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Mutagenicity study of butachlor and its metabolites using *Salmonella typhimurium*

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Butachlor is the most commonly used herbicide in Taiwan and many other countries. It has been reported to be an indirect mutagen and carcinogen in various in vitro assay systems. Previous investigation has also demonstrated that butachlor stimulates cell proliferation, transforms normal embryonic cells, and induces stomach tumors in Spraque-Dawley rats. However, the mechanism of butachlor carcinogenicity is still not clear. In order to clarify the toxicologic and carcinogenic properties of butachlor, we proposed a metabolic pathway, and synthesized the authentic metabolites by chemical methods. In addition, we tested the mutagenicity of butachlor and these metabolites on *Salmonella typhimurium*. The results indicate that butachlor might manifest its carcinogenicity via the mutagenicity of its metabolic products. Although the molecular mechanism of butachlor-induced cellular toxicity is still not clear, it is likely that the cellular transformation ability of butachlor is partly associated with its mutagenicity.

Key words: Acetamides, acetanilides, butachlor, mutagenicity tests, *Salmonella typhimurium*

Butachlor (2-chloro-2',6'-diethyl-N-[butoxymethyl] acetanilide) is a pre-emergence herbicide belonging to the chloroacetanilide family and is widely used to control grasses in the rice producing countries of Asia and South America [1]. More than 8000 tons of butachlor are being used every year in Taiwan, and much more in other Asian countries [2]. The structure of this compound is similar to that of alachlor (2-chloro-2',6'-diethyl-N-[methoxymethyl] acetanilide), another chloroacetanilide herbicide that has also been used in large quantity to control weeds, primarily in agricultural crops including corn and soybeans in the United States [3]. These 2 herbicides differ only in having a butoxyl and methoxyl group, respectively, on the N-methyl moiety. Alachlor is a potential human carcinogen inducing lung tumor in mice and stomach, thyroid, and nasal turbinate tumors in rats [3]. Butachlor is not used in the United States and little information on its toxicology is available.

Butachlor has been reported to be an indirect mutagen. Its mutagenicity on *Salmonella typhimurium* strain TA 100 requires the addition of rat liver extract (S9) [4]. It also requires S9 to induce chromosome aberrations in Chinese hamster ovary cells [5]. Moreover, butachlor causes sister chromatid exchanges in rat tracheal epithelial cells [6]. Previous findings have suggested that butachlor stimulates cell proliferation and transforms normal embryonic cells [7]. In addition, butachlor has also been reported to induce stomach tumors in Spraque-Dawley rats [8,9]. All of these findings suggest that butachlor is similar to alachor not only in chemical structure but also in properties. Thus, butachlor is very possibly a carcinogen in a similar way to alachlor. Although previous investigations have all demonstrated that butachlor may be a carcinogen, it is still very popularly used for weed control in Asia countries [10,11]. Previous investigations have revealed that there are detectable levels of butachlor in soil during the spring application period [12]. Despite the high rates of butachlor application, and possible environmental contamination from it, there is still little toxicologic information available about it.

Previously, we have demonstrated that butachlor can be metabolized through the mercapturic acid pathway to form butachlor acetylcysteine conjugate (BACC) [13]. Both alachlor and butachlor have been suggested as carcinogens that are involved in a complex metabolic

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activation pathway that leads to a DNA-reactive dialkylbenzoquinone imine [14]. In order to test this hypothesis that the carcinogenicity of butachlor involves further metabolic intermediates, here we proposed a consequential metabolic pathway of butachlor from BACC to the DNA-reactive dialkylbenzoquinone imine. Important intermediates in this pathway are 2,6-diethylaniline (DEA), which is bioactivated through subsequent dealkylation and oxidation to form 3,5-diethyl-p-amino-phenol (DEAP, OH-DEA) and the proposed carcinogenic product 3,5-diethyl benzoquinone 4-imine (DEBQI). In this report, the metabolic intermediates were chemically synthesized, identified by gas chromatography/mass spectroscopy and their mutagenicity tested on various *S. typhimurium* strains.

