a factor of 50 higher compared to the control at 30 µg/ml of 246TCP and only a factor of 5 of the commercial fungicide mixture.

In the study of pulp mill effluent by a bacterial assay (17) the mutagenic response was a factor 3.6 times higher at 1 mg pulp mill extract/ml level as compared to the background level. In the study of Kinae et al. (18) high DNA damaging potency was obtained by 246TCP and 3456TeCG at 0.5 and 0.1 mg/disk level.

It is important to know the mutagenic activity of pulp industry effluents which are complex mixtures but it is also important to know the mutagenicity of single chlorinated phenols and related compounds because they seem to appear from new sources to the environment like in the precipitation of snow and concentrate in animal tissues, too (1).

Chlorophenols did not give positive response in the *Salmonella typhimurium* and *Saccharomyces cerevisiae* assays (15, 16) although one chloroguaiacol and -catechol gave positive response in the yeast. However, both a chlorophenol and chloroguaiacol gave positive response in *Bacillus subtilis*.

It is obvious that several *in vitro* systems, including the mammalian cell assay should be used simultaneously in the study of the mutagenicity of chlorinated phenols and related compounds. The final evaluation of their mutagenic potency and risk to the environment can, however, be ascertained when more data of their dilution in waters and the concentration coefficients in the tissues of different organisms is available.

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Inability of chlorophenols to induce 6-thioguanine-resistant mutants in V79 Chinese hamster cells

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Summary

The induction of mutation at the hypoxanthine–guanine phosphoribosyl transferase locus and cytotoxicities of 6 different chlorophenols (2,4- and 2,6-dichlorophenol, 2,4,5- and 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol) were examined in V79 Chinese hamster cells without exogenous metabolic activation. The chlorophenols were cytotoxic to V79 cells, but failed to produce significant increases in the frequency of 6-thioguanine-resistant mutants.

Chlorophenols are used extensively as wood preservatives (fungicides), bactericides, slimicides, herbicides and as intermediates in the manufacture of other pesticides. The annual world-wide production of chlorophenols has been estimated to be about 200 000 ton (Paasivirta, 1978). Chlorine bleaching of pulp produces chlorophenol-containing effluents (Lindström and Nordin, 1976). Furthermore, penta-chlorophenol and various di-, tri- and tetra-chlorophenols have been identified as mammalian metabolites of hexachlorocyclohexane (IARC, 1979; Munir et al., 1984).

In humans, soft tissue sarcoma, malignant lymphoma and leukemia have been associated with occupational exposure to 2,4,5-tri- and penta-chlorophenol, and to chlorophenol formulations containing mainly 2,4,6-tri-, 2,3,4,6-tetra- and penta-chlorophenol (IARC, 1982). In animal studies, 2,4,6-trichlorophenol (96–97% pure) has been shown to produce lymphomas and leukemias in male Fisher rats, and hepatocellular carcinomas or adenomas in both male and female B6C3F1 mice (National Cancer Institute, 1979). Long-term bioassays of pentachlorophenol by the U.S. National Toxicology Program are in progress (Huff, 1982).

The genotoxicity of chlorophenols has not been well characterized. 2,4,6-Trichlorophenol was reported to exhibit high DNA-damaging potency without exogenous metabolic activation in *Bacillus subtilis* rec-assay (Kinae et al., 1981). 2,4-Di- and 2,4,5-tri-chlorophenol induced stickiness and lagging of chromosomes, and chromosome fragmentation in *Vicia faba* (Amer and Ali, 1974). In *Saccharomyces cerevisiae*, in the absence of exogenous activation, 2,6-di- and 2,4,5-tri-chlorophenol failed to induce reverse mutation or mitotic gene conversion (Nestmann and Lee, 1983), whereas 2,4,6-trichlorophenol induced forward mutation but did not induce mitotic gene conversion, and pentachlorophenol induced both forward mutation and mitotic gene conversion (Fahrig et al., 1978). Pentachlorophenol failed to induce sex-linked recessive lethals in *Drosophila melanogaster* (Vogel and Chandler, 1974), and there was con-

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flicting evidence with regard to the production of chromosomal aberrations in workers exposed to pentachlorophenol (Wyllie et al., 1975; Bauchinger et al., 1982).

