Mercapturic Acid Formation in the Metabolism of 2-Chloro-4-Ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-Triazine in the Rat

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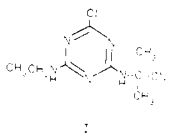
2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine, a herbicidal triazine, is rapidly metabolized after oral ingestion by the rat. The radioactivity of a single oral dose of the [^{14}C]labeled herbicide was excreted largely in the urine (40.6%) and feces (47.2%). Eight metabolites were detected in the urine. Two major metabolites were isolated from the urine of 12 rats and identified as N-acetyl-S-[4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazinyl-2]-L-cysteine and 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine. These metabolites accounted for 40 and 15%, respectively, of the total urinary metabolites. Thus N-desethylation and mercapturic acid formation are major routes in the metabolism of the herbicide. *s*-Triazinyl mercapturic acids have not hitherto been reported as metabolites of 2-chlorotriazines.

The metabolism of *s*-triazine herbicides, particularly 2-chlorotriazines, in mammals has not been extensively studied. Investigations of the metabolism of simazine, atrazine, and propazine in the rat and rabbit (Böhme and Bär, 1967) and of propazine in the goat and sheep (Robbins *et al.*, 1968), suggest the triazine ring is not cleaved, N-dealkylation occurs readily, and the carbon chlorine bond is stable (but is hydrolyzed in the plant). N-substituting groups, *e.g.*, isopropyl, are partially oxidized to carboxylic acid derivatives. Sixteen metabolites of ring [^{14}C]labeled propazine, most unidentified, have been found in goat urine. The metabolism of an oral dose of the herbicidal *s*-triazine, 2-chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine (I) (Bladex) in the rat is described in this paper.



I

MATERIALS

2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine. The compound was obtained from the Woodstock Agricultural Research Centre, with a specific activity of 14 μCi per mg. 2-Chloro-4,6-bis(ethylamino)-*s*-triazine was an impurity in the preparation to the extent of 3.3% w/w. Thin-layer chromatography on silica gel F_{254} (Merck) using 30% v/v acetone in hexane as eluting solvent also revealed the presence of a number of more polar impurities amounting to less than 1% of the total activity. The required compound was separated from these impurities by preparative thin-layer chromatography. The [^{14}C]herbicide was detected by autoradiography and by ultraviolet absorption. The section of the plate containing the compound was cut out and the compound eluted with chloroform. Thin-layer chromatography followed by autoradiography showed the radiochemical purity of the final material to be better than 99%. Ethyl-labeled herbicide was obtained from the Woodstock Agricultural Research Centre.

Reference Triazine Derivatives. 2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine and 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine were obtained from Degussa-Wolfgang, Forschung Chemie Organische, Hanau-Main, West Germany.

Paper Chromatography. Analytical and preparative paper chromatography were carried out on Whatman No. 1 and 3 grade papers, respectively, using n-butanol-2N ammonia (1 to 1, v/v, upper phase) as solvent in all cases.

Thin-Layer Chromatography. Merck prepared plates of Kieselgel F_{254} (Merck, Darmstadt), analytical and preparative types, were used for thin-layer chromatography except where stated to the contrary. Compounds were located by viewing in ultraviolet light or by spraying with a *t*-butyl hypochlorite-starch-potassium iodide reagent (Whitenberg, 1967) which detected compounds containing an N-H group.

Paper Electrophoresis. Paper electrophoresis was carried out on a Shandon High Voltage apparatus at 100 v/cm, in three buffer systems: 0.05M sodium potassium phosphate, pH 10; pyridine (100 ml), acetic acid (10 ml), water (2.5 liters), pH 6.1; formic acid (78 ml), acetic acid (148 ml), water (2.5 liters), pH 2.0. Glucose was used as marker to correct for endosmotic flow.

Column Chromatography. Merck silica gel (0.2 to 0.5 mm particle size) was mixed with AnalaR acetone and poured to give a column 3 cm × 50 cm.

