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Genetic Effects of Chlorinated Anilines and Azobenzenes on *Salmonella typhimurium*

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Abstract. The mutagenicity of 19 herbicide-derived chlorinated azobenzenes and structurally related chlorinated anilines and nitrobenzenes was assayed towards several strains of *S. typhimurium*, using the plate incorporation method and the fluctuation test, in the presence or in the absence of liver post-mitochondrial fractions, in aerobic and anaerobic conditions.

Positive results were obtained with 4,4′-dichloroazobenzene, 4,4′-dichloroazoxybenzene, 3,4-dichloronitrobenzene and, to a much lesser extent, with 3,4,3′,4′-tetrachloroazobenzene. No mutagenic effect was observed with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in any condition.

Numerous investigations (Bartha and Pramer 1967; Bartha *et al.* 1968; Bartha and Pramer 1970; Chisaka and Kearney 1970; Bordeleau *et al.* 1972; Kaufman *et al.* 1972) have shown that various important chloroanilide herbicides can be degraded in soil by microbial acylamidases with the release of chlorinated aniline moieties which are further transformed by soil fungal peroxidases to stable chlorinated azobenzene residues. These azo compounds have been shown (Still 1969) to be absorbed by plant roots and translocated to shoots and grains; therefore, they could eventually be consumed by animals or man.

Moreover, during the synthesis of chlorinated anilines or their subsequent conversion into herbicides, trace amounts of chloroazo- and chloroazoxybenzenes are formed as unwanted contaminants, some of which, such as 3,4,3′,4′-tetrachloroazobenzene (TCAB) and 3,4,3′,4′-tetrachloroazoxybenzene (TCAOB), have been responsible for three outbreaks of chloracne among chemical workers (Taylor *et al.* 1977).

TCAB and TCAOB present many similarities with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDBF), two well-known acnegen persistent contaminants formed during the production of the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) (Kimbrough 1974) and polychlorobiphenyls (PCBs) (Curley *et al.* 1975); they are

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considered as two of the most potent toxic and teratogenic molecules known. All four compounds are approximately isosteric and are potent inducers of hepatic microsomal enzymes (Poland *et al.* 1976; Saint-Ruf *et al.* 1979).

TCAB has been reported to increase the back mutation frequency of the $meth_3$ locus in *Aspergillus nidulans* (Prasad 1970) and to be weakly mutagenic towards *Salmonella typhimurium* TA 98 (Hsia *et al.* 1977).

Similarly, high concentrations of TCDD have been shown to induce high mutation frequencies in *S. typhimurium* TA 1532, which is known to revert by frameshift mutation (Hussain *et al.* 1972).

The present study was initiated to determine the possible genetic effects of structurally related chlorinated anilines and chlorinated azobenzenes on *Salmonella typhimurium* using either the classical Ames test (Ames *et al.* 1975) or the fluctuation test as developed by Green *et al.* (1977). In order to ascertain the possible role of liver microsomal azoreductases in the mutagenic activity of chlorinated azobenzenes, the assays were performed both in aerobic and anaerobic conditions as well as in the presence or absence of S-9 fraction (9000 × g supernatant) of rat liver homogenate.

Materials and Methods

Chemicals

All commercial products were of the purest grade available. 3,4-dichloronitrobenzene (DCNB), 4-dichloroaniline, 1-chloro, 2-nitro, and 1-chloro-4-nitrobenzenes and 4,4′-dichloroazoxybenzene (DCAOB) were obtained from Aldrich Europ, Janssen Pharmaceutica, Belgium; 3,4-dichloroaniline (DCA) was obtained from Fluka.

4,4′-dichloroazobenzene (DCAB) (m.p. 185°C), 3,4,3′,4′-tetrachloroazobenzene (TCAB) (m.p. 160°C), 2,3,2′,3′-tetrachloroazobenzene (m.p. 204°C), 2,4,2′,4′-tetrachloroazobenzene (m.p. 170°C), 2,5,2′,5′-tetrachloroazobenzene (m.p. 191°C), 3,5,3′,5′-tetrachloroazobenzene (m.p. 198°C), 2,4,2′,4′-tetrabromoazobenzene (m.p. 186°C), 3,3′-dichloro-4,4′-dimethylazobenzene (m.p. 132°C), 2,3,4,2′,3′,4′-hexachloroazobenzene (m.p. 250°C), 2,4,5,2′,4′,5′-hexachloroazobenzene (m.p. 216°C), and 3,4,5,3′,4′,5′-hexachloroazobenzene (m.p. 285°C) were prepared by the treatment of the corresponding chlorinated and brominated anilines with active manganese dioxide (purchased from Merck-Schuchardt) according to the procedure described by Wheeler and Gonzales 1964).

