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CHEMICAL CHANGES OF ORGANIC COMPOUNDS IN CHLORINATED WATER

XI*. THIN-LAYER CHROMATOGRAPHIC FRACTIONATION OF AMES MUTAGENIC COMPOUNDS IN CHLORINE-TREATED 4-METHYLPHENOL SOLUTION

SUKEO ONODERA*, MIKA YAMASHITA, SHUNJI ISHIKURA and SHIZUO SUZUKI
Faculty of Pharmaceutical Sciences, Tokyo University of Science, 12, Ichigaya-funagawara-machi, Shinjuku-ku, Tokyo 162 (Japan)
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SUMMARY

The diethyl ether extract from an aqueous solution of 4-methylphenol after treatment with hypochlorite was mutagenic to the Ames *Salmonella* test strain TA100 in the absence of liver homogenate. Gas chromatography–mass spectrometry (GC–MS) showed the occurrence of, at least, twenty compounds in the extract: chloro-4-methylphenols, chlorohydroxy-4-methylquinones and chlorinated 4-methylphenol dimers. The diethyl ether extract was fractionated into several fractions by silica gel and polyamide thin-layer chromatography (TLC). The fractionated components were then examined for mutagenicity by means of Ames assays, and were identified by GC–MS. TLC fractionation of the extract revealed that the major components present in the extract are not mutagenic, but minor components (less than 4% of the total extract) are mutagenic. GC–MS analysis indicated the presence of chlorinated 4-methylphenol dimers in the fraction which exhibited the highest mutagenicity.

INTRODUCTION

Ever since it was reported that chlorination of drinking water sources and waste waters produces mutagenic substances[1,2], the interest in their precursors and chemical composition in unchlorinated and chlorinated waters has increased. In the past few years, several publications have described the identification and testing of mutagenic compounds found in various chlorinated waters[3–25]. Several volatile compounds exhibiting mutagenicity have been identified as low-molecular-weight chloroacetones, vinyl chloride and other chemicals by using gas chromatography–mass spectrometry (GC–MS)[26–33], but many of the non-volatile mutagenic substances have not been fully characterized.

* For Part X, see ref. 35.

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The presence of these uncharacterized mutagens in drinking water has been directly linked with water chlorination practices[5,20,21,23–25]. It seems that they are also formed as a result of the reaction of chlorine with phenolic compounds which may be present in the water sources. Direct support for this hypothesis has been provided by Rapson *et al.*[10] and by our recent studies which demonstrated that phenols, naphthols and phenylphenols react with chlorine to form direct-acting mutagenic products[15,22]. We now present more detailed findings on the characteristics of the mutagens formed by the reaction of chlorine with 4-methylphenol in dilute aqueous solution.

EXPERIMENTAL

Materials

4-Methylphenol, 2-chloro-4-methylphenol, 2,6-dichloro-4-methylphenol, trichloroacetic acid and chloromaleic acid used for product identification and mutagenicity tests were commercial products. An hypochlorite solution was prepared by diluting sodium hypochlorite solution (*ca.* 10% available Cl; Nakarai Chemicals, Kyoto, Japan) with 1 *M* buffer solution of pH 7. The hypochlorite concentration was determined by iodometric titration. Silica gel F_{254} (20 × 20 cm, thickness 0.25 mm) and Polyamide 11 F_{254} (20 × 20 cm, thickness 0.15 mm) pre-coated thin-layer chromatography (TLC) plates (E. Merck, Darmstadt, F.R.G.) were used to fractionate the diethyl ether extract.

Treatment of aqueous 4-methylphenol solution with chlorine and extraction of reaction mixture

Aqueous 4-methylphenol (0.5 mmol/l) was treated with 20 equiv. of chlorine at 20°C for 1 h at pH 7. The unreacted chlorine was removed by addition of sodium thiosulphate solution. The reaction mixture was then acidified to pH 2.0 with 0.1 *M* hydrochloric acid before extracting with three 200-ml portions of diethyl ether. The extracts were dried over anhydrous sodium sulphate and concentrated under vacuum at 40°C to volumes suitable for silica and polyamide TLC, GC and GC–MS analyses. A part of the diethyl ether extract was resuspended in 2 ml of dimethyl sulphoxide for mutagenicity assays.