The mutagenicity of chlorophenols in mammalian cells has not been determined. However, the chlorophenol derivative 2,4-D (2,4-dichlorophenoxyacetic acid) was shown to be mutagenic without exogenous activation in V79 Chinese hamster cells (Ahmed et al., 1977). To extend the previous genotoxicity studies, we examined the induction of mutation to 6-thioguanine resistance in V79 Chinese hamster cells by 6 different chlorophenols.

Materials and methods

Chemicals

2,4- and 2,6-dichlorophenol, 2,4,5- and 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol and pentachlorophenol were purchased from Fluka AG, Buchs, Switzerland, purified by recrystallization and determined to be > 99.5% pure by GC. Chlorinated dibenzo-*p*-dioxins were not detected (limit of detection, 0.05 ppm). Ethyl methanesulfonate (EMS) was obtained from Sigma Chemical Co., St. Louis, MO. Stock solutions of the chlorophenols (50 mg/ml in acetone) and EMS (50 mg/ml in water) were prepared immediately before use.

Cell culture

V79 Chinese hamster cells, originally obtained from Dr. Eliezer Huberman (Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN), were cloned to reduce the spontaneous background frequency of 6-thioguanine-resistant mutants and stored in ampoules frozen in liquid nitrogen. No HATG (hypoxanthine, aminopterin, thymidine, glycine) treatments were performed. Prior to each experiment, an ampoule of cells was thawed and used. The cells were grown in Dulbecco's modified Eagle's medium (K.C. Biological, Inc., Lenexa, KS) supplemented with 10% fetal calf serum (Gibco Ltd.; Paisley, Scotland), 2 mM glutamine (K.C. Biological), 100 IU/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml fungizone (Gibco Ltd.) at 37°C with a humidified atmosphere of 10% CO_2 in air. Cell culture dishes were purchased from Falcon Co., Oxnard, CA.

Mutation assay

The forward mutation assay on 6-thioguanine resistance in V79 cells was based on the replating method described by McMillan and Fox (1979). 1×10^6 cells were plated in 100-mm dishes in 9 ml of medium and cultured for 24 h. The test chemical was then added to the dishes in 1 ml of medium to give the desired final concentrations. For each concentration, two dishes were used. Each experiment included a solvent control and a positive control of EMS at 200 μg/ml. The maximum solvent concentration was 1%. This level had no effect on cell growth or spontaneous mutant frequency. After 24 h of treatment, cells were dissociated with a trypsin–EDTA solution, and cell survival was determined by plating 200 cells in 60-mm dishes (4 dishes/dose) in 4 ml of medium and staining the dishes with Giemsa 6 days later. Colonies of at least 50 cells were counted and survival was calculated relative to the solvent control.

At the time cells were plated for survival, 1–2 $\times 10^6$ cells of each treatment condition were plated in 100-mm dishes in 10 ml of medium to allow phenotypic expression. Cells were subcultured every 48 h, maintaining 1×10^6 cells at each subculture. After a 6-day expression time, which is considered to be adequate in general (Bradley et al., 1981), 1×10^5 cells were plated in 100-mm dishes (10 dishes/dose) in 9 ml of medium, and 6-thioguanine (Sigma Chemical Co.) was added in 1 ml of medium 4 h later to give a final concentration of 10 μg/ml. The number of mutant colonies was determined 10 days later, after Giemsa staining. To determine cell viability at the time of mutant selection, 200 cells were plated in 60-mm dishes (4 dishes/dose) in 4 ml of medium, and the colonies were stained with Giemsa and counted 6 days later. Mutant frequencies were expressed as the numbers of mutants per 10^6 viable cells.