Materials and Methods

Chemicals

Butachlor was purchased from Chem Services (West Chester, PA, USA), and diethylaniline from Fluka Chemie AG (Buchs, Switzerland). All other chemicals were the highest grade available and purchased from regional suppliers.

Metabolite synthesis

2,6-diethylaniline acetate (DEAA) was synthesized from DEA with acetanhydride to form a precipitate [15]. The precipitate was then filtered and dissolved in ethanol. The same volume of distilled water was added to this solution to reprecipitate the DEAA product, which was then heated at 70°C to dissolve it, after which the solution was placed at room temperature until crystal formation. Then acetic anhydride was added to dissolve the DEAA crystals, followed by cold sulfuric acid and the mixture was then kept on ice for 10 min. Then pre-mixed nitric acid and sulfuric acid (7:10) were added drop by drop into the solution and the mixture was kept at 20 to 25°C for 40 min to allow the reaction to take place. Ice water was then added to form a yellow precipitate, which, after filtration, was dissolved in 80% ethanol to form NO_2-DEAA. NH_2-DEAA was prepared by hydration of NO_2-DEAA by the methods described by Feng and Wratten 1987 [16]. OH-DEAA and OH-DEA were prepared by deacetylation method described by Adams et al 1982 [15]. For OH-DEA synthesis, an alternative method was used. In brief, direct hydroxylation of DEA by hydroxyl free radical followed by dichloromethane extraction was carried out as described previously [17].

Gas chromatography/mass spectrometry

A Hewlett-Packard Model 5890 microprocessor-controlled gas chromatograph interfaced to a Hewlett-Packard Model 5971A mass selective detector was used for the identification of the synthesized standard compounds. Separation was carried out using a fused silica capillary column coated with 5% methyl silicone gum phase. Helium was used as the carrier gas at an inlet pressure of 30 kPa. The injection port was kept at 250°C, while the gas chromatography/mass spectrometry interface was maintained at 280°C. The column temperature was increased from 80°C to 220°C at a rate of 12°C per min after 3 min at 80°C. An aliquot (1 μL) of each sample was injected without any further treatment into the injection port in the splitless mode.

Rat microsome preparation (S9)

S9 were prepared from male Spraque-Dawley rats purchased from the Animal Service Center of the National Science Council, Taiwan. Briefly, the animals were stimulated by intraperitoneal administration with 100 mg alachlor 1254 in 0.5 mL corn oil. Five days later, the animals were sacrificed and the livers were removed, weighed, and placed in ice-cold 50 mM potassium phosphate (pH 7.5) containing 0.1 mM ethylenediamine tetra-acetic acid (EDTA) and 1.15% potassium chloride. The livers were then minced with scissors, homogenized with a Polyron homogenizer, and then centrifuged at 10,000 *g* for 15 min. The supernatant was filtered through glass wool and centrifuged at 100,000 *g* for 1 h. The microsomal pellet was re-suspended in 50 mM potassium phosphate (pH 7.5) containing 0.1 mM EDTA and 0.25 M sucrose. Aliquots were stored at –80°C until use. Protein concentration was determined using a Bio-Red reagent (Bio-Rad Co, Redmond, WA, USA) prior to use.

S. typhimurium mutagenicity assay

S. typhimurium strain TA97 and TA98, which detects frameshift mutagens, and strain TA100, which detects base-pair substitution mutagens, were used for the Ames test. Butachlor and its metabolites at various concentrations were assayed. The *Salmonella* mutation test was carried out using a microsuspension assay that was modified to provide enhanced sensitivity compared to the standard plate-incorporation method. Briefly, *S. typhimurium* test strains were grown in Oxoid nutrient broth No. 2 at 37°C with shaking for about 10 h. The bacterial cultures were checked to make sure that

their optical density at 650 nm was between 0.4-0.6 before being used. The S9-dependent or -independent mutagenicity was determined as follows. The reaction mixtures, containing 100 μL of various concentration of compounds and 800 μL of phosphate-buffered saline with or without S9 reaction mixture and 100 μL of bacterial solution (approximately 0.6-1.0 × 10^7 bacteria per assay), were placed in test tubes on a rotary shaker and incubated at 37°C for 30 min. Rat liver S9 (10% v/v in S9 reaction mixture) was used as the activating system. After incubation, 2.4 mL of top agar, containing 0.05 mM each of histidine and biotin, was added to each test tube and the test tube contents were poured onto Vogel-Bonner minimal agar plates. The plates were inverted, incubated at 37°C for 48 h, and then colony numbers were counted. The number of histidine revertant colonies was manually counted, and the results were expressed as the number of revertant colonies per plate. All assays were performed on duplicate plates. Spontaneous revertants were not subtracted from the number of revertants obtained for each assay.