Assay of Radioactivity. Radioactivity on chromatograms was located with a Desaga radioscanner. Radioactivity was measured by liquid scintillation counting in either a dioxan-based scintillator mixture (Bray, 1960) or a phenylethylamine-methanol-toluene mixture (Dobbs, 1963) using a Packard Tricarb liquid scintillation spectrometer, Model 3003.

Nuclear Magnetic Resonance (nmr) Spectroscopy. Nmr spectra were measured on a Varian Associates HA 100 Spectrometer. Compounds were dissolved in the usual range of deuterated solvents, but the spectrum of the isolated major metabolite was measured only in D_2O because of the necessity to recover the samples unchanged and uncontaminated.

Mass Spectrometry. Mass spectra were measured using a modified MS 9 mass spectrometer (Associated Electrical Industries).

Animal Experiments. Young adult rats (Carworth Farm E strain, maintained as a specific pathogen-free colony in this laboratory), weighing between 200 and 250 g, were used.

Three male and three female rats were each dosed by stomach tube with 2-chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine (0.8 mg; 14 μCi per

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Table I. Excretion and Retention of Radioactivity in Rats after Oral Administration of 2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine

Animal No.	Sex	Recovery of ^{14}C (% of administered dose) over a 4 day collection period					
		Urine	Feces	Carcass	Skin	Gut	Total
1	M	38.6	36.5	1.6	0.7	14.3	91.7
2	M	43.0	47.1	2.1	0.8	0.3	93.3
3	M	42.1	45.2	2.3	1.0	0.3	90.9
	Mean	41.2	42.9	2.0	0.8	5.0	91.9
	SEM	1.35	3.24	0.21	0.09	4.68	0.70
4	F	35.2	55.9	2.0	0.4	0.3	93.8
5	F	42.0	48.8	2.0	0.5	0.9	94.2
6	F	43.0	49.8	2.2	0.5	0.3	95.8
	Mean	40.1	51.5	2.1	0.5	0.5	94.5
	SEM	2.44	2.21	0.09	0.01	0.22	0.59

mg) in 1 ml of arachis oil. The rats were housed in glass metabolism cages (Jencons Ltd.) as described by Wright *et al.* (1965) and had free access to food and water. Urine and feces were collected daily. One male rat and one female rat were housed in closed cages ventilated with air at 300 to 400 ml per min; the exhaled CO_2 was collected by drawing it through two traps in series, each containing 400 ml of 4N NaOH. The traps were replaced daily. After 4 days all animals were killed by ether anesthesia and were skinned (the feet, muzzle, and tail remaining with the skin). The alimentary tract from the anus to just above the stomach was then removed. Urine and the contents of the carbon dioxide traps were processed for radioactive counting daily, and the remaining samples were stored at −20° C until required. For the isolation of metabolites, 12 female rats (Carworth Farm E strain) were housed in glass metabolism cages and each rat dosed by stomach tube four times (once every 48 hr) with 1 ml of arachis oil containing 12 5 mg (2.71 μCi) of [^{14}C]labeled herbicide.

One young male rat was dosed orally with 2-chloro-4-[^{14}C]-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine (1.22 mg, specific activity 8.55 μCi per mg). Excreted radioactivity was measured as described above.

Measurement of Radioactivity in Samples. Measurements of radioactivity in urine, feces, gut, skin plus hair, and remaining carcass were made by the methods described by Wright *et al.* (1965).

RESULTS

Excretion and Retention of Radioactivity after Oral Administration of 2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine. The amount of radioactivity, expressed as a percentage of the administered dose, recovered from the urine, feces, and tissues of the rats after 4 days is shown in Table I. The major portion of the dose was excreted in 4 days via the urine (40.6%) and feces (47.2%). Only 3.0% of the dose remained in the animal after 4 days. The total recovery of radioactivity was 93.2%. In a separate experiment using one rat of each sex, no radioactive carbon dioxide was detected in the expired gases. A more detailed breakdown of the excretion of radioactivity in urine and feces is shown in Table II. The excretion of radioactivity from the [1-^{14}C]ethyl-labeled analog was as follows: urine, 17.1; feces, 26.3; carbon dioxide, 47.9; remaining in carcass, 5.3%.