A chemical was considered to be mutagenic if the mutant frequency exceeded the 99% upper confidence limit of the historical background mutant frequency and a dose-related increase in the mutagenicity was observed. The historical background mutant frequency (the solvent control

frequencies in 12 independent experiments including those reported here) was $7.0 \times 10^{-6} \pm 4.5 \times 10^{-6}$ (mean ± standard deviation). The 99% confidence interval calculated by multiplying the standard deviation by the appropriate t value of the Student's t distribution (one-sided) requiring 99% confidence was 12.2×10^{-6}, and the 99% upper confidence limit was 19.2×10^{-6}.

Results and discussion

The results are presented in Table 1. Each of the 6 chlorophenols tested reduced the plating efficiency (% cell survival) in a dose-dependent manner. This cytotoxic effect can be attributed to the ability of pentachlorophenol and other chlorophenols to inhibit oxidative phosphorylation (Farquharson et al., 1958; Weinbach et al., 1965). At the concentrations tested, none of the chlorophenols produced significant increases in the frequency of 6-thioguanine-resistant mutants. Evidence that the cells in these experiments were responsive was shown by EMS at 200 μg/ml. In a separate experiment, EMS induced marked dose-related increases in 6-thioguanine resistance.

There have been few studies regarding the induction of gene mutation by chlorophenols. Our negative results are in accordance with the findings of Nestmann and Lee (1983), who showed that 2,6-di- and 2,4,5-tri-chlorophenol were inactive in inducing reverse mutation in *S. cerevisiae*, and of Vogel and Chandler (1974), who reported that pentachlorophenol failed to induce sex-linked recessive lethals in *D. melanogaster*. Fahrig et al. (1978) found that 2,4,6-tri- and penta-chlorophenol induced forward mutation to cycloheximide resistance in *S. cerevisiae* without exogenous metabolic activation. However, the biochemical basis of cycloheximide resistance is poorly understood, and this mutation system is not considered to be completely reliable (Zimmermann et al., 1984).

Most human and animal carcinogens have been found to cause gene mutation, chromosome breakage or other types of DNA lesions. We point out that the apparent lack of induction of gene mutation by chlorophenols does not necessarily mean the lack of genotoxicity of these chemicals. In this respect, it is interesting to note the findings of

TABLE 1

INDUCTION OF MUTATION TO 6-THIOGUANINE (TG) RESISTANCE IN V79 CHINESE HAMSTER CELLS BY TREATMENT WITH CHLOROPHENOLS FOR 24 h

Chemical	Dose (μg/ml)	Survival (%) [a]	Number of TG-resistant mutants/10^6 viable cells
2,4-Dichloro-phenol	0	100	12
	12.5	108	6
	25	80	6
	50	18	0
EMS [b]	200	84	965
2,6-Dichloro-phenol	0	100	4
	125	88	0
	250	82	0
	500	17	6
EMS	200	62	584
2,4,5-Trichloro-phenol	0	100	9
	6.25	90	3
	12.5	63	6
	25	45	5
	50	17	7
EMS	200	64	1262
2,4,6-Trichloro-phenol	0	100	2
	12.5	85	3
	25	79	4
	50	72	2
	100	53	0
EMS	200	79	631
2,3,4,6-Tetra-chlorophenol	0	100	1
	12.5	101	7
	25	88	16
	50	34	7
	100	10	9
EMS	200	88	1181
Pentachloro-phenol	0	100	16
	6.25	90	10
	12.5	73	0
	25	53	0
	50	27	7
EMS	200	55	966
EMS	0	100	8
	50	74	180
	100	73	259
	200	53	613
	400	13	1367

[a] The average absolute plating efficiency of control cells was 75%, range 62–89%.

[b] Positive control, ethyl methanesulfonate.

