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Genotoxicity Assessment of Atrazine and Some Major Metabolites in the Ames Test

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The herbicidal properites of s-triazines were discovered in the 1950's by J.R. Geigy, Ltd., Basel, Switzerland (Esser et al. 1975). Since then, many such compounds have been synthesized and evaluated for herbicidal activity, and several major products established for agriculture (e.g., atrazine, simazine, cyanazine, ametryn, and prometryn). One such compound, atrazine, is a widely-used selective herbicide for control of grassy and broadleaf weeds in maize, sorghum, rangeland, sugarcane, and other crops, as well as a nonselective herbicide in non-cropland situations (Anonymous 1983). A recent report (Anonymous 1987) indicated that atrazine is the second most widely used pesticide in the United States, with about 79 million pounds of active ingredient applied annually. The wide use of herbicides in this class over the past 30 years has resulted in human and environmental exposure and prompted questions about their toxicological effects on target and nontarget species, including plants and animals.

In studies on the mutagenic potential of s-triazine herbicides, various assay systems have been employed and the compounds tested varied in purity from commercially formulated products to high purity compounds. Consequently, results concerning the mutagenic potential of these compounds have also varied. In intact plant tissues, mitotic chromosome aberrations have been reported to be caused by atrazine and simazine (Wuu and Grant 1966; Strovev 1968 and 1970; Wuu and Grant 1967; Liang and Liang 1972). Increased frequency of sister chromatid exchanges in maize caused by atrazine have also been published (Chou and Weber 1981). However, in other studies, neither atrazine nor simazine induced mitotic aberrations in several plant species (Sawamura 1965; Muller et al. 1972).

Results concerning s-triazine toxicology in microbial genotocity assays were generally all negative. Atrazine did not induce reverse mutations at the his locus in five Salmonella strains (Sieler 1973); induce point mutations in Salmonella typhimurium or in a T4 bacteriophage (Anderson et al. 1972); or

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cause any genotoxic effects in four Salmonella strains (Lusby et al. 1979). Simazine and atrazine did not induce point mutations in S. typhimurium (Shirasu et al. 1976). Several s-triazines, including atrazine and simazine, did not cause gene conversion in Saccharomyces cerevisiae (Siebert and Lemperle 1974). Negative genotoxic activity was also reported for atrazine tested in a gene conversion assay in Aspergillus nidulans with or without in vitro mammalian metabolic activation (Bertoldi et al. 1980). When 16 s-triazines, including atrazine, simazine, and triethylene-melamine were tested in several S. typhimurium strains with and without S9 activation, only triethylene-melamine was found mutagenic (Lusby et al. 1979). Positive genotoxic effects in microbial assays include a very weak mutagenic response by 2-hydroxy-4,6-bis(ethyamino)-s-triazine and 2-chloro-4,6-diamino-s-triazine in S. typhimurium (Sieler 1977).

In contrast to the preponderance of negative genotoxic effects found for s-triazines in microbial assays with or without in vitro mammalian metabolism, several positive effects have been found when s-triazines have been activated via plant metabolism. Metabolism of atrazine by Nicotiana alata cells produced a product that induced somatic segregation and mutation in Aspergillus nidulans (Benigni et al. 1979). In in vivo plant (maize) studies, atrazine was reported to be activated into an unidentified compound(s) that induced gene reversion and conversion in S. cerevisiae (Plewa and Gentile 1975 , 1976). Isolation of an active mutagen fraction produced by maize metabolism of atrazine also reportedly induced gene conversion in S. cerevisiae (Singh et al. 1982). Recently a water-soluble extract from maize plants treated with atrazine was mutagenic in one strain (TA100) of S. typhimurium (Means et al. 1988). Use of ^{14}C-labeled atrazine, led these researchers to suggest that this mutagen was an atrazine metabolite, although its identity was not determined. Atrazine metabolism in plants has received considerable attention over the years since its development and several major metabolites formed relatively early during metabolic degradation have been identified, as summarized in reviews (Esser et al. 1975; Ashton and Crafts 1981). The structures of some major plant metabolites of atrazine, plus benzoxazinone which catalyzes atrazine degradation in plants, are outlined in Figure 1. Atrazine metabolism is a complex and dynamic process, dependent on factors including initial herbicide dose, rate of absorption and translocation, plant age, and rate of plant growth. Many metabolites formed relatively early in atrazine degradation are continually transformed into water-soluble and insoluble residues which are difficult to extract and identify quantitatively. However, several water-soluble atrazine metabolites were extractable and quantifiable up to 30 days after plants were pulse-treated with atrazine (Lamoureux et al. 1973). In all of the above positive reports of genotoxicity of atrazine in maize, plants were exposed to atrazine during germination through the 3-leaf stage (2-3 weeks) when plants were havested and extracted. This indicated that the active mutagen could be an early metabolite in the atrazine degradation pathway. The objectives of our experiments were to examine atrazine and several of its known major metabolites in plants in the S. typhimurium mutagenicity assay using three bacterial strains. We wanted to test these compounds in high purity and at a range of doses to help clarify some of the results obtained by other laboratories