Examination of Urinary Metabolites. A preliminary investigation of freeze-dried, methanol-extracted urine by thin-layer chromatography revealed eight metabolites, one of which constituted 60% of the radioactivity in the urine. The urine from 12 female rats dosed orally with a total of 525 mg (114 μCi) of the [^{14}C]labeled herbicide was found to contain 30.1 μCi of radioactivity or 140 mg equivalents of metabolites. The urine was freeze-dried to give 50.1 g of solid which was extracted three times with 300 ml of methanol at room temperature. The extract was evaporated to a syrup (23.6 g) which contained 120 mg equivalents of metabolites. This extract was analyzed by paper chromatography. A radioscan of the chromatogram (Figure 1) shows that the major metabolite (C) constituted about 40% of the urinary radioactivity. It is not surprising that the proportion of the major metabolite varied from that found in the balance study since the herbicide was administered at 62 times the level used in the latter.

The methanol extract (7 g) dissolved in 10 ml of methanol was applied to a silica gel column and eluted with acetone and then with increasing concentrations of methanol in acetone. The process was carried out three times using 21 g of the extract. A fraction of the radioactivity (13%) was eluted between 10–30% methanol, and the major fraction (60%) was eluted between 70–100% methanol. Total recovery from the column was 90%. Paper chromatographic analysis of the early fraction revealed a radiochemically pure component, R_f 0.85 (*i.e.*, E, Figure 1). This component was stored at −20° C until further purification. The major component was com-

Figure 1. Paper chromatographic analysis of the urinary metabolites of 2-chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine

Table II. Daily Levels of Radioactivity in Urine and Feces after Oral Administration of 2-Chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-[2,4,6-^{14}C]-*s*-triazine to rats

Animal No.	Sex	Elimination of ^{14}C (% of administered dose) 0–24 hr	24–48 hr	48–72 hr	72–96 hr	Total
			Urine			
1	M	31.3	4.6	1.8	0.9	38.6
2	M	38.7	3.0	0.9	0.3	42.9
3	M	31.7	9.8	0.4	0.2	42.1
	Mean	33.9	5.8	1.0	0.5	41.2
	SEM	2.39	2.03	0.39	0.20	1.34
4	F	28.6	5.5	0.8	0.3	35.2
5	F	36.8	3.3	1.6	0.4	42.1
6	F	34.2	7.9	0.6	0.2	42.9
	Mean	33.2	5.6	1.0	0.3	40.1
	SEM	2.40	1.35	0.30	0.06	2.44
			Feces			
1	M	18.6	8.2	7.7	2.2	36.7
2	M	20.2	12.9	10.2	3.8	47.1
3	M	16.9	22.2	4.7	1.3	45.1
	Mean	18.6	14.4	7.5	2.4	42.9
	SEM	0.9	4.13	1.56	0.73	3.24
4	F	32.3	20.3	3.0	0.3	55.9
5	F	4.7	12.8	21.2	10.1	48.8
6	F	16.9	30.2	2.4	0.3	49.8
	Mean	18.0	21.1	8.9	3.6	51.6
	SEM	7.97	5.03	6.15	3.38	2.20

posed mainly of the major metabolite C, contaminated with more polar radioactive material. The diagnostic test for metabolite C consisted of thin-layer chromatography using a solvent composed of ethyl acetate : formic acid : water (70 : 4 : 4, v/v), in which C migrated at R_f 0.5. Unless otherwise stated, this solvent was used throughout.