Bauchinger et al. (1982), who reported that peripheral lymphocytes of pentachlorophenol-exposed workers showed a significant increase in chromosome-type aberrations (dicentrics and acentrics) but not in chromatid-type aberrations, such as breaks or exchanges. Further studies will be required to clarify the genotoxic properties of chlorophenols, as well as their suspected human and animal carcinogenicity.

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A comparison of chromosome aberration induction by 25 compounds tested by two Chinese hamster cell (CHL and CHO) systems in culture

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Summary

Twenty-five chemicals were tested for the induction of chromosomal aberrations in 2 cultured mammalian cell sysems, Chinese hamster lung cells (CHL) and Chinese hamster ovary cells (CHO). This study was carried out to provide a data set that would permit an assessment of the extent to which the 2 systems agree in the results produced. Results presented for the 2 systems in this paper are not based on the same criteria but rather on the criteria standardly used in each of the systems. In tests conducted in the absence of S9 mix, 7 chemicals gave positive results in both systems and 12 were negative in both. In tests with S9 mix, 5 were positive in both systems and 9 were negative in both. When the overall results including tests both with and without S9 mix were considered, the 2 systems agreed on 15 results, 11 positives and 4 negatives. A review of the test conditions and data suggests that disagreements in test results were more often due to differences in the protocols used in these 2 systems than to a difference in the sensitivities of the 2 cell lines.

Chromosomal aberration tests using cultured mammalian cells have been widely used in primary screening for environmental mutagens and/or carcinogens and in evaluating the genotoxicity of chemicals including drugs, food additives, cosmetics, pesticides, and industrial chemicals.

For the chromosomal aberration test in vitro, primary cells (e.g., human lymphocytes) as well as established cell line cells (e.g., Chinese hamster fibroblasts) are commonly used (Evans et al., 1980). In the U.S.A., under the National Toxicology Program (NTP), a Chinese hamster ovary cell line, CHO cells, has been used for in vitro cytogenetic studies on a variety of chemicals since 1980. Chemicals have been tested under code for their ability to induce chromosomal aberrations with and without rat liver microsome fraction (S9) (Galloway et al., 1985, 1987). In Japan, a different protocol using Chinese hamster lung cells, CHL cells, has been used for screening chemicals and test data on more than 700 substances have been

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accumulated during the past 10 years (Ishidate and Odashima, 1977; Ishidate, 1988).

A recent review and comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures (Ishidate et al., 1988) shows that there are qualitative differences in test results when chemicals are tested in different cell lines. These differences in results are more often due to differences in the test protocols, e.g., treatment time, harvest time, or the conditions used for metabolic activation, etc., than to hypersensitivity of a particular cell type. Related questions are whether or not clastogenic effects seen only at very high dose levels are artifactual and if so, whether the CHL and CHO cells are particularly prone to such effects (Ishidate and Harnois, 1987). These problems are important not only when the data are evaluated qualitatively as well as quantitatively, but also when guidelines for genotoxicity tests are prepared by governmental authorities for regulatory purposes.

The present study was conducted to compare the results obtained in CHL and CHO cells on 25 test chemicals. Some of the CHO test results were previously published by the NTP and the U.S. National Institute of Environmental Health Sciences (NIEHS). All experiments with CHL cells were performed at the Division of Genetics and Mutagenesis, National Institute of Hygienic Sciences, Tokyo (Japan), while the experiments with CHO cells were conducted at laboratories under contract to the NTP.