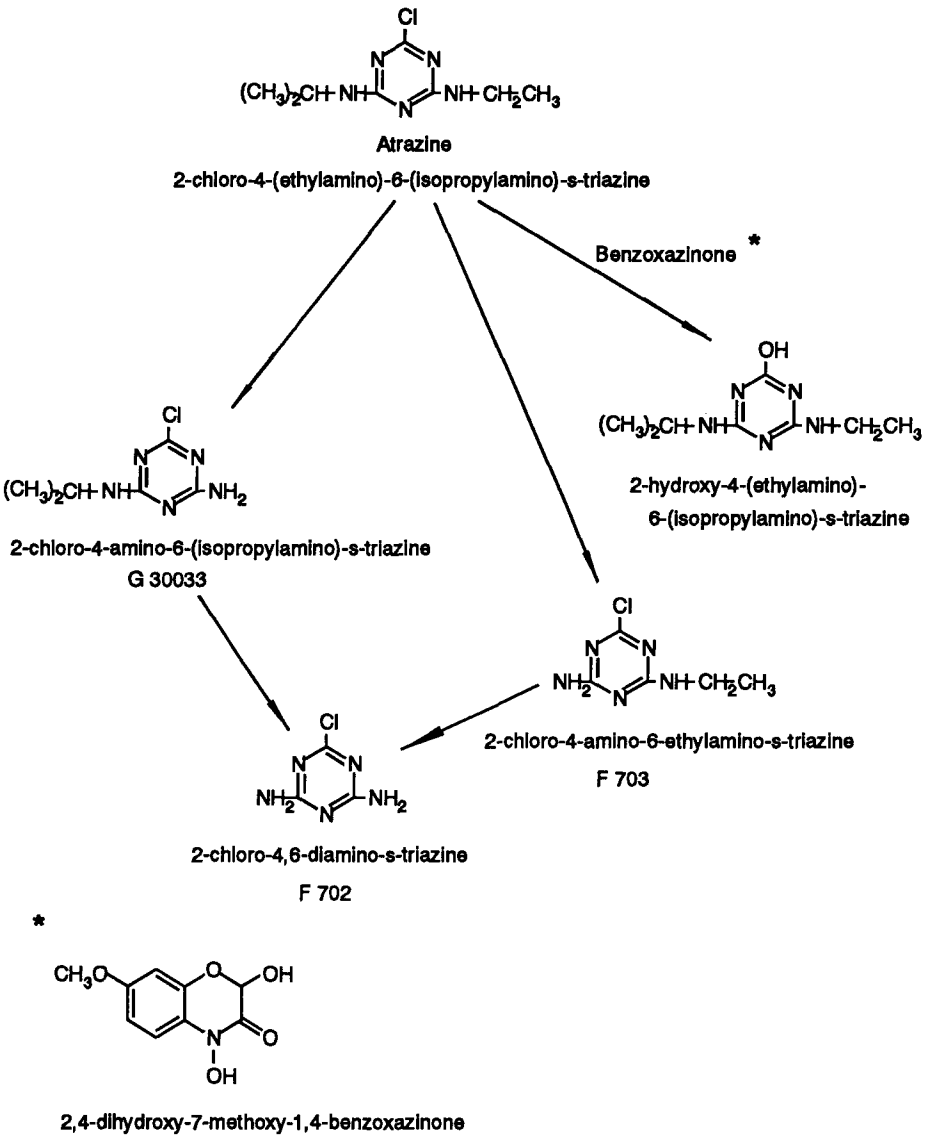

Figure 1. Structures of atrazine and some of its major metabolites.

and to possibly identify the actual atrazine mutagenic metabolite suggested by others (Means et al. 1988). Furthermore, we are not aware of any published report evaluating the mutagenic potential of this important herbicide and its major metabolites in a single genotoxicity assay.