Results

Synthesis of butachlor metabolites

We proposed a pathway for butachlor metabolism similar to that in previous reports of alachlor metabolism. In order to test whether butachlor manifested its carcinogenicity via the mutagenicity of its metabolites, the intermediates in this proposed pathway (Fig. 1) were synthesized chemically step by step and the crystal product of each step was tested for melting point and then separated by thin layer chromatography (data not shown) as a purity test. After the preliminary purity tests, we used gas chromatography/mass spectrometry to determine the structure of these products. DEAA, the first step product, was demonstrated to have a melting point of the 138-140°C and gave a single spot on thin layer chromatography analysis. According to the results of the gas chromatography/mass spectrometry analysis, it consisted of a single peak with a molecular weight demonstrated to be 191.1. In mass spectrometry, all the breakdown fragments in this analysis satisfied the structure characteristics of DEAA (Fig. 2A). This suggested that this product is DEAA. NO_2-DEAA is a light yellow crystal, melting point 154-155°C. The molecular weight of this product was determined to be 236, and the mass spectrometry also demonstrated its structural characteristics (Fig. 2B).

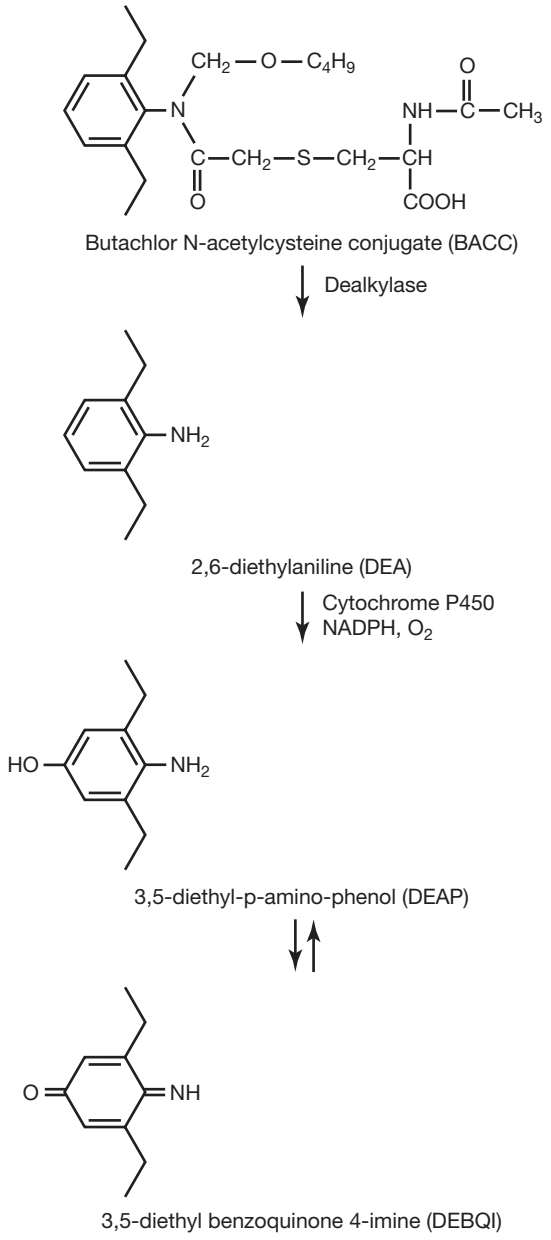

Fig. 1. Proposed metabolic pathway of butachlor from its N-acetylcysteine conjugate butachlor acetylcysteine conjugate.