The remainder of the methanol extract (2.5 g) was subjected to preparative paper chromatography and the major metabolite eluted with water. This sample was used for preliminary chromatographic and electrophoretic analysis.

Paper Electrophoresis. The metabolite was analyzed by paper electrophoresis, following purification by paper chromatography. The results established that the molecule was amphoteric, presumably containing the expected basic groups of the original herbicide and an acidic function added during metabolism.

Further Purification of the Major Urinary Metabolite (C). (a) Preparative Thin-Layer Chromatography. The major radioactive fraction from the silicic acid columns was applied to 24 Merck preparative plates, which were then developed with ethyl acetate : formic acid : water (70 : 4 : 4, v/v). The major radioactive band (R_f *ca.* 0.4) was eluted with 40% aqueous methanol.

(b) Paper Chromatography. The metabolite from stage a was further purified by preparative chromatography on six sheets of Whatman No. 3 paper. The radioactive band of the chromatogram, R_f 0.3, was eluted with water, and the solution was evaporated to afford 63 mg of material containing 22 mg equivalents of metabolite C.

(c) Final Stages. Half of the material from stage b was further purified by the thin-layer chromatographic procedure described above, but using two 20 × 20 cm plates spread with Merck silica gel HR, pre-washed with acetone, to give 24.3 mg of syrup containing 10 mg equivalents of metabolite. Preparative chromatography on one sheet of Whatman No. 3 paper, prewashed successively with water and distilled butanol-ammonia solvent, afforded 18.7 mg of material containing 10 mg equivalents of metabolite. Thin-layer and paper chromatographic analyses showed this compound to be radiochemically pure. Electrophoresis at pH 10.0 revealed a trace of nonradioactive impurity in the sample. This sample was used without further purification for structural investigations by physical methods.

Properties of Metabolite C. Nmr Spectroscopy. The nmr spectrum of metabolite C in D_2O is shown in Figure 2. Two major signals appear at δ 1.88 and δ 2.05 p.p.m. Com-

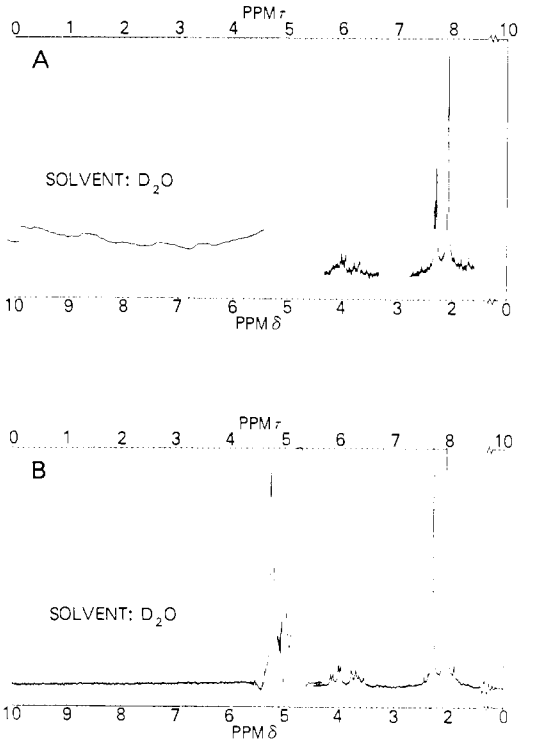


Figure 2. Nmr spectra of (A) metabolite C and (B) N-acetyl-S-[4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazinyl-2]-L-cysteine (II)

