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August 4, 1994

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Dear Sir/Madam:

In May, 1991 the International Diatomite Producers Association (IDPA) reported to the EPA pursuant to Section 8(e) of the Toxic Substances Control Act (15 U.S.C. 2607(e)) the preliminary findings of a cohort mortality study of certain workers in the diatomite industry. Supplemental information was provided in October, 1992 and again in October 1993 (Reference; EPA Document Control Number: 8EHQ-92-1237 SUPP).

In January of 1993 an *In Vitro Toxicology of Size-Selected Fractions of Diatomaceous Earth* was sponsored by and conducted at Schuller International's Mountain Technical Center by G.A. Hart and T. W. Hesterberg. It is our understanding that the study report was previously provided to EPA on an F.Y.I. basis by the investigators. With this letter we are providing an additional copy of that document along with a recent critique of the study by Brooke T. Mossman, Ph.D. of the University of Vermont. The latter is also being submitted to you on an F.Y.I. basis.

The findings of this study provide neither definitive nor significant information regarding the toxicity of diatomaceous earth. While perhaps of some future interest, it is clear that at least in regards to diatomaceous earth, in vitro testing continues to be experimental and not a predictor of pathogenic potential to humans. As such we do not believe that this new information meets the requirements of a TSCA 8(e) submission.

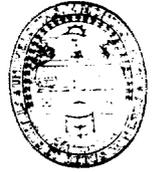
Sincerely,

Mel J. Mirliss
Executive Director

Note:
as per phone call - MR/TDAS
Submitter wants to submit
this to the EPA as an FYI.
(not related to any other submission)

The University of Vermont

DEPARTMENT OF PATHOLOGY
 MEDICAL ALUMNI BUILDING, BURLINGTON, VERMONT 05405-0068
 TEL: (802) 656-2210
 FAX: (802) 656-8892



October 19, 1993

Mr. Mel Merlis
 Executive Director, IDPA
 26 Windjammer Court
 Long Beach, CA 90803

FAX: (310)-598-8109

Dear Mel,

I have read the study on the in vitro toxicology of diatomaceous earth products completed by Dr. Tom Hesterberg of Mountain Technical Center. In general, it is a well performed study assessing the toxic effects of natural (Nat) and flux-calcined (FC) diatomaceous earth fractions with the two crystalline silica reference dusts, cristobalite and α -quartz, in Chinese hamster ovary cells (CHO). Titanium dioxide (TiO_2) and crocidolite asbestos were used as negative and positive controls, respectively, although it should be emphasized that these controls were not used in all assays here. For example, the data on crocidolite, including its physical characterization, was reported previously by Hart et al., 1992a in references. The procedures and bioassays used here were identical to those described in the Hart publication in which the cytotoxicity of 4 preparations of refractory ceramic fibers correlated with the fibrogenic and carcinogenic potential of these preparations in chronic inhalation studies. However, both crocidolite and chrysotile asbestos were used as positive controls in some (but not all) of the Hart et al. (1992) study and TiO_2 was not used as a negative control.

Several points should be taken into consideration in the interpretation of results from the May 26, 1993 study on diatomaceous earth products. First, as Tom correctly states in the second paragraph of his letter, bioassays here were not performed on lung cells which may be targets of mineral-induced disease in man, i.e. lung epithelial cells and fibroblasts or mesothelial cells. Secondly, the increased toxic effects of Nat may reflect increased particles/ μg or its increased surface area for cell contact. In this regard, it would be useful to perform surface area determinations on all preparations (a BET test using nitrogen adsorption can be performed by Micromeritics or other companies quite inexpensively), and to calculate all results on a surface area basis. The induction of both toxic effects and nuclear abnormalities might be related to fibers longer than 8 microns and less than .25 microns, i.e. Stanton fibers (see enclosed paper by Yegles et al), but I am perplexed by the Table II summary characterization of fibers indicating that cristobalite contained 21% fibers with 13% of those > 7.5 microns as the SEMs in Figure 1 do not appear to show any fibers. Characterization of only 100 particles may give a prejudicial

view and a rule of thumb used in our inhalation work is to measure at least 200. TEM is preferable to SEM because fine, thin asbestos fibers may not be visible using the latter technique. Moreover, I think it important to verify that the size selection process used here for diatomaceous earth (DE) products did not select for increased numbers of fibers in the respirable DE preparations. Since fibrous geometry and size are regarded as important in both toxic, carcinogenic and fibrogenic effects of mineral dusts, it will be important to verify that fibers are an extremely small fraction of the bulk products used in the workplace.

An extremely relevant point is the use of positive and negative dusts for comparisons with DE products. For example, it can be argued that even ultrafine TiO_2 (or any inert dust) can produce tumors in animals at overload concentrations used in inhalation studies. If you use the chrysotile (as opposed to crocidolite) asbestos data from the Hart et al., 1992 paper in Figure 3a or 8 (I've sketched it in on these figures), there is no question that the silica and DE preparations are significantly less active in their toxic potential. Moreover, if you compare the data from Appendix 5 (Induction of Nuclear Abnormalities) of the DE study with Table II data on crocidolite and chrysotile asbestos from the Hart et al., 1992 study, crocidolite causes increased (this should be verified by statistics) numbers of micronuclei, polynuclei and nuclear abnormalities at $5 \mu\text{g}/\text{cm}^2$ and chrysotile at $1 \mu\text{g}/\text{cm}^2$ whereas DE products have less striking effects which may not be significant at 3 and $5 \mu\text{g}/\text{cm}^2$. In essence, if chrysotile were used as a positive control here instead of crocidolite, the entire first paragraph of page 3 of the report would be incorrect. As it stands, this sends up a "red flag". Tom is correct in that further testing and characterization of DE products might be merited, but based upon findings here, it is probably premature to embark upon inhalation testing. It should be emphasized that the nuclear alterations might not represent genotoxic, but pyknotic (cell-death related abnormalities) and that any future bioassays should include: 1) chrysotile asbestos as a more appropriate positive control; 2) TiO_2 ; and 3) a control (negative) fiber such as wollastonite which is not associated with disease in man. These controls will be necessary to validate this and other in vitro assays.

Please do not hesitate to call if you have further questions.

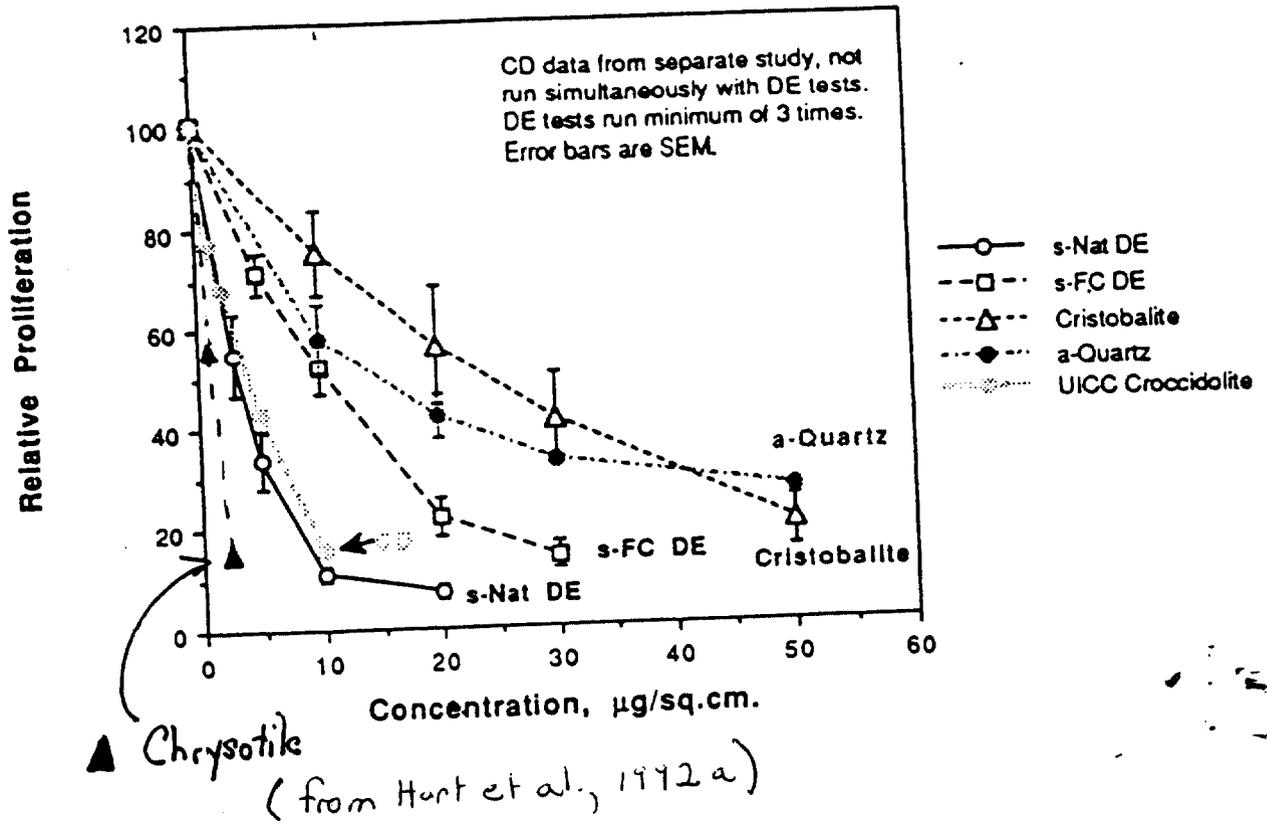
Sincerely,



Brooke T. Mossman
Professor

Enclosure

Fig. 3a. Inhibition of Proliferation
(CHO K1 Cells)



Appendix 5. Induction of Nuclear Abnormalities

Averages from two separate tests.

Particulate	Concentration µg/sq.cm.	Percent w. Mncis.* ± Std. Dev.	Percent Polynuc.** ± Std. Dev.	Percent Abnormal ± Std. Dev.
Control	0	2 ± 1	1 ± 1	3 ± 2
s-Nat DE	3	4 ± 1	8 ± 5	11 ± 6
	5	5 ± 1	11 ± 8	15 ± 7
	10	5 ± 2	19 ± 11	23 ± 9
	20	10 ± 5	23 ± 10	32 ± 4
s-FC DE	5	5 ± 0	10 ± 4	13 ± 4
	10	6 ± 1	14 ± 4	19 ± 5
	20	8 ± 2	23 ± 4	27 ± 3
	30	9 ± 1	32 ± 10	36 ± 7
Cristobalite	5	4 ± 1	5 ± 0	9 ± 0
	10	6 ± 1	6 ± 2	12 ± 4
	20	6 ± 2	9 ± 3	14 ± 1
	30	7 ± 3	12 ± 1	18 ± 3
alpha-Quartz	5	4 ± 0	3 ± 1	7 ± 1
	10	7 ± 0	6 ± 0	12 ± 1
	20	4 ± 1	6 ± 2	9 ± 1
	30	6 ± 2	6 ± 1	11 ± 2

* with one or more micronuclei.

**with two or more normal-size nuclei; may also have micronuclei.

0 (cont)

2 ± 0

0 ± 1

3 ± 1

9 ± 2

21 ± 0

28 ± 1

2

17

23

10

29

28

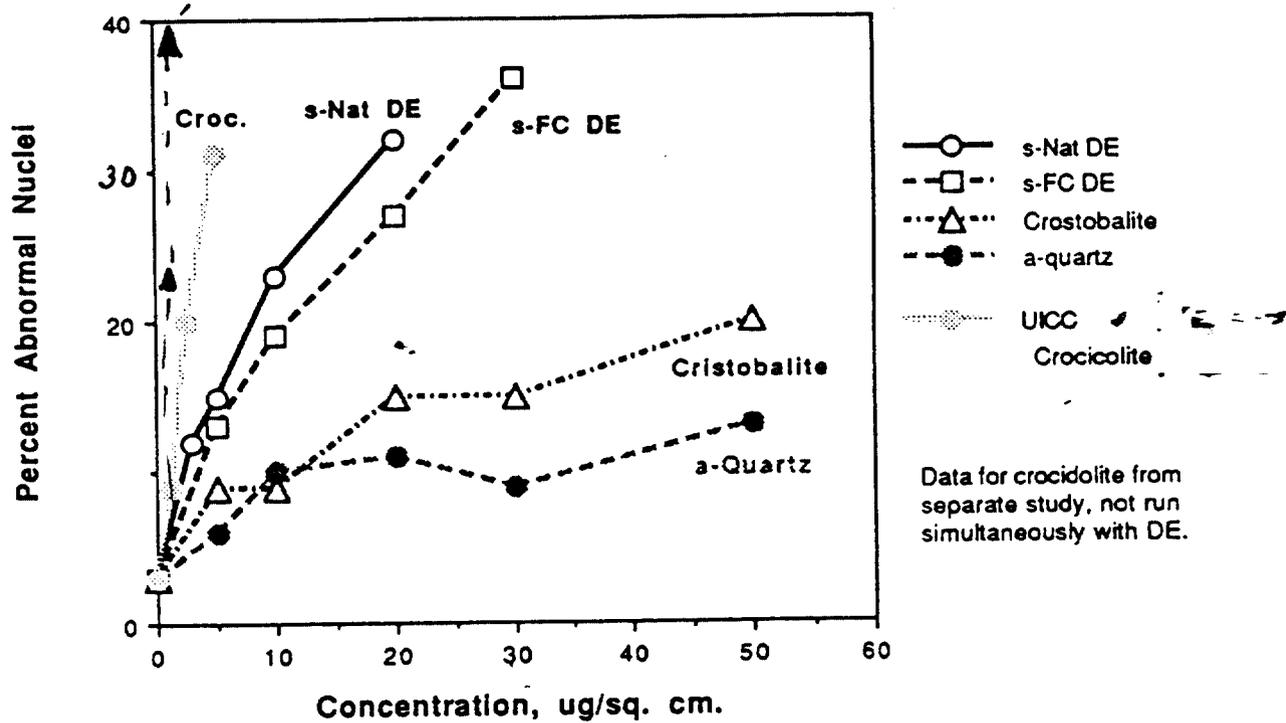
From Hart et al., 1972

UICC
Crocidolite

UICC
Chrysotile

Chrysotile (from Hart et al., 1992 a)

Fig. 8. Induction of Nuclear Abnormalities (CHO Cells)



Data for crocidolite from separate study, not run simultaneously with DE.

Induction of Metaphase and Anaphase/Telophase Abnormalities by Asbestos Fibers in Rat Pleural Mesothelial Cells *In Vitro*

Michel Yegles, Laure Saint-Etienne, Annie Renier, Xavier Janson, and Marie-Claude Jaurand
INSERM U139, Hôpital Henri Mondor, Créteil Cedex, and LEPI, DASES, Paris, France

The cytogenetic effects of asbestos fibers on rat pleural mesothelial cells were studied *in vitro*. Crocidolite UICC significantly enhanced aneuploidy and produced few structural chromosome aberrations, whereas anatase, an isomorphic particle, induced no numerical or structural changes. Mitomycin C (300 nM) produced a tenfold increase in abnormal anaphases compared with controls. Asbestos produced anaphase/telophase abnormalities in a concentration-dependent manner. The majority of the abnormalities involved lagging chromosomes. Crocidolite UICC induced abnormalities at a dose of 7.0 $\mu\text{g}/\text{cm}^2$, whereas Canadian chrysotile did so at 1.0 to 2.0 $\mu\text{g}/\text{cm}^2$. When the response was assessed by the number of long and thin fibers per cm^2 (length $> 8 \mu\text{m}$; diameter $\leq 0.25 \mu\text{m}$), crocidolite UICC produced more abnormalities than Canadian chrysotile at all concentrations. On a per-weight basis, these findings differ from those obtained after intrapleural inoculation, as crocidolite induced more mesotheliomas than chrysotile; however, on a per-fiber basis, the *in vitro* and *in vivo* effects were similar. These results show that anaphase/telophase analysis is sensitive and complementary to metaphase analysis, and suggest that asbestos might produce cell transformation by inducing chromosome missegregation and aneuploidy.

Lung cancer and pleural mesothelioma occur frequently in asbestos-exposed populations. Neoplastic transformation of mesothelial cells can be induced by exposure to asbestos both *in vivo* (1, 2) and *in vitro* (3). An understanding of the mechanisms of cell transformation is important not only from a fundamental point of view but to an increasing use of other fiber types as well (4). It has been found that human malignant mesothelioma cells show both losses and gains of specific chromosomes (5, 6). Human mesothelial cells treated *in vitro* with asbestos were found to be aneuploid (7), and asbestos-induced mesotheliomas in rats show specific chromosomal translocations (8). Moreover, trisomy 1 seems to be involved in spontaneous peritoneal mesotheliomas in Fisher 344 rats (9) and possibly in the transformation of pleural mesothelial cell (10). Lastly, human and rodent mesotheliomas exhibit nonrandom karyotype abnormalities (11). Chromosome changes may thus be important in asbestos-induced transformation of mesothelial cells. Asbestos produces aneuploidy and chromosome abnormalities in a wide range of mammalian cells (12). Aneuploidy induced by fibers seems to be due to chromosomal missegregation during anaphase. As fibers are internalized and accumulate in the perinuclear region (13), their presence during

mitosis could produce interactions with the microtubules and chromosomes (14, 15). Hesterberg and Barrett (16) reported that crocidolite produced a 20-fold increase in abnormal anaphases in Syrian embryo hamster cells. In Chinese hamster V79 cells, chrysotile and crocidolite both induce abnormal segregation of chromosomes during anaphase (17).

So far, only Canadian chrysotile has been used to study chromosomal changes induced by asbestos in rat pleural mesothelial cells (RPMC) (18). This fiber type is a strong inducer of numerical chromosome changes and produces small but significant structural chromosome alterations, but it is not known whether anaphase or telophase abnormalities occur.

In the present study, we report data on the cytogenetic effects of Canadian chrysotile, crocidolite, and anatase (TiO_2) on RPMC anaphases and telophases. Mitomycin C, known for its ability to produce DNA recombination (19), was used as a positive reference. Investigations of aneuploidy and chromosomal aberrations were carried out with crocidolite and anatase.

Materials and Methods

Mineral Samples

Crocidolite, an amphibole fiber, was obtained from the Union Internationale Contre le Cancer (UICC), and Canadian chrysotile fibers were provided by the Société Nationale de l'Amiante (Sherbrooke, Quebec, Canada). Anatase (titanium dioxide, TiO_2) was a gift from INERIS (Verneuil-en-Halatte, France).

(Received in original form November 9, 1992 and in final form February 18, 1993)

Address correspondence to: Marie-Claude Jaurand, INSERM U139, CHU Henri Mondor, 94010 Créteil Cedex, France.

Abbreviations: rat pleural mesothelial cell(s), RPMC; titanium dioxide, TiO_2 .

Am. J. Respir. Cell Mol. Biol. Vol. 9, pp. 186-191, 1993

TABLE 1
Particle granulometry

Fibers	Number of Asbestos or TiO ₂ particles ($\times 10^6/\mu\text{g}$)		Total
	Length > 8 μm , Diameter \leq 0.25 μm		
Canadian chrysotile	1.5		34.5
Crocidolite UICC	0.07		5.3
Anatase	—		2,200.0

Asbestos and anatase were suspended at 1 mg/ml in culture medium and sonicated for 5 min at 2 W (20 KHz). Granulometric studies were performed by means of electron microscopy. An aliquot of the fiber suspension was filtered through a Nuclepore filter, which was then dissolved on electron microscope grids. The length and diameter of the fibers were determined. Following incubation with cells, the particle concentration is expressed as $\mu\text{g}/\text{cm}^2$ of culture dish to take into account particle setting; 1 $\mu\text{g}/\text{cm}^2$ is equivalent to 5 $\mu\text{g}/\text{ml}$ of medium for the metaphase analysis and to 3 $\mu\text{g}/\text{ml}$ for the anaphase/telophase analysis.

Chemicals

Mitomycine C was from Sanofi (Choay, Paris, France), colchicine was from Sigma Chemical Co. (St. Louis, MO), and ethanol (Normapur, Paris, France), methanol, and acetic acid were from Prolabo (Paris, France).

Cell Culture

RPMC established in our laboratory were used (20). The cells were grown as a monolayer in Ham's F10 medium

(GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (Boehringer, France), 50 $\mu\text{g}/\text{ml}$ streptomycin, and 100 U/ml penicillin (GIBCO). The medium was buffered with 1 mM HEPES (GIBCO), at pH 7.3 to 7.4. Confluent monolayers were routinely subcultured by detachment with 0.25% trypsin + 0.02% EDTA (Eurobio, Les Ulis, France). RPMC were mycoplasma free.

The cells maintained a stable phenotype until about 15 to 25 passages (40 to 70 doublings), depending on the culture conditions, and were used routinely up to 20 passages.

Aneuploidy and Chromosomal Aberration Test

This test was performed as described elsewhere (18). RPMC (8×10^5) were seeded in 25-cm² plastic flasks (Costar, Cambridge, MA) and incubated for 24 h before treatment. Particles (anatase: 2, 5, and 10 $\mu\text{g}/\text{cm}^2$; crocidolite: 1, 2, 4, and 6 $\mu\text{g}/\text{cm}^2$) were added to the cultures, which were incubated for an additional 48 h. Colchicine was then added (final concentration, 0.4 $\mu\text{g}/\text{ml}$) for 20 min.

Cells were detached with 0.25% trypsin + 0.02% EDTA, centrifuged (1,400 rpm for 10 min), then resuspended and incubated for 20 min in 0.075 M KCl at 37°C. They were fixed 3 times with 3:1 ethanol/acetic acid. For each treatment, 50 well-spread metaphases were examined.

Aneuploidy was determined by measuring chromosome number, and chromosomal aberrations were scored according to Savage (21). Aberrations were classified according to chromatid gaps (g), chromatid breaks (c), chromosomal breaks (C), and minutes (M).

Analyses were performed in blinded conditions. Aneuploidy and the number of aberrant cells were compared with controls by using the chi-square test.

TABLE 2
Aneuploidy analysis of RPMC exposed to crocidolite or anatase

Agent		Experiment No.	Number of Metaphases with the Indicated Number of Chromosomes						% of Aneuploidy
$\mu\text{g}/\text{cm}^2$	Part./cm ² ($\times 10^6$)		< 38	38-40	41	42	43	> 43	
Crocidolite									
0	0	1	3	1	0	42	4	0	16
		2	0	2	14	33	0	1	33
1	3.0	1	1	1	1	45	2	0	10
2	6.0	1	2	1	3	44	0	0	12
4	8.0	1	14	7	6	23	0	0	54*
		2	4	11	17	17	1	0	66†
6	12.0	2	4	8	15	22	1	0	56‡
Anatase									
0	0	1	1	1	0	39	3	6	22
		2	0	3	11	36	0	0	28
2	4,400	1	0	4	6	36	1	4	28
		2	0	1	11	36	1	1	28
5	11,000	1	1	2	0	39	3	5	
		2	0	1	10	38	1	0	22
10	22,000	1	0	0	6	36	2	6	28
		2	0	3	11	36	0	0	28

Definition of abbreviation: Part. = particles or fibers.

* $P < 0.001$ (chi-square test).

† $P < 0.01$ (chi-square test).

‡ $P < 0.05$ (chi-square test).

TABLE 3
Analysis of aberrations of RPMC treated with crocidolite or anatase

$\mu\text{g}/\text{cm}^2$	Agent		Experiment No.	Number of Aberrations				
	Part./ cm^2 ($\times 10^6$)			c	g	C	M	Total
Crocidolite								
0	0.0		1	0	1	0	0	1
			2	0	0	0	0	0
1	3.0		1	0	1	0	1	2
2	6.0		1	1	1	0	0	2
4	12.0		1	1	0	0	0	1
			2	0	1	0	1	2
6	18.0		2	0	1	4	1	6*
Anatase								
0	0		1	0	0	0	0	0
			2	0	0	0	0	0
2	4,400		1	0	0	0	0	0
			2	0	0	0	0	0
5	11,000		1	0	0	0	0	0
			2	0	0	0	0	0
10	22,000		1	0	0	0	1	1
			2	0	0	0	1	1

Definition of abbreviations: Part. = particles or fibers; c = chromatid break; g = chromatid gap; C = chromosome break; M = minute.
* $P < 0.05$ (Fisher's exact test).

Anaphase/Telophase Abnormalities Test

Cells (3×10^5) were plated into 9-cm² slide flasks (Nunc, Copenhagen, Denmark) in 3 ml of medium and incubated for 24 h before treatment. Agents (mitomycin C: 0.3 μM ; anatase: 0, 5, 7.5, and 10 $\mu\text{g}/\text{cm}^2$; Canadian chrysotile: 0, 0.5, 1.0, 1.5, and 2.0 $\mu\text{g}/\text{cm}^2$; crocidolite: 0, 0.9, 1.8, 3.5, and 7 $\mu\text{g}/\text{cm}^2$) were added to the medium, and the cultures were incubated for 48 h at 37°C.

Cells were fixed and stained using a modified method of Wissinger and associates (22), then fixed with fresh methanol:acetic acid (3:1). The flasks were then opened and air-dried overnight. The slides were placed in 5% perchloric acid at 4°C for 24 h to remove RNA, then rinsed several times in distilled water for 10 min and air-dried overnight. As Brilliant Blue R and safranin O gave inadequate contrast, we applied 3% Giemsa for 10 min. Only cells with clearly separated poles were scored. An anaphase/telophase was considered abnormal if the cell contained lagging chromatin, bridges, or asymmetric segregation. "Lagging chromatin" included chromatids, chromosomes, and fragments. A bridge was a side-arm bridge in which the sister chromatids were still partially connected. Asymmetric segregation was recorded when the two poles were disproportionate in size or when a multipolar segregation occurred. A total of 150 anaphases/telophases were scored for each treatment. Fisher's test was used to test differences between results obtained from controls and treatments.

Results

Granulometric Studies of Fibers

The number of fibers per unit weight is given in Table 1.

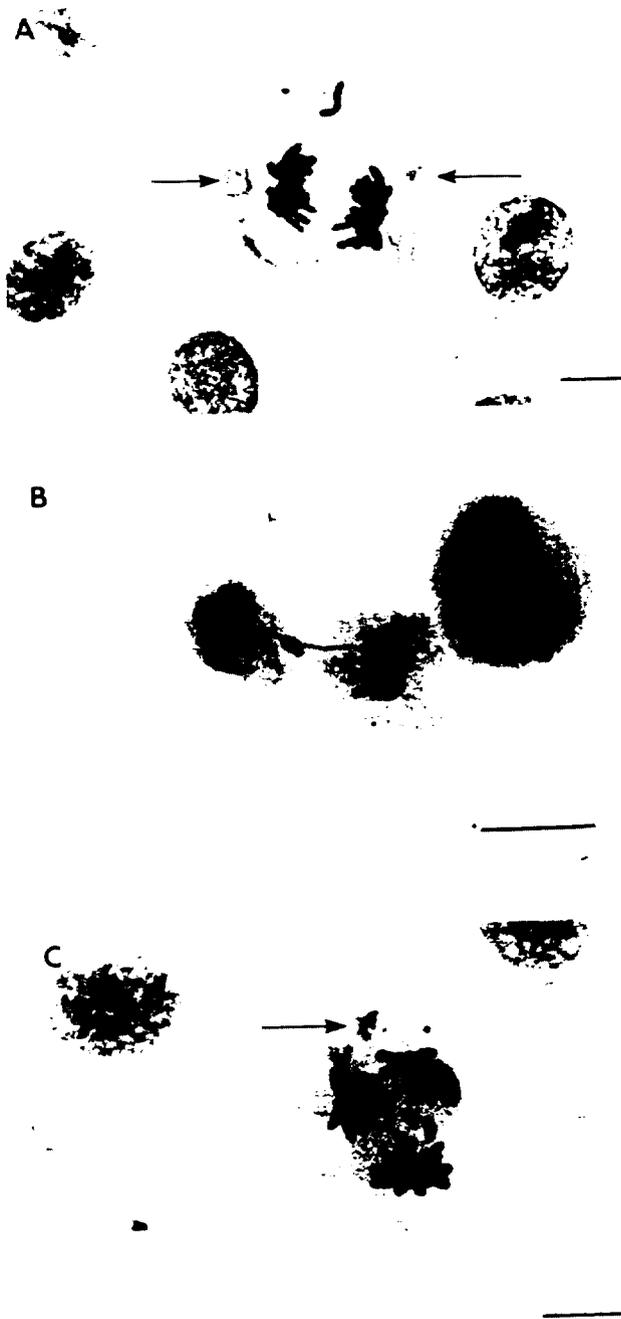


Figure 1. Micrographs of various types of anaphase/telophase abnormalities in RPMC treated with asbestos: (A) lagging chromatin; (B) bridge plus lagging chromatin; (C) multipolar segregation plus lagging chromatin. Bar = 10 μm . Fibers can be seen in the cells (arrows).

Metaphase Analysis

Aneuploidy. Compared with control cultures (Table 2), there was no significant increase in aneuploidy after treatment with anatase (2, 5, and 10 $\mu\text{g}/\text{cm}^2$). Crocidolite UICC induced a significant increase in metaphases at 4 and 6 $\mu\text{g}/\text{cm}^2$, with numerical chromosome changes ($P < 0.01$).

Chromosome aberrations. In the control cultures, $0.5 \pm$

TABLE 4
Anaphase and telophase analysis of RPMC exposed to mitomycine C

Dose (nM)	Experiment No.	Normal Mitosis (%)	Abnormal Mitosis (%)			
			Lagging Chromatin	Bridge	Asymmetric Segregation	Total
0	1	98.0	2.0	0.0	0.0	2.0
	2	94.7	4.7	0.6	0.0	5.4
300	1	46.7	43.3	3.3	4.7	51.3*
	2	46.0	38.7	4.7	4.0	54.0*

* Statistically significant values of $P < 0.001$ (chi-square test).

1% of cells showed chromosome aberrations (mean of four experiments) (Table 3). Anatase did not induce chromosomal changes, whereas crocidolite UICC did so only at $6 \mu\text{g}/\text{cm}^2$ (mainly chromosome breaks).

Anaphase/Telophase Analysis

Figure 1 shows various types of abnormal anaphases in RPMC cultures. Control cultures showed a high proportion ($96 \pm 1.6\%$) of normal anaphase/telophases (mean of nine experiments) (Tables 4 to 7).

Mitomycin C (300 nM) produced a 10-fold increase in abnormal anaphases (mainly lagging chromosomes) compared with the untreated controls (Table 4). Anatase did not significantly increase abnormal mitoses (Table 5).

Crocidolite UICC (Table 6) induced a significant enhancement of anaphase abnormalities at $7.0 \mu\text{g}/\text{cm}^2$, whereas Canadian chrysotile (Table 7) did so at 1.0, 1.5, and $2.0 \mu\text{g}/\text{cm}^2$. Thus, when we compared the effects of chrysotile and crocidolite on a per-weight basis (Figure 2), more abnormalities were obtained with chrysotile. In terms of the number of total fibers per cm^2 , crocidolite UICC at 37.1×10^6 total fibers/ cm^2 ($7 \mu\text{g}/\text{cm}^2$) induced about 20% of abnormal anaphases, whereas Canadian chrysotile at 69.0×10^6 total fibers/ cm^2 ($2.0 \mu\text{g}/\text{cm}^2$) produced 26.7% of abnormal anaphases (Tables 6 and 7). Thus, considering the number of total fibers per cm^2 (Figure 2), crocidolite produced approximately the same percentage of abnormalities as chrysotile at all the concentrations tested. In contrast, on the basis of the number of fibers defined by Stanton and colleagues

TABLE 5
Anaphase and telophase analysis of RPMC exposed to anatase

Dose $\mu\text{g}/\text{cm}^2$	Part./ cm^2 ($\times 10^6$)	Experiment No.	Normal Mitosis (%)	Abnormal Mitosis (%)			
				Lagging Chromatin	Bridge	Asymmetric Segregation	Total
0.0	0.0	1	96.7	3.3	0.0	0.0	3.3
		2	93.3	6.7	0.0	0.0	6.7
5.0	11,000	1	97.3	2.0	0.7	0.0	2.7
		2	96.0	2.7	0.7	0.7	4.0
7.5	16,000	1	96.0	4.0	0.0	0.0	4.0
		2	94.7	3.3	0.7	1.3	5.3
10.0	22,000	1	96.7	2.7	0.7	0.0	3.3
		2	94.3	4.7	1.3	0.0	6.0

Definition of abbreviation: Part. = particles or fibers.

TABLE 6
Anaphase and telophase analysis of RPMC exposed to UICC crocidolite

Dose $\mu\text{g}/\text{cm}^2$	fibers/ cm^2 ($\times 10^6$)	Experiment No.	Normal Mitosis (%)	Abnormal Mitosis (%)			Total
				Lagging Chromatin	Bridge	Asymmetric Segregation	
0.0	0.0	1	96.0	2.7	0.7	0.7	4.3
		2	97.8	2.7	0.0	0.0	2.5
		3	94.7	4.7	0.7	0.0	5.3
0.9	2.7	2	96.0	2.7	0.7	0.7	4.0
		3	96.0	2.7	1.3	0.0	4.0
1.8	5.4	1	91.3	7.3	0.7	0.7	8.7
		2	85.3	10.0	3.3	1.3	14.7*
		3	91.4	4.6	1.3	2.6	8.6
3.5	10.5	1	90.7	6.7	1.3	1.3	9.3
		2	86.0	7.3	2.0	4.7	14.0*
		3	88.7	8.0	0.7	2.7	11.3
7.0	21.0	1	84.7	10.7	2.0	2.7	15.3*
		2	76.7	12.0	2.0	9.3	23.3*
		3	79.3	8.0	1.3	11.3	20.7*

* $P < 0.001$ (chi-square test).

(23) as the most carcinogenic (length $> 8 \mu\text{m}$, diameter $< 0.25 \mu\text{m}$), crocidolite was more efficient than chrysotile. Furthermore, the two fibers acted in a concentration-dependent manner (Figure 2). The predominant abnormality was lagging chromatin, mainly involving whole chromosomes. Bridges were significantly enhanced with mitomycin C but not with crocidolite; chrysotile produce a dose-dependent enhancement in one experiment.

Discussion

We studied the induction of metaphase and anaphase/telophase abnormalities in RPMC by asbestos fibers. Anatase did not produce metaphase abnormalities, while crocidolite significantly enhanced aneuploidy but produced few structural chromosome aberrations. This is in agreement with a study of V79 cells (17) in which 6% of treated cells showed aberrations after incubation with approximately $10 \mu\text{g}/\text{cm}^2$

TABLE 7
Anaphase and telophase analysis of RPMC exposed to Canadian chrysotile

Dose $\mu\text{g}/\text{cm}^2$	fibers/ cm^2 ($\times 10^6$)	Experiment No.	Normal Mitosis (%)	Abnormal Mitosis (%)			Total
				Lagging Chromatin	Bridge	Asymmetric Segregation	
0.0	0.0	1	96.7	2.7	0.7	0.0	3.3
		2	97.3	2.0	0.7	0.0	2.7
0.5	17.3	1	88.7	8.7	2.0	1.3	12.0*
		2	92.7	5.3	1.3	0.7	4.0
1.0	34.5	1	88.0	11.7	0.0	0.7	12.0*
		2	88.7	8.7	2.0	0.7	11.3†
1.5	51.2	1	72.0	22.0	4.0	1.3	28.0‡
		2	75.3	20.7	2.7	0.7	24.7‡
2.0	69.0	1	74.0	23.3	2.0	0.7	26.0‡
		2	72.7	19.3	7.3	0.7	27.3‡

* Statistically significant values of $P < 0.01$ (chi-square test).

† Statistically significant values of $P < 0.05$ (chi-square test).

‡ Statistically significant values of $P < 0.001$ (chi-square test).

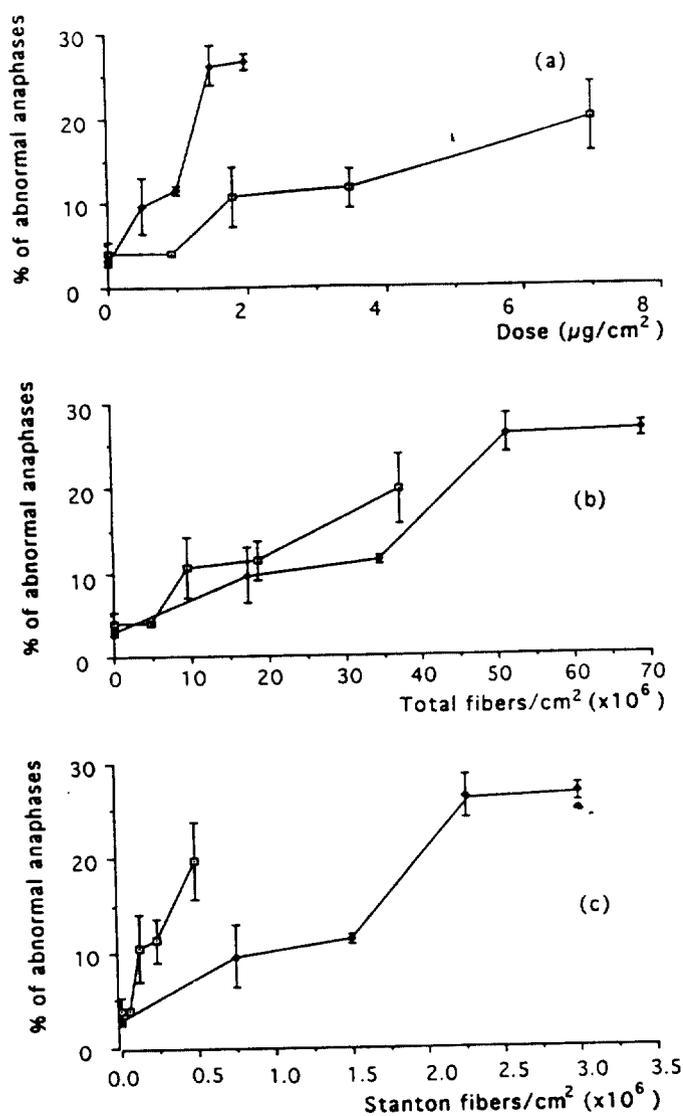


Figure 2. Anaphase/telophase response of RPMC according to the fiber amount: (a) weight; (b) total number of fibers; (c) Stanton fibers. Data shown following treatment with (squares) crocidolite UICC and (diamonds) Canadian chrysotile.

crocidolite. In contrast, 19% of Chinese hamster lung cells showed structural aberrations after treatment with $5 \mu\text{g}/\text{cm}^2$ crocidolite (24) and rates of up to 30% have been reported with ovarian cells (25). These differences might be due to the cell type and/or the culture medium. However, it should be noted that our experiments were not carried out in the best conditions to observe chromosomal aberrations since the cells were mainly at their second mitosis. A significant decrease in the number of diploid RPMC (42 chromosomes) was observed after exposure to 4 and $6 \mu\text{g}/\text{cm}^2$ crocidolite and mostly associated with hypodiploidy; similar results have been reported by Lechner and associates (7) with human mesothelial cells treated with amosite. In a previous study, we observed a significant decrease in the number of diploid RPMC with $0.4 \mu\text{g}/\text{cm}^2$ chrysotile, confirming that chrysotile is more effective on a per-weight basis (17).

Studies of anaphase/telophase showed that Canadian chry-

sotile and crocidolite UICC produced abnormal segregation of chromosomes in RPMC. Canadian chrysotile was more mutagenic than crocidolite UICC on a per-weight basis; in contrast, on the basis of the number of fibers with critical dimensions as defined by Stanton and colleagues (23), crocidolite was more efficient. Mitomycin C was a very powerful inducer of abnormal anaphase/telophase, confirming that DNA recombination can produce chromosome mis-segregation in these cells. Anatase, an isomorphous particle, produced no increase in abnormal anaphases, in agreement with the metaphase analysis in which no aneuploidy was detectable.

In similar experiments carried out with Syrian hamster embryo cells (16), crocidolite (3×10^6 fibers/ cm^2) produced 18% of abnormal anaphases (compared with 0.8% in controls), whereas with Chinese hamster lung cells V79 (17) crocidolite induced 4.9% of abnormal anaphases at 4.2×10^6 fibers/ cm^2 and 9.7% at 4.2×10^7 fibers/ cm^2 (compared with 2.3% in controls). In our experiments, we observed a rate similar to that obtained with V79 cells at low concentrations, but it reached about 20% when the amount of fibers was increased eightfold. RPMC thus seem to be less sensitive to crocidolite than Syrian hamster embryo cells, but more sensitive than V79 cells. Pakekar and co-workers (17) have reported that the results of anaphase analysis are limited by the reduction in anaphase figures, but this was not observed with RPMC at the concentrations we tested. Despite a concentration-dependent reduction in the number of mitotic figures due to an increasingly cytotoxic effect of the fibers, it was possible to analyze 150 anaphase/telophases in each group.

Our results show that two types of asbestos fibers inducing mesothelioma in animals produce mitotic damage in mesothelial cells, contrary to a noncarcinogenic compound, anatase (26). The asbestos samples studied here have been tested in rats by intrapleural inoculation to determine their carcinogenic potency. After injection of 20 mg, the tumor yields were 56% for crocidolite (27) and 29% for Canadian chrysotile (28) (0% for both controls). Moreover, a given number of amphibole fibers (length $> 8 \mu\text{m}$; diameter $\leq 0.25 \mu\text{m}$) produced more mesotheliomas than did chrysotile (29). The results obtained in the anaphase/telophase test showed the same order of toxicity for chrysotile and crocidolite only when the number of fibers with critical dimensions as defined by Stanton and colleagues (23) was taken into consideration. There is, thus, a qualitative correlation between *in vitro* and *in vivo* findings when comparisons are made on the basis of the relevant number of fibers. We have found differences in the degree of fiber dispersion in the various methods used to prepare fibers for animal and cell studies. This is especially true with chrysotile, where the different preparations have been found to vary by a factor of 7, probably due to the presence of protein in the medium, which favors the opening of bundles. Generally, only one high dose of fiber is tested for tumor induction *in vivo*; in contrast, a range of concentrations is studied *in vitro* using relatively low doses. A single intrapleural inoculation generally deposits 20 mg at the surface of the pleura, while a maximum of 2 mg is deposited on a surface area of 200 cm^2 *in vitro*, which approximately corresponds to the total rat pleural surface (30).

It has been suggested that neoplastic transformation by asbestos is a multistep process (31), and several mechanisms such as chromosome missegregation (16, 17), chromosomal mutations (32, 33), and DNA change (34) by production of oxygen reactive species (35) may be involved.

It is not clear whether chromosome missegregation caused by fibers is due to direct physical effects with normal cell division and/or to a chemical effect on the mitosis apparatus. As reported by Wang and colleagues (14), chromosomes are frequently in close contact with long, thin UICC chrysotile fibers *in vitro*; such chromosome-asbestos interactions were less frequent with crocidolite UICC than with chrysotile. Rieder and associates (15) proposed that chromosome missegregation may be due to steric phenomena (physical interaction of long fibers trapped in the spindle or with kinetochore, the motor of chromosome segregation, or with the chromosomes) or to charge-charge interactions of fibers with the mitosis apparatus. These interactions seem to be dependent on fiber dimensions and could conceivably interfere with chromosome segregation. These findings suggest a mechanism at the chromosomal level whereby asbestos might contribute to the initiating/progression phase of cell transformation.

Finally, our results confirm that anaphase/telophase analysis is a sensitive and complementary assay to metaphase analysis (chromosome structural aberrations and aneuploidy). A systematic study of other fibers with different properties may provide a better understanding of the mechanism of aneuploidy induction.

Acknowledgments: The writers thank Laurence Kheuang (INSERM U139) for the photography.

References

- Wagner, J. C., G. Berry, and V. Timbrell. 1973. Mesothelioma in rats after inoculation with asbestos and other material. *Br. J. Cancer* 28:173-185.
- Davis, J. M. G., S. T. Beckett, R. E. Bolton, P. Collings, and A. P. Middleton. 1978. Mass number of fibers in the pathogenesis of asbestos related lung diseases in rats. *Br. J. Cancer* 37:637-647.
- Patérou, M. J., J. Bignon, and M. C. Jaurand. 1985. *In vitro* transformation of rat pleural mesothelial cells by chrysotile fibers and/or benzopyrene. *Carcinogenesis* 6:523-529.
- Bignon, J., J. Peto, and R. Saracci. 1989. Non-occupational exposure to mineral fibers. IARC Scientific Publication No. 90. IARC, Lyon, France.
- Tiainen, M., L. Tammilehto, T. Tuomi, and S. Knuutila. 1989. Chromosomal abnormalities and their correlations with asbestos exposure and survival in patients with mesothelioma. *Br. J. Cancer* 60:618-626.
- Hagemeyer, A., M. A. Versnel, E. Van Drunen, M. Moret, M. J. Bouts, T. H. Van der Kwast, and H. C. Hoogsteden. 1990. Cytogenetic analysis of malignant mesothelioma. *Cancer Genet. Cytogenet.* 47:1-28.
- Lechner, J. F., T. Tokiwa, M. LaVeck, W. F. Benedict, S. Banks-Schlegel, H. Yeager, A. Banerjee, and C. C. Harris. 1985. Asbestos-associated chromosomal changes in human mesothelial cells. *Proc. Natl. Acad. Sci. USA* 82:3884-3888.
- Libbus, B. L., and J. E. Craighead. 1988. Chromosomal translocations with specific breakpoints in asbestos-induced rat mesothelioma. *Cancer Res.* 48:6455-6461.
- Funaki, K., J. Everitt, E. Bermudez, and C. Walker. 1991. Trisomy of rat chromosome-1 associated with mesothelial cell transformation. *Cancer Res.* 51:4059-4066.
- Jaurand, M. C., L. Saint-Etienne, A. Van der Meeren, S. Endo-Capron, A. Renier, and J. Bignon. 1991. Neoplastic transformation of rodent cells. In Cellular and Molecular Aspects of Fiber Carcinogenesis. C. C. Harris, J. F. Lechner, and B. R. Brinkley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 131-147.
- Walker, C., J. Everitt, and J. C. Barrett. 1992. Possible cellular and molecular mechanisms for asbestos carcinogenicity. *Am. J. Ind. Med.* 21:253-273.
- Jaurand, M. C. 1991. Mechanisms of action of fibers in carcinogenesis. In Asbestos Related Cancer. M. Sluysers, editor. Ellis Horwood Ltd., Chichester, UK. 42-60.
- Cole, R. W., J. G. Ault, J. H. Hayden, and C. L. Rieder. 1991. Crocidolite asbestos fibers undergo size-dependent microtubule-mediated transport after endocytosis in vertebrate lung epithelial cells. *Cancer Res.* 51:4941-4947.
- Wang, N. S., M. C. Jaurand, L. Magne, L. Kheuang, M. C. Pinchon, and J. Bignon. 1987. The interactions between asbestos fibers and metaphase chromosomes of rat pleural mesothelial cells in culture. A scanning and transmission electron microscopic study. *Am. J. Pathol.* 126:343-349.
- Rieder, C. L., G. Sluder, and B. R. Brinkley. 1991. Some possible routes for asbestos-induced aneuploidy during mitosis in vertebrate cells. In Cellular and Molecular Aspects of Fiber Carcinogenesis. C. C. Harris, J. F. Lechner, and B. R. Brinkley, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1-26.
- Hesterberg, T. W., and J. C. Barrett. 1985. Induction by asbestos of anaphase abnormalities: mechanism for aneuploidy induction and possible carcinogenesis. *Carcinogenesis* 6:473-475.
- Palekar, L. D., J. F. Eyre, B. M. Most, and D. L. Coffin. 1987. Metaphase and anaphase analysis of V79 cells exposed to erionite, UICC chrysotile and crocidolite UICC. *Carcinogenesis* 8:553-560.
- Jaurand, M. C., L. Kheuang, L. Magne, and J. Bignon. 1986. Chromosomal changes induced by chrysotile fibres or benzo-3,4-pyrene in rat pleural mesothelial cells. *Mutat. Res.* 169:141-148.
- Latt, S. A. 1974. Sister chromatid exchanges induce human chromosome damage and repair: detection by fluorescence and induction by mitomycin C. *Proc. Natl. Acad. Sci. USA* 71:3162-3164.
- Jaurand, M. C., J. F. Bernaudin, A. Renier, H. Kaplan, and J. Bignon. 1981. Rat pleural mesothelial cells in culture. *In Vitro* 2:98-102.
- Savage, J. R. 1975. Classification and relationships of induced chromosomal structural changes. *J. Med. Genet.* 12:103-122.
- Wissinger, W. L., D. E. Estervig, and R. J. Wang. 1981. A differential staining technique for simultaneous visualization of mitotic spindle and chromosomes in mammalian cells. *Stain Technology* 56:221-226.
- Stanton, M. F., M. Layard, A. Tegeris, A. Miller, M. May, E. Morgan, and A. Smith. 1981. Relation of particle size to carcinogenicity in amphibole asbestos and other fibrous mineral. *J. Natl. Cancer Inst.* 67:965-975.
- Huang, S. L., D. Saggiaro, H. Michelmann, and H. V. Malling. 1978. Genetic effects of crocidolite asbestos in Chinese hamster lung cells. *Mutat. Res.* 57:225-232.
- Sincock, A. M., J. D. A. Delhanty, and G. Casey. 1982. A comparison of the cytogenetic response to asbestos and glass fibres in Chinese hamster and human cell lines. *Mutat. Res.* 101:257-268.
- Pott, F., U. Ziem, F. J. Reiffer, F. Huth, H. Ernst, and U. Mohr. 1987. Carcinogenicity studies on fibres, metal compounds, and some other dusts in rats. *Exp. Pathol.* 32:129-152.
- Jaurand, M. C., J. Fleury, G. Monchaux, M. Nebut, and J. Bignon. 1987. Pleural carcinogenic potency of mineral fibers (asbestos, attapulgite) and their cytotoxicity on cultured cells. *J. Natl. Cancer Inst.* 79:797-804.
- Van der Merren, A., J. Fleury, M. Nebut, G. Monchaux, X. Janson, and M. C. Jaurand. 1992. Mesothelioma in rats following intrapleural injection of chrysotile and phosphorylated chrysotile (chrysophosphate). *Int. J. Cancer* 50:937-942.
- Jaurand, M. C. 1991. Observations on the carcinogenicity of asbestos fibers. *Ann. NY Acad. Sci.* 643:258-271.
- Rubin, J., M. Clawson, A. Planch, and Q. Jones. 1988. Measurements of peritoneal surface area in man and rat. *Am. J. Med. Sci.* 295:453-458.
- Barrett, J. C., P. W. Lamb, and R. W. Wiseman. 1989. Multiple mechanisms for the carcinogenic effects of asbestos and other mineral fibers. *Environ. Health Perspect.* 81:81-89.
- Hei, T. K., Z. Y. He, C. Q. Piao, and C. A. Waldren. 1991. The mutagenicity of mineral fibers. In Mechanisms in Fibre Carcinogenesis. R. C. Brown, editor. NATO Asi Series A. Plenum Press, New York. 223:319-325.
- Hei, T. K., C. Q. Piao, Z. Y. He, D. Vannais, and C. A. Waldren. 1992. *Cancer Res.* 52:6305-6309.
- Renier, A., F. Lévy, F. Pilière, and M. C. Jaurand. 1990. Unscheduled DNA synthesis in rat pleural mesothelial cells treated with mineral fibres. *Mutat. Res.* 241:361-367.
- Lund, L. G., and A. E. Aust. 1991. Iron-catalyzed reactions may be responsible for the biochemical and biological effects of asbestos. *BioFactors* 3:83-89.

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May 26, 1993

Document Control Officer
Chemical Information Division
Office of Toxic Substances (WH-557)
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401 M Street, S.W.
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To Whom It May Concern:

The following report is being sent to you on an F.Y.I. basis.

A study of the *in vitro* toxicology of diatomaceous earth (DE) products has been completed at Mountain Technical Center (MTC) of Schuller International, Inc. Findings are summarized below; complete report is attached.

At this time, there are no known *in vitro* tests that have been validated to predict the pathogenic potential of respirable dusts to the whole organism. It is not known to what, if any, extent the results from *in vitro* tests reflect what happens in the whole organism.

In Vitro toxic effects of four siliceous dusts were evaluated in cultures of Chinese hamster ovary (CHO) cells using four toxic endpoints: inhibition of proliferation, colony forming efficiency, cell viability as measured by intracellular esterase activity, and micronucleus induction. Natural and flux-calcined (heated to >2000°F with soda ash; decreases surface area by melting and closing smaller porosities) diatomaceous earth (DE) products were size-separated to provide two respirable subpopulations of the bulk product. These two respirable subpopulations were used as the test dusts for the *in vitro* studies; they are designated Nat and FC, respectively. Toxic effects of Nat and FC were compared with those of two pure crystalline silica reference dusts, α -quartz and cristobalite, as well as TiO_2 and UICC crocidolite asbestos (CD). All four silica dusts elicited a qualitatively similar, concentration-dependent response pattern in the CHO cells, consisting of particle uptake, visible nuclear alterations (micro-, multiple-, and/or misshapen nuclei), reduction in

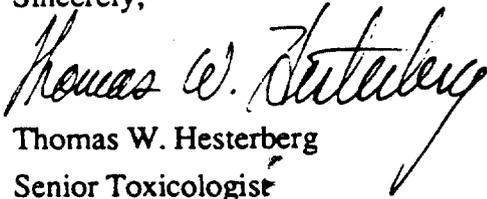
Mountain Technical Center
10100 West Ute Avenue (80127)
P.O. Box 625005
Littleton, CO 80162-5005
Tel 303 978-5200

SCHULLER

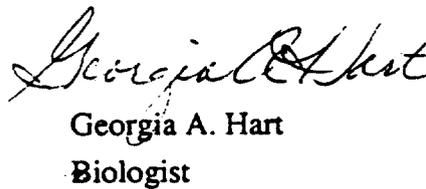
A Unit of Schuller International, Inc.

cell proliferation and colony formation, and no evidence of decreased cell viability. This same pattern has been observed in CHO and other cultured cells exposed to crocidolite and other mineral and vitreous fibers. However, the severity of toxic response varied among the test particulates. When the EC50's (concentration which reduced cell proliferation to 50% of negative controls) of the 4 silica dusts were calculated as $\mu\text{g}/\text{cm}^2$, Nat was the most toxic ($\text{EC50} = 4 \mu\text{g}/\text{cm}^2$) and cristobalite was least toxic ($\text{EC50} = 25 \mu\text{g}/\text{cm}^2$). The ranking shifted when concentration was calculated as number of particles/ cm^2 : FC was the most toxic ($\text{EC50} = 860 \times 10^3$), Nat and cristobalite were similar ($\text{EC50} = \sim 2000 \times 10^3$), and a-Quartz was least toxic ($\text{EC50} = 8400 \times 10^3$). In an attempt to determine the causes of these differences in toxicity, various parameters (crystallinity, surface area, particle dimensions, % fibrous particles) of the four dusts were compared. Natural DE is primarily amorphous while flux calcined DE is 40% crystalline and the two reference dusts are >96% crystalline. The number of particles/ μg was 6.5-fold greater in Nat than in FC, and Nat was estimated to have a 10-fold greater surface area than FC. One possible cause of toxic differences could be a combination of surface area and surface chemistry resulting in different adsorbance capacities of the various particulates for cellular constituents. Another possible cause of toxic differences could be percentage of particles that are fibrous (length/diameter ≥ 3). When EC50's are calculated as number of fibers longer than $7.5 \mu\text{m}$ per cm^2 , the EC50's for Nat, FC, cristobalite and CD are very similar to each other (214, 259, 214 and 384, respectively, $\times 10^3$). A-quartz and TiO_2 had no fibrous particles. Further investigations of DE materials are needed to test these hypotheses. A clarification of the relationships between toxicity and particle characteristics could provide a major step toward an understanding of the cellular mechanisms of particulate toxicology.

Sincerely,



Thomas W. Hesterberg
Senior Toxicologist
Health Safety and Environment



Georgia A. Hart
Biologist

cc: R. Anderson, A. Jankousky, R. Versen, J. Chase, R. Batson, M. Merliss
(International Diatomaceous Earth Producers Assoc.), M. Fleischman (Celite Corp.), V.
Vu (US EPA)

**In Vitro Toxicology of
Size-Selected Fractions of
Diatomaceous Earth
Products**

G. A. Hart and T. W. Hesterberg

May 1993

Mountain Technical Center, Schuller Int.

In Vitro Toxicology Laboratory

Littleton, Colorado

In Vitro Toxicology of Size-Selected Fractions of Diatomaceous Earth Products

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In Vitro Toxicology of Size-Selected Fractions of Diatomaceous Earth Products

G.A. Hart and T.W. Hesterberg
Mountain Technical Center, Schuller International, Littleton, Colorado

ABSTRACT

In Vitro toxic effects of four silicon dusts were evaluated in cultures of Chinese hamster ovary cells (CHO) using four toxic endpoints: inhibition of proliferation, colony forming efficiency, viability as measured by intracellular esterase activity, and micronucleus induction. Natural and flux-calcined diatomaceous earth (DE) products were size-separated to provide two respirable test dusts, Nat and FC, respectively. Toxic effects of the two diatomaceous earth dusts were compared with those of two pure crystalline silica reference dusts (α-quartz and cristobalite), TiO₂ and UICC crocidolite asbestos (CD). All four silica dusts elicited a similar, concentration-dependent response pattern in the CHO cells, consisting of particle uptake, visible nuclear alterations (micro-, multiple-, and/or misshapen nuclei), reduction in cell proliferation and colony formation, and no evidence of decreased cell viability. This same pattern has been observed in CHO and other cell cultures exposed to crocidolite and other mineral fibers. However, the severity of toxic response varied among the test particulates. When the EC50's (concentration which reduced proliferation to 50% of negative controls) of the 4 silica dusts were calculated as μg/cm², Nat was the most toxic (EC50 = 4 μg/cm²) and cristobalite was least toxic (EC50 = 25 μg/cm²). The ranking shifted when concentration was calculated as number of particles/cm² -- FC was the most toxic (EC50 = 860 x 10³), Nat and cristobalite were similar (EC50 = ~2000 x 10³), and α-Quartz was least toxic (EC50 = 8400 x 10³). In an attempt to determine the causes of these differences in toxicity, various parameters (crystallinity, surface area, particle dimensions, % fibrous particles) of the four dusts were compared. Natural DE is primarily amorphous while flux calcined DE is 40% crystalline and the two reference dusts are >96% crystalline. The number of particles/μg was 6.5-fold greater in Nat than in FC, and Nat was estimated to have a 10-fold greater surface area than FC. One possible cause of toxic differences could be a combination of surface area and surface chemistry resulting in different adsorbencies of the various particulates for cellular constituents. Another possible cause of toxic differences could be percentage of particles that are fibrous (length/diameter ≥ 3). When EC50's are calculated as number of fibers longer than 7.5 μm per cm², the EC50's for Nat, FC, cristobalite and CD are very similar to each other (214, 259, 214 and 384, respectively, x 10³). α-quartz and TiO₂ had no fibrous particles. Further investigations of DE materials are needed to test these hypotheses. A clarification of the relationships between toxicity and particle characteristic could provide a major step toward an understanding of the cellular mechanisms of particulate toxicology.

INTRODUCTION

In vitro cytotoxicity of particulates has in some cases paralleled in vivo pathogenesis (Hart et al., 1992). However, the in vitro responses of cells to a toxin may vary greatly depending upon the cell type and the species of origin. Therefore, no single in vitro test system has been demonstrated to predict the toxicity of a substance in the whole animal. Furthermore, some in vivo correlates of particulate toxicity cannot at this time be measured in vitro, such as lung deposition and clearance and long term biopersistence. Therefore, a battery of in vitro tests is recommended as a means of screening materials for their toxicologic potential, followed by short term animal inhalation studies to determine lung deposition and clearance, and finally long term animal inhalation studies to determine biopersistence and pathogenesis (Hesterberg et al., 1991).

The purpose of the present study was to develop data which might contribute to a better understanding of the cellular mechanisms of particulate toxicity. To this end, the study utilized size-selected, respirable diatomite subfractions which were well characterized in terms of particle number/ μg , size, shape and crystallinity. These subfractions should be further characterized for surface area and chemical composition.

The in vitro systems herein described utilized four different assays, each of which measures a different parameter of toxicity: cell proliferation, viability, colony forming efficiency and abnormal nucleus induction (methods described in *Appendix*). In vitro cell responses to size-selected sub-fractions of two diatomite types (natural and flux calcined) were compared with responses to two reference chemicals (cristobalite and α -quartz), all of which were composed of particles within the respirable range. Two tests also included titanium dioxide particles, which is generally considered to be a nontoxic, nuisance dust.

In these assays, the in vitro toxicity of size-selected diatomites is quantitatively similar to that of crocidolite asbestos. DE also induced abnormal nuclei that were similar to those induced by CD and other vitreous fibers (Hart et al., 1992a, 1992b); however, some qualitative differences were noted between DE-induced and fiber-induced abnormalities, respectively (see *Results*). In contrast to the diatomites, the in vitro toxicity of the two reference crystalline silica particulates (cristobalite and quartz) was relatively low and similar to that observed with titanium dioxide.

Suggestions for follow-up in vitro research include the use of lung cell types derived from the actual target tissues of particle-induced respiratory disease in humans and animals (lung epithelium, macrophages, mesothelium and/or fibroblasts). To determine the particle characteristics that are responsible for the toxic effects, assays could be conducted using an assortment of different DE dusts that have been altered in various ways, e.g., particle size and shape, total surface area, and chemical composition (see *Discussion*).

TEST PARTICULATES

Selection of Test Particles, Rationale

Natural and flux calcined diatomaceous earth (DE) were obtained from Celite Corporation and subjected to a size-separation process in the MTC Particle Preparation Laboratory (see next section). These two diatomite forms have some similarities as well as important differences (Table I.). Both are composed of fine, porous silicon dioxide particles in a variety of sizes and shapes (see photographs, Figure 1A-H.)). The Materials Safety and Data Sheets (MSDS) provided by Celite for these two products show a major difference in crystallinity. Natural DE is mostly amorphous silica, with less than 3% crystalline quartz. Flux calcined DE has a much higher crystalline content; it also contains no more than 3% quartz but can be as much as 60% cristobalite (Table I). This agrees with the crystallinity analysis performed on the bulk products at MTC: natural DE had 3.98% quartz and no cristobalite; flux calcined DE had 1.95% quartz and 39.59% cristobalite.

Other differences between the two forms of DE that could influence toxicity could exist in surface area, porosity, density, size, shape and surface chemistry. Surface area in bulk natural DE is 10- to 20-fold greater (roughly 20 cubic meters/gram) than in flux calcined (approximately 1-2 cubic meters/gram). Porosity is generally believed to be less prevalent and finer in flux calcined than in natural DE (verbal communication, Garth Coombs, Schuller Int., Inc.). Density (number of particles/unit mass), size and shape were evaluated for the size-selected test particles (Table II.) and are discussed in *Results*. Surface chemistry was not analyzed in the present study.

The alpha-quartz and cristobalite particles used in this study are respirable reference chemicals from the U.S. National Bureau of Standards (NBS). These two particulates are

two different, pure crystalline forms of silicon dioxide (96% and 98% purity, respectively). Quartz and cristobalite were selected for use in the study in order to evaluate the toxic impact of crystalline versus amorphous silicon dioxide.

The toxic effects of DE are compared with the toxicity of Union Internationale Contre Cancer (UICC) crocidolite asbestos (CD), which was evaluated in the MTC In Vitro Laboratory in a separate study. CD was obtained from V. Timbrell*. Titanium Dioxide was obtained from the U.S. National Bureau of Standards, Standard Reference No. 154a.

Test Article Preparation and Characterization

Natural diatomite and flux calcined diatomite products were subjected to a size-selection process at MTC to provide a test material of each that is composed of particulates within the respirable size range. The size-selected fractions of these two materials are designated S-Nat and S-FC. CD and the three reference chemicals, cristobalite, alpha-quartz and titanium dioxide, were not size-selected because they were in the respirable size range as supplied.

Because particle size and geometry are known to be critical determinants of toxicity, especially with fibrous materials, length, width and aspect ratio (length/width) were determined for each of the test articles using scanning electron microscopy (SEM). Length and width were determined for each of 100 particles by measuring first the longest axis and then the perpendicular axis that appeared to be representative of the width of the particle. Data are summarized in Table II and graphed in Figure 2. S-Nat and S-FC are composed of particles in a variety of shapes and sizes, some of which (38% and 36%, respectively) are more or less fibrous (fibrous is here defined as having an aspect ratio ≥ 3). In Figure

* Dr. V. Timbrell, Pneumoconiosis Research Unit, Llandough Hospital, Penarth, Glamorgan, U.K.

REFERENCES

- Clark, G. 1981. Staining Procedures, Fourth Edition. Waverly Press, Inc.
- Hart, G.A., Newman, M.M., Bunn, W.B. and Hesterberg, T.W. Cytotoxicity of refractory ceramic fibers to chinese hamster ovary cells in culture. 1992a. *Toxicology In Vitro* 6(4):317-362.
- Hart, G.A., M.M. Newman, W.B. Bunn, and T.W. Hesterberg Importance of fiber dimensions in the cytotoxicity of mineral fibers to chinese hamster ovary cells in culture. The Toxicologist, March, 1992b.
- Hesterberg, T.W., and Barrett, J.C. 1985. Induction by asbestos fibers of anaphase abnormalities: mechanism for aneuploidy induction and possibly carcinogenesis. *Carcinogenesis* 6:473-475.
- Hesterberg, T.W., R. Mast, E.E. McConnell, O. Vogel, J. Chevalier, D.M. Bernstein and R. Anderson. Chronic inhalation toxicity and oncogenicity study of refractory ceramic fibers in Fisher 344 rats. The Toxicologist 11:85(254), 1991.
- Hesterberg, T.W., Vu, V., McConnell, E.E., Chase, G.R., Bunn, W. B. and Anderson, R.. Use of Animal Models to Study Man-made Fiber Carcinogenesis. In "Cellular and Molecular Aspects of Fiber Carcinogenesis", Current Communications in Cell and Molecular Biology, (eds. C. Harris, J. Lechner, and B. Brinkley), Cold Spring Harbor Laboratory Press, pp. 183-205, 1991.
- Puck, T.T. *J. Exp. Med.* 108:945, 1958.
- Sincock, A. M., Delhanty, J. D. A., and Casey, G. 1982. A comparison of the cytogenetic response to asbestos and glass fibre in Chinese hamster and human cell lines. Demonstration of growth inhibition in primary human fibroblasts. *Mutat. Res.* 101, 257-268.

ACKNOWLEDGEMENTS

The data in this report was compiled through the cooperation of the following Mountain Technical Center Laboratories. Individuals who were particularly instrumental in the technical work are mentioned:

MTC Particle Preparation Laboratory, W. Miller

Size separation of diatomite test particles: Barry Fitzpatrick.

MTC Microstructural Analysis Laboratory, R. Hamilton

SEM dimensional analysis and photography of diatomite test particles: Frank D'Ovidio, John Strothers and Cathy Smith.

MTC In Vitro Laboratory, G.A. Hart

PCOM and Fluorescent microphotographs: Lisa Kathman.

Technical Support for in vitro testing: Lisa Kathman and Mildred Newman .

Study Director: T.W. Hesterberg, Ph.D.

Study Supervisor: G.A. Hart, M.A.

Figure 1. SEM Microphotographs of Diatomite Test Articles: A.-B.: s-Na: DE, magnified X 1000 & 2500, respectively. C.-D.: s-FC DE, magnified X 1000 & 2500, respectively.

