of substrate-specific metabolizing enzymes in the liver, it was surprising to find that (1) patterns of metabolism were unchanged and (2) rates of metabolism corresponded more closely to the total P-450 level rather than to the level of some specific isozyme. These experimental results suggest that atrazine is a substrate with similar binding affinities for several P-450 isozymes producing comparable rates and product ratios or that it reacts with an active oxygen species produced

by many, or all, P-450 isozymes.

Mechanisms of Phase I Atrazine Metabolism. Several observations indicate that P-450-dependent activity is responsible for the phase I reactions of atrazine and the other triazine herbicides in microsomal incubations. First, there is an absolute requirement for NADPH. Second, atrazine produces a dose-dependent inhibition of the P-450-mediated O-demethylation of *p*-nitroanisole (data not shown). Third, PB, a known P-450 inducer, increases triazine metabolism rates. P-450 is also known to mediate a large number of N-dealkylation reactions similar to those observed for the triazine herbicides in other substrates such as the drug, aminopyrene, and the carbamate insecticides (Hodgson and Dauterman, 1980). Further studies (data not shown) with purified P-450IIB1, the primary phenobarbital-inducible form of P-450, in a reconstituted system with cytochrome P-450 reductase and the necessary cofactors (dilaurolylphosphatidylcholine and NADPH) produced the same metabolites in the same ratios as were found with the analogous PB-induced microsomal system.

Although isozymes of P-450 have broad and overlapping specificities with regard to xenobiotic metabolism, they generally show substrate preferences, at least insofar as the preferred and most rapidly oxidized substrate is concerned. The PB and 3-MC induction studies in rats sugggest that the rate of atrazine metabolism corresponds to total P-450 level rather than to the concentration of a specific isozyme.

P-450 reactions are thought to involve an active oxygen species, presumably generated and rapidly consumed within the enzyme–substrate binding region (Ingleman-Sundberg et al., 1982). When atrazine was stirred at room temperature with Fenton's reagent, a hydroxyl-radical-generating mixture of ferrous iron and hydrogen peroxide in diluted acid (Fenton, 1894; Walling, 1975), N-dealkylation was the primary reaction, just as had been observed in the microsomal systems. The 2-hydroxy product was also generated, as has been noted in resistant plant species (Shimabukuro, 1968; Kneusli et al., 1969). Atrazine plus Fenton's reagent yielded 16% of the double-dealkylated product plus 10% of the 2-hydroxy product(s) and oxidized iron (as a yellow-brown precipitate). These data support the involvement of an active oxygen species (and of P-450) in the metabolism of atrazine. It is noteworthy that the enzymatic N-dealkylation reaction is site specific (e.g., giving a specific monodealkylated product), while Fenton's reagent oxidizes the available molecular sites without preference.

Studies in which either mannitol (a hydroxyl free-radical scavenger) or superoxide dismutase (a superoxide scavenger) was added to an in vitro system of atrazine and rat hepatic microsomes produced no inhibition of atrazine metabolism. There was minimal inhibition of atrazine metabolism when ascorbic acid, a general free-radical scavenger, was added to the in vitro system. These data indicate that, at least in the in vitro system, a radical-mediated oxidation of atrazine by P-450 cannot be demonstrated.

SUMMARY

All species tested produced the deisopropylated and the deethyl metabolites of atrazine, the deethylated metabolite of simazine, and the dealkylated and hydroxyl products from terbutryn, suggesting a common reaction pathway. There was considerable variation among species in the rates of metabolism and in the site specificity of the reaction; that is, the ratios of primary metabolites varied considerably among species for atrazine and terbutryn. In Sprague-Dawley and Fischer rats, microsomal phase I metabolism of atrazine produced 3–4 times more of the deisopropylated product than of the deethyl product. Further metabolism of these primary metabolites led to the didealkylated product, but the deisopropyl compound gave rise to about 20-fold more of the didealkylated product than of the deethyl compound.

Microsomal preparations from Sprague-Dawley and Fischer rats provided comparable rates and patterns of phase I and phase II metabolism of atrazine, when data were normalized to the amount of product per nanomole of P-450 or per milligram of protein. However, on a per gram of liver or a per gram of body weight basis, Fischer rats metabolized atrazine much more rapidly. The apparent K_m was 27.5 μM for Sprague-Dawley and 27.8 μM for Fischer rat hepatic microsomes. No binding spectra with the oxidized microsomes were detected in either strain. No sex-related differences in metabolism were noted in any of the species tested.

Phase I metabolism of atrazine was shown to be P-450 mediated. Phase II metabolism proceeded via glutathione conjugation of atrazine and, to a lesser extent, via the glutathione conjugation of the monodealkylated products.

Induction studies with PB and 3-MC in Sprague-Dawley and Fischer rats suggest that metabolism can be catalyzed by several different isozymes of P-450 and that the reactions are qualitatively and quantitatively similar. In addition, studies with Fenton's reagent indicate that a free-radical mechanism may be involved in the phase I metabolism of these compounds.

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Fate of 3-Phenoxybenzaldehyde: Diphenyl Ether Cleavage, a Major Metabolic Route in Chicken†

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The fate of 3-phenoxybenzaldehyde was investigated in chicken after oral administration of the unlabeled compound for 3 days, followed by a single dose of ald-^{14}C-labeled material. Approximately 98% of the administered radiocarbon was eliminated within 24 h. Radiocarbon metabolites were excreted free as well as conjugated. Free metabolites identified in excreta were 3-hydroxybenzoic acid (8%), 3-phenoxybenzoic acid (5%), and 2′- and/or 4′-hydroxy-3-phenoxybenzoic acid (traces, <1%). Only a small amount (8%) of 3-hydroxybenzoic acid was eliminated as glucuronic acid and sulfate conjugates. Hydrolysis of the polar metabolites with 2 N HCl produced 37–42% 3-hydroxybenzoic acid. Similarly, 3-hydroxybenzoic acid (both free and conjugates) was also detected in the excreta of hens fed deltamethrin or fenvalerate. The diphenyl ether cleavage of 3-phenoxybenzaldehyde is a major route, probably via 3-phenoxybenzoic acid, in chickens.

3-Phenoxybenzaldehyde (3-PBald) is a transitory metabolite of several pyrethroids including the cyano-substituted derivatives (Miyamoto et al., 1981). In the latter case, formation of 3-PBald is a two-step process that involves (i) generation of the cyanohydrin by the ester cleavage and (ii) rapid elimination of a molecule of HCN from the cyanohydrin to produce 3-PBald. The aldehyde oxidizes rapidly into 3-phenoxybenzoic acid (3-PBacid). Comparative metabolism studies of 3-PBacid, including that generated during metabolism of pyrethroids, have reported considerable species variation. For example, the acid undergoes glutamic acid conjugation in the cow (Gaughan et al., 1978a), glycine conjugation in the goat (Ivie and Hunt, 1980), glycylvaline dipeptide conjugation in the mallard duck (Huckle et al., 1981b, and sulfate conjugation of 3-(4-hydroxyphenoxy)benzoic acid in the rat (Huckle et al., 1981a). In the chicken, Huckle et al. (1982) identified α-N-acetyl-δ-*N*-(3-phenoxybenzoyl)-L-ornithine as the principal excretory metabolite. These workers observed rapid and quantitative elimination of radiocarbon in the excreta of chickens given a single dose of 3-phenoxy[*ring*-U-^{14}C]benzoic acid. Although 63.5% of the excreted radioactivity was extractable in methanol, only about half of the labeled material was identifiable (Huckle et al., 1982). Similarly, very little radiocarbon from [*alcohol*-^{14}C]permethrin

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