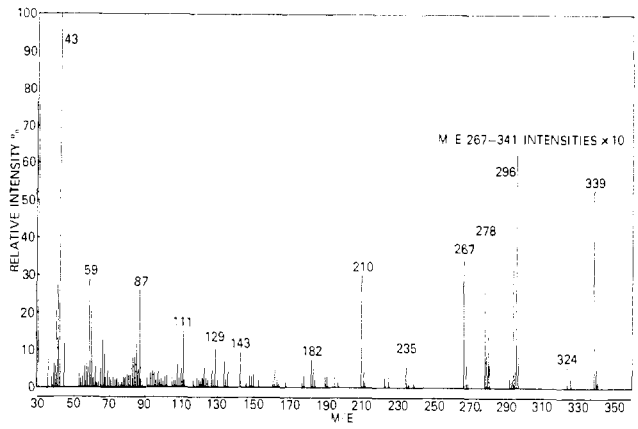
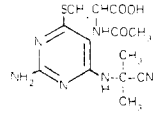


Figure 3. Mass spectrum of metabolite C

parison with some related triazines showed that (CH_3CCH_3) was present in the molecule (δ 1.88). The second major signal (δ 2.05) was suggestive of an acetyl group (CH_3CO). The N-ethyl group was proved conclusively to be absent from the molecule.

Mass Spectrometry. The mass spectrum of metabolite C is illustrated in Figure 3. The parent mass peak gave a molecular weight of 339 for the metabolite. This was considerably higher than the molecular weights of the original herbicide and related compounds, suggesting that a group had been added to the molecule during metabolism. If the 2-chlorine atom of the triazine was replaced by N-acetyl cysteine (mercapturic acid formation) a metabolite with structure II and molecular weight 339 would be formed. Further tests were carried out to test this possibility.

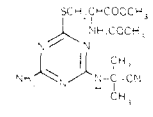


II

The Presence of Sulfur in Metabolite C. A 20-μg portion of metabolite C was purified further by paper electrophoresis at pH 10 by removing a small amount of nonradioactive impurity. The spot, located by ultraviolet absorption, was cut from the paper and found, by the sodium formate fusion test (Feigl, 1960), to contain sulfur.

The Methylation of Metabolite C. Part of the sample of metabolite C remaining from stage b of the further purification sequence above, containing 4.5 mg equivalents, was dissolved in ethanol and methylated by the addition of a solution of diazomethane in ether. Thin-layer chromatography after 1 hr indicated that metabolite C (R_f 0.5) was converted quantitatively into a less polar compound R_f 0.85, which was purified by thin-layer chromatography in chloroform:acetone (70:30, v/v). The product was eluted with acetone and analyzed by nmr and mass spectrometry.

Properties of the Methyl Ester of Metabolite C. Nmr Spectroscopy. The nmr spectrum of the methylated metabolite in $CDCl_3$ is shown in Figure 4. The spectrum shows that a methyl group had been introduced into the molecule, probably at a carboxylic acid group ($COOCH_3$, δ 3.75). The spectrum, as a whole, was in agreement with the expected structure III:



III

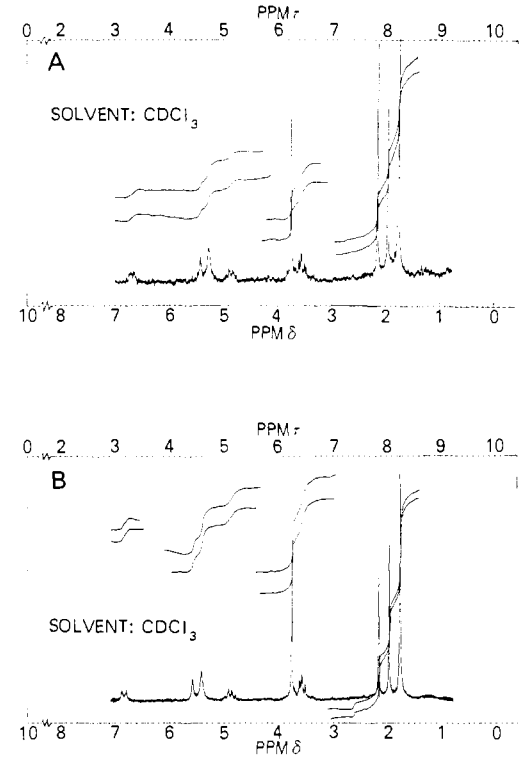


Figure 4. Nmr spectra of (A) methylated metabolite C and (B) the methyl ester of compound II (*i.e.*, III)