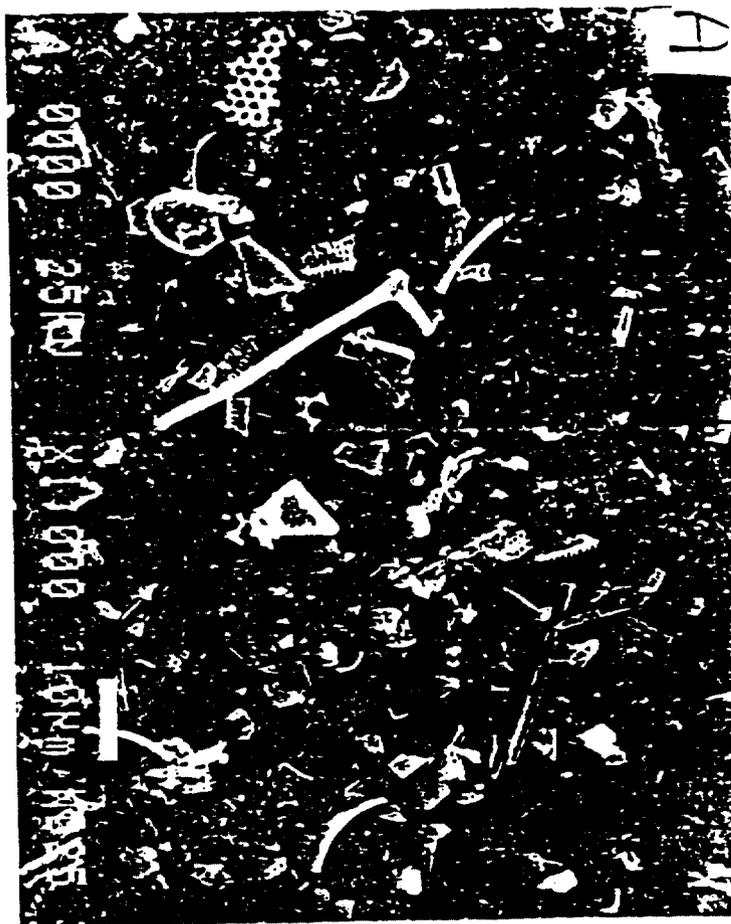
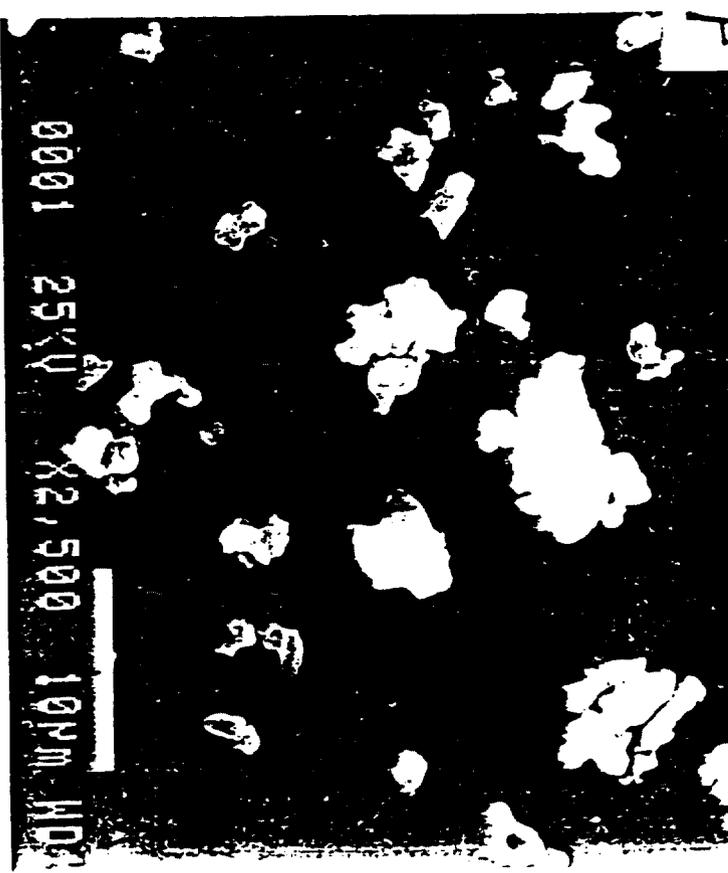
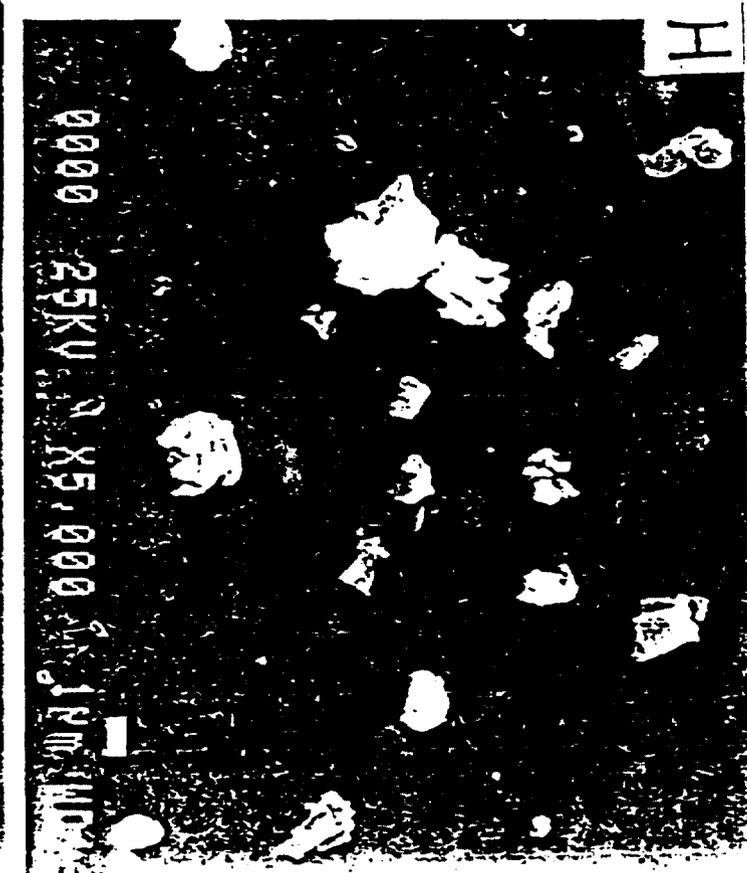
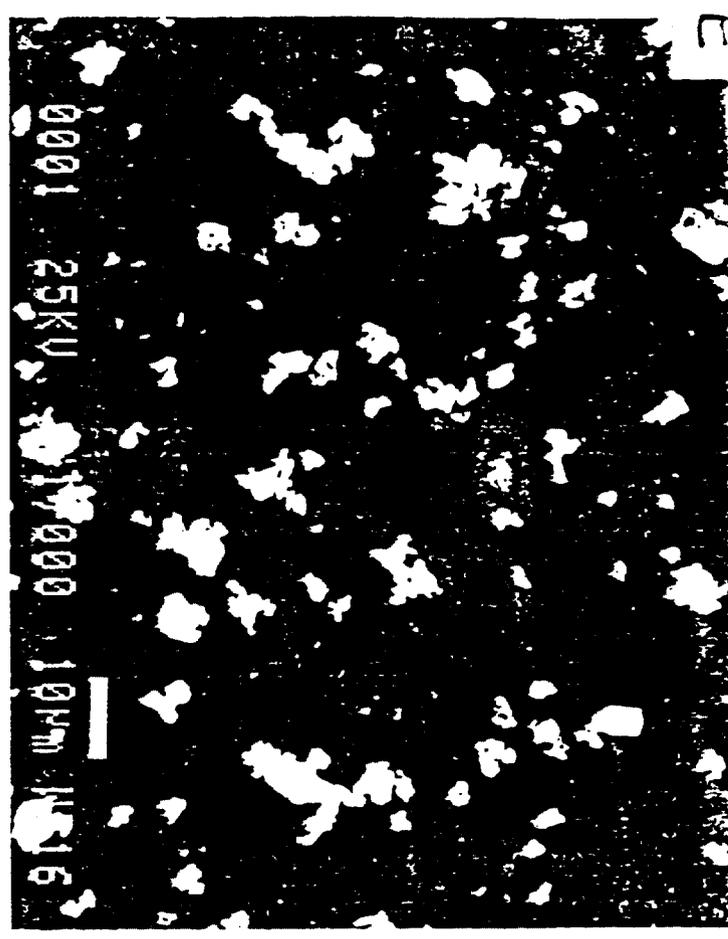
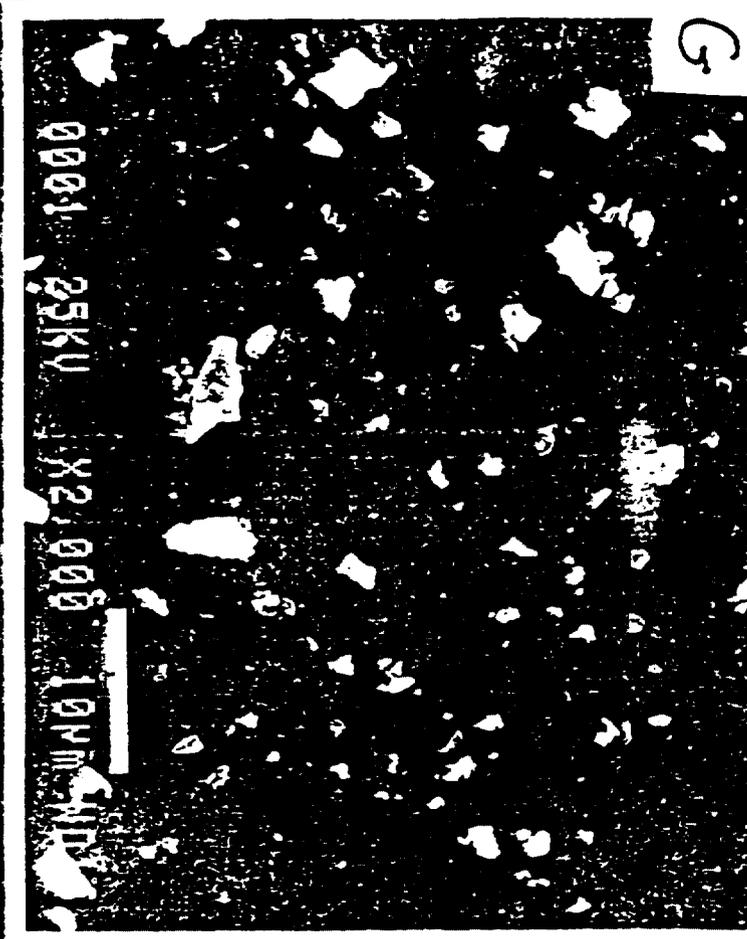


Figure 1. SEM Microphotographs of Crystalline Silica Reference Particles: E.-F.: Cristobalite, magnified X 1000 & 2500, respectively. G.-H.: alpha-Quartz, magnified X 2000 & 5000, respectively.



1, it can be seen that S-Nat has a population of particles that are similar in size to those of S-FC, but the former also contains a second population of particles that are much smaller than those of the latter. Average dimensions of the constituent particles of natural DE are $3.8 \pm 3.9 \mu\text{m} \times 1.3 \pm 1.0 \mu\text{m}$ and of flux calcined DE are $5.9 \pm 4.1 \mu\text{m} \times 2.1 \pm 1.5 \mu\text{m}$. Cristobalite particles fall roughly in the size ranges of the diatomites and a portion (21%) of these are fibrous (aspect ratio ≥ 3), while alpha-quartz particles are much smaller and do not have a significant portion of fibrous shapes. Figure 1 also includes an electron micrograph of titanium dioxide particles at approximately 1000X.

The toxicity of fibers may be influenced more by particle number than by particle mass (Hart et al., 1992a and 1992b). The same may be true for non-fibrous particulates. Therefore the number of particles/ μg was determined for each of the test articles (Table II). Note that the number of particles/ μg is roughly eight times greater for S-Nat and quartz (514,000 and 525,000 particles/ μg , respectively) than for S-FC and cristobalite (78,000 and 75,000 particles/ μg , respectively), Table II. The higher number of particles/ μg in S-Nat and quartz could be related to smaller average particle sizes, particularly the smaller diameters of these two materials (1.3 and 1.1 μm , respectively). Differences in porosity may also play a role in that flux calcined DE particles may be less porous and therefore heavier, resulting in fewer particles per unit mass. Differences in porosity would also result in differences in surface area, which could in turn impact toxic effect if surface chemistry is involved in toxicity (e.g., adsorption and subsequent disruption of cellular components). Future studies should determine the surface area of the size-selected particulates as well as the bulk starting material. Surface chemistry should also be analyzed.

METHODS AND MATERIALS

The four assays used to assess the in vitro cytotoxicity of the test articles are described below. Methodologies are summarized in the *Appendix*.

Cell Proliferation Assays

Two different assays were used to measure the potential of the test material to inhibit cell proliferation: the "Inhibition of Cell Proliferation" (ICP) and the "Colony Forming Efficiency" (CFE) assay. Each test included 3-4 concentrations of each test material, negative controls of cells in growth medium only, and 3 replicates of each exposure. Each of the two assays was conducted a minimum of three times. These two assays do not distinguish between the cessation of cell division and the outright killing of cells.

Cell Viability Assay

The esterase activity viability (EAV) assay was utilized to assess cell viability. Cultures of CHO-K1 cells were exposed to a high concentration of the test article for three days and harvested as described for the ICP assay. Suspensions of the harvested cells were treated with carboxyfluorescein (CF), a non-fluorescent ester, which is taken up by both viable and non-viable cells. The intracellular CF is cleaved by cytoplasmic esterases and then becomes both polar and fluorescent. The polar compound accumulates in the viable cells because the intact membranes of healthy cells do not permit polar molecules to escape. The non-viable cells, with damaged membranes, lose the dye as rapidly as it forms and therefore do not fluoresce.

Nuclear Abnormality Assay

The impact of the test articles on nuclear material was assessed by an assay which measures the induction of nuclear abnormalities (INA). This assay was developed from a standard genotoxicity assay which measures the incidence of micronucleus formation. A micronucleus is believed to form as a result of chemical or mechanical breakage of chromosomes or disturbance of chromosome migration during cell division. In previous cell culture studies with asbestos, small groups of chromosomes or chromosome fragments were displaced from the two masses of chromosomes which had migrated to the opposite poles of the dividing cell (Hesterberg and Barrett, 1985). These 'lost' chromosomes become incorporated in their own nuclear membrane and appear as a small, secondary nucleus alongside the main cell nucleus.

Micronucleus formation, with few other visible nuclear deformities, is typically induced in CHO cells as a result of treatment with a soluble chemical carcinogen such as mitomycin. However, in vitro exposure to certain particulates, such as asbestos and some other mineral fibers, induces a range of nuclear abnormalities, including micronuclei, multiple nuclei, and many-lobed nuclei. Other studies have shown a dose-dependent increase in structural and numerical chromosomal changes with increasing concentrations of asbestos or other mineral fibers (Sincock, et al., 1982). In our laboratory, the incidence of nuclear abnormalities increases with increasing CD concentration, suggesting that these abnormalities are related to chromosomal changes induced by the fibers. Therefore, the INA assay used in this study determines the incidence of all of the above nuclear abnormalities.

Inhibition of Proliferation with Particle-Exposed Medium versus Particulates

Toxic effects observed following exposure to the test particles could be a result of one or more phenomena, including: (1) direct contact between particles and cells, in which particle size, shape and/or surface chemistry impacts cell structure; (2) removal of essential growth substances from the culture medium as a result of the adsorption of these substances onto the surface of the test particles; or (3) release of toxic chemicals into the growth medium as a result of test particle leaching. The second two possibilities were tested using proliferation assays as described below.

To test the toxicity of the particle-exposed medium from each of the respective test particles, growth media were prepared with a high concentration of each of the particles and incubated approximately 24 hrs. at 37°C with gentle rocking. For negative controls, growth medium without test particles was incubated similarly. All five aliquots of medium (with or without particulates) were centrifuged, and the supernatant of each was filtered through Millipore filter (a 0.45 μm filter was used in the first test; a 0.22 μm filter was used in the second test). The filtrate was then added to culture dishes containing actively growing cells. Other cultures were exposed to growth medium containing the test particles. Incubation, harvesting and counting procedures were followed as described in the *Appendix* for the ICP test. The filtrate was examined for the presence of particles. Because 200,000-400,000 particles/ml were observed in the filtrates of the first test (data not shown), a .22 μm filter was used in the second test. Filtrates in the second test had approximately 2,000-4,000 very fine particles/ml (unfiltered diatomite suspensions used in this test contained approximately 8×10^6 S-FC particles/ml or 40×10^6 S-Nat particles/ml).

Test System: CHO-K1 Cell Line

The Chinese hamster ovary (CHO-K1) cell line was selected for these studies for several reasons. Used in our laboratory in over 100 assays on 20 different mineral fiber samples, these cells have proven to be highly phagocytic (readily ingest or take up particles), highly proliferative (dividing once or twice per day) and sensitive to toxic particulates such as asbestos. Furthermore, the relative severity of toxic effects induced by four different refractory ceramic fibers in our in vitro CHO cell studies shows concordance with the in vivo progression of fibrosis and the incidence of lung tumors observed in long term animal inhalation studies (Hart et al., 1992a). The CHO-K1 cell line is an immortalized cell line derived from cultured hamster ovary cells (Puck, 1958). It is an anchorage-dependent (adheres to the bottom of the culture dish), epithelia-like cell type.

RESULTS

Cell Proliferation and Viability

Data from the ICP and CFE tests using the CHO-K1 test system (Figure 3 and 4) show a similar pattern: S-Nat is most toxic, S-FC is intermediate, and the respective toxicities of the two pure crystalline forms of silica (cristobalite and quartz) are relatively low. Figure 3 compares the ICP curves of the two DE's with the curves for UICC crocidolite asbestos (CD). (Tests for CD followed the same protocol but were not conducted simultaneously with the DE ICP tests.) Note that these comparisons are made on the basis of mass/cm² and not by number of particles/cm². In this test system, the cell proliferation curve for cultures exposed to S-Nat is similar to the curve for cultures exposed to CD. The EC50 (concentration which reduced proliferation to 50% of negative controls) of each test particulate is provided in Table II.

The inhibition of proliferation induced by particulate suspensions contrasts sharply with the inhibition induced by the filtrate from these suspensions. When particles were removed from the medium by a .22 μ m filter and then added to the cell cultures, virtually no inhibition was observed (Figure 5). However, when a .45 μ m filter was used (one test), a slight depression in cell numbers was observed for both DE's (data not included). This was probably due to the quantity of fine particles observed in the filtrate.

CHO-K1 cell viability, as measured by the EAV test, was not appreciably decreased by exposure to high concentrations of the test particulates (Figure 6 and Table III). This contrasts sharply with the large differences in cell proliferation observed over the same three days of particulate exposure (Figure 7). Note that in this test, cristobalite-exposure is higher (50 μ g/cm²) than that of the other four test articles (20 μ g/cm².) Cell numbers in S-

Nat- and CD-exposed cultures show almost no increase over the original number of cells plated; cristobalite, at more than twice the mass concentration of the other test particulates, is the least inhibitory and S-FC is intermediate.

Nuclear Abnormality Assay

The results from the nuclear abnormality assay (Figure 8) agree with the results from the ICP and CFE assays; the highest incidence of nuclear abnormalities was observed in the S-Nat cultures, the incidence with S-FC was second highest, and the two crystalline reference particulates had relatively low incidences.

The types of visible cell damage induced by each of these four particulates can be seen in the photomicrographs of acridine orange-stained cells (Figures 9-14). The photographs are in pairs: the first was taken with fluorescence microscopy, and the second depicts the same field with phase contrast optical microscopy (PCOM). In the fluorescent photographs, the cytoplasm of the cells is orange and the nuclei are yellow. In the PCOM photographs, the cells appear as opalescent bodies in tones of blue, yellow and green.

Unexposed CHO-K1 cells, growing in culture medium without any test particulates, are seen in Figure 9A and B. Note the micronucleus (arrow). In unexposed CHO-K1 cells the background incidence of nuclear abnormalities ranges from 2-7%. One or two small, distinct micronuclei per cell is typical of genotoxic chemical exposure; however, as can be seen in the fluorescent photomicrographs, nuclear abnormalities induced by particulates is typically far more diverse, including multiple and grossly distorted nuclei as well as micronuclei.

Shapes, sizes and locations of the test particles can be seen in the PCOM photographs of the exposed cultures, even though fixing and staining procedures wash away a portion of the particles. The many fibrous shapes as well as non-fibrous particles of both diatomite dusts are visible in Figures 10B, 10D, 10F and 11B. This is in contrast to the two crystalline silica reference particulates, in which only non-fibrous particles can be seen (Figures 12B and 13B). The two DE's are also similar in that each has a population of large particles as well as fine particles, however S-Nat clearly has more of the latter. Likewise, the range of sizes present in the two crystalline reference particulates appears similar but the distribution does not: quartz has many more small particles than does cristobalite. Finally, note that the cells have accumulated large numbers of particles, most of which appear to be within the cells.

A review of the fluorescent photographs of exposed cultures gives the impression of a range of gross cytoplasmic and nuclear abnormalities. Cells with multiple and irregularly shaped nuclei are seen. Visible cellular abnormalities appear less severe in the cristobalite- and quartz-exposed cultures. In the S-Nat-exposed cultures, many nuclei appear to have little or no cytoplasm (orange-fluorescing) remaining around them (Fig 10C, E). Note the orange-fluorescing particles in these photographs. With PCOM (Figure 10D, F) these same particles appear to be a non-fibrous subpopulation of the diatomite. The same pattern of fluorescence was observed in S-Nat that was incubated in medium without cells and stained with acridine orange; therefore it was concluded that this phenomenon was not a result of cellular interactions with the particles. In contrast to the S-Nat photographs, those of S-FC (Figure 9) do not have any of these orange-fluorescing particles.

As with the ICP data, the INA curves for S-Nat and S-FC are comparable to the INA curve for UICC crocidolite asbestos (when concentrations are calculated as mass/unit surface area). Qualitative differences as well as similarities can be observed by comparing cells

exposed to the CD fibers (Figure 14) with cells exposed to diatomite particles. Acridine orange stained CD-exposed cells give a general impression of nuclear disruptions with little effect on the cytoplasm. Multiple nuclei, including one or more micronuclei are common, and to a lesser extent, enlarged cells with multi-lobed-appearing nuclei are observed. On the other hand, cells exposed to the diatomites (Figures 15 and 16, 1000 X) appear to have morphological distortions and both nuclear and cytoplasmic membranes are indistinct. These abnormalities are not observed in the photographs of cells exposed to each of the two reference particulates, Cristobalite and α -Quartz.

DISCUSSION

At this time, no known in vitro test systems can be used to predict the whole-organism effects of inhaled particles. Thus, the findings of this study should not be misconstrued to be, in any way, indicative of what possible effects the test particulates could have on an organism following inhalation exposure. However, the in vitro toxicity of diatomites to cultured CHO-K1 cells was sufficiently clear to indicate that further testing is needed to determine the potential toxicity of DE.

Assays measuring cell proliferation, colony forming efficiency, and the induction of nuclear abnormalities, showed striking, concentration-related toxicity of DE to cultured CHO-K1 cells. Cell viability, on the other hand, was only slightly decreased by diatomite exposure. Toxicity from soluble chemicals was ruled out when no effects were observed in cells exposed to filtered DE-leachate. These observations suggest that the mechanism of toxicity is primarily a disruption of cell division resulting from the cellular uptake (phagocytosis) of particles. CHO-K1 cells divide rapidly and, according to our observations, readily take up a variety of different types of particles by phagocytosis, two features which make them sensitive to this type of toxic mechanism.

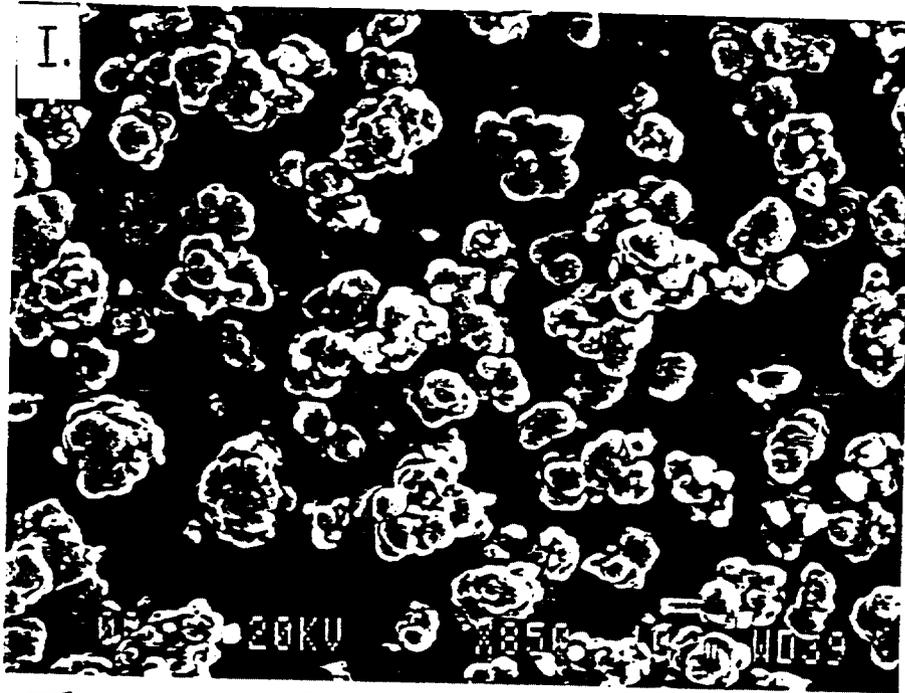
The several particulates evaluated in the present study were not equally toxic. In an attempt to determine the causes of these differences in toxicity, various parameters (crystallinity, surface area, particles/ μg , dimensions, % fibrous particles) of the four dusts were compared (Tables I and II). Crystallinity was not directly related to toxicity; S-Nat was the most toxic of the silica dusts, but it is primarily amorphous. Nor was particle number directly related to toxicity; the number of particles/ μg was similar in S-Nat and quartz, yet the former was considerably more toxic than the latter. One possible cause of toxic differences could be a combination of surface area and surface chemistry resulting in

different adsorbencies of the various particulates for cellular constituents. S-Nat was more toxic than FC, and S-Nat is estimated to have a 10-fold greater surface area than FC. Another possible cause of toxic differences could be percentage of particles that are fibrous (length/diameter ≥ 3). When EC50's are calculated as number of fibers longer than 7.5 μm per cm^2 , the EC50's for Nat, FC, cristobalite and CD are very similar to each other (214, 259, 214 and 384, respectively, $\times 10^3$). Quartz and TiO_2 had no fibrous particles. Further investigations of DE materials are needed to test these hypotheses. A clarification of the relationships between toxicity and particle characteristic could provide a major step toward an understanding of the cellular mechanisms of particulate toxicology.

Further in vitro testing is advised, utilizing cultured cells derived from lung tissues, such as normal rat alveolar macrophages, rat or human airway epithelium and/or lung mesothelium. Lung macrophages are a key cell in the pathogenesis of respirable particles in the whole organism, particularly in lung clearance. These highly phagocytic and mobile cells ingest particles in the lung, subject them to digestive and corrosive secretions, and attempt to carry the particles up and out of the respiratory tract. Particles that chemically or physically interfere with these clearance functions may be more pathogenic to the organism than particles which the macrophages are able to either degrade or carry out of the lungs. Lung mesothelial cells may be a target tissue of inhalation toxicants in that pulmonary mesothelioma has been associated with whole-animal exposure to some mineral fibers. (Hesterberg et al., 1991). Airway epithelium may provide a protective barrier to some particulates and may therefore be less sensitive than other tissues to the toxic effects of some particulates. All of these lung cell types are available for testing in the MTC In Vitro Laboratory, including both human and rodent cell lines as well as normal rodent and human primary cells.

Other DE test particulates should also be evaluated, to measure the impact of surface area, surface chemistry and particle size, shape and number of particles/unit culture surface area. In vitro testing of different size-separated fractions of the several diatomaceous earth products as well as size-selected reference chemical particulates could help clarify the source(s) of the observed toxic effects.

Fig 1. I. Titanium Dioxide
magnification estimated at approximately 1000.



Titanium Dioxide

Fig. 2. Comparison of Particle Lengths

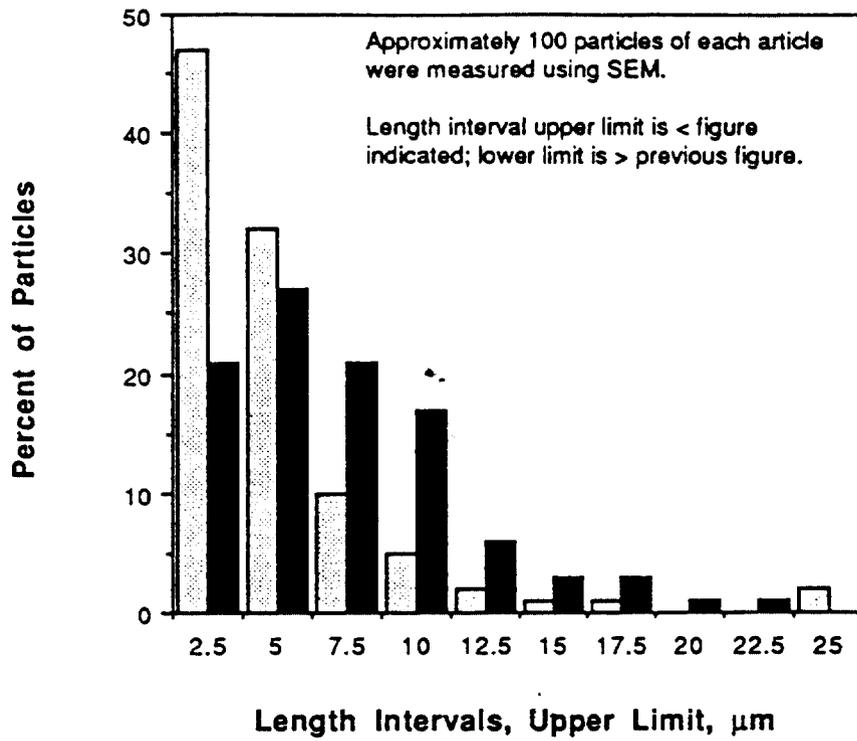


Fig. 3a. Inhibition of Proliferation (CHO K1 Cells)

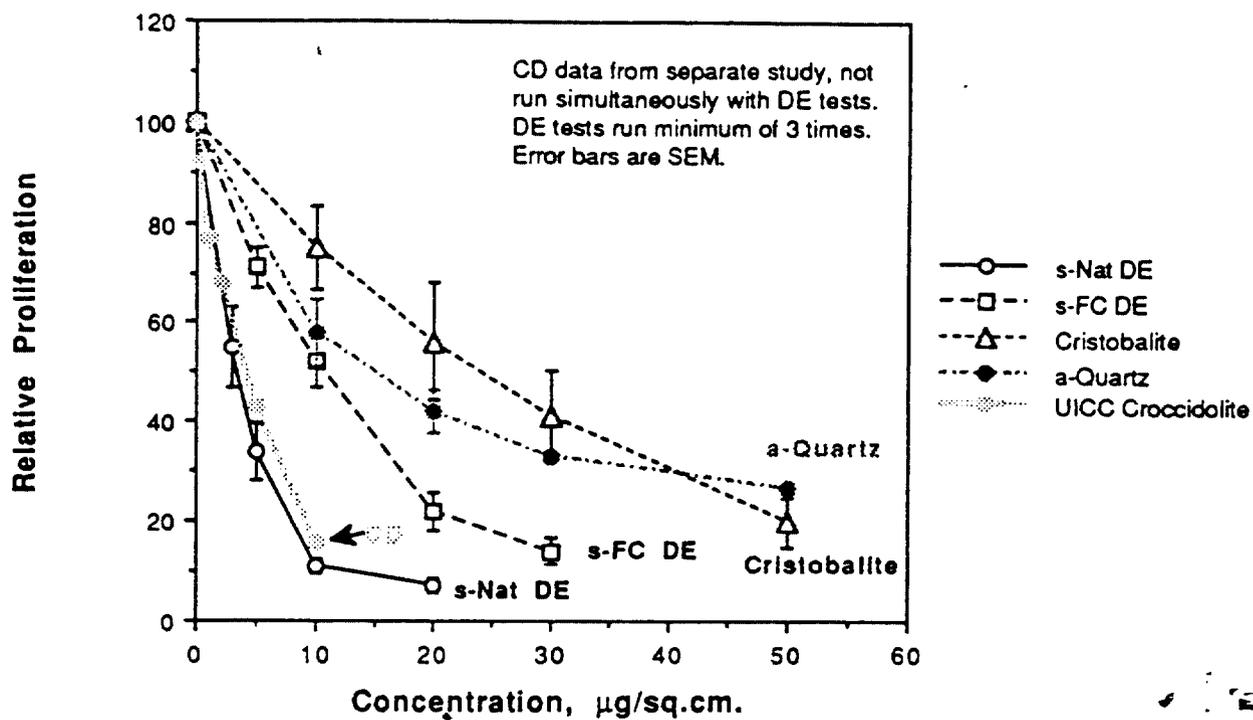


Fig. 3 b. Inhibition of Proliferation Cristobalite, a-Quartz and Titanium Dioxide

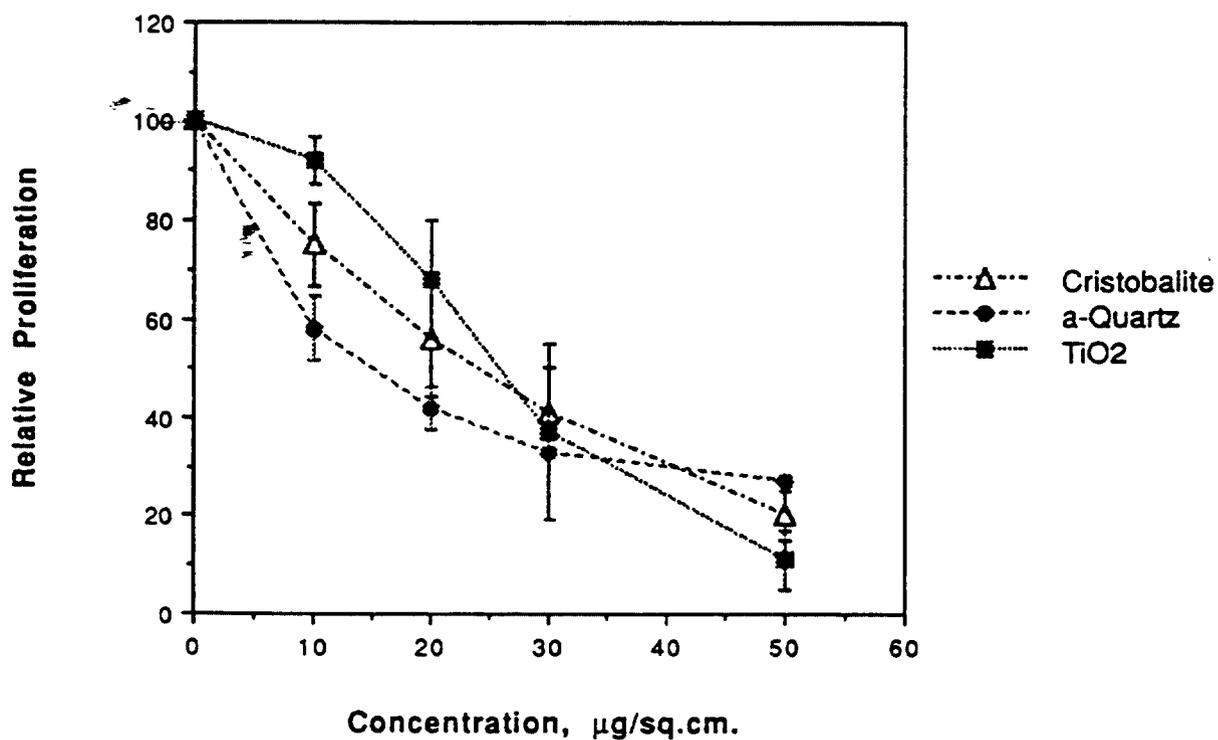


Fig. 4. Colony Forming Efficiency (CHO K1 Cells)

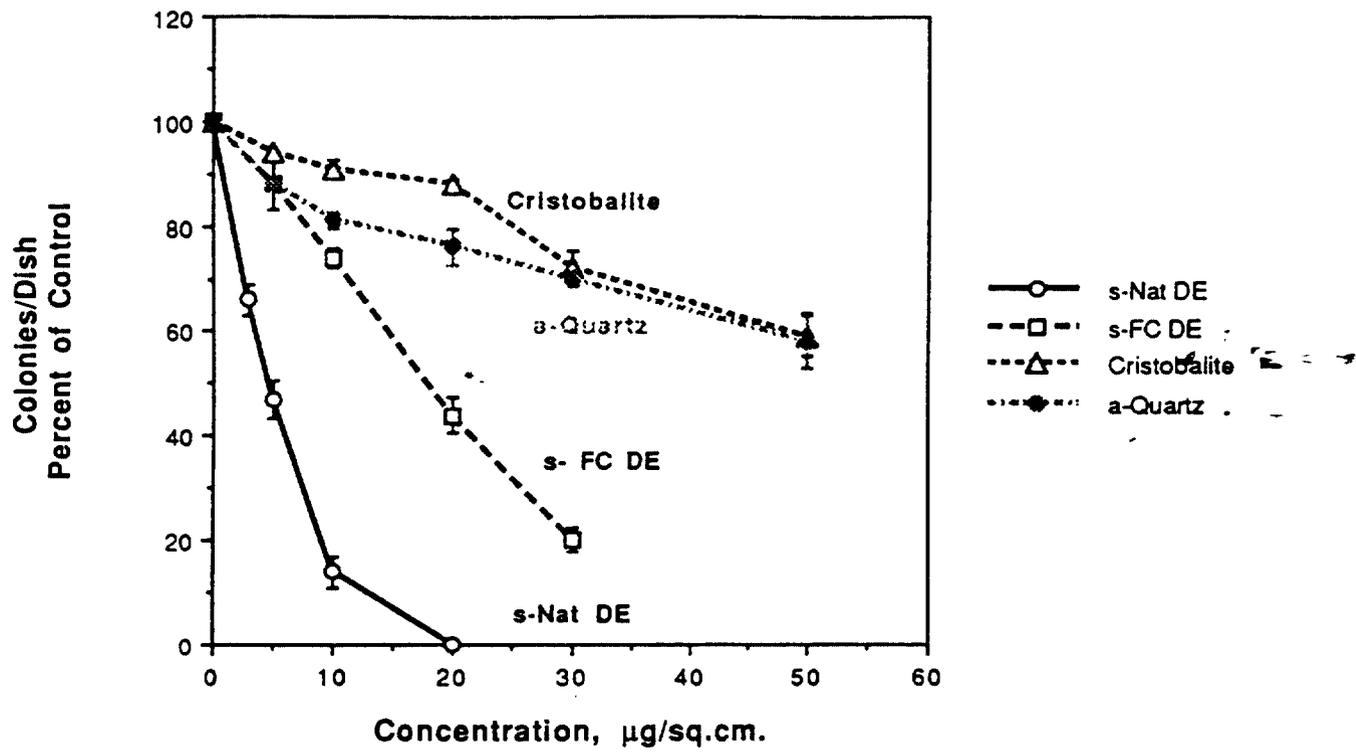


Fig. 5. Inhibition of Cell Proliferation with Particulates v. Particle-Exposed Medium

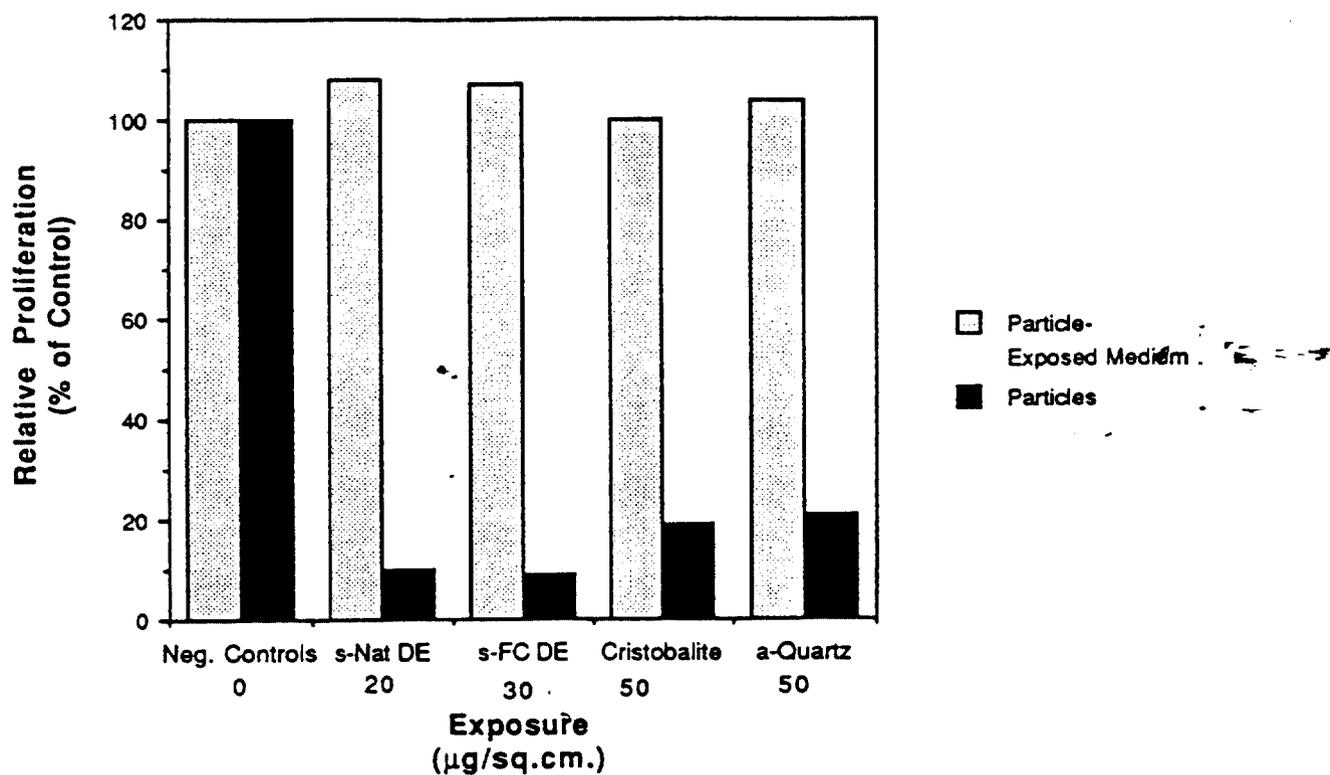


Fig. 6. Cell Viability

After 1, 2 and 3 Days of Particle Exposure.

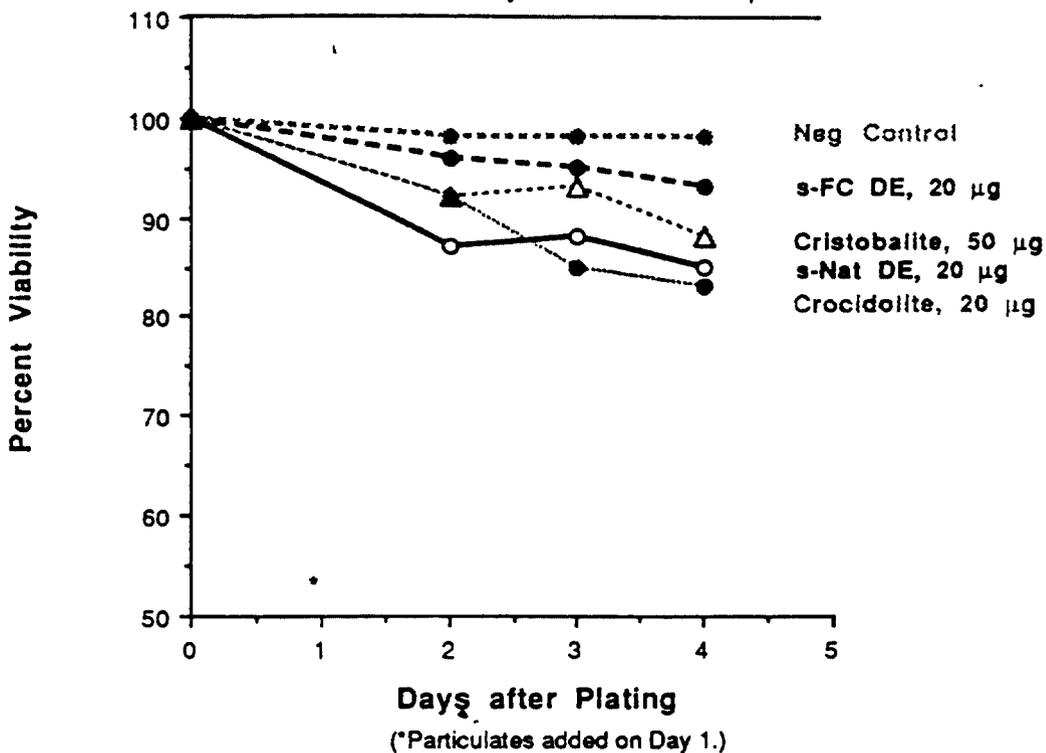


Fig. 7. Cell Proliferation

After 1, 2 and 3 Days of Particle Exposure.

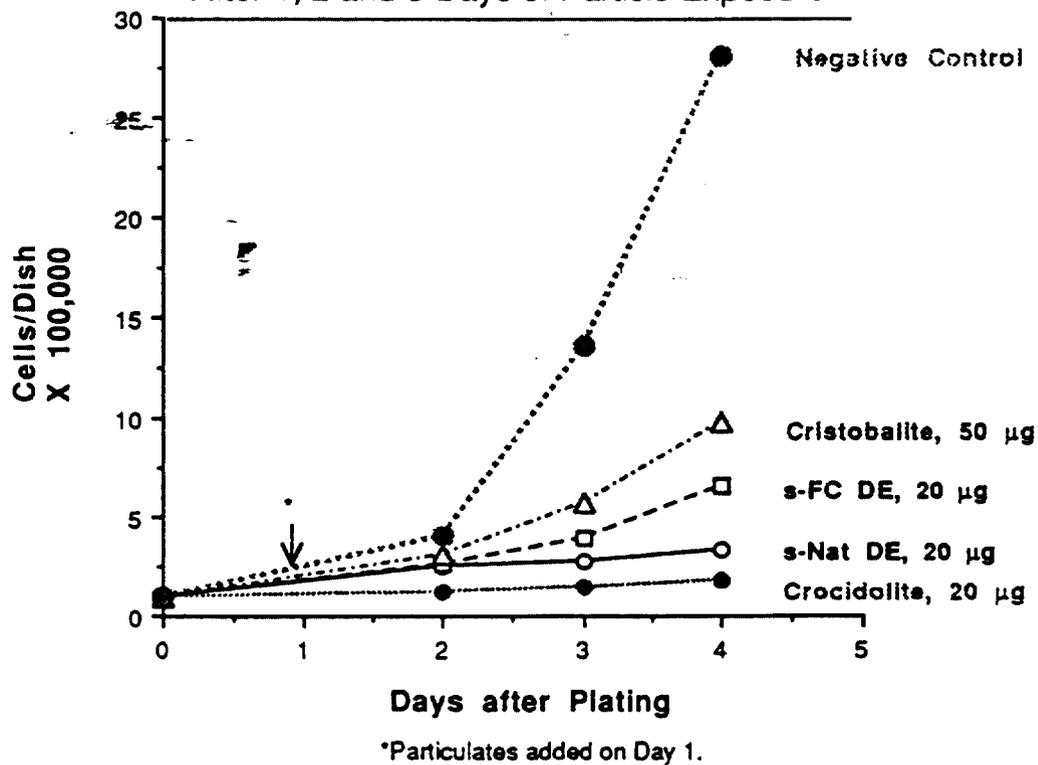


Fig. 8. Induction of Nuclear Abnormalities (CHO Cells)

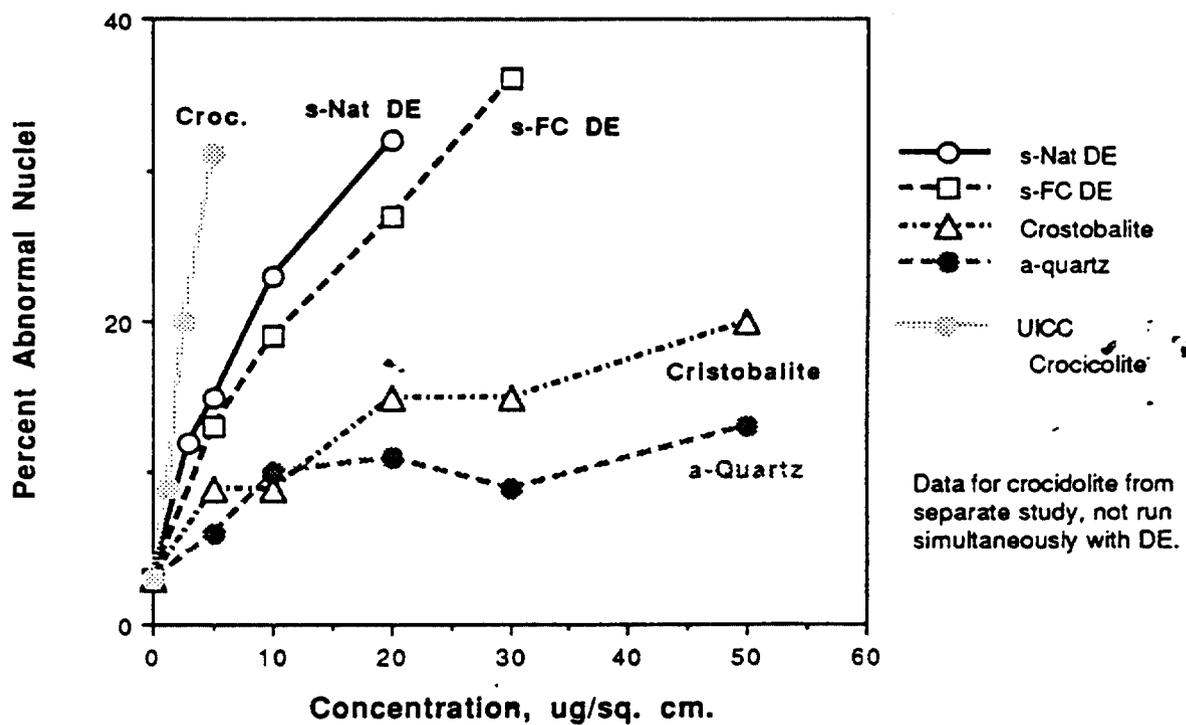


Fig. 9. Normal, unexposed CHO-K1 cells growing in culture. A.: Fluorescent light. B: PCOM. White arrow indicates micronucleus.

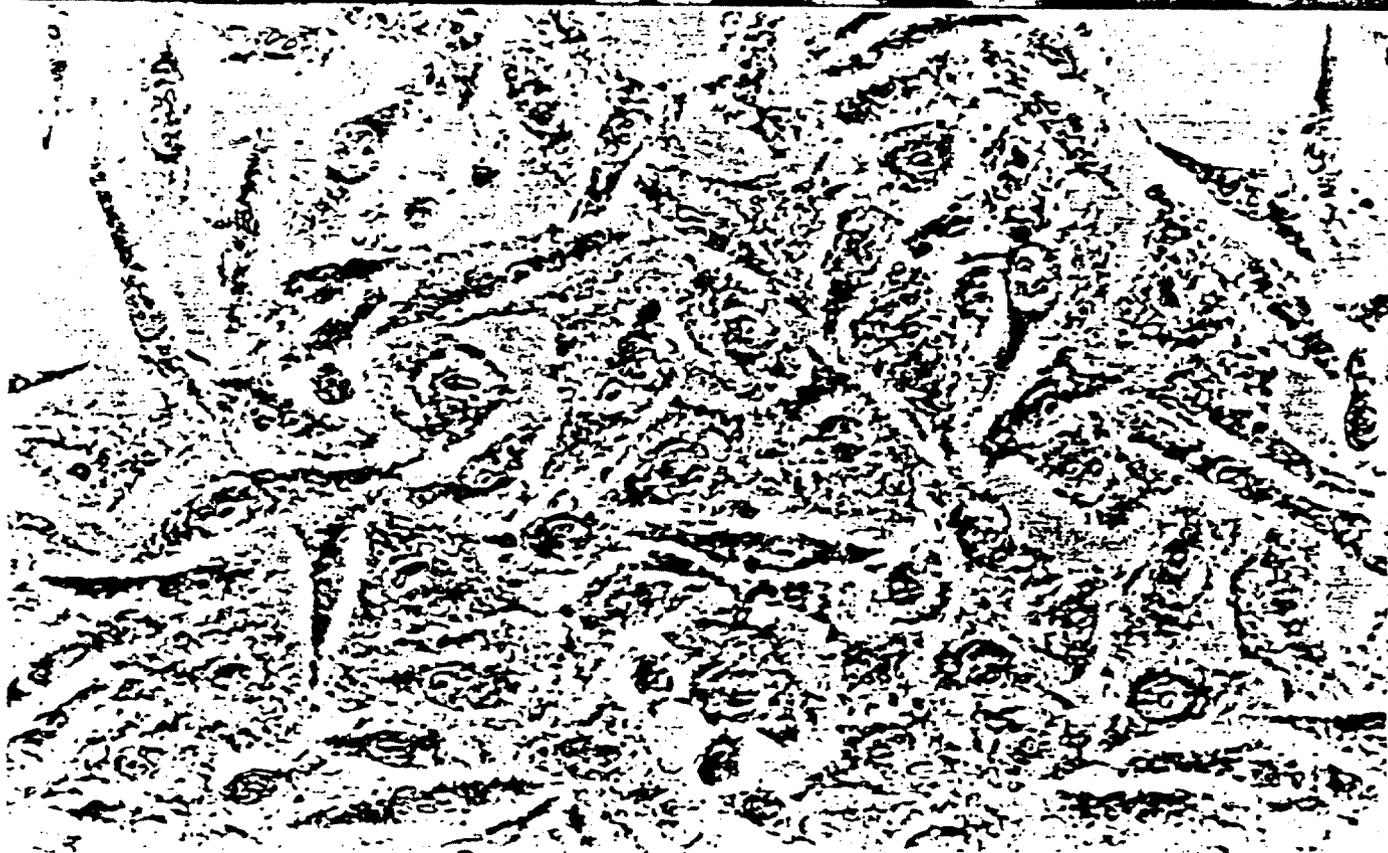
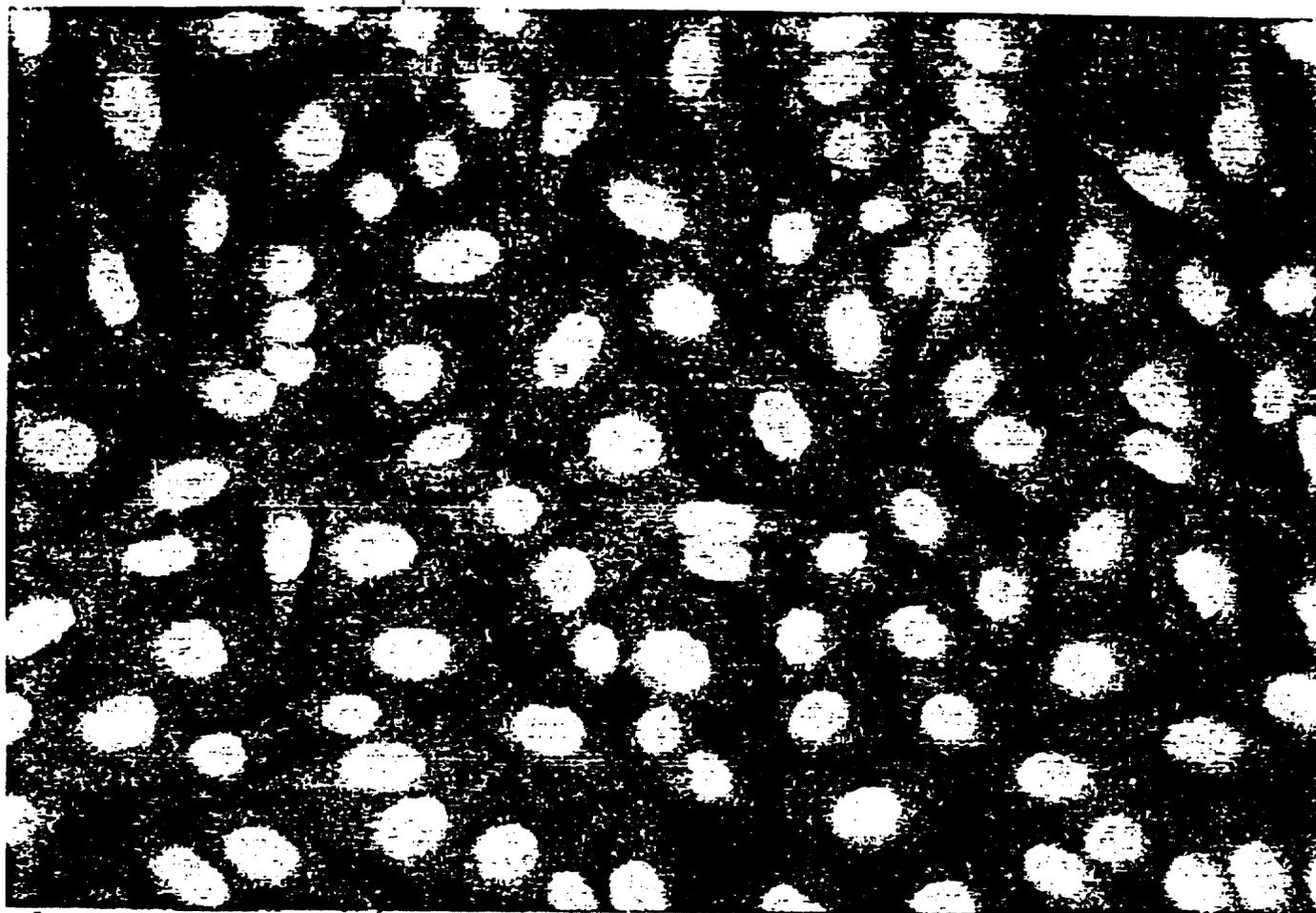


Fig. 10A-B. CHO-K1 cells exposed to s-Nat DE, 10 μ g sq.cm. A: Fluorescent light.
B: PCCM. X400.

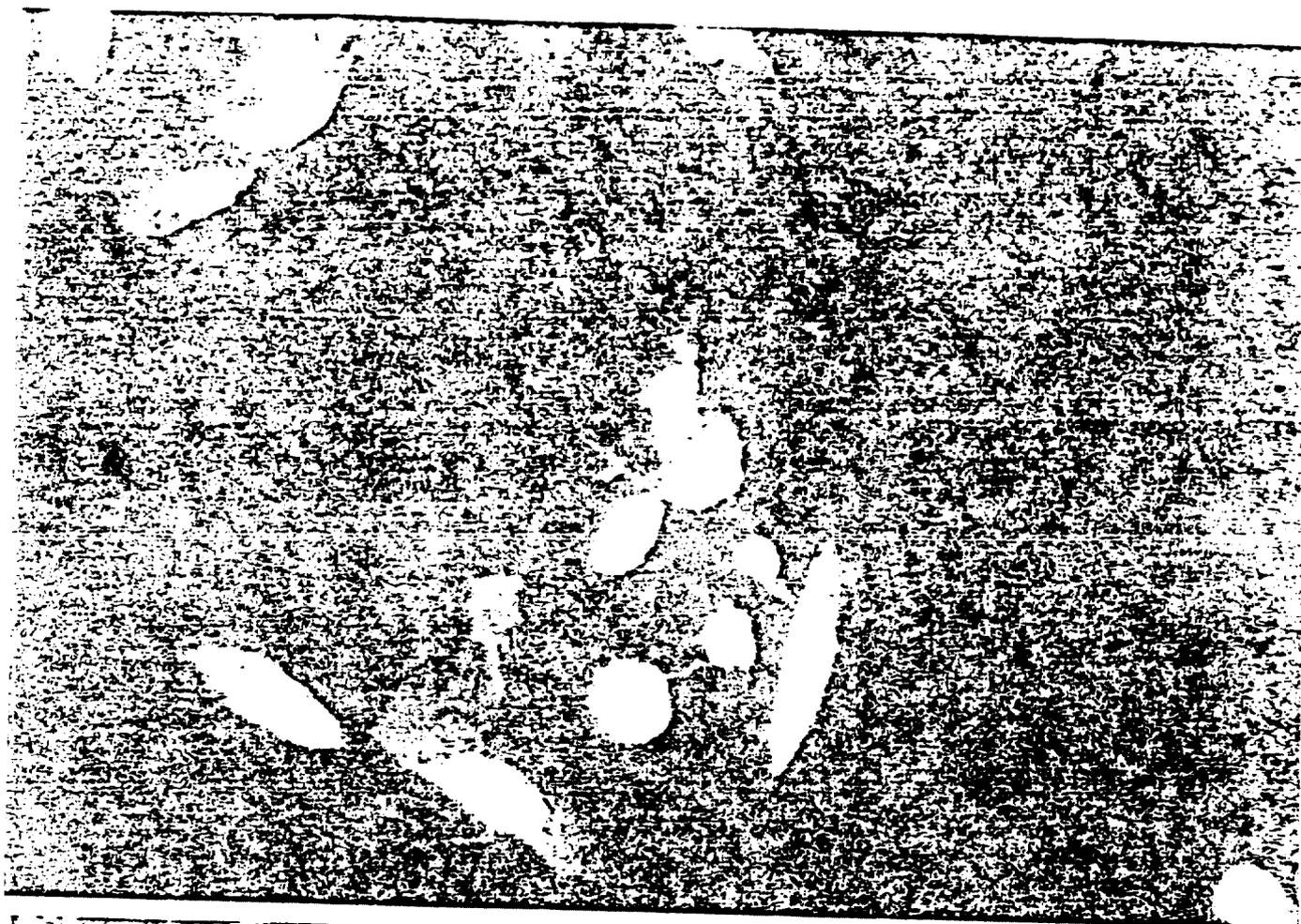


Fig. 10. C-D. CHO-K1 cells exposed to s-Nat DE, 20 $\mu\text{g}/\text{sq. cm}$. C.: Fluorescent light. Note orange particles separate from gold nuclear material. D: PCOM. Note cells are associated with numerous particles which appear to be in and on the cells. X400.

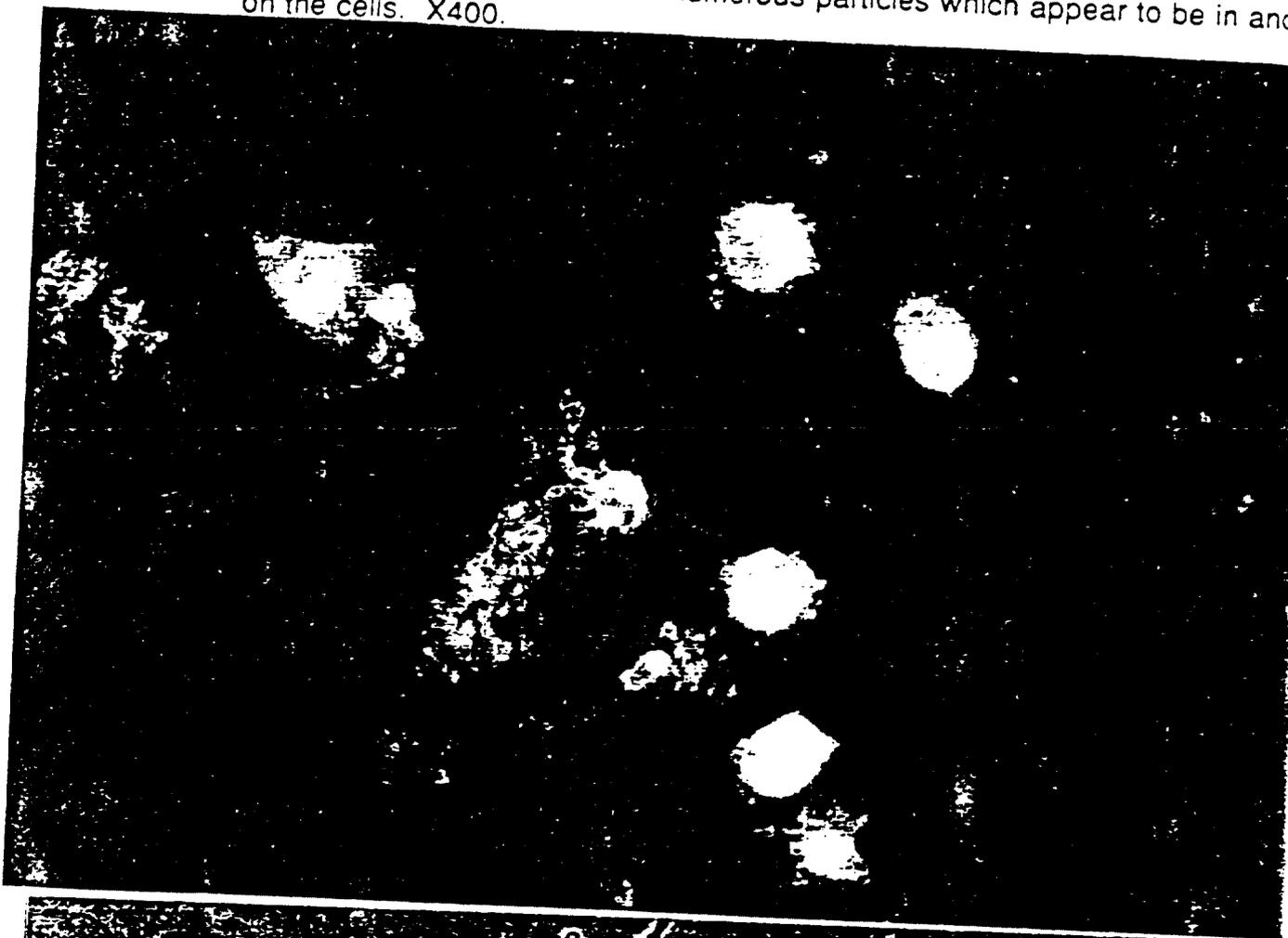


Fig. 10. E-F. CHO-K1 cells exposed to s-Nat DE, 20 $\mu\text{g}/\text{sq. cm}$. E.: Fluorescent light.. Note cytoplasm flouresces yellow rather than orange. F: PCOM. X400.

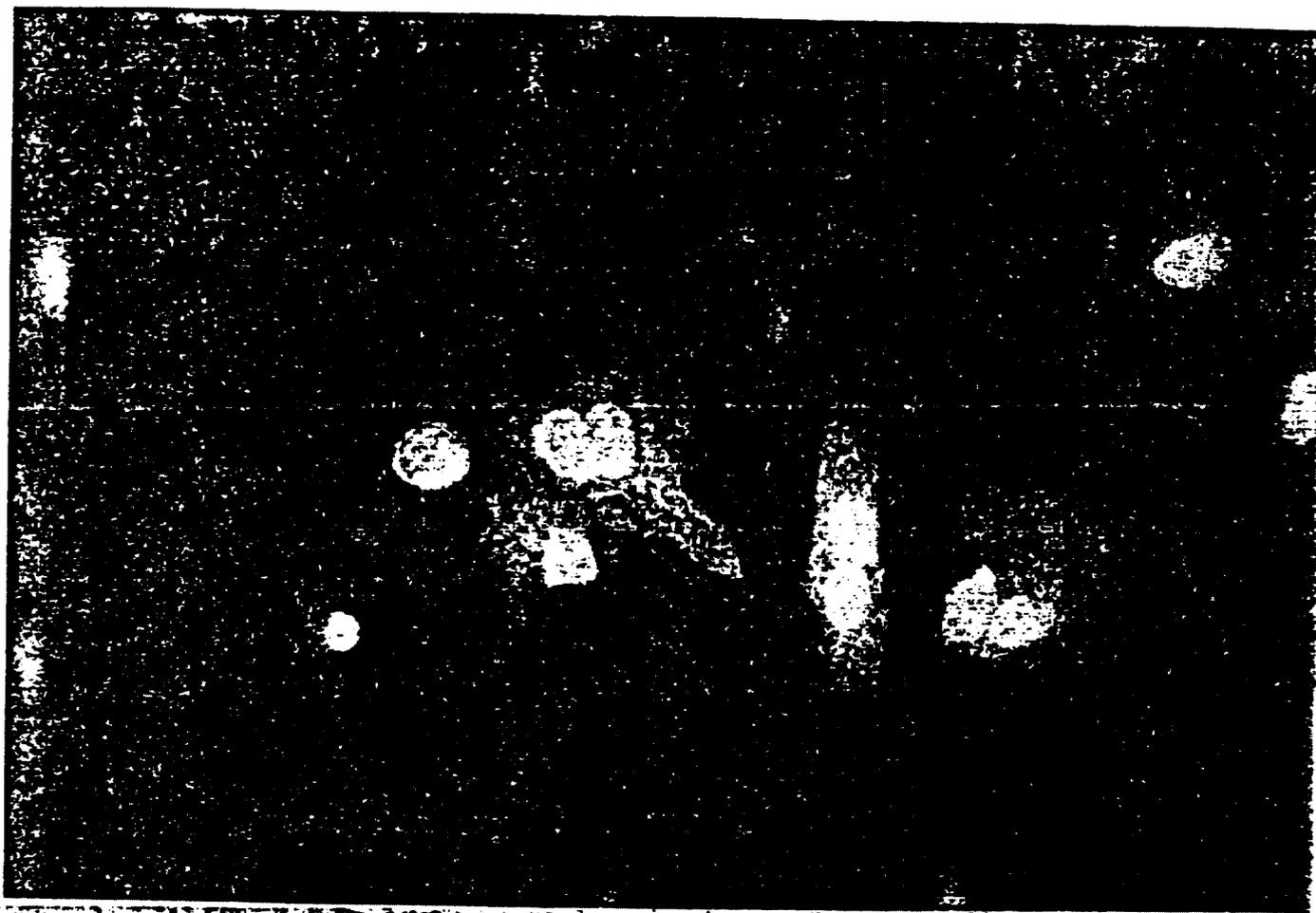


Fig. 11. A-B. CHO-K1 cells exposed to s-FC DE, 30 $\mu\text{g}/\text{sq. cm}$. A.: Fluorescent light.
B: PCOM. X400.