The third synthesized product, NH_2-DEAA, is a milky white amorphous powder, melting point 185-186°C; gas chromatography/mass spectrometry analysis demonstrated its molecular weight to be 206 and the breakdown fragments in this analysis satisfied its structural characteristics (Fig. 2C). OH-DEAA, the fourth product, isolated by silica gel chromatography after its synthesis, was demonstrated to have a molecular weight of 207. Analysis results for the gas chromatography/mass spectrometry are shown in Fig. 2D. The final step is the synthesis of OH-DEA (DEAP), the precursor of the

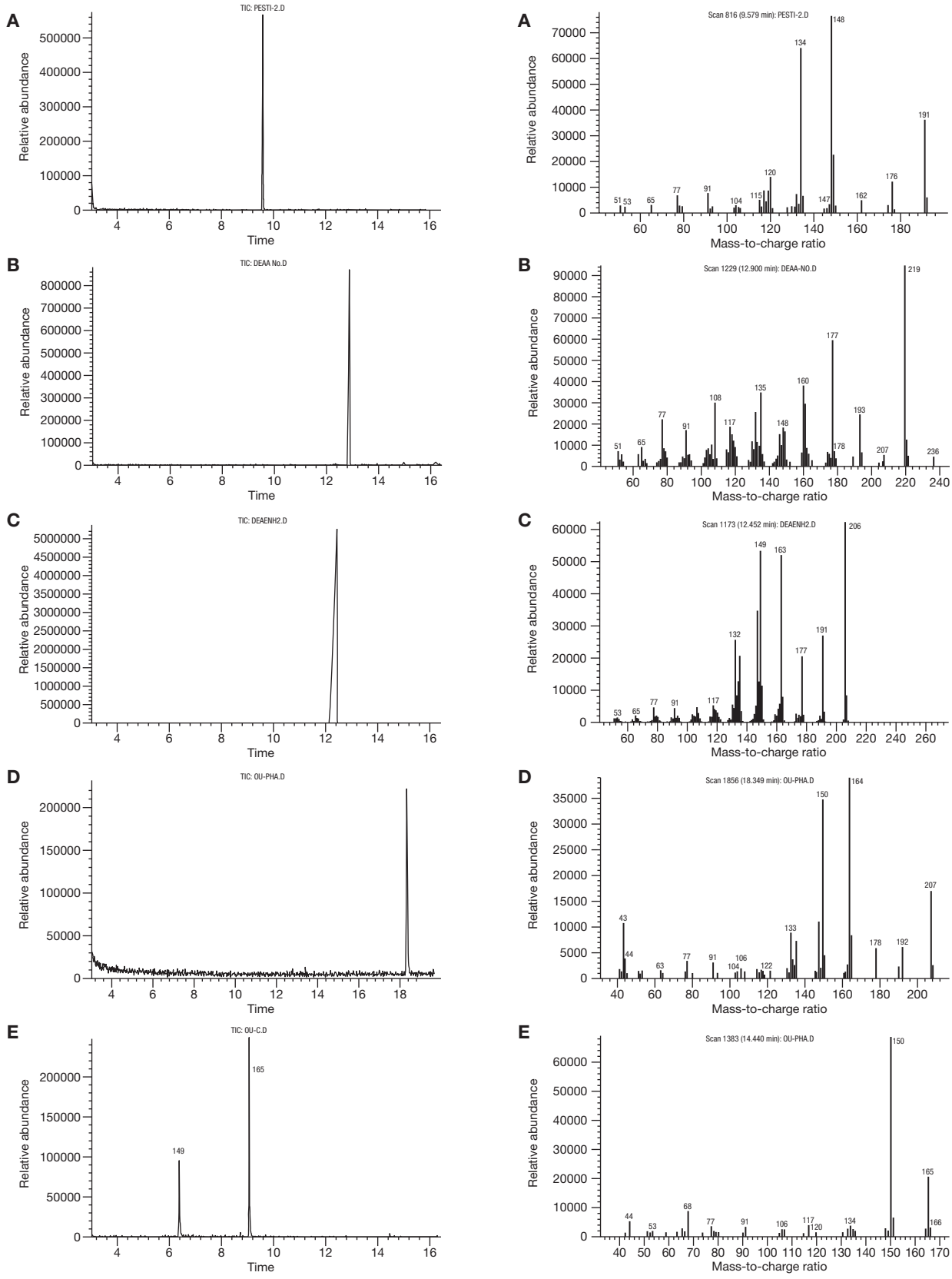

Fig. 2. Gas chromatography (left) and mass spectrometry (right) analysis of synthesized metabolites. A) 2,6-diethylaniline acetate (DEAA); B) NO_2-DEAA; C) NH_2-DEAA; D) OH-DEAA; E) 3,5-diethyl-p-amino-phenol (OH-DEA).

proposed DNA reactive intermediate DEBQI. The results of this procedure produced more than 1 product, one of them was demonstrated to be DEAP with a molecular weight 165 (Fig. 2E). OH-DEA is an unstable compound, which can spontaneously transform into the DNA reactive final product DEBQI; the reaction can also be reversed to produce the parent compound DEA. Thus, as it is very difficult to isolate the pure OH-DEA product. We did not carry out any further purification and the reaction product mixture was used for the mutagenicity assay.

Salmonella mutagenicity assay

Various *Salmonella* strains were used to test mutagenicity of the butachlor metabolites DEA, and OH-DEA (DEAP). The mutagenicity assays were conducted by incubating the 2 synthesized intermediates and their precursor butachlor with the *S. typhimurium* strains, both with and without the rat liver microsome fraction (S9). *S. typhimurium* strains TA97 and TA98, which detect frameshift mutagens, and strain TA100, which detects base-pair substitution mutagens, were used for these tests. The results demonstrated that butachlor is a mutagen for TA98 without the addition of S9 ($p<0.05$), and for TA100 when S9 