MATERIALS AND METHODS

Salmonella typhimurium strains TA97, TA98, and TA100 were provided

by Dr. Bruce Ames, Berkley, CA. Mutagenicity assays were conducted following procedures previously described by Ames et al. (1975). Positive controls [0.5 μg sodium azide (NaAz), 50 μg 9-aminoacridine (9AA), or 1 μg 2-nitrofluorene (NF)/plate] were included in all assays. DMSO was the solvent for all chemicals and was also used as a solvent control. All experiments were repeated at least once, and each experiment was triplicated. The number of revertant colonies for each treatment was calculated from the means of triplicated plates.

All triazine herbicides or metabolites were analytical reference standards (U.S. Environmental Protection Agency), except G-30033 [2-chloro-4-amino-6-(isopropylamino)-s-triazine], which was provided by Ciba Geigy Corp. All compounds used were of 99% or greater purity. Structures of these high-purity compounds are shown in Figure 1, which also schematically outlines atrazine metabolism in plants.

RESULTS AND DISCUSSION

In two separate Salmonella mutagenicity assays for each compound, high purity atrazine, hydroxyatrazine, and 2-chloro-4-amino-6- (isopropylamino)-s-triazine were all found nonmutagenic in 3 bacterial strains when tested at 0.01 to 10.0 μmol per plate (Table 1). Positive mutagenic chemical controls in the 3 strains indicate the assays (run at different times) were valid. In repeat experiments, similar results were obtained for atrazine and the metabolites tested (data not shown). Two additional major metabolites [2-chloro-4-amino-6-diamino-s-triazine (F702) and 2-chloro-4-amino-6-(ethylamino)-s-triazine (F703)], also tested over a 1000-fold concentration range, showed no genotoxic activity (Table 2). These assays were run simultaneously and the same positive mutagenic chemical controls were used. Other strains (TA1535, TA1537, & TA1538) were also examined in our laboratory in a battery of assays with atrazine and these metabolites. No mutagenic responses were noted in any of the tests (data not shown to avoid redunancy). Another atrazine metabolite, the glutathione conjugate, was not available for analysis. Atrazine and its major metabolites tested here were nontoxic at these concentrations to the test organisms and exhibited no genotoxicity, even at very high doses.

Generally, in vitro tests for mutagenicity have shown atrazine to be negative, even when used with the mammalian hepatic microsomal system (S9), as summarized by Adler (1980) and more recently reported by Kappas (1988). These results indicate that mammalian liver S9 preparations do not activate atrazine via metabolism to form mutagenic compounds. Mammalian metabolism (rats and rabbits) of atrazine has been shown to produce at least three metabolites identical to those produced in plants (Figure 1), with the exception of hydroxyatrazine (Menzie 1969). A fourth plant metabolite of atrazine, the glutathione conjugate, is also formed in mammals via soluble hepatic glutathione-S-transferases (Dauterman and Muecke 1974; Hutson et al. 1970). These findings may indicate that atrazine poses no major biohazard with respect to mammalian food chains. Because of these results, we did not use the S9 system in our tests.

Table 1. Mutagenicity assay of atrazine[1], hydroxyatrazine[2], and G-30033[3].

Treatment, µmol/plate	Number of His+ Revertants/Plate[4] TA100	TA97	TA98
Atrazine			
0.260 9AA	-	811 a[5]	-
0.005 2NF	-	-	467 a
0.008 NaAz	583 a	-	-
0	148 b	235 b	18 b
0.010	192 b	214 b	21 b
0.100	149 b	266 b	21 b
1.000	179 b	233 b	20 b
10.000	152 b	245 b	17 b
Hydroxyatrazine			
0.260 9AA	-	691 a	-
0.005 2NF	-	-	235 a
0.008 NaAz	473 a	-	-
0	99 b	142 b	23 b
0.010	100 b	144 b	21 b
0.100	82 b	146 b	22 b
1.000	94 b	144 b	22 b
10.000	95 b	125 b	20 b
G-30033			
0.260 9AA	-	844 a	-
0.005 2NF	-	-	410 a
0.008 NaAz	561 a	-	-
0	226 b	185 b	26 b
0.010	205 b	194 b	22 b
0.100	226 b	194 b	24 b
1.000	204 b	200 b	22 b
10.000	208 b	182 b	23 b

[1] 10.0 µmol = 2157 µg; [2] 2-hydroxy-4-(ethylamino) 6-(isopropylamino)-s-triazine, 10.0 µmol = 1970 µg; [3] 2-chloro-4-amino-6-(isopropylamino)-s-triazine, 10.0 µmol = 1876 µg; [4] Each number is the average of 3 determinations. Repeat assays were conducted, also using 3 plates/dose, which gave similar results. [5] Numbers in vertical columns and within treatments followed by the same letter are not significantly different at the 95% confidence level, as determined by the overlap of 2 standard errors of the means.