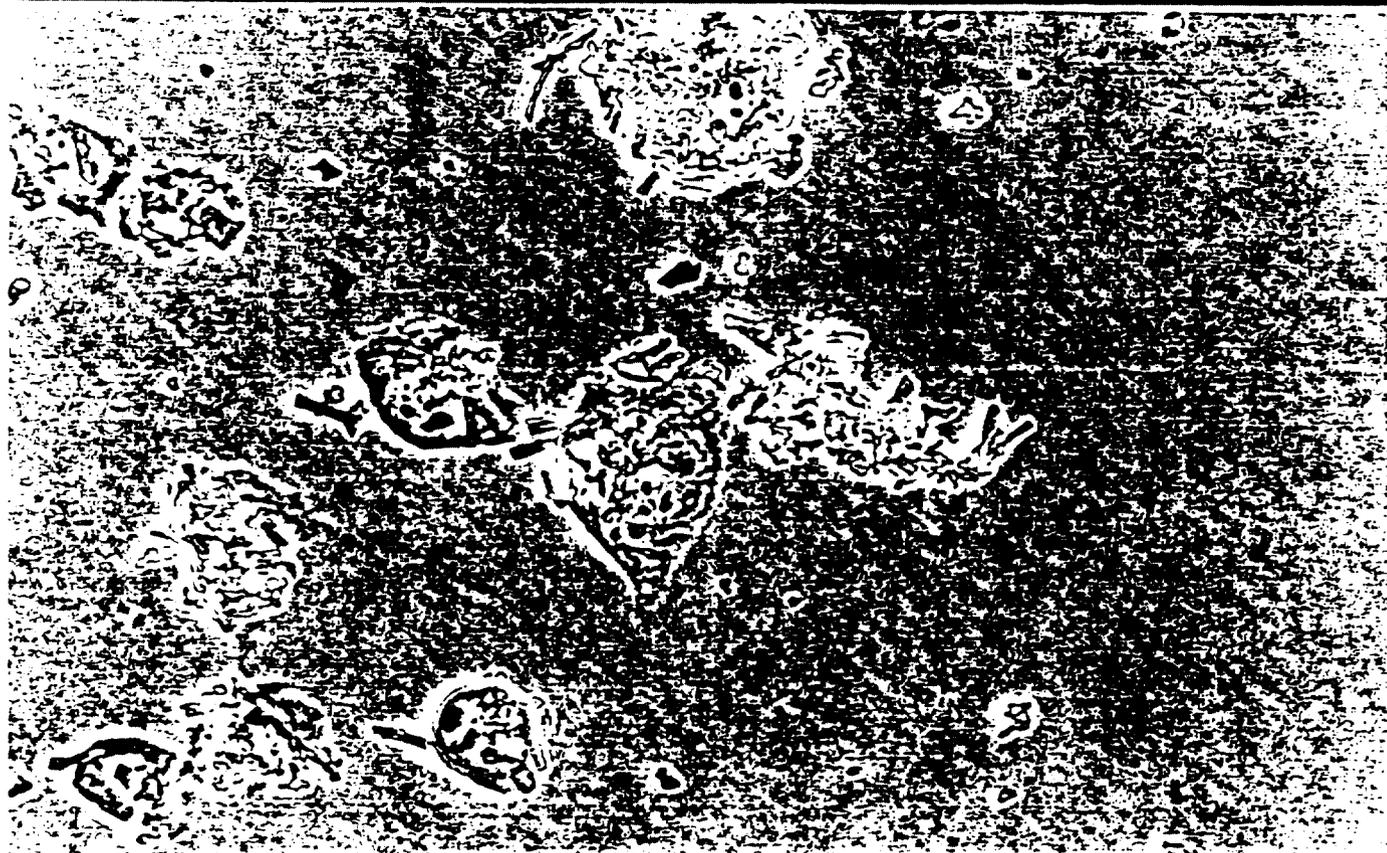
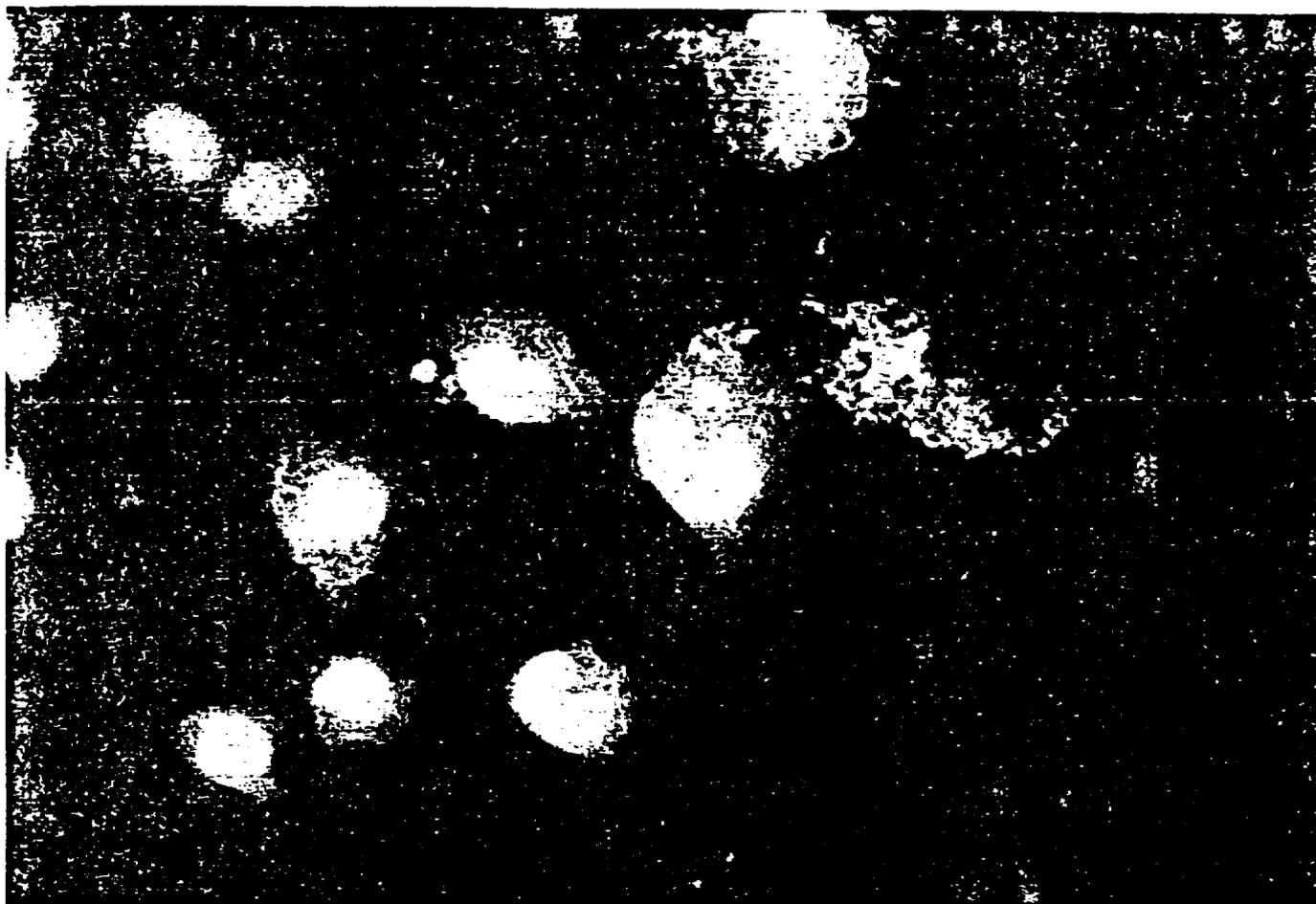


Fig. 12. A-B. CHO-K1 cells exposed to cristobalite, 30 $\mu\text{g}/\text{sq. cm}$. A: Fluorescent light. B: PCOM. X400.

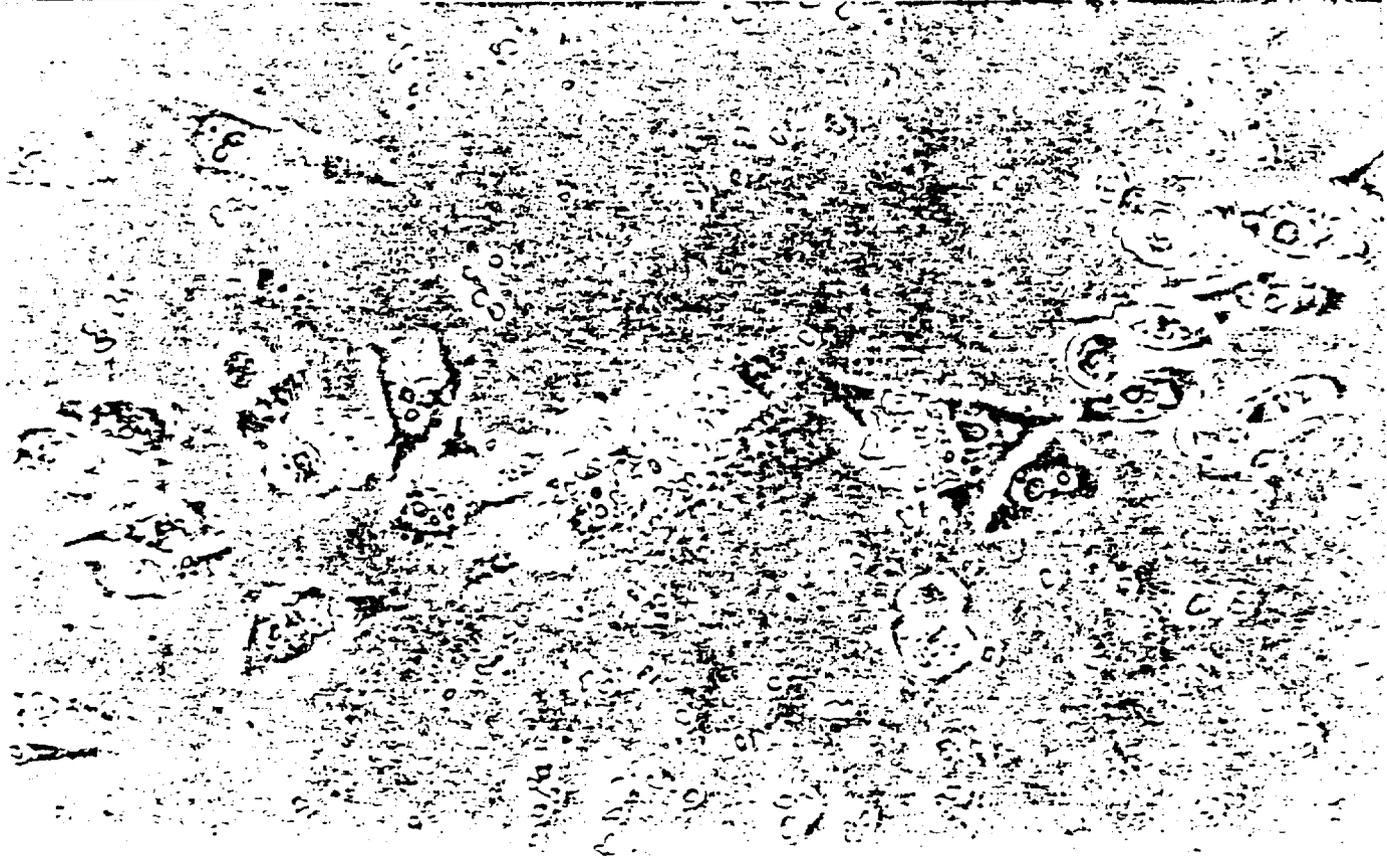
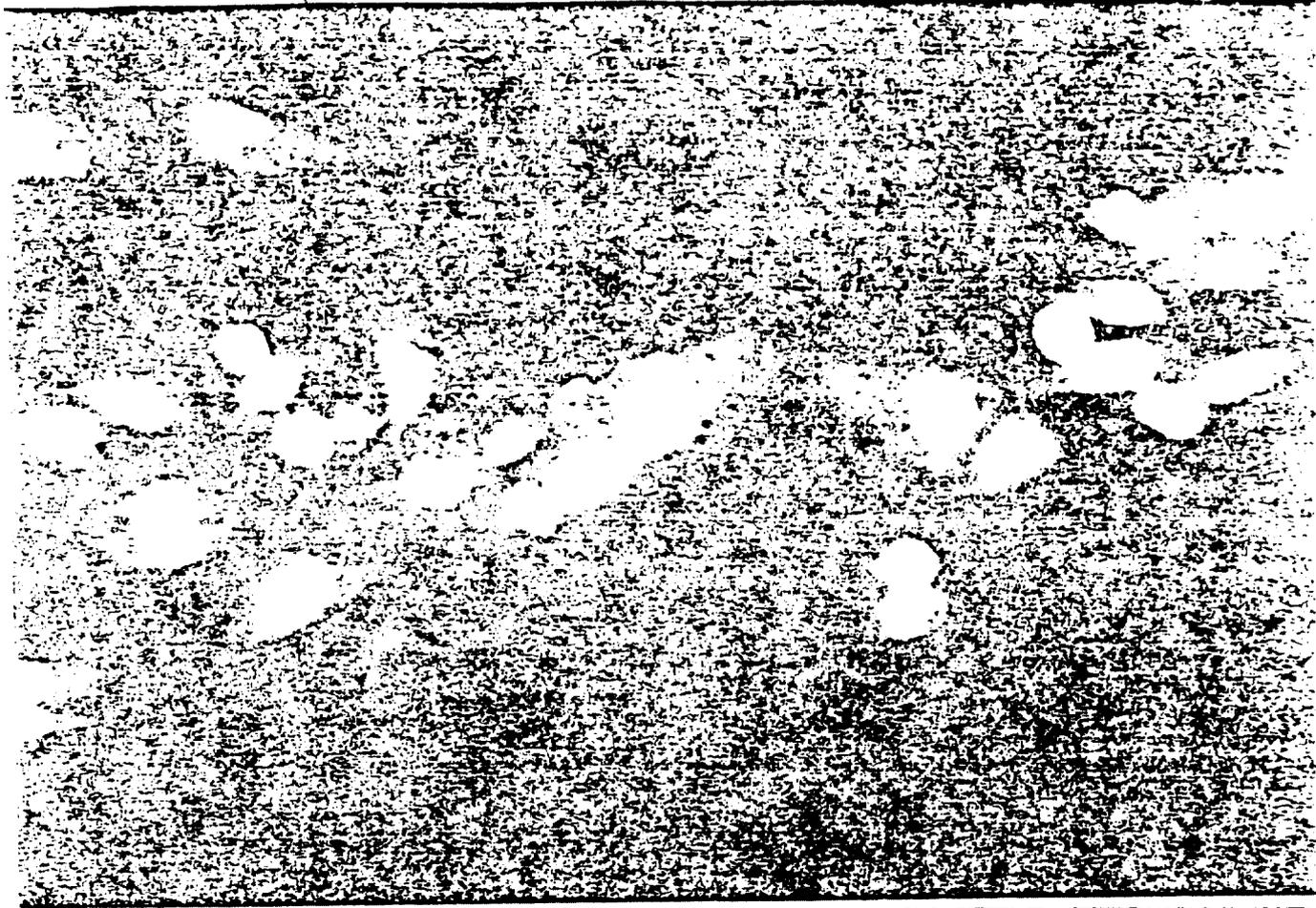


Fig. 13. A-B. CHO-K1 cells exposed to alpha-quartz, 30 $\mu\text{g}/\text{sq. cm}$. A: Fluorescent light. B: PCOM. X400.

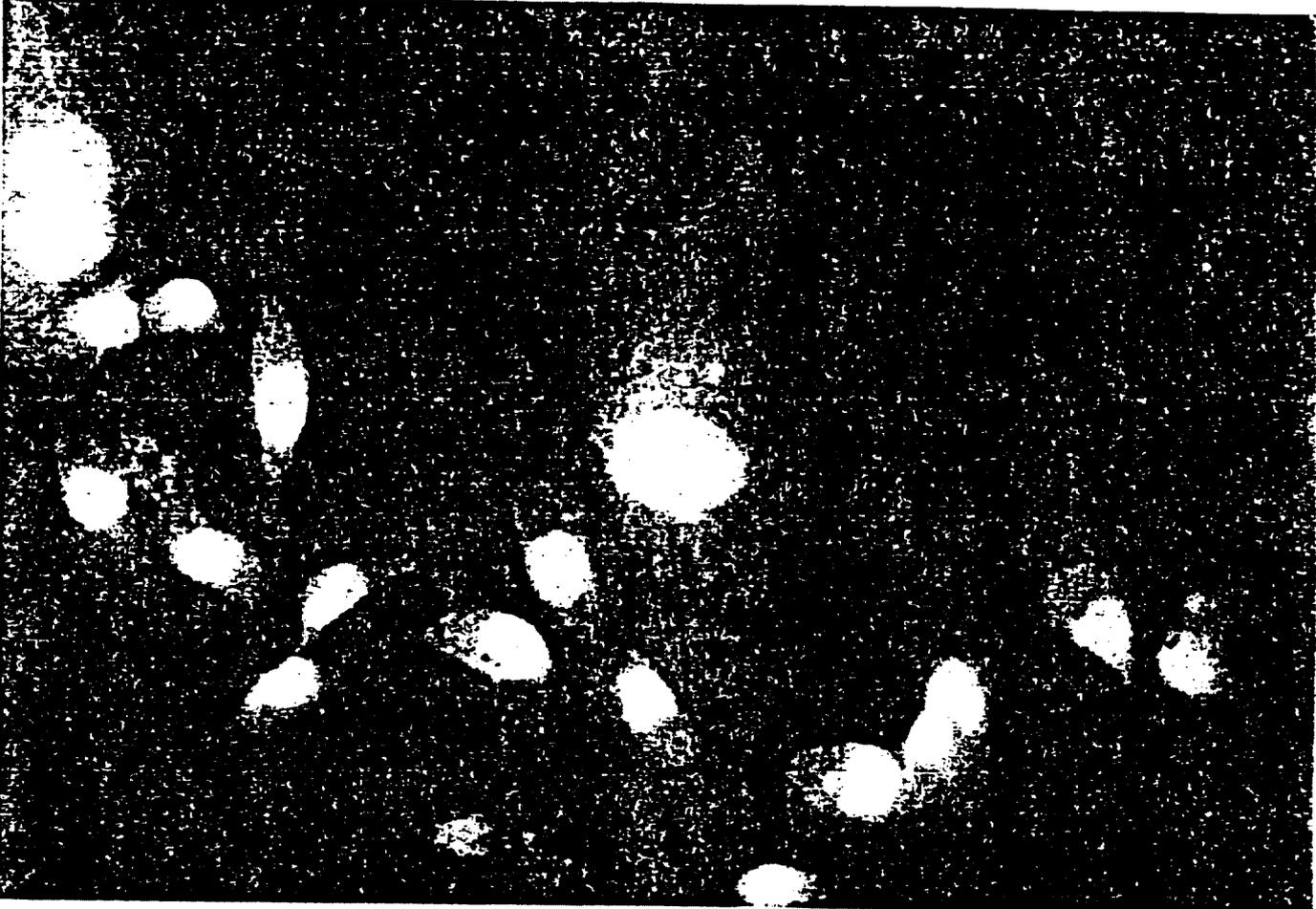


Fig. 14. A-B. CHO-K1 cells exposed to UICC Crocidolite, 5 $\mu\text{g}/\text{sq. cm}$. A.:
Fluorescent light. B: PCOM. X400.

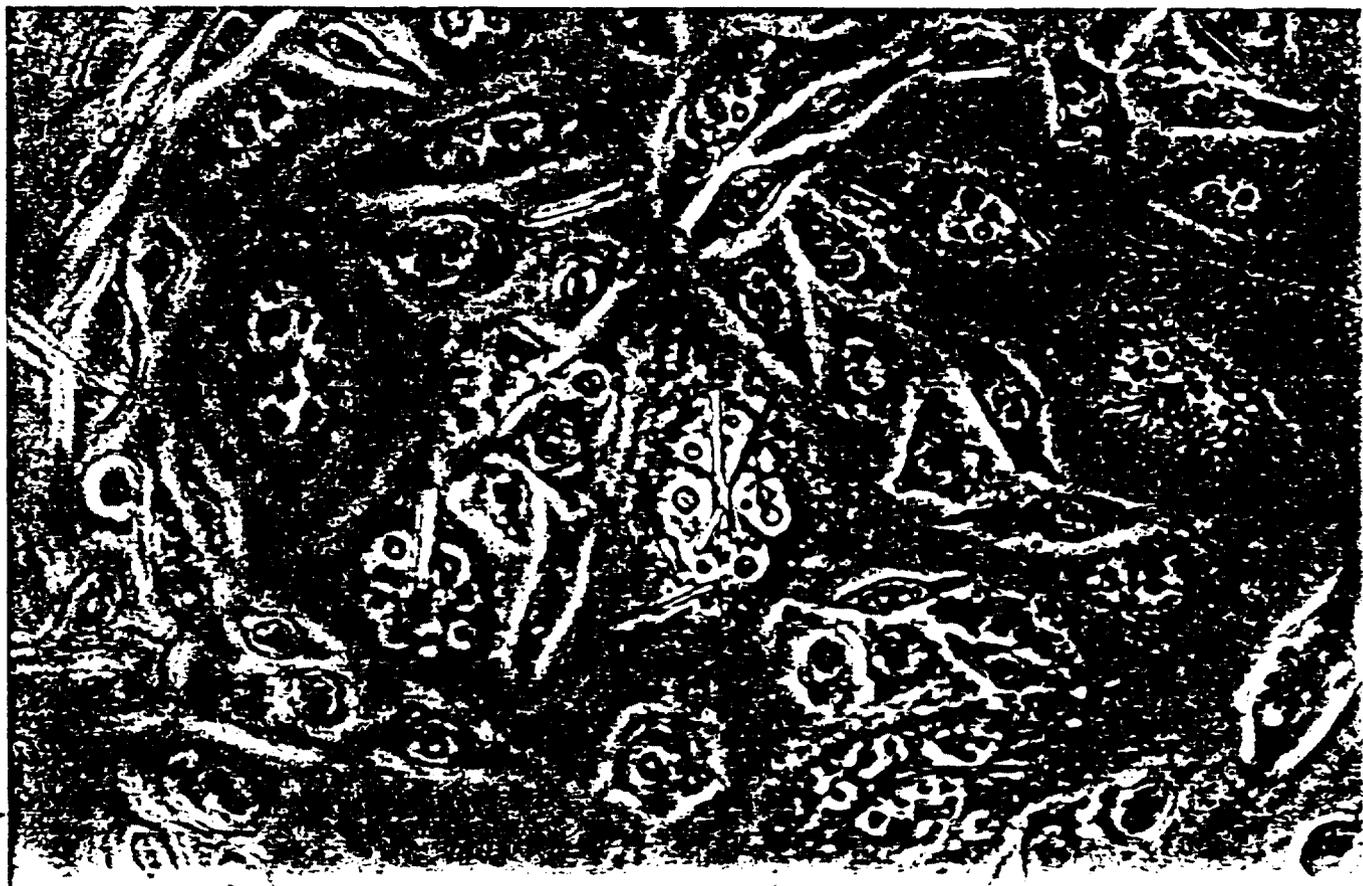
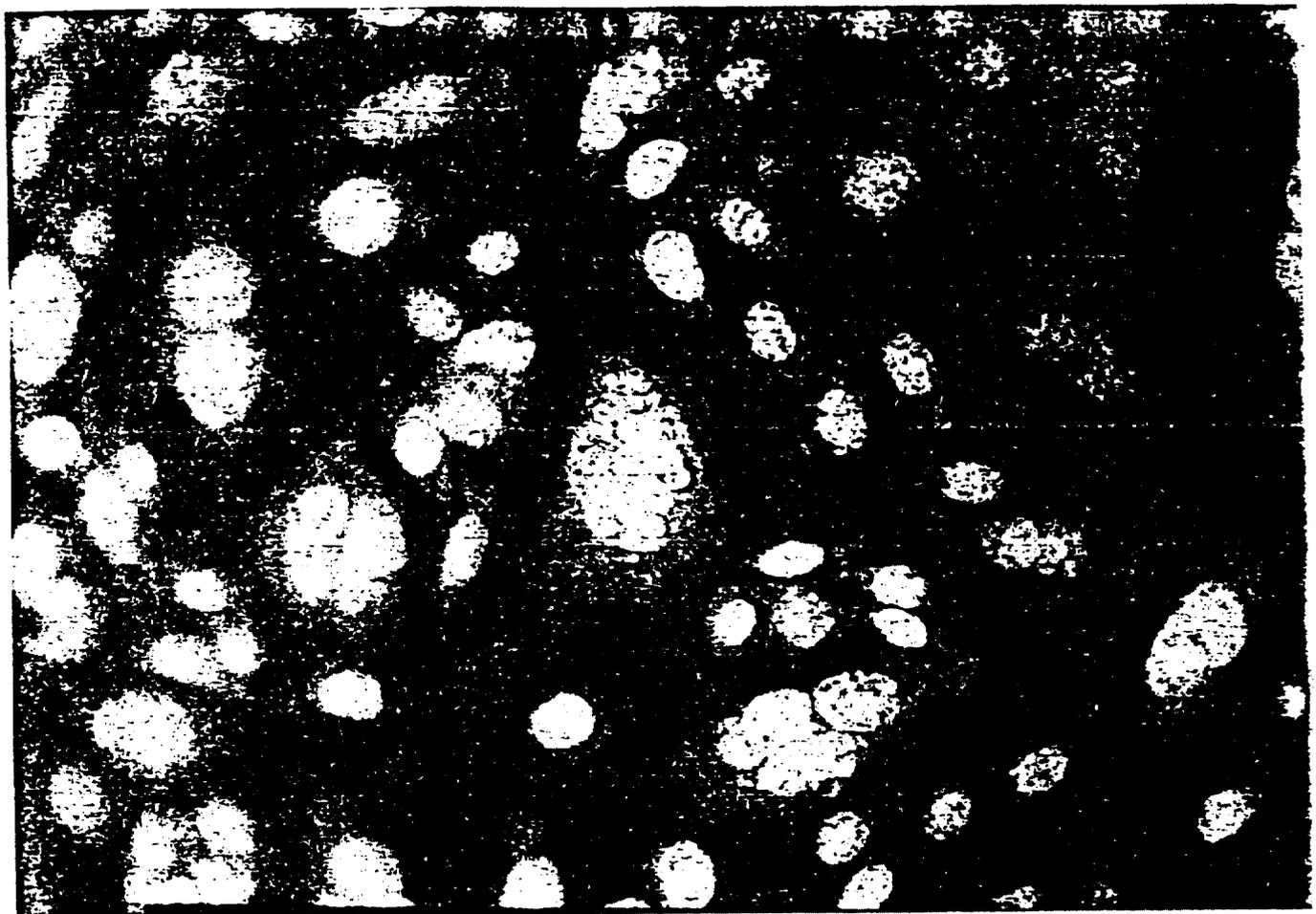


Table I. Test Particulates, Description of Bulk Materials

Generic Name	Trade Name or ID Number	Supplier	Chem. Composition Supplier	Crystallinity			
				Supplier Information		MTC Analysis	
				Cristobalite	Quartz	Cristobalite	Quartz
Natural DE	Celite 500	Celite	Silica		<3%	none detected	3.98%
Flux Calcined DE	Hyflo Super Cel Celite 513	Celite	Silica	<60%	<3%	39.59%	1.95%
Cristobalite	Std. Ref. 1879	NBS*	Silica	96%		not done	not done
alpha-Quartz	Std. Ref. 1878	NBS*	Silica		98%	not done	not done

*U.S. National Bureau of Standards

Table II. Comparison of Effective Concentration-50, EC 50 CHO-K1 Cells

EC50 is the concentration which reduces cell proliferation to 50% of negative (unexposed) control cultures.

Test Article	Surface Area	% Crystallinity	Avg. Length	% Fibrous*	% longer than 7.5 μ m	Particles/ μ g (x 1,000)	Effective Concentration-50		
							μ g/cm ²	Part./cm ² (x 1,000)	F. \geq 7.5/cm ² (x 1,000)
s-Nat DE	-20 cu.M./g	<4% Quartz	3.8	38%	11%	514	4	2060	214
s-FC DE	-2 cu.M./g	~40% Cristobalite	5.9	36%	31%	78	11	860	259
a-Quartz	?	>96% Quartz	2.0	6%	0	525	16	8400	0
Cristobalite	?	>96% Cristobalite	4.6	21%	13%	75	25	1880	214
UICC Crocidolite	?	0	1.8	~100%	4%	2400	4	9600	384
Titanium Dioxide	?	0	n/d	n/d	n/d	n/d	26		

*Fibrous defined as having aspect ratio \geq 3.

Appendix 1. Test Particles, Dimensions
 Measured using SEM.

Particulate	Diameter Range μm	Diameter Avg. μm	Diameter Std. Dev.	Length Range μm	Length Avg. μm	Length Std. Dev.	Aspect Ratio**	
							Avg.	% > 3
s-Nat DE*	0.2-5.8	1.3	1.0	0.9-23.3	3.8	3.9	3.65	38%
s-FC DE*	0.4-8.1	2.1	1.5	0.7-20.7	5.9	4.1	3.50	36%
Cristobalite	0.4-7.8	2.1	1.5	0.7-24.7	4.6	3.9	2.28	21%
alpha-Quartz	0.4-5.1	1.1	0.8	0.5- 8.1	2.0	1.4	1.93	6%

*Dimensions were analyzed after size-selection processing by MTC Fiber Preparation Laboratory.

**Length/Width

Appendix 2. Particle Dimensions

Approximately 100 particles of each article were measured using SEM.

Intervals		s-Nat. DE	s-FC DE	Cristobalite	a-Quartz
Length, μm		Percent	Percent	Percent	Percent
\geq	$<$				
0.0	2.5	47	21	31	78
2.5	5.0	32	27	38	18
5.0	7.5	10	21	18	2
7.5	10.0	5	17	1	2
10.0	12.5	2	6	8	
12.5	15.0	1	3	2	
15.0	17.5	1	3	0	
17.5	20.0	0	1	1	
20.0	22.5	0	1	0	
22.5	25.0	2		1	

Diameter, μm					
\geq	$<$				
0.0	0.5		4		
0.5	1.0	15	18	5	14
1.0	1.5	32	17	23	52
1.5	2.0	25	16	18	25
2.0	2.5	11	15	17	10
2.5	3.0	8	7	11	2
3.0	3.5	3	7	5	3
3.5	4.0	1	5	5	1
4.0	4.5	3	1	5	2
4.5	5.0	0	5	3	
5.0	5.5	0	1	3	
5.5	6.0	0	0	1	
6.0	6.5	2	0	3	
6.5	7.0		1	0	
7.0	7.5		1	0	
7.5	8.0		0	0	
8.0	8.5		1	2	

Appendix 3. In Vitro Tests

Abbreviation	Name/Description
ICP	Inhibition of Cell Proliferation
	Sixty mm culture dishes are seeded with 100,000 cells in 5 ml complete medium (CM). Cells are allowed to settle to the bottom and, if cell type is anchorage-dependent, cells are allowed to attach before test article is added. Test Particulates are added to cultures in one ml complete medium/dish. Negative control cultures receive 1 ml CM/dish. Each exposure group is set up in triplicate. After three days exposure, at 37°C and 5% CO ₂ , cells are harvested with 0.05% Trypsin and 0.02% EDTA in Hanks balanced salt solution (Irvine, Santa Ana, CA) and counted using a Coulter counter. Relative proliferation is determined by dividing the number of cells present in each exposed culture by the number of cells present in unexposed cultures.
CFE	Colony Forming Efficiency
	Sixty mm culture dishes are seeded with 200 cells in 5 ml complete medium and exposed as described for ICP above. Each exposure is set up in triplicate. After 5 days exposure, visible colonies appear. Colonies are stained with 0.4% w/v Giemsa in buffered methanol (Sigma, St. Louis, Missouri). Colonies are counted using a stereoscope at low power. CFE is determined by dividing the number of colonies in each exposed culture by the number of colonies in unexposed cultures.
INA	Induction of Nuclear Abnormalities
	Cultures are prepared as described for ICP above. After two days exposure, culture dishes are fixed with methanol/acetic acid (3:1, v/v) and stained with 0.01% Acridine orange (as described in Clark, 1981). Using a microscope fitted with epifluorescence, the percentage of cells containing micronuclei and/or other visible nuclear abnormalities is determined for each culture dish. Visible nuclear abnormalities include multiple nuclei and lobed nuclei.
EAV	Esterase Activity Viability Test
	Cultures are prepared and exposed as described for ICP above. After 3 days exposure, cells are harvested and resuspended in saline. 5(6)-Carboxyfluorescein diacetate (Sigma, St. Louis, Mo.) is added to each of the cell suspensions (5 µl stock / ml of cell suspension; stock is .1% carboxyfluorescein in acetone diluted 1:1 in saline just before use). After 1--30 minutes at room temperature, hemacytometer counts of the cell suspensions are made using both fluorescence and phase contrast optical microscopy (PCOM). Percent viable cells is determined by dividing the number of fluorescing cells (viable cells) by the number of cells visible with PCOM (total cells).

Appendix 4. Easterase Activity, Viability, CHO-K1 Cells.

Three days exposure to high concentration of test article.
 Each figure is the average of 4 hemacytometer counts/culture.
 Data = Cells/Culture Dish, X 10,000.

Test Articles	Concentration µg/sq.cm.	Total Cells/Culture			Fluorescent Cells			Percent Viable			Relative Prolif. (% of Control) Avg.	
		Test No.			Test No.			Test No.				
		#1.	#2.	#3.	#1.	#2.	#3.	#1.	#2.	#3.		
Neg Control	0	317	376	458	316	370	446	100	98	97	98	100
s-Nat DE	20	35	34	43	28	29	35	80	87	81	83	11
s-FC DE	20		89	59		83	54		94	92	93	18
Cristobalite	50	53	103	55	48	87	51	91	84	93	89	18
a-Quartz	50	76			74			97			97	24
Crocidolite	20		22	24		18	20		80	83	81	6

Appendix 5. Induction of Nuclear Abnormalities

Averages from two separate tests.

Fiber	Concentration μg/sq.cm.	Percent w. Mncls.* ± Std. Dev.	Percent Polynuc.** ± Std. Dev.	Percent Abnormal ± Std. Dev.
Control	0	2 ± 1	1 ± 1	3 ± 2
s-Nat DE	3	4 ± 1	8 ± 5	11 ± 6
	5	5 ± 1	11 ± 8	15 ± 7
	10	5 ± 2	19 ± 11	23 ± 9
	20	10 ± 5	23 ± 10	32 ± 4
s-FC DE	5	5 ± 0	10 ± 4	13 ± 4
	10	6 ± 1	14 ± 4	19 ± 5
	20	8 ± 2	23 ± 4	27 ± 3
	30	9 ± 1	32 ± 10	36 ± 7
Cristobalite	5	4 ± 1	5 ± 0	9 ± 0
	10	6 ± 1	6 ± 2	12 ± 4
	20	6 ± 2	9 ± 3	14 ± 1
	30	7 ± 3	12 ± 1	18 ± 3
alpha-Quartz	5	4 ± 0	3 ± 1	7 ± 1
	10	7 ± 0	6 ± 0	12 ± 1
	20	4 ± 1	6 ± 2	9 ± 1
	30	6 ± 2	6 ± 1	11 ± 2

* with one or more micronuclei.

**with two or more normal-size nuclei; may also have micronuclei.

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COMPANY SANITIZED

September 16, 1994

Lynn Moos Vlier
Deputy Director
Chemical Management Division
Environmental Protection Agency
401 M Street, S.W.
Washington, D.C. 20460

Dear Ms. Moos:

In response to the questions you raised after our meeting on [redacted], I offer the following additional information. The product being contemplated by [redacted] and the Brookhaven Laboratories, although still in the early stages of research and development, can best be characterized as a [redacted] which, in many ways will be similar to [redacted] that have been available in the United States for many years. The key difference between the contemplated product and currently available [redacted] is that the contemplated product, if successful, will [redacted]

[redacted], while still allowing the [redacted]. As [redacted] presently contemplated, the [redacted] will be designed to work on a variety of [redacted]

At this point, three stages of work are envisioned before any commercial application of the [redacted] can be considered. The first stage, which encompasses the work currently underway, involves the [redacted] itself. This process will require the use of [redacted] to which the [redacted]. The initial [redacted]

[redacted] contemplated are [redacted]. As the parties explained in our [redacted] meeting, these [redacted] are to be made under controlled conditions by trained laboratory personnel, following all state and federal regulations.

The sole purpose for generating these . We believe that the which is to be generated will be substantially similar to other currently, as well as historically, generated in laboratories in the United States. References to the use of such mediums have already been provided to Mr. Hassur of EPA by . Since we believe that substantially similiar

, we believe that the do not fall under the . Neither do the

are neither . Similiarly, since the

would not apply to the and Brookhaven activity. Since the research and development work which would occur would be under the direction of , the project would not present an

Assuming that the laboratory trials are successful, the next stage presently envisioned would focus on the on a larger scale. At first, this would most likely take place in . Ultimately, if the performs in accordance with the parties' expectations, are contemplated. At all points during this stage of work, state and federal regulations will be strictly followed. We expect that the relevant regulations will be those which currently exist for the

The final step currently envisioned by the parties will involve the to insure that they meet with all necessary regulatory standards . Each of these tests will obviously vary according to

We greatly appreciate EPA's consideration of this matter. Please let me know if I may be of any further assistance.

Sincerely,

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COMPANY SANITIZED

September 16, 1994

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Deputy Director
Chemical Management Division
Environmental Protection Agency
401 M Street, S.W.
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_____ , while still allowing the _____ . As _____ will be designed to _____ presently contemplated, the _____ work on a variety of _____

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Sincerely,



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ATTACHMENT IV

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TERATOGENIC STUDY IN RATS EXPOSED TO HALON 1301 BY INHALATION

Haskell Laboratory Report No. 499-78

Medical Research Project No. 2777

Report by:

A. S. Rogers

A. S. Rogers
Technician, Pathobiology

R. Culik

R. Culik
Senior Research Pathologist

C. D. Crowe

C. D. Crowe, Toxicologist
Acute Investigations Section

Approved by :

Henry J. Trochimowicz

H. J. Trochimowicz
Staff Toxicologist

J. G. Aftosis

J. G. Aftosis, D.V.M.
Manager, Pathobiology Division

Reviewed by :

Christiann Barba

Christiann M. Barba, Auditor
Quality Assurance Committee

ASR:RC:CDG:HJT:JGA:CMB:ljm

Dates: Initiation April 20, 1978

Completion September 7, 1978

TERATOGENIC STUDY IN RATS EXPOSED TO HALON 1301 BY INHALATION

Haskell Laboratory Report No. 499-78

Medical Research Project No. 2777

This study was conducted by Ms. Carole Doleba Crowe under the direction of Dr. H. J. Trochimowicz. Laparotomy, necropsy and gross pathology of the dams and gross examination of the fetuses were carried out by Dr. R. Culik, Mrs. Jean A. Hostetler, Ms. Ann S. Rogers, Mr. A. H. Stenholm, Mr. W. I. Swan and Mr. F. L. Ulmer. Clearing and Alizarin staining of the skeletons, and inspection of the fetuses were done by Dr. R. Culik, Ms. Ann S. Rogers, Ms. Alice V. Erwin, and Mr. A. H. Stenholm. Statistical evaluation of the data was carried out by Mr. W. E. Payerweather. The above work was supervised by Dr. J. G. Aftosmis.

Rooms in which rats were housed were controlled for temperature (72-74° F) and humidity (50%). Food¹ and water were offered ad libitum except when exposed. During the 10-day exposure period the animals were fed one hour following exposure until exposure the next day.

Rats in the test groups were exposed to nominal concentrations of 1000, 10,000 or 50,000 ppm (v/v, in air) of Halon 1301 vapors on days 6 through 15 of gestation for 6 hours daily in 600-liter Rochester-type inhalation chambers. Control rats were exposed simultaneously to room air in a 1.4 m³ stainless steel and glass dynamic chamber.

Rats were observed daily for clinical signs of toxicity and changes in behavior. They were weighed to within one gram on day of arrival, twice weekly thereafter and again on the day of sacrifice.

The desired vapor concentrations were obtained by adding a metered flow of Halon 1301 gas to the primary chamber air supply.

Chamber concentrations were routinely monitored at approximately one-half hour intervals using a Varian Aerograph Model 600D gas chromatograph equipped with a flame ionization detector. A 1' x 1/8" O.D. stainless steel column packed with 10% SE-30 on 60/80 mesh Chromosorb[®] W HP was maintained at 50°C for the analyses.

<u>Design Levels</u>	<u>Analytical Results</u>
Halon 1301	(Mean ± SD)
1,000 ppm	962 ± 57
10,000 ppm	10,196 ± 1,514
50,000 ppm	49,505 ± 4,753

¹ Purina Rat Chow, Purina Company, St. Louis, Missouri

TERATOGENIC STUDY IN RATS EXPOSED TO HALON 1301 BY INHALATION

Haskell Laboratory Report No. 499-78

Medical Research Project No. 2777

INTRODUCTION

The purpose of this study was to evaluate the teratogenic potential of monobromotrifluoromethane (Halon 1301) when inhaled by pregnant rats during the period of fetal organogenesis.

TEST MATERIAL

Test material was a nonflammable compressed gas (colorless liquid at 25°C in cylinder) with a purity of 99.99 +%. It was submitted by Robert A. Gorski of the Petrochemicals Department. Other names for this product are FC-13B1 and FC 1301. It was assigned Haskell No. 12206, MR-2777-002.

PROCEDURE

Test animals were Charles River CD (ChR-CD) primigravida albino rats bred at Charles River Breeding Laboratories, Inc., North Wilmington, Mass. One-half of the rats were bred on April 17, 1978 and the other half on April 18, 1978. All rats were received in one shipment on April 20, 1978 as three and two days pregnant*. The morning sperm was found in the vagina was counted as Day 1 of gestation.

The 108 rats were randomly assigned to one of four exposure groups, each group consisting of two lots according to breeding date. There were 27 rats per group, housed individually in suspended stainless steel wire mesh cages and identified by cage card number.

* All data are presented for the sum total as a unit.

All rats were sacrificed by chloroform inhalation on the twenty-first day of gestation. At sacrifice, the abdominal wall of the female was opened and both ovaries and uterus were removed and inspected. Next the uterus was opened and the fetuses removed and examined. The following observations and measurements were recorded:

1. Number of corpora lutea in each ovary
2. Number of implantation sites in each horn
3. Number and location of all live and dead fetuses.
4. Number and location of resorptions
5. Weight of each live fetus (to 0.01g)
6. Crown-rump length of each live fetus (to 0.01 cm)
7. Any gross anomaly which could be observed under a long focal length lens of 2½ x.

About one-half of the fetuses from each litter were preserved in 95% alcohol for subsequent maceration in 1% aqueous KOH, clearing and staining with Alizarin Red and examination to detect skeletal abnormalities. The remaining fetuses were fixed in Bouin's fluid for free-hand razor-blade sectioning by the Wilson method (1) with the modification described by Barrow and Taylor (2) and examination under a dissecting microscope for visceral and neural anomalies. Measurement of the lens of each eye was recorded as a part of this examination. The uterus and ovaries of rats in all groups were examined for gross changes and those of pregnant rats were preserved in Bouin's fluid for possible histologic examination. Other tissues and organs were examined grossly and discarded if found to be normal.

-
- (1) Wilson, J. G. (1965) Methods for administering agents and detecting malformations in experimental animals. In: Teratology: Principles and Techniques. J. G. Wilson and J. Warkany, eds., Univ. of Chicago, pp. 262-277.
 - (2) Barrow, Mark V. and Taylor, W. Jape (1969). A rapid method for detecting malformations in rat fetuses. Journal of Morphology, 127:291-306.

STATISTICAL EVALUATION

For statistical evaluation of the data, the litter was considered the experimental unit of treatment and observation. The Fisher Exact Probability test was used to evaluate the incidence of resorptions and abnormalities among litters. Maternal and fetal body weights and measurements were treated statistically by analysis of variance and least significant difference (LSD) tests. The number of corpora lutea, implantations and live fetuses per litter were analyzed by the Wilcoxon rank sum test. In all cases two tailed significance tests were performed and significance was judged at the 0.05 probability level.

RESULTS

1. Body Weight

No meaningful differences in average body weight gains were found between the control and any test group.

2. Clinical Signs

Minor hair loss occurred in two rats, one each in the 0 and 50,000 ppm groups. Two rats in the high level (50,000 ppm) developed brown crusty sores. One rat in the high level group was blind in the right eye on arrival. She was removed from the test for further observation and is not included in the final tabulation of the data.

3. Gross Changes in Organs of Dams at Necropsy

No gross pathological changes were observed in the ovaries and uterus or vital organs and tissues of pregnant treated females.

4. Pregnancy Outcome and Fetal Development

A summary of the effect of exposure to Halon 1301 on the dams, and on the outcome of pregnancy and fetal development is presented in Table I.

Although the mean body weight of pregnant females in the high-level group was lower than the mean of the control group, the difference was not statistically significant and the actual gains in body weight were similar.

Parameters measuring the outcome of pregnancy and fetal development, including the number of litters with partial and total resorptions, and the body measurements of the fetuses were not different from those of the concurrent controls.

5. Fetal Anomalies and Malformations

The type and the incidence of external, visceral and skeletal anomalies and malformations among litters in the control and treated groups and the number of fetuses examined are summarized in Table II.

Small subcutaneous hematomas and petechial hemorrhages on various parts of the body were found in fetuses from litters of all groups including the control group. Two runts were found, one in the control group and one in the intermediate level.

Visceral examination showed a slight incidence of hydronephrosis and undescended testes in all groups and a very slight incidence of hydroureter in the control and the mid-level groups. One fetus in the control group was found with hydronephrosis and hydrocephalus. In the group receiving 10,000 ppm Halon 1301, one fetus showed hydrocephalus plus a herniated diaphragm, one had a blood-filled peritoneum, and another showed hydronephrosis and inguinal hernia. All three fetuses were from different litters.

No malformations or major abnormalities of the skeletal system were noted. The small incidence of minor anomalies and common variants listed in the table was similar in all groups. These variations are

biological and are related to chronological age of the fetuses (delayed ossification, bipartite centra) or to their genetic background (wavy and 14th full or rudimentary ribs) and not to the treatment.

SUMMARY AND CONCLUSIONS

1. Exposure of pregnant rats to Halon 1301 vapors from day six through fifteen of gestation at levels of 1000, 10,000 and 50,000 ppm had no effect on their body weight gains. No compound-related clinical signs of toxicity or changes in behavior were noted.
2. The outcome of pregnancy measured by the number of implantation sites, resorptions and live fetuses, was not adversely affected by the exposure.
3. Exposure did not affect embryonal development as measured by weight and crown-rump length of the fetuses.
4. Only three fetuses, each one from a different litter, were found with malformations. All three were from dams exposed to the intermediate level (10,000 ppm) of Halon 1301. These defects were not treatment-related. They are considered as spontaneous, congenital malformations of genetic origin seen in this strain of rats at about the same frequency as in this study.
5. Under the conditions of this test, Halon 1301 was not embryotoxic or teratogenic when inhaled by pregnant ChR-CD rats.

TABLE I

EFFECT OF HALON 1301 ON THE OUTCOME OF PREGNANCY AND FETAL DEVELOPMENT OF THE RAT

	Air Concentration of Halon 1301 (1)		
	0 ppm	1000 ppm	50,000 ppm
Females bred	27	27	26
Females pregnant (%)	21 (77.8)	23 (85.2)	22 (84.6)
Corpora lutea/pregnant female	12.8 ± 3.2	13.4 ± 1.9	12.5 ± 2.2
Implantations/litter (2)	10.7 ± 2.6	10.5 ± 2.1	10.0 ± 2.7
Live fetuses/litter (2)	9.9 ± 2.7	10.0 ± 2.1	9.5 ± 2.7
Litters with early resorptions (%)	12 (57.1)	7 (30.4)	10 (45.5)
Litters with late resorptions (%)	1 (4.8)	0 (0)	0 (0)
Litter with dead fetuses (%)	0 (0)	0 (0)	0 (0)
Litters with partial resorptions (%)	13 (61.9)	7 (30.4)	10 (45.5)
Litters totally resorbed (%)	0 (0)	0 (0)	0 (0)
Resorptions/litter with resorptions (2)	1.2 ± 0.7	1.6 ± 0.6	1.2 ± 0.4
Initial body weight of pregnant female (g) (2)	223 ± 11	225 ± 13	219 ± 13
Final body weight of pregnant female (g) (2)	375 ± 38	380 ± 23	365 ± 20
Fetal crown-rump length (cm) (3)	4.2 ± 0.3	4.2 ± 0.3	4.2 ± 0.3
Fetal weight (g) (3)	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1

(1) Administered by inhalation, 6 hrs./day on days 6 through 15 of gestation; sacrificed on day 21.

(2) Mean ± 1 standard deviation.

(3) Mean of litter means ± 1 standard deviation.

INCIDENCE OF ANOMALIES IN LITTERS (FETUSES) AFTER MATERNAL EXPOSURE TO HALON 1301 (1)

Types of Anomalies	Concentrations		
	0 (Control)	1000 ppm	50,000 pp
<u>Gross</u>			
No. litters (fetuses) examined	21 (207)	23 (231)	22 (208)
Petechial hemorrhage	10 (15)	8 (14)	3 (5)
Subcutaneous hematoma	3 (4)	8 (9)	6 (6)
Agnathia	-	-	-
Runts	1 (1)	-	-
<u>Visceral</u>			
No. litters (fetuses) examined	21 (100)	23 (109)	22 (101)
Hydronephrosis	2 (2)	3 (3)	2 (2)
Undescended testes	2 (2)	2 (2)	4 (4)
Hydrourter	1 (1)	-	-
Hydronephrosis and hydrocephalus	1 (1)	-	-
Liver peliosis	4 (4)	1 (1)	-
Peritoneum - fluid filled	-	-	-
Hydronephrosis and inguinal hernia	-	-	-
<u>Skeletal</u>			
No. litters (fetuses) examined	21 (107)	23 (122)	22 (107)
14th Rudimentary rib(s)	16 (45)	21 (60)	20 (56)
Sternebrae unossified	5 (9)	11 (13)	12 (20)
Centra bipartite	2 (2)	2 (2)	2 (2)
14th Rib(s)	-	3 (3)	2 (3)
Wavy rib(s)	-	2 (2)	3 (3)

(1) Administered by inhalation, 6 hrs./day on days 6 through 15 of gestation; sacrificed on day 21.



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ATTACHMENT V

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E. I. du Pont de Nemours and Company
Haskell Laboratory for Toxicology and Industrial Medicine

HASKELL LABORATORY REPORT NO. 980-76 MR NO. 2365-001

Material Tested: Methane, Bromotrifluoro-

Haskell No. 10,261

Other Code: Halon 1301

MUTAGENIC ACTIVITY OF METHANE, BROMOTRIFLUORO-
IN THE SALMONELLA/MICROSOME ASSAY

Materials and Methods: Five histidine auxotrophs of *Salmonella typhimurium* were used in the assays. Strains TA 1535 and TA 100 are used to detect base-pair substitution mutations whereas strains TA 1537, TA 1538 and TA 98 are used to detect frameshift mutations.

The tests are performed in the presence and absence of a rat-liver homogenate activation system (S-9 mix). In the absence of metabolic activation, approximately 10^8 bacteria are added to 2 ml top agar (0.6% agar, 0.6% NaCl, 0.05 mM L-histidine, 0.05 mM biotin). The solution is mixed and poured on the surface of a Davis minimal agar plate prior to exposure to the test gas. The metabolically activated system involves the addition of 0.5 ml of S-9 mixture to the bacteria-top agar mixture. The S-9 mix contains per ml: 0.3 ml of the 9000 X 8 supernatant of homogenized rat liver, 8 mM MgCl₂, 0.05 M KCl, 5 mM glucose-6-phosphate, 4 mM NADP and 100 mM sodium phosphate (pH 7.4). This mixture is added directly to the top agar immediately before it is poured over the minimal agar plate.

The minimal plates with the bacteria overlay were exposed to the test gas in 9-liter glass chambers. The chambers, with Teflon® stopcocks and Viton® o-ring gaskets, were specially designed for this purpose. The open petri plates were held to the chamber by stainless steel racks, ten plates per chamber. The test gas was mixed with air from the compressed air line and introduced into the chambers through a flow-meter system.

After the gas-air mixture had flowed through the exposure chamber for 5 volume changes, the chambers were closed and placed in a 37°C incubator for either 6, 48, or 72 hours. At the end of the exposure period the chambers were flushed with air for several minutes and the plates removed. The 6 hour exposure plates were returned to the incubator to complete the 48 hour incubation period. The 48 hour and 72 hour exposure plates were counted immediately after removal from the chamber.

The concentration of the test gas in the exposure chambers was measured using a "Varian Aerograph" Model 600-D gas chromatograph, the measured values agreed well with the values indicated from the flow-meter system. The concentrations of

Exposure levels of up to 40% were tested in the activated and nonactivated assays.

Tables I, II, and III.

Methane, Bromotrifluoro- was tested in Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 at levels of 10%, 20%, 30% and 40%. The test gas was not mutagenic in the microbial assay either in the presence or absence of a liver microsomal system, i.e., it did not significantly increase the spontaneous mutation frequency.

Report by: Antje Koops

Antje Koops
Molecular Biologist

Approved by: Byron E. Butterworth

Byron E. Butterworth
Chief, Molecular Biology Section

383:25

date: January 7, 1977

TABLE I

MUTAGENIC ACTIVITY OF METHANE, BROMOTRIFLUORO- IN SALMONELLA TYPHIMURIUM STRAINS TA 1535 AND TA 100, AT 6 AND 48 HOUR EXPOSURE PERIODS, WITH AND WITHOUT METABOLIC ACTIVATION

Gas Added	Histidine ⁺ Revertants Per Plate*			
	6 Hour Exposure Period		48 Hour Exposure Period	
	TA 1535	TA 100	TA 1535	TA 100
0% Methane, Bromotrifluoro- with activation	44	148	49	162
without activation	20	136	29	116
10% Methane, Bromotrifluoro- with activation	40	130	48	139
without activation	16	127	26	129
20% Methane, Bromotrifluoro- with activation	37	143	51	138
without activation	24	110	20	117
15% Methane, Chlorofluoro (positive control)				
with activation	1143	2522	-	-
without activation	846	1363	-	-

* Average number of revertants from 3 plates

TABLE II

MUTAGENIC ACTIVITY OF METHANE, BROMOTRIFLUORO- IN SALMONELLA TYPHIMURIUM STRAINS TA 1535, TA 1537, TA 1538, TA 98 AND TA 100 AT 48 AND 72 HOUR EXPOSURE PERIODS WITH AND WITHOUT METABOLIC ACTIVATION

Gas Added	Histidine ⁺ Revertants Per Plate*				
	TA 1535	TA 1537	TA 1538	TA 98	TA 100
48 Hour Exposure					
Methane, Bromotrifluoro- 0% with activation	16	8	15	16	81
without activation	8	10	9	18	63
20% with activation	34	14	18	38	131
without activation	23	11	6	25	83
30% with activation	42	10	13	30	161
without activation	18	13	3	35	92
40% with activation	42	15	8	25	90
without activation	16	8	4	18	89
72 Hour Exposure					
Methane, Bromotrifluoro- 0% with activation	59	6	23	28	150
without activation	24	11	7	14	98
20% with activation	55	7	20	34	110
without activation	26	10	7	27	148
6 Hour Exposure					
Methane, Chlorofluoro- 15% with activation	1375	-	-	-	1924
without activation	769	-	-	-	1284

* Average number of revertants from 2 plates

TABLE III

MUTAGENIC ACTIVITY OF METHANE, BROMOTRIFLUORO- IN SALMONELLA TYPHIMURIUM
STRAIN TA 1535 AT 48 HOUR EXPOSURE PERIODS WITH AND WITHOUT METABOLIC ACTIVATION

Gas Added	Histidine ⁺ Revertants Per Plate*	
	TA 1535	
Methane, Bromotrifluoro-		
0% with activation	15	(43)**
without activation	12	(19)
20% with activation	16	(24)
without activation	13	(19)
30% with activation	16	(63)
without activation	12	(16)
Methane, Chlorofluoro-		
10% with activation	3330	(3608)
without activation	2537	(2711)

* Average number of revertants from 10 plates.

** Values in parentheses are from the 48 hour exposure plates which were reincubated at 37°C for an additional 24 hours to give 48 hour exposure with a total of 72 hour incubation.

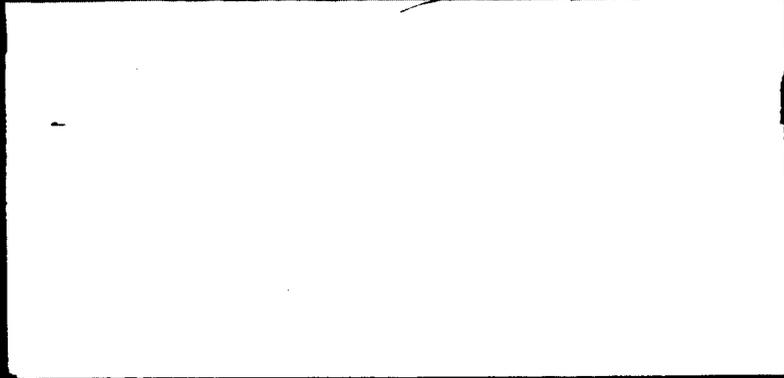


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ATTACHMENT III

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TOXICITY REVIEW

HALON 1301

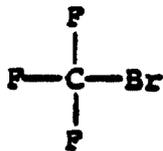


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This review reflects the available toxicity literature, both published and unpublished. Studies have not been evaluated for scientific merit. Contact Haskell Laboratory if you have questions.

Common Name: Halon 1301
Chemical Name: Methane, bromotrifluoro-
Synonyms: Freon® 13B1, FC-13B1
CAS Registry No.: 75-63-8
Chemical Structure:



Physical and Chemical Properties:

Description:	Colorless gas
Molecular Weight:	148.9
Boiling Point:	-57.8°C @ 760 mm Hg
Melting Point:	-168°C
Density/Specific Gravity:	1.57 g/cc (liquid at 70°F)
Vapor Pressure:	56.5 psig @ 0°F 199 psig @ 70°F 300 psig @ 100°F
Flash Point/Flammability:	Nonflammable
Solubility:	300 ppm in water @ 77°F
Conversion Factors:	1 mg/L - 164 ppm ₃ 1 ppm = 6.1 mg/m ³

Exposure Standards:

TLV® = 1000 ppm; STEL = 1200 ppm (1).
OSHA 8-hour TWA = 1000 ppm (2).
See Related Reference 52 for additional information.

DOT Classification:

Nonflammable gas; DOT shipping name monobromotrifluoromethane (44).

EPA RCRA Status

None.

FDA Status:

None.

TSCA Inventory:

Yes.

TOXICITY

A. Acute

1. Oral

- Not applicable.

2. Skin and Eyes

- Liquid Halon 1301 may freeze the skin (frostbite) on contact. Such contact should be avoided by wearing protective clothing and eye protection (3).

3. Inhalation

a) Animal Studies

<u>Concentration (ppm)</u>	<u>Duration of Exposure</u>	<u>Animal Species</u>	<u>Effect(s)</u>	<u>Refer- ence</u>
850,000	2 hours	Mice Guinea pigs	Lethal	4
832,000	15 minutes	Rats	Lethal	5
800,000	4 hours	Rats	3/70 deaths	40
800,000	2 hours	Rats Rabbits Mice Guinea pigs	No deaths Toxic effects: drowsiness, tremors, ataxia and convulsions	4

<u>Concentration (ppm)</u>	<u>Duration of Exposure</u>	<u>Animal Species</u>	<u>Effect(s)</u>	<u>Refer- ence</u>
800,000	30 minutes	Rats, Mice	Lethal	10
770,000	1 hour	Rats	No deaths	6
600,000	2 hours	Mice, Rats, Guinea Pigs, Rabbits	No deaths Hypoactivity Slow and deep respiration	4
560,000	1 hour	Rats	No deaths	6
500,000	2 hours	Rats, Mice, Guinea Pigs, Rabbits	Slight initial CNS, depression Normal behavior thereafter	4
500,000	26 minutes	Rats	Drowsiness	7
400,000	2 hours	Mice, Rats, Guinea Pigs, Rabbits	No effect	4
370,000	7 hours	Cats Guinea Pigs Mice Rats Rabbits	Lethal (1/1) Lethal (6/6) Survived Survived Survived	8
370,000	7 hours	Guinea Pigs	No deaths	11
360,000	3.5 hours	Cats Guinea Pigs Mice Rats Rabbits	Survived Lethal (5/6) Survived Survived Survived	8
360,000	1.33 hours	Cats Guinea Pigs Mice Rats Rabbits	Survived Survived Survived Survived Survived	8
300,000	2 hours	Mice, Rats Guinea Pigs Rabbits	No effect	4

<u>Concentration (ppm)</u>	<u>Duration of Exposure</u>	<u>Animal Species</u>	<u>Effect(s)</u>	<u>Refer- ence</u>
300,000	40 minutes	Rats	No effect Pathology normal	40
200,000	2 hours	Monkeys Rabbits Guinea Pigs Rats	Glassy eyes (1/2) Restlessness (4/4) Lacrimation (1/6) Restlessness (10/10) Normal recovery	40
200,000	2 hours	Guinea Pigs	No effect Normal Pathology	9
200,000	2 hours	Rats	No effects Normal Pathology	40
180,000	7 hours	Rabbits Cats Mice Rats Guinea Pigs	Lethal (1/4) Survived Survived Survived Survived	8
150,000	2 hours	Monkeys Rabbits Guinea Pigs Rats	Glassy eyes (1/2) No effect No effect No effect	40
90,000	7 hours	Cats, Mice Rats, Rabbits, Guinea Pigs	No effect	8
46,000-50,000	2 hours	Guinea Pigs	No effect	9
44,000	2 hours	Guinea Pigs	No effect	8
24,000-28,000	2 hours	Guinea Pigs	No effect	9
9,000-13,000	2 hours	Guinea Pigs	No effect	9

- Pathological examination of animals killed by Halon 1301 exposure revealed edema and hemorrhage in the lungs, severe pneumonitis and tracheitis and congestion of the liver, spleen and kidneys. No cellular changes were seen in these organs (5). Examination of other animals which survived Halon 1301 exposures failed to reveal any significant changes (40).

- Hematological examination of the monkeys exposed to 10 or 20% Halon 1301 failed to reveal any significant changes (40).
- Seven monkeys trained on continuous and discrete avoidance performance tasks were exposed to Halon 1301 concentrations ranging from 10.5 to 42%. Significant performance decrements were observed in all monkeys during 20-25% exposures. Higher concentrations resulted in impaired performance to the point of complete disruption of operant behavior in some monkeys. No visible signs of CNS depression or analgesia accompanied this loss of ability to perform conditioned performance tasks (12).
- See Related References 45, 47, 49 and 50 for more information.

b) Human Exposures

<u>Concentration (percent)</u>	<u>Duration of Exposure</u>	<u>Effect(s)</u>	<u>Reference</u>
15	1 minute	Severe dizziness and marked paresthesia. Increased heart rate and T-wave depression (EKG). Recovery was rapid and complete within 5 minutes.	13
12	1 minute	Severe dizziness and mild paresthesia in 1/2 subjects. T-wave depression and increased heart rate.	13
10	1 minute	No effect for the first 30 second followed by slight dizziness and paresthesia (1/2). Heart rate increased and T-wave was depressed in 1/2 subjects.	13
10	3-3.5 minutes	Light headedness increasing to near unconsciousness. Slight disturbance tests (balance and reaction time). No EKG changes were observed.	40

10	20 minutes	Euphoria (2/6) Light-headedness (3/6) Paresthesia (1/6) Tinnitus (1/6) Slight to moderate eye/ nose irritation (2/6) Pulmonary discomfort (2/6)	14
9	2 minutes	After 1 minute of exposure dizziness was felt which increased in intensity. Increased heart rate, no EKG changes.	13
7	3 minutes	Exposure was during flight at 5,000-20,000 feet. No adverse subjective biomedical or EKG effects were reported.	16
7	3 minutes	Dizziness, faintness and drowsiness (6/8). Dif- ferent altitudes had no effect on subjective symptoms. No compound- related EKG changes.	15
7	3-3.5 minutes	Light-headedness. No EKG changes.	40
6	3 minutes	Slight paresthesia, dizziness. No EKG changes. Increased heart rate.	13
5	3-3.5 minutes	No effects observed. EKGs normal.	40
5	20 minutes	Euphoria (2/4) Light headedness (2/4) Pressure in the ears (1/4) or in the head (1/4) Slight eye and nose irritation (1/4) Slight pulmonary discomfort (1/4)	14

4	3 minutes	Dizziness, faintness and drowsiness (3/8). Changes in altitude had no effect on symptoms. No compound-related EKG changes.	15
4	3 minutes	Exposure was during flight at 5,000-20,000 feet. No adverse subjective biomedical or EKG effects were reported.	16
3	3-3.5 minutes	No effects observed. EKGs normal.	40
1	3-3.5 minutes	No effects observed. EKGs normal.	40

- Ten volunteers were exposed to inhalation of CBrF_3 from an anesthesia machine. These experiments consisted of a series of relatively short exposures to different concentrations of CBrF_3 . Heart rate, blood pressure and electrocardiographic tracings were monitored. Cardiac arrhythmias were not observed to develop during exposure of 5 subjects for a total of 10 to 17 minutes to 4-6.7% nor during the additional exposures of 5 subjects to 7-9.6% for 5 to 7 minutes. These subjects reported reactions including dizziness, tingling of extremities, light-headedness and a fear of imminent loss of consciousness. Five subjects were exposed to 8.2-15.7% for total exposure times of 10-31 minutes. Only one of these subjects developed a serious cardiac arrhythmia. The subjective sensations which were reported to have developed during these serial exposures to CBrF_3 were on the central nervous system. These sensations ranged from an increased awareness of sound, visual disturbances (e.g. flashing before eyes), tingling of extremities and numbness of body to the sensation of the room rocking back and forth or spinning, drowsiness, and the fear of impending unconsciousness (14).
- Three volunteers, one visually handicapped, were exposed to 0.1, 4.3, 4.5 and 7.1% of Halon 1301 for up to 30 minutes. The subjects' health and feelings were monitored before, during, and up to 2 hours after exposure. Included before each exposure were

a complete physical examination (blood pressure, body temperature, electrocardiogram [ECG], pulmonary function, urinalysis, clinical blood chemistry & symptoms) and mood evaluation. During and after each exposure, the subjects performed various tasks to measure effects of exposure on eye-hand coordination, short-term memory, reaction time, depth perception, balance, ability to walk on a treadmill, write, and speak. Finally, spontaneous electroencephalograms (EEGs) were made immediately after exposure on each subject. At 0.1% Halon 1301, no significant deviations from normal were detected in any of the subjects. At 4.3 and 4.5%, subjects experienced sensations of light-headedness, dizziness, and euphoria 2 minutes into exposure. At 9 minutes into exposure at 4.5%, only the visually handicapped subject showed signs of impaired balance; at 30 minutes after exposure, his response was normal. A second subject showed mild impairment in balance at 30 minutes. At 7.1%, one subject showed mild changes in tests of balance during exposure, while the visually handicapped subject performed unsatisfactorily in the balance and eye-hand coordination tests. Sensations of light-headedness, dizziness, and euphoria also were noted, persisting up to 60 minutes after exposure. Analysis of ECG tracings did not show any abnormalities caused by the exposures. One of the subjects showed slight changes in brain wave activity (EEG) at 7.1%, with a pattern representative of slight anesthesia. All untoward physiological and subjective responses were reversible shortly after exposure. Results of the other health tests were within normal limits (40)

B. Extended Inhalation Studies

- A single cat survived two 7-hour exposures to 18% Halon 1301. Guinea pigs exposed to the same concentration died after 2 exposures as did 1/4 rabbits. All of the 10 rats and 10 mice similarly exposed survived. Five 7-hour exposures to 8.72% killed 4/6 guinea pigs. Mice, rats, cats and rabbits were not killed except for 2 natural deaths. Five 7-hour exposures to 4.44% was responsible for the death of 1/10 mice, but no other deaths were attributable to the Halon 1301 exposure. Dogs exposed to either 9 or 18% for 7 hours on each of 2 consecutive days developed no signs of intoxication. Acute hemorrhagic pneumonitis and degeneration of

the liver and kidneys were found among animals that died as the result of exposure (8).