is added to the test system ($p<0.01$) [Table 1]. The mutagenicity of butachlor on TA100 without S9 is significant only at 1000 μM, but at >200 μM with S9. We suggest that butachlor may be mutagenic against TA100 with or without S9, but that the cytotoxicity increases as the butachlor concentration is raised and this might attenuate its mutagenicity. The mutagenicity results are similar to those of previous studies [4]. DEA, the first proposed metabolite from BACC, was also found to be mutagenic against *S. typhimurium* strains TA97 and TA98 (Table 2, $p<0.05$). Since this compound is a metabolic intermediate, we did not assay its mutagenicity with rat liver S9 fraction but we employed 3 more *S. typhimurium* strains (TA1537, TA1538, and TA1535) for further tests. These 3 strains are the parent strains of TA97, TA98 and TA100, respectively. According to the results, DEA was also mutagenic against strains TA1537, TA1538 and TA1535 ($p<0.01$). It should be noted that the mutagenicity is lower than that of the well-known mutagen N-methyl-N'-nitro-N-nitrosoguanidine. Finally, we tested the mutagenicity of the predicted DNA-reactive metabolite OH-DEA. OH-DEA was found to mutate TA98 when S9 was not added, and TA98 as well as TA100 when S9 were added to the reaction system (Table 3). This result is similar to that of butachlor and suggests that the mutagenicity against TA100 was attenuated by the cytotoxicity. Across these mutagenicity tests, OH-DEA or its further metabolites in the reaction mixture were the most mutagenic. These results support our hypothesis that although butachlor is not a strong mutagenic chemical in itself, it is convertible into a stronger mutagen via metabolic activation in this case by the S9 microsome fraction.

Discussion

We have synthesized the 5 proposed metabolic intermediates of butachlor by chemical methods from its initial product DEA to the final DNA-reactive product OH-DEA. The melting point determinations, thin layer chromatography separation and gas chromatography

