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MUTAGENICITY OF CHLORINATED CYCLOPENTADIENES DUE TO METABOLIC ACTIVATION

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Abstract—Chlorinated cyclopentadienes of which hexachlorocyclopentadiene is used for pesticide synthesis is suggested to undergo metabolic conversion forming acylating and possibly mutagenic tetrachlorocyclopentadienone. Tetra-, penta-, and hexachlorocyclopentadiene differ in the chlorine substitution at C-1. Oxygen insertion into C-1, which results in the formation of the dienone should depend on the degree of chlorine substitution at this position. The dienone is not stable and can only be isolated as the dimer. To detect its formation an *in vitro*-test system, comprising mouse liver microsomes for metabolic activation and *E. coli* K 12 (343/113) to detect mutagenicity, has been used.

According to the previous suggestions tetrachlorocyclopentadiene and pentachlorocyclopentadiene were highly mutagenic after metabolic activation, whereas hexachlorocyclopentadiene was not.

Chlorinated cyclopentadienes are used for pesticide synthesis [1]. Although workers who are involved in pesticide synthesis may be exposed to those compounds, virtually no toxicological evaluation of these chemicals has been performed. Maximal allowable concentrations are only given for the non-halogenated cyclopentadienes. Similar to the metabolic activation of dichloromethane [2], oxygen insertion at C-1 of chlorinated cyclopentadienes might occur leading to the formation of tetrachlorocyclopentadienone in the presence of liver microsomal enzymes.

In analogy to the stabilizing effect of halogen substitution of ethylenes [3], oxygen attack is unlikely to occur at halogenated sites of the molecules. This also implies that tetrachlorocyclopentadiene and pentachlorocyclopentadiene should be more susceptible to oxygen insertion at C-1 than hexachlorocyclopentadiene which carries two chlorine atoms at this position. Thus, quantitative differences should be expected in the formation of the dienone from the three chlorinated cyclopentadienes.

However, due to its high reactivity tetrachlorocyclopentadienone has only been isolated as the dimer [4]. In anology to perchlorodihydropentalenone [5] it is a vinylogous acid chloride with probably acylating properties and may be mutagenic.

To detect the formation of the dienone and to demonstrate its potential mutagenic properties, a test system has been used which comprises mouse liver microsomes for metabolic conversion of the dienes to the dienone and a suitable *E. coli* strain to detect mutagenic activity of the reaction product formed. The results demonstrate that addition of tetrachlorocyclopentadiene and pentachlorocyclopentadiene to the test system increases mutation frequency severalfold, whereas hexachlorocyclopentadiene has no such effect.

MATERIALS AND METHODS

Hexachlorocyclopentadiene was purchased from BASF, Ludwigshafen, Germany. Pentachlorocyclopentadiene was synthesized by Dr. Lahaniatis, Institut für Ökologische Chemie der GSF, Neuherberg, as described by McBee *et al.* [6]. Tetrachlorocyclopentadiene was synthesized according to Roedig and Hörnig [7]. The compounds were gaschromatographically pure. The melting points corresponded to those given in the literature (hexachlorocyclopentadiene 83–84° [8], pentachlorocyclopentadiene 84–86° [9], tetrachlorocyclopentadiene 62–63° [10].

Chemicals used in the bacterial metabolizing test system to detect mutagenicity such as Bacto agar, Bacto nutrient broth, were from Difco Laboratories; amino acids, galactose, glucose and methyltryptophane, DL-isocitrate-Na_3 from E. Merck AG, Darmstadt; isocitrate dehydrogenase and NADP from Boehringer, Mannheim.

Mutagenic activity was tested in a liquid *in vitro* system using *Escherichia coli* K 12 (343/113) [11, 12], as previously described [13, 14]. In the test procedure 4 to 8 × 10^8 cells of a growing culture (3–4 hr) were suspended in 1.35 ml incubate containing 7.5 mg microsomal protein, isolated from mouse liver, a NADPH-generating system consisting of 7.0 mM $MgCl_2$, 4.5 mM NADP, 19.0 mM DL-isocitrate, 2 U isocitrate dehydrogenase in 0.1 M phosphate buffer pH 7.4 and different concentrations of the test compounds. This mixture was incubated in a shaking water bath at 37° in the dark for 60 or 120 min.

Subsequently, 7 ml of ice cold saline were added,

Table 1. Mutation frequency in different systems of *Escherichia coli* K-12 after incubation with hexa-, penta- and tetrachlorocyclopentadiene in the presence of mouse liver microsomes ($\bar{x} + s_x$)

Concentration Cyclopentadiene (M)	Colony-forming units per ml ($\times 10^8$)	Survival %	Mutant colonies per plate gal$^-$	mtr$^+$	nad$^-$	arg$^-$	Mutant colonies per plate per survivors gal$^-$	mtr$^+$	nad$^-$	arg$^-$
Hexachloro-										
None	3.05 ± 0.1	100	52 ± 2	16 ± 2	2 ± 1	53 ± 2	52	16	2	53
2.7×10^{-3}	2.14 ± 0.16	70	53 ± 6	10 ± 9	2 ± 2	48 ± 10	76	14	3	69
2.7×10^{-4}	2.43 ± 0·09	80	47 ± 5	13 ± 3	2 ± 1	50 ± 11	59	16	3	63
Pentachloro-										
None	4.57 ± 0.20	100	135 ± 14	17 ± 3	2 ± 1	55 ± 4	135	17	2	55
2.4×10^{-3}	3.99 ± 0.31	87	126 ± 42	19 ± 5	25 ± 9	242 ± 16	145	22	29	278
2.4×10^{-4}	4.68 ± 0.25	102	146 ± 9	19 ± 2	4 ± 2	76 ± 12	144	19	4	75
2.4×10^{-5}	4.71 ± 0.16	103	133 ± 11	17 ± 4	3 ± 1	63 ± 6	133	17	3	61
Tetrachloro-										
None	8.56 ± 0.41	100	138 ± 4	15 ± 1	2 ± 1	70 ± 2	138	15	2	70
1.0×10^{-3}	5.90 ± 0.34	69	107 ± 10	38 ± 7	17 ± 4	283 ± 33	155	55	25	410
1.0×10^{-4}	6.85 ± 0.38	80	127 ± 10	33 ± 3	14 ± 3	231 ± 25	159	41	18	288
1.0×10^{-5}	7.36 ± 0.47	86	128 ± 6	15 ± 6	5 ± 2	130 ± 14	149	17	6	151