Fractionation of mutagenic extract by TLC

First, the diethyl ether extract (about 200 mg) was fractionated into six fractions (A–F) by TLC on silica gel plates using acetone–hexane (1:1, v/v) as a developing solvent. The detection of the spots on the plates was performed by UV irradiation. The plates were also sprayed with 0.5% bromocresol green solution in order to detect acidic products. The separated zones were scraped off by using special recovery tubes (Wako, Osaka, Japan) and the adsorbed substances were then eluted with diethyl ether. The R_F values of the fractions and the amounts of sample recovered from each fraction are shown in Table I. The recoveries (%, w/w) of individual fractions were calculated from:

$$[(\text{Amount of sample recovered})/(\text{amount of sample applied})] \cdot 100$$

TABLE I

RESULTS OF TLC OF THE DIETHYL ETHER EXTRACT ON SILICA GEL WITH ACETONE–HEXANE (1:1, v/v), AND MUTATION TESTS WITH STRAIN TA 100 IN THE ABSENCE OF S9 FOR EACH FRACTION AND SEVERAL STANDARD COMPOUNDS

Fraction No. and standard compound	R_F *values*	*Amount of sample recovered*		*Net revertants (· 10⁴)*		
		mg	*% (w/w)**	*per mg*	*per fraction (%)***	
Original extract	–	244	100	2.2	537	100
A	0.00–0.08	0.2	0.08	0.1	0.1	0.02
B	0.08–0.26	0.7	0.28	0.1	0.1	0.02
C	0.26–0.48	0.2	0.08	0.1	0.1	0.02
D	0.48–0.65	14.6	5.98	0.5	6.6	1.23
E	0.65–0.90	24.7	10.12	1.1	27.4	5.10
F	0.90–1.00	58.9	24.14	3.3	191.4	35.60
4-Methylphenol	0.90–0.95				ND***	
2-Chloro-4-methylphenol	0.90–0.96				ND	
2,6-Dichloro-4-methylphenol	0.92–0.97				ND	
Trichloroacetic acid	0.00–0.18				ND	
Chloromaleic acid	0.00–0.25				ND	

* [(Amount of sample recovered)/(amount of sample applied)] · 100.

** Mutagenicity derived from the initial and linear portion of the dose–response curve for each sample (Fig. 7).

*** Not detected in the tested range of 1–100 μg per plate.

In the second step, fraction F, which exhibited strong mutagenicity, was further fractionated into nine fractions [F(a)–F(i)] by TLC on polyamide plates, developed with chloroform. Detection was performed as described above, as were scraping off of the separated zones and the elution of the adsorbed substances. The R_F values of

TABLE II

RESULTS OF TLC OF FRACTION F (TABLE I) ON POLYAMIDE WITH CHLOROFORM, AND MUTATION TESTS FOR EACH COMPONENT WITH STRAIN TA 100 IN THE ABSENCE OF S9

Details as in Table I.

Fraction No.	R_F *values*	*Amount of sample recovered*		*Net revertants (· 10⁴)*		
		mg	*% (w/w)*	*per mg*	*per fraction (%)*	
Fraction F		166	100	3.3	598	100
F(a)	0.00–0.10	trace	–	0.1	0.1	–
F(b)	0.10–0.20	2.6	1.56	0.5	1.3	0.2
F(c)	0.20–0.60	11.4	6.86	2.5	28.5	4.8
F(d)	0.60–0.71	20.8	12.52	0.3	6.2	1.0
F(e)	0.71–0.78	35.2	21.20	0.3	10.6	1.8
F(f)	0.79–0.83	16.0	3.64	2.2	35.2	5.9
F(g)	0.85–0.87	4.5	1.70	6.1	27.5	4.6
F(h)	0.90–0.92	11.2	6.74	6.5	72.8	12.6
F(i)	0.93–1.00	14.2	8.56	12.5	181.3	30.3

the components and the amounts recovered from each fraction are shown in Table II.

Gas chromatography–mass spectrometry

A Shimazu GC-6A gas chromatograph equipped with a flame ionization detector and a 2 m × 0.3 cm I.D. glass column packed with 10% Apiezon L on Chromosorb W AW DMCS (60–80 mesh) was programmed from 180 to 280°C at 5°C/min. The carrier gas (nitrogen) flow-rate was 50 ml/min. A Shimazu Model Chromatopac-1A data system was used to determine the retention times and the areas of peaks on the chromatograms.