Table 1. Mutagenicity of butachlor on *Salmonella typhimurium*

Butachlor (μM)	Revertants per plate[a]					
	Without S9			With S9		
	TA97	TA98	TA100	TA97	TA98	TA100
0	110 ± 11	10 ± 2	92 ± 7	134 ± 12	18 ± 2	89 ± 5
50	116 ± 9	10 ± 2	69 ± 6	142 ± 12	15 ± 1	96 ± 4
100	113 ± 6	15 ± 2[c]	79 ± 6	132 ± 10	19 ± 2	97 ± 3
150	114 ± 6	15 ± 2[c]	87 ± 6	142 ± 11	15 ± 4	100 ± 10
200	117 ± 8	14 ± 1[c]	96 ± 8	127 ± 9	17 ± 2	113 ± 8[c]
1000	112 ± 6	19 ± 3[c]	120 ± 8[c]	137 ± 11	16 ± 2	130 ± 7[c]
MNNG[b]	214 ± 15	27 ± 2	1487 ± 181	593 ± 53	30 ± 2	3240 ± 72

Abbreviation: MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

[a]Values are mean ± standard deviation from more than 3 different experiments. Spontaneous revertants were not substracted from the number of revertants obtained for each assay.

[b]Positive control: 2 μg MNNG per plate was used as positive control.

[c]Indicates a significant increase from control (butachlor 0 μM) [$p<0.05$].

Table 2. Mutagenicity of 2,6-diethylaniline (DEA) on *Salmonella typhimurium*

DEA (μM)	Revertants per plate[a]					
	TA97	TA98	TA100	TA1537	TA1538	TA1535
0	103 ± 8	21 ± 4	69 ± 8	19 ± 2	19 ± 2	20 ± 2
50	107 ± 9	29 ± 3[c]	64 ± 7	9 ± 1[c]	4 ± 1[c]	30 ± 4[c]
100	85 ± 6[c]	6 ± 1[c]	39 ± 6	2 ± 1[c]	4 ± 1[c]	7 ± 1[c]
150	89 ± 7[c]	7 ± 2[c]	58 ± 8	3 ± 2[c]	12 ± 2[c]	5 ± 2[c]
200	87 ± 6[c]	30 ± 3[c]	69 ± 9	27 ± 2[c]	39 ± 4[c]	28 ± 3[c]
MNNG[b]	223 ± 16	40 ± 4	2040 ± 180	62 ± 8	15 ± 2	3422 ± 206

Abbreviation: MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

[a]Values are mean ± standard deviation from more than 3 different experiments. Spontaneous revertants were not substracted from the number of revertants obtained for each assay.

[b]Positive control: 2 μg MNNG per plate was used as positive control.

[c]Indicates a significant increase from control (butachlor 0 μM) [$p<0.05$].

have all demonstrated that the syntheses were successful. However, the yield of the final product OH-DEA was less than expected. Since the final product is unstable, we did not purify it.

In order to use these products as standard materials for further analysis, it will be important in the future to increase the production rate of this compound and improve the purification method. Since DEAA, NO_2-DEAA, NH_2-DEAA are the intermediates produced by chemical synthesis and not real metabolites of butachlor, we didn't use them for mutagenicity assays. Our results demonstrated that the OH-DEA mixture is much more mutagenic than its parent compound butachlor and DEA, especially when S9 was added to the reaction. Since we do not have enough purified OH-DEA for DEBQI synthesis, the previous assay (OH-DEA with S9) was an alternative to test the mutagenicity of the further OH-DEA metabolites. S9 contains various enzymes for metabolism and it is assumed that OH-DEA in the reaction mixture would be metabolized by oxidation to the final compound DEBQI, which is the ultimate mutagen produced in this metabolic pathway. In the mutagenicity tables, a bipolar effect and a decrease in colony number were seen when the chemical concentration was raised. This is because the chemicals would seem to have both mutagenicity and cytotoxicity effects at the same time. Both cytotoxicity and mutagenicity increased when the concentration of chemicals was raised. The bacterial death caused by cytotoxicity would attenuate the mutagenicity since fewer colonies are found. The cytotoxicity and mutagenicity of butachlor, DEA, and OH-DEA for each strain of *Salmonella* varied and the colony number of revertants is a balance of the cytotoxicity and mutagenicity of the chemicals in each assay. Thus, some data show a decrease and other data show a bipolar

Table 3. Mutagenicity of 3,5-diethyl-p-amino-phenol (OH-DEA) on *Salmonella typhimurium*

