Metabolism of Atrazine by the Soluble Fraction (105000g) from Chicken Liver Homogenates

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The soluble fraction (105000g) from chicken liver homogenates contains a heat-labile, glutathione-dependent enzyme(s) which metabolizes atrazine in in vitro incubations. This is accomplished by conjugation with glutathione and subsequent hydrolysis and partial N-dealkylation to the hydroxy and dealkylated analogues. Evidence is also presented for some dechlorination of the chloro-s-triazine to hydroxy-s-triazine. However, enzymatic hydrolysis of atrazine is the predominant reaction in the soluble fraction from chicken liver homogenates.

The metabolism of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine], an s-triazine herbicide, used extensively in the culture of corn, has been studied in the chicken (Foster et al., 1972; Foster and Khan, 1976; Khan and Foster, 1976). Residues of unchanged atrazine and some hydroxy and dealkylated metabolites were found to be present in excreta and various body tissues after the chickens had been fed an atrazine fortified ration (Foster and Khan, 1976; Khan and Foster, 1976). The largest concentration of hydroxyatrazine and deethylhydroxyatrazine was found in the liver. Partial N-dealkylation and hydrolysis were proposed as major pathways for atrazine metabolism in the chicken. Dauterman and Muecke (1974) reported that in vitro metabolism of atrazine by rat liver involved dealkylation in the microsomal fraction and conjugation with glutathione (GSH) in the soluble fraction. However, there was no evidence for dechlorination of atrazine to hydroxyatrazine.

The soluble fraction from chicken liver homogenates has been shown to contain GSH-dependent enzyme systems, which are implicated in the metabolic breakdown of the insecticides lindane (Foster and Saha, 1978) and tetrachlorvinphos (Akhtar and Foster, 1977). The present study is a continuation of the investigation of the metabolism of atrazine by chickens and reports on the in vitro degradation of atrazine by the soluble fraction (105000g) from chicken liver homogenates.

EXPERIMENTAL SECTION

Chemicals. All solvents were of pesticide grade (Caledon Laboratories Ltd., Georgetown, Ontario, Canada) and used as received. Reference standards of atrazine and metabolites were gifts from Ciba Geigy Limited, Switzerland.

The radiopurity of ring-labeled [^{14}C]atrazine (sp act., 0.44 mCi/mg) was checked on precoated silica gel G (Fisher Scientific Co.) TLC plates which had been previously washed with methanol. The plates were developed in a benzene/acetic acid/water (60:40:3), v/v/v) system and air-dried. The spots were located by spraying with 0.1 M silver nitrate, followed by 0.1 M potassium dichromate. The R_f for atrazine under these conditions was 0.765. Further purity of the [^{14}C]atrazine was established by comparison of spectroscopic (NMR and mass spectrum) and gas chromatographic retention time data with those for authentic standard. A stock solution was prepared by dissolving 6.8 mg of [^{14}C]atrazine in 1.0 mL of acetone.

Gas Chromatography (GC). The gas chromatograph was a Pye, Series 104, Model 64, fitted with an alkali flame ionization detector having an RbCl annulus. The column was a 1.5 m × 0.4 cm i.d. glass tube packed with 3% Carbowax 20 M coated on 80–100 mesh Chromosorb WHP. The operating conditions were: on-column injections; injector port temperature turned off; column and detector temperatures were 220 and 270 °C, respectively. The nitrogen carrier gas, hydrogen, and air flow rates were 60, 35, and 300 mL/min, respectively.

Gas Chromatography–Mass Spectrometry (GC–MS). A Finnigan Model 3100 mass spectrometer connected to a Finnigan Model 9500 gas chromatograph by means of a jet separator was used. The mass spectrometer was interfaced with a Model 6100 computer-controlled data acquisition system. A 1.5 m × 0.4 cm i.d. glass column packed with 3% Carbowax 20 M coated on 80–100 mesh Chromosorb WHP was used for gas chromatographic separation. The ionization energy, in general, was 70 eV. A synthetic mixture of the suspected metabolites was prepared from the reference compounds and the mass spectra obtained. The samples were analyzed under identical conditions and the mass spectra of the metabolites were compared with those of the reference compounds.

Measurement of Radioactivity. The radioactivity of aliquots of extracts was determined in a Packard Tri-Carb liquid scintillation spectrometer, Model 3320, using an external standard and correcting the data for quenching. The activity was measured in a scintillation solution containing 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene (5 g, 50 mg, 1000 mL).