The following assignments for the chemical shifts (Figure 4) could be made, based on experience with related triazines and with N,S-diacetyl cysteine:

Signal	*Relative proton count*	*Proposed assignment*
1.77	6.0 (used as reference)	$(CH_3)_2C$
1.97	3.0	$NHCOCH_3$
2.16	4.05	acetone
3.50, 3.56, 3.61	2.0	SCH_2
3.75	3.1	$O.COCH_3$
4.84, 4.91	1.0	$NH.CH$
5.28	2.0	NH_2-triazine
5.43	1.0	NH-triazine
6.6, 6.8	0.9	NH(cysteine)

The spectrum is thus consistent with structure III. The anomalous aspect of the spectrum is the signal at δ 2.16, apparently accounting for four protons. This, however, was found to be due to acetone which may form an adduct with III, since it could not be completely removed by evaporation.

Mass Spectrometry. The molecular weight of the parent ion of the methyl ester was 353 (see Figure 5). This supports the suggestion that diazomethane introduced one CH_3 group into II to form III. The cracking patterns shown for metabolite C (Figure 3) and for its methyl ester (Figure 5) are consistent with the proposed structures. An accurate mass determination was carried out on the 353 peak derived from the methyl ester. The result, 353.126045, is at variance from that expected for III (353.126999) by only 2.7 p.p.m. The agreement is well within the expected experimental error and proves that the elemental formula for the methyl ester is that of III ($C_{15}H_{19}N_7O_3S$).

The Synthesis of N-Acetyl-S-[4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazinyl-2]-L-cysteine (II). N,S-Di-