An Hitachi M-80 combined mass spectrometer–gas chromatograph equipped with an Hitachi M-003 data-processing system was used for the qualitative analyses of samples under the following conditions. The ion source was operated at 250°C with a trap current of 70 μA and an electron energy of 70 eV. A glass column (2 m × 0.3 cm I.D.) packed with 5% SE-30 on Chromosorb W AW DMCS (60–80 mesh) was used for the GC separation of the diethyl ether extracts and TLC-separated components. The oven temperature of the gas chromatograph was programmed from 220 to 300°C at 5°C/min. Compounds in the diethyl ether extract and TLC-separated components were identified by comparison of their retention times and mass spectra with those those of authentic compounds.

Mutagenicity tests

The mutagenicity of the samples was tested according to the method of Ames *et al.*[34] with minor modifications. Liver homogenate (S9) was prepared from male Sprague-Dawley rats, which had been pre-treated with polychlorinated biphenyls to activate enzymes. *Salmonella typhimurium* strains TA 98 and TA 100 were used throughout the experiments. The samples were dissolved in dimethyl sulphoxide and pre-incubated with a test strain at 30°C for 30 min (prior to plating) with and without S9. A buffer was used when S9 was not employed. After addition of the test samples, the plates were incubated at 37°C for 2 d. The assay was performed in triplicate for each sample. The mutagenic activity is expressed as the mean value of revertants.

RESULTS AND DISCUSSION

The previous Part[35] revealed that treatment with chlorine of methylphenol solutions (*o*-, *m*- and *p*-cresols) in dilute aqueous solution produces a series of highly chlorinated compounds, including chlorinated methylphenols, polychlorinated polyhydroxy-methylphenols and polychlorinated methylphenol dimers. In addition Owada and Matsushima[36] have demonstrated that the mutagenic substances (positive to strain TA100 without S9) produced from the reaction of methylphenol with hypochlorite in dilute aqueous solution are non-volatile and lipid soluble.Since these mutagenic substances were not characterized in the previous papers[35,36], further GC–MS studies combined with TLC fractionation of the mutagenic diethyl ether extract were performed in the present work.

GC and GC–MS studies of original diethyl ether extracts

A typical GC (with flame-ionization detection) trace of a mutagenic diethyl

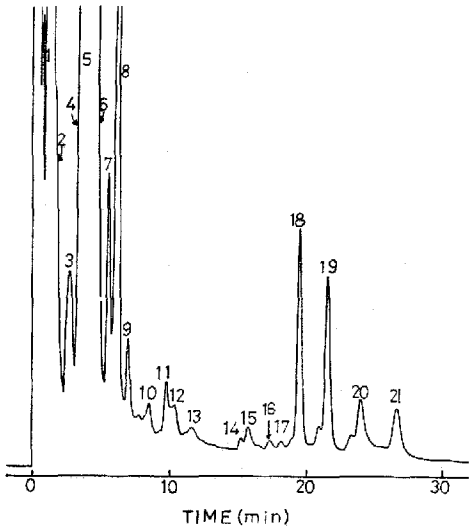


Fig. 1. Typical gas chromatogram (with flame ionization detection) of a mutagenic diethyl ether extract of 4-methylphenol solution (0.5 m*M*) after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) at 20°C for 1 h. Main compounds as in Table III. For GC conditions, see Experimental.

ether extract of 4-methylphenol after treatment with hypochlorite (20 equiv. of chlorine per mol of compound) is shown in Fig. 1. It indicates occurrence of, at least, twenty products in the extract. Some of the peaks were identified on the basis of the retention times of known compounds. Compounds corresponding to other peaks were determined from the mass spectrum of each peak (Figs. 2–4).

Fig. 2A shows the mass spectrum of peak 5, which is the main GC peak of the mutagenic extract. The molecular ion (M^+ is at *m/e* 242 with three chlorine atoms and the most abundant fragment ion is at *m/e* 123 ($C_5H_5Cl_2O$), which arises by loss of COCl and CO from the molecular ion, indicating the occurrence of $C_7H_5Cl_3O_3$ in the extract. Although the compound, $C_7H_5Cl_3O_3$, corresponding to peak 5 is considered from the nature of the reaction of 4-methylphenol with chlorine and its mass fragmentation pattern to be probably a trichlorodihydroxylated methylcyclohexenone, it could not be identified unambiguously. The mass spectrum of peak 8 (Fig. 2B) gave the same fragment ions at *m/e* 179, 123, 87 and 53 as those of peak 5. Although the compound corresponding to peak 8 is considered to be analogous to that corresponding to peak 5, its exact nature is not clear.