The radioactivity, which remained in air-dried filter paper residues from incubation mixture extracts, was determined by combustion in a Packard Sample Oxidizer, Model 306 (Packard Instrument Canada Ltd.). The $^{14}CO_2$, which was produced, was absorbed in and admixed with appropriate volumes of Carbo-Sorb and Permafluor V (Packard Instrument Canada Ltd.) and the radioactivity was measured as above.

Enzyme Preparation. Single Comb White Leghorn hens, approximately 1.5 years old at 70% production, were killed by cervical dislocation. The livers were removed, washed in distilled water, and placed on crushed ice. A homogenate was prepared in ice-cold 0.134 M phosphate buffer, pH 7.4 (8 g/40 mL), in a glass–Teflon homogenizer. The homogenate was centrifuged at 2500 rpm for 5 min at 4 °C. The precipitate was discarded and the supernatant centrifuged at 10 000 rpm for 10 min at 4 °C. The

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precipitate (mitochondria) was discarded and the supernatant centrifuged at 40 000 rpm (105000g) for 30 min at 4 °C. The precipitate (microsomes) was discarded and the clear supernatant or soluble fraction was the enzyme preparation. A fresh preparation was made on the day each series of incubations was carried out.

In Vitro Incubations. Incubations were carried out in glass-stoppered Erlenmeyer flasks (50 mL). A typical incubation mixture contained: enzyme preparation (4.5 mL), reduced glutathione (GSH) (0.5 mL), [^{14}C]atrazine (680 μg, 17.35 × 10^4 dpm), and atrazine (134 μg). Control flasks contained no enzyme or GSH and the volume was kept constant by adding an appropriate amount of 0.134 M phosphate buffer, pH 7.4. An equivalent volume of acetone was added to flasks which contained no atrazine. GSH was prepared, just prior to its addition to the incubation flasks, by mixing 0.0027 g of glutathione with 0.05 N NaOH (0.05 mL) and neutralizing the mixture with 0.134 M phosphate buffer, pH 7.4 (0.45 mL). The flasks were gassed with dry N_2, stoppered, and incubated for specified time intervals at 37.5 °C. The reaction was terminated by freezing at −20 °C in a dry ice/alcohol mixture and taken to dryness by freeze-drying.

Extraction of Atrazine and Metabolites. The dried material was extracted by shaking with methanol (4 × 25 mL), and the extracts were filtered through Whatman No. 1 filter paper into round-bottom flasks (300 mL). The incubation flask was finally washed with acetone (2 × 10 mL) and the washes were added to the filter paper. The sample residue on the filter paper was washed once more with acetone (1 × 10 mL). The filter paper and residues were air-dried and retained for further analyses. The combined filtrate was taken to dryness on a rotary evaporator at room temperature.

The dried filtrate was redissolved in several portions of anhydrous chloroform (5–10 mL) and placed on an acidic alumina column (20 g, acidic aluminum oxide, Woelm, activity I, 24 mm × 70 mm, and prewashed with chloroform) which had been topped with 10 mm of anhydrous Na_2SO_4. The column was first eluted with 200 mL of dried chloroform (eluate I) and then with 200 mL of methanol (eluate II).

Eluate I was taken to dryness on a rotary evaporator at room temperature. The residue was redissolved in methanol (2 mL). Radioactivity was quantitated by liquid scintillation counting and atrazine and metabolites were determined by GC.

Eluate II was concentrated to about 2 mL on a rotary evaporator at room temperature and an excess of freshly prepared diazomethane solution (prepared from Diazald, Aldrich Company, Inc., Milwaukee, Wis.) was added until the yellow color persisted (Khan et al., 1975). The flask was stoppered and the contents allowed to stand at room temperature for about 3 h with occasional shaking. The mixture was taken to dryness in a stream of dry air. The residue was dissolved in methanol (5 mL), the radioactivity quantitated by scintillation counting, and an aliquot analyzed by GC.

The recovery of atrazine was shown to be nearly quantitative by following the described procedure. However, recoveries of the hydroxy metabolites were low (30–55%) partly because of the low efficiency of the methylation technique which resulted in only a 50–70% yield of methoxy analogues.

RESULTS AND DISCUSSION

The radioactivity data from preliminary incubations (Table I) indicate that addition of phosphate buffer (0.134 M, pH 7.4) resulted in about 10% degradation of atrazine.