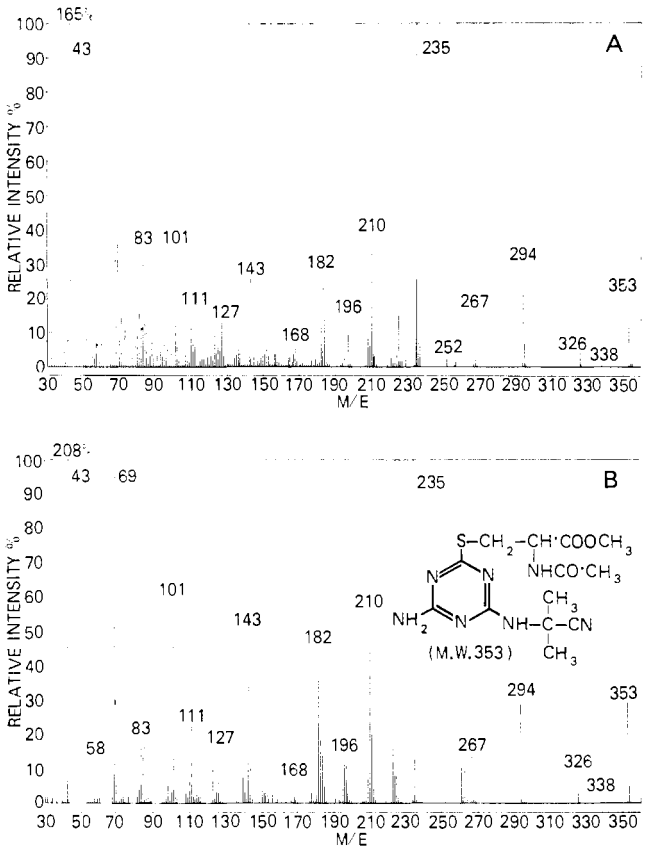


Figure 5. Mass spectra of (A) methylated metabolite C and (B) compound III

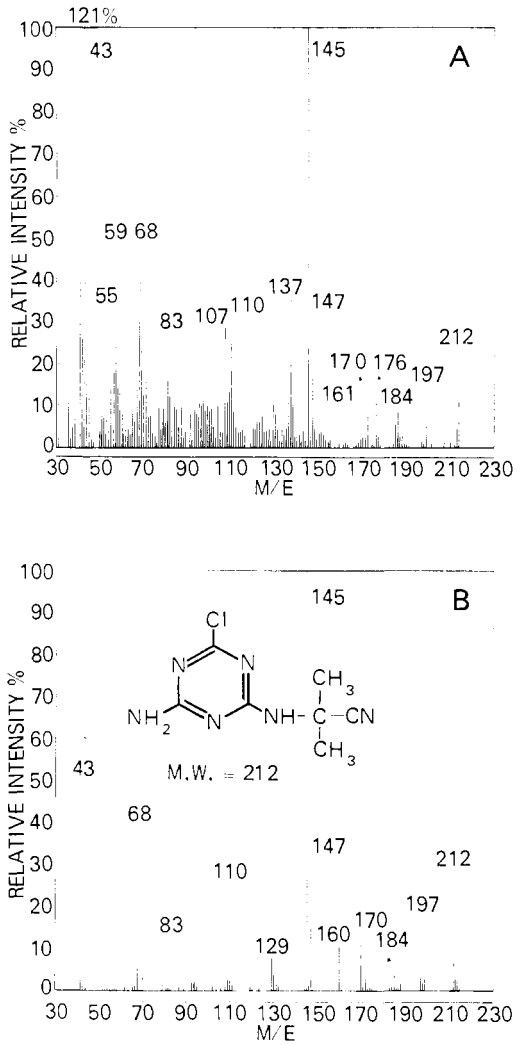
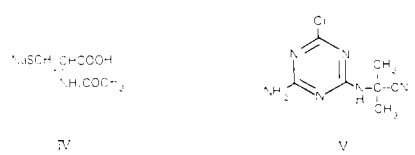


Figure 6. Mass spectra of (A) metabolite E and (B) 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine

acetyl-L-cysteine (Smith and Gorin, 1961) (2 g) was dissolved in 10 ml of anhydrous methanol and 10 ml of M sodium methoxide in methanol was added. After 2 hr, the solution was evaporated to dryness and the resulting S-sodio derivatives of N-acetyl-L-cysteine (IV) was used without further purification. 2-Chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine (V) (340 mg) and the sodio-derivative of N-acetyl cysteine (340 mg) were refluxed for 3/4 hr in 4 ml of dimethylformamide. The mixture was cooled and streaked on to eight sheets of Whatman No. 3 paper and developed in the butanol-ammonia solvent. The product (R_f 0.25) was separated from the starting materials (R_f 0.85 and 0.05) and another major product (R_f 0.5), eluted with water and rechromatographed on four prewashed sheets of paper to afford 135 mg of a syrup. Thin-layer chromatography in ethyl acetate:formic acid:water (70:4:4, v/v) (R_f 0.5), and nmr spectroscopy in D_2O (Figure 2) suggested that the synthetic material possessed the structure II. This was confirmed by methylation with diazomethane to a methyl ester indistinguishable from methylated metabolite C on thin-layer chromatography and possessing identical nmr and mass spectra (Figures 4 and 5, respectively).



IV V

Metabolite E. The first radioactive component eluted from the preparative silica gel columns was radiochemically pure when analyzed by paper chromatography (R_f 0.85) and was identical with metabolite E (Figure 1). Thin layer chromatography in ethyl acetate:formic acid:water (70:4:4, v/v); acetone:methanol (80:20, v/v); chloroform:acetone (60:40, v/v); acetonitrile:ether:acetic acid (60:30:10, v/v); benzene:acetone:acetic acid (80:15:5, v/v), showed that metabolite E was probably 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine. The metabolite was purified by (i) preparative paper chromatography (R_f 0.85) and (ii) chromatography on one prewashed Merck prepared plate in chloroform:acetone (90:10, v/v) (R_f 0.34). The isolated metabolite (*ca.* 1 mg) was analyzed by mass spectrometry and its spectrum found to be identical with the spectrum of authentic 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine (Figure 6), thus confirming the structure of E as 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine.