Fig. 3A illustrates the mass spectrum of a dichlorinated compound, corresponding to peak 18 from a diethyl ether extract of chlorine-treated 4-methylphenol solution. The molecular ion (M^+) is at *m/e* 282 and several fragment ions occur at *m/e* 267 ($M^+ - CH_3$), 219 ($M^+ - Cl - CO$), 212 ($M^+ - 2Cl$) and 146 ($M^+ - 2Cl - C_4H_2O$), indicating the occurrence of a 4-methylphenol dimer with two chlorine atoms. The mass spectrum of peak 19 (Fig. 3B) was interpreted as being that of a 4-methylphenol dimer with three chlorine atoms. The same molecular ion, but a different fragmentation pattern, was also observed for peak 20 (Fig. 4A). This leads to the conclusion that two isomers of a 4-methylphenol dimer with three chlorine atoms are present in the diethyl ether extract from chlorine-treated 4-methylphenol

solution. Fig. 4B shows that peak 21 with the molecular ion at *m/e* 350 is a 4-methylphenol dimer with four chlorine atoms.

A summary of the chlorinated products identified or determined from their GC retention times and mass spectra is presented in Table III.

TLC fractionation of mutagenic diethyl ether extract

A preliminary fractionation of the mutagenic diethyl ether extract from the chlorinated 4-methylphenol solution was performed by TLC using silica gel plates and subsequently polyamide plates. The TLC results for the original extract and fraction F, and mutagenicity tests with strain TA100 in the absence of S9, are shown in Tables I and II, and presented graphically in Figs. 5 and 6.

When the original diethyl ether extract was fractionated on silica gel plates (Fig. 5), the recovery in the fractions was approximately 41% (w/w) of the amount

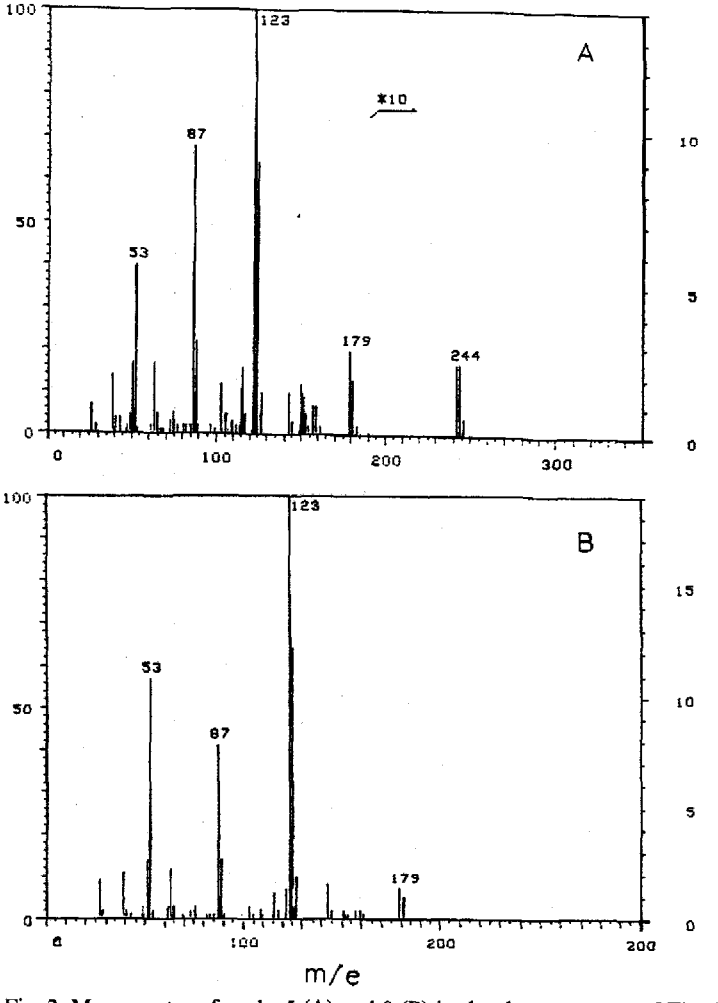


Fig. 2. Mass spectra of peaks 5 (A) and 8 (B) in the chromatogram of Fig. 1.