3,4,3′,4′-tetrachloroazoxybenzene (TCAOB) (m.p. 140°C) was obtained by the controlled reduction of 3,4-dichloronitrobenzene by $LiAlH_4$ in anhydrous ether (Corbett and Holt 1963).

2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) was prepared as described elsewhere (Buu-Hoï *et al.* 1971).

TCDD and the chlorinated azobenzenes were dissolved in dimethylsulfoxide (DMSO); the chlorinated anilines and nitrobenzenes were dissolved in absolute ethanol.

Animals

Adult male Wistar rats (200-250 g) were fed a (U.A.R. (AO3)-Animalabo, Bruxelles) diet. Drug treatments were administered as follows: phenobarbital sodium 0.1% in drinking water given *ad lib.* during seven days prior to sacrifice; Aroclor® 1254 in corn oil, 500 mg/kg body weight, intraperitoneally, five days prior to sacrifice; 3-methylcholanthrene in soja oil, 40 mg/kg body weight, intraperitoneally, 24 hr prior to sacrifice.

Salmonella typhimurium strains TA 98, TA 100, TA 1530, TA 1535, TA 1537, TA 1538, TA 1532, TA 1950, TA 1975, TA 1978, G 46 were obtained through the courtesy of Dr. Bruce Ames (University of California, Berkeley).

Mutagenicity Assays

Metabolic Activating System

The post-mitochondrial (S9) fractions were obtained from two or three pooled rat livers, the homogenate (3 ml of 0.15 M KCl/g wet liver) of which was centrifuged as described (Ames *et al.* 1975). Preparation of the S9 mix was made according to Ames *et al.* (1975) by adding $MgCl_2$ (8 μ mol/ml mix), KCl (33 μmol/ml mix), sodium phosphate (100 μmol/ml mix), glucose-6-phosphate (5 μmol/ml mix) and $NADP^+$ (4 μmol/ml mix). 50 μl, 100 μl, 200 μl or 300 μl (75 mg wet liver)/ml mix of S9 were utilized.

Plate Tests

Tests were performed in duplicate by mixing substrate dilutions (0.1 ml/plate), 1-7 $\times$ 10^7 bacteria from an overnight culture in nutrient broth (Difco)/plate, S9 mix (0.5 ml/plate) in histidine-biotin-supplemented top agar (2 ml/plate) which was afterwards layered on minimal glucose agar (Vogel Bonner E medium) in petri dishes. The plates were incubated for 48 hr at 37°C in the dark and the numbers of his + revertant colonies were calculated.

The toxicity of the substrate was evaluated by determining the bacterial survival: the operations were similar except that the number of bacteria was lower (10^4-10^5 fold dilution) and that top agar was poured on nutrient agar (Difco).

The assays in anaerobic conditions (20, 21) were carried out in a BBL GasPak Anaerobic system.

Bacterial Fluctuation Tests

Tests were performed in triplicate by using a modification of the method proposed by Green *et al.* 1977.

In a sterile tube kept in iced water, were successively introduced: liquid minimal glucose medium (Vogel Bonner E Medium) supplemented with histidine and biotin (0.005 mM) (4 ml); 2-8 $\times$ 10^7 bacteria from an overnight culture in nutrient broth (Difco), substrate dilution (0.1 ml), S9 mix (300 μl S9/ml mix) (1 ml). The homogenized mixture was distributed in 50 sterile tubes (0.1 ml/tube). The racks of 50 tubes were incubated at 37°C in the dark for 3 hr, 2 ml of histidine-biotin supplemented liquid minimal glucose medium containing bromocresol purple (BCP) (5 μg/ml) as pH indicator were then added to each tube.

After incubation for 72 hr at 37°C in the dark, the numbers of positive growing tubes (yellow)/rack were counted.

In the absence of S9 mix, the protocol was simplified as follows: 2-8 $\times$ 10^7 bacteria from an overnight culture in nutrient broth (Difco) and substrate dilution (0.1 ml) were added to histidine-biotin (0.005 mM) supplemented liquid minimal glucose medium containing BCP (100 ml). The homogenous mixture was distributed at constant volume into 50 tubes. The racks were incubated at 37°C in the dark for 72 hr. The numbers of positive tubes/rack were counted.