Materials and methods

The test chemicals (see Table 1 for names and CAS numbers, and Fig. 1 for structures) were chosen from a list of chemicals selected by the NTP for Salmonella mutagenicity testing in 1985. The group of chemicals is not a random selection and contains a large number of chlorophenols and phenylenediamines. Samples were supplied under code by the NTP chemical repository (Radian Corp., Austin, TX, U.S.A.). They were dissolved immediately before use in culture medium (M), physiological saline (S), dimethyl sulfoxide (DMSO), ethanol (E), acetone (A) or carboxymethylcellulose (CMC). The name of the laboratory where the test was conducted is also indicated in Table 1 (NIHS for National Institute of Hygienic Sciences, Tokyo 158, Japan; HB for Hazleton Biotechnologies, Kensington, MD 20895, U.S.A.; LB for Litton Bionetics Inc., Kensington, MD 20895, U.S.A.; EHRT for Environmental Health Research and Testing Inc., Lexington, KY 45244, U.S.A.; and BSC for Bioassay Systems Corp., Woburn, MA, U.S.A.).

Experimental protocols in CHL and CHO systems were as described below. Major differences in experimental protocols are summarized in Table 2.

CHL system

The CHL test protocol has been described in detail in the *Data Book of Chromosomal Aberration Tests In Vitro* (Ishidate, 1988). The CHL cells were originally established from the lung of a female newborn Chinese hamster (Koyama et al., 1970), and the clonal cell line (CHL-CL-11) has been stocked in liquid nitrogen at the NIHS Cell Bank (JCRB0030). The cells were grown in a monolayer in Eagle's minimum essential medium (MEM, Gibco) containing no antibiotics and supplemented with 10% calf serum. The modal chromosome number was 25 and more than 80% of cells had 25 chromosomes. The doubling time was estimated to be 15–17 h.

To select the maximum test chemical concentration, a preliminary test was conducted on each test chemical as follows: 1.2×10^4 cells were seeded in a 35-mm plastic dish with 2 ml of culture medium. The test chemical, at different concentrations (usually diluted at 2-fold intervals), was added on the 3rd day of culture. After an additional 2 days, the medium was discarded and the cells were washed with physiological saline. The cells were then fixed with 10% formalin solution and stained with 0.1% crystal violet solution. The concentration needed for 50% cell growth inhibition was approximated using a cell densitometer (Monocellater, Olympus Co. Ltd., Japan). For the chromosomal aberration test, 3 serial concentrations, including one above and one below the 50% growth inhibition dose, were generally used. For the negative control, untreated and/or solvent-treated cells were used. For the positive control, mitomycin C (CAS No. 50-07-7)