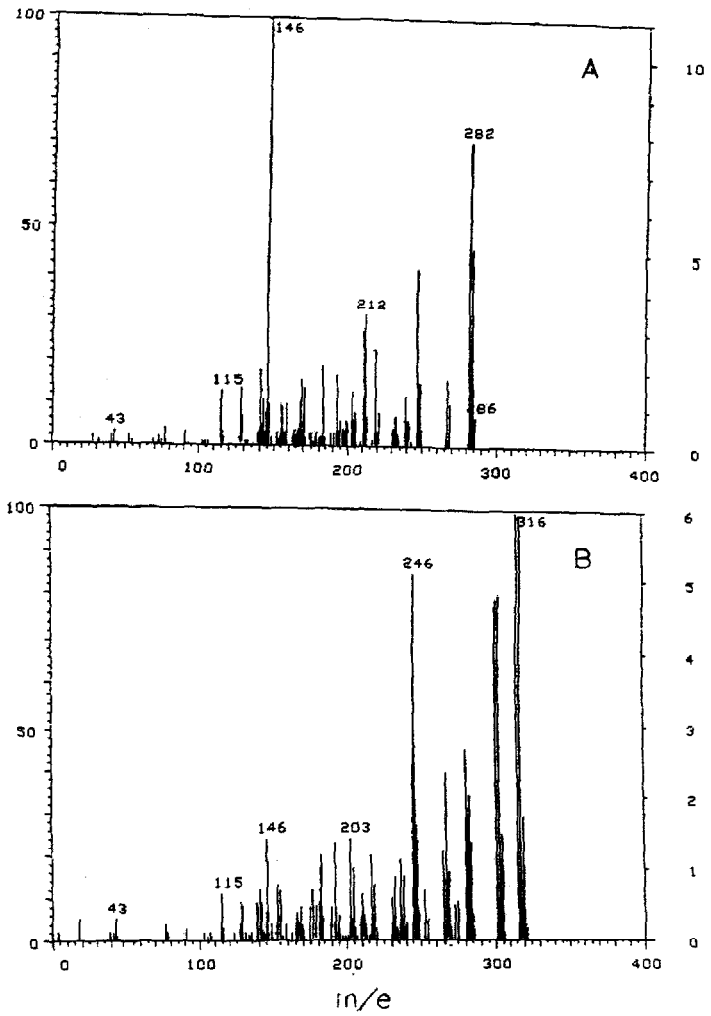


Fig. 3. Mass spectra of peaks 18 (A) and 19 (B) in the chromatogram of Fig. 1.

of sample applied, while the mutagenicity recovered from the silica gel plates was 42% of the overall activity applied (Table I). These results indicate that both mutagenic and non-mutagenic substances in the extract are strongly adsorbed on the silica gel plates. However, about 85% of the total mutagenicity of samples recovered from the silica gel plates was found to be concentrated in fraction F. This suggests that the mutagenic materials in the extract are of intermediate polarity or are non-polar.

Since GC analysis showed the occurrence of chloro-4-methylphenols, chloro-4-methylquinones and chloro-4-methylphenol dimers, fraction F was further fractionated into nine fractions [F(a)–F(i)] on polyamide plates (Fig. 6). The recovery in the fractions was approximately 62% (w/w) of the amount of sample applied, while the mutagenicity recovered from the plates was 61% of the overall activity applied