Table I. In Vitro Metabolism of Atrazine by the Soluble Fraction (105000g) from Chicken Liver Homogenate with [^{14}C]Atrazine as the Substrate

flask[a]	incubation time, h	radioactivity, %[b] eluate I	eluate II	filter paper residues	recov. of total radioact., %
1	0	90.2	8.0	0.5	98.7
2	5	80.6	13.7	0.2	94.5
3	5	57.6	19.0	1.8	78.4

[a] Incubation flasks 1 and 2 contained 0.134 M phosphate buffer, pH 7.4 (5.0 mL); atrazine (100 μL, 134 μg); and [^{14}C]atrazine (100 μL, 680 μg, 17.35 × 10^4 dpm). Flask 3 contained reduced glutathione (0.5 mL); soluble fraction (4.5 mL); atrazine (100 μL, 134 μg); and [^{14}C]atrazine (100 μL, 680 μg, 17.35 × 10^4 dpm). The flasks were gassed with dry nitrogen and incubated at 37.5 °C for the indicated times. [b] Percentage of total radioactivity is the average of duplicate incubations carried out 1 week apart.

Most of the degraded material was present in the form of hydroxy analogues as evidenced by the extraction of about 8% of the radioactivity in eluate II. Incubation of atrazine in phosphate buffer for 5 h resulted in further degradation (20% of the herbicide). More than half of the degraded material was converted to hydroxy metabolites. When atrazine was incubated with enzyme preparaton for 5 h in the presence of phosphate buffer and GSH, the herbicide was degraded to an even greater extent (44%). Slightly less than half the radioactivity was extracted in eluate II, which indicates the conversion of atrazine to the hydroxy metabolites. In addition, it was noted that the recovery of total radioactivity from enzyme incubation was lower than from buffer incubation (Table I). In an identical incubation, which was carried out in flasks with center wells containing rolls made from strips of filter paper (Whatman No. 1, 1 × 8 cm) to which Carbosorb was added at the incubation period, it was shown that an insignificant amount (<0.1%) of atrazine was totally metabolized to $^{14}CO_2$ during 25 h. It was considered probable that some of the herbicide or its breakdown products was bound to protein from the soluble fraction or was conjugated with GSH and was not extracted by the technique employed in this study. However, attempts to combust aliquots of the alumina packings to $^{14}CO_2$ and thus obtain data about unextracted radioactivity remaining on the columns were not satisfactory. No further attempt was made to account for the missing activity. Finally, it should also be noted that the radioactivity associated with the air-dried filter paper residues was considerably greater from the enzyme incubated samples than that from control samples.

Gas chromatographic and GC–MS analyses of eluate I from the incubation flasks (Table II) showed the presence of only unchanged atrazine. Eluate II from the buffered incubation samples (flasks 1 and 2) contained only hydroxyatrazine. However, eluate II from the enzymatic incubation samples (flask 3) contained greater concentrations of hydroxyatrazine in addition to small amounts of two dealkylated hydroxy metabolites (Table II). This indicates that metabolism of atrazine by an enzyme(s) in the soluble fraction of chicken liver homogenates proceeded via hydrolysis accompanied by partial N-dealkylation. These results are consistent with previous findings which indicate that partial N-dealkylation and hydrolysis were major pathways of atrazine metabolism in the chicken. It should also be noted that enzymatic dealkylation by the soluble fraction of chicken liver

Table II. Quantitation of Unchanged Atrazine and Metabolites after Incubation of the Herbicide with the Soluble Fraction from Chicken Liver Homogenates

		concentration, μg[b]			
			eluate II		
flask[a]	incubation time, h	eluate I atrazine	hydroxyatrazine	deethylhydroxyatrazine	deisopropylhydroxyatrazine
1	0	611	50.4		
2	5	546	86.8		
3	5	390	120.4	1.8	0.6

[a] Incubation flasks 1 and 2 contained 0.134 M phosphate buffer, pH 7.4, (5.0 mL); atrazine (100 μL, 134 μg); and [^{14}C]atrazine (100 μL, 680 μg, 17.35 × 10^4 dpm). Flask 3 contained reduced glutathione (0.5 mL); soluble fraction (4.5 mL); atrazine (100 μL, 134 μg); and [^{14}C]atrazine (100 μL, 680 μg, 17.35 × 10^4 dpm). The flasks were gassed with dry nitrogen and incubated at 37.5 °C for the indicated times. [b] Concentrations are the average of duplicate incubations carried out 1 week apart.

homogenate occurred on either of the alkylamino groups resulting in the formation of 2-hydroxy-4-amino- or 2-hydroxy-7-amino-*s*-triazine structures.