Results

Plate Incorporation Assays

Substantially negative results were obtained when the following compounds 4-chloroaniline, 1-chloro,2-nitrobenzene, 1-chloro,4-nitrobenzene, DCA, TCAB, TCAOB, 2,5,2′,5′-tetrachloroazobenzene, 3,3′-dichloro-4,4′-dimethylazobenzene, 3,5,3′,5′-tetrachloroazobenzene, 2,3,2′,3′-tetrachloroazobenzene, 2,4,2′,4′-tetrachloroazobenzene, 2,4,2′,4′-tetrabromoazobenzene, 2,3,4,2′,3′,4′-hexachloroazobenzene, 2,4,5,2′,4′,5′-hexachloroazobenzene,

3,4,5,3′,4′,5′-hexachloroazobenzene and TCDD, were assayed towards the various experimental strains.

The assays were performed both in the absence and in the presence of a liver microsomal fraction; incubations were carried out in aerobic as well as in anaerobic conditions and the concentrations varied from 1 μg up to 2,000 μg per plate.

In any case, the number of his + revertants/plate was greater than two- to threefold the number of spontaneous revertants. DCNB, when utilized at high doses produces a slight but statistically significant mutagenic activity towards TA 1530 in the absence of S9 mix and towards TA 100 and TA 1538 both in the absence and in the presence of S9 mix Aroclor 1254® (Table 1). However, in this concentration range, the cytotoxic effect was marked (about 100% at 1,200 μg/plate).

DCAB and DCAOB exhibited mutagenic effects towards strains TA 1538, TA 98 and TA 100 in the presence of S9 mix Arochlor 1254. The effect was particularly marked on TA 98. TA 1538 seemed to be particularly sensitive to DCAB (Figure 1). Moreover, the reversion rate of DCAB and DCAOB was related to the S9 mix composition (Table 2).

As shown in Figure 2, positive liver mediated mutagenic effects were observed only when those liver post mitochondrial fractions were obtained from Aroclor 1254® or 3-MC pretreated animals. Negative results were obtained with liver fractions from control or phenobarbitone pretreated animals.

Fluctuation Tests

Except for the results presented in Table 3, all the assays performed with the compounds listed above either in the absence or in the presence of fortified S9 fractions were negative (P = NS). DCAB and DCAOB, which were clearly mutagenic in the plate incorporation tests were not submitted to the fluctuation tests.

DCNB shows a weak mutagenic activity toward strains TA 100 and TA 1538; the number of revertants is slightly enhanced in the presence of a fortified S9 fraction.

A very weak mutagenic activity was observed with TCAB in the presence of S9 mix Aroclor 1254®, in a very narrow range of concentrations. However, the statistical significance of that results is debatable.

Discussion

Among the 19 herbicide-derived chlorinated azobenzenes and structurally related chlorinated anilines and nitrobenzenes assayed with the *S. typhimurium* test system developed by Ames (1975), two (DCAB and DCAOB) were found to be mutagenic in the "classical" plate incorporation test after incubation in aerobic conditions, in the presence of fortified liver post mitochondrial fractions obtained from rats pretreated with Aroclor 1254® or 3-MC. The mutagenicity of a third one, DCNB, detected by the plate incorporation method was confirmed in fluctuation tests performed aerobically with and without fortified S9.

Table 1. Mutagenic activity of DCNB

Strain	TA 1530		Number of His + rev./plate TA 1535		TA 100		TA 1537		TA 1538		TA 98	
μg/plate	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
0	24	24	13	10	123	121	7	8	14	22	26	35
1	32	12	11	14	154	138	6	11	9	24	19	33
10	31	15	11	18	148	145	7	7	8	28	27	33
100	30	18	13	16	190	172	7	7	16	33	26	37
200	30	10	10	11	206	204	5	9	13	60	14	42
500	85	15	16	16	191	321	15(T)	12	30	81	22	39
800	62	20	19(T)	19		308	12(T)	13(T)	47(T)	70(T)	38(T)	54(T)
1,000	63	16(T)	25(T)	14(T)	234(T)	323(T)	2(T)	11(T)	71(T)	82(T)	26(T)	63(T)
1,500	35(T)	30(T)	5(T)	(T)	2(T)	382(T)	(T)	6(T)	18(T)	(T)	9(T)	45(T)

(a) Without S9mix; (b) with S9 mix Aroclor 1254®

(T) Cytotoxic effect. Incubation in aerobic conditions.
Concentration of S9 mix: 100 μl S9/ml mix.