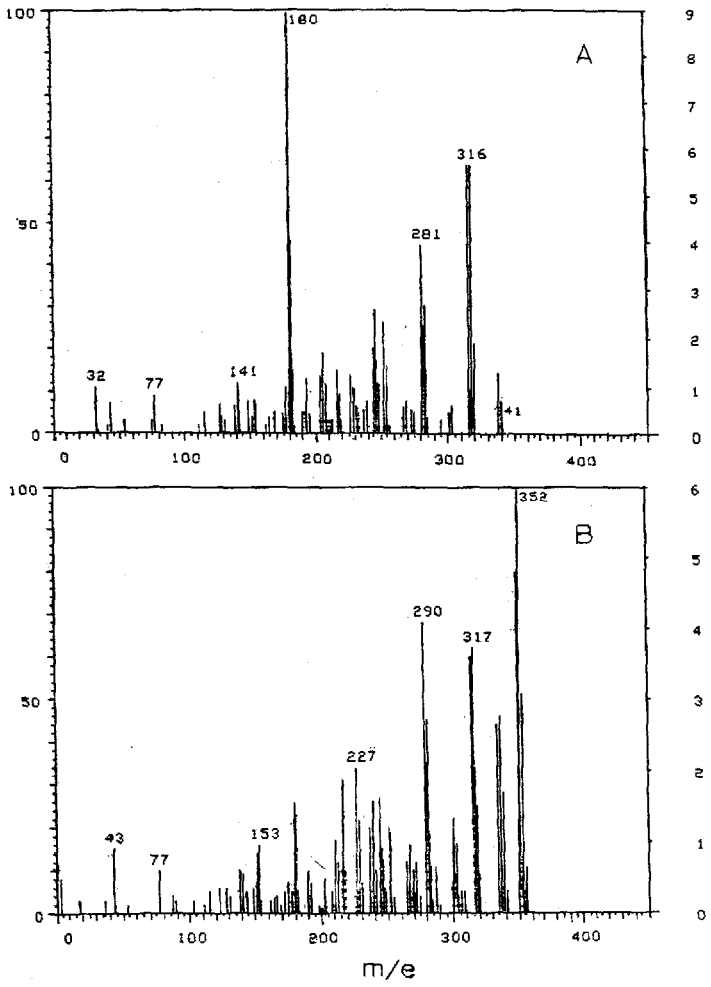


Fig. 4. Mass spectra of peaks 20 (A) and 21 (B) in the chromatogram of Fig. 1.

(Table II). These results indicate that both mutagenic and non-mutagenic substances are strongly adsorbed on the polyamide plates.

The dose–response curves of the samples are shown in Fig. 7. Among these components, fraction F(i) showed the strongest mutagenic response. Its mutagenicity was approximately 50% of the overall activity recovered from the polyamide plates. Fractions F(c), F(f), F(g) and F(h) also exhibited weak mutagenicity. About 50% of the mutagenicity of the original diethyl ether extract obtained from the chlorinated 4-methylphenol solution was thus accounted for by fraction F(i).

GC and GC–MS studies of the polyamide TLC fractions

In order to obtain further information on the mutagenic components present in the diethyl ether extract of chlorine-treated 4-methylphenol solution, the components [fractions F(a)–F(i)] obtained by polyamide TLC were investigated by means

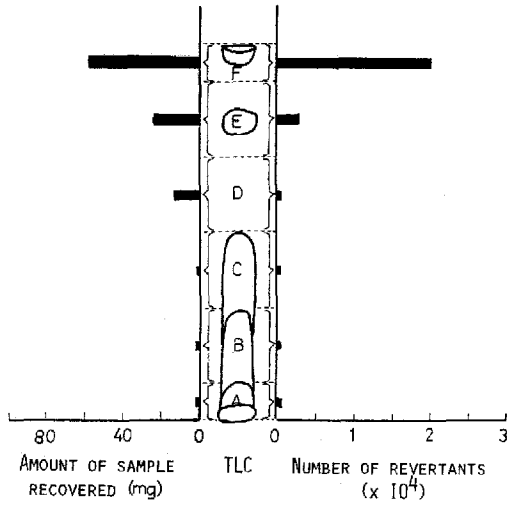


Fig. 5. Fractionation of a mutagenic diethyl ether extract of chlorine-treated 4-methylphenol solution by silica gel TLC. For TLC conditions and mutagenicity tests, see Experimental.

of GC and GC–MS. Fig. 8 shows the gas chromatograms (flame ionization detection) of fractions F(a), F(c), F(e), F(g) and F(i). The results of mutagenicity tests and GC determinations on the polyamide TLC fractions are shown in Fig. 6.

Fraction F(c), which exhibited a weak mutagenic response, consists of a compound with molecular formula $C_7H_5Cl_3O_3$, and the unknown compound corresponding to peak 8. Fraction F(e), which exhibited a very slight mutagenic response, consists of a compound with molecular formula $C_7H_5Cl_3O_3$. This leads to the con-

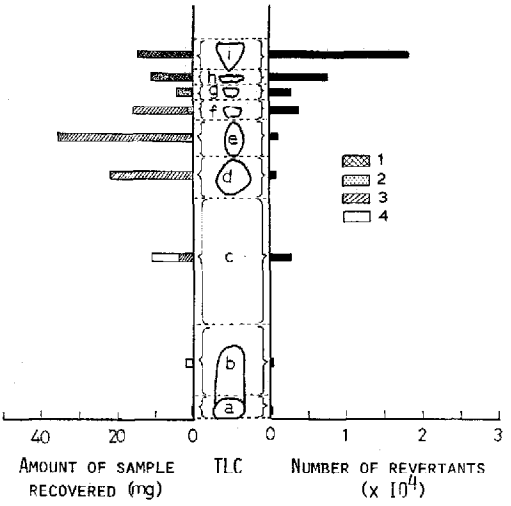
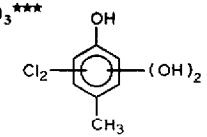
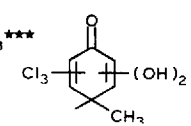
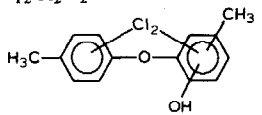
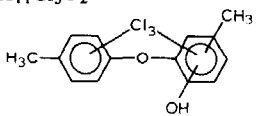
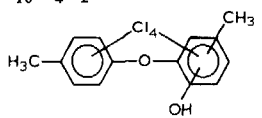