- Exposure of groups of 3 rats, 3 guinea pigs, 1 dog and 3 cats to 20-60% Halon 1301 for up to 70 hours did not produce symptoms of toxicity (17).
- A group of 20 rats and 20 guinea pigs was exposed to 5.1% Halon 1301 continuously for 10 days. No toxic effects or pathologic or histopathologic changes were observed. Hematologic determinations also failed to reveal any adverse compound-related changes (6).
- A group of 20 mice, 10 rats and 10 guinea pigs was exposed to 50% of Halon 1301 in air, 2 hours daily for 15 days. No significant effects were observed (4).
- Rats were exposed to 5% of Halon 1301, 23 hours daily for 30 days. Studies of blood did not reveal any accumulation of Halon 1301. There was no elevation in the rate of excretion of fluoride ion in the urine. No gross pathological changes were found (18).
- Rats and dogs were exposed to 2.3% of Halon 1301 in air for 6 hours daily, 5 days per week for up to 18 weeks. At no time did the animals show any signs of intoxication. Upon autopsy the animals showed no evidence of pulmonary edema or necrosis as had been seen in acute lethal studies. There was a moderate diffuse congestion of the entire respiratory tract but no other significant changes. All other organs appeared normal (19).

Halon 1301 Blood Levels

- Beagle dogs with cannulas surgically implanted in the common carotid artery and external jugular vein were exposed to 5%, 7.5%, and 10% Halon 1301 for 60 minutes. Blood samples were withdrawn from the cannulas before, during, and after exposure and analyzed for the Halon 1301 concentration. Blood levels of Halon 1301 increased rapidly during the first ten minutes of exposure, plateaued within twenty minutes, and declined rapidly after exposure. The mean blood concentrations at equilibrium were directly proportional to inspired levels: at an inspired concentration of 5% - arterial 19.2 ug/mL,

venous 14.6 ug/mL; at an inspired concentration of 7.5% - arterial 30.6 ug/mL, venous 28.4 ug; and at an inspired concentration of 10% - arterial 40.2 ug/mL, venous 32.1 ug/mL. During exposure arterial concentration was greater than venous concentration, but after exposure the venous concentration slightly exceeded the arterial concentration (40 , 41).

- Anesthetized rats were exposed to 5% of Halon 1301 for 30 minutes and blood levels of Halon 1301 determined. The following blood levels were found at the indicated post-exposure times:

<u>Time</u>	<u>Level</u>
0	5.6 ug/g
15 minutes	0.62 ug/g
1 hour	0.35 ug/g
2 hour	0.05 ug/g
4 hour	0.07 ug/g

(18)

- Groups of 15 rats were exposed for 1-5 minutes to 70% Halon 1301. Rats were removed at various times for collection of brain tissue, heart and intracardiac blood samples. During the first minutes of exposure, the Halon 1301 concentration in the brain and heart rose rapidly. Following the end of exposure, the concentrations in brain and heart fell rapidly. The Halon 1301 concentrations in the samples of intracardiac blood were similar to those of the hearts obtained at the corresponding time intervals (20).

Cardiac Studies

a) Cardiac Arrhythmias

- It has been known for some time that inhalation of vapors from certain organic materials, which include such compounds as carbon tetrachloride and gasoline, can make the heart muscle abnormally reactive to elevated adrenalin levels with resulting cardiac arrhythmias. These arrhythmias are frequently ventricular in origin and may result in sudden death. This phenomenon is commonly referred to as cardiac sensitization. Since Halon 1301 is used as a fire extinguishing agent and fire is a life-threatening emergency which can be expected to result in high circulating levels of adrenalin in persons attempting to extinguish the fire or those

whose escape may be blocked, several investigators have studied the effects of adrenalin plus Halon 1301 exposure on the cardiovascular system.

- EC50* (dogs) = 200,000 ppm (23).
- Development of spontaneous cardiac arrhythmias were not observed in the electrocardiographic tracings obtained during exposure of 1 dog to 10% (100,000 ppm) CBrF₃ for 20 minutes, exposure of 4 dogs to 20% (200,000 ppm) for 15 minutes, and exposure of 5 dogs to 30% (300,000 ppm) for 15 minutes. Salivation and whole body trembling occurred during exposures to 30% and to a lesser extent during exposures to 20%. Weakness and inability to stand was reported only for those dogs exposed to 20%. Recovery following exposure to 30% occurred within 1-2 minutes after exposure was ended. The author does not report whether the CBrF₃ was mixed with air or with oxygen. Cardiac sensitization to intravenously injected epinephrine during 5-minute exposures to 10% or to 20% CBrF₃ was evidenced by increases in the number of ectopic beats in 2/2 exposed to 10% and in 1/2 exposed to 20% and by ventricular tachycardia in 1/2 exposed to 20% (13).
- Dogs were exposed to 5, 7.5 and 10% of Halon 1301 for 30-60 minutes. The test animals received an injection of epinephrine both before and after the exposure. None of the dogs exposed to 5.0% developed cardiac arrhythmias. Two of 12 dogs exposed to 7.5% showed a marked response after a 30-minute exposure. Following a 30-minute exposure to 10% Halon 1301 there was one definite and one questionable response in 12 dogs (40).
- A dog was exposed to 10% Halon 1301 for a few minutes following an injection of epinephrine. A challenge injection followed the exposure. Some effect on the heart was seen (40).
- Halon 1301 did not cause cardiac sensitization of the beagle heart below a concentration of 10% in air (40). Further experimentation showed that the minimum effect level is closer to 7.5% than 10% (40).

* EC50 is the concentration causing cardiac arrhythmias in 50% of the animals. Exposure was for 5 minutes followed by an exogenous dose of epinephrine.

- Groups of 8 dogs were exposed to 10, 20 or 40% Halon 1301 in air. Exposure times were 15, 27 and 55 minutes, respectively. During exposure the chambers were darkened and the dogs subject for 5 minutes to fright-inducing stimuli. Those dogs exposed to 10% Halon 1301 demonstrated increased alertness, those exposed to 20% developed tremors without increased alertness and those exposed to 40% howled, salivated and developed dyspnea and tremors (14).
- Spontaneous cardiac arrhythmias developed within 1-3 minutes of exposure to 40% Halon 1301. Arrhythmias could be produced in those animals not developing spontaneous arrhythmias by the intravenous injection of a pressor dose of epinephrine. Larger doses caused ventricular fibrillation with cardiac arrest in dogs and, commonly, spontaneous defibrillation in monkeys (21).
- Anesthetized guinea pigs, cats and dogs were exposed to 20% Halon 1301. This exposure increased the arrhythmogenic effect of i.v. infusion of epinephrine (24).
- Five anesthetized monkeys were exposed, successively, for 10 minutes each, to 10-80% Halon 1301. Spontaneous ventricular arrhythmias developed during the first 5 minutes of exposure to 30% and higher. Cardiac arrhythmias spontaneously appearing in monkeys exposed to CBrF_3 required a minimal blood pressure threshold for their production. The blood pressure threshold varied as an inverse function of the log of the CBrF_3 concentration to which monkeys in acid-base balance were exposed. Acidosis decreased the threshold and alkalosis increased the threshold at concentrations of 10 and 20% CBrF_3 but were without effect at 30% or greater concentrations. Epinephrine decreased the blood pressure threshold required to trigger arrhythmias but was not necessary for their production as it is in the case of cyclopropane (25).
- No significant difference in the percentage of marked responses was seen when dogs with myocardial infarctions were used instead of healthy dogs (40, 42).

- Groups of rats were exposed to one of the following conditions:

- 1) simulated altitude with added Halon 1301 (27 rats);
- 2) simulated altitude without Halon 1301 (9 rats);
- 3) simulated altitude with added Halon 1301 and injected epinephrine (27 rats);
- 4) simulated altitude without Halon 1301 but with injected epinephrine (9 rats).

No rats died during any chamber exposure. Three animals developed cardiac arrhythmias during inhalation of Halon 1301. One rat breathing 24 percent Halon 1301 at a simulated altitude of 5,000 feet, and one exposed to 16 percent CBrF_3 at 389 mm Hg, developed premature atrial contractions about one minute after the Halon 1301 was admitted to the chamber. Indications of bundle branch blocks appeared as the exposure continued. In both cases, these changes disappeared when the CBrF_3 -air mixture was replaced with room air during the chamber descents. Premature atrial contractions were also noted in the electrocardiogram of one rat breathing 24 percent Halon 1301 at 632 mm Hg. This animal had received an epinephrine injection before the exposure. Normal EKG tracings reappeared when the rat was returned to ambient conditions. No other prolonged cardiac arrhythmias were noted on the electrocardiograms from any other rats. Histological examination of the lungs from rats sacrificed immediately after exposure showed no pathologic changes which could be directly related to breathing Halon 1301 or exposure to hypobaric conditions. In this experiment epinephrine was administered intramuscularly to rats rather than the usual intravenous route. The use of i.m. administration would be expected to produce much lower blood levels of epinephrine than i.v. injection. The observation of only 1/27 premature atrial contractions after exposure to 24% Halon 1301 at 632 mm Hg may reflect the low dose of epinephrine rather than any species different (22).

- Rats were subjected to 79% Halon 1301 plus 21% oxygen and challenged with subtoxic doses of epinephrine. CNS effects were the major

observations in rats, 17/20 showed respiratory arrest within 40 minutes and several exhibited erratic convulsive behavior prior to exhibiting respiratory difficulties (26).

b) Heart Rate And Blood Pressure

- Inhalation of 80% Halon 1301 caused blood pressure falls in many cases and a marked elevated diastolic pressure in rhesus monkeys. Inhalation of 70 percent Halon 1301 triggered cardiac arrhythmias. These arrhythmias were dependent on the maintenance of a minimum blood pressure. Halon 1301 (80%) inhalation in dogs which had undergone a left lumbar sympathectomy caused a large increase in right femoral artery blood flow. Concurrently, mean arterial blood pressure, peak systolic blood pressure fell while left ventricular and diastolic pressure rose (27).
- Dogs were exposed to 50 and 75% Halon 1301 for 10-minute periods. Recordings were made and arterial and coronary sinus blood samples were obtained before, during and after the exposures. A small decrease in the vigor of myocardial contraction, decreases in vasoconstrictor tone and mean arterial blood pressure, an increase in aortic blood flow, an increase in myocardial lactate utilization and a decrease in myocardial oxygen consumption were observed as functions of the Halon 1301 concentration. Conclusions were than under the conditions of this experiment (1), a decrease in pressure-volume work done by the left ventricular myocardium during Halon 1301 exposure resulted in a decrease in myocardial oxygen consumption; (2) myocardial metabolism was little affected; and (3) decreased peripheral vascular flow resistance was accompanied by increased cardiac output (28).
- To investigate the effect of Halon 1301 on blood pressure 10 anesthetized dogs were exposed to air or 70% Halon 1301 for 50 minutes. The blood pressure of the treated dogs was significantly lower than pre- and postexposure levels and also lower than for air treated controls. No significant differences were seen for cardiac output. Heart rate was significantly lower (29).
- Halon 1301 was administered to dogs and the following parameters recorded: pulmonary

resistance, pulmonary compliance, respiratory minute volume, heart rate and aortic blood pressure. Halon 1301 was shown to produce an increase in pulmonary resistance a decrease in compliance an increase in respiratory minute volume, very little effect on heart rate even at concentrations up to 20%, and a mild hypertensive effect (4% increase at 20% concentration). Heart-lung experiments in surgically prepared dogs showed little change in myocardial contraction even at high concentrations. Experiments with rats showed Halon 1301 capable of producing apnea with atrioventricular conduction blocks occurring during this period of apnea. Halon 1301 also produced a rise in pulmonary resistance and fall in compliance as it did in the dog experiments (40).

- Exposure of dogs and monkeys to 10-80% of Halon 1301 caused cardiovascular and CNS effects which increased in severity with increasing concentration. An initial fall in blood pressure of 10-20 mm Hg at the lower concentrations and 40-60 mm Hg at the higher concentrations was observed. Epileptiform convulsions were seen in about 50% of the dogs exposed to 50-80% Halon 1301 while conscious. Conscious monkeys, on the other hand, became lethargic, and no convulsions were seen (21).
- The mechanism of the decrease in mean arterial blood pressure in the dog during Halon 1301 exposure was a decrease vasomotor tone resulting from ganglionic blockade. No direct vascular smooth muscle effect of CBrF_3 was observed. When arterial blood from one dog was perfused through the hind leg at constant flow rate through a hind limb of another dog, exposure of the donor to 67-70% CBrF_3 was accompanied by a reversible decrease in the donor's mean arterial blood pressure, but the perfusion pressure was unaltered (30).
- When anesthetized dogs were exposed to Halon 1301 the animals myocardial contractility decreased inversely with the concentration of Halon 1301 (31).
- Halon 1301 significantly decreased total peripheral resistance and myocardial contractility in anesthetized open-chested dogs and monkeys resulting in a reversible hypotension during exposure (32).

- Groups of dogs were exposed to 27-75% Halon 1301 in oxygen or 27-75% mixture of O₂ and N₂. Exposure to Halon 1301 may result in disturbances of myocardial energy metabolism that are connected to myocardial performance. The exposure resulted in a progressive rise of plasma glucose concentrations that persisted for at least 30 minutes post exposure (33).
- Exposure of dogs to 27, 51 or 75% Halon 1301 produced no myocardial tissue hypoxia, slight metabolic acidosis, normal pyruvate, lactate, and coronary sinus blood glucose, decreased mean arterial blood pressure and myocardial contraction strength and elevated arterial blood glucose (34).
- Twelve anesthetized cats were subjected under hyperbarbic conditions to 5-minute inhalation of 5% Halon 1301. This exposure was associated with:
 - 1) Cardiac arrhythmias in 10/12 cats;
 - 2) Fall in systolic blood pressure (average decrease -18.4 mm Hg) in 10/10 cats and a fall in diastolic pressure (average decrease - 19 mm Hg) in 9/10 cats (35).
- A anesthetized cat was exposed to 80% Halon 1301 and 20% oxygen. This exposure did not materially modify the physiologic equilibrium of the cat. The following changes were observed:
 - Slight activation of respiration;
 - Decreased arterial blood pressure (1 mm Hg);
 - Slight decrease in oxygen consumption;
 - CNS depression (hypnotic). (4)
- Anesthetized dogs and monkeys were exposed to 70 or 80% of Halon 1301 and EEGs recorded. The most significant findings were (1) dominance of the EEGs by 6-9 Hz waves beginning 2-3 minutes after beginning exposure to CBrF₃ and (2) a nearly normal susceptibility of the EEG to activation by auditory and photic stimuli during exposure to CBrF₃. Several lines of evidence were explored which suggested that the central nervous system effects of CBrF₃ may be the result of the induction of functional changes at the rhinencephalic level (36).

- To determine if the negative inotropic effect of Halon 1301 was accompanied by altered myocardial metabolism, urethane anesthetized-guinea pigs were exposed for 30 minutes and myocardial ATP, ADP, AMP and creatine phosphate levels were determined. A concentration of 75% Halon 1301, produced a 34% decrease in contractibility. Blood levels stabilized within 2.5 minutes. Blood pressure and heart rate were decreased. High energy phosphate levels were not altered in a fashion commensurate with the theory that a block in energy metabolism was responsible for the production of the induced negative inotropic effect (43).
- See Related References 46, 48 and 51 for more information.

C. Carcinogenic Potential

- No evidence of a carcinogenic potential has been found. However, the longest study conducted was an 18-week inhalation study (19).

D. Mutagenic Potential

- Halon 1301 was tested in Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA-100 at levels up to 40%. The gas was not mutagenic either in the presence or absence of a liver microsomal system (40).

E. Embryotoxic Potential

- Groups of 27 pregnant rats were exposed 6 hours a day on days 6-15 of gestation to 962 + 57, 10,196 + 1514 or 49,505 + 4753 ppm of Halon 1301. No compound-related clinical signs of toxicity or changes in behavior were noted. The outcome of pregnancy, measured by the number of implantation sites, resorptions and live fetuses, were not adversely affected by the exposure. Exposure did not affect embryonal development as measured by weight and crown-rump length of the fetuses. Three fetuses were found with malformations. All three were from dams exposed at the intermediate level, however, these effects were not considered compound-related. Under the conditions of this test, Halon 1301 was not embryotoxic or teratogenic (40).

F. Other Reproduction Studies

- None.

G. Aquatic

- None.

H. Human Exposure

- See Section A.2.b. for details of controlled inhalation experiments in human volunteers.

I. Epidemiology

- No information available.

J. Metabolism

- See Related References 53-55.

K. Pyrolysis Studies

- Halon 1301 decomposes upon contact with flames or hot surfaces above 1000°F. While this action appears necessary for the product to function effectively as a fire extinguishing agent, it also results in the formation of several new compounds whose properties are considerably different from Halon 1301. The common decomposition products of Halon 1301 are: HF, HBr, Br₂, carbonyl halides, consisting of carbonyl fluoride (COF₂) and carbonyl bromide (COBr₂). The quantities of these materials in the post-extinguishment atmosphere depend upon several factors, such as the size of the fire, type of fuel, enclosure size, degree of ventilation, and rapidity of flame extinguishment. In practical fire tests, the predominant decomposition products are HF and HBr. Free bromine and carbonyl halides have not been detected in significant quantities and post-extinguishment atmospheres.
- ALC (15-minute exposure, rats) = 14,000 ppm (5).
- The toxicity of pyrolyzed Halon 1301 appears to be due to the HF formed. The 15-minute LC50 for HF is 2700 ppm. At the LC50 determined for pyrolyzed Halon 1301 (2300 ppm), 2480 ppm of HF is formed.
(37)

- The passage of Halon 1301 through an Inconel tube heated to 482°C did not result in any detectable decomposition of the compound, nor did the exposure of the animals to the effluent material result in any deviation in the mortality rates seen with undecomposed material. When the tube is heated to 593°C, some decomposition of the compound and an increase in mortality was seen. The incidence of mortality was dependent on the Halon 1301 concentration. Mild degrees of pneumonitis were found in all exposed species, except rats, which were exposed to 482°C fumes. Acute chemical pneumonitis and degeneration of the liver, kidneys and brain were found in all species exposed to fumes generated at temperatures greater than 482°C (8).
- Deaths as the result of inhalation of pyrolysis products of Halon 1301 characteristically resulted from pulmonary hemorrhage and edema. The 15-minute LC50 value for pyrolyzed Halon 1301 calculated from these experiments was 2300 ppm. The pathologic response and delayed death patterns are similar to those seen from HF (38).
- Groups of animals were exposed for 30 minutes to atmospheres containing the pyrolysis products of Halon 1301. None of the animals died after being exposed at concentrations up to 80% of 800°C pyrolysis products. Signs of toxicity included dyspnea and prostration. When exposed to vapor products at 1000°C, all of the animals were killed at concentrations greater than 1%. At 0.75% approximately 20% of the exposed rats and 90% of the mice were killed. At 0.5%, none of the rats and 50% of the mice died (4).
- When Halon 1301 is applied to ordinary fires such as gasoline or wood, the resulting decomposition products may include HF, HBr, carbonyl halides and in some cases free bromine in concentrations depending on the quantity used. When applied in quantities of 2.3 pounds in a 720 cubic foot room for durations of the order of 5 to 30 minutes Halon 1301 caused some injury to guinea pigs, but was not lethal (9).
- With the exception of initial excitement, no symptoms indicative of a toxic action were observed during exposure of 20 mice and 40 rats to 1.07% Halon 1301 in air for 15 minutes. Very few deaths occurred during gasoline fires, however, the trachea and lungs were found to be congested (39).

REFERENCES

1. "ACGIH TLV® Booklet" (1981).
2. Code of Federal Regulations, Title 29 Section 1910.1000.
3. Du Pont Bulletin B-29D, "Du Pont Halon 1301 Fire Extinguishment" (1977) (C-1205).
4. Paulet, G., Arch. Mal. Prof., 23, 341-347 (1962) (J-2142).
5. Comstock, C. C. et al., Army Chemical Center, Report No. 23 (1950) (J-31).
6. Unpublished Data, Hazleton Laboratories, Reported by McHale, E. T., Atlantic Research Corporation, U. S. Army Res. Office Contract DAHC 19-71-C-0026 (J-1638).
7. Unpublished Data, ICI Ltd. (1954) (J-102).
8. Treon, J. P. et al., Unpublished Data, Kettering Laboratory (1957) (J-1868).
9. Dufour, R. E., Underwriters' Laboratories, Inc., Report NC 445 (J-1493).
10. Caujolle, F., Bull. Ins., Ind du Froid, 21 (1964) (J-1683).
11. Engibous, D. L. and T. R. Torkelson, Dow Chemical Company. Data reported in U.S.N.T.I.S. Report PB 161942.
12. Carter, V. L. Jr., et al., Tox. Appl. Pharmacol., 17(3), 648-655 (1970).
13. Clark, D. G., Unpublished Data, ICI Ltd. (1970) (J-4064).
14. Hine, C. H. et al., Proc. 4th Ann. Conf. Atmospheric Cont. Conf. Spaces, AMRL-TR-68-175, 127-144 (1968) (J-2418).
15. Call, D. W., Aerosp. Med., 44, 202-204 (1973) (J-2409).
16. Smith, D. G. and D. J. Harris, Ibid., 198-201 (J-2408).
17. Scholz, J. and W. Weigand, Z. Arbeitsmed. Arbeitsschutz, 14(6), 129-131 (1964) (CA 62:2166h). (J-2439).
18. Griffin, T. B. et al., Appr. Halogenated Fire Ext. Agents, Proc. Symp., 136-147 (1972) (J-2383).

REFERENCES (Cont'd.)

19. Comstock, C. C. et al., U. S. Army Chemical Corps, Med. Div. Report No. 5030-180 (1953) (J-476).
20. Van Stee, E. W. and K. C. Back, Aerosp. Res. Lab., Wright-Patterson Air Force Base, AMRL-TR-70-139 (1970) (CA 75:74289).
21. Van Stee, E. W. and K. C. Back, Tox. Appl. Pharmacol., 15, 164-174 (1969).
22. Call, D. W., Appr. Halogenated Fire Ext. Agents, Proc. Symp., 127-135 (1972) (J-2383).
23. Clark, D. G. and D. J. Tinston, Brit. J. Pharmacol., 49(2), 355-357 (1973).
24. Wills, J. H. et al., Tox. Appl. Pharmacol., 22, 305-306 (1972).
25. Van Stee, E. W. and K. C. Back, Aerospace Med. Res. Lab., AMRL-TR-68-188 (J-2370).
26. Rhoden, R. A. and K. L. Gabriel, Tox. Appl. Pharmacol., 25, 469 (1973).
27. Van Stee, E. W. et al., Proc. Annu. Conf. Environ. Toxicol., 5th, AMRL-TR-74-125, 155-167 (1974) (J-2836).
28. Back, K. C. and E. W. Van Stee, Tox. Appl. Pharmacol., 25, 469 (1973).
29. Van Stee, E. W. et al., Proc. 4th Ann. Conf. Atmos. Cont. Conf. Spaces, AMRL-TR-68-175, 113-126 (1968).
30. Van Stee, E. W. and K. C. Back, Tox. Appl. Pharmacol., 23(3), 428-442 (1972).
31. Van Stee, E. W. et al., Ibid., 26(4), 549-558 (1973).
32. Van Stee, E. W. and K. C. Back, Aerosp. Med. Res. Lab, Wright-Patterson Air Force Base, AMRL-TDR-68-182 (1971) (J-2369).
33. Van Stee, E. W. et al., Tox. Appl. Pharmacol., 34(1), 62-71 (1975).
34. Van Stee, E. W. et al., Proc. Annu. Conf. Environ. Toxicol., 4th, 65-83 (1973) (J-5247).

RELATED REFERENCES

Inhalation

45. Dimov, D., Arh. Hig. Rada Toksikol., 23(2), 153-5 (1972) (CA 79:944).
"Toxicology of bromotrifluoromethane"
46. Back, K. C. and E. W. Van Stee, Wright-Patterson Air Force Base, AGARD Rep. R-599 (CA 78:132355) (J-5248).
"Cardiovascular and nervous system effects of bromotrifluoromethane" A review.
47. Van Stee, E. W., Wright-Patterson AFB, U.S.N.T.I.S. Report No. AD-A011538 (1974) (CA 84:26504).
"Review of the toxicology of halogenated fire extinguishing agents"
48. Toy, P. A. et al., Tox. Appl. Pharmacol., 38(1), 7-17 (1976).
"The effects of three halogenated alkanes on excitation and contraction in the isolated, perfused rabbit heart"
49. Kappus, H., et al., Funct. Glutathione Liver Kidney, [Pap. Konf. Ges. Biol. Chem], 25th, 176-182 (1978) (CA 91:118086).
"Lipid peroxidation induced by ethanol and halogenated hydrocarbons in vivo as measured by ethane exhalation"
50. Karpov, B. D., Tr. Leningr. Sanit.-Gig. Med. Inst., 111, 10-14 (1975) (CA 89:79501).
"Materials for the toxicology of some bromofluorohydrocarbons of the methane and ethane series"
51. Back, K. C. and E. W. Van Stee, Int. Encycl. Pharmacol. Ther., Section 102 (Pharmacol. Methods Toxicol.), 103-113 (1979) (CA 92:70440) (J-).
"Various techniques for evaluating cardiodynamic function using chronically instrumented canine models"

RELATED REFERENCES (Cont'd.)

Industrial Hygiene

52. Bales, R. E., NIOSH Pub. No. 79-101 (1978) (CA 90:209347)
(J-).

"Fluorocarbons. An industrial hygiene survey of worker exposure in four facilities"

Metabolism/Biochemical

53. Wolf, C. R. et al., Mol. Pharmacol., 13(4), 698-705 (1977)
(CA 87:63685).

"The reduction of polyhalogenated methanes by liver microsomal cytochrome P450"

54. Van Stee, E. W. et al., Wright-Patterson AFB, U.S.N.T.I.S.
Report AD-751428 (1971) (CA 78:132421).

"Halogenated hydrocarbons and drug metabolism. Effect of fluorocarbons on hexobarbital sleeping and zoxazolamine paralysis times in mice"

55. Young, W. and J. A. Parker, Combust. Toxicol., 2(4), 286-97
(1975) (CA 84:100431).

"Effect of fluorocarbon on acetylcholinesterase activity and some counter measures"

Richard C. Graham:md
March 6, 1978

Updated:
Richard C. Graham:jrg
November 18, 1981

R. C. Graham

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August 26, 1994

Ms. Sineta Wooten
Document Control Officer
Environmental Protection Agency
Chemical Management Division
401 M Street, S.W.
Washington, D.C. 20460

Dear Ms. Wooten:

Enclosed are the materials you requested. The attached Statement of Work is considered by _____ to be proprietary information, including proprietary information which embodies trade secrets or commercial or financial information which is privileged or confidential under the Freedom of Information Act (5 USC 522 (B) (4)) and, consequently, the disclosure of which is prohibited by the provisions of section 14 of the Toxic Substances Control Act.

Sincerely,

64940000016

APPENDIX A

STATEMENT OF WORK

64940000016

TITLE:

TECHNOLOGY AREA:

BROOKHAVEN PI(S): DR. LEON PETRAKIS
 DEPARTMENT OF APPLIED SCIENCE
 TELEPHONE: (516) 282-3037
 FAX: (516) 282-4130
 EMAIL: petrakis@bnlnx1.bnl.gov

INDUSTRY PARTNER:

OBJECTIVE:

To develop a process for a chemical agent which in which the
 which the development of a process for application that a included is the
 in such a way

COMMERCIAL IMPACT:

is the single biggest expense
 accounting for approximately Therefore, a
 process which would result in would eliminate the
 and would have a tremendous cost advantage over traditional
 approaches.

Funds for

will come from

will come from the

\$ Thousand

FY94

FY95

FY96

Total:

Total
Detailed

budget attached.

INDUSTRY CONTRIBUTION:

It is anticipated that spending for this project will be
be an industry cost sharing of of the total project costs.

At this level, there will

\$ Thousand

FY94

FY95

FY96

Total

Total Funds-In: \$

Total In-Kind:

PROJECT DESCRIPTION:

Background

The is being conducted in the United States. A
cooperative R&D effort in which and
commercialization expertise at is combined with the analytical.
and and testing capabilities at Brookhaven
National Laboratory provides an opportunity to improve the In the

commercialization phase of the proposed program, both partners will be able to provide significant expertise regarding the regulatory and risk assessment issues that will arise.

Technical Approach

Phase I of the project will begin with a study. These will identify promising leading to The ability of to produce similar results and the mechanical, physical and chemical characteristics of the will be determined next. In order to assess the effectiveness of the in these studies, state-of-the-art analytical equipment and methods available at BNL will be used in a collaborative manner.

In Phase II of the project, will be used for studying the effectiveness of various combinations of while producing These studies will include tasks that require the acquisition of and chemical of the laboratory. The chemical will be acquired through BNL capabilities will be used to determine and information by and. These will complement test data acquired in the laboratories.

Data needed to for the on such as will be obtained in Phase III. Studies to quantify will be conducted. Based upon these results, an will be selected for use in small-scale tests. These will verify that obtained in Phase I and Phase II are reproducible, and will establish technical feasibility for a process.

The construction and testing of prototype and subsequently, engineering-scale, will take place in Phase IV. This phase will culminate in under conditions that simulate those at customer sites. Data needed to establish regulatory agency compliance as well as costs, will be derived from these activities which are intended to assess the commercial feasibility.

It is particularly significant for the proposed program that BNL possesses in a wide variety of, and therefore, has a technical and O&M staff fully trained with these materials. The BNL technical staff routinely perform and for analysis by.

projects could be safely performed at BNL on a variety of technology. monitored for technology and technology.

The following is a list of sample characterization tools at BNL that will be employed as needed in studying the

and to identify and to in the

As these techniques will be especially prominent in the program, a few added statements about them are in order here. is one of the most powerful characterization tools for the identification and quantification of of In order to facilitate a complete analysis, it is particularly important that sufficient achieved to minimize a from the different and

In addition, as high a level as possible is desirable, as this effectively sets a The best way to achieve both of these goals is to collect using a such as the at BNL. This technique will be utilized in the CRADA as one of the primary characterization tools the any intermediate products and the The information obtained from will be critical for understanding the and will be used in the development of better It will also help to quantify any and to This information is needed to ascertain whether the process has

Another set of important experiments will of the process. This will produce important which will be used to optimize the complimentary information is a related technique which is readily available at BNL. It provides and is particularly good for studying and for yielding a better quantification such as the samples.

including to look at and chemistry.

determine and to look at

changes in the chemistry or structure of the material.

to determine that the composition.

Milestones and Anticipated Results

A number of significant technical challenges will need to be overcome in the development of
The proposed new process contemplates the development

which
with and a
to should be
effective in the presence . Second a may be required
which can be maintain the and
during Finally, a delivery system will be developed which allows the
to be applied to in such a way that
The complete system and
must be safe and easy to use.

The most preferred process will result in a enough to
under Federal regulations, while retaining the performance
characteristics essential for continued service. An alternative process would
Federal standards but would the material
requiring the

Research conducted thus far by has found the following:

- literature, does occur in over a as described in the patent
addition of other components, The
their addition can have an important effect on the and the timing of
and the observed level of
- shown to readily , implying that the as a model system, has been
under continuous However, the
- substantially and reduced its with a In this experiment,
was maintained addition.

A research program aimed at developing the proposed process has been outlined and is described below. There are four research goals:

- (I) Development that will be effective with in the
- (II) Development of a the integrity
while allowing a high level achieved;

- (III) Development of _____ which will allow _____ and _____ to _____
- (IV) Scale-up of the complete system to full-scale laboratory tests.

Procedures for Interaction

Technical management for the overall project will be under the direction of _____ with BNL's efforts under the direction of its Principal Investigator. Both organizations will participate concurrently in each phase of the R&D effort. This will serve to increase _____ options considered, confirm the reproducibility of _____ and provide scientific, technological and commercial inputs into all aspects of the program.

Product evaluations up to full-scale will be performed jointly.

Efforts to obtain regulatory agency approvals for the developed processes and to determine the commercial viability of the processes will be managed by _____. BNL will provide advice and assistance in identifying the compliance requirements and in _____

Close communications between the two organizations will be maintained via telephone, e-mail, and fax messages. In addition, a monthly review meeting will be held at the site that is most appropriate for that month's agenda.

Reports

The parties will jointly prepare annual reports at the end of years one and two of the project and a final report at the conclusion of the project.

TASKS AND DELIVERABLES:

Goal A:

<u>Task No.</u>	<u>Due Date</u>	<u>Task Description</u>	<u>BNL</u>	<u> </u>
1	06/94	Develop a method for quantitative determination This work is essential for all phases of the work with , providing a means of measuring the extent It is anticipated that refinement of the analytical technique employed will be an ongoing effort and will involve both BNL and analytical resources.	X	X
2	07/94	Complete laboratory evaluation which have undergone limited commercial-scale testing, will be fully assessed. This will be done to determine the United states and to explore whether they might form the basis of an improved system. These tests will be conducted both		X
3	03/95	Determine whether development with This task involves the	X	X

Goal C:

<u>Task No.</u>	<u>Due Date</u>	<u>Task Description</u>	<u>BNL</u>	<u> </u>
1	07/94	Implement an accurate method	X	X
2	12/94	Determine the effect of existing application methods for	X	X
3	09/95	Investigate the use of which can allow the application	X	X

the need for is an important component of the improved economics of the proposed new process. An attempt will be made to develop a

The approach will be to first evaluate and then, if needed, evaluate other approaches. BNL expertise in will play a key role in this Task 3.

Goal D: Assess the commercial feasibility of the new process through large-scale testing.

<u>Task No.</u>	<u>Due Date</u>	<u>Task Description</u>	<u>BNL</u>	<u> </u>
1	03/96	Complete a controlled full-scale test of a commercially feasible system and	X	X

Once a viable bench-scale process has been demonstrated which meets the criteria set out above, a full-scale test will be conducted under controlled conditions to prove the technical performance of the process and allow a better estimation of the commercial feasibility for the large-scale testing

Detailed Description of Significant BNL Research Responsibilities Under Each Project Goal With Associated Costs

Goal A: Task 1

Perform physical and chemical analysis of samples supplied by appropriate analytical methods. The analysis will be

and identification of reaction products. The procedures

This is an ongoing task which will be repeated for each new chemical process introduced and tested first in the laboratories. It will be an interactive task inasmuch as the delivered analyses will help to determine the next steps at

Deliverable schedule: Development of a procedure

Analysis of samples supplied by (ongoing).

Cost:

Goal A: Task 4

Characterize reaction products from the most reaction paths. The techniques described above, and additional techniques, will be used to fully describe the chemical reactions and identify the chemical composition, reaction products, and identify the reaction products. . . . should all be determined for the to address regulatory issues.

Deliverable schedule: Report describing the reaction steps and the full characterization of all reaction products (6/95).

Cost:

Goal B: Task 1

Characterize of properties of

Use this information to

both with and without the addition to the

Deliverable schedule: Establish a technique for
with and without
(12/94).

Use the above technique to

Cost:

Goal E: Task 2

which will maintain the

The

may be organic or inorganic, and should be chosen

The effect of
performances will be assessed by This work will utilize the analytical techniques
developed Goal A, Task 1, as well developed
under Goal B, Task 1. measurements will follow procedures to be
developed by

Deliverable schedule:

which will maintain the
during treatment and
to take place (7/95).

Cost:

Goal C: Task 1

Develop test procedures for accurately determining

proposed must be usable for small scale
must be capable of being implemented by , even if
at BNL. Although a method which accurately determines
an improved technique which
preferred.

The test methods
The technique
must take place

Deliverable schedule: Develop a technique which can be
implemented by and BNL for small-scale testing (6/94).

Based on feedback from and BNL, develop an improved
(if needed) (1/95).

Cost:

Goal C: Task 3

Investigate the use

The optimum
be considered. Analytical techniques developed by BNL
in this work, along with developed methods for
at laboratory-scale The proposed system must be effective

Deliverable schedule: Develop a which will

Cost:

Goal D Task 1

In cooperation with , identify a
the laboratory. The , BNL, or some
other facility. Both and BNL will seek the necessary regulatory approvals

Techniques for
will be supplied by BNL. Procedures for
will be responsibility of

Deliverable schedule: In cooperation with complete a full-scale laboratory
evaluator

Cost:

BENEFITS TO BNL AND PARTNER:

BNL

- 1.
- 2.

-
1. Access to extensive BNL technical capabilities.

2. New commercial product with large national and international market potential

APPLICATION TO DOE/ER PROGRAMS:

The work performed in this program has a very high probability of stimulating new ideas in research will evolve that will be appropriate for submission to the for FY 96 funding. It is anticipated that proposals for nonproprietary

ENVIRONMENTAL, HEALTH & SAFETY ISSUES:

samples will be conducted following RNL's established safety and environmental protection procedures for such materials.

FYI

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August 31, 1994

Document Processing Center (TS-790)
Attn: Section 8(e) Coordinator
Office of Toxic Substances
Environmental Protection Agency
401 M Street S. W.
Washington, DC 20460

Dear Sir:

RE: FOR YOUR INFORMATION (FYI) UNDER TSCA SECTION 8(e)

We are submitting an FYI on a chemical substance Tri-*p*-tolylamine (synonym: 4-Methyl-N,N-bis(4-methylphenyl)benzenamine) CAS No. 1159-53-1 under Section 8(e) of the Toxic Substances Control Act.

The chemical structure of this compound is $(CH_3C_6H_5)_3N$. [] has purchased, for research and development purposes, approximately [] of this material since 1992 [] for use as an ingredient in a developmental [] product. A copy of the [] Material Safety Data Sheet is attached. []

[] obtained a test report "Final Report - Test for TPA Enrichment in Carp" (Final Report for Bioaccumulation Test of Tri-*p*-tolylamine in Fish, conducted at Kurume Research Laboratory, Japan), written in Japanese, [] on July 28, 1994. The report was immediately submitted for English translation and was completed on August 16, 1994. We received the translated report on August 18, 1994. The report indicates that the Bioconcentration Factors (BCF) in an 8-week fish bioaccumulation test were 3,650-13,900 and 6,440-17,800 at test concentrations 10 $\mu\text{g/L}$ and 1 $\mu\text{g/L}$, respectively. In the same report, it is indicated that the 48-hour LC_{50} in fish was >200 mg/L (non-toxic at this level). Based on TSCA Section 8(e), "Measurements and indicators of pronounced bioaccumulation heretofore unknown to the Administrator (including bioaccumulation in fish beyond 5,000 times water concentration in a 30-day exposure or having an *n*-octanol/water partition coefficient greater than 25,000) should be reported **when coupled with potential for widespread exposure and any non-trivial adverse effect.**" Although the BCFs during week 4 (approximately 30 days) of the test exceeded 5,000, there is no widespread exposure or any known non-trivial adverse effect with the intended use of this material. [] is reporting the environmental effect of this material as an FYI, since we do not believe that it represents any significant risk.

SANITIZED

[] has provided two test reports on tri-*p*-tolylamine: (1) Acute oral toxicity to the rat: The oral LD₅₀ is greater than 5.0 g/kg (practically non-toxic). (2) Four-week oral toxicity study in the rat with two-week recovery period: There were no changes in clinical signs, bodyweight gains, food consumption, clinical pathology, organ weight, and macroscopic/microscopic pathology, that were considered to be related to treatment of the test material at up to 1,000 mg/kg/day. It was concluded that 1,000 mg/kg/day represents the no-observed effect level. In addition, [] has conducted two genetic toxicology assays (Ames test and in vitro chromosomal aberration assay in Chinese hamster ovary cells) on tri-*p*-tolylamine. Both assays gave a negative result (we have not obtained the test reports).

Based on the above information, we believe that there is no significant risk to human health or the environment, relative to our intended use of tri-*p*-tolylamine. Therefore, we are reporting the result of the bioaccumulation studies as an FYI.

Documents Attached:

1. Final Report - Test for TPA Enrichment in Carp (in Japanese)
2. Final Report - Test for TPA Enrichment in Carp" (Final Report for Bioaccumulation Test of Tri-*p*-tolylamine in Fish) (translated in English).
3. Acute oral toxicity to the rat
4. Four-week oral toxicity study in the rat with two-week recovery period
5. Material Safety Data Sheet of Tri-*p*-tolylamine

Any questions regarding this submission should be addressed to the undersigned at [].

Sincerely yours,

[
Manager
]

Attachments

Attachment 1
SANITIZED VERSION

受理番号	S92-2274
試験番号	42274

最終報告書

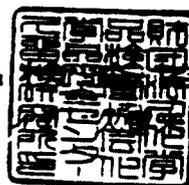
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TPAのコイにおける濃縮度試験

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平成 5年 3月23日

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研究所

陳 述 書

財団法人 化学品検査協会
化学品安全センター久留米研究所

試験委託者 []

試験の表題 TPAのコイにおける濃縮度試験

試験番号 42274

上記試験は、「新規化学物質に係る試験及び指定化学物質に係る有害性の調査の項目等を定める命令第4条に規定する試験施設について」（環保業第39号、薬発第229号、59基局第85号、昭和59年3月31日、昭和63年11月18日改正）に定める「新規化学物質に係る試験及び指定化学物質に係る有害性の調査の項目等を定める命令第4条に規定する試験施設に関する基準」及び「OECD Principles of Good Laboratory Practice」(May 12, 1981)に従って実施したものです。

平成 5年 3月 23日

運営管理者 勝浦 洋 

信頼性保証書

財団法人 化学品検査協会
化学品安全センター久留米研究所

試験委託者 []

試験の表題 TPAのコイにおける濃縮度試験

試験番号 42274

上記試験は財団法人化学品検査協会化学品安全センター久留米研究所の信頼性保証部門が監査及び査察を実施しており、監査又は査察を行った日付並びに運営管理者及び試験責任者に報告を行った日付は以下の通りです。

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平成 5年 3月23日	平成 5年 3月23日	平成 5年 3月23日

本最終報告書は、試験の方法が正確に記載されており、内容が試験計画及び標準操作手順に従い、かつ、生データを正確に反映していることを保証します。

平成 5年 3月23日

信頼性保証業務担当者

岡 敏 雄 

平成 5年 3月23日

信頼性保証部門責任者

内 島 達 

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1. 試験の表題

TPAのコイにおける濃縮度試験

2. 試験条件

2.1 急性毒性試験

- | | |
|-----------|------------------|
| (1) 供試魚 | ヒメダカ |
| (2) ばく露期間 | 48時間 |
| (3) ばく露方法 | 半止水式(8~16時間毎に換水) |

2.2 濃縮度試験

- | | |
|-----------|-------------------------------|
| (1) 供試魚 | コイ |
| (2) 試験濃度 | 第1濃度区 10 µg/l
第2濃度区 1 µg/l |
| (3) ばく露期間 | 8週間 |
| (4) ばく露方法 | 連続流水式 |
| (5) 分析方法 | 高速液体クロマトグラフィー |

3. 試験結果

- | | |
|--------------------|--|
| (1) 被験物質の48時間LC50値 | 200 mg/l以上 |
| (2) 濃縮倍率 | 第1濃度区 3650~13900倍
第2濃度区 6440~17800倍 |

4. 被験物質の安定性

被験物質は保管条件下及び試験条件下で安定であることを確認した。

最終報告書

試験番号 42274

1. 表 題 T P A のコイにおける濃縮度試験
2. 試験委託者 名称 []
住所 []
3. 試験施設 名称 財団法人 化学品検査協会
化学品安全センター久留米研究所
住所 (〒830) 福岡県久留米市中央町19-14
TEL (0942) 34-1500
運営管理者 勝 浦 洋
4. 試験目的 T P A のコイにおける濃縮性の程度について知見を得る。
5. 試験方法 「新規化学物質に係る試験の方法について」(環保業第5号、薬発第615号、49基局第392号、昭和49年7月13日)に規定する〈魚介類の体内における化学物質の濃縮度試験〉及び「OECD Guidelines for Testing of Chemicals」(May 12, 1981)に定める'305C. Bioaccumulation : Degree of Bioconcentration in Fish'に準拠した。
6. 優良試験所
基準への適合 「新規化学物質に係る試験及び指定化学物質に係る有害性の調査の項目等を定める命令第4条に規定する試験施設について」(環保業第39号、薬発第229号、59基局第85号、昭和59年3月31日、昭和63年11月18日改正)に定める「新規化学物質に係る試験及び指定化学物質に係る有害性の調査の項目等を定める命令第4条に規定する試験施設に関する基準」(以下「GLP基準」という。)及び「OECD Principles of Good Laboratory Practice」(May 12, 1981)に適合して行った。

7. 試験期間

- (1) 試験開始日 平成 5年 1月 8日
(2) ばく露開始日 平成 5年 1月12日
(3) ばく露終了日 平成 5年 3月 9日
(4) 試験終了日 平成 5年 3月23日

8. 試験関係者

試験責任者	<u>藤本一馬</u>
試験担当者	<u>石坂千恵</u>
飼育管理責任者	<u>川島康郎</u>
急性毒性試験担当者	<u>川島康郎</u>
試験資料管理部門責任者	<u>高橋明</u>

9. 最終報告書作成日

平成 5年 3月23日
作成者 石坂千恵

10. 最終報告書の承認

平成 5年 3月23日
試験責任者 氏名 藤本一馬 

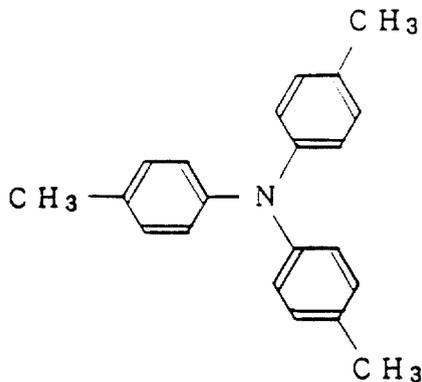
11. 被験物質

本報告書においてTPAは、次の名称及び構造式等を有するものとする。

11.1 名称^{*1} Tri (4-methyl phenyl) amine

11.2 構造式等^{*1}

構造式



分子式 $C_{21}H_{21}N$

分子量 287.40

11.3 純度^{*1} 99.9%以上

11.4 提供者及びロット番号^{*1}

(1) 提供者 []

(2) ロット番号 920602

*1 試験委託者提供資料による。

11.5 被験物質の確認

試験委託者提供の赤外吸収スペクトルと当研究所の当該測定スペクトルとが一致することを確認した（図-15, 参考資料2参照）。

11.6 物理化学的性状

外 観	無色結晶	
融 点 ^{*1}	116℃	
溶解性	水	10mg/ℓ以下
	ヘキサン	61g/ℓ
	クロロホルム	100g/ℓ以上（目視）
	酢酸エチル	100g/ℓ以上（目視）
	メタノール	490mg/ℓ

*1 試験委託者提供資料による。

11.7 保管条件及び保管条件下での安定性

- (1) 保管条件 冷暗所
- (2) 安定性確認 ばく露開始前及び終了後に被験物質の赤外吸収スペクトルを測定した結果（図-15参照）、両スペクトルは一致し、保管条件下で安定であることを確認した。

11.8 試験条件下での安定性

ばく露開始前に予備検討を行い、試験条件下で安定であることを確認した。

12. 急性毒性試験

12.1 試験方法

「工場排水試験方法，魚類による急性毒試験」(JIS K 0102-1986 の 71.) の方法に準じて行った。

12.2 供試魚

- | | |
|-----------------|--|
| (1) 魚 種 | ヒメダカ <u>Oryzias latipes</u> |
| (2) 供給源 | 中島養魚場
(住所 〒 869-01 熊本県玉名郡長洲町大明神) |
| (3) 蕃養条件
期間等 | 魚の入手時に目視観察をして異状のあるものを除去し、蕃養槽で薬浴後、流水状態で27日間飼育した。 |
| 薬浴 | 20 mg/l エルバージュ (上野製薬製) 溶液及び 7 g/l 塩化ナトリウム溶液を用いて止水状態で24時間薬浴を行った。 |
| (4) じゅん化条件 | じゅん化槽でじゅん化し、その間異状のあるものは除去し、最終的には 25 ± 2℃ の水温の流水状態で56日間飼育した。 |
| (5) 体 重 | 平均 0.298 |
| (6) 全 長 | 平均 3.2 cm |
| (7) 検 定 | 田端健二 ^{*2} の方法に準じ、塩化第二水銀検定合格魚と同一ロット (TFO-921119) のものを試験に供した。 |

*2 用水と廃水, 14.1297-1303 (1972)

12.3 試験用水

(1) 種類

久留米研究所敷地内で揚水した地下水

(2) 分析及び水質確認

当研究所にて水温、pH及び溶存酸素は連続測定を行った。また、全硬度、蒸発残留物、化学的酸素要求量、遊離塩素及びアンモニア態窒素並びに有機リン、シアンイオン、重金属等の有害物質は6ヶ月に1回定期的に分析した。試験用水を試験に供する場合、分析した項目が全硬度、蒸発残留物については「水道法に基づく水質基準」(昭和53年8月31日厚生省令第56号)、その他のものについては「水産用水基準」(社団法人日本水産資源保護協会 昭和58年3月)に記載されている濃度以下であることを確認した(参考資料1参照)。

12.4 試験条件

(1) 試験水槽	円型ガラス製水槽
(2) 試験液量	4ℓ / 濃度区
(3) 試験水温	25 ± 2℃
(4) 溶存酸素濃度	ばく露開始時 7.8 mg/ℓ ばく露終了時 6.1 mg/ℓ
(5) pH	ばく露開始時 7.6 ばく露終了時 7.5
(6) 供試魚数	10尾 / 濃度区
(7) ばく露期間	48時間
(8) ばく露方法	半止水式(8~16時間毎に換水)
(9) 照明	試験期間中、遮光下にて試験を行った。

12.5 原液調製法

(1) 分散剤

HCO-40

(2) 調製方法

被験物質をアセトンに超音波照射しながら溶解させ、これに50倍量のHCO-40を加えて良く攪拌し、アセトンを留去した後、イオン交換水に溶解させて1000mg/lの原液を調製した。

12.6 試験の実施

(1) 実施場所 115LC50室

(2) 試験実施日 平成5年1月18日～平成5年1月20日

12.7 48時間LC50値の算出

Doudoroff法で行った。

12.8 試験結果

被験物質の48時間LC50値 200mg/l以上 (図-3参照)

13. 濃縮度試験の実施、

13.1 供試魚

- | | | |
|---------------------------------|----|---|
| (1) 魚 | 種 | コイ <u>Cyprinus carpio</u> |
| (2) 供給源 | | 杉島養魚場
(住所 〒 866 熊本県八代市郡築一番町 123-2)
供試魚受入日 平成 4年11月13日 |
| (3) 蕃養条件 | | |
| 期間等 | | 魚の入手時に目視観察をして異状のあるものを除去し、
受入槽で薬浴後、流水状態で4日間飼育した。 |
| 薬浴 | | 50 mg/l水産用テラマイシン散(台糖ファイザー製)
溶液及び7 g/l塩化ナトリウム溶液を用いて止水状態で
24時間薬浴を行った。 |
| (4) じゅん化条件 | | じゅん化槽でじゅん化し、その間異状のあるものは除去
し、最終的には $25 \pm 2^\circ\text{C}$ の水温の流水状態で29日間
飼育した。さらに試験水槽へ移し、同温度の流水状態で
7日間飼育した。
じゅん化終了日 平成 4年12月22日 |
| (5) ばく露開始時の体重、体長等 ^{*3} | | |
| 体 重 | 平均 | 22.8 g |
| 体 長 | 平均 | 9.6 cm |
| 脂質含有率 | 平均 | 3.7 % |
| | | *3 ロット(TFC-921113-I)の測定値 |
| (6) 餌 | 料 | |
| 種 類 | | コイ用ペレット状配合飼料 |
| 製 造 元 | | 日本配合飼料株式会社 |
| 給餌方法 | | 供試魚体重の約2%相当量を1日2回に分けて給餌した。
ただし、供試魚の採取前日は給餌を止めた。 |

13.2 試験用水

12.3に同じ。

13.3 試験及び環境条件

- | | | |
|-------------|---|----------------------|
| (1) 試験水供給方法 | 当研究所組立流水式装置を用いた。 | |
| (2) 試験水槽 | 100ℓ容ガラス製水槽 | |
| (3) 試験水量 | 原液4ml/分及び試験用水800ml/分の割合で
1158ℓ/日を試験水槽に供した。 | |
| (4) 試験温度 | 25±2℃ | |
| (5) 溶存酸素濃度 | 第1濃度区 | 7.0～8.0mg/ℓ (図-11参照) |
| | 第2濃度区 | 6.9～7.8mg/ℓ (図-12参照) |
| | 対照区 | 7.5～8.2mg/ℓ (図-13参照) |
| (6) 供試魚数 | 第1及び第2濃度区 | 19尾 (ばく露開始時) |
| | 対照区 | 5尾 (ばく露開始時) |
| (7) ばく露期間 | 8週間 | |
| (8) 実施場所 | 213アクアトロン室 | |

13.4 原液調製法

(1) 分散剤

12.5の(1)に同じ。

(2) 調製方法

・第1濃度区

12.5の(2)と同様の方法で2mg/ℓの原液を調製した。

・第2濃度区

12.5の(2)と同様の方法で0.2mg/ℓの原液を調製した。

・対照区

HCO-40を100mg/ℓとなるようにイオン交換水に溶解させて、
原液を調製した。

以上を25ℓ容のガラス製原液タンクより試験水槽に供給した。

13.5 試験濃度

48時間LC50予備値及び被験物質の分析感度を考慮して、

第1濃度区 10μg/ℓ

第2濃度区 1μg/ℓ

に被験物質濃度を設定した。同時に、空試験として対照区を設定した。

13.6 試験水及び供試魚分析

13.6.1 分析回数

試験水分析は第1、第2濃度区ともばく露期間中、毎週2回計16回行い、1回当りの分析試料は1点とした。また、供試魚分析は第1、第2濃度区ともばく露開始後、2、4、6及び8週の計4回行い、1回当りの分析試料は2尾とした。対照区はばく露開始前及びばく露終了時に行い、1回当りの分析試料は2尾とした。

13.6.2 分析試料の前処理

(1) 試験水

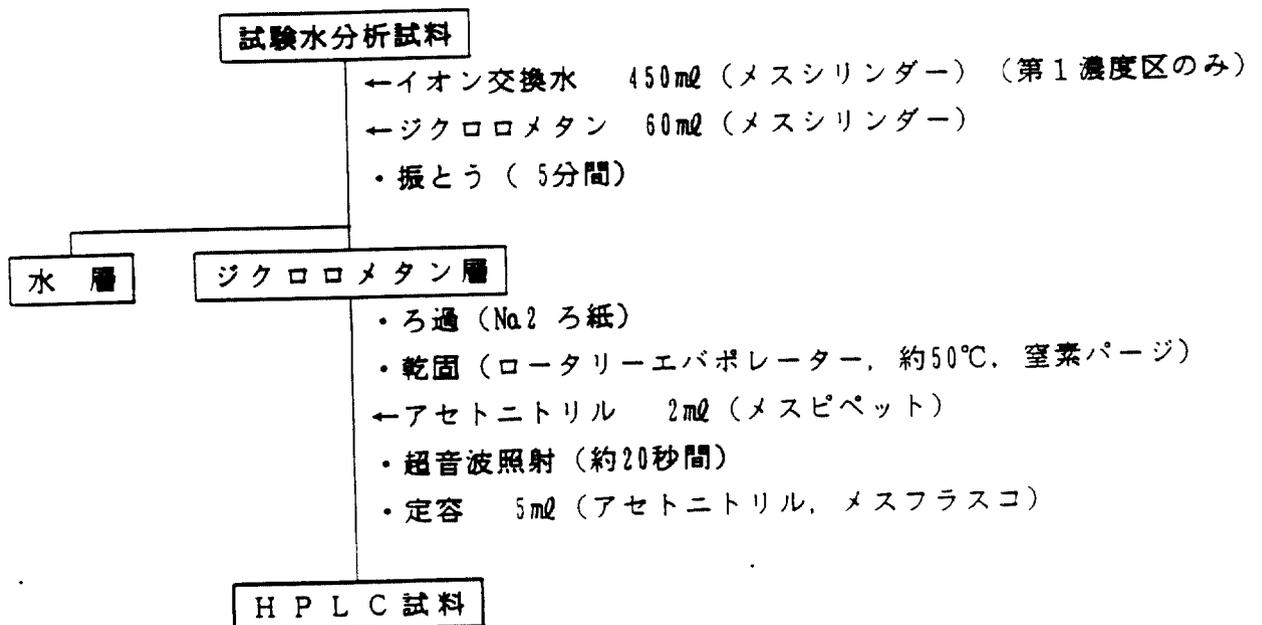
試験水槽から

第1濃度区 50ml

第2濃度区 500ml

を採取し、以下のフロースキームに従って前処理操作を行い、高速液体クロマトグラフィー（HPLC）試料とした。

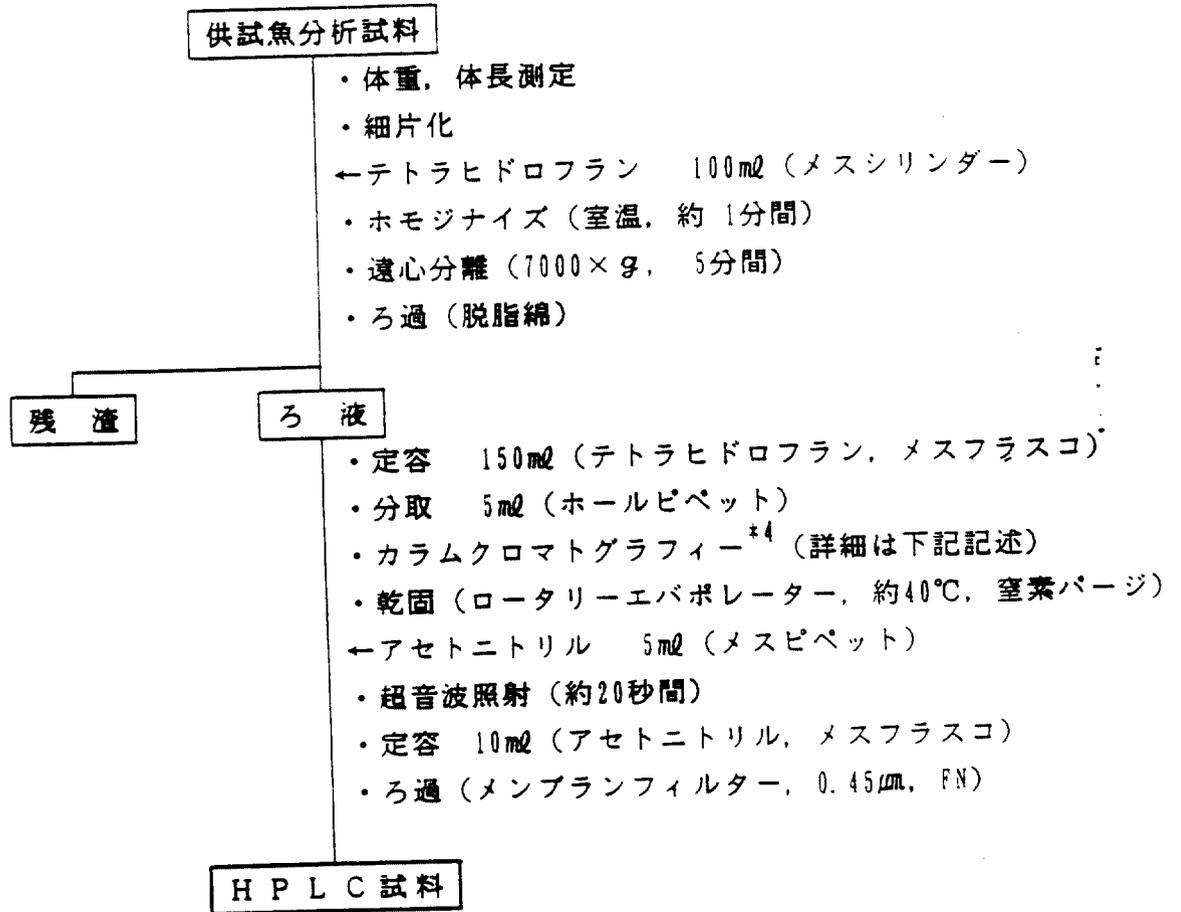
フロースキーム



(2) 供試魚

試験水槽から供試魚を採取し、以下のフロースキームに従って前処理操作を行い、HPLC試料とした。

フロースキーム



*4 カラムクロマトグラフの条件

セップバック 塩基性アルミナ

負荷法 試料液全量を負荷した。

溶出法 第1溶出液 テトラヒドロフラン 5ml

被験物質は負荷分及び第1溶出液で溶出した。

13.6.3 定量分析

13.6.2の前処理を行って得られたHPLC試料は、下記の定量条件に基づき高速液体クロマトグラフィーにより定量を行った。供試魚分析の定量はHPLC試料を適宜希釈し、直線性の確認された濃度範囲になるように被験物質濃度を調整した。最終定容液中の被験物質濃度は、クロマトグラム上の被験物質のピーク面積を濃度既知の標準溶液のピーク面積と比較し、比例計算して求めた(表-4, 5, 図-6, 表-7, 8, 9, 図-8, 9, 10参照)。

(1) 定量条件

機 器	高速液体クロマトグラフ
ポンプ	島津製作所製 LC-6A
検出器	島津製作所製 SPD-6AV
カラム	L-column ODS 15cm×4.6mmφ ステンレス製
溶 離 液	アセトニトリル
流 量	1.0ml/min
測定波長	300nm (図-14参照)
注 入 量	30μl
感 度	
検出器	0.005ABU/FS
記録計	レンジ 1.2mV

(2) 標準溶液の調製

分析試料中の被験物質濃度を求めるための標準溶液の調製は次のように行った。

被験物質0.1gを精密にはかりとり、テトラヒドロフランに溶解して1000mg/lの標準原液を調製した。これをアセトニトリルで希釈して0.1mg/lの標準溶液とした。

(3) 検量線の作成

(2)の標準溶液調製法と同様にして0.05、0.1及び0.2mg/lの標準溶液を調製した。これらを(1)の定量条件に従って分析し、得られたそれぞれのクロマトグラム上のピーク面積と濃度により検量線を作成した。

検量線より被験物質ピーク面積の検出下限は、ノイズレベルを考慮して150W・sec(被験物質濃度4.9μg/l)とした(図-4参照)。

13.6.4 回収試験及びブランク試験

(1) 方法

前述した試験水及び供試魚分析操作における被験物質の回収率を求めるため、回収試験用試験水及び魚体ホモジネートに被験物質分散液を添加し、13.6.2及び13.6.3の操作に準じて回収試験を行った。また、被験物質を加えない回収試験用試験水及び魚体ホモジネートについて、回収試験の場合と同じ操作によりブランク試験を行った。回収試験及びブランク試験は、2点について測定した。この結果、ブランク試験においてクロマトグラム上、被験物質ピーク位置にはピークは認められなかった。分析操作における各2点の回収率及び平均回収率は下記に示すとおりであり、平均回収率を分析試料中の被験物質濃度を求める場合の補正值とした（表-3, 6、図-5, 7参照）。

(2) 結果

分析操作における回収率

試験水分析（被験物質0.5 µg添加）

91.3%, 93.4% 平均92.3%

供試魚分析（被験物質30 µg添加）

94.0%, 92.4% 平均93.2%

13.6.5 分析試料中の被験物質濃度の算出及び定量下限

(1) 試験水分析試料中の被験物質濃度の算出

表-4, 5の計算式に従って計算し、計算結果は有効数字3ケタに丸めて表示した。

(2) 試験水中の被験物質の定量下限濃度

13.6.3 (3)の検量線作成で求めた被験物質の検出下限より、試験水中の被験物質の定量下限濃度^{*5}はそれぞれ、

第1濃度区 0.53 µg/l

第2濃度区 0.053 µg/l

と算出される。

(3) 供試魚分析試料中の被験物質濃度の算出

表-7, 8, 9の計算式に従って計算し、計算結果は有効数字3ケタに丸めて表示した。

(4) 供試魚中の被験物質の定量下限濃度

13.6.3 (3)の検量線作成で求めた被験物質の検出下限より、供試魚中の被験物質の定量下限濃度^{*5}は供試魚体重を30gとしたとき53ng/gと算出される。

$$*5 \text{ 被験物質定量下限濃度 } (\mu\text{g}/\text{l} \text{ 又は } \text{ng}/\text{g}) = \frac{A}{\frac{B}{100} \times \frac{C \times E}{D}}$$

A : 検量線上検出下限濃度 ($\mu\text{g}/\text{l}$)

B : 回収率 (%)

C : 試験水採取量 (ml) 又は 供試魚体重 (g)

D : 最終液量 (ml)

E : 分取比

計算結果は有効数字2ケタに丸めた。

13.7 濃縮倍率 (BCF) の算出

表-7, 8, 9の計算式に従って計算し、計算結果は有効数字3ケタに丸めて表示した。

なお、13.6.5 (4)で求めた供試魚中の被験物質定量下限濃度より、下記の倍率を越えて濃縮されたとき濃縮倍率の算出が可能となる。

第1濃度区 6.0倍

第2濃度区 65倍

13.8 数値の取扱い

数値の丸め方は、JIS Z 8202-1985 参考3 規則Bの方法に従った。

14. 試験結果

14.1 試験水中の被験物質濃度

試験水中の被験物質濃度を表-1に示す。

表-1 試験水中の被験物質濃度 (ばく露開始時からの測定値の平均値)
(単位 $\mu\text{g}/\text{L}$)

	2 週	4 週	6 週	8 週	付 表	付 図
第1濃度区	8.21	8.39	8.69	8.80	表-4	図-6
第2濃度区	0.781	0.779	0.796	0.810	表-5	

14.2 濃縮倍率

濃縮倍率を表-2に示す。

表-2 濃 縮 倍 率

	2 週	4 週	6 週	8 週	付 表	付 図
第1濃度区	3650	4910	10100	10800	表-7	図-8
	4290	6830	9820	13900		
第2濃度区	7450	12900	15100	17800	表-8	図-9
	6440	11300	17700	13400		