Underlined figures differ from control values.

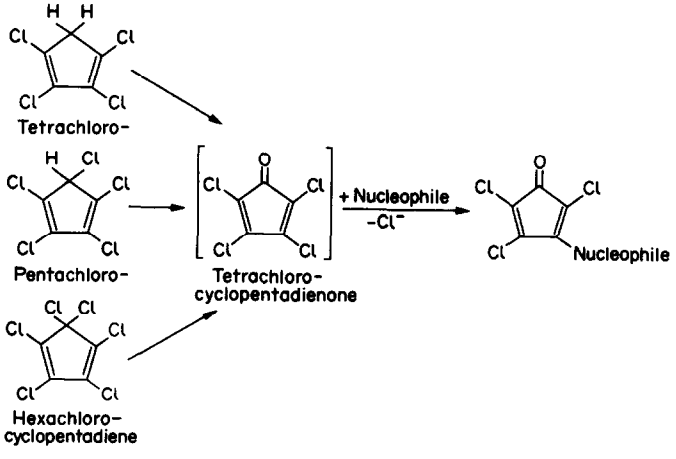

Fig. 1. Proposed metabolic formation of reactive tetrachlorocyclopentadienone from chlorinated tetrachlorocyclopentadienes.

and the tubes were centrifuged twice at 7000 *g*. The pellet was resuspended in 1.35 ml saline. Aliquots of 0.1 ml or 0.1 ml of 10^{-1} or 10^{-2} dilutions were plated on selective media to detect mutants. Cytotoxicity of the test compounds was determined by plating 0.1 ml of a 10^{-5} dilution of complete medium. Colonies of survivors on complete medium were counted after 20–24 hr, colonies of mutants on the selective media after 36–72 hr. The results given in Table 1 represent the mean values of three plates of a representative experiment.

Liver microsomes have been isolated from male mice pretreated for 10 days with 0.1 per cent phenobarbital in the drinking water.

RESULTS AND DISCUSSION

Tetra-, penta- and hexachlorocyclopentadienes at concentrations above 10^{-3} M were cytotoxic to *E. coli*. At these concentrations the survival rate of the bacteria was reduced by 20–30 per cent. Consequently, for mutagenicity testing no more than 2.7×10^{-3} M hexachloro-, 2.4×10^{-3} M pentachloro-, and 10^{-3} M tetrachlorocyclopentadiene were used (Table 1).

When the compounds were incubated in the test system without metabolically active microsomes no increase in spontaneous mutation frequency could be observed indicating that they are not mutagenic *per se*.

However, in the presence of the complete metabolizing system, tetrachloro- and pentachlorocyclopentadiene significantly increased mutation frequency in the MTR$^+$ (induction of methyl triptophane resistance)- and the arg$^-$ systems (Table 1, underlined numbers), whereas hexachlorocyclopentadienone did not.

We conclude from these experiments that metabolic insertion of oxygen into the C-1 position of hexachlorocyclopentadiene is hindered by the presence of two chlorine atoms, whereas no or only one chlorine atom at this position permits oxygen insertion (Fig. 1). A further differentiation on the availability of the

C-1 position to the oxygen attack on tetra- and pentachlorocyclopentadiene has not been possible because both compounds are unstable as well as insoluble. Within 10 min after addition of the compounds to the incubation system, the mixture turned dark, indicating degradation or dimerization [15]. These dimers were not mutagenic when determined with or without metabolic activation at concentrations up to 2.7×10^{-3} M (data not given in the table).

In analogy to perchlorodihydropentalenone we suggest that tetrachlorocyclopentadienone is an acylating agent [5] which may induce base pair substitutions. This agrees with our findings that the arg^--system and the MTR^+-system are affected. The two mutation systems are known to be changed by mutants causing base pair substitutions [11]. However, tetrachlorocyclopentadiene also increased mutation frequency in the nad^--system which can only be reverted by frameshift mutagens [11]. This system has been introduced by treatment of the wild strain with the frameshift mutagen ICR-191 [2-methoxy-6-chloro-9-(2-chloroethylaminopropyl)-amino acridine]. Neither our investigations nor observations of others, however, present any evidence that frameshift mutagens such as benzo(a)pyrene, methylcholanthrene or aminofluorene do revert the nad^--system. We therefore cannot explain the positive effect seen with tetrachloro- and pentachlorocyclopentadiene on those systems.

Of the three chlorinated cyclopentadienes only the hexachlorocyclopentadiene is used in the manufacture of pesticides. According to the present investigations this compound is not metabolized to mutagenic species. However, tetra- and pentachlorocyclopentadiene undergo microsomal metabolism forming mutagenic reactants, possibly tetrachlorocyclopentadienone. In view of the positive mutagenicity results, long-term animal studies with those compounds are required.

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