OH-DEA (μg/plate)	Revertants per plate[a]					
	Without S9			With S9		
	TA97	TA98	TA100	TA97	TA98	TA100
0	116 ± 7	10 ± 2	91 ± 12	144 ± 12	18 ± 1	80 ± 4
6	122 ± 8	18 ± 2[c]	65 ± 6	178 ± 18	24 ± 2[c]	107 ± 5[c]
12	115 ± 7	21 ± 3[c]	67 ± 7	171 ± 17	33 ± 2[c]	108 ± 5[c]
18	129 ± 6	21 ± 2[c]	93 ± 7	187 ± 18[c]	36 ± 3[c]	131 ± 6[c]
24	114 ± 7	26 ± 3[c]	99 ± 6	177 ± 19	36 + TC[c]	123 ± 5[c]
72	112 ± 6	24 ± 3[c]	137 ± 11[c]	168 ± 17	39 + TC[c]	156 ± 6[c]
MNNG[b]	433 ± 24	29 ± 2	1819 ± 152	475 ± 32	34 ± 3	2974 ± 60

Abbreviations: TC = tiny colonies; MNNG = N-methyl-N'-nitro-N-nitrosoguanidine

[a]Values are mean ± standard deviation from more than 3 different experiments. Spontaneous revertants were not substracted from the number of revertants obtained for each assay.

[b]Positive control: 2 μg MNNG per plate was used as positive control.

[c]Indicates a significant increase from control (butachlor 0 μM) [$p<0.05$].

effect. According to the results of mutagenicity assays on the *S. typhimurium* strains, butachlor is likely to manifest mutagenicity via its metabolic products.

Although alachlor has been classified as a probable human carcinogen, there has been no such classification of butachlor up to the present. The classification of alachlor as a probable human carcinogen is based on findings of an increased incidence of tumors in rats chronically administered alachlor. One proposed mechanism of alachlor carcinogenicity involves its bioactivation to a DNA-reactive metabolite. Alachlor and butachlor are both chloroacetanilides. Chloroacetanilides have been shown to be extensively metabolized in the liver using labeled compounds incubated with subcellular fractions obtained from animal models [13,18,19], and it has been found that the metabolism of chloroacetanilides in rodents is very complex [20]. Although butachlor was also demonstrated to induce tumors in animals, its carcinogenic mechanism is still unclear. Our results showed that although butachlor is not a strong mutagen, its metabolites are highly mutagenic and this may provide a possible mechanism for butachlor carcinogenicity. Based on Table 1 and Table 2, a biphasic change in mutagenicity was detected. We have interpreted this as being due to concurrent increasing mutagenicity and cytotoxicity, since butachlor was found to be cytotoxic to cells at higher concentrations [7]. The cytotoxicity and mutagenicity could be varied in different bacteria strains.

Since butachlor has been used widely in many countries, an extensive study of its safety is very important. In addition to the mutagenicity and carcinogenicity problems, butachlor may have other toxicologic implications, for example, it could act as an environmental hormone [21]. Various chemicals such as pesticides, plasticizers and persistent pollutants are highly suspected of having endocrine-disrupting effects in animals and humans. These environmental endocrine-disrupting chemicals have been reported to be the cause of reproductive aberrations and teratogenic effects in the bald eagles of the Great Lakes [22], and to cause reproductive disorders and testicular cancer in humans [23,24]. Alachlor has been shown to mimic estrogen and is capable of binding to the estrogen receptor, causing transcription from estrogen response elements in DNA and causing the proliferation of MCF-7 cells in vitro [25]. Based on the similarity of butachlor and alachlor in their chemical structure and properties, it is highly probable that butachlor has the same endocrine disrupting properties. Accumulating evidence suggests that steroid hormones regulate apoptosis in hormone-responsive tissue. Alachlor and butachlor therefore may be considered to be environmental estrogens that can mimic the function or activity of endogenous estrogen 17beta-estradiol. These environmental estrogens may function as endocrine disrupters both in wildlife and humans, leading to developmental defects, disease and potentially cancer. Whether butachlor acts as an environmental estrogen is therefore of interest and under investigation at the present.

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