Fig. 6. Fractionation of the most mutagenic fraction F (from Fig. 5) by polyamide TLC. For TLC conditions and mutagenicity tests, see Experimental. 1 = Polychlorinated 4-methylphenol dimers; 2 = 2,6-dichloro-4-methylphenol; 3 = compound with molecular formula $C_7H_5Cl_3O_3$; 4 = compound corresponding to peak 8 in Fig. 1.

TABLE III

PRODUCTS OF 4-METHYLPHENOL WITH HYPOCHLORITE IN DILUTE AQUEOUS SOLUTION AT 20°C

GC peak No. (Fig. 1)	*Compound*	*m/e (order of ion intensity)*	*% in extract**	*Distribution on polyamide plate*
1	4-Methylphenol**	108 (M^+)	12.40	–
2	2-Chloro-4-methylphenol**	142 (M^+)	9.30	–
3	$C_7H_6Cl_2O_3$*** 	89 ($M^+ - 2Cl - 3CO$) 53 ($M^+ - 2Cl - 3CO - C_3H$), 123 ($M^+ - 2CO - CHO$), 208 (M^+)	1.00	–
4	2,6-Dichloro-4-methylphenol**	141 ($M^+ - Cl$), 176 (M^+), 77 ($M^+ - 2Cl - CHO$)	8.70	F(g)
5	$C_7H_5Cl_3O_3$*** 	123 ($M^+ - COCl - 2CO$), 87 ($M^+ - COCl - CHO - CO - Cl$), 53 ($M^+ - COCl - 2CO - 2Cl$), 179 ($M^+ - COCl$), 242 ($M^+$)	57.40	F(e)

8	Unknown	123, 53, 87, 179	4.38	F(c)
18	$C_{14}H_{12}Cl_2O_2$*** 	146 ($M^+ - 2Cl - C_4H_2O$), 282 (M^+), 247 ($M^+ - Cl$), 212 ($M^+ - 2Cl$), 219 ($M^+ - Cl - CO$), 267 ($M^+ - CH_3$)	1.40	F(i)
19	$C_{14}H_{11}Cl_3O_2$*** 	316 (M^+), 246 ($M^+ - 2Cl$), 301 ($M^+ - CH_3$), 281 ($M^+ - Cl$), 267 ($M^+ - Cl - CH_3$), 146 ($M^+ - 3Cl - C_4HO$)	1.24	F(i)
20	Isomer of compound 19***	180 ($M^+ - 3Cl - CH_3O$), 316 (M^+), 281 ($M^+ - Cl$), 246 ($M^+ - 2Cl$), 253 ($M^+ - COCl$)	0.59	F(i)
21	$C_{14}H_{10}Cl_4O_2$*** 	350 (M^+), 280 ($M^+ - 2Cl$), 315 ($M^+ - Cl$), 335 ($M^+ - CH_3$)	0.51	F(i)

* Yield derived from GC peak area, relative to total area of all peaks observed (including any residual starting material).
** Complete identification based on MS interpretation and confirmed by comparison with a reference spectrum and the retention time of the substance.
*** Tentative structure; identification based on MS interpretation.

clusion that the major product, $C_7H_5Cl_3O_3$ (about 60%, w/w), present in the diethyl ether extract is not mutagenic, but the minor component corresponding to peak 8 (4.4% , w/w) is suspected to be weak mutagenic substance.

2,6-Dichloro-4-methylphenol corresponding to peak 4 was distributed in fractions F(f) to F(h), with the highest purity in fraction F(g) (Fig. 6), but this compound exhibited no mutagenic response (Table I). On the other hand, chlorinated 4-methylphenol dimers were distributed in fractions F(g) to F(i) in increasing amount and mutagenicity (Fig. 6). The gas chromatogram of the fraction with highest mutagenicity, F(i), however, showed that a mixture of di-, tri- and tetrachlorinated 4-methylphenol dimers was present (Fig. 8). Therefore, it seems that at least one of the chlorinated 4-methylphenol dimers formed by the reaction of 4-methylphenol with hypochlorite in water contributes to the mutagenicity of the chlorinated 4-methylphenol solution.