表-2の濃縮倍率とばく露期間との相関を図-1及び図-2に示した。
また、被験物質のコイに対する濃縮性の程度は、濃縮倍率で第1濃度区において3650~13900倍、第2濃度区において6440~17800倍であった。

供試魚は外観観察等の結果、異常は認められなかった。

また、試験水中の平均被験物質濃度は表-1に示されるように、設定値の約80%程度が保持された。

15. 試資料の保管

15.1 被験物質

保管用被験物質約20gを保管用容器に入れ密栓後、「GLP基準」第32条に定める期間、当研究所試料保管室に保管する。

15.2 生データ、資料等

試験により得られた分析結果、測定結果、観察結果、その他試験ノート等最終報告書の作成に用いた生データ、試験計画書、依頼書、調査表、資料等は最終報告書と共に、「GLP基準」第32条に定める期間、当研究所資料保管室に保管する。

16. 備考

16.1 試験に使用した主要な装置・機器、特殊器具、試薬等

(1) 試験系（飼育施設）に係わる装置

原液供給用微量定量ポンプ : 東京理化学器械製 型 GMW
溶存酸素測定装置 : 飯島精密工業製 型 552

(2) 分析及び原液調製に使用した装置・機器、特殊器具、試薬

装置・機器

高速液体クロマトグラフ : 13頁参照
ロータリーエバポレーター : 東京理化学器械製 型 N-1
振とう機 : 入江商会製 TS式
大洋科学工業製 型 SR-IIW
ホモジナイザー : キネマチカ社製
遠心分離機 : 日立製作所製 型 20PR-52

特殊器具

セップバック 塩基性アルミナ : 日本ミリポア・リミテッド製
メンブランフィルター : 野村マイクロサイエンス製
型 FN

試薬

ジクロロメタン : キシダ化学製 試薬一級
アセトニトリル : 和光純薬工業製 HPLC用
テトラヒドロフラン : ナカライテスク製 試薬一級
アセトン : 和光純薬工業製 試薬一級
HCO-40 : 日光ケミカルズ製

17. 表及び図の内容

表の内容

表-1	試験水中の被験物質濃度（測定値） [本文中記載]
表-2	濃縮倍率 [本文中記載]
表-3	回収試験及びブランク試験（試験水分析）計算表
表-4	第1濃度区試験水分析計算表
表-5	第2濃度区試験水分析計算表
表-6	回収試験及びブランク試験（供試魚分析）計算表
表-7	第1濃度区供試魚分析計算表
表-8	第2濃度区供試魚分析計算表
表-9	対照区供試魚分析計算表
参考資料1	試験用水の水質測定表

図の内容

- 図-1 ばく露期間-濃縮倍率相関図 (第1濃度区)
 - 図-2 ばく露期間-濃縮倍率相関図 (第2濃度区)
 - 図-3 急性毒性試験における被験物質濃度-死亡率曲線
 - 図-4 検量線用HPLCチャート及び検量線
 - 図-5 回収試験及びブランク試験 (試験水分析) HPLCチャート
 - 図-6 試験水分析HPLCチャート
 - 図-7 回収試験及びブランク試験 (供試魚分析) HPLCチャート
 - 図-8 第1濃度区供試魚分析HPLCチャート
 - 図-9 第2濃度区供試魚分析HPLCチャート
 - 図-10 対照区供試魚分析HPLCチャート
 - 図-11 第1濃度区試験水中の溶存酸素濃度
 - 図-12 第2濃度区試験水中の溶存酸素濃度
 - 図-13 対照区試験水中の溶存酸素濃度
 - 図-14 被験物質の紫外吸収スペクトル
 - 図-15-1 ばく露開始前の被験物質の赤外吸収スペクトル
 - 図-15-2 ばく露終了後の被験物質の赤外吸収スペクトル
- 参考資料2 試験委託者提供の赤外吸収スペクトル

Table-4-1

Calculation table for analysis of test water
(Level 1)

		Test No.	42274
Sample description	A	I	J
Standard 0.1mg/L	2754		
Test water at 2nd day	2176	8.56	
Standard 0.1mg/L	2784		
Test water at 7th day	2066	8.04	
Standard 0.1mg/L	2733		
Test water at 9th day	2046	8.11	
Standard 0.1mg/L	2623		
Test water at 11th day	1975	8.15	8.21
Standard 0.1mg/L	2721		
Test water at 15th day	2013	8.01	
Standard 0.1mg/L	2774		
Test water at 18th day	2118	8.27	
Standard 0.1mg/L	2790		
Test water at 22nd day	2403	9.33	
Standard 0.1mg/L	2764		
Test water at 25th day	2214	8.67	8.39

A : Peak area ($\mu\text{V}\cdot\text{sec}$)	
A(std):Standard solution	A(t):Sample
B : Ratio of portion used for analysis	1
C : Final volume	5mL
F : Recovery rate	92.3%
H : Volume of test water taken out	50ml
I : Concentration of test substance in test water ($\mu\text{g/L}$)	
$I = P \times A(t) / A(\text{std}) / B \times C / F \times 100 / H$	
J : Average concentration of test substance in test water ($\mu\text{g/L}$)	
$J = \{I(1) \dots I(n)\} / n$	
n: Number of test water analysis	
I(1): First analysis of test water	I(n): Last analysis of test water
P : Concentration of standard solution	0.1mg/L

Table-3

**Calculation table for recovery and blank test
(Analysis of test water)**

Test No. 42274

Sample description	A	B	C	D	E	F
Standard 0.1mg/L	2861					
Recovery a	2612	1	5	-	0.456	91.3%
Recovery b	2672	1	5	-	0.467	93.4%
						Average
						92.3%
Standard 0.1mg/L	2944					
Blank a	n.d.	1	5	-	-	
Blank b	n.d.	1	5	-	-	

a,b : individual sample

A : Peak area ($\mu\text{V}\cdot\text{sec}$)

B : Ratio of portion used for analysis

C : Final volume (mL)

D : Amount of blank in test water (μg)

E : Amount of test substance recovered (μg)

$$E = P \times A(t) / A(\text{std}) \times 1 / B \times C - D$$

F : Recovery rate (%)

$$F = E / Q \times 100$$

P : Concentration of test substance in standard solution (mg/L)

Q : Amount of test substance added ($0.5\mu\text{g}$)

January 8, 1993

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Table-4-2

Calculation table for analysis of test water
(Level 1)

Test No. 42274

Sample description	A	I	J
Standard 0.1mg/L	2857		
Test water at 29th day	2524	9.57	
Standard 0.1mg/L	2739		
Test water at 32nd day	2488	9.84	
Standard 0.1mg/L	2776		
Test water at 36th day	2188	8.54	
Standard 0.1mg/L	2659		
Test water at 39th day	2272	9.25	8.69
Standard 0.1mg/L	2829		
Test water at 43rd day	2454	9.39	
Standard 0.1mg/L	2925		
Test water at 46th day	2645	9.79	
Standard 0.1mg/L	2981		
Test water at 50th day	2496	9.07	
Standard 0.1mg/L	2801		
Test water at 53rd day	2120	8.20	8.80

A : Peak area ($\mu\text{V}\cdot\text{sec}$)	
A(std):Standard solution	A(t):Sample
B: Ratio of portion used for analysis	1
C: Final volume	5mL
F: Recovery rate	92.3%
H: Volume of test water taken out	50ml
I : Concentration of test substance in test water ($\mu\text{g/L}$)	
$I = P \times A(t) / A(\text{std}) / B \times C / F \times 100 / H$	
J : Average concentration of test substance in test water ($\mu\text{g/L}$)	
$J = \{I(1) \dots I(n)\} / n$	
n: Number of test water analysis	
I(1): First analysis of test water	I(n): Last analysis of test water
P : Concentration of standard solution	0.1mg/L

March 10, 1993

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Table-5-1

Calculation table for analysis of test water
(Level 2)

Sample description	Test No. 42274		
	A	I	J
Standard 0.1mg/L	2754		
Test water at 2nd day	1940	0.763	
Standard 0.1mg/L	2784		
Test water at 7th day	2128	0.828	
Standard 0.1mg/L	2733		
Test water at 9th day	1796	0.712	
Standard 0.1mg/L	2623		
Test water at 11th day	1991	0.822	0.781
Standard 0.1mg/L	2721		
Test water at 15th day	1942	0.773	
Standard 0.1mg/L	2774		
Test water at 18th day	1866	0.728	
Standard 0.1mg/L	2790		
Test water at 22nd day	2070	0.803	
Standard 0.1mg/L	2764		
Test water at 25th day	2048	0.802	0.779

A : Peak area ($\mu\text{V}\cdot\text{sec}$)	
A(std):Standard solution	A(t):Sample
B: Ratio of portion used for analysis	1
C: Final volume	5mL
F: Recovery rate	92.3%
H: Volume of test water taken out	500ml
I: Concentration of test substance in test water ($\mu\text{g/L}$)	
$I = P \times A(t) / A(\text{std}) / B \times C / F \times 100 / H$	
J: Average concentration of test substance in test water ($\mu\text{g/L}$)	
$J = \{I(1) \dots I(n)\} / n$	
n: Number of test water analysis	
I(1): First analysis of test water	I(n): Last analysis of test water
P: Concentration of standard solution	0.1mg/L

Table-5-2

Calculation table for analysis of test water
(Level 2)

Sample description	Test No. 42274		
	A	I	J
Standard 0.1mg/L	2857		
Test water at 29th day	2605	0.987	
Standard 0.1mg/L	2739		
Test water at 32nd day	1703	0.673	
Standard 0.1mg/L	2776		
Test water at 36th day	2117	0.826	
Standard 0.1mg/L	2659		
Test water at 39th day	2036	0.829	0.796
Standard 0.1mg/L	2829		
Test water at 43rd day	2332	0.893	
Standard 0.1mg/L	2925		
Test water at 46th day	2259	0.836	
Standard 0.1mg/L	2981		
Test water at 50th day	2526	0.918	
Standard 0.1mg/L	2801		
Test water at 53rd day	1972	0.762	0.810

A : Peak area ($\mu\text{V}\cdot\text{sec}$)	
A(std):Standard solution	A(t):Sample
B: Ratio of portion used for analysis	1
C: Final volume	5mL
F: Recovery rate	92.3%
H: Volume of test water taken out	500ml
I : Concentration of test substance in test water ($\mu\text{g/L}$)	
$I = P \times A(t) / A(\text{std}) / B \times C / F \times 100 / H$	
J : Average concentration of test substance in test water ($\mu\text{g/L}$)	
$J = \{I(1) \dots I(n)\} / n$	
n: Number of test water analysis	
I(1): First analysis of test water	I(n): Last analysis of test water
P : Concentration of standard solution	0.1mg/L

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Table-6

**Calculation table for recovery and blank test
(Analysis of test fish)**

Sample description	A	B	C	D	E	G
Standard 0.1mg/L	2682					
Recovery a	2520	5/150	10	-	28.2	94.0%
Recovery b	2479	5/150	10	-	27.7	92.4%
						Average
						93.2%
Standard 0.1mg/L	2697					:
Blank a	n.d.	5/150	10	-	-	:
Blank b	n.d.	5/150	10	-	-	:

a,b ; individual sample

A : Peak area ($\mu\text{V}\cdot\text{sec}$)

B : Ratio of portion used for analysis

C : Final volume (mL)

D : Amount of blank in test fish (μg)

E : Amount of test substance recovered (μg)

$$E = P \times A(t) / A(\text{std}) \times 1 / B \times C - D$$

F : Recovery rate (%)

$$F = E / Q \times 100$$

P : Concentration of test substance in standard solution (mg/L)

Q : Amount of test substance added ($30\mu\text{g}$)

January 8, 1993

Name C Ishizaka

Table-7

Calculation table for analysis of test fish

(Level 1)

Sample description	A	D	G	K	H	J
Standard 0.1mg/L	2877					
Test fish after 2 weeks a	1727	50	32.2	30.0	8.21	3650
Test fish after 2 weeks b	1989	50	31.6	35.2	8.21	4290
Standard 0.1mg/L	2848					
Test fish after 4 weeks a	2027	50	27.8	41.2	8.39	4910
Test fish after 4 weeks b	2961	50	29.2	57.3	8.39	6830
Standard 0.1mg/L	2740					
Test fish after 6 weeks a	2576	100	34.3	88.2	8.69	10100
Test fish after 6 weeks b	2339	100	32.2	85.3	8.69	9820
Standard 0.1mg/L	2904					
Test fish after 8 weeks a	2651	100	30.9	95.1	8.80	10800
Test fish after 8 weeks b	3502	100	31.8	122	8.80	13900

A : Peak area ($\mu\text{V}\cdot\text{sec}$)	
A(std):Standard solution	A(t):Sample
B : Ratio of portion used for analysis	5 / 150
C : Final volume	10mL
D : Dilution factor	
E : Average concentration of blank in analysis of control	
F : Recovery rate	93.2%
G : Weight of test fish (g)	
K : Concentration of test substance in test fish ($\mu\text{g/g}$)	
$K = (P \times A(t) / A(\text{std}) / B \times D \times C / G - E) / F \times 100$	
H : Average concentration of test substance in test water ($\mu\text{g/L}$)	
J : BCF	
$J = (P \times A(t) / A(\text{std}) / B \times D \times C / G - E) / F \times 100 / H$	
P : Concentration of test substance in standard solution	0.1mg/L

March 17, 1993

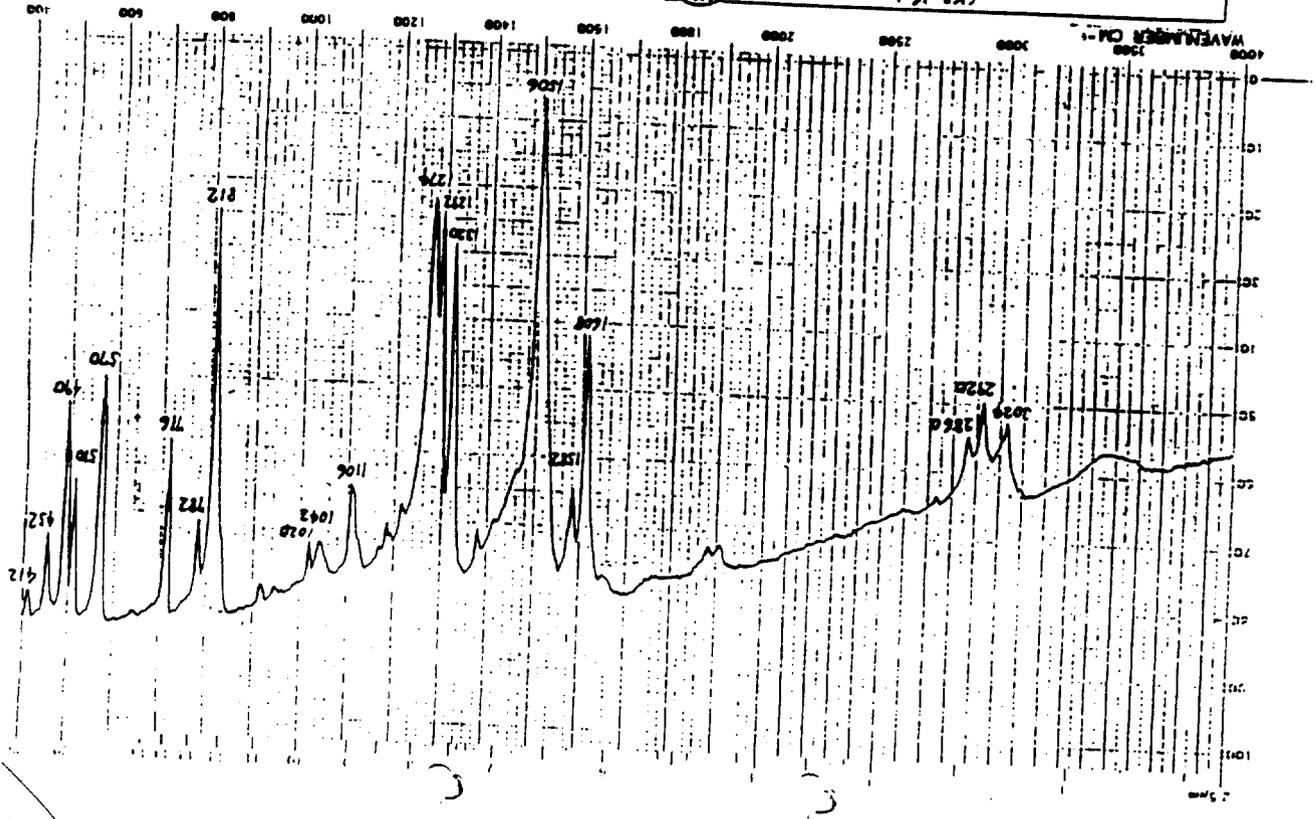
Name C Ishizaka

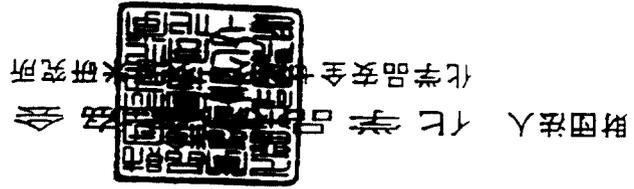
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Reference IR spectrum supplied by the operator

Tri-n-butylamine (Lot. 920602) (K2.16)





平成 5年 3月 31日

2-1

T P A の 部 位 別 試 験

参 考 資 料

T P A の コ イ に お け る 濃 縮 度 試 験 (試 験 番 号 4 2 2 7 4)

Table-8

Calculation table for analysis of test fish

(Level 2)

Sample description	A	D	G	K	Test No. 42274	
					H	J
Standard 0.1mg/L	2836					
Test fish after 2 weeks a	1395	10	27.2	5.82	0.781	7450
Test fish after 2 weeks b	1325	10	29.9	5.03	0.781	6440
Standard 0.1mg/L	2810					
Test fish after 4 weeks a	2706	10	30.8	10.1	0.779	12900
Test fish after 4 weeks b	2142	10	27.8	8.83	0.779	11300
Standard 0.1mg/L	2696					
Test fish after 6 weeks a	3123	10	31.0	12.0	0.796	15100
Test fish after 6 weeks b	4005	10	33.9	14.1	0.796	17700
Standard 0.1mg/L	2845					
Test fish after 8 weeks a	3330	10	26.1	14.4	0.810	17800
Test fish after 8 weeks b	2839	10	29.7	10.8	0.810	13400

A : Peak area ($\mu\text{V}\cdot\text{sec}$)
 A(std):Standard solution A(t):Sample
 B : Ratio of portion used for analysis 5/150
 C : Final volume 10mL
 D : Dilution factor
 E : Average concentration of blank in analysis of control
 F : Recovery rate 93.2%
 G : Weight of test fish (g)
 K : Concentration of test substance in test fish ($\mu\text{g/g}$)
 $K = \{P \times A(t) / A(\text{std}) / B \times D \times C / G - E\} / F \times 100$
 H : Average concentration of test substance in test water ($\mu\text{g/L}$)
 J : BCF
 $J = \{P \times A(t) / A(\text{std}) / B \times D \times C / G - E\} / F \times 100 / H$
 P : Concentration of test substance in standard solution 0.1mg/L

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Table-9

Calculation table for analysis of test fish
(Control)

Test No. 42274

Sample description	A	E	G	I
Standard 0.1mg/L	2875			
Before the initiation of exposure a	n.d.	-	29.1	-
Before the initiation of exposure b	n.d.	-	27.8	-
Standard 0.1mg/L	2774			
After the termination of exposure a	n.d.	-	30.7	-
After the termination of exposure b	n.d.	-	29.0	-
<p>A : Peak area ($\mu\text{V}\cdot\text{sec}$)</p> <p style="padding-left: 40px;">A(std):Standard solution A(t):Sample</p> <p>B : Ratio of portion used for analysis 5/150</p> <p>C : Final volume 10mL</p> <p>E : Amount of blank in analysis of control (μg)</p> <p style="padding-left: 40px;">$E = P \times A(t) / A(\text{std}) \times B \times C$</p> <p>G : Weight of test fish (g)</p> <p>I : Concentration of blank in test fish ($\mu\text{g/g}$)</p> <p style="padding-left: 40px;">$I = E / G$</p> <p>P : Concentration of test substance in standard solution 0.1mg/L</p>				

March 16, 1993

Name C. Ishizaka

Reference 1 Analytical results of dilution water

Sampling date February 2, 1993

Item	Unit	Measured value	Standard value	Detection limit
Total hardness (Ca, Mg)	mg/L	120	< 300*1	
Evaporated residue	mg/L	296	< 500*1	
Chemical oxygen demand	mg/L	0.7	< 5*2	
Chloride ion	mg/L	n.d.	< 0.02 *2	0.01
Ammonia nitrogen	mg/L	0.04	< 1*2	
Cyanide ion	mg/L	n.d.	n.d.*2	
Organic phosphorus	mg/L	n.d.	n.d.*2	
Iron	mg/L	0.04	< 1.0*2	
Mercury	mg/L	n.d.	n.d.*2	< 0.0005
Cadmium	mg/L	n.d.	< 0.01 *2	< 0.01
Cr ⁺⁶	mg/L	n.d.	< 0.05 *2	< 0.04
Lead	mg/L	n.d.	< 0.1*2	< 0.01

*1 Ministerial ordinance of the Ministry of Public Welfare No.56 (August 31, 1978)

*2 Water quality criteria for fisheries (Shadanzoin Nihon Suisansigen Hogokyokai, March 1983)

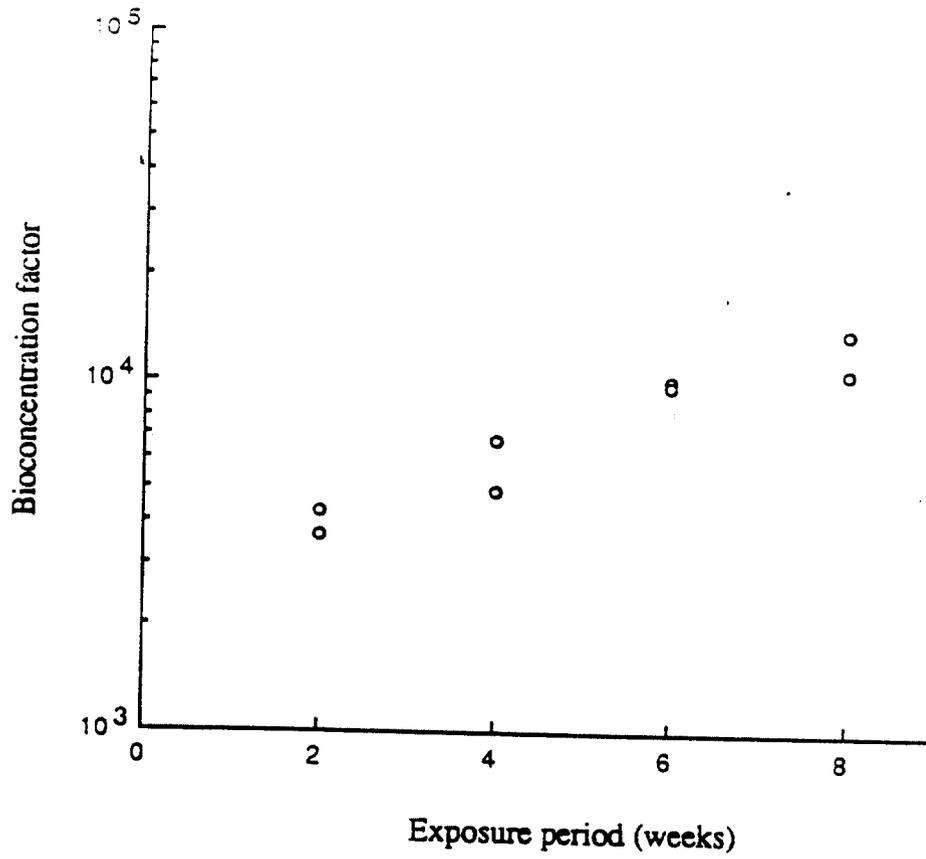


Fig. 1 Correlation between exposure period and bioconcentration factor (Level 1)

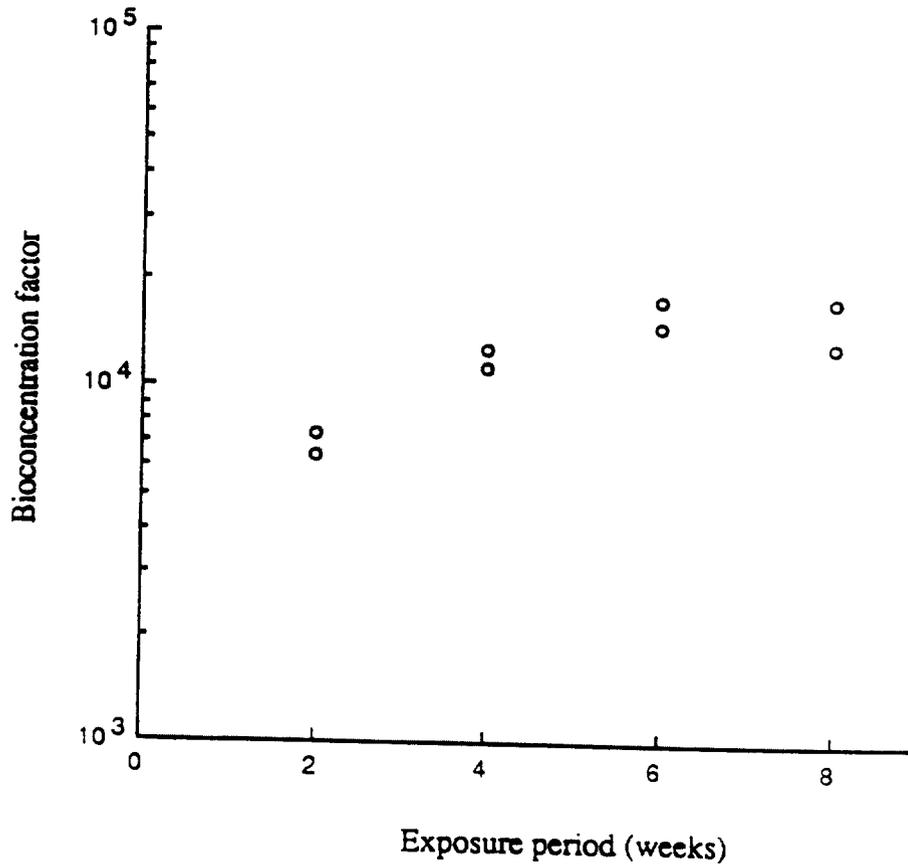
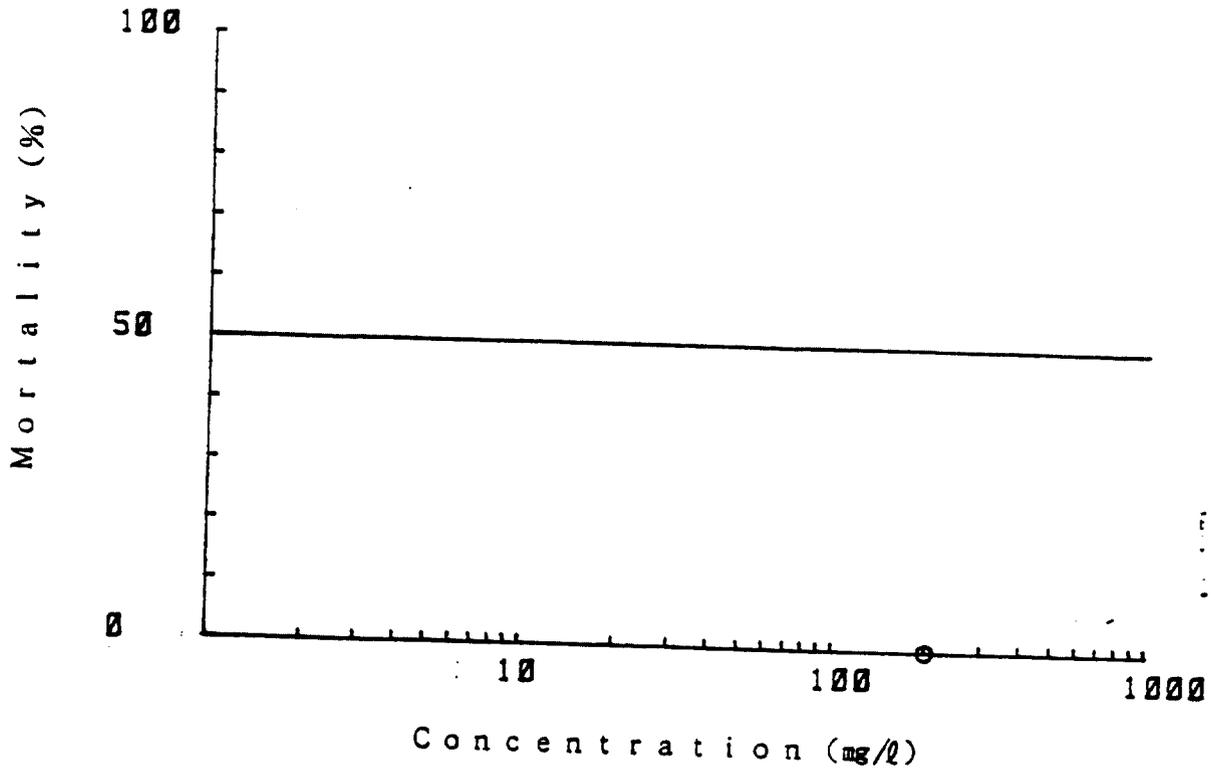


Fig. 2 Correlation between exposure period and bioconcentration factor (Level 2)

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Test No 42274

48 hr LC50 = >200 mg/l



Cumulative Mortality

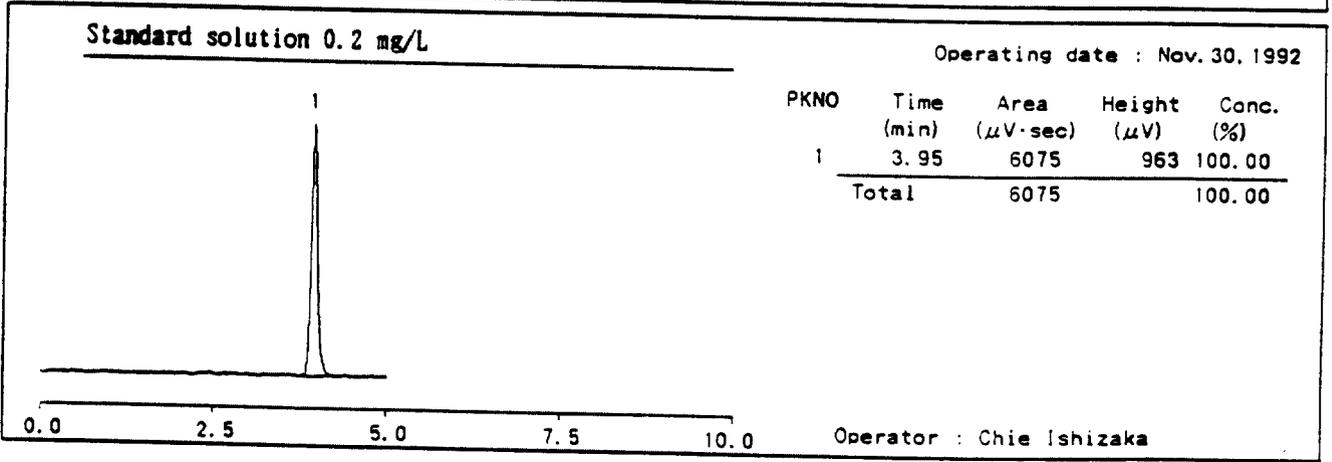
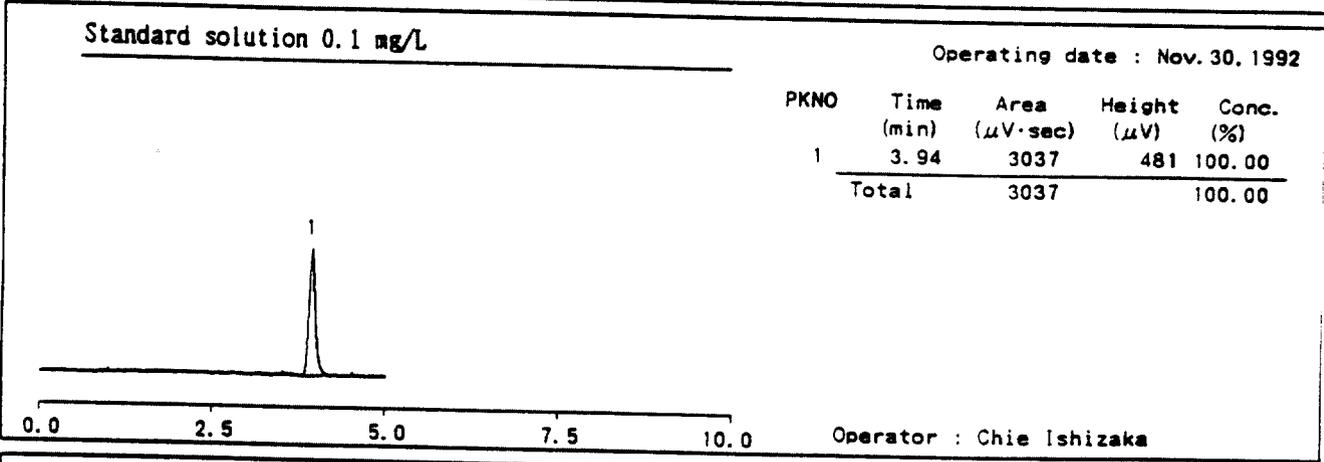
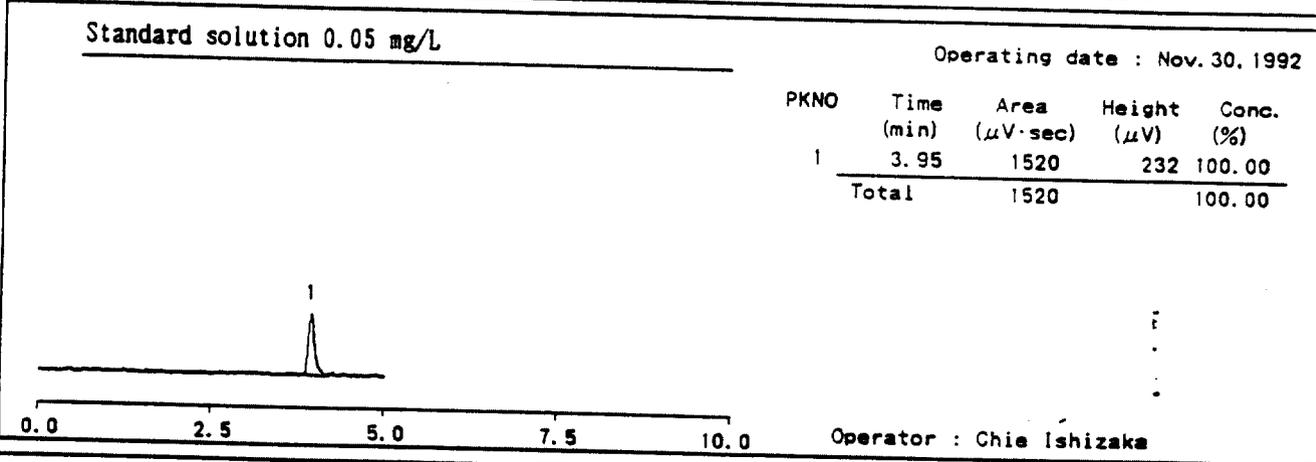
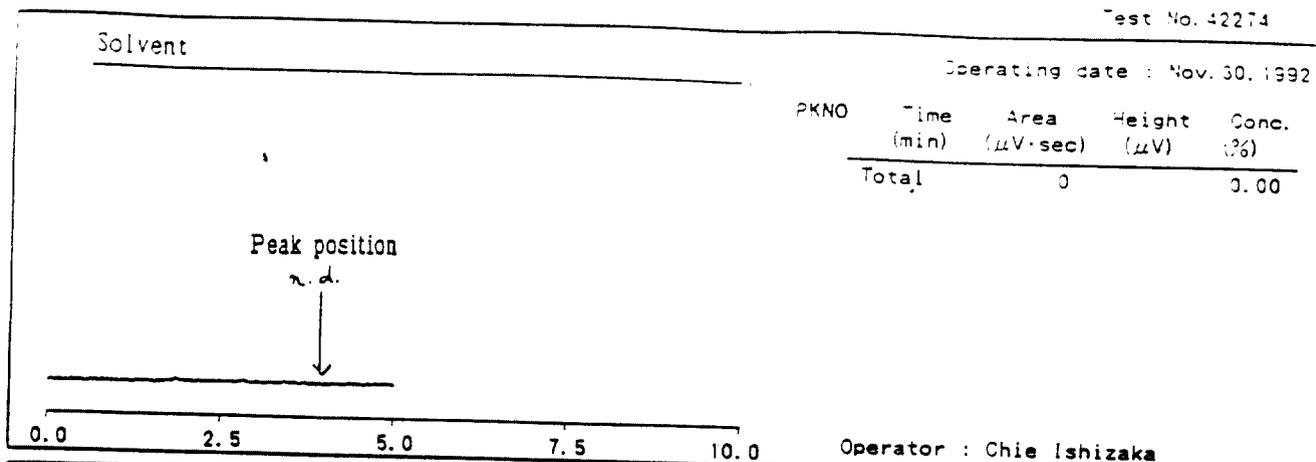
Concentration (mg/l)	Cumulative Mortality (%)		
	2 hours	24 hours	48 hours
Control	-	0	0
200	-	0	0

Fig. 3

Concentration-Mortality Curve

BEST COPY AVAILABLE

Test No. 42274

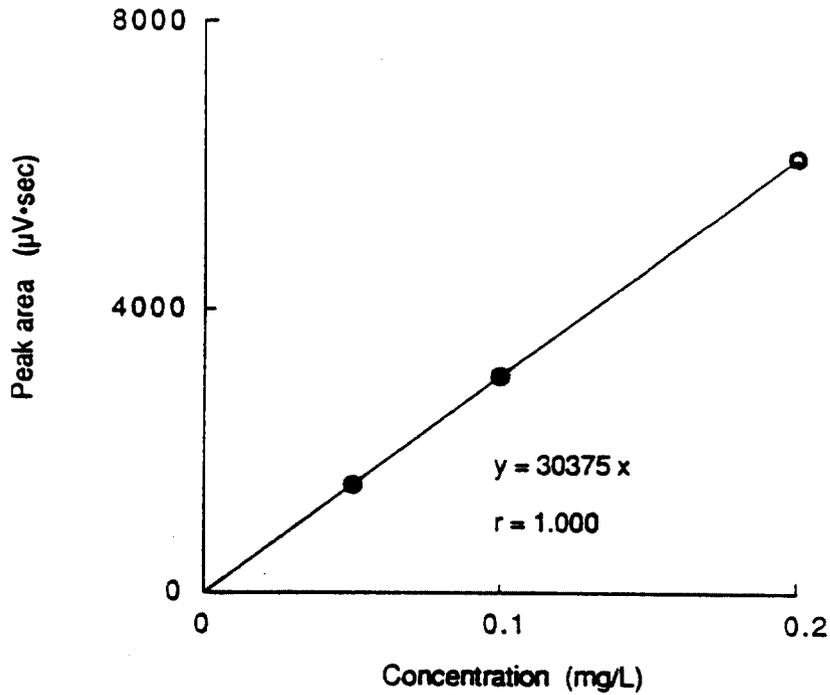


Date : Dec. 3, 1992

Name : Chie Ishizaka

Fig. 4 - 1 Analytical chart of HPLC analysis

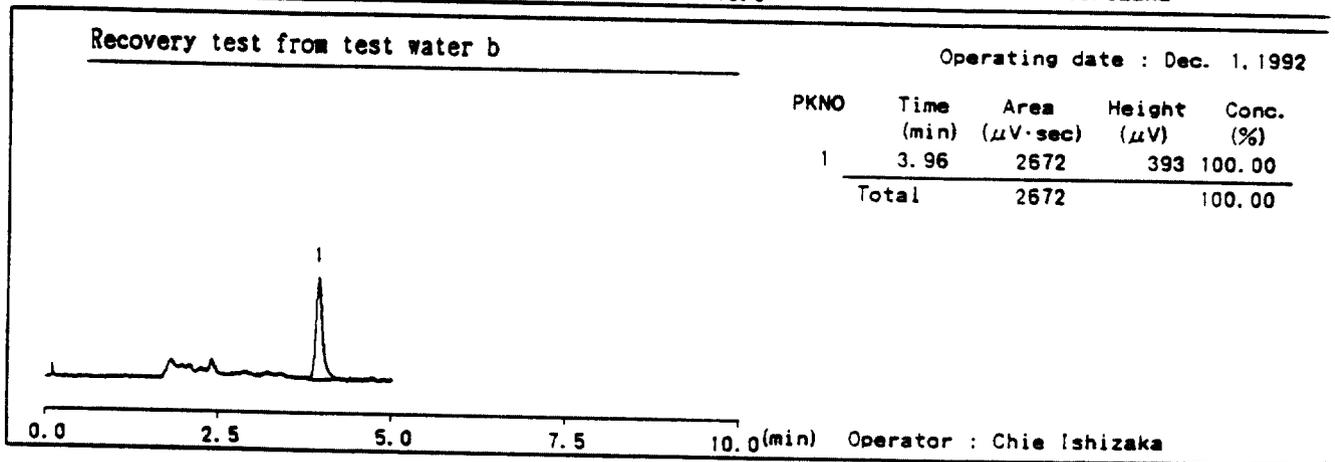
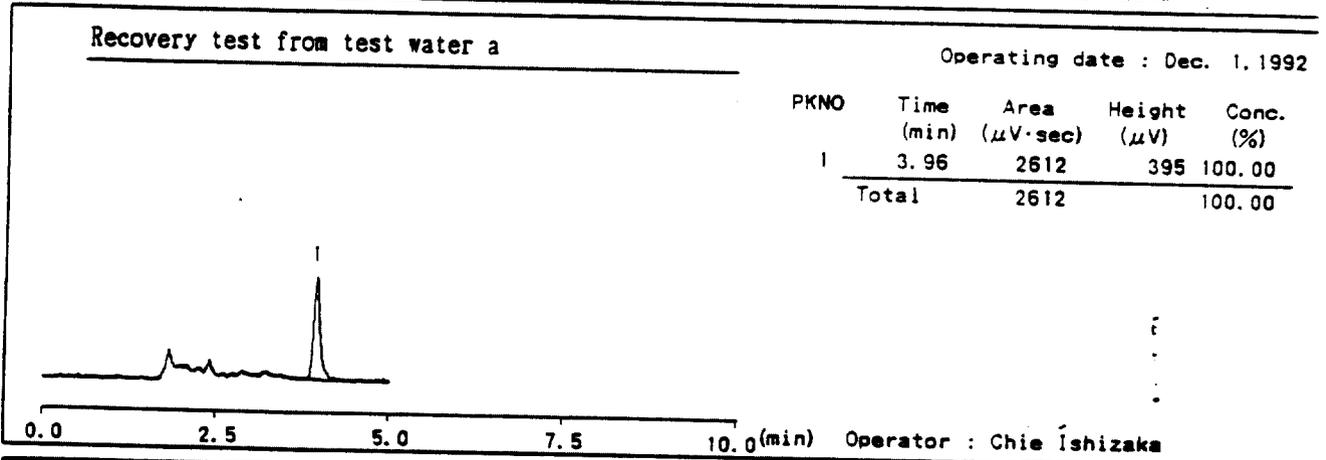
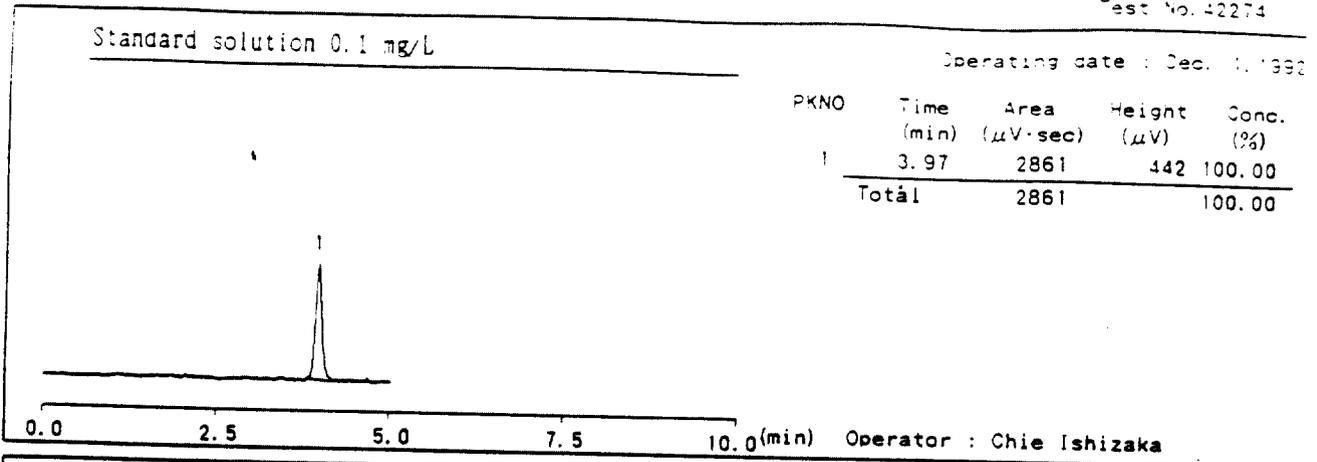
Calibration curve of 42274 by HPLC



Conditions of HPLC

Instrument	LC-6A,SPD-6AV
Sample	TPA
Solvent	Acetonitrile
	I.V. 30 (µL)
Column	L-column ODS (L 15cm I.D. 4.6 mm)
Eluent	Acetonitrile
Flow rate	1.0 ml/min
Detector	UV
Wave length	300 nm
Sensitivity	0.005 ABU/FS
Rec. rang	1.2 mV

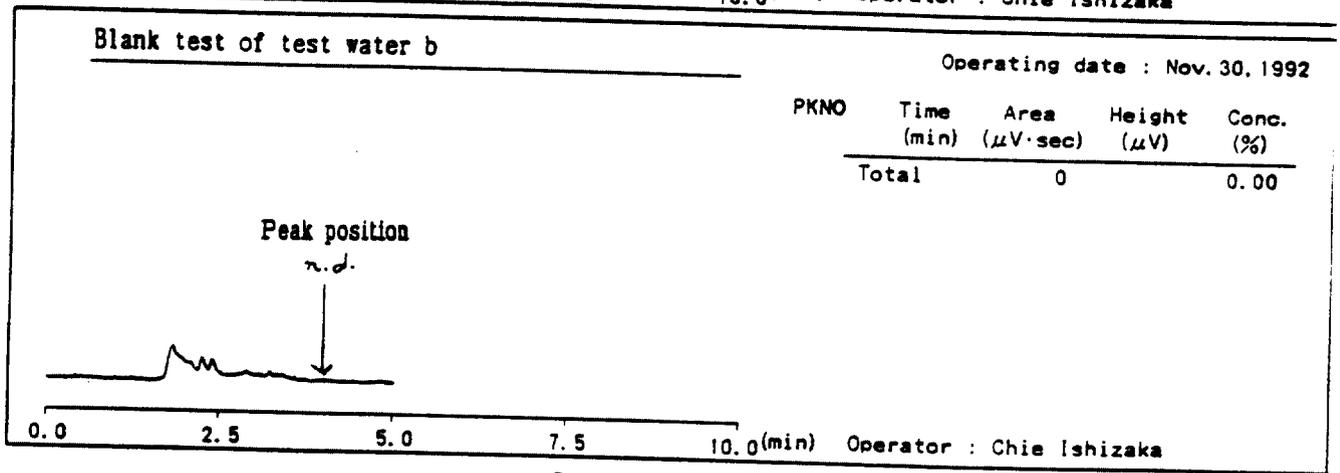
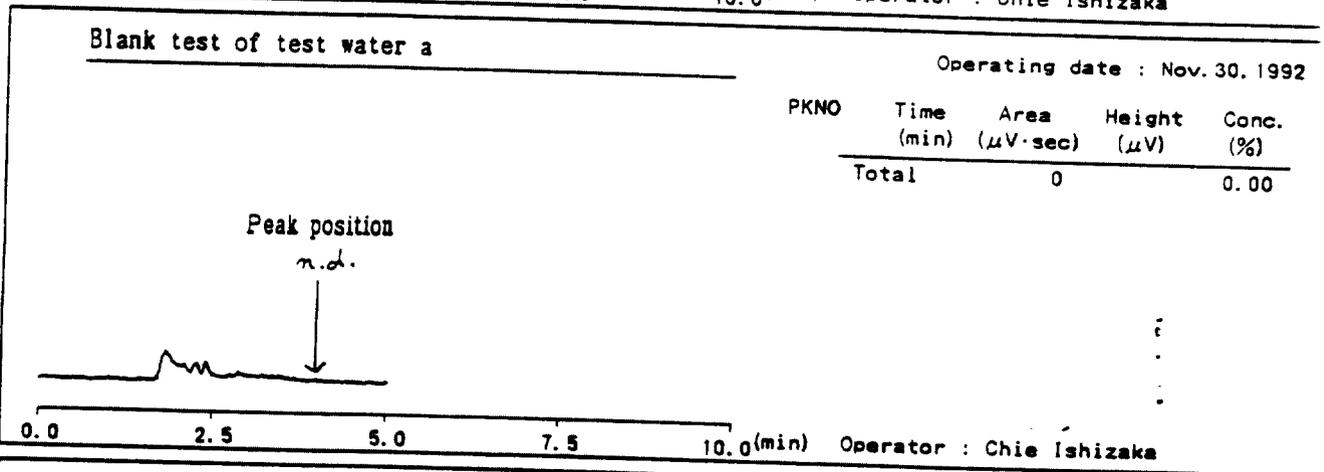
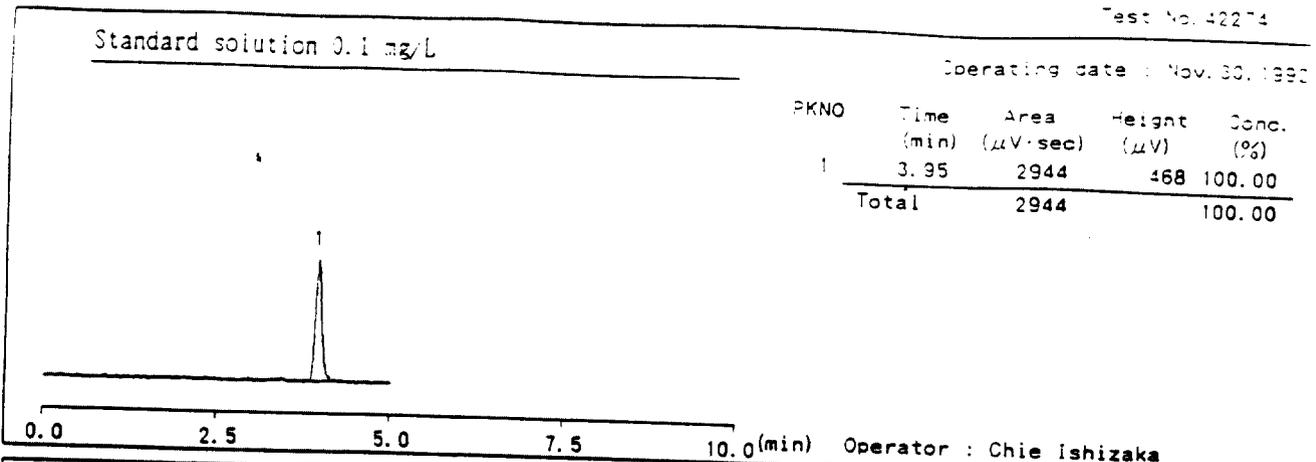
Fig. 4 - 2 Calibration curve and conditions of HPLC analysis
Test No. 42274



Date : Dec. 3, 1992

Name : C Ishizaka

Fig. 5 - 1 Recovery and blank test (analysis of test water)



Date : Dec. 3, 1992

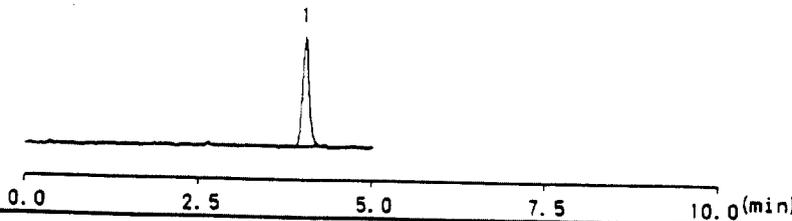
Name : C. Ishizaka

Fig. 5 - 2 Recovery and blank test (analysis of test water)

Standard solution 0.1 mg/L

Operating date : Jan. 13, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.03	2754	420	100.00
Total		2754		100.00

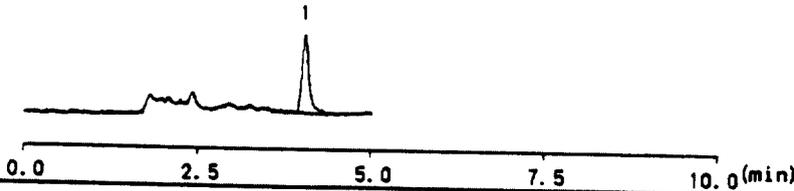


Operator : Chie Ishizaka

Test water at 2nd day (Level 1)

Operating date : Jan. 13, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.04	2176	304	100.00
Total		2176		100.00

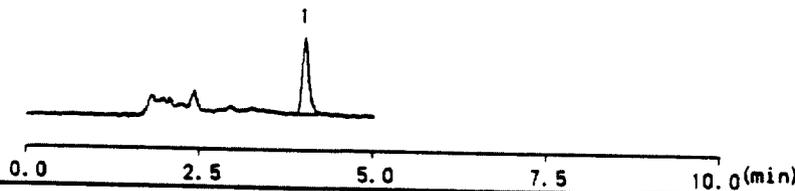


Operator : Chie Ishizaka

Test water at 2nd day (Level 2)

Operating date : Jan. 13, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.02	1940	293	100.00
Total		1940		100.00

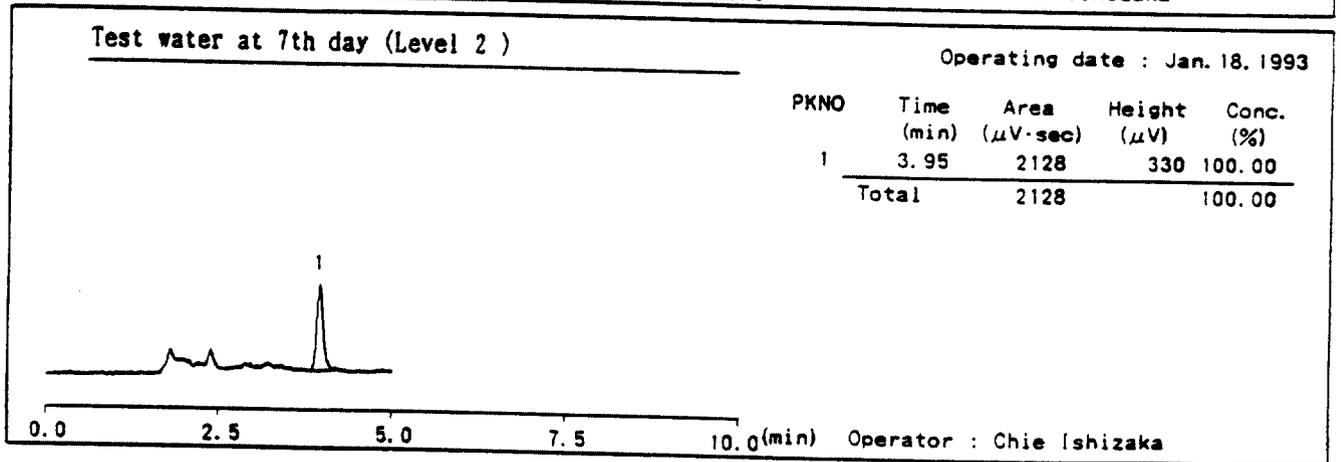
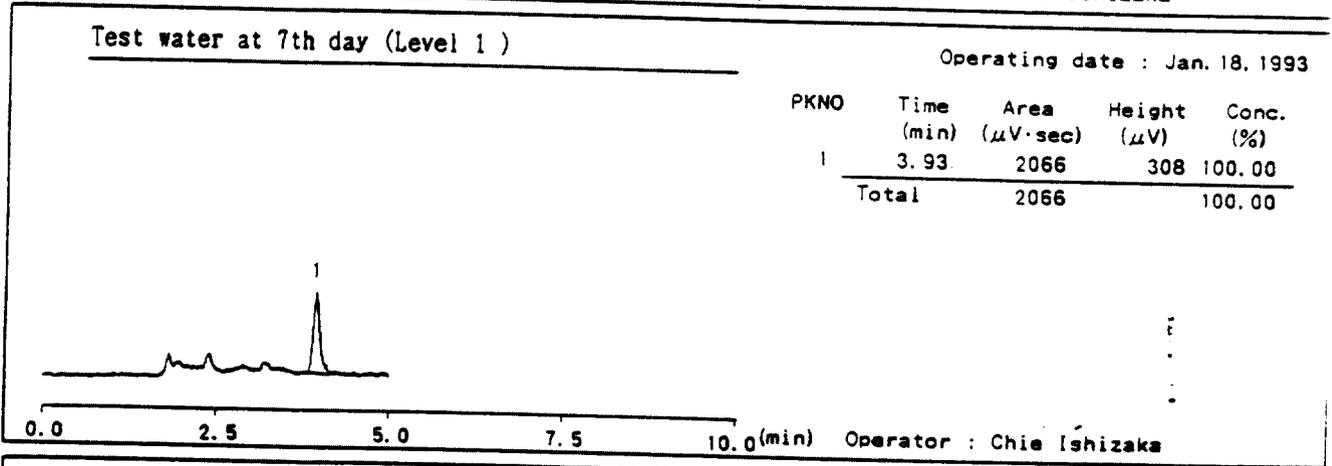
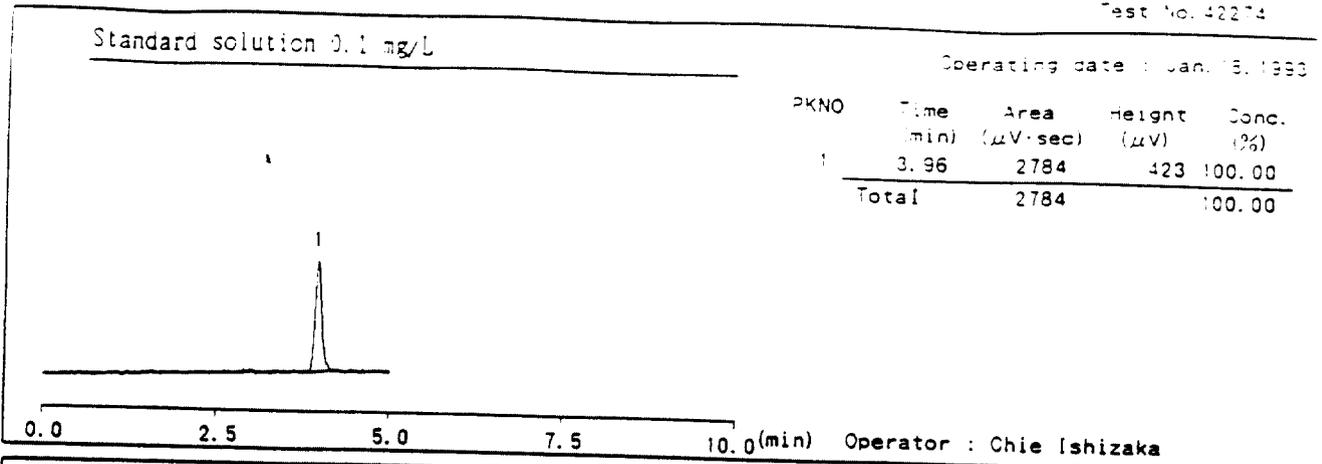


Operator : Chie Ishizaka

Date : Jan. 14, 1993

Name : C. Ishizaka

Fig. 6 - 1 Analysis of test water



Date : Jan. 20, 1993

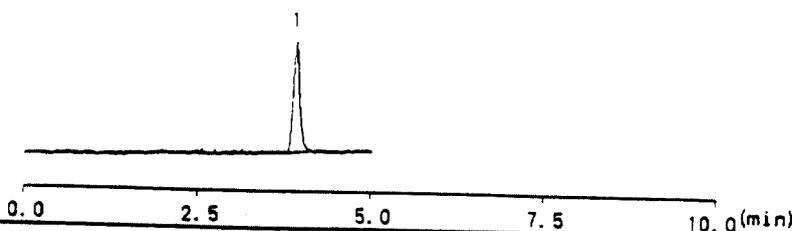
Name : C. Ishizaka

Fig. 6 - 2 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Jan. 20, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.90	2733	421	100.00
Total		2733		100.00

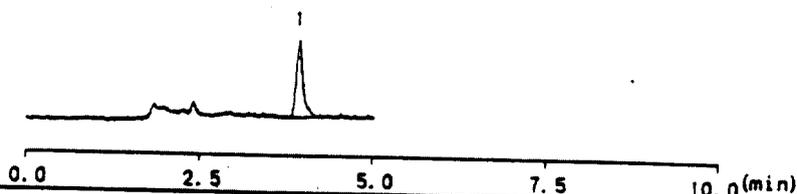


Operator : Chie Ishizaka

Test water at 9th day (Level 1)

Operating date : Jan. 20, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2046	298	100.00
Total		2046		100.00

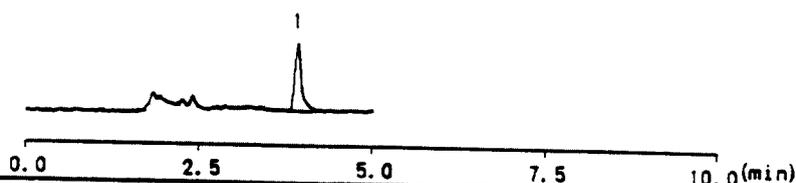


Operator : Chie Ishizaka

Test water at 9th day (Level 2)

Operating date : Jan. 20, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	1796	258	100.00
Total		1796		100.00



Operator : Chie Ishizaka

Date : Jan. 21, 1993

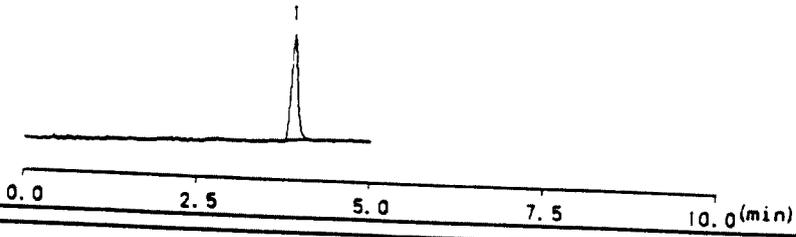
Name : C. Ishizaka

Fig. 6 - 3 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Jan. 22, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.88	2623	409	100.00
Total		2623		100.00

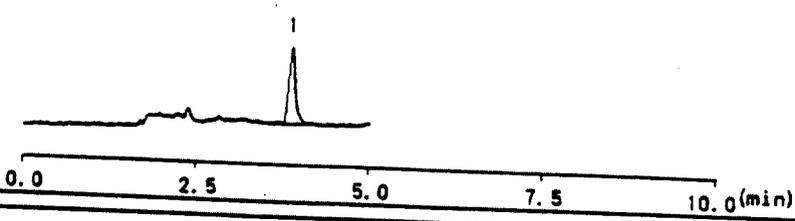


Operator : Chie Ishizaka

Test water at 11th day (Level 1)

Operating date : Jan. 22, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.86	1975	296	100.00
Total		1975		100.00

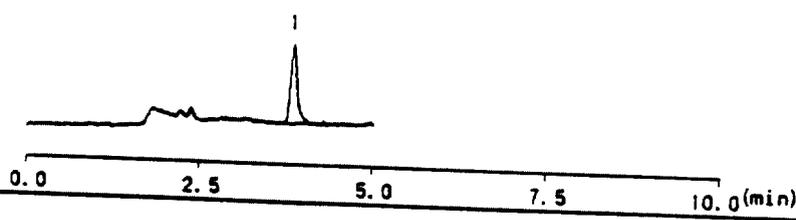


Operator : Chie Ishizaka

Test water at 11th day (Level 2)

Operating date : Jan. 22, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.85	1991	306	100.00
Total		1991		100.00



Operator : Chie Ishizaka

Date : Jan. 22, 1993

Name : C. Ishizaka

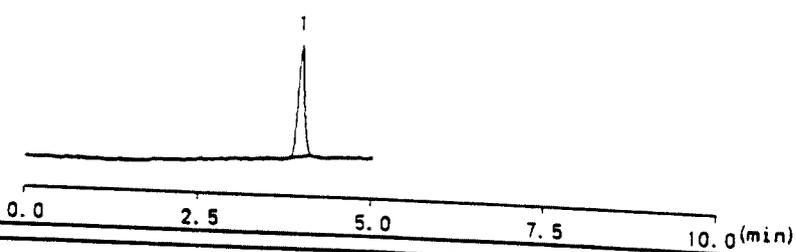
Fig. 6 - 4 Analysis of test water

Standard solution 0.1 mg/L

Test No. 42274

Operating date : Jan. 25, 1993

PKNO	Time (min)	Area ($\mu V \cdot sec$)	Height (μV)	Conc. (%)
1	3.94	2721	428	100.00
Total		2721		100.00

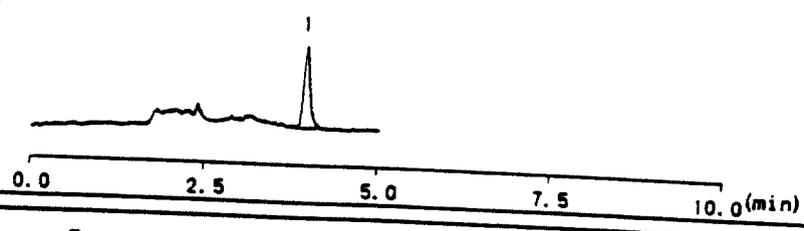


Operator : Chie Ishizaka

Test water at 15th day (Level 1)

Operating date : Jan. 26, 1993

PKNO	Time (min)	Area ($\mu V \cdot sec$)	Height (μV)	Conc. (%)
1	3.95	2013	314	100.00
Total		2013		100.00

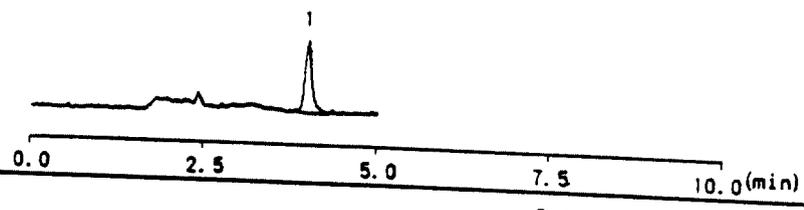


Operator : Chie Ishizaka

Test water at 15th day (Level 2)

Operating date : Jan. 26, 1993

PKNO	Time (min)	Area ($\mu V \cdot sec$)	Height (μV)	Conc. (%)
1	4.00	1942	275	100.00
Total		1942		100.00



Operator : Chie Ishizaka

Date : Jan. 26, 1993

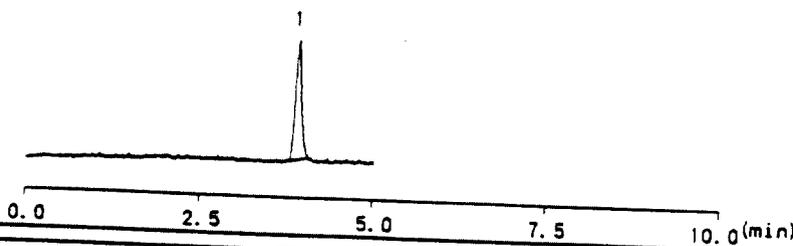
Name : C. Ishizaka

Fig. 6 - 5 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Jan. 29, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.89	2774	457	100.00
Total		2774		100.00