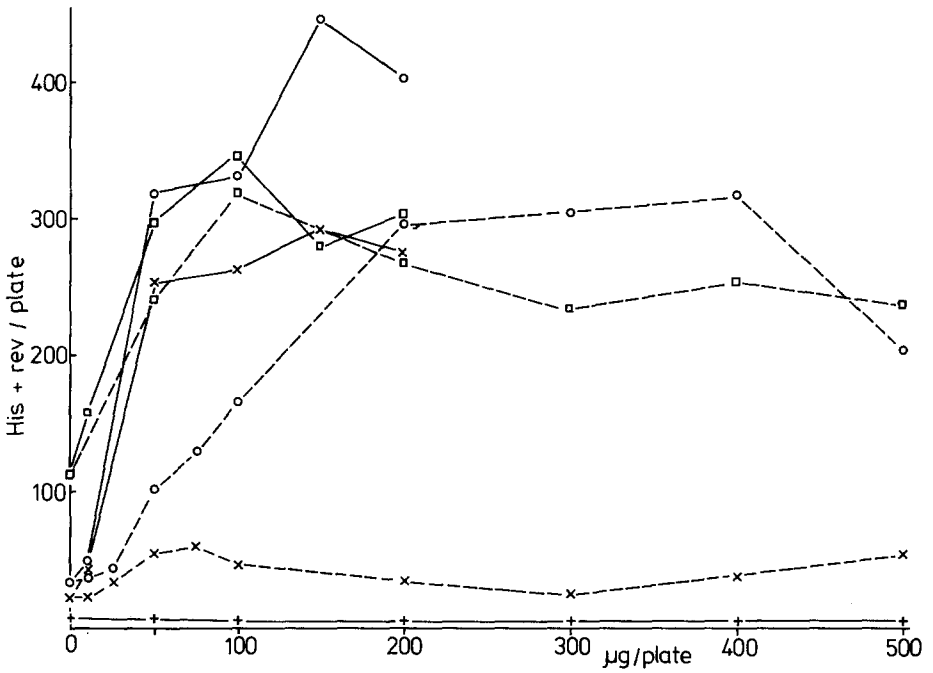

Fig. 1. Mutagenic activity of DCAB (straight lines) and DCAOB (dotted lines) towards TA 1538 (×), TA 98 (○), TA 100 (□) and TA 1535 (+) in the presence of S9 mix Aroclor 1254® (100 µl S9/ml mix). Incubations were carried out in aerobic conditions

Finally, a scarcely significant mutagenic effect of TCAB was observed on TA 1538 and TA 1532 in the presence of fortified S9 fractions, using the fluctuation test.

All assays carried out in anaerobic conditions were negative, which suggests that the microsomal azoreductases do not play a major role in the possible activation of chlorinated azobenzenes to mutagenic intermediates.

Moreover, our results demonstrating a very weak mutagenic activity of TCAB as well as a total inactivity of TCDD towards *S. typhimurium* are in disagreement with previously reported data (Hsia 1977; Hussain *et al.* 1972). In the particular case of TCDD, it must be noted that the positive results described elsewhere (Hussain *et al.* 1972) were obtained at concentrations well above the solubility limit of the compound, which makes the significance of the results obtained by those authors questionable. Nevertheless, the dichlorinated derivatives (DCNB, DCAB and DCAOB) have been shown to be mutagenic toward several strains of *S. typhimurium*.

Recent reports indicate that it is more and more evident that most chemical carcinogens are mutagens and that many mutagens, whose carcinogenicity has not yet been investigated, have in fact now been shown to be carcinogens (Bartsch 1976); therefore, the somatic-cell mutation theory of carcinogenesis has regained more attention.