Some information about the identification of the mutagenic compounds and the availability of suitable methods for analyzing such compounds is necessary to establish their fate and stability. Several compounds, *viz.*, chloroacetones, 2-chloropropenal and chlorinated hydroxyfuranone, have been cited as important mutagens present in kraft chlorination effluents[26,27,31]. Two simple oxidation products, *o*-benzoquinone and chloro-*o*-benzoquinone, have been demonstrated to be mutagenic substances present in chlorine-treated catechol solution[12]. Recently Onodera *et al.*[22] demonstrated that the treatment of 4-phenylphenol with chlorine in aqueous solution produces compounds with mutagenicity towards *S. typhimurium* strain TA100 in the absence of S9, and the oxidation products, 4-phenyl-*o*-benzoquinone and dichlorinated [4-phenyl-*o*-benzoquinone], identified in the solution are mutagenic[33].

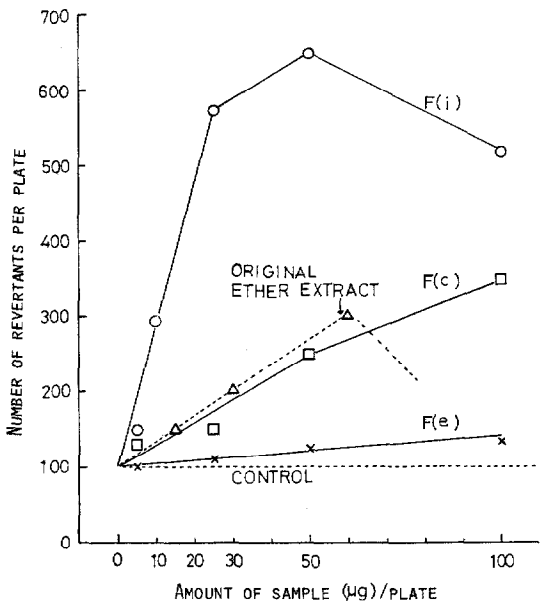


Fig. 7. Mutagenicity of the original diethyl ether extract and polyamide TLC fractions F(c), F(e) and F(i) against *S. typhimurium* strain TA100 without S9. Each point represents the average of triplicate determinations. At higher doses per plate the extract was toxic to the tested strain.

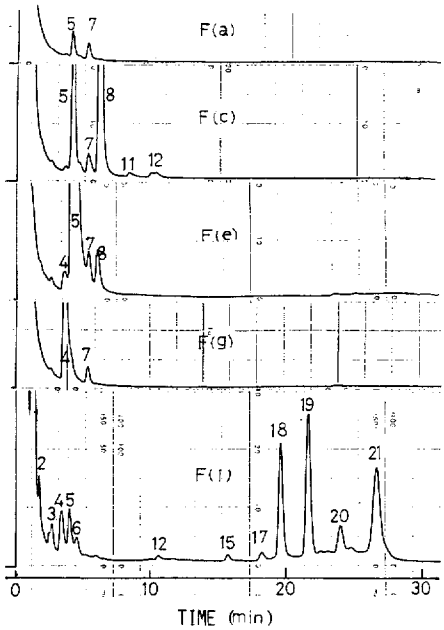


Fig. 8. Gas chromatograms (flame ionization detection) of polyamide TLC fractions F(a), F(c), F(e), F(g) and F(i). Main compounds as in Table III. For GC conditions, see Experimental.

In the present work, the compounds responsible for the mutagenic response could not be completely isolated from the diethyl ether-extractable products of the reaction of 4-methylphenol with hypochlorite in water. However, repeated fractionations by TLC revealed that the major components present in the extract are not mutagenic, but minor components which comprise less than 4% of the total amount of the extract are mutagenic (Tables I–III and Figs. 5 and 6). Over 50% of the mutagenicity of the original ether extract obtained from the chlorinated 4-methylphenol solution can be accounted for by the activity of this minor component [fraction F(i)]. Furthermore, GC–MS studies of the most mutagenic fractions, F(g) to F(i), suggested that the mutagenicity of the extract is probably due to the chlorinated 4-methylphenol dimers, which have yet to be identified.

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