TABLE 1

TEST CHEMICALS, CAS NUMBERS, SOLVENTS USED AND LABORATORIES WHERE THE TESTS WERE CONDUCTED

Chemical No.	Test chemical	CAS No.	Solvent used [a]		Laboratory [b]	
			CHL	CHO	CHL	CHO
1	2,3,4-Trichlorophenol	15950-66-0	DMSO	DMSO	NIHS	HB(4)
2	2,3,6-Trichlorophenol	933-75-5	DMSO	DMSO	NIHS	LB(4)
3	3,4,5-Trichlorophenol	609-19-8	DMSO	DMSO	NIHS	HB(4)
4	2,3,4,5-Tetrachlorophenol	4901-51-3	DMSO	DMSO	NIHS	LB(4)
5	2,3,4,6-Tetrachlorophenol	58-90-2	DMSO	DMSO	NIHS	LB(4)
6	2,3,5,6-Tetrachlorophenol	935-95-5	DMSO	DMSO	NIHS	LB(4)
7	*o*-Phenylenediamine	95-54-5	DMSO	DMSO	NIHS	HB(4)
8	*m*-Phenylenediamine	108-45-2	DMSO	DMSO	NIHS	HB(4)
9	2,6-Toluenediamine dihydrochloride	15481-70-6	Saline	DMSO	NIHS	EHRT(1)
10	*N*,*N*-Dimethyl-*p*-phenylenediamine	99-98-9	DMSO	DMSO	NIHS	EHRT(4)
11	*N*,*N*,*N*′,*N*′-Tetramethyl-*p*-phenylenediamine	100-22-1	DMSO	DMSO	NIHS	EHRT(4)
12	*N*,*N*-Diethyl-*p*-phenylenediamine	93-05-0	DMSO	DMSO	NIHS	EHRT(4)
13	*N*,*N*′-Di-*sec*.-butyl-*p*-phenylenediamine	101-96-2	DMSO	DMSO	NIHS	EHRT(4)
14	*N*,*N*′-Diphenyl-*p*-phenylenediamine	74-31-7	DMSO	DMSO	NIHS	EHRT(4)
15	*N*,*N*′-Di-2-naphthyl-*p*-phenylenediamine	93-46-9	DMSO	DMSO	NIHS	EHRT(4)
16	*N*-Phenyl-1-naphthylamine	90-30-2	DMSO	DMSO	NIHS	BSC(3)
17	*N*-Phenyl-2-naphthylamine	135-88-6	DMSO	DMSO	NIHS	BSC(4)
18	*p*-Isopropoxydiphenylamine	101-73-5	DMSO	DMSO	NIHS	BSC(3)
19	4,4′-Dimethoxydiphenylamine	101-70-2	DMSO	DMSO	NIHS	BSC(3)
20	4,4′-Dioctyldiphenylamine	101-67-7	EtOH	Acet	NIHS	BSC(3)
21	*N*-Nitrosodiphenylamine	86-30-6	DMSO	DMSO	NIHS	BSC(3)
22	Tris(2,3-epxoypropyl)isocyanurate	2451-62-9	DMSO	DMSO	NIHS	BSC(3)
23	Triallyl isocyanurate	1025-15-6	DMSO	DMSO	NIHS	BSC(3)
24	Chromium carbonyl	13007-92-6	CMC	Acet	NIHS	LB(4)
25	1-(1,2-Dibromoethyl)-3,4-dibromocyclohexane	3322-93-8	DMSO	DMSO	NIHS	BSC(2)

[a] DMSO, *dimethyl sulfoxide*; *Saline*, *physiological saline*; *EtOH*, *ethanol*; *CMC*, *sodium carboxymethyl cellulose*; *Acet*, *acetone*.

[b] NIHS, National Institute of Hygienic Sciences (Tokyo, Japan); HB, Hazléton Biotechnologies (U.S.A.); LB, Litton Bionetics (U.S.A.); EHRT, Environmental Health Research and Testing (U.S.A.); BSC, Bioassay Systems Corp. (U.S.A.).

(1) Data originally published in Gulati et al. (1989).

(2) Data originally published in Loveday et al. (1989).

(3) Data originally published in Loveday et al. (1990).

(4) Complete data, including SCE data, will be published by the NTP in their series of manuscripts on in vitro test results.

or *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (CAS No. 70-25-7) was used in the test without S9 mix, while benzo[*a*]pyrene (CAS No. 50-32-8) or dimethylnitrosamine (CAS No. 62-75-9) was used in the test with S9 mix.

In the test without metabolic activation, the cells were grown in the presence of the test agent for 24 h or 48 h. Colcemid (0.2 μg/ml final concentration) was added to the culture medium 2 h before harvesting. Chromosome preparations were made by the air-drying technique. In the system with metabolic activation, cells were treated with the test agent and S9 mix for 6 h. S9 was prepared from the livers of male Fischer rats pretreated with PCB (KC-400). 10 ml of S9 mix consisted of 3 ml of S9, 2 ml of 20 mM HEPES buffer solution (pH 7.2), 1 ml of 50 mM $MgCl_2$, 1 ml of 330 mM KCl, 1 ml of 50 mM glucose 6-phosphate, 1 ml of 40 mM NADP and 1 ml of distilled water. The final concentration of S9 in the medium was adjusted to 5% (0.5 ml of S9 mix and 2.5 ml of culture medium including calf serum). Cells treated only with the test agent (without S9 mix) for 6 h also served as a control. After 6-h treatment, the reaction mixture was replaced with fresh culture medium, and the cells grown for an additional 18 h. 2 h before harvesting, Colcemid was added to the culture medium.