Discussion. The results show that 2-chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine is rapidly metabolized in the rat. Excretion of significant quantities of metabolite in the feces, even on the fourth day, suggests that a proportion of the material may be excreted via the bile and may undergo enterohepatic circulation. The excretion rate of radioactivity in the feces was slower than that in the urine. Only 3% of the administered radioactivity remained in the animals after 4 days. The total recovery of radioactivity

was 93.2%. The absence of $^{14}CO_2$ from the ring-labeled herbicide in the expired gases suggests that the triazine ring remains intact during metabolism. On the other hand, the large amount of radioactive carbon dioxide expired during the metabolism of the [1-^{14}C]ethyl-labeled herbicide suggested that N-desethylation was a major route of degradation of the compound.

The major radioactive metabolite in the urine of the treated rats was found to be N-acetyl-S-[4-amino-6-(1-methyl-1-cyanoethylamino)-s-triazinyl-2]-L-cysteine (II). The evidence presented in the Results section for this structure is primarily based on mass spectrometry and on nmr spectroscopy of the isolated metabolite. Methylation of the metabolite helped to confirm the structure because the resultant methyl ester (III) was more suitable than the metabolite for both nmr and mass spectrometry. The synthesis of II and III from the appropriate triazine and cysteine derivatives serves to confirm the assignment of structure. Experiments attempting to crystallize synthetic II itself or as its barium salt or picrate were unsuccessful.

A second metabolite was identified as 2-chloro-4-amino-6-(1-methyl-1-cyanoethylamino)-s-triazine (V).

It would be inappropriate at this stage to speculate on the possible pathways of metabolism of 2-chloro-4-ethylamino-6-(1-methyl-1-cyanoethylamino)-*s*-triazine in the rat. However, it is clear that N-desethylation and mercapturic acid formation are major routes in the metabolism of the herbicide. N-Dealkylation by liver microsomal enzymes is a well established reaction of foreign compounds in the mammal (Gillette, 1963). It is reasonably well established that mercapturic acid formation from organic halides is a multistage sequence of enzyme catalyzed reactions initially involving the glutathione-dependent cleavage of the C-Cl bond (Boyland and Chasseaud, 1969), glutathione acting as the acceptor of the transferred aryl or alkyl group. In the case of the 2-chloro-triazine herbicide, the substituted diamino-*s*-triazinyl group would be transferred to glutathione. It is becoming increasingly clear that glutathione plays a role in a large number of detoxication reactions. Several enzymes which catalyze the transfer of various groups to gluthathione have been reported in the literature: glutathione S-aryl transferase (Grover and Sims, 1964), glutathione S-alkyl transferase, using methyl iodide as substrate (Johnson, 1966), glutathione S-alkyl transferase, using phosphoric acid triesters as substrates (Hutson, Pickering, and Donninger, 1968), glutathione S-epoxido transferase (Boyland and Williams, 1965), and recently an enzyme catalyzed reaction between glutathione and α,β-unsaturated carbonyl compounds (Boyland and Chasseaud, 1968). It will be of interest to investigate the nature of the enzyme involved in the transfer of triazinyl groups. The superficial similarity between the benzene and *s*-triazine rings suggests that glutathione-S-aryl transferase could be involved in the metabolism of 2-chloro-triazines.

This is the first reported isolation of a triazinyl mercapturic acid but it is possible that other herbicides of the 2-chloro-triazine type, *e.g.*, atrazine, propazine, and simazine, are metabolized in the mammal by this route.

ACKNOWLEDGMENT

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