In order to ascertain whether enzymatic degradation is glutathione dependent, atrazine was incubated in phosphate buffer for 5 h with a fresh enzyme preparation (Table III). The data indicate that the fresh enzyme preparation contained sufficient GSH for 21% of the atrazine to be metabolized. However, addition of GSH to the incubation media increased metabolism to about 42% in this period. When the enzyme preparation was dialyzed against 0.134 M phosphate buffer, pH 7.4, for 14 h, considerably less metabolism of atrazine occurred. However, addition of GSH (17.57 μmol) to the dialyzed preparation restored the activity to the original level. The data in Table III also show that incubation of atrazine with boiled enzyme (heated to boiling for 2 min) did not result in degradation of the herbicide. Furthermore, addition of GSH to the incubation media containing boiled enzyme had no effect on the metabolism of atrazine. These observations clearly demonstrate that the involvement of GSH in the metabolism of atrazine is purely enzymatic in nature.

These studies indicate that the soluble fraction (105000*g*) from chicken liver homogenates contains a heat-labile, glutathione-dependent enzyme(s) which in in vitro incubation, metabolizes atrazine. Since only a small amount of the dealkylated hydroxy metabolites was detected, dealkylation is not considered to be the major enzymatic reaction in this study. This is in agreement with the findings of Dauterman and Muecke (1974) for rat liver preparations. The data also indicate that the enzyme(s) in the soluble fraction, which are involved in the metabolism of atrazine, are glutathione dependent. The

Table III. Effect of Reduced Glutathione on the Enzymatic Metabolism of Atrazine by the Soluble Fraction of Chicken Liver Homogenates

incubation conditions[a]			eluate I, unchanged atrazine, %
buffer mL	soluble fraction, mL	GSH mL	
5.5			100[b]
5.0		0.5	102
1.0	4.5		79.1
0.5	4.5	0.5	58.3
1.0	4.5[c]		97.3
0.5	4.5[c]	0.5	72.2
	4.5[c]	1.0	60.6
1.0	4.5[d]		100
0.5	4.5[d]	0.5	100

[a] Reaction conditions were the same as recorded in text, 804 μg of atrazine, time 5 h. [b] The amount of recovered atrazine at 0 h incubation in buffer, which in general was 10% lower than the amount added, was taken as 100%. The value of atrazine in eluate I of different incubations is a comparative value with respect to 0 h incubation. [c] Dialyzed against 0.134 M phosphate buffer, pH 7.4, for 14 h at 4 °C. [d] Heated to boil for 2 min.

mechanism probably involves conjugation with glutathione (Akhtar, 1978a,b) and subsequent hydrolysis to form the hydroxy metabolites (Hutson et al., 1976). Thus the data provide evidence for the presence of a dechlorinase in the soluble fraction, which, in the presence of glutathione, results in the dechlorination of the chloro-*s*-triazine to hydroxy-*s*-triazine. Dauterman and Muecke (1974) did not report dechlorinase activity in rat liver preparations.

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LITERATURE CITED

Akhtar, M. H., *J. Agric. Food Chem.* **26**, 932 (1978a).
Akhtar, M. H., *J. Environ. Sci. Health*, in press (1978b).
Akhtar, M. H., Foster, T. S., *J. Agric. Food Chem.* **25**, 1017 (1977).
Dauterman, W. C., Muecke, W., *Pestic. Biochem. Physiol.* **4**, 212 (1974).
Foster, T. S., Khan, S. U., *J. Agric. Food Chem.* **24**, 566 (1976).
Foster, T. S., Morley, H. V., Purkayastha, R., Greenhalgh, R., Hunt, J. R., *J. Econ. Entomol.* **65**, 982 (1972).
Foster, T. S., Saha, J. G., *J. Environ. Sci. Health*, **B13**, 25 (1978).
Hutson, D. H., Holmes, D. S., Crawford, M. J., *Chemosphere* **2**, 79 (1976).
Khan, S. U., Foster, T. S., *J. Agric. Food Chem.* **24**, 768 (1976).
Khan, S. U., Greenhalgh, R., Cochrane, W. P., *J. Agric. Food Chem.* **23**, 430 (1975).

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