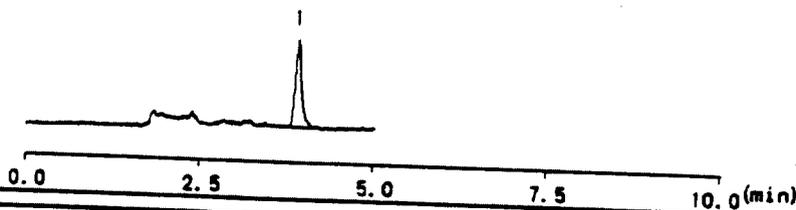


Operator : Chie Ishizaka

Test water at 18th day (Level 1)

Operating date : Jan. 29, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.89	2118	335	100.00
Total		2118		100.00

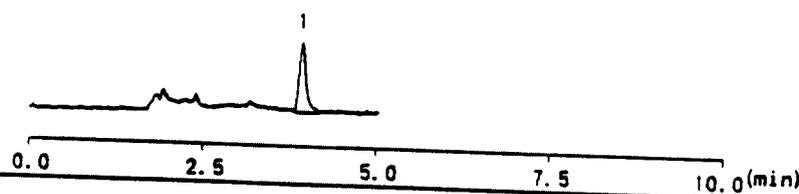


Operator : Chie Ishizaka

Test water at 18th day (Level 2)

Operating date : Jan. 29, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.92	1866	265	100.00
Total		1866		100.00



Operator : Chie Ishizaka

Date : Feb. 1, 1993

Name : C Ishizaka

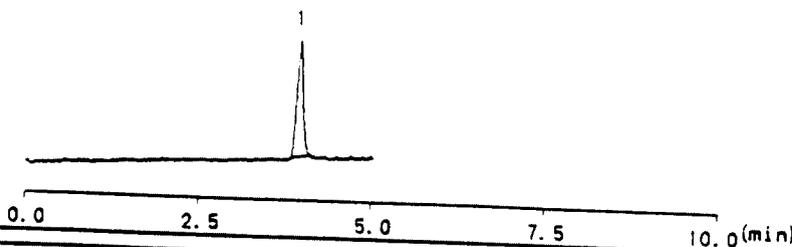
Fig. 6 - 6 Analysis of test water

Test No. 42274

Standard solution 0.1 mg/L

Operating date : Feb. 2. 93

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2790	445	100.00
Total		2790		100.00

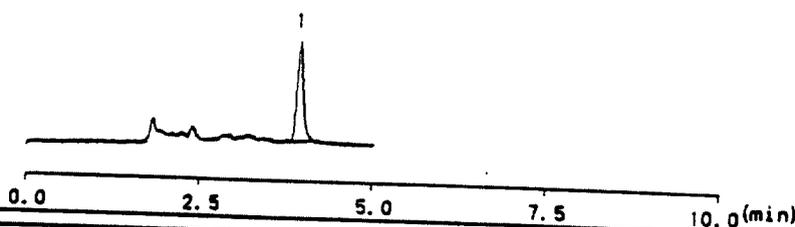


Operator : Chie Ishizaka

Test water at 22nd day (Level 1)

Operating date : Feb. 2, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2403	377	100.00
Total		2403		100.00

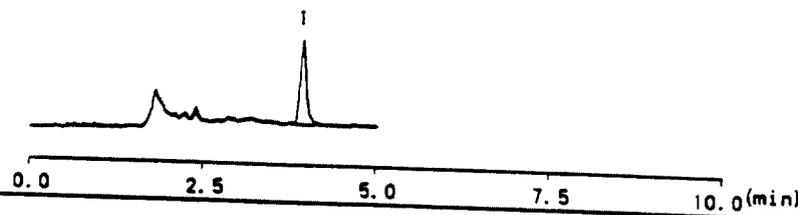


Operator : Chie Ishizaka

Test water at 22nd day (Level 2)

Operating date : Feb. 2, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2070	321	100.00
Total		2070		100.00



Operator : Chie Ishizaka

Date : Feb. 5, 1993

Name : C. Ishizaka

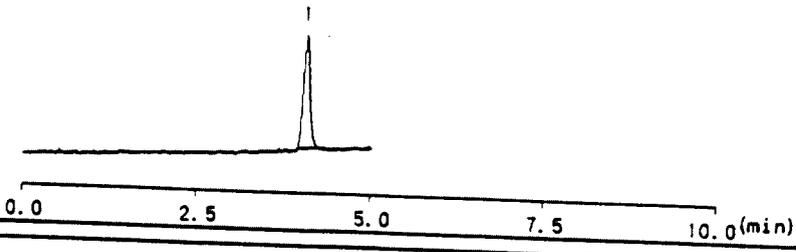
Fig. 6 - 7 Analysis of test water

Test No. 42274

Standard solution 0.1 mg/L

Operating date : Feb. 5, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.05	2764	432	100.00
Total		2764		100.00

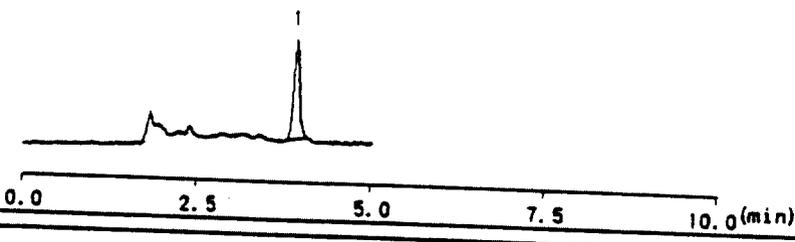


Operator : Chie Ishizaka

Test water at 25th day (Level 1)

Operating date : Feb. 5, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.91	2214	384	100.00
Total		2214		100.00

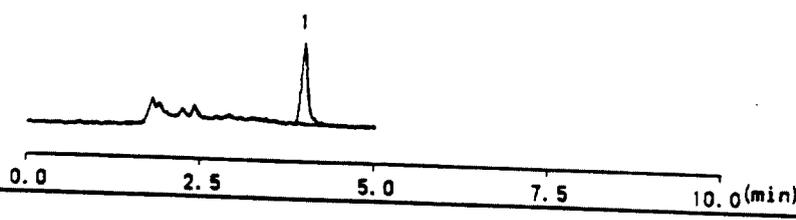


Operator : Chie Ishizaka

Test water at 25th day (Level 2)

Operating date : Feb. 5, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	2048	305	100.00
Total		2048		100.00



Operator : Chie Ishizaka

Date : Feb. 5, 1993

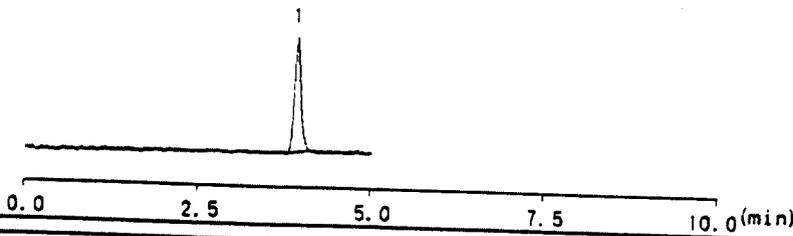
Name : C Ishizaka

Fig. 6 - 8 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.92	2857	438	100.00
Total		2857		100.00

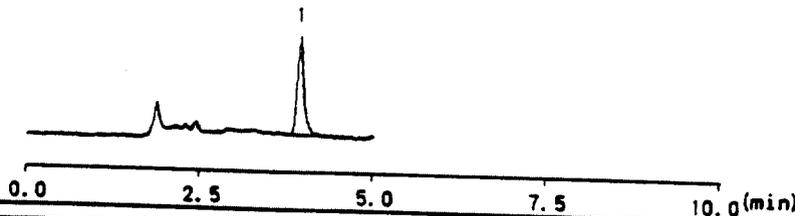


Operator : Chie Ishizaka

Test water at 29th day (Level 1)

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2524	379	100.00
Total		2524		100.00

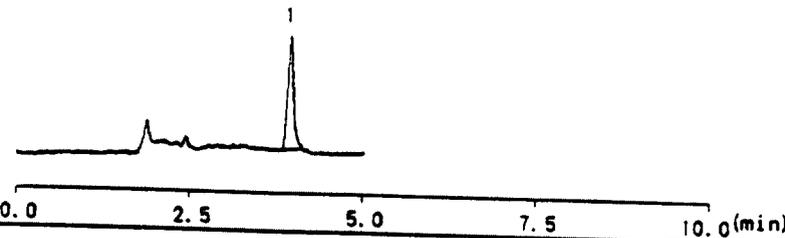


Operator : Chie Ishizaka

Test water at 29th day (Level 2)

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.93	2605	432	100.00
Total		2605		100.00



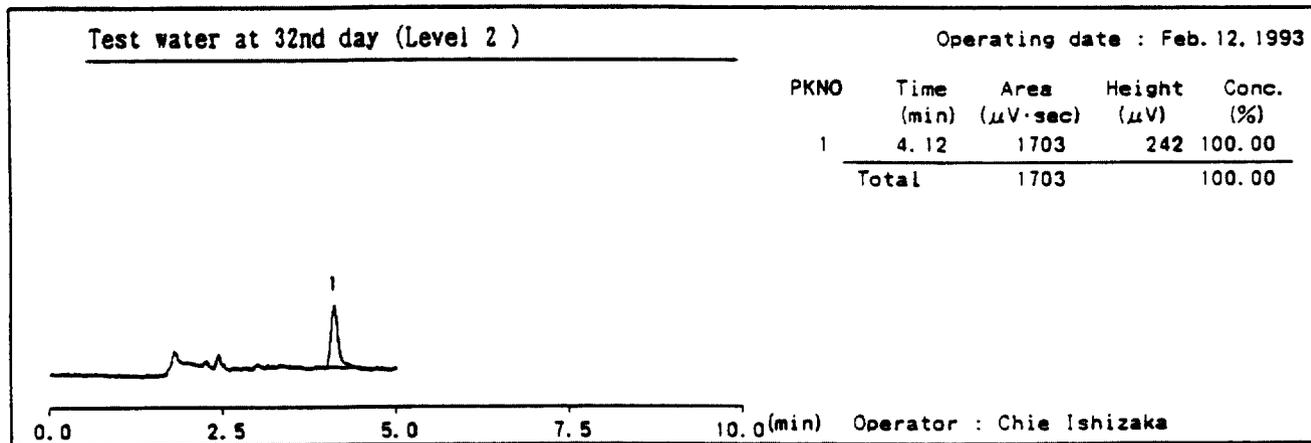
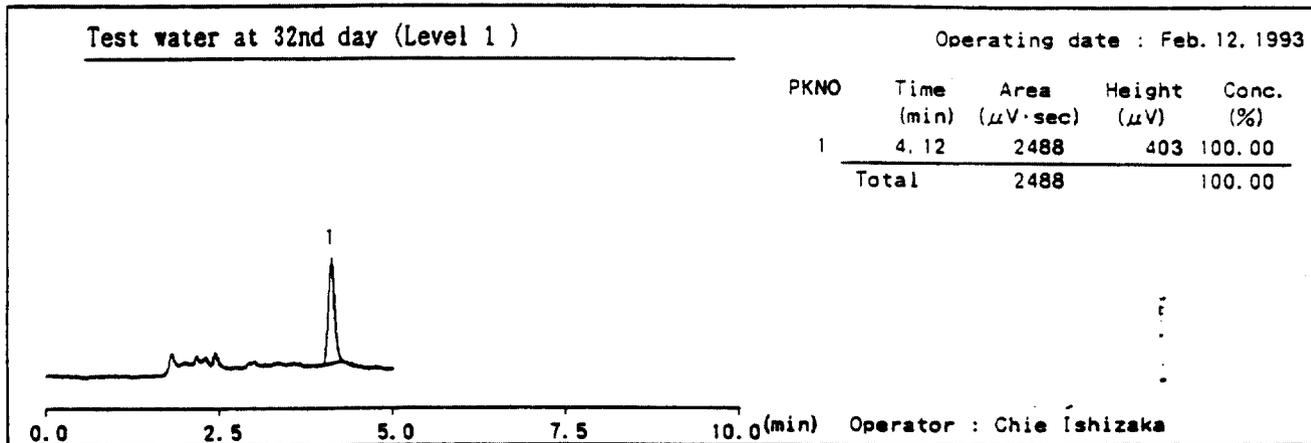
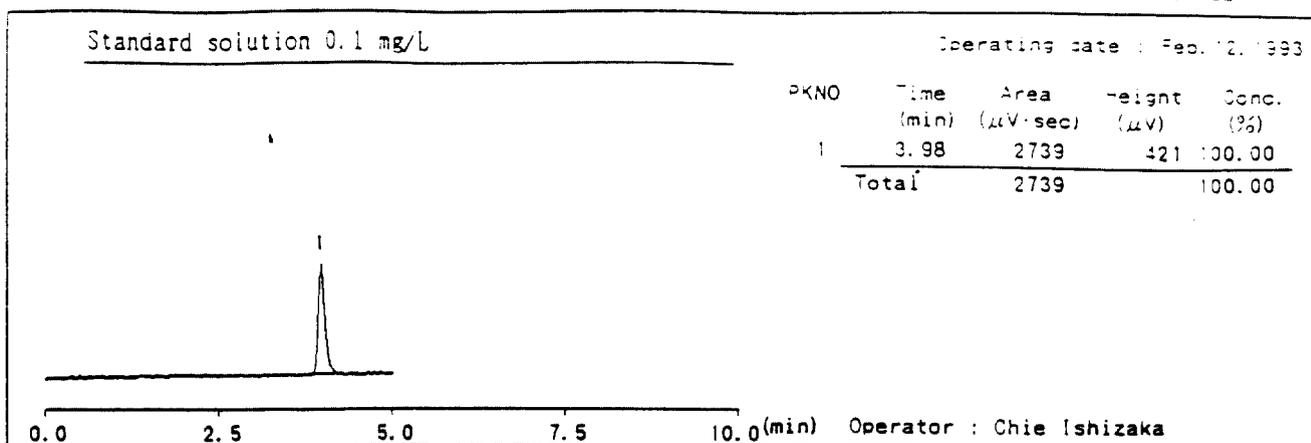
Operator : Chie Ishizaka

Date : Feb. 10, 1993

Name : C Ishizaka

Fig. 6 - 9

Analysis of test water



Date : Mar. 17. 1993

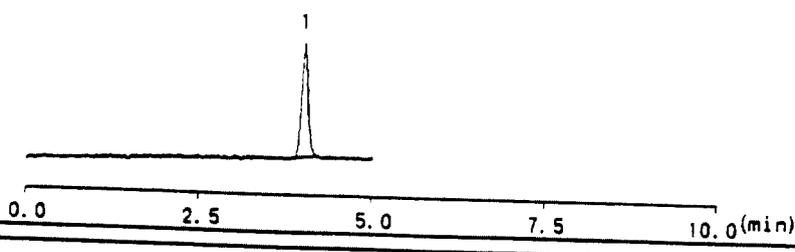
Name : C Ishizaka

Fig. 6 -10 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Feb. 16, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.00	2776	434	100.00
Total		2776		100.00

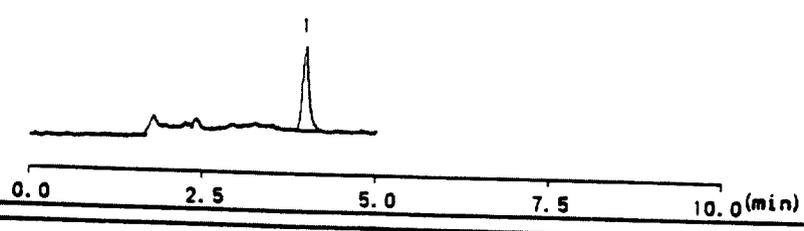


Operator : Chie Ishizaka

Test water at 36th day (Level 1)

Operating date : Feb. 16, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.97	2188	323	100.00
Total		2188		100.00

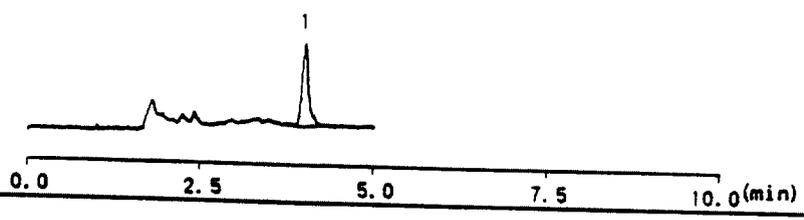


Operator : Chie Ishizaka

Test water at 36th day (Level 2)

Operating date : Feb. 16, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.99	2117	315	100.00
Total		2117		100.00



Operator : Chie Ishizaka

Date : Feb. 22, 1993

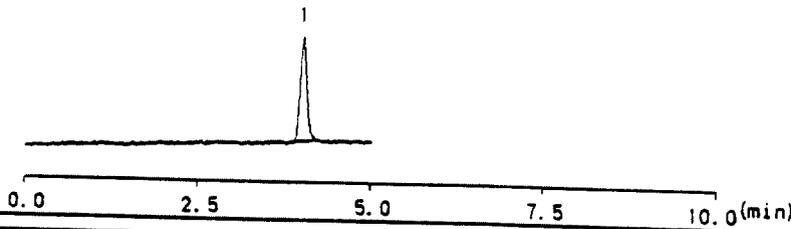
Name : C. Ishizaka

Fig. 6 -11 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Feb. 19, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.00	2659	401	100.00
Total		2659		100.00

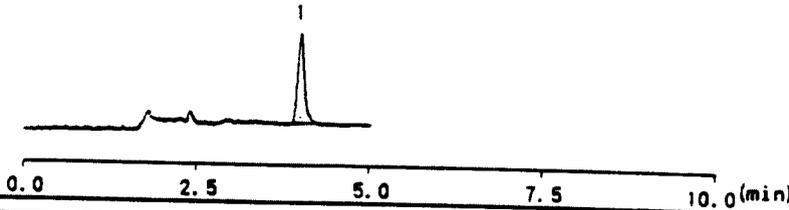


Operator : Chie Ishizaka

Test water at 39th day (Level 1)

Operating date : Feb. 19, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.99	2272	341	100.00
Total		2272		100.00

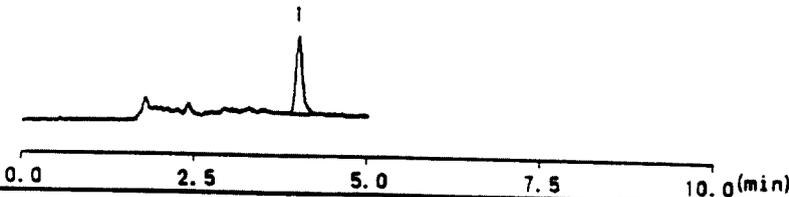


Operator : Chie Ishizaka

Test water at 39th day (Level 2)

Operating date : Feb. 19, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.00	2036	300	100.00
Total		2036		100.00

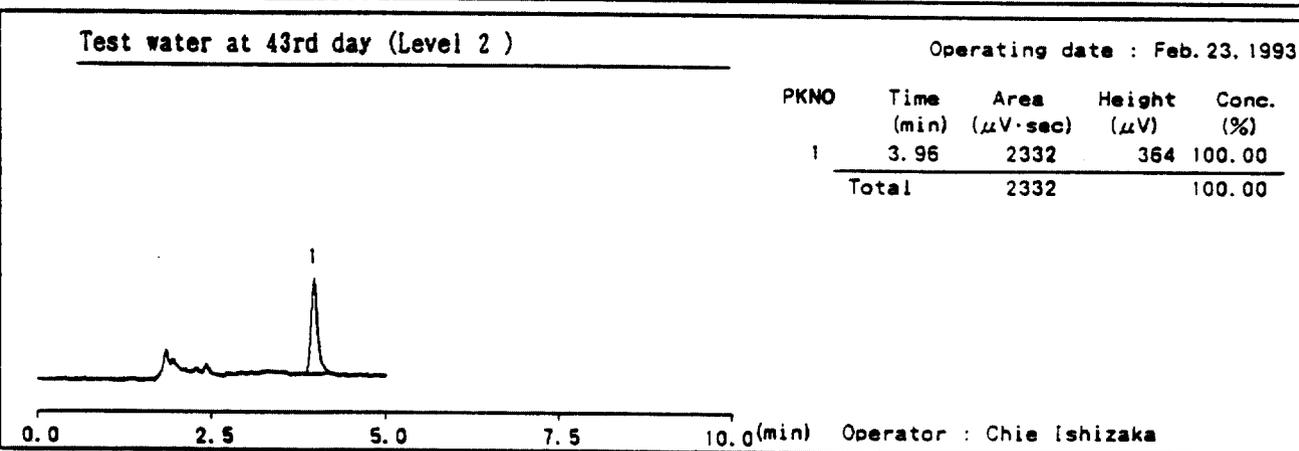
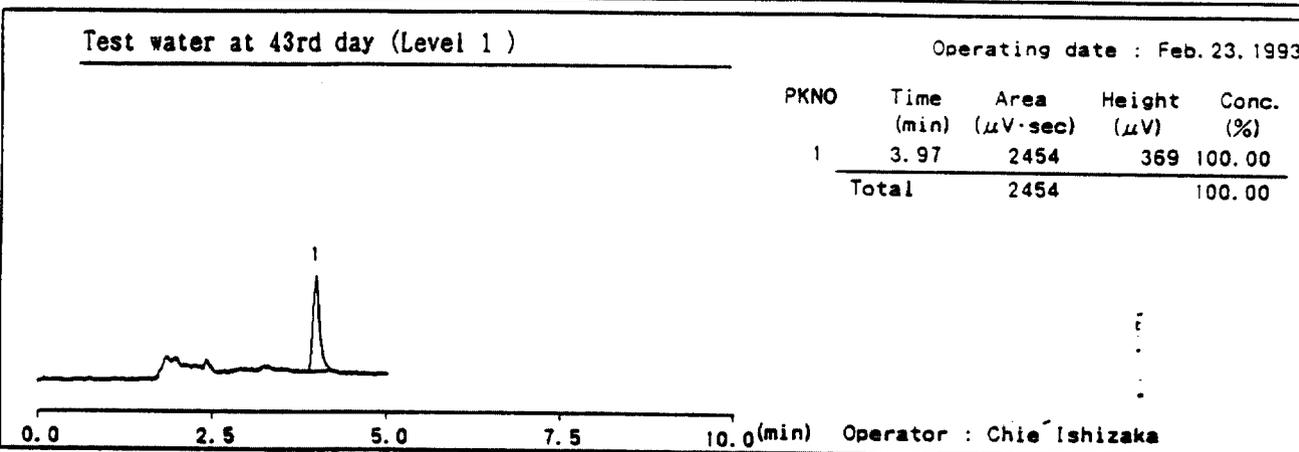
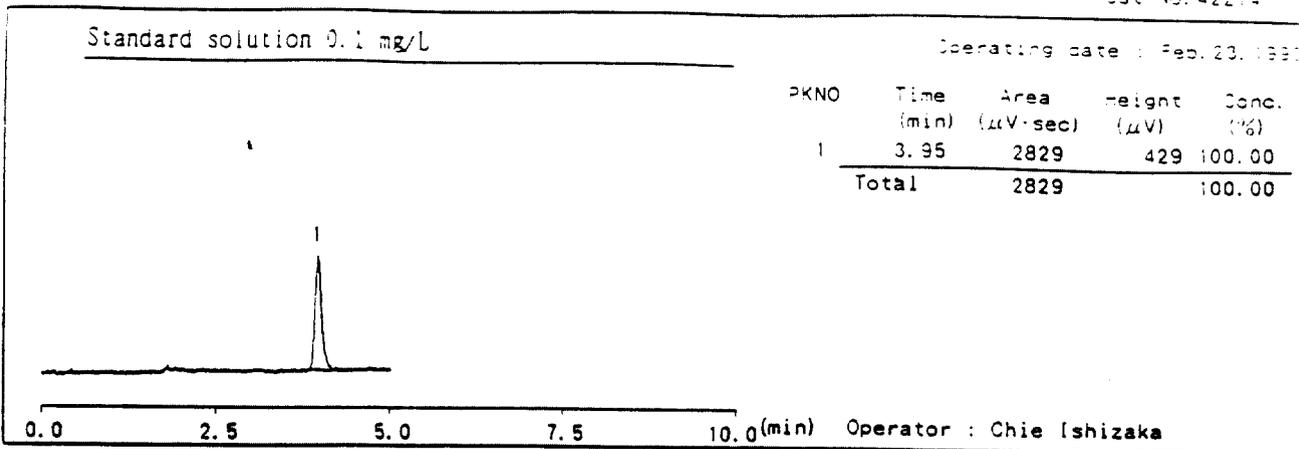


Operator : Chie Ishizaka

Date : Feb. 22, 1993

Name : C Ishizaka

Fig. 6 -12 Analysis of test water



Date : Feb. 27, 1993

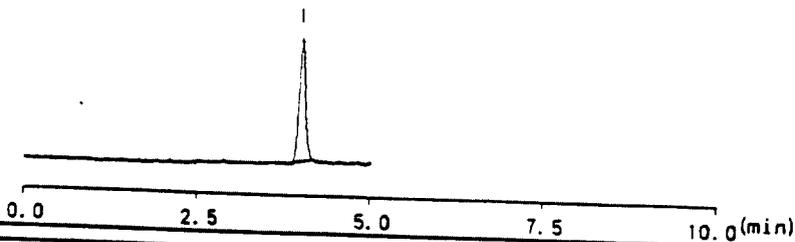
Name : C. Ishizaka

Fig. 6 -13 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Feb. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.99	2925	470	100.00
Total		2925		100.00

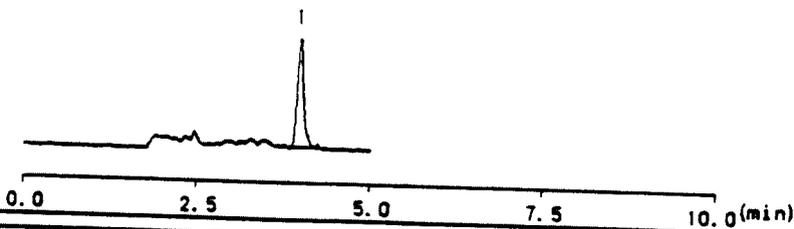


Operator : Chie Ishizaka

Test water at 46th day (Level 1)

Operating date : Feb. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	2645	415	100.00
Total		2645		100.00

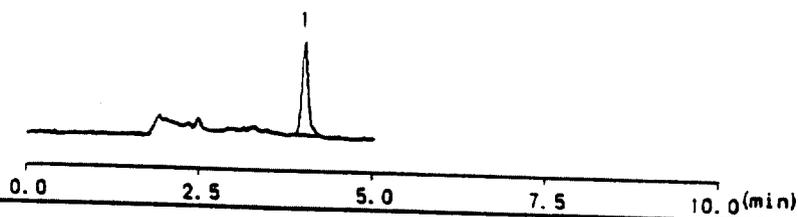


Operator : Chie Ishizaka

Test water at 46th day (Level 2)

Operating date : Feb. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	4.01	2259	352	100.00
Total		2259		100.00

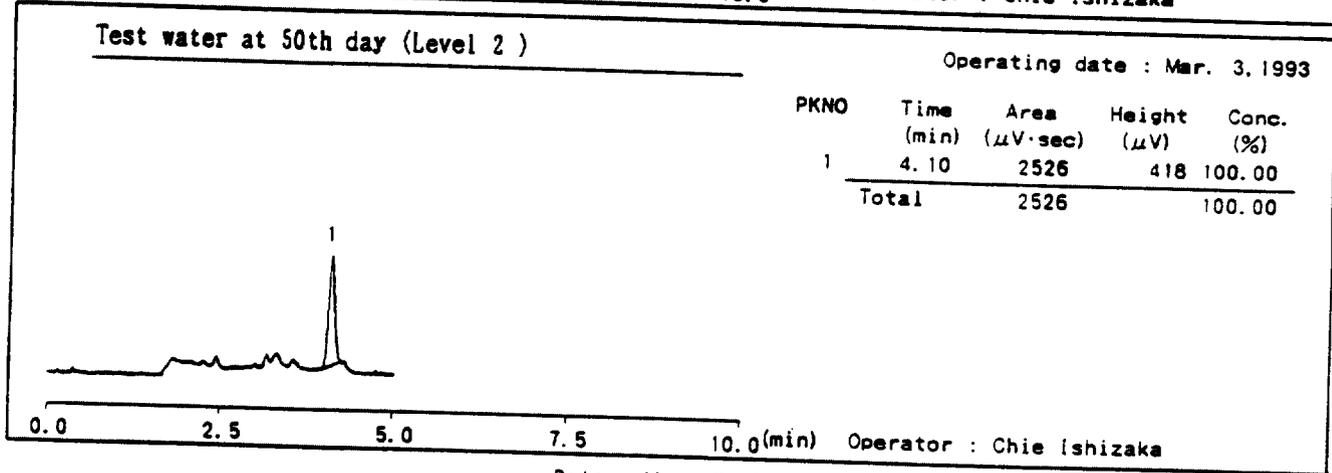
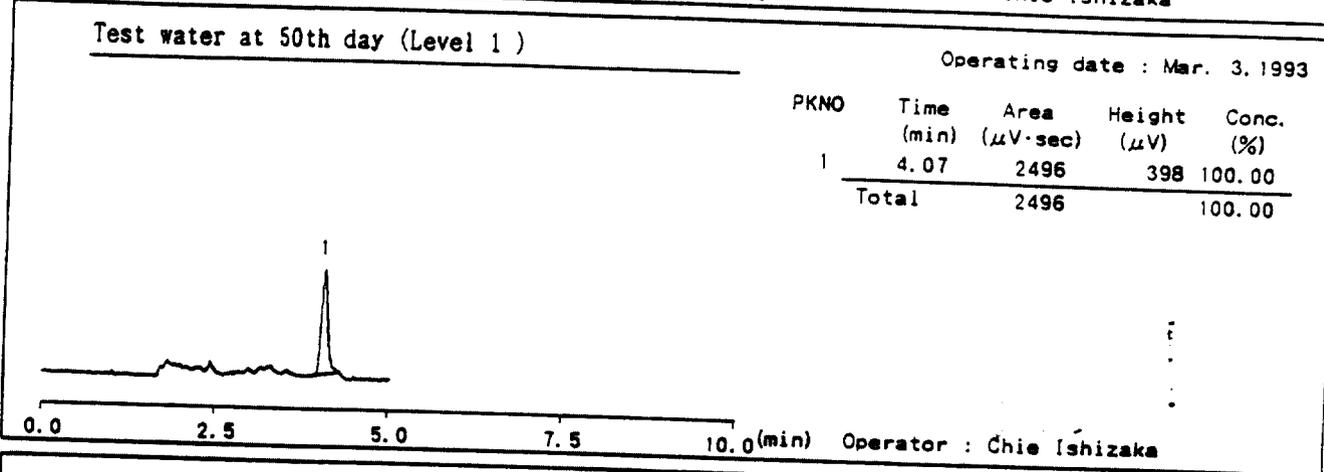
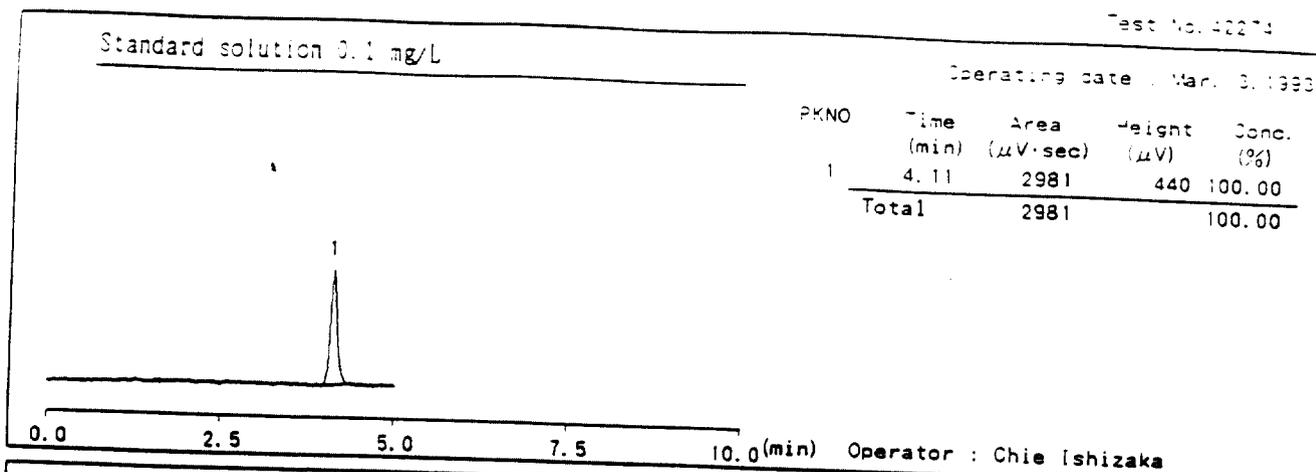


Operator : Chie Ishizaka

Date : Feb. 27, 1993

Name : C. Ishizaka

Fig. 6 -14 Analysis of test water



Date : Mar. 9, 1993

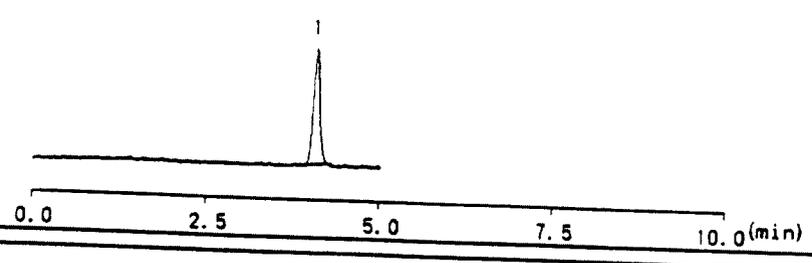
Name : C. Ishizaka

Fig. 6 -15 Analysis of test water

Standard solution 0.1 mg/L

Operating date : Mar. 5, 1993

PKNO	Time (min)	Area (μ V-sec)	Height (μ V)	Conc. (%)
1	4.07	2801	438	100.00
Total		2801		100.00

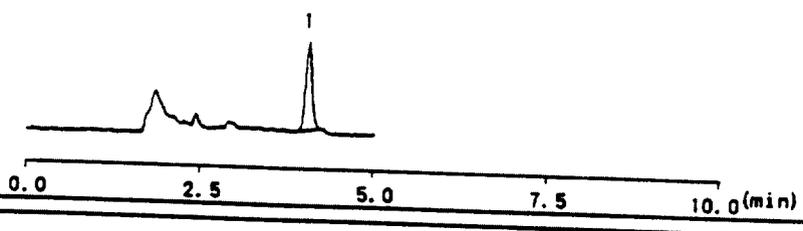


Operator : Chie Ishizaka

Test water at 53rd day (Level 1)

Operating date : Mar. 5, 1993

PKNO	Time (min)	Area (μ V-sec)	Height (μ V)	Conc. (%)
1	4.05	2120	335	100.00
Total		2120		100.00

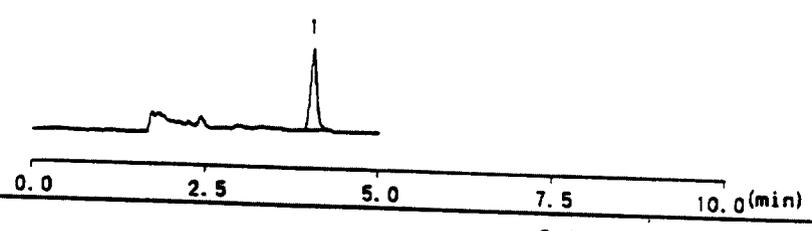


Operator : Chie Ishizaka

Test water at 53rd day (Level 2)

Operating date : Mar. 5, 1993

PKNO	Time (min)	Area (μ V-sec)	Height (μ V)	Conc. (%)
1	4.05	1972	312	100.00
Total		1972		100.00



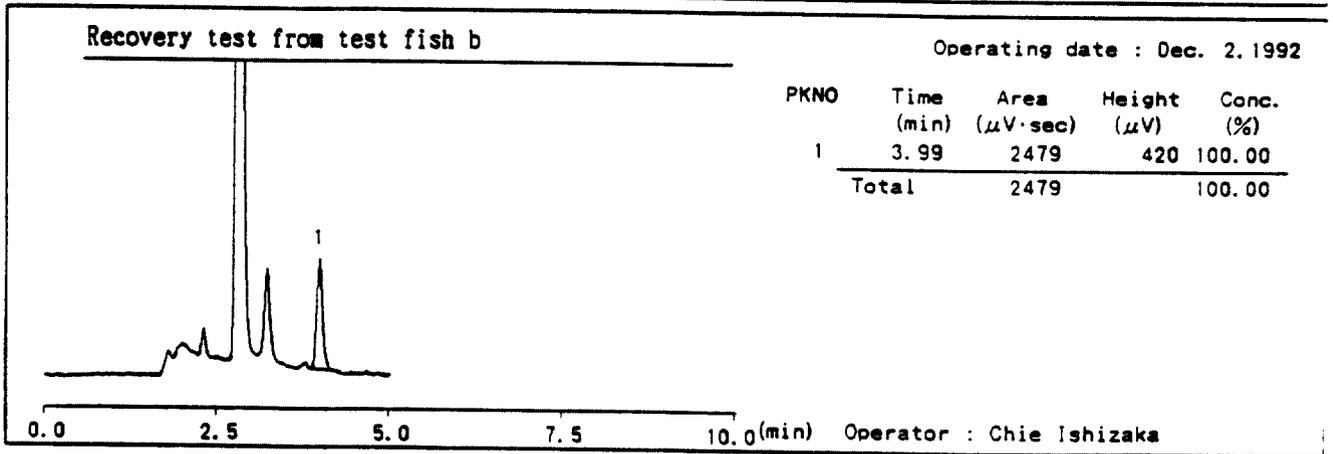
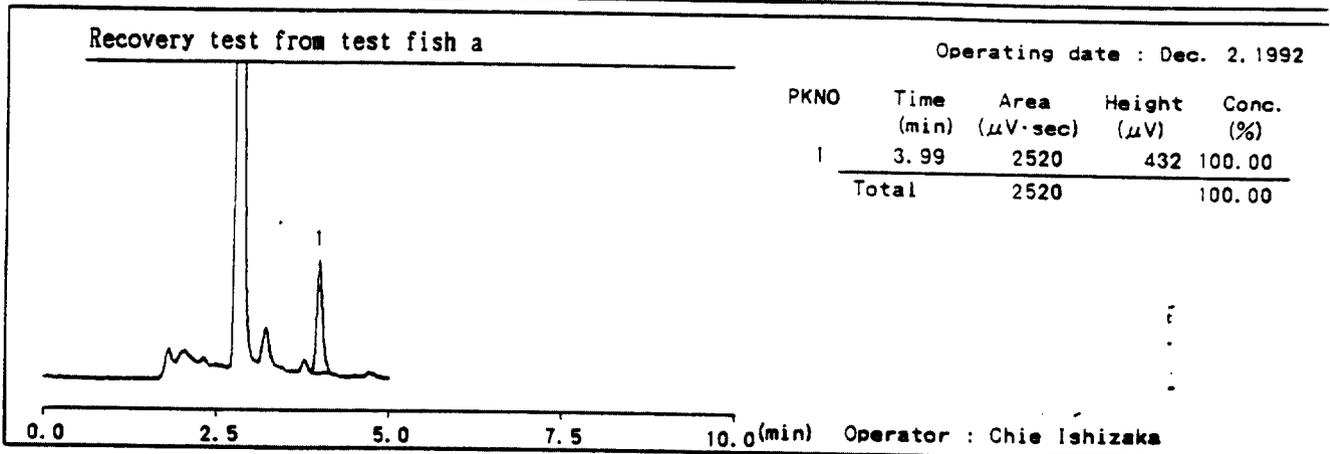
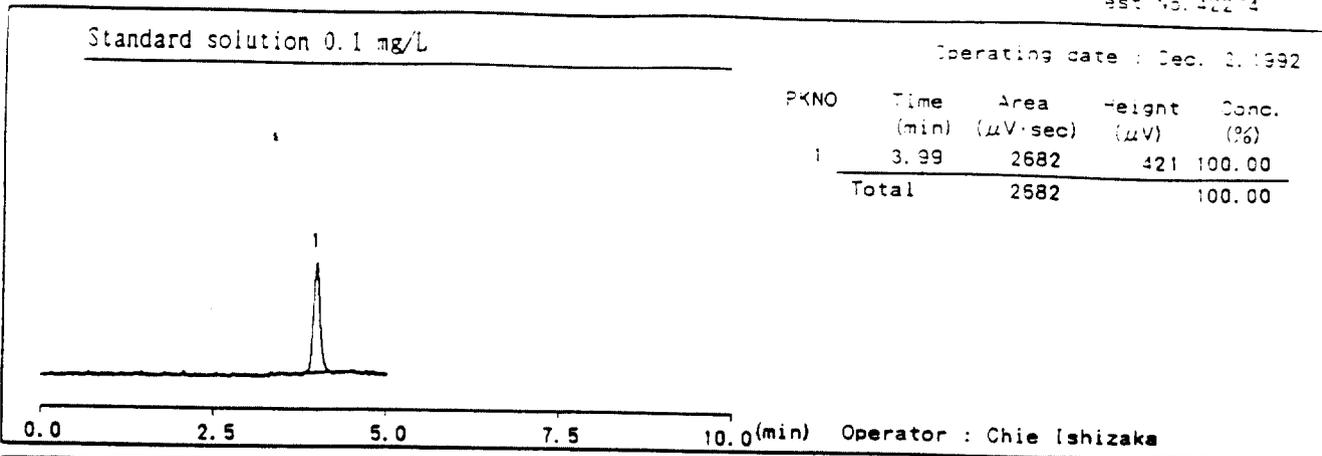
Operator : Chie Ishizaka

Date : Mar. 9, 1993

Name : C. Ishizaka

Fig. 6-16

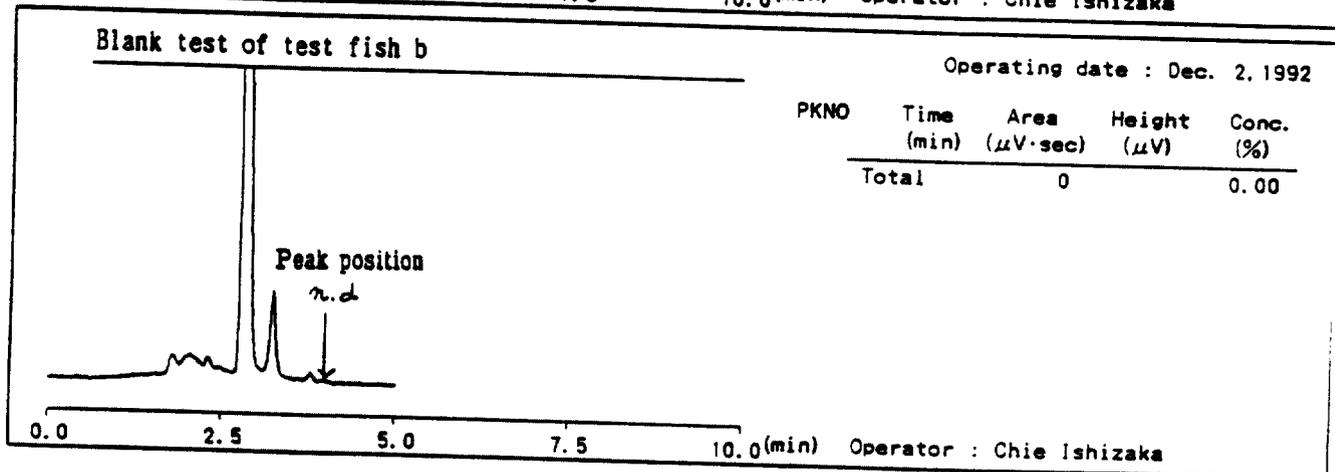
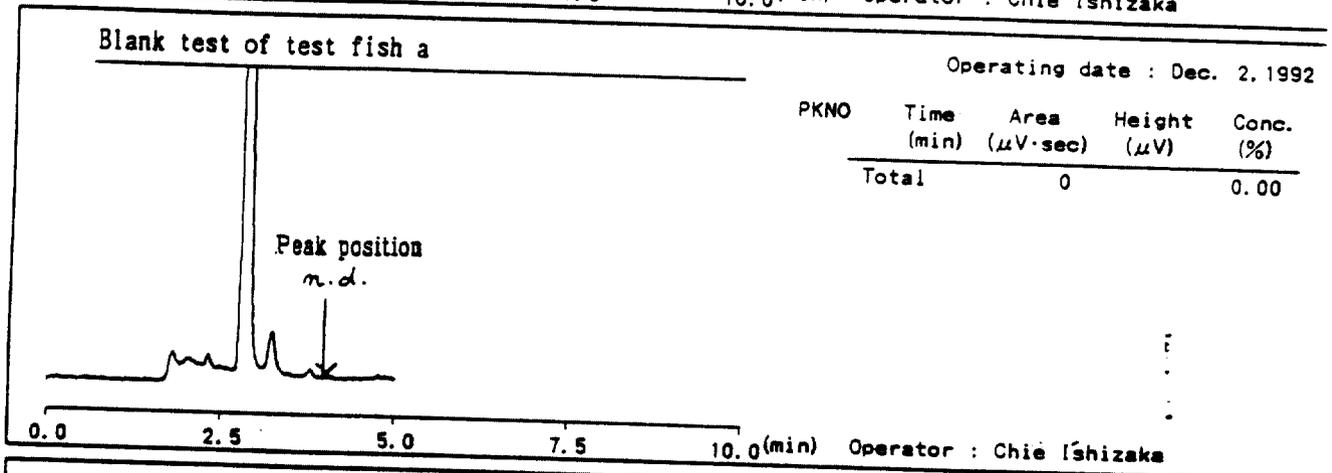
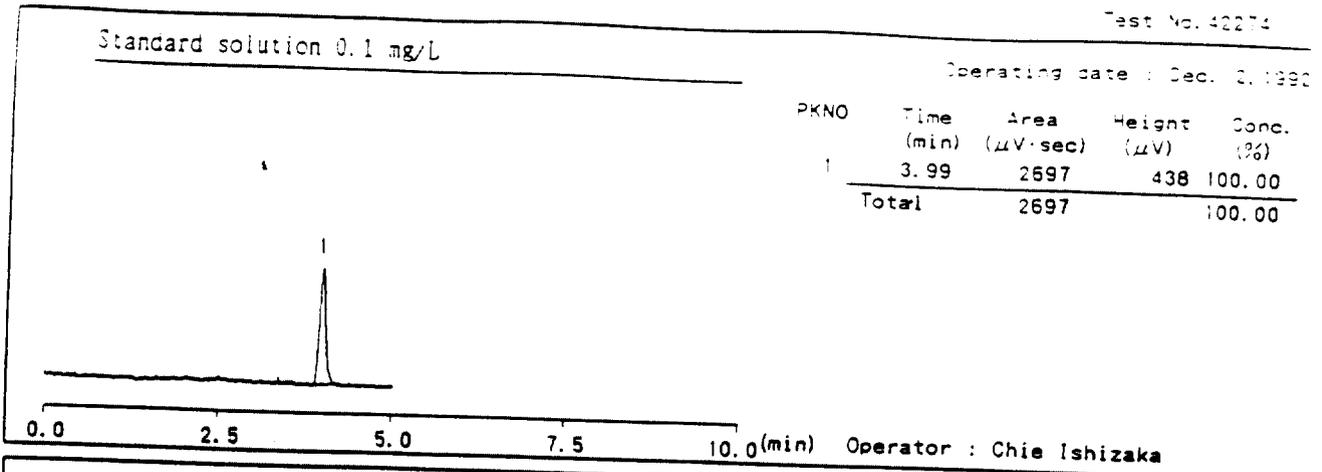
Analysis of test water



Date : Dec. 3, 1992

Name : C. Ishizaka

Fig. 7 - 1 Recovery and blank test (analysis of test fish)



Date : Dec. 3, 1992

Name : C. Ishizaka

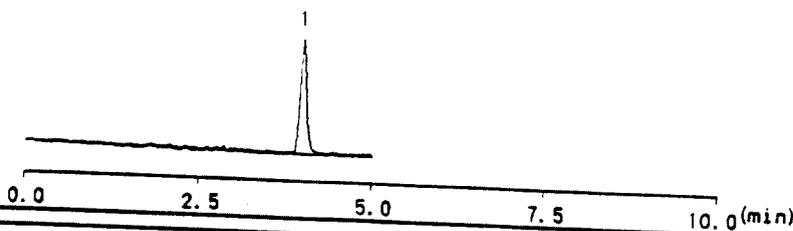
Fig. 7 - 2 Recovery and blank test (analysis of test fish)

Test No. 42274

Standard solution 0.1 mg/L

Operating date : Jan. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	2877	439	100.00
Total		2877		100.00

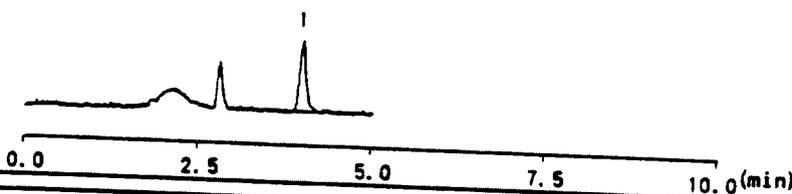


Operator : Chie Ishizaka

Test fish after 2 weeks (Level 1-a)

Operating date : Jan. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	1727	270	100.00
Total		1727		100.00

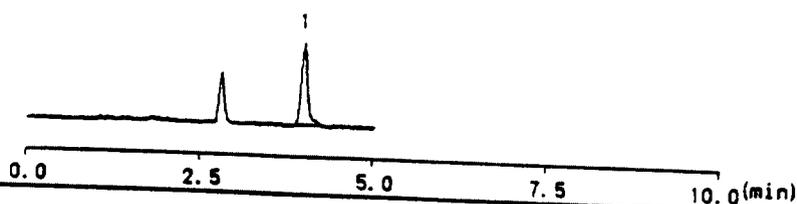


Operator : Chie Ishizaka

Test fish after 2 weeks (Level 1-b)

Operating date : Jan. 26, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	1989	310	100.00
Total		1989		100.00



Operator : Chie Ishizaka

Date : Feb. 1, 1993

Name : C. Ishizaka

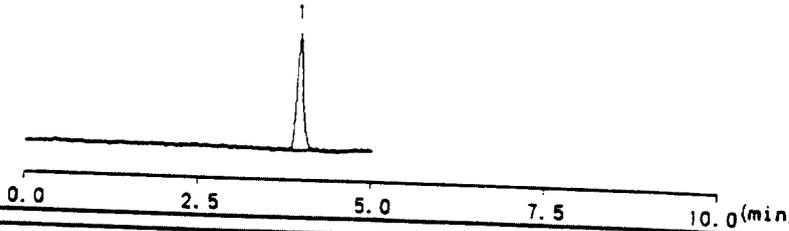
Fig. 8 - 1

Analysis of test fish (Level 1)

Standard solution 0.1 mg/L

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.94	2848	445	100.00
Total		2848		100.00

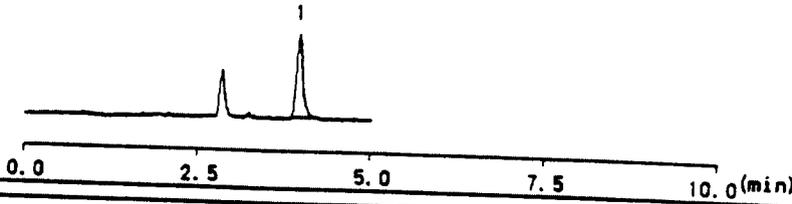


Operator : Chie Ishizaka

Test fish after 4 weeks (Level 1-a)

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.94	2027	320	100.00
Total		2027		100.00

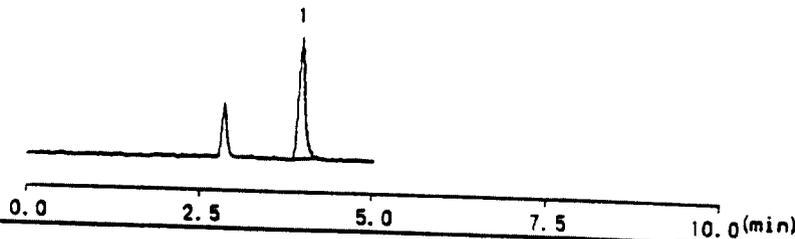


Operator : Chie Ishizaka

Test fish after 4 weeks (Level 1-b)

Operating date : Feb. 9, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.95	2961	463	100.00
Total		2961		100.00



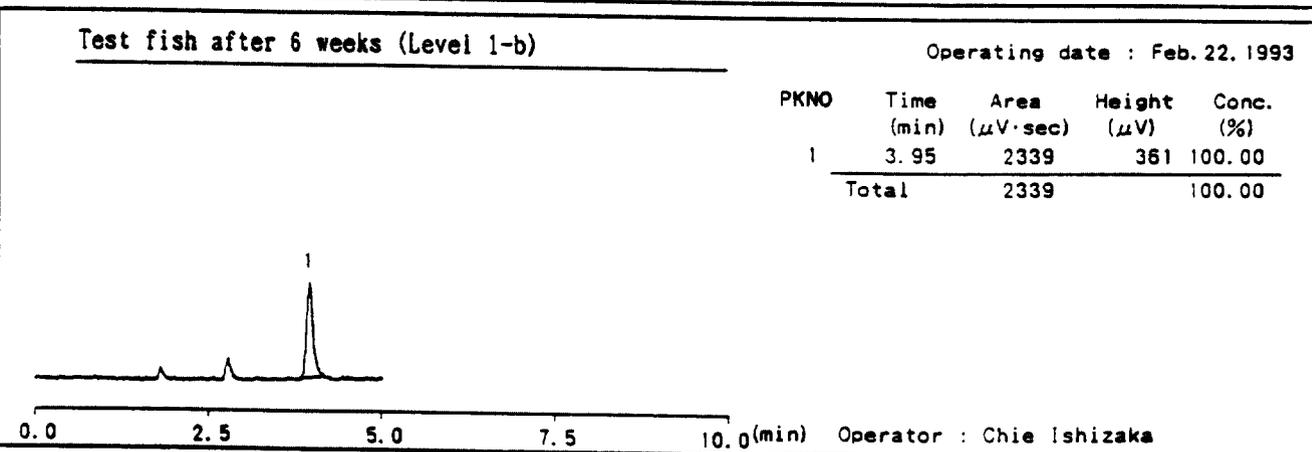
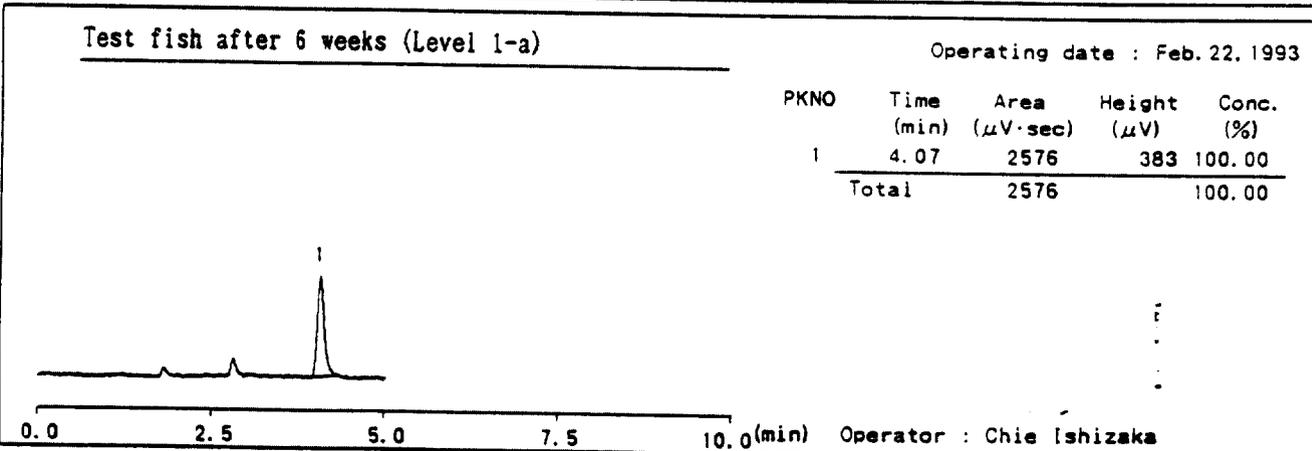
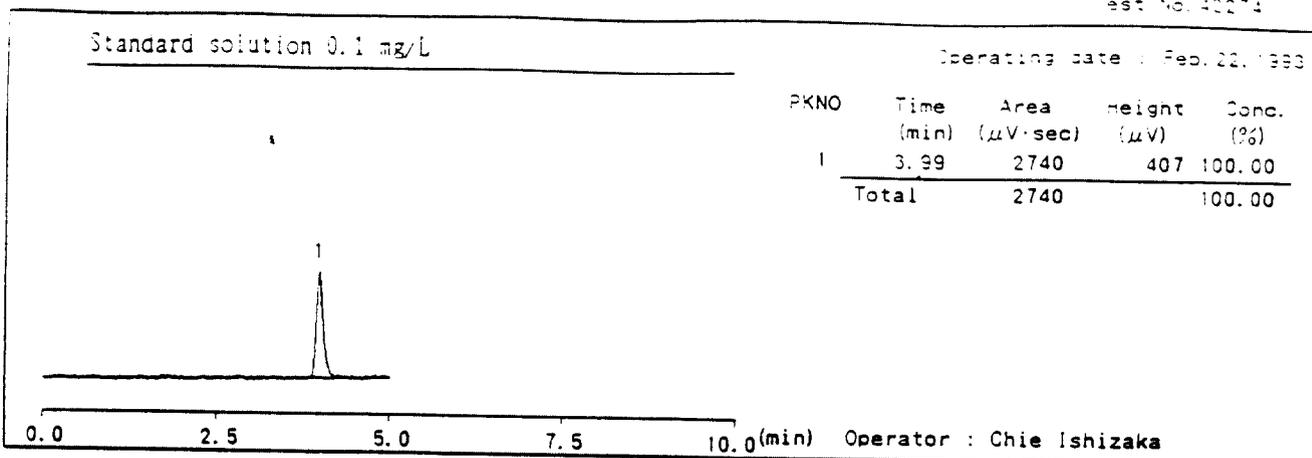
Operator : Chie Ishizaka

Date : Feb. 10, 1993

Name : C Ishizaka

Fig. 8 - 2

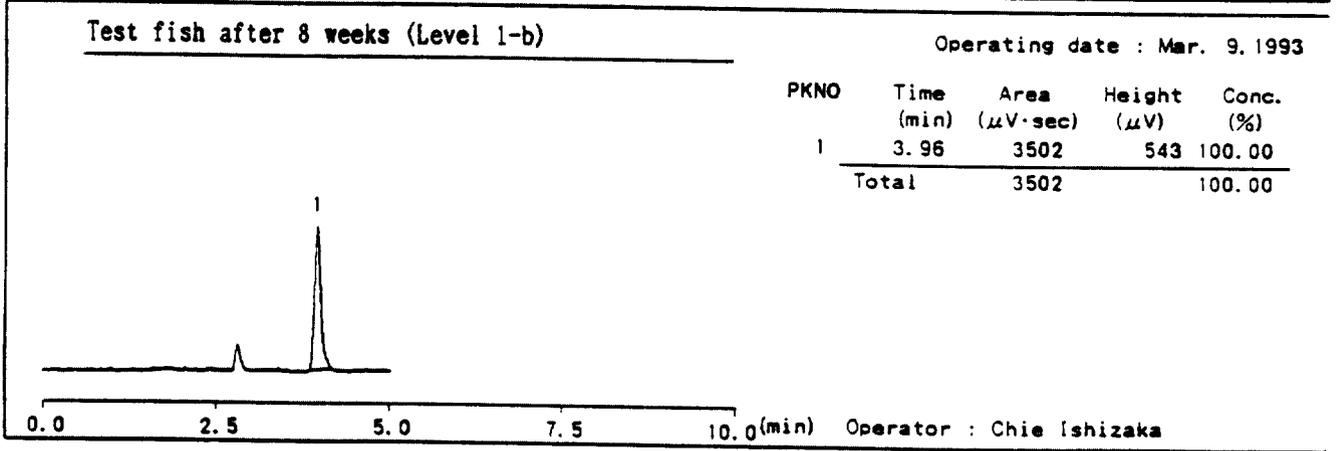
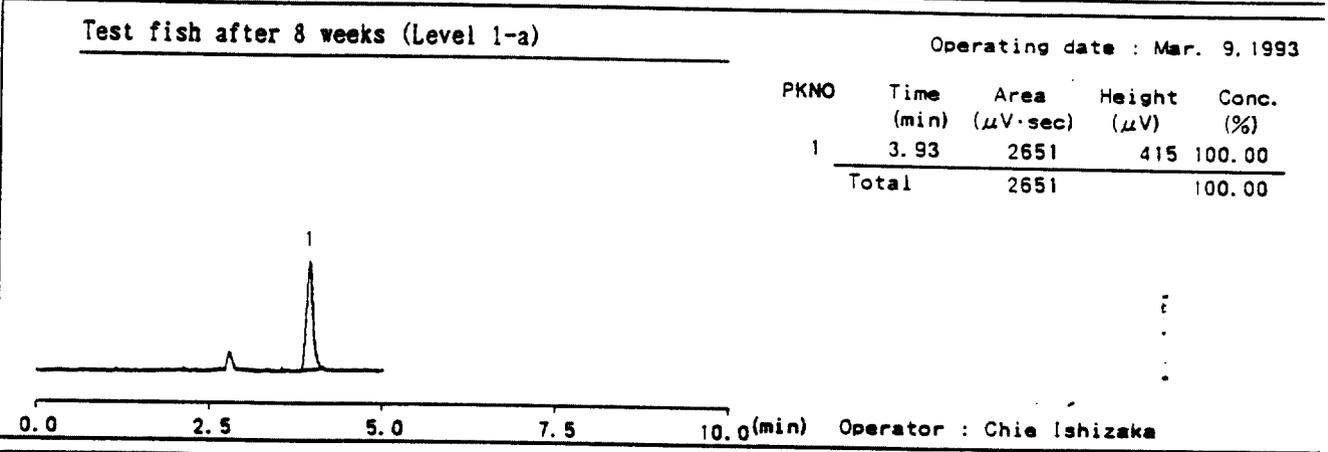
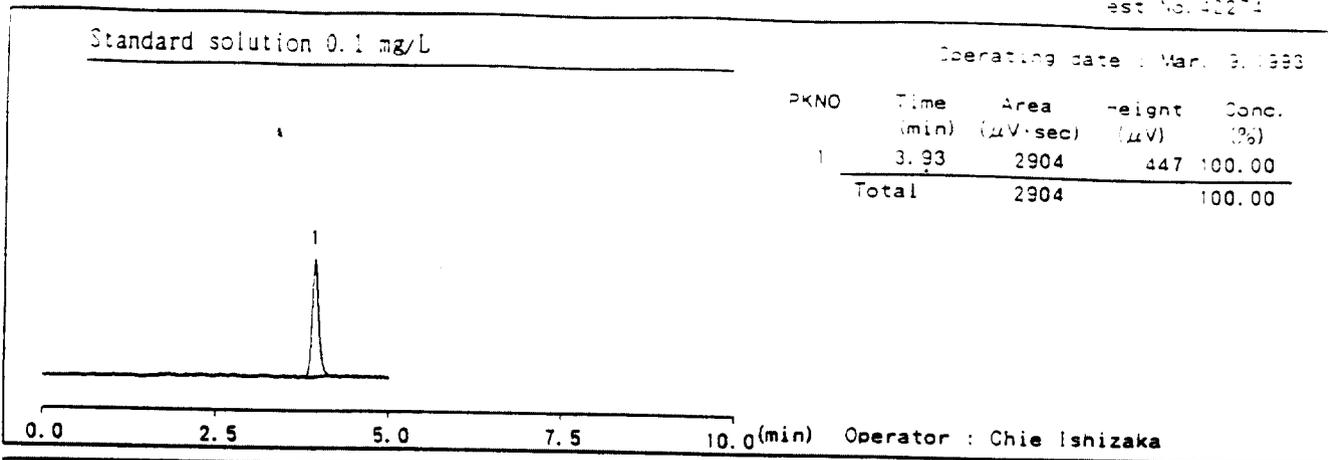
Analysis of test fish (Level 1)



Date : Feb. 22, 1993

Name : C Ishizaka

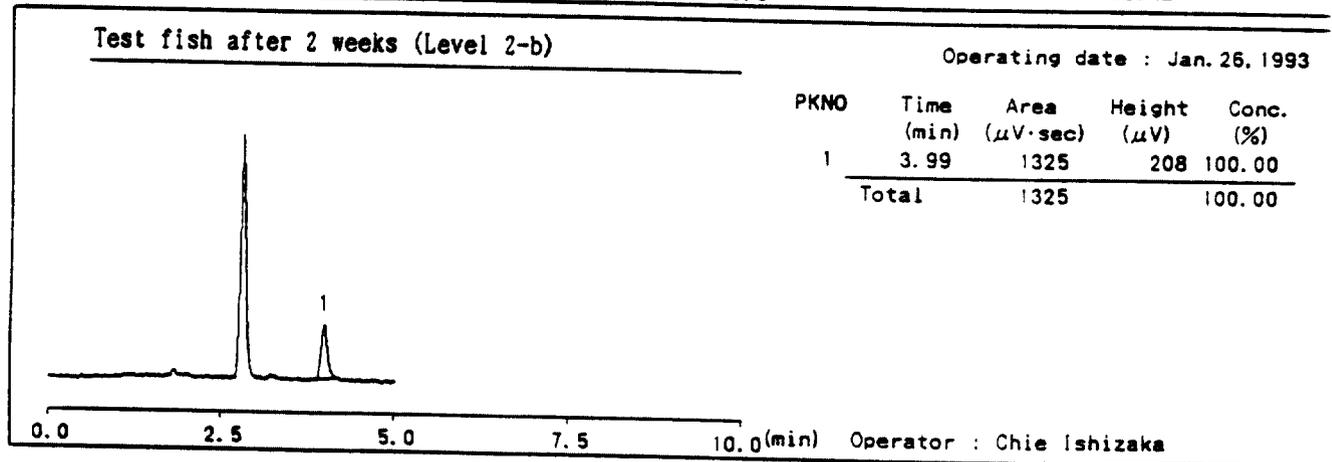
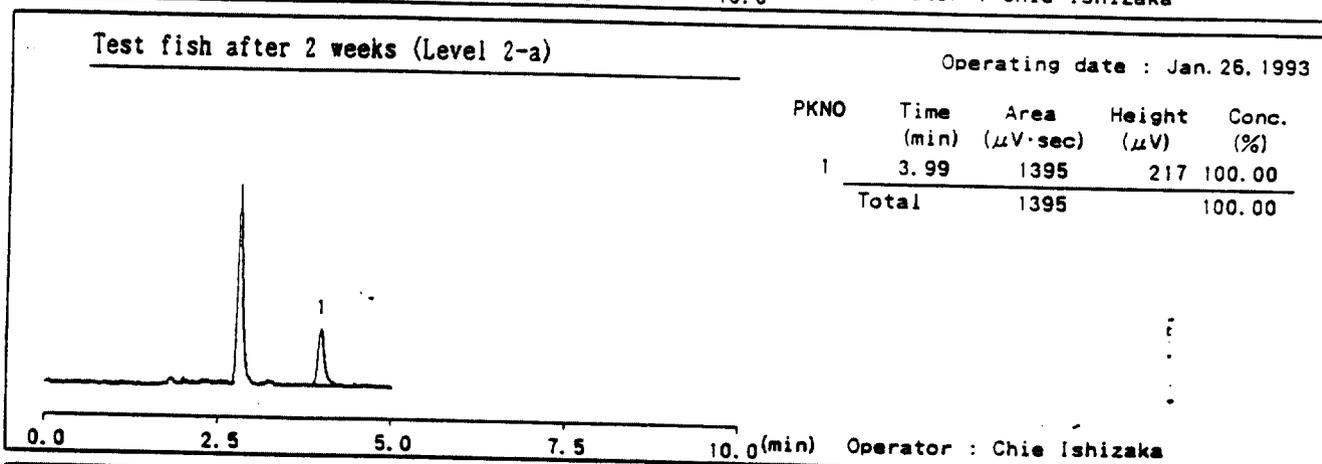
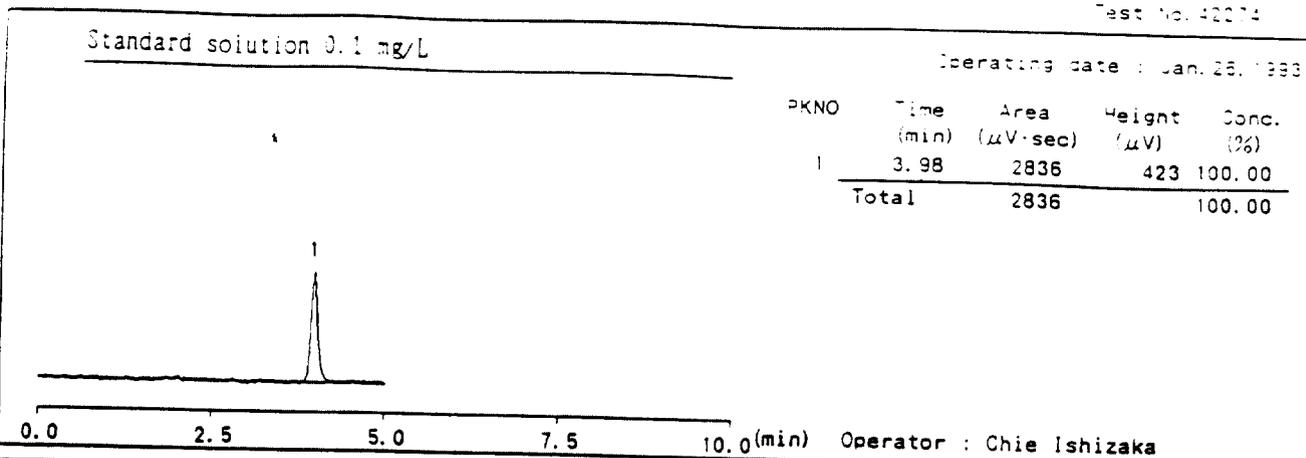
Fig. 8 - 3 Analysis of test fish (Level 1)