A recent evaluation of six short-term tests for detecting organic chemical carcinogens (Purchase *et al.* 1976) using 120 organic chemicals, including 58

Table 2. Effect of the S9 mix composition on the mutagenicity of DCAB (a) and DCAOB (b) towards TA 98[a]

N° of His + rev./plate										
	Composition of S9 mix Aroclor 1254®: μl S9/ml mix									
μg/plate	50 μl		100 μl		150 μl		200 μl		300 μl	
	a	b	a	b	a	b	a	b	a	b
0	28	28	30	30	29	27	43	43	42	42
10	78	39	49	34	51	33				
50	177	77	319	103	269	67	368		391	
100	210	112	333	161	515	122	702	206	852	113
150	218		452		502		684		742	
200	221		412	301	603	238	736	333	734	236
300				306		308		284		331
400				315		201		330		313
500		110		204		275		349		279

[a] Experimental conditions are described in Materials and Methods. Incubations were carried out in aerobic conditions

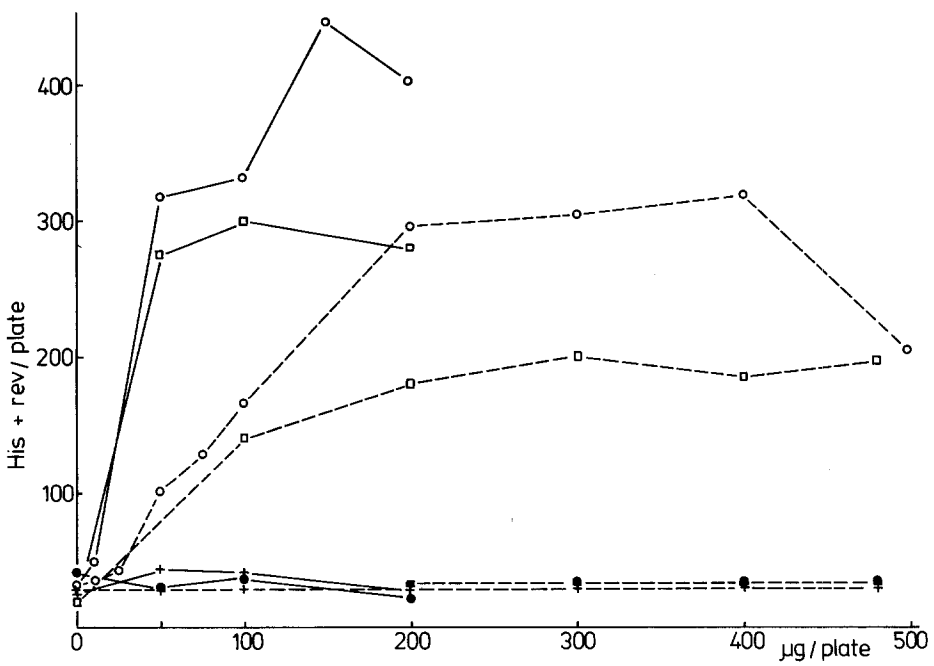

Fig. 2. Effects of the pretreatments applied to rats on the mutagenicity of DCAB (straight lines) and DCAOB (dotted lines) towards TA 98: Congrol (+), Phenobarbital (●), Aroclor 1254® (○), Methyl-3-cholanthrene (□). Concentration of S9 in the S9 mix: 100 μl S9/ml mix. Incubations were carried out in aerobic conditions

Table 3. Results of fluctuation tests[a]

Strain	S9 mix	μg/ml	N° of experiment	Average number of positive tubes per rack Control	Treated	Significance (P)
DCNB						
TA 100	None	5	3	19.3	25	NS
		10			37.6	<0.001
		15			37.3	<0.001
	Aroclor 1254®	5	3	15.9	38.6	<0.001
		10			46	<0.001
		15			47.3	<0.001
	Phenobarbital	5	1	25	31	NS
		10			43	<0.001
		15			29	NS
TA 1538	Aroclor 1254®	5	3	10.6	30	<0.001
		8			28.3	<0.001
		10			27.6	<0.001
		15			26	<0.001
TCAB						
TA 1538	Aroclor 1254®	1.25	2	12	23.5	<0.05
TA 1532	Aroclor 1254®	1.25	2	9	10	NS
		2.50			17	<0.05
		3.75			16	NS

[a] Experimental conditions are described in Materials and Methods

known human or animal carcinogens, disclosed a 93% of accurate predictions using the rat liver microsome test in vitro with *Salmonella typhimurium* strains, as utilized in the present study.

The good correlation observed between carcinogenic and mutagenic activity indicate that the compounds which were found to be mutagenic in the present study could present a possible carcinogenic activity in animals and man.

Therefore, in order to assess their possible health hazard, the possible formation of those compounds, by degradation of related herbicides in the environment, should be carefully evaluated.

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