Our results are in agreement with other results demonstrating that atrazine is a non-mutagenic compound (Anderson et al. 1972; Sieler 1973; Lusby et al. 1979; Kappas 1988). Using very similar extraction methodology, Sumner et al. (1984) found no alteration of endogenous mutagenic compounds in extracts of 30-day old or mature maize after treatment with commercially-formulated atrazine, contrary to other reports (Plewa and Gentile 1975, 1976; Means et al. 1988).

Table 2. Mutagenicity assay of atrazine metabolites F702[1] and F703[2].

Treatment, µmol/plate	Number of His+ Revertants/Plate[3] TA100	TA97	TA98
0.260 9AA	-	849 a[4]	-
0.005 2NF	-	-	386 a
0.008 NaAz	417 a	-	-
0	132 b	175 b	17 b
F702			
0.010	109 b	176 b	17 b
0.100	114 b	161 b	14 b
1.000	97 b	160 b	16 b
10.000	98 b	110 b	16 b
F703			
0.010	122 b	255 b	21 b
0.100	124 b	235 b	19 b
1.000	116 b	246 b	23 b
10.000	138 b	171 b	20 b

[1] F702 = 2-chloro-4,6-diamino-s-triazine; [2] F703 = 2-chloro-4-amino-6-(ethylamino)-s-triazine; [3] Each number is the average of 3 plates. Another assay was conducted, also using 3 plates/dose, which gave similar results. [4] Numbers in vertical columns and within treatments followed by the same letter are not significantly different at the 95% confidence level, as determined by the overlap of 2 standard errors of the means.

Results using this treatment/extraction protocol from our laboratory also contradict the findings of these latter researchers (data not shown).

Triazine-resistant maize was found to contain a compound [2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3(4H)-one] which is able to rapidly inactivate s-triazines (see Figure 1). This compound occurs in other plants and has insecticidal and fungicidal properties (Esser et al. 1975). It also has been shown to be a naturally occurring mutagen in S. typhimurium assays (Hashimoto et al. 1979). Levels of these compounds (benzoxazinones) may be altered by plants under stress, i.e., inadequate nutrient conditions, general herbicide and herbicide metabolite impact on enzymatic and nonenzymatic pathways. These naturally-occurring mutagenic compounds, as well as the levels of other compounds altered by atrazine treatment, may interfer with assays that examine extracts from atrazine-treated plants, especially if extraction procedures are non-specific (Sumner et al. 1984).

Since the parent atrazine compound and four of its major metabolites were nontoxic and nonmutagenic to several S. typhimurium strains at extremely high doses, it is unlikely that they are the mutagens previously reported to be present in AAtrex^{R}-treated plants (Means et al. 1988). It is also doubtful that the mutagen isolated by these researchers is a glutathione conjugate (untested in

our experiments), since their mutagen(s) was stable to UV light, high temperature (100 C), 5% sodium hypochlorite, and 10% hydrogen peroxide, and the glutathione conjugate is relatively unstable (Homer LeBaron, pers. comm.).

REFERENCES

Adler, ID (1980) A review of the coordinated research effort on the comparison of test systems for the detection of mutagenic effects sponsored by the EEC. Mutation Res 74:77-93

Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res 31:347-346

Anderson KJ, Leight DG, Takahashi MT (1972) Evaluation of herbicides for possible mutagenic properties. J Agr Food Chem 20:649-656

Anonymous (1983) In: Herbicide Handbook of the Weed Science Society of America, 5th edition. WSSA, Champaign, IL, pp 30-35

Anonymous (1987) National pesticide use analyzed. Alternat Agric News 5:1-4

Ashton FM, Crafts AS (1981) Mode of Action of Herbicides. John Wiley and Sons, New York, chap 19

Benigni R, Bignami M, Camoni I, Carere A, Conti G, Iachetta R, Moapurzo G, Ortali VA (1979) A new in vitro method for testing plant metabolism in mutagenicity studies. J Toxicol Environ Health 5:809-819