Date : Mar. 9, 1993

Name : C Ishizaka

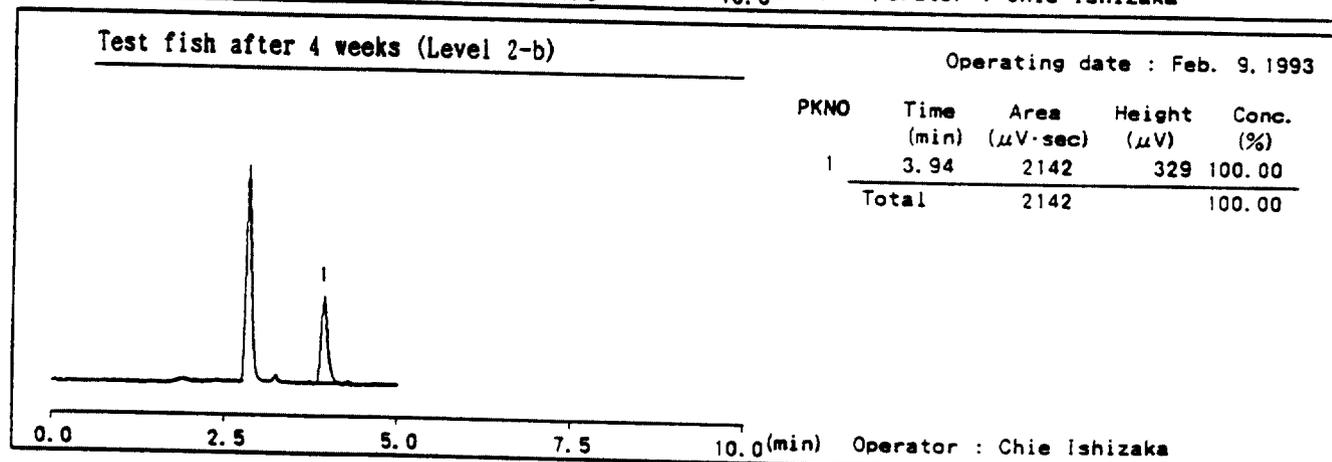
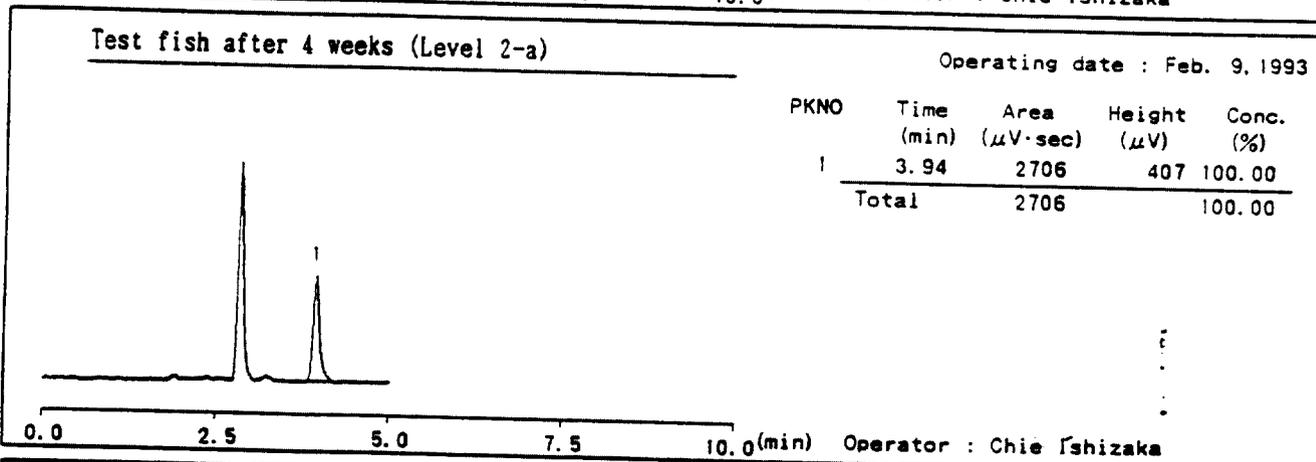
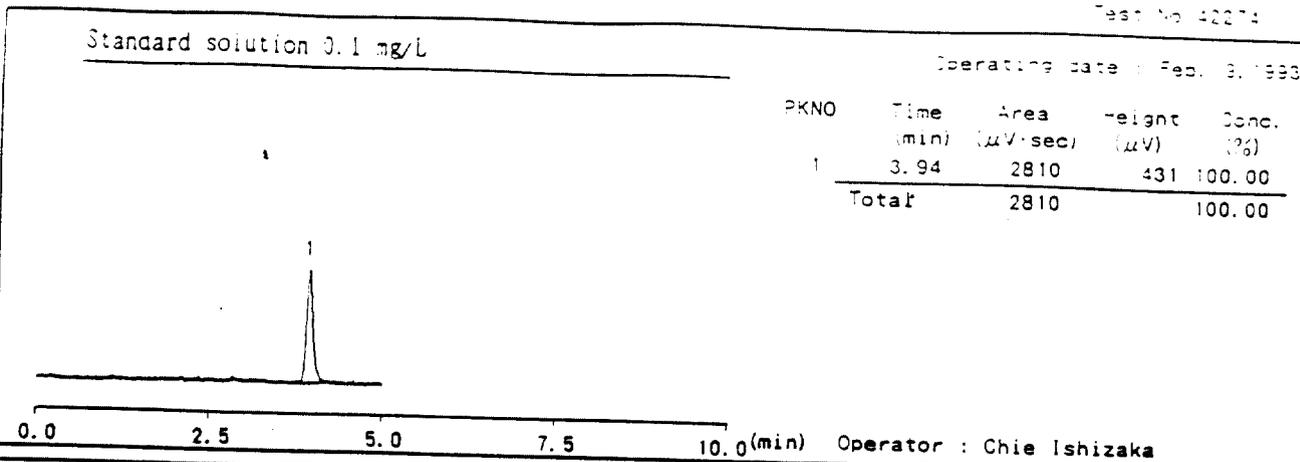
Fig. 8 - 4 Analysis of test fish (Level 1)



Date : Feb. 1, 1993

Name : C Ishizaka

Fig. 9 - 1 Analysis of test fish (Level 2)



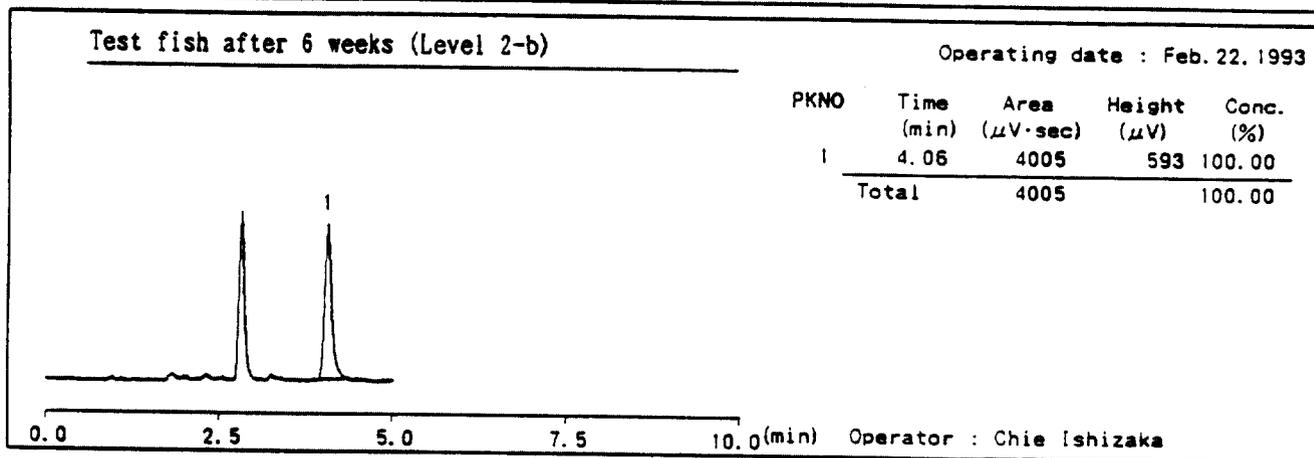
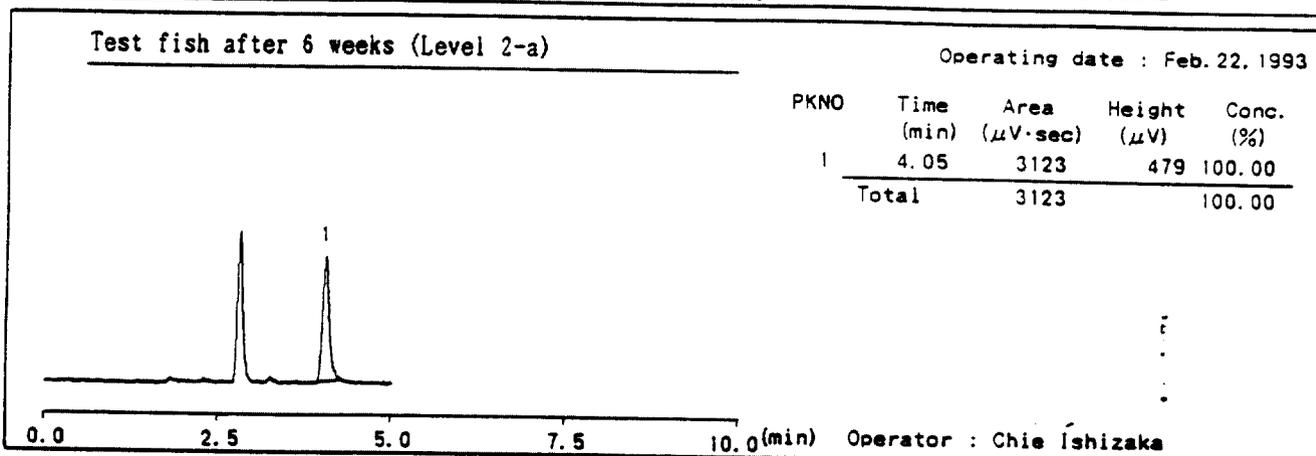
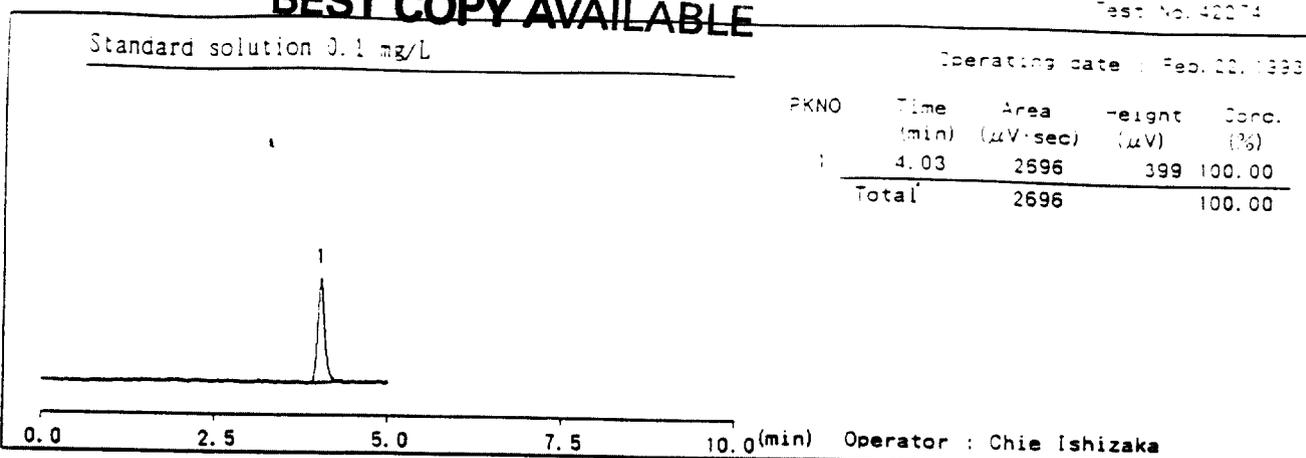
Date : Feb. 10, 1993

Name : C. Ishizaka

Fig. 9 - 2 Analysis of test fish (Level 2)

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Test No. 42274

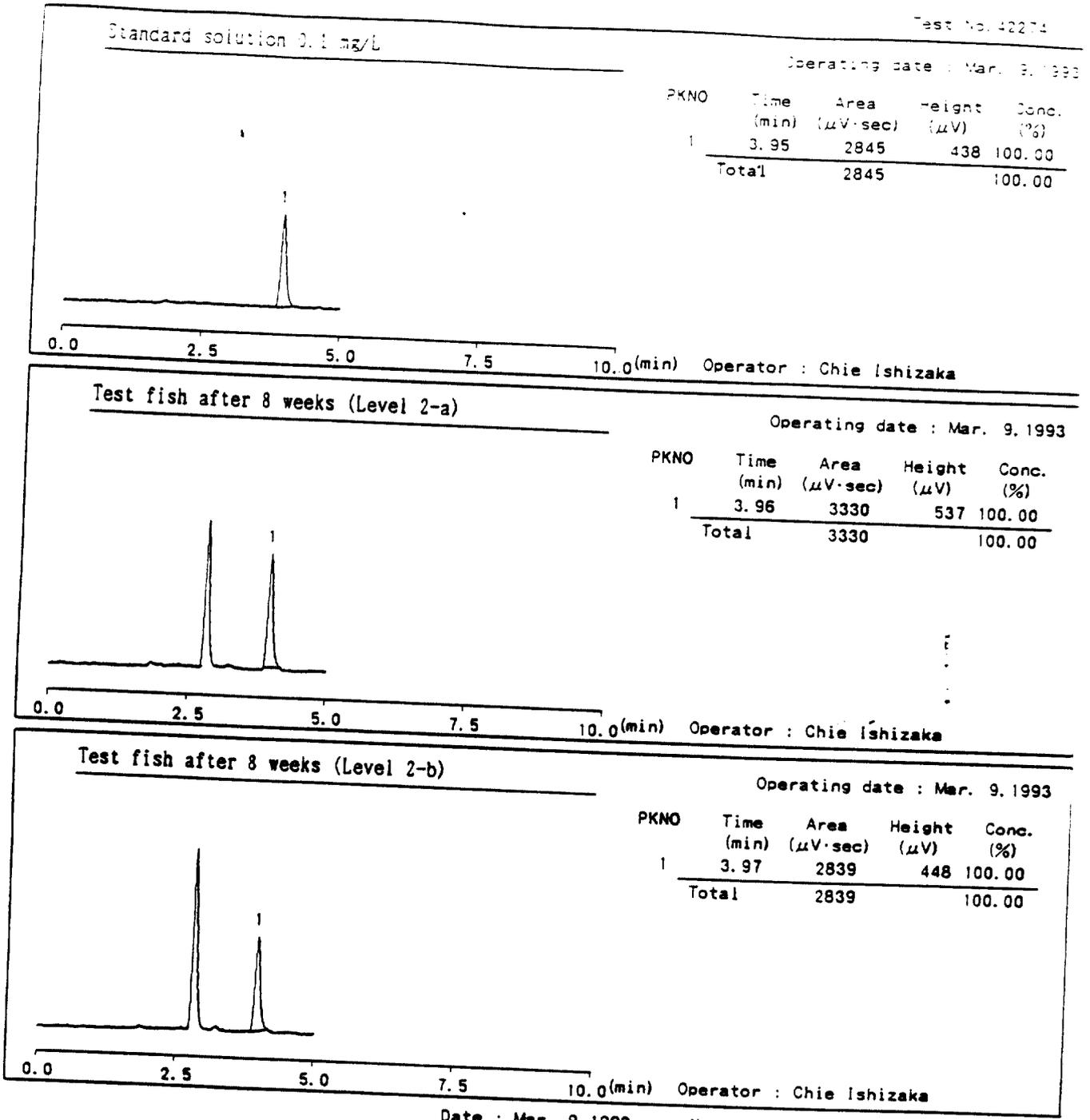


Date : Feb. 22, 1993

Name : C Ishizaka

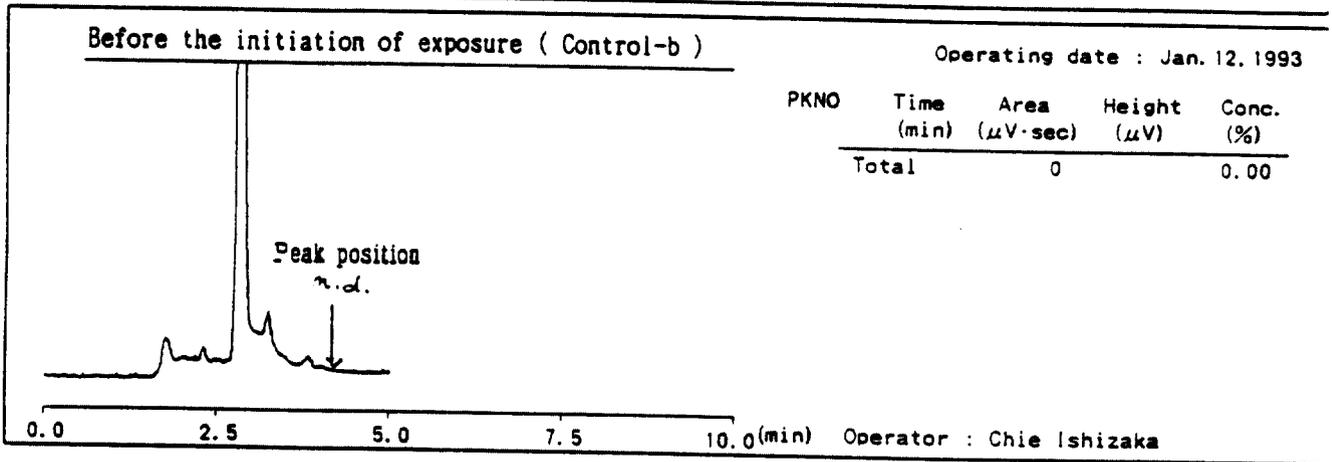
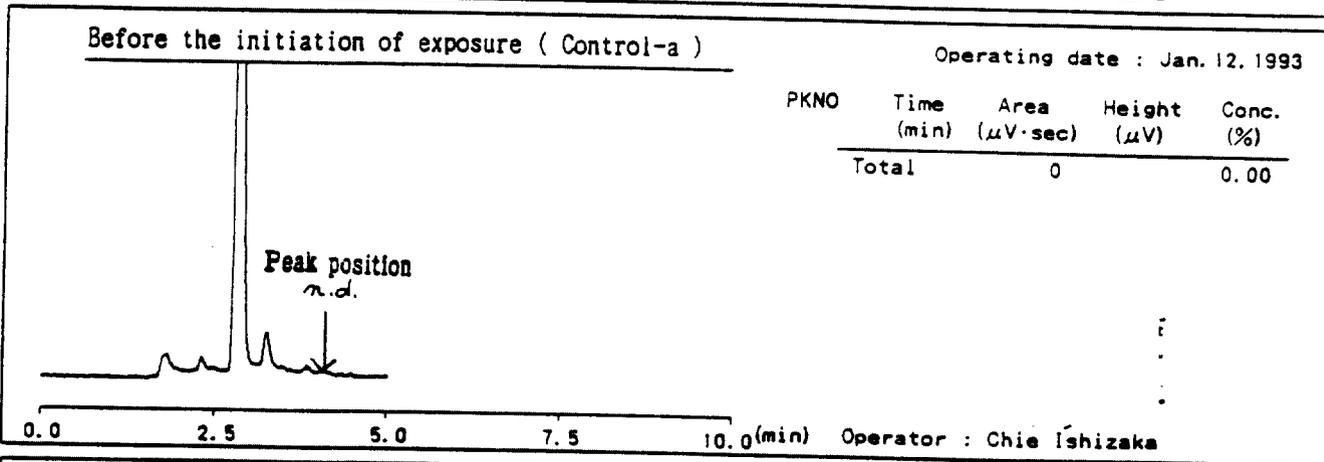
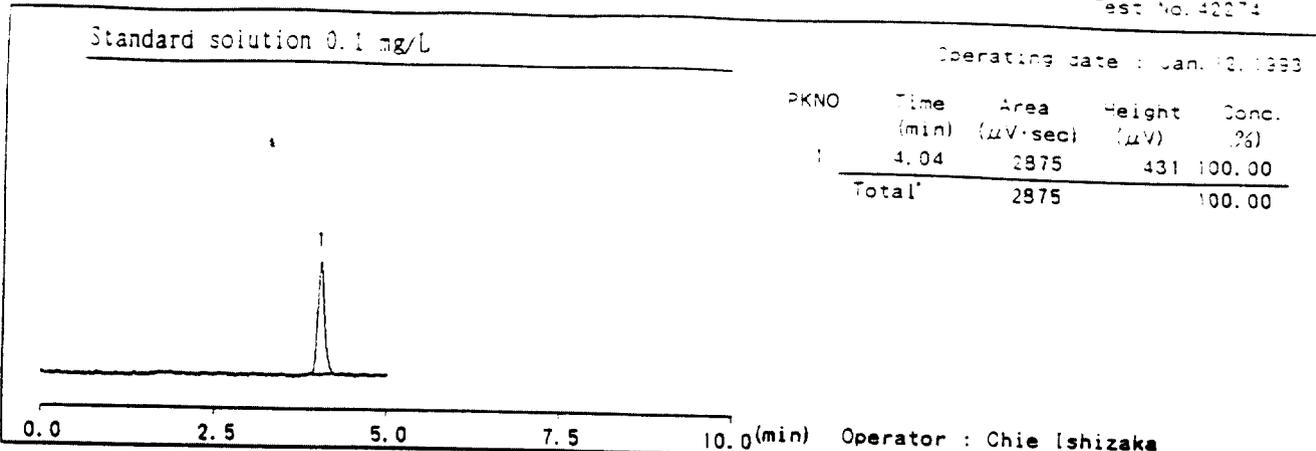
Fig. 9 - 3 Analysis of test fish (Level 2)

BEST COPY AVAILABLE



Name : C. Ishizaka

Fig. 9 - 4 Analysis of test fish (Level 2)

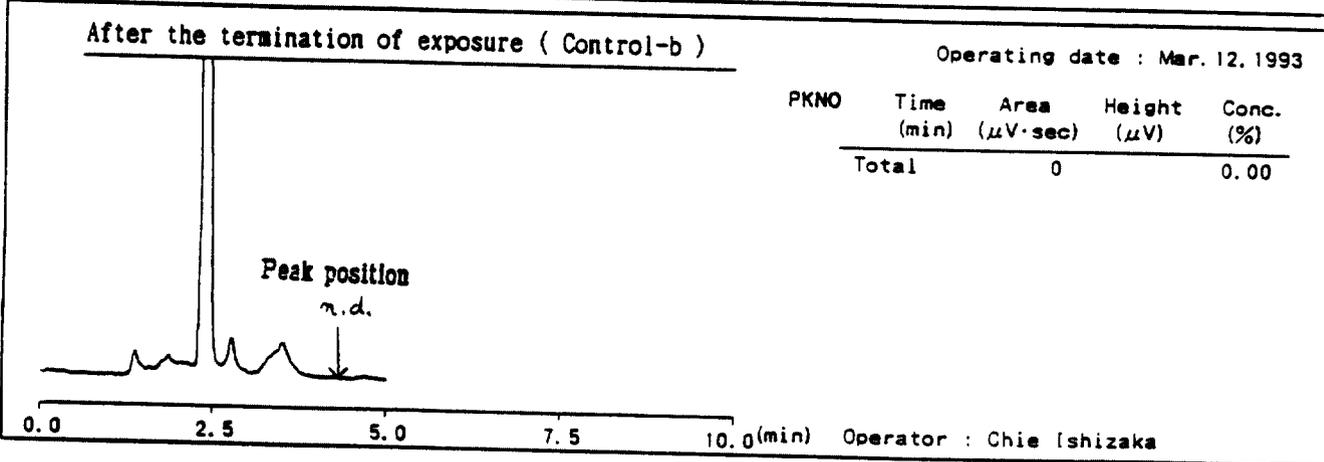
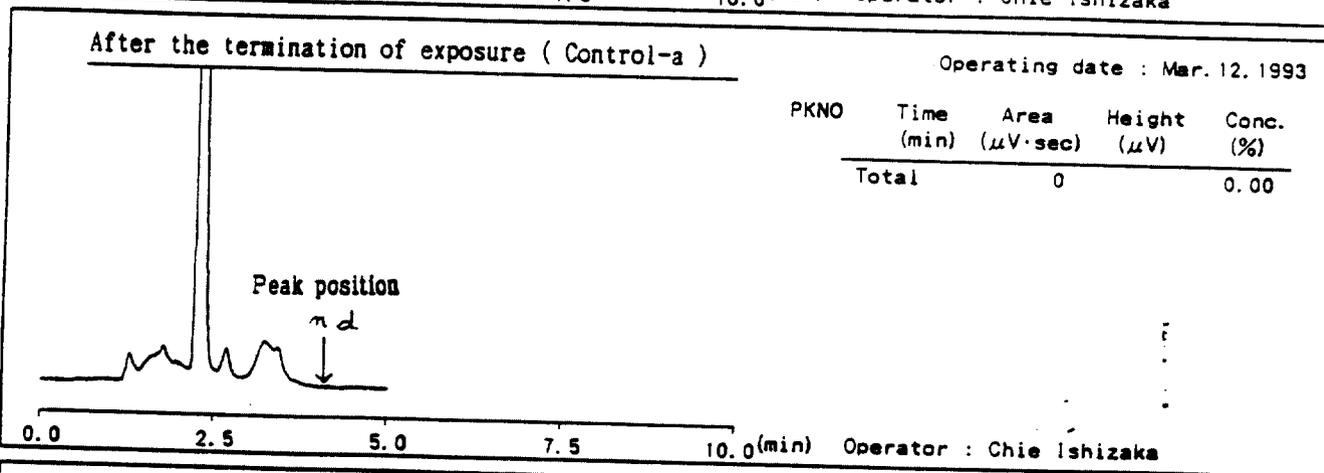
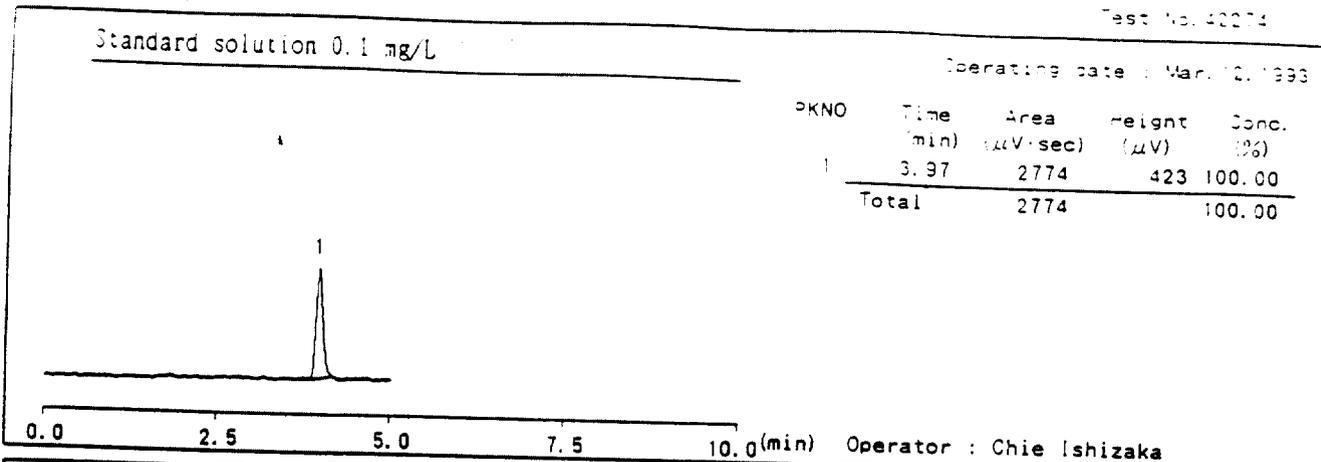


Date : Feb. 1, 1993

Name : C Ishizaka

Fig.10 - 1 Analysis of test fish (Control)

Test No. 42274



Date : Mar. 17, 1993

Name : C. Ishizaka

Fig.10 - 2 Analysis of test fish (Control)

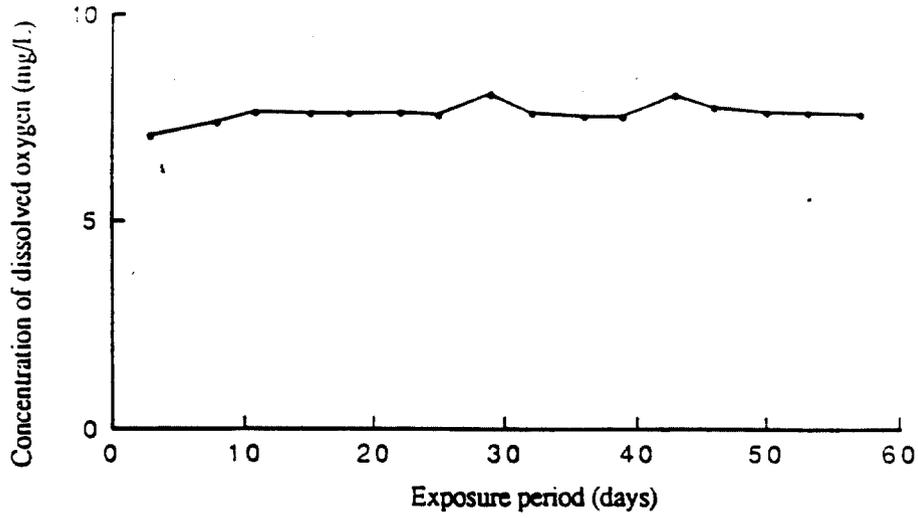


Fig. 11 Concentration of dissolved oxygen (Level 1)

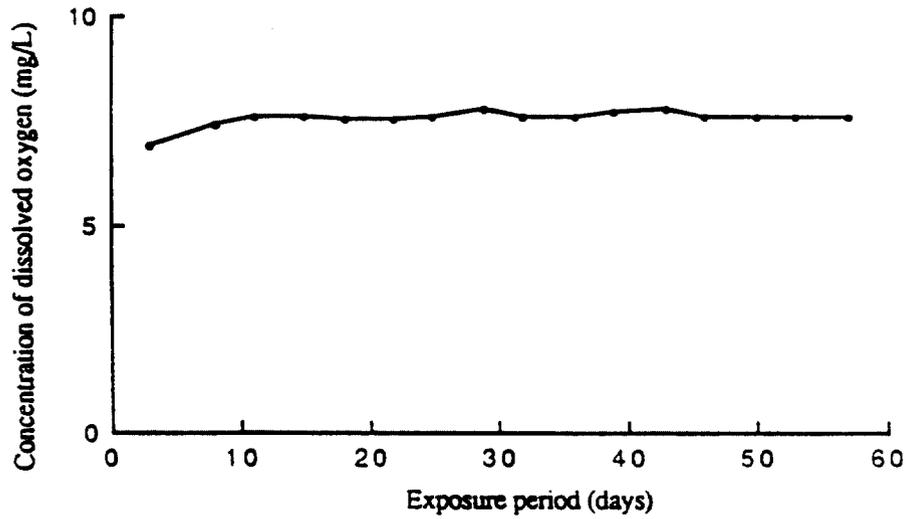


Fig. 12 Concentration of dissolved oxygen (Level 2)

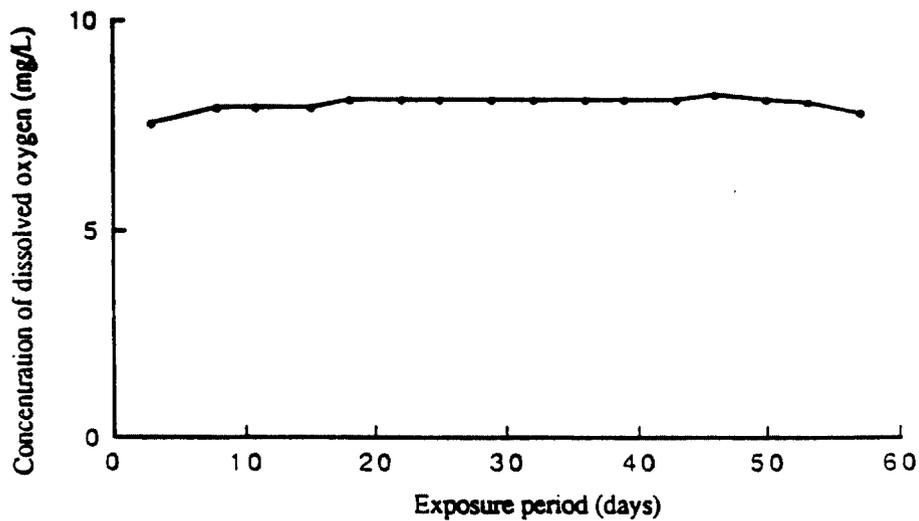


Fig. 13 Concentration of dissolved oxygen (Control)

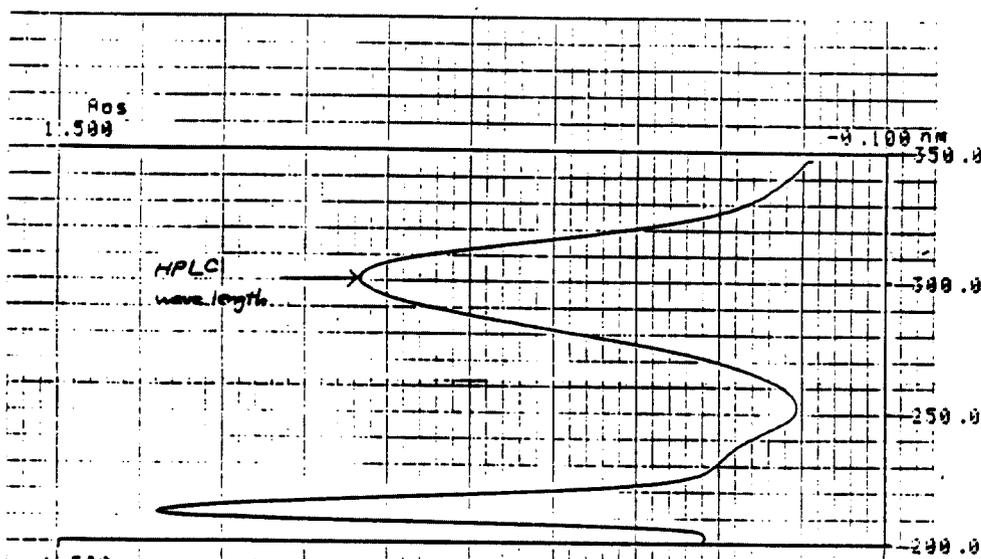
BEST COPY AVAILABLE

Fig. 1

V15

Test No. <u>42274</u>	Date <u>Oct. 2</u> 1992	Instrument <u>HITACHI 150-20</u>
Sample <u>TPA</u>		Slit _____ nm
Solvent <u>Acetonitrile</u>		Scan Speed _____ nm/min
Reference <u>Acetonitrile</u>		Range _____ mV
Photometric Mode <u>Ab</u> %T		Scan-Chart Speed _____ mm/min
Cell <u>10 mm x 10 mm (glass QUARTZ)</u>		Note: UV absorption spectrum
Wave Length <u>200 nm ~ 350 nm</u>		
Scale Limit _____		
Operator <u>C. Ishizaka</u>		

Chemicals Inspection & Testing Institute, Japan Chemical Biotesting Center



1.500 Abs 0.100 nm

350.0

300.0

250.0

200.0

0.100

HPLC wave length

Remarks: 42274 '92.10.2

Scan Speed: 200.0 nm/min Response: Medium No. of Repeat: 1

Cycle Time: xxx

length (nm)

PEAK DETECTION

Peak Window 0.001 Abs

DTTCa 1

No.	WL (nm)	Peak (Abs)	WL (nm)	Valley (Abs)
1	300.0	0.915		
2			252.4	0.071
3	210.8	1.500		
4			202.0	0.247

HITACHI CHART NO. 228-1050

HITACHI CHART NO. 228-1050

BEST COPY AVAILABLE

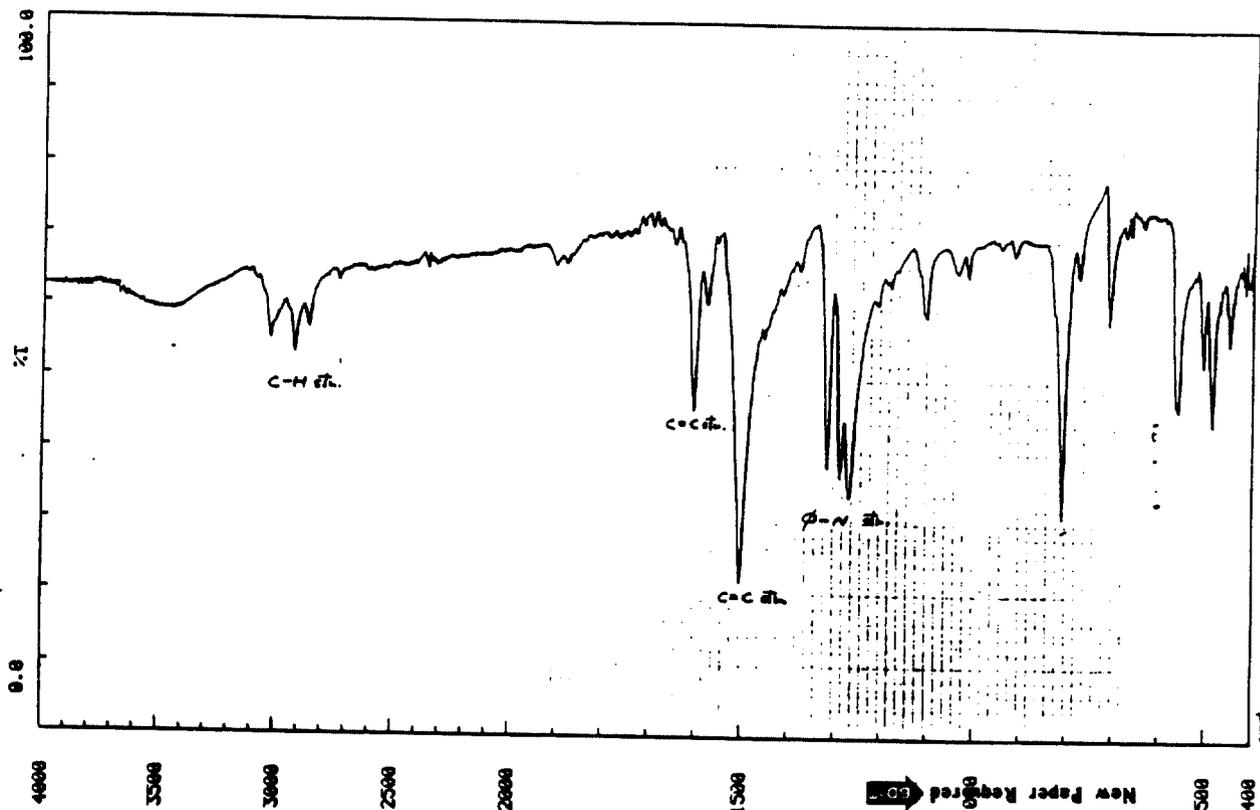


Fig. 15-1

IR spectrum of test substance measured before the initiation of exposure to fish.

Memory no. : 2

KBr tablet

Data mode	%T
Slit Program	W
Scan speed	2
Accumulations	1
# of spectra	1

DATE : '93. 1. 12

OPERATOR : C. Ishizaka

SAMPLE : 42274

V scale 4000 ~ 400.

Ord. scale 0.0 ~ 100.0 %T

Sampling step 2:1 (cm⁻¹)

Grating change 300.0 cm⁻¹

Interval time 0 min

New Paper Required 50%

参 考 資 料

試験番号 42274

1. 表 題 TPAのコイにおける濃縮度試験

2. 試験委託者 名称 []

住所 []

3. 試験施設 名称 財団法人 化学品検査協会
化学品安全センター久留米研究所
住所 (〒830) 福岡県久留米市中央町19-14
TEL (0942) 34-1500
運営管理者 勝 浦 洋

4. 試験目的 TPAの部位別試験

5. 試験期間 部位別試験実施日 平成 5年 3月15日

6. 報告書作成日 平成 5年 3月31日

7. 報告書の承認

試験責任者

平成 5年 3月31日
氏名 藤本 一馬 

8. 部位別試験

被験物質が魚体のいずれの部位に濃縮されているかの知見を得ることを目的とし、実施した。

8週間ばく露した供試魚を外皮（頭部を除く皮、うろこ、ひれ、消化管、えら）、頭部、内臓（消化管以外の臓器）及び可食部（前記の部分を除いた残部）に大別し、各重量を測った後、各部位中の被験物質を分析した。分析法は濃縮度試験の分析法に準じた（表-1, 2, 図-1, 2参照）。

各部位中の被験物質濃度及び濃縮倍率は、有効数字3ケタに丸めて表示した。なお、数値の丸め方はJIS Z 8202-1985 参考3 規則Bの方法に従った。

部位別試験結果

	部 位	各部位中の被験物質濃度 ($\mu\text{g/g}$)	濃縮倍率	付 表	付 図
第1濃度区	外 皮	143	16200	表-1	図-1
		146	16500		
	頭 部	184	20900		
		163	18500		
	内 臓	174	19700		
		230	26200		
可食部	70.4	8010			
	73.2	8320			
第2濃度区	外 皮	12.9	15900	表-2	図-2
		13.5	16700		
	頭 部	26.6	32900		
		30.5	37700		
	内 臓	16.5	20300		
		20.1	24900		
	可食部	10.4	12900		
		10.1	12500		

添付資料

表 - 1 部位別試験第 1 濃度区供試魚分析計算表

表 - 2 部位別試験第 2 濃度区供試魚分析計算表

図 - 1 部位別試験第 1 濃度区供試魚分析 H P L C チャート

図 - 2 部位別試験第 2 濃度区供試魚分析 H P L C チャート

Table-1

Calculation table for analysis of parts of test fish

(Level 1)

Test No. 42274

Sample description	A	D	G	K	H	J
Standard 0.1mg/L	2777					
Teguments* a	2614	25	5.3	143	8.80	16200
Teguments* b	2762	25	5.5	146	8.80	16500
* Including intestine and gill						
Standard 0.1mg/L	2951					
Head a	1435	100	8.5	184	8.80	20900
Head b	1271	100	8.5	163	8.80	18500
Standard 0.1mg/L	2901					
Viscera a	845	50	2.7	174	8.80	19700
Viscera b	913	50	2.2	230	8.80	26200
Standard 0.1mg/L	2866					
Remainder parts a	4791	25	19.1	70.4	8.80	8010
Remainder parts b	4612	25	17.7	73.2	8.80	8320

A : Peak area ($\mu\text{V}\cdot\text{sec}$)

A(std):Standard solution A(t):Sample

B : Ratio of portion used for analysis 5/150

C : Final volume 10mL

D : Dilution factor

E : Average concentration of blank in analysis of control

F : Recovery rate 93.2%

G : Weight of part (g)

K : Concentration of test substance in test fish ($\mu\text{g/g}$)

$$K = P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E$$

H : Average concentration of test substance in test water ($\mu\text{g/l}$)

J : BCF

$$J = (P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E) / H$$

P : Concentration of test substance in standard solution

0.1mg/L

March 16, 1993

Name C. Ishizaka

Table-2

Calculation table for analysis of parts of test fish

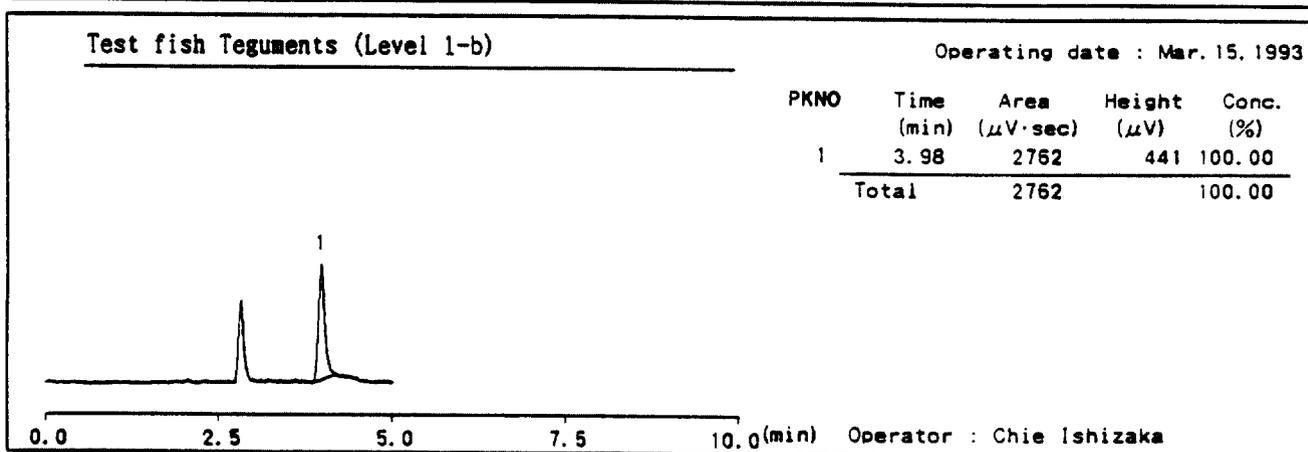
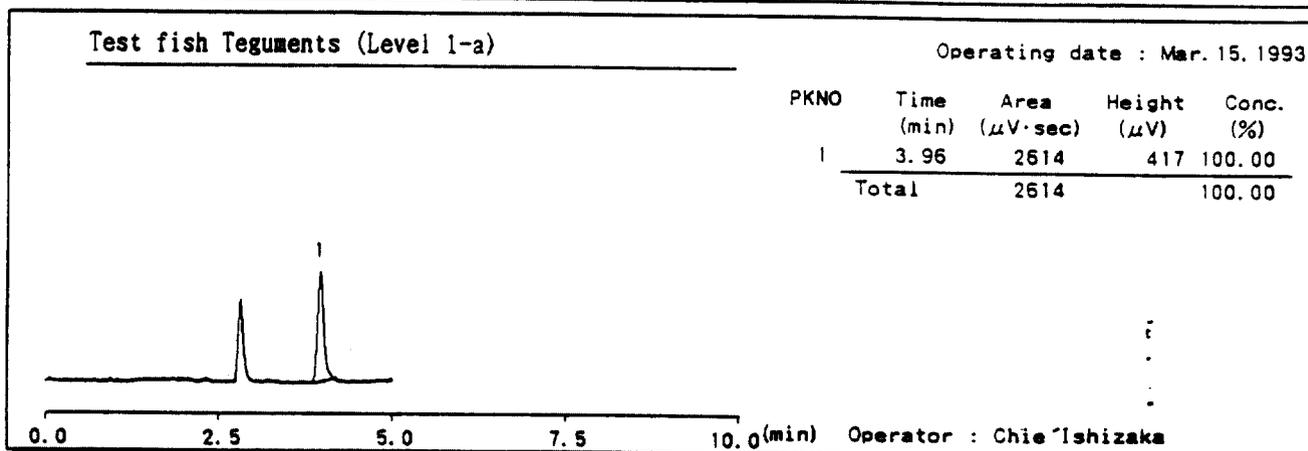
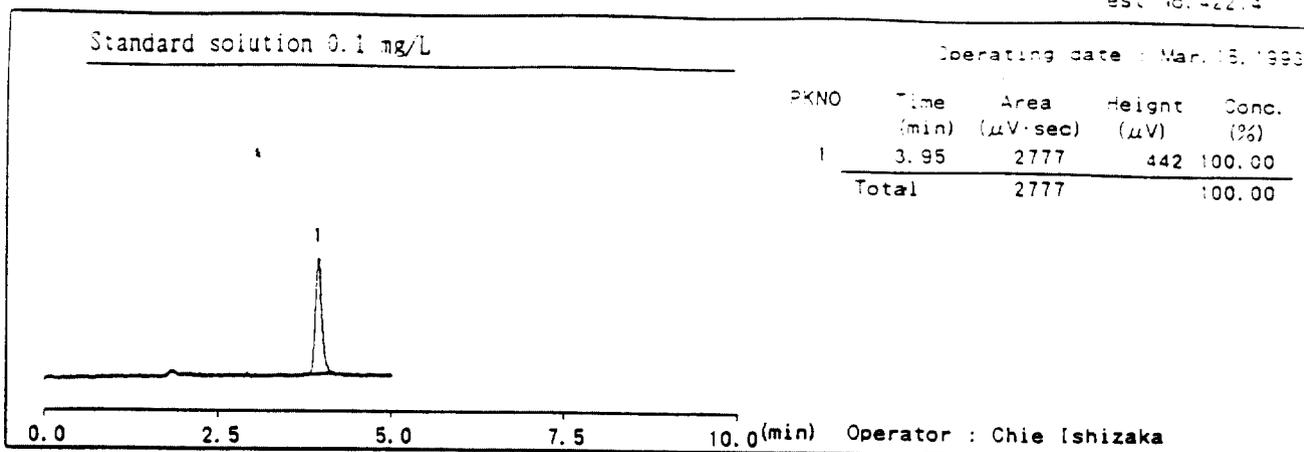
(Level 2)

Test No. 42274

Sample description	A	D	G	K	H	J
Standard 0.1mg/L	2989					
Teguments* a	2688	2	4.5	12.9	0.810	15900
Teguments* b	3068	2	4.9	13.5	0.810	16700
* Including intestine and gill						
Standard 0.1mg/L	2809					
Head a	1789	10	7.7	26.6	0.810	32900
Head b	2264	10	8.5	30.5	0.810	37700
Standard 0.1mg/L	2919					
Viscera a	448	5	1.5	16.5	0.810	20800
Viscera b	730	5	2.0	20.1	0.810	24900
Standard 0.1mg/L	2824					
Remainder parts a	2822	5	15.4	10.4	0.810	12900
Remainder parts b	3075	5	17.3	10.1	0.810	12500
<p>A : Peak area ($\mu\text{V}\cdot\text{sec}$) A(std):Standard solution A(t):Sample</p> <p>B : Ratio of portion used for analysis 5/150</p> <p>C : Final volume 10mL</p> <p>D : Dilution factor</p> <p>E : Average concentration of blank in analysis of control</p> <p>F : Recovery rate 93.2%</p> <p>G : Weight of part (g)</p> <p>K : Concentration of test substance in test fish ($\mu\text{g/g}$) $K = \frac{P \times A(t)}{A(\text{std}) / B \times D \times C / F \times 100 / G - E}$</p> <p>H : Average concentration of test substance in test water ($\mu\text{g/l}$)</p> <p>J : BCF $J = \frac{P \times A(t)}{A(\text{std}) / B \times D \times C / F \times 100 / G - E} / H$</p> <p>P : Concentration of test substance in standard solution 0.1mg/L</p>						

March 30,1993

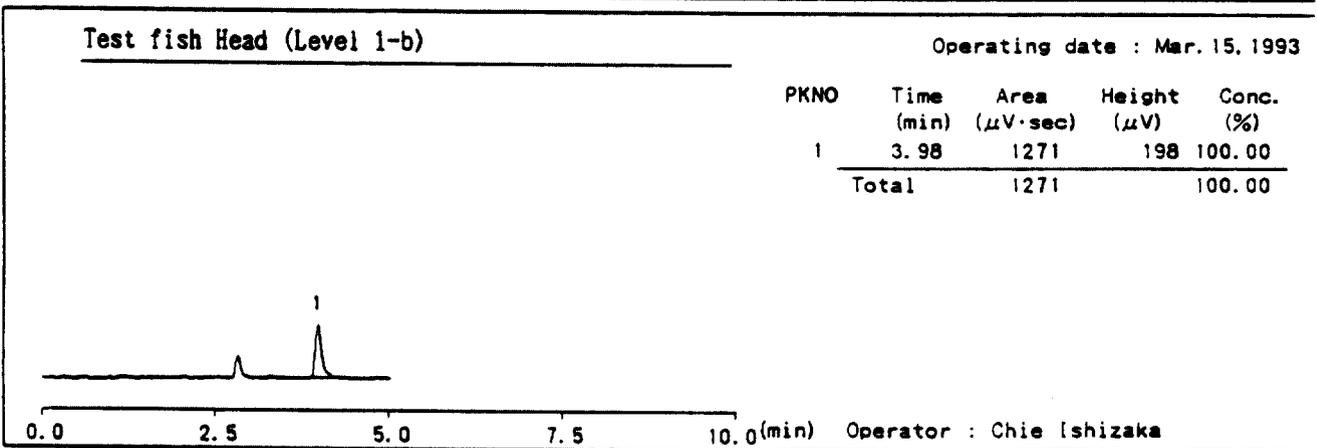
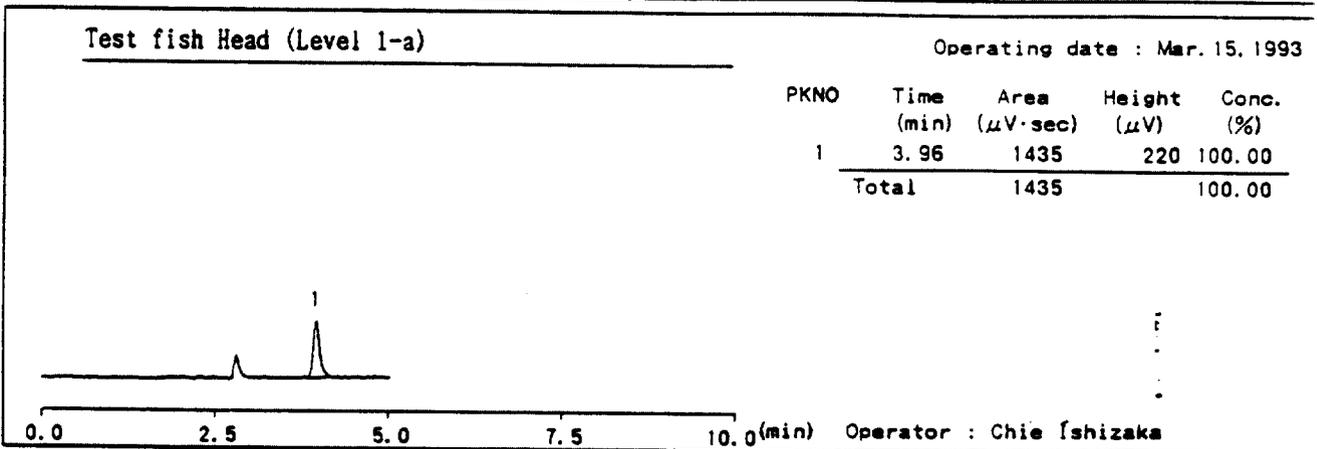
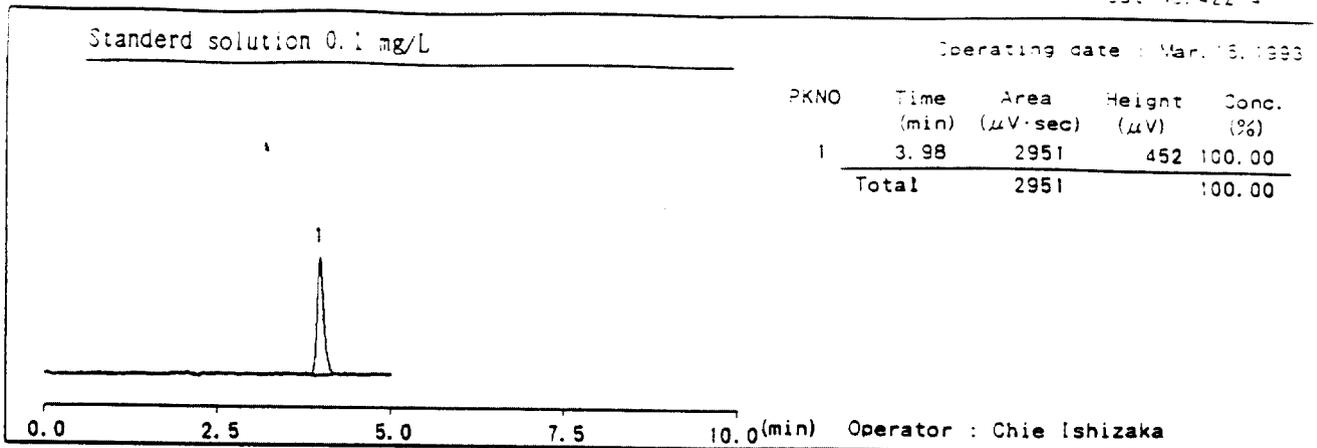
Name C. Ishizaka



Date : Mar. 16, 1993

Name : C. Ishizaka

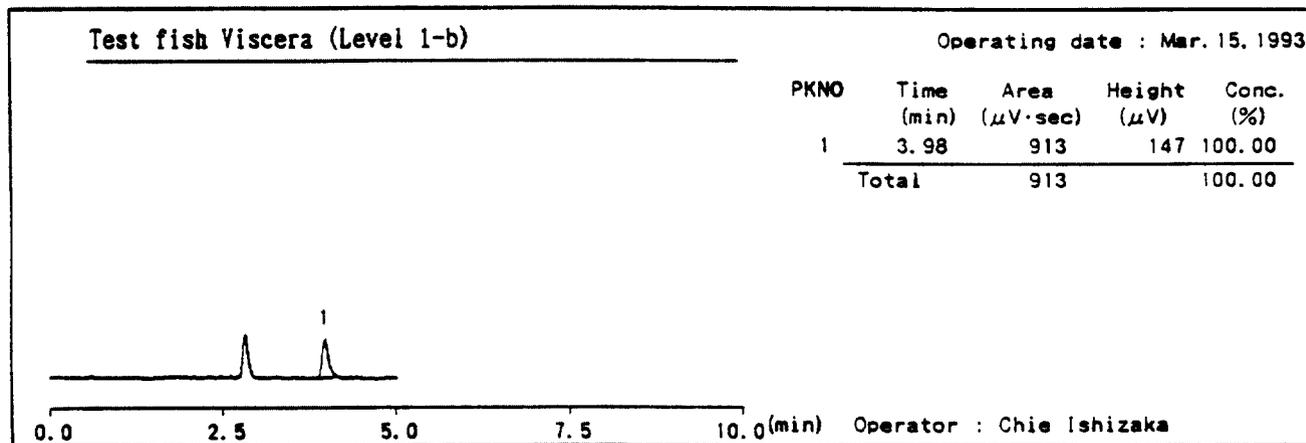
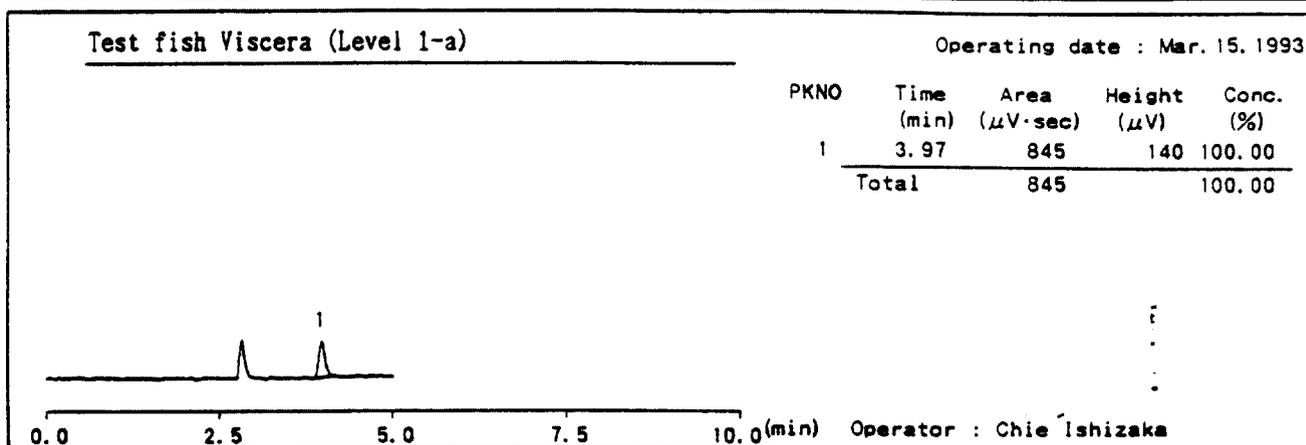
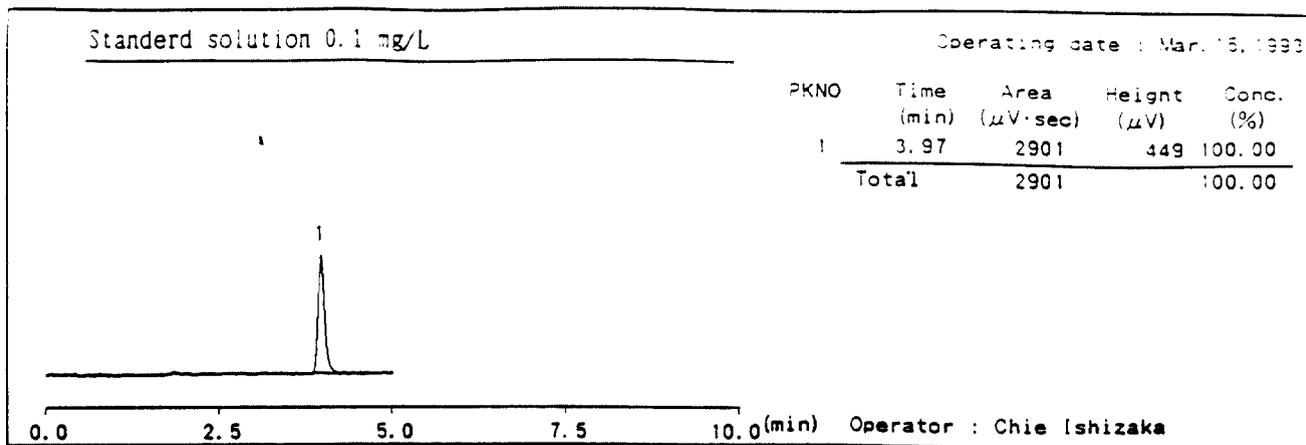
Fig. 1 - 1 Analysis of parts of test fish (Level 1)



Date : Mar. 16, 1993

Name : C. Ishizaka

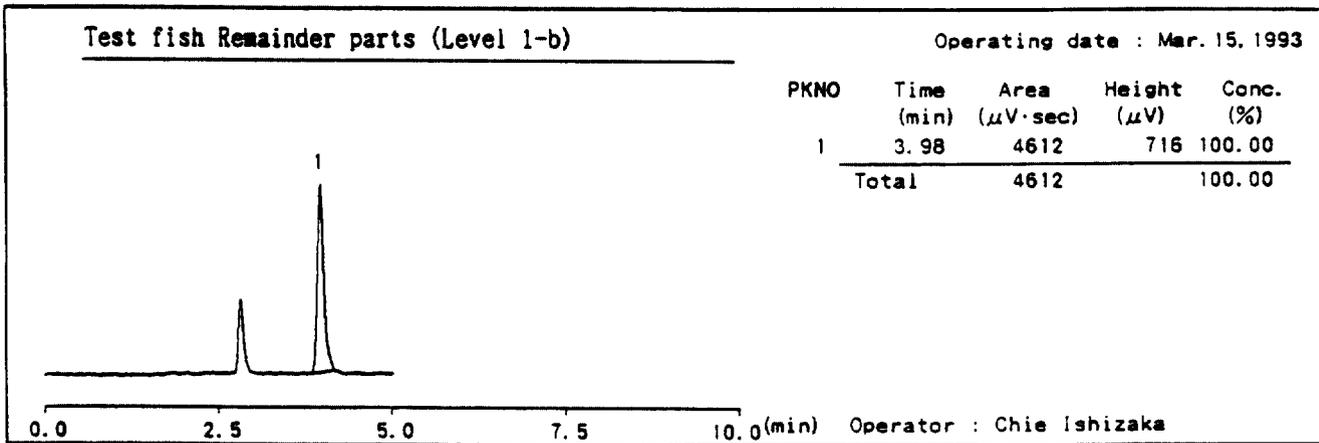
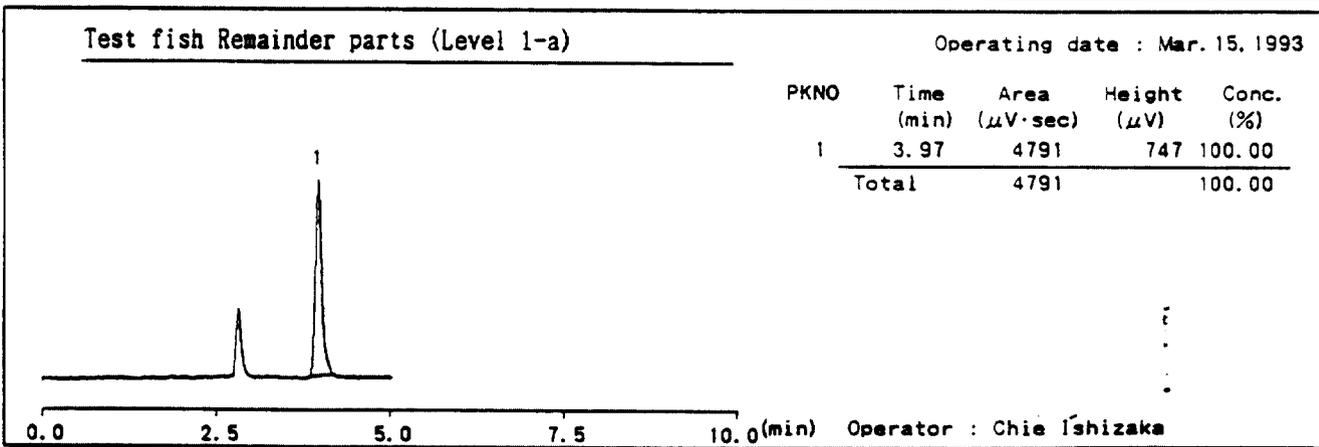
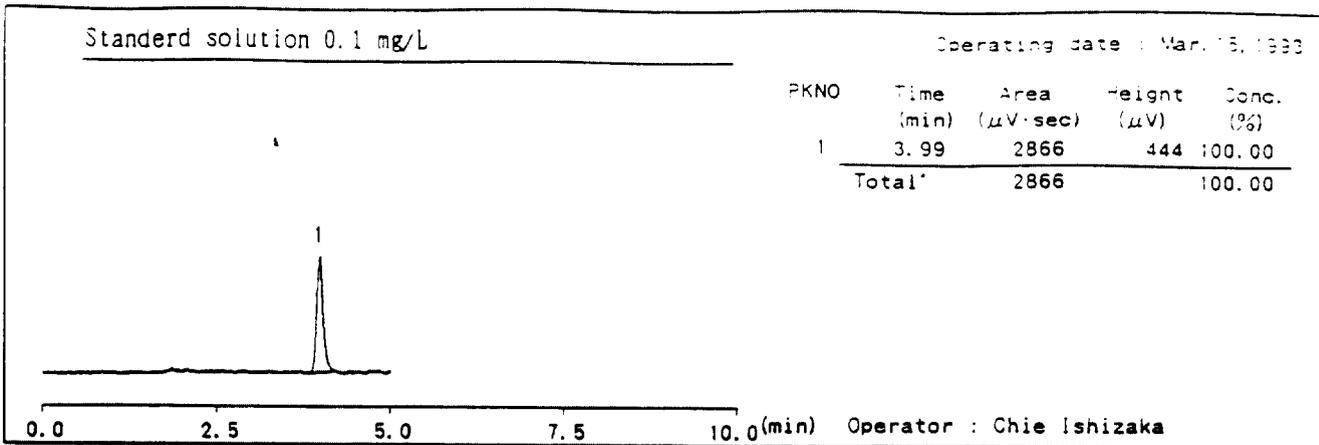
Fig. 1 - 2 Analysis of parts of test fish (Level 1)