Bertoldi M de, Griselli M, Giovannetti M, Barale R (1980) Mutagenicity of pesticides evaluated by means of gene conversion in Saccharomyces cerevisiae and Aspergellus nidulans. Environ Mutag 2:359-370

Chou TS, Weber DF (1981) The effect of atrazine on sister-chromatid exchange in maize. Genetics 97:521

Dauterman WC and Muecke W (1974) In vitro metabolism of atrazine by rat liver. Pestic Biochem Physiol 4:212-219

Esser HO, Dupuis G, Ebert E, Marco GJ, Vogel C (1975) s-Triazines. In: Kearney PC, Kaufman DD (eds) Herbicides: Chemistry, Degradation and Mode of Action, 2nd edition. Marcel Dekker Inc, New York, chap 2

Hashimoto Y, Shudo K, Okamoto T, Takahashi Y, Sugimura T (1979) Mutagenicities of 4-hydroxy-1,4-benzoxazinones naturally occurring in maize plants and of related compounds. Mutation Res 66:191-194

Hutson DH, Hoadley EC, Griffiths MH, Donninger C (1970) Mercapturic acid formation in the metabolism of 2-chloro-4-ethylamino-6- (1-methyl-1-cyanoethylamino)-s-triazine in the rat. J Agr Food Chem 18:507-512

Kappas A (1988) On the mutagenic and recombinogenic activity of certain herbicides in Salmonella typhimurium and in Aspergillus nidulans. Mutation Res 204:615-621

Lamoureux GL, Stafford LE, Shimabukuro RH, Zaylskie RG (1973) Atrazine metabolism in sorghum: catabolism of the glutathione conjugate of atrazine. J Agr Food Chem 21:1020-1030

Liang GHL, Liang TYS (1972) Effects of atrazine on chromosomal behavior in sorghum. Can J Genet Cytol 14:423-427

Lusby DF, Simmons I, McGuire PM (1979) Variation in mutagenecity of s-triazine compounds tested on four Salmonella strains. Environ Mutagen 1:287-290

Means JC, Plewa MJ, Gentile JM (1988) Assessment of the mutagenicity of fractions from s-triazine-tested Zea mays. Mutation Res 197:325-336
Menzie CA (1969) Metabolism of Pesticides. USDI, Bur Sport Fisheries and Wildlife Special Scientific Report - Wildlife No 127. Washington, DC, p 314
Muller A, Ebert E, Gast A (1972) Cytogenetic studies with atrazine (2-chloro-4-ethylamino-6-isopropylamino- s-triazine) on plants. Experientia 28:704-705
Plewa MJ, Gentile JM (1975) A maize-microbe bioassay for detecting proximal mutagenicity in agricultural chemicals. Maize Genet Coop Newslett 49:40-43
Plewa MJ, Gentile JM (1976) Mutagenicity of atrazine: A maize-microbe bioassay. Mutation Res 38:287-292
Sawamura S (1965) Cytological studies on the effect of herbicides in plant cells in vivo, II. Non-hormonic herbicides. Cytologia 30:325-348
Shirasu Y, Moriua M, Kato K, Furuhasi A, Kada T (1976) Mutagenicity screening of pesticides in the mocrobial system. Mutation Res 40:19-30
Siebert D, Lemperle E (1974) Genetic effects of herbicides: induction of mitotic gene conversion in Saccharomyces cerevisiae. Mutation Res 22:111-120
Sieler JP (1973) A survey on the mutagenecity of various pesticides. Experientia 29:622-623
Singh I, Lusby AF, McGuire PM (1982) Mutagenicity of HPLC fractions from extracts of AAtrex-treated corn. Environ Mutagenesis 4:45-53
Strovev VS (1968) Cytogenetic activity of the herbicides simazine and maleic acid hydrazide. Genetika 6:130-132
Strovev VS (1970) Cytogenetic activity of the herbicides atrazine, chloro-IPC, and paraquat. Genetika 6:31-37
Sumner DD, Cassidy JE, Szolics IM, Marco GH, Bakshi KS, Brusick DJ (1984) Evaluation of the mutagenic potential of corn (Zea mays L.) grown in untreated and atrazine (AAtrex) treated soil in the field. Drug Chem Toxicol 7:243-257
Wuu KD, Grant WF (1966) Morphological and somatic chromosomal aberrations induced by pesticides in barley (Hordeum vulgare). Can J Genet Cytol 8:481-501
Wuu KD, Grant WF (1967) Chromosomal aberrations induced by pesticides in meiotic cells of barley. Cytologia 32:31-41

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