Date : Mar. 16, 1993

Name : C. Ishizaka

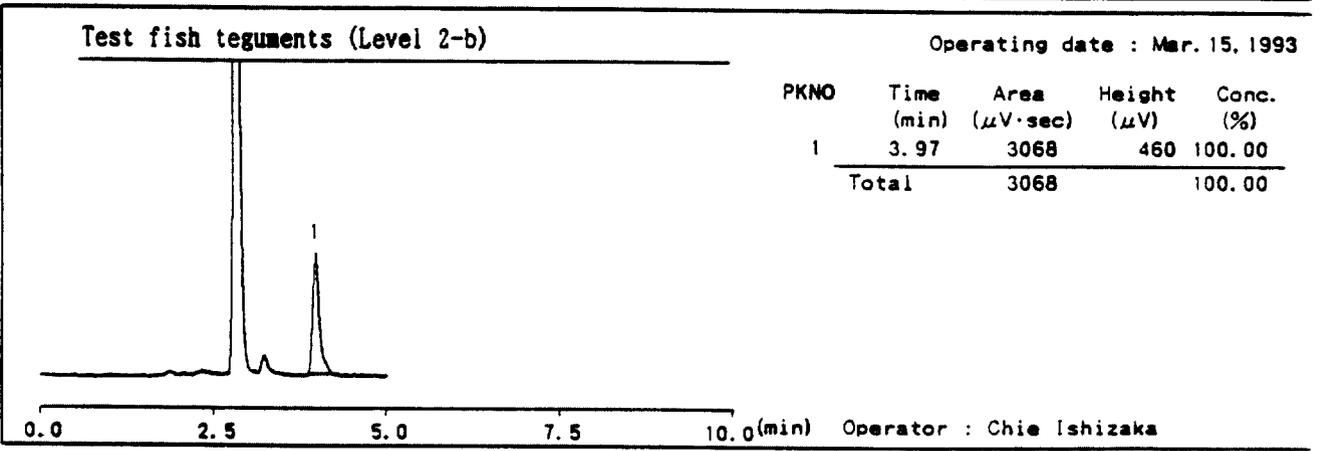
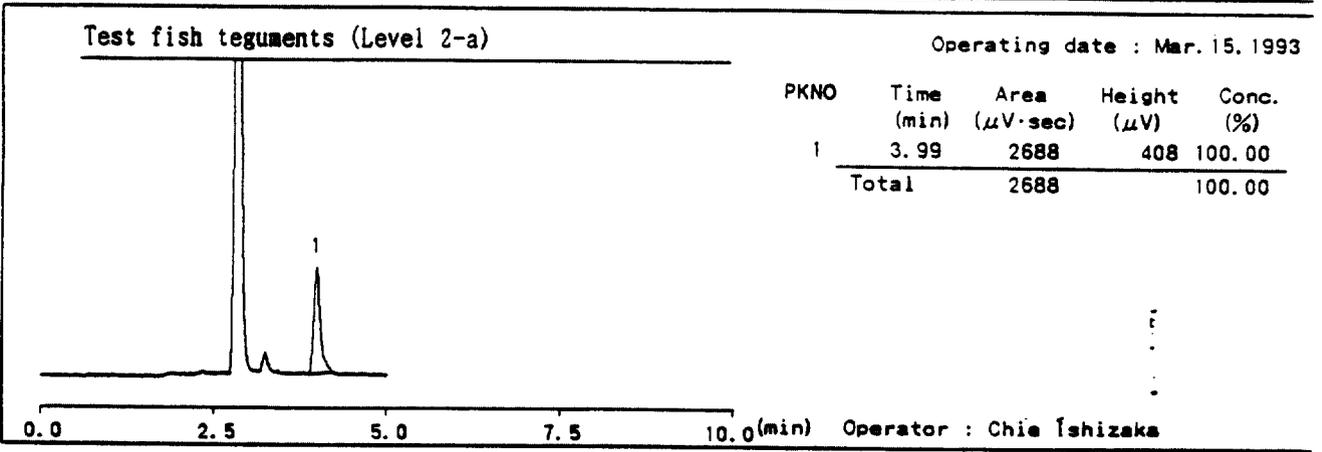
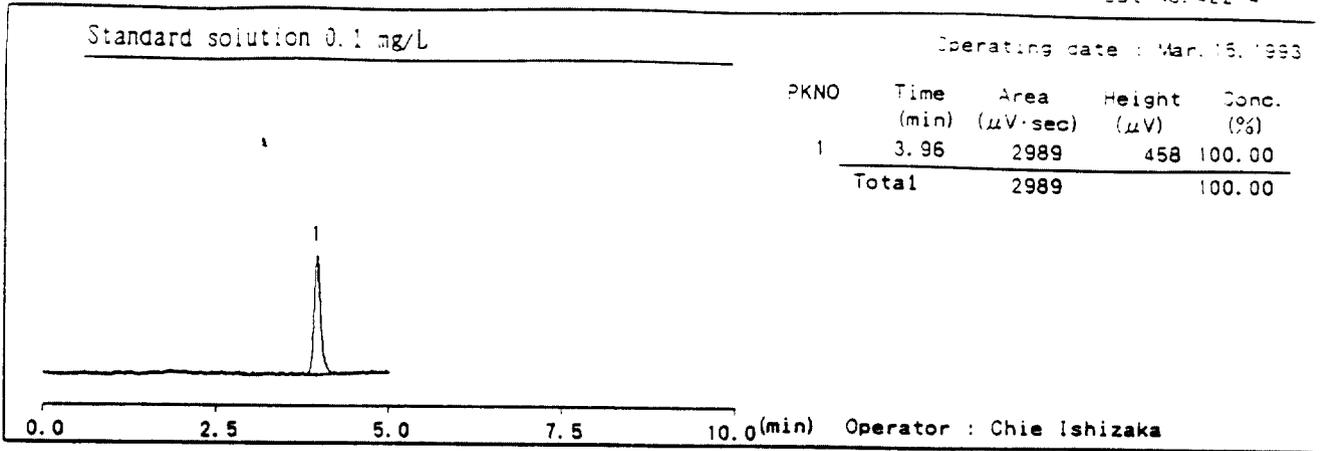
Fig. 1 - 3 Analysis of parts of test fish (Level 1)



Date : Mar. 16, 1993

Name : C Ishizaka

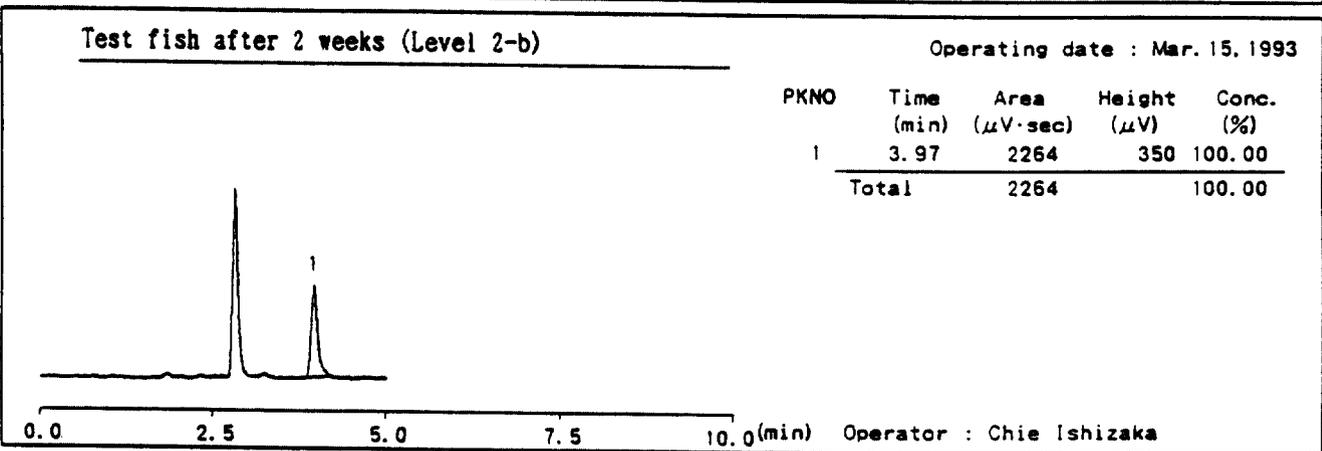
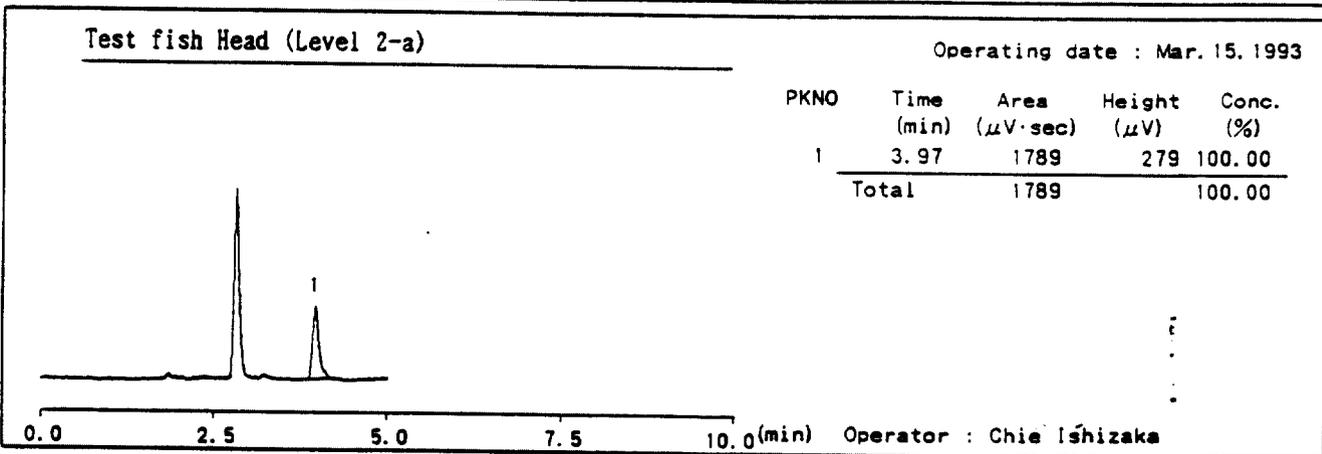
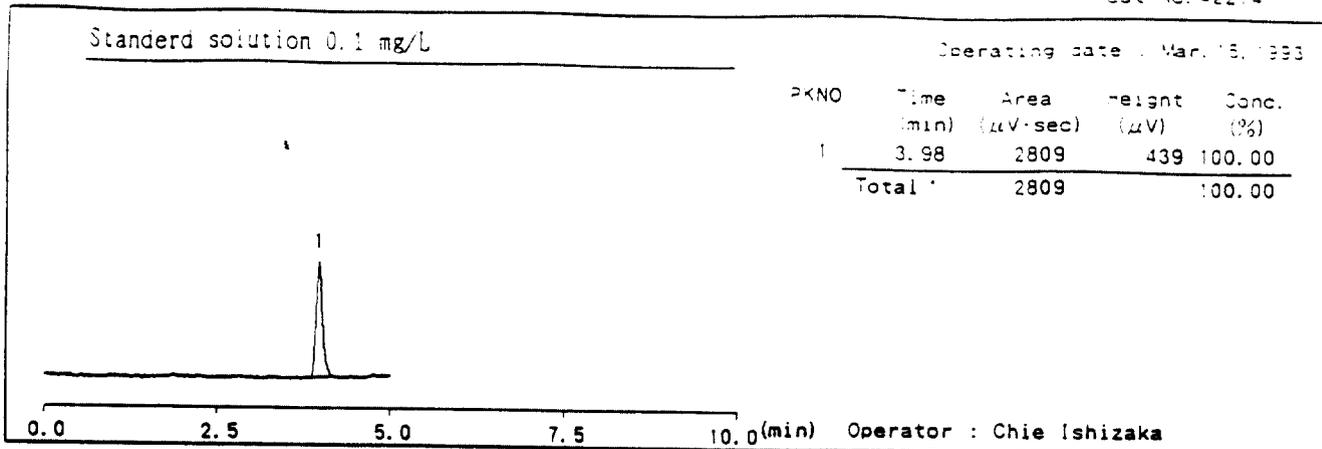
Fig. 1 - 4 Analysis of parts of test fish (Level 1)



Date : Mar. 16, 1993

Name : C Ishizaka

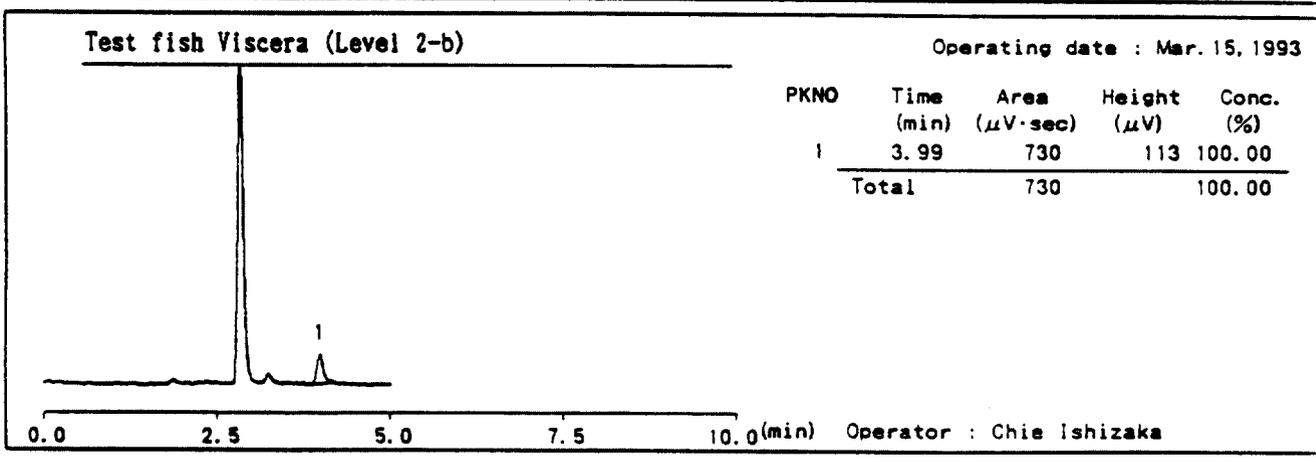
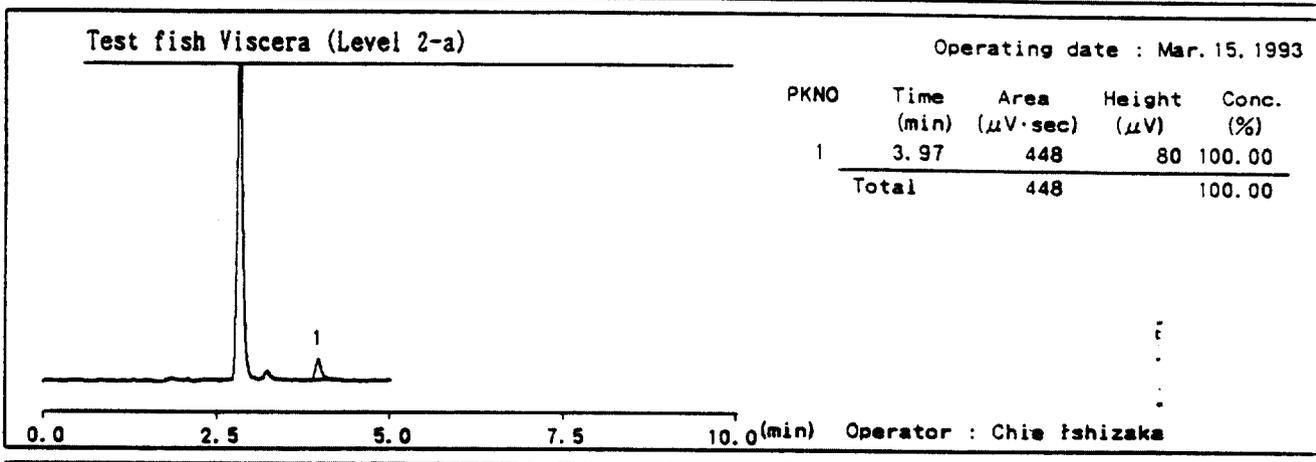
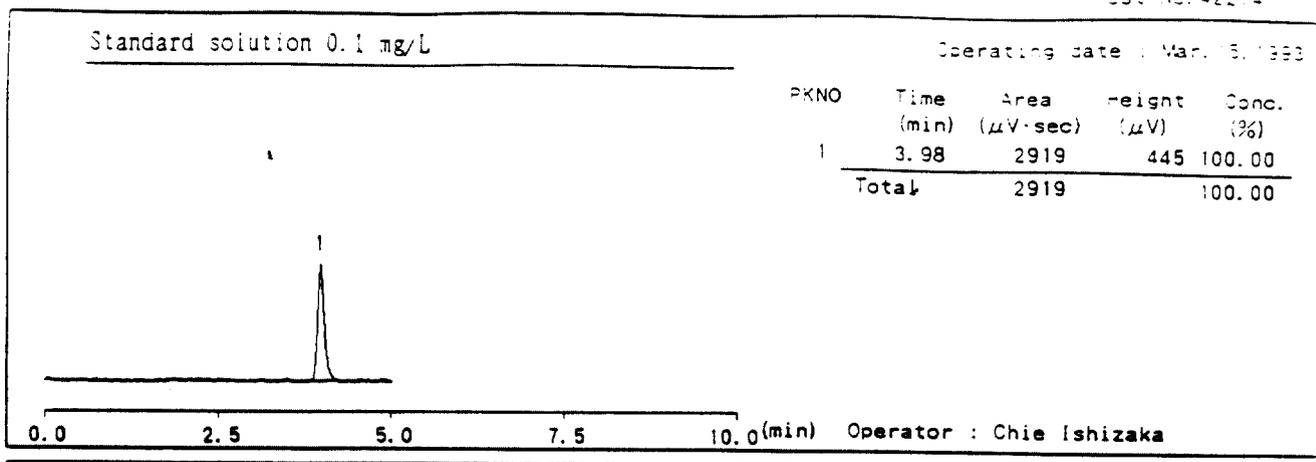
Fig. 2 - 1 Analysis of parts of test fish (Level 2)



Date : Mar. 16, 1993

Name : C. Ishizaka

Fig. 2 - 2 Analysis of parts of test fish (Level 2)



Date : Mar. 16, 1993

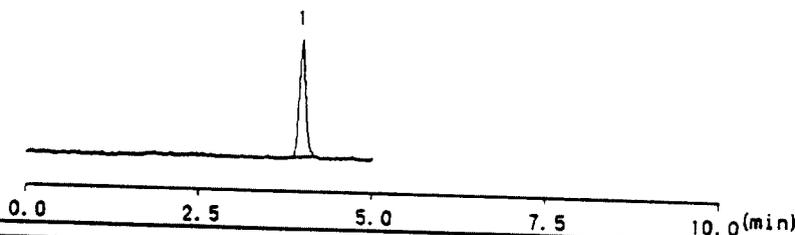
Name : C. Ishizaka

Fig. 2 - 3 Analysis of parts of test fish (Level 2)

Standard solution 0.1 mg/L

Operating date : Mar. 15, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.97	2824	448	100.00
Total		2824		100.00

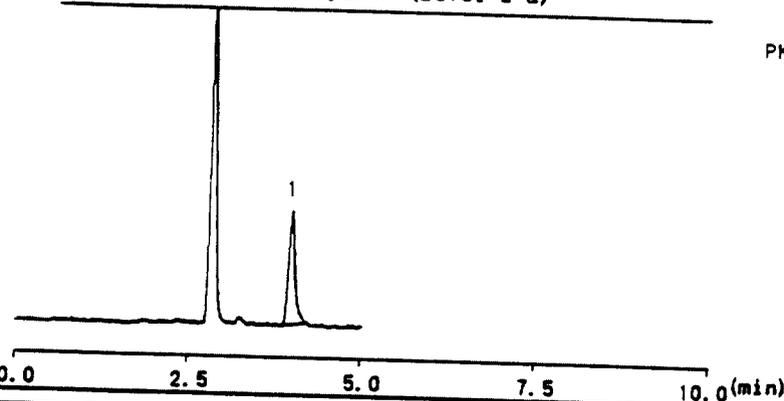


Operator : Chie Ishizaka

Test fish Remainder parts (Level 2-a)

Operating date : Mar. 15, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.99	2822	432	100.00
Total		2822		100.00

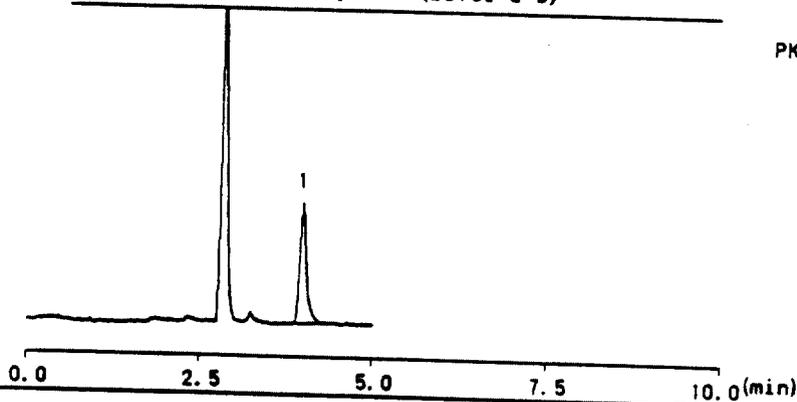


Operator : Chie Ishizaka

Test fish Remainder parts (Level 2-b)

Operating date : Mar. 15, 1993

PKNO	Time (min)	Area ($\mu\text{V}\cdot\text{sec}$)	Height (μV)	Conc. (%)
1	3.98	3075	457	100.00
Total		3075		100.00



Operator : Chie Ishizaka

Date : Mar. 16, 1993

Name : C. Ishizaka

Fig. 2 - 4

Analysis of parts of test fish (Level 2)

参 考 資 料

試験番号 42274

1. 表 題 TPAのコイにおける濃縮度試験

2. 試験委託者 名称 []

住 所 []

3. 試験施設 名称 財団法人 化学品検査協会
化学品安全センター久留米研究所
住 所 (〒830) 福岡県久留米市中央町19-14
TEL (0942) 34-1500
運営管理者 勝 浦 洋

4. 試験目的 TPAの排泄試験

5. 試験期間 (1) 排泄開始日 平成 5年 3月15日

(2) 排泄終了日 平成 5年 3月29日

6. 報告書作成日 平成 5年 3月31日

7. 報告書の承認

試験責任者

平成 5年 3月 31日
氏 名 藤本 一馬 

8. 排泄試験

被験物質が供試魚中から排泄される過程を調べることを目的とし、実施した。

8週間ばく露した供試魚を試験用水（被験物質及び分散剤を含まない水）に移し、供試魚中の被験物質を経時的に分析した。

〈試験及び環境条件〉

試験水槽 100ℓ 容ガラス製水槽

試験水量 試験用水800ml／分の割合で1152ℓ／日を試験水槽に供した。

試験温度 25±2℃

供試魚における被験物質の濃度は、供試魚中の被験物質の分析結果に基づき、次の式により算出した。

$$CF_n = \frac{F_n}{W}$$

CF_n : 排泄開始後 n 日の供試魚中の被験物質濃度

F_n : 排泄開始後 n 日の供試魚中の被験物質絶対量

W : 魚体重

供試魚中の被験物質絶対量及び被験物質濃度は、有効数字3ケタに丸めて表示した。
なお、数値の丸め方はJIS Z 8202-1985 参考3 規則B の方法に従った。

供試魚中の被験物質の残留率は次のように算出した。

ばく露終了時（8週）の供試魚中被験物質濃度の平均値（2尾）を100として、排泄試験開始4、8及び14日後の供試魚中被験物質の残留率（%）を算出した（表-1、2、図-1、2参照）。

残留率

（単位 %）

	4 日 後	8 日 後	1 4 日 後	付 表	付 図
第 1 濃 度 区	117 77.9	50.6 86.8	86.6 73.4	表 - 1	図 - 1
第 2 濃 度 区	58.5 161	115 143	73.1 69.7	表 - 2	図 - 2

添付資料

表 - 1 排泄試験第 1 濃度区供試魚分析計算表

表 - 2 排泄試験第 2 濃度区供試魚分析計算表

図 - 1 排泄試験第 1 濃度区供試魚分析 H P L C チャート

図 - 2 排泄試験第 2 濃度区供試魚分析 H P L C チャート

図 - 3 排泄曲線（第 1 濃度区）

図 - 4 排泄曲線（第 2 濃度区）

Table-1

**Calculation table for analysis of test fish
Depuration test (Level 1)**

Sample description	A	D	G	K	M
Standard 0.1mg/L	2877				
Test fish after 4 days a	3636	100	32.1	127	117%
Test fish after 4 days b	2239	100	29.6	84.6	77.9%
Standard 0.1mg/L	2767				
Test fish after 8 days a	1562	100	33.1	54.9	50.6%
Test fish after 8 days b	2285	100	28.2	94.3	86.8%
Standard 0.1mg/L	2971				
Test fish after 14 days a	4585	50	26.4	94.1	86.6%
Test fish after 14 days b	4133	50	28.1	79.7	73.4%

A :	Peak area ($\mu\text{V}\cdot\text{sec}$)	
	A(std):Standard solution	A(t):Sample
B :	Ratio of portion used for analysis	5/150
C :	Final volume	10mL
D :	Dilution factor	
E :	Average concentration of blank in analysis of control	
F :	Recovery rate	93.2%
G :	Weight of test fish (g)	
K :	Concentration of test substance in test fish ($\mu\text{g/g}$)	
	$K = P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E$	
L :	Average concentration of test substance in test fish after 8 weeks ($\mu\text{g/g}$)	109
M :	Residual rate (%)	
	$M = (P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E) / L$	
P :	Concentration of test substance in standard solution	0.1mg/L

March 29, 1993

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Table-2

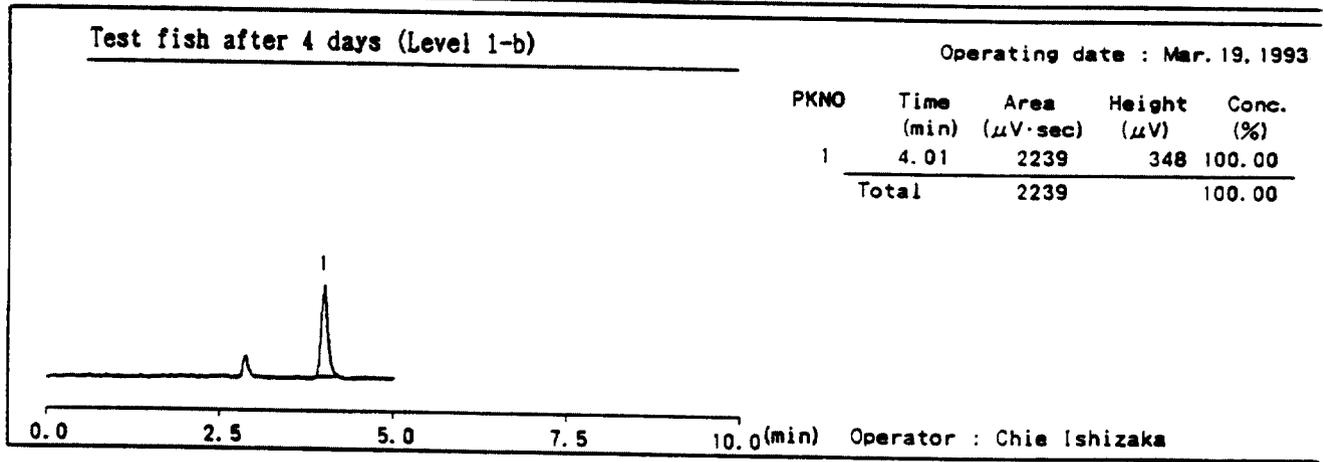
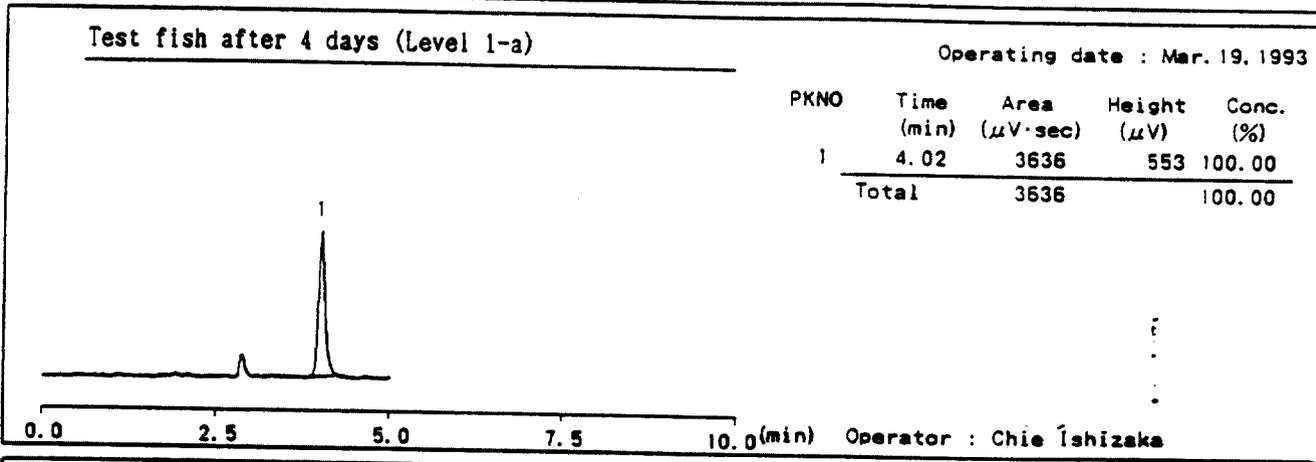
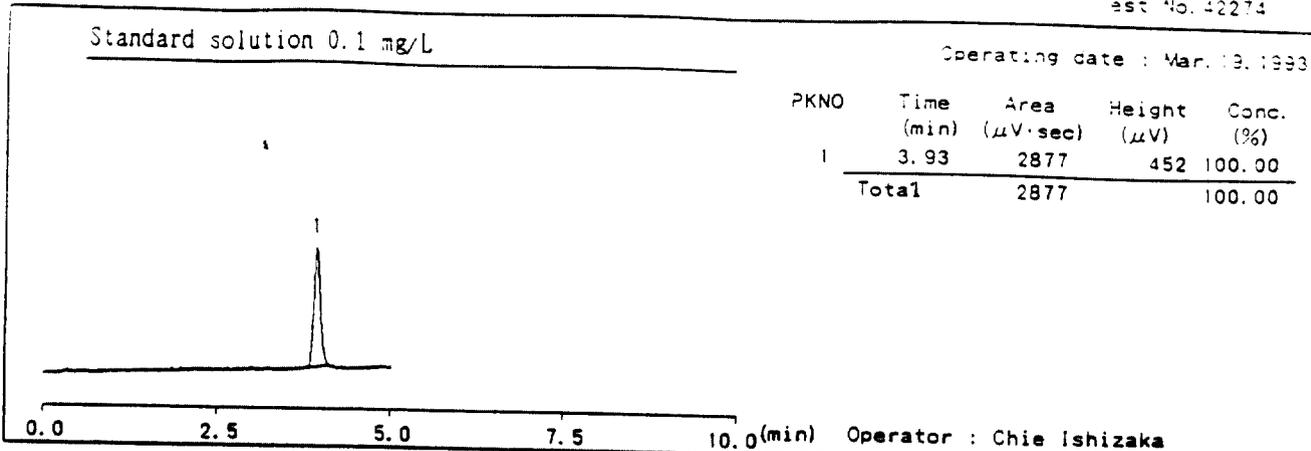
**Calculation table for analysis of test fish
Depuration test (Level 2)**

Sample description	Test No. 42274				
	A	D	G	K	M
Standard 0.1mg/L	2938				
Test fish after 4 days a	2128	10	31.6	7.38	58.5%
Test fish after 4 days b	3146	20	33.9	20.3	161%
Standard 0.1mg/L	2813				
Test fish after 8 days a	3846	10	30.3	14.5	115%
Test fish after 8 days b	4774	10	30.3	18.0	143%
Standard 0.1mg/L	2761				
Test fish after 14 days a	2257	10	28.5	9.23	73.1
Test fish after 14 days b	2075	10	27.5	8.80	69.7%

A :	Peak area ($\mu\text{V}\cdot\text{sec}$)	
	A(std):Standard solution	A(t):Sample
B :	Ratio of portion used for analysis	5/150
C :	Final volume	10mL
D :	Dilution factor	
E :	Average concentration of blank in analysis of control	
F :	Recovery rate	93.2%
G :	Weight of test fish (g)	
K :	Concentration of test substance in test fish ($\mu\text{g/g}$)	
	$K = P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E$	
L :	Average concentration of test substance in test fish after 8 weeks ($\mu\text{g/g}$)	12.6
M :	Residual rate (%)	
	$M = (P \times A(t) / A(\text{std}) / B \times D \times C / F \times 100 / G - E) / L$	
P :	Concentration of test substance in standard solution	0.1mg/L

March 29, 1993

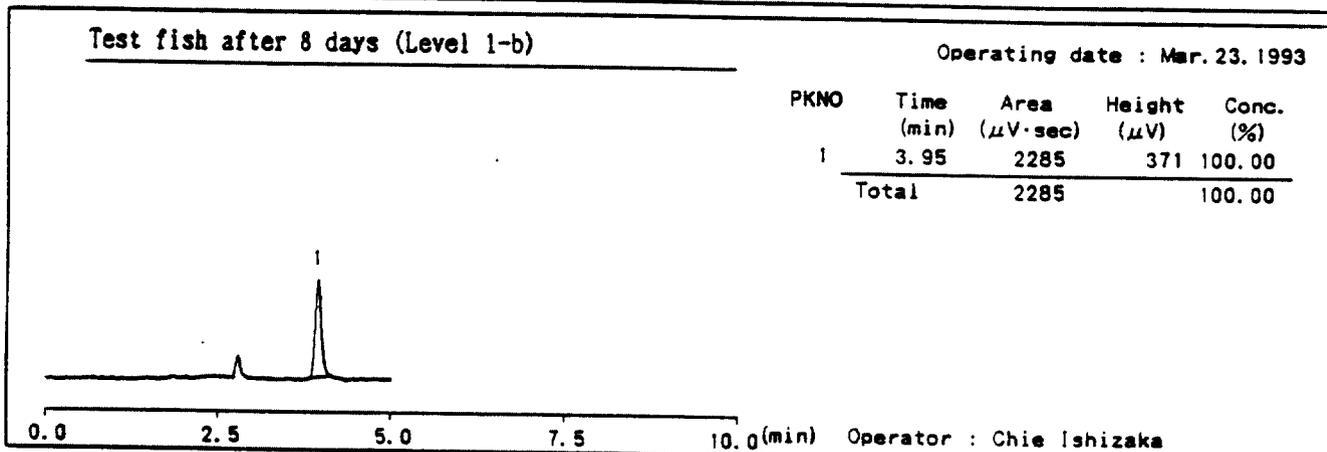
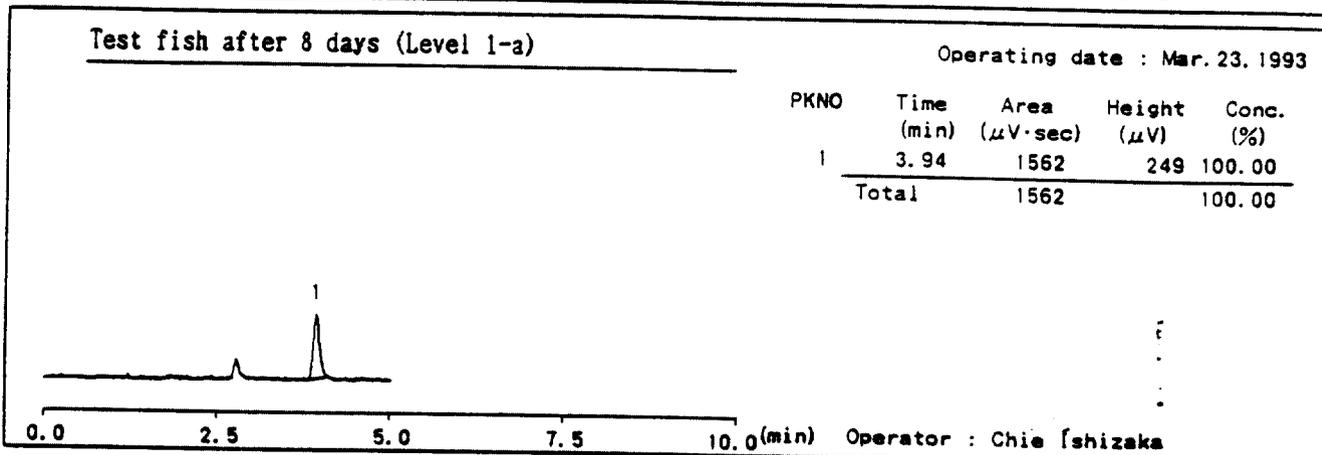
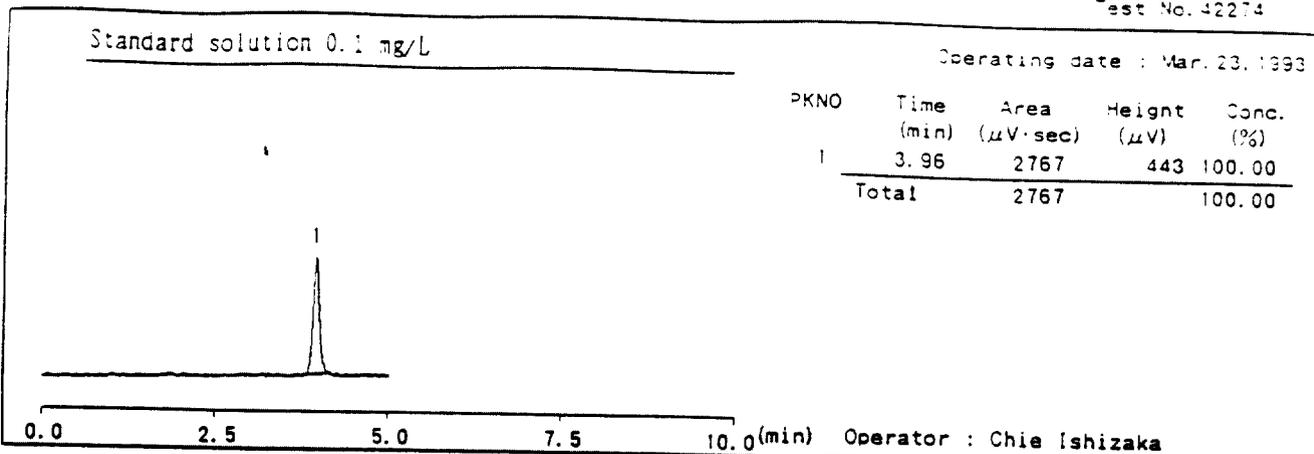
Name C. Ishizuka



Date : Mar. 19, 1993

Name : C Ishizaka

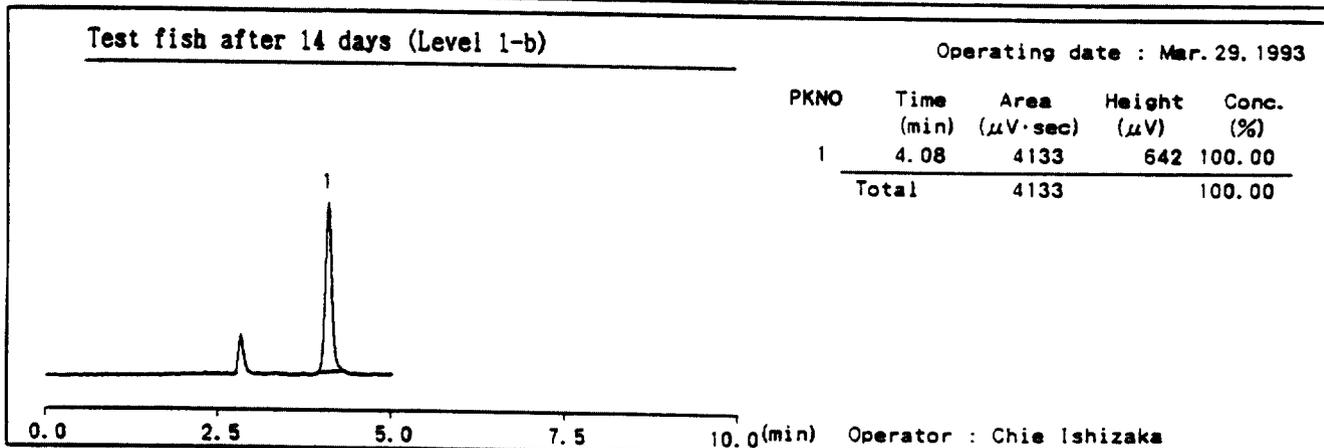
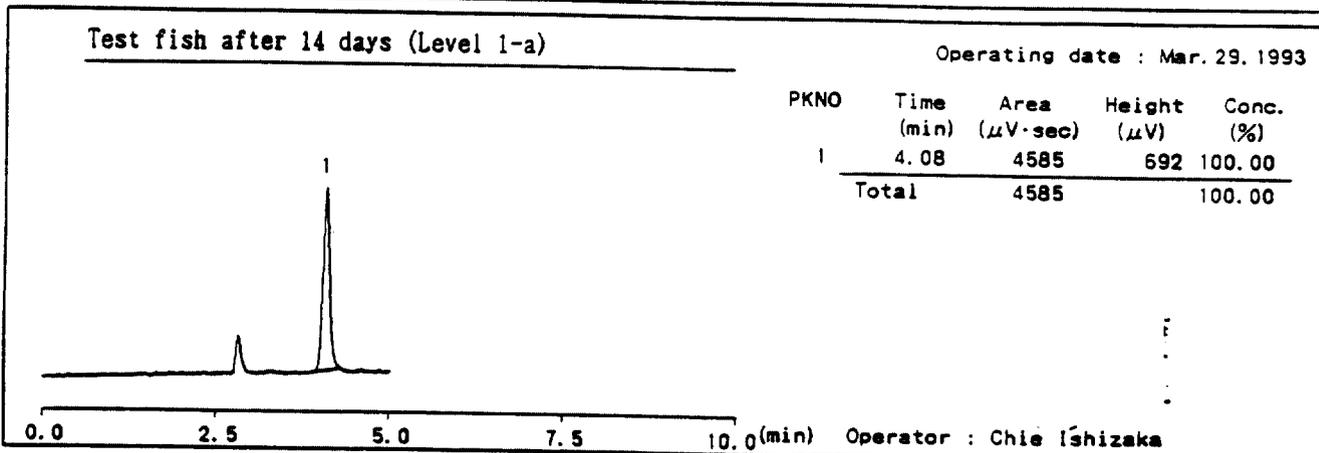
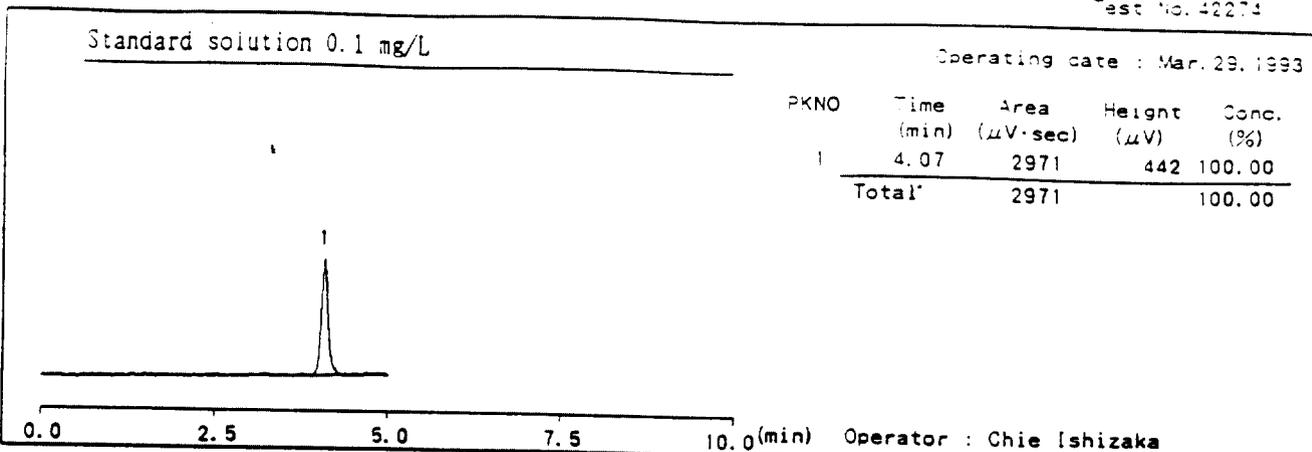
Fig. 1 - 1 Depuration test of test fish (Level 1)



Date : Mar. 23, 1993

Name : C Ishizaka

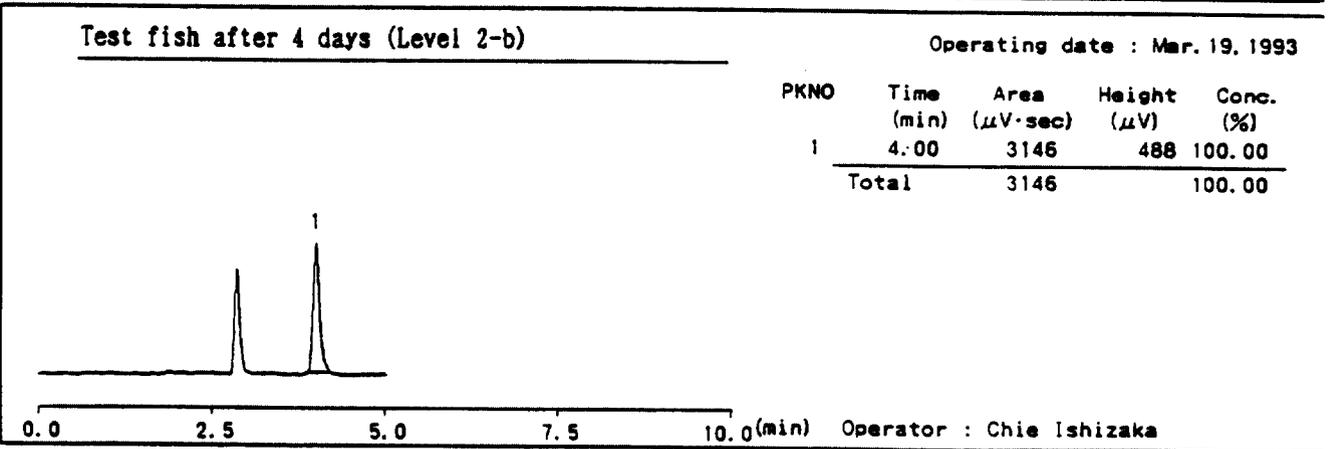
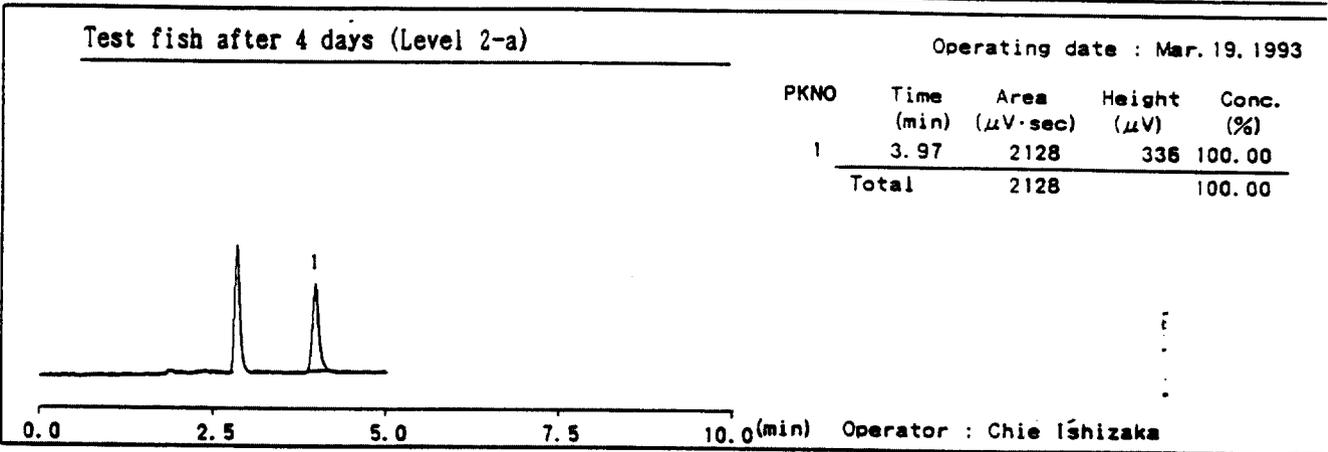
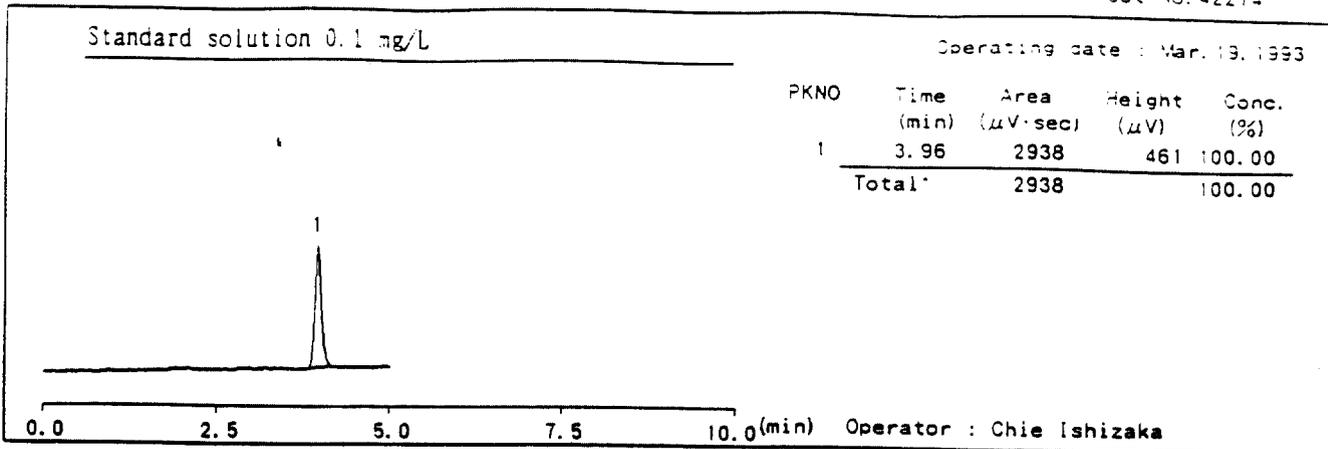
Fig. 1 - 2 Depuration test of test fish (Level 1)



Date : Mar. 29, 1993

Name : C. Ishizaka

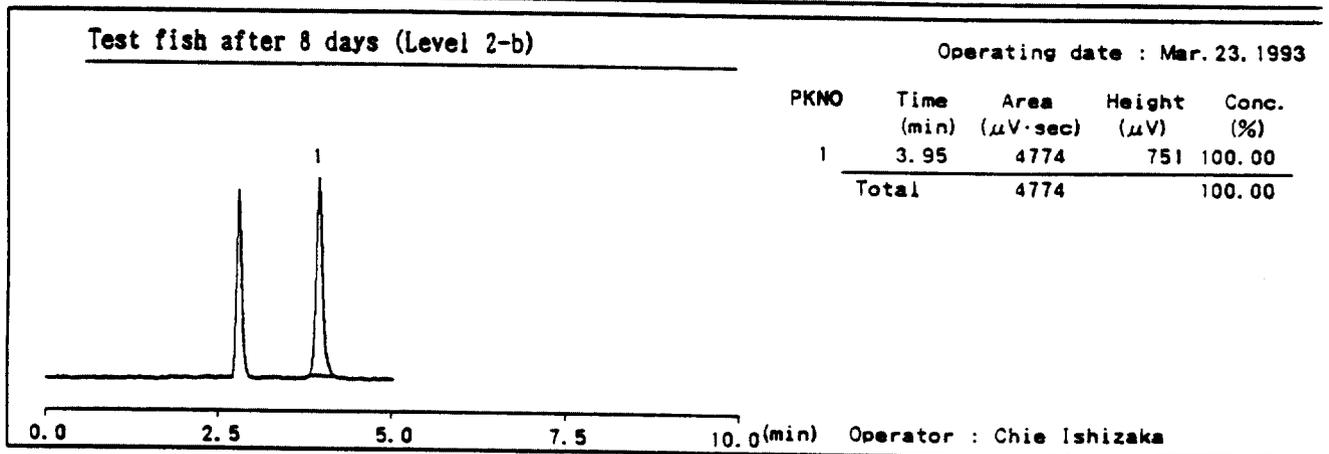
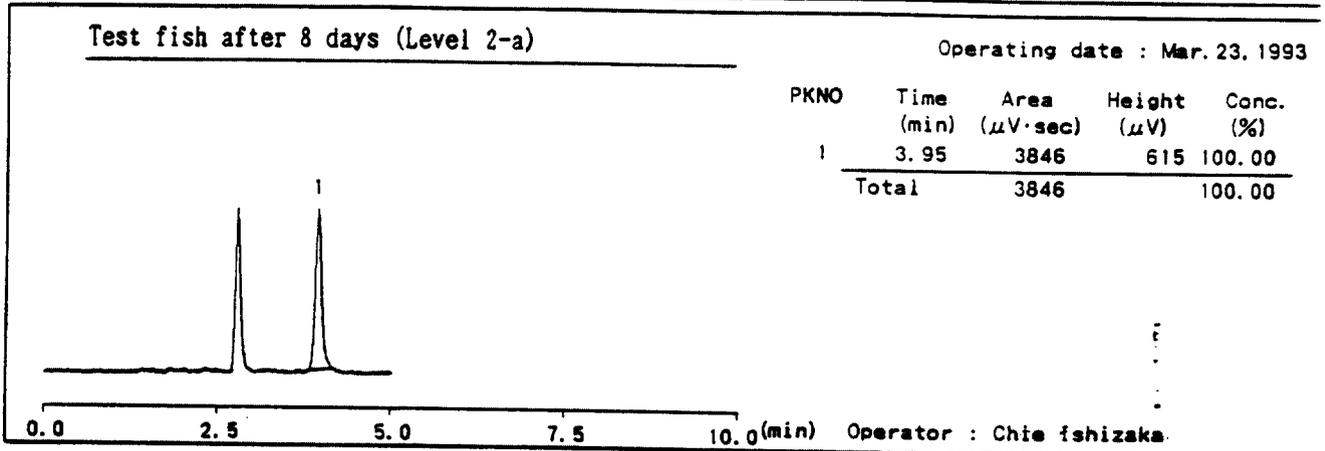
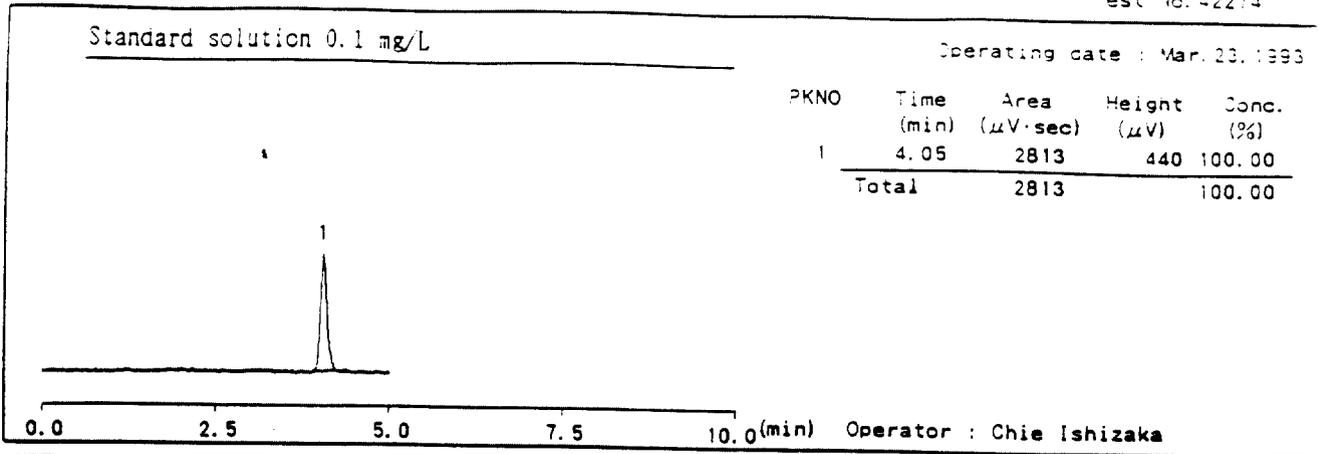
Fig. 1 - 3 Depuration test of test fish (Level 1)



Date : Mar. 19, 1993

Name : C Ishizaka

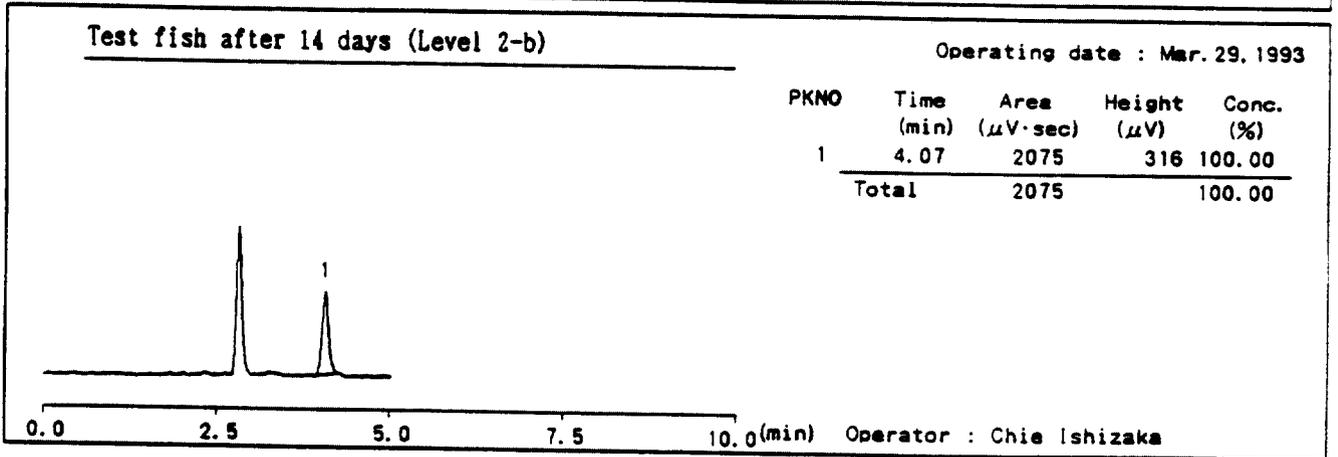
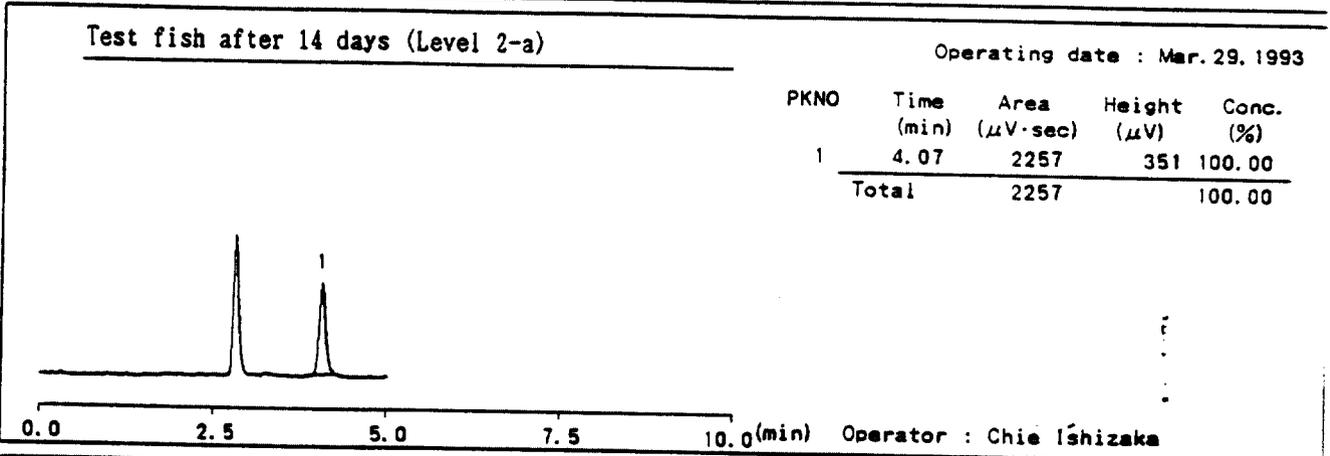
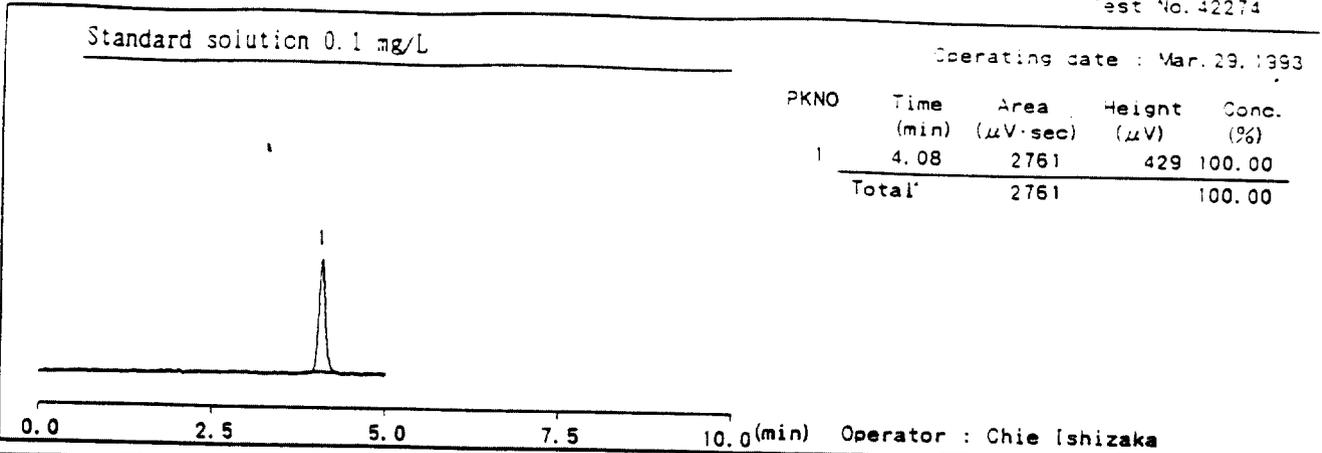
Fig. 2 - 1 Depuration test of test fish (Level 2)



Date : Mar. 23, 1993

Name : C. Ishizaka

Fig. 2 - 2 Depuration test of test fish (Level 2)



Date : Mar. 29, 1993

Name : C Ishizaka

Fig. 2 - 3 Depuration test of test fish (Level 2)

Test No.42274

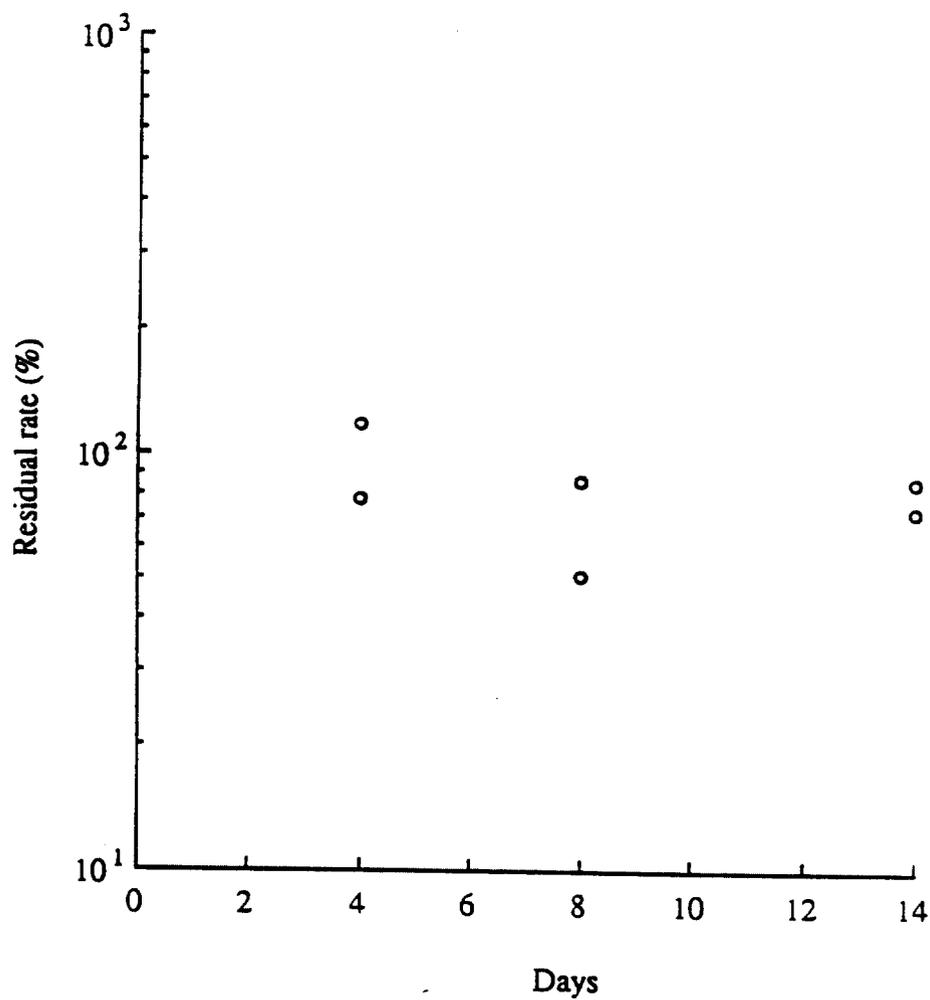


Fig. 3 Depuration curve (Level 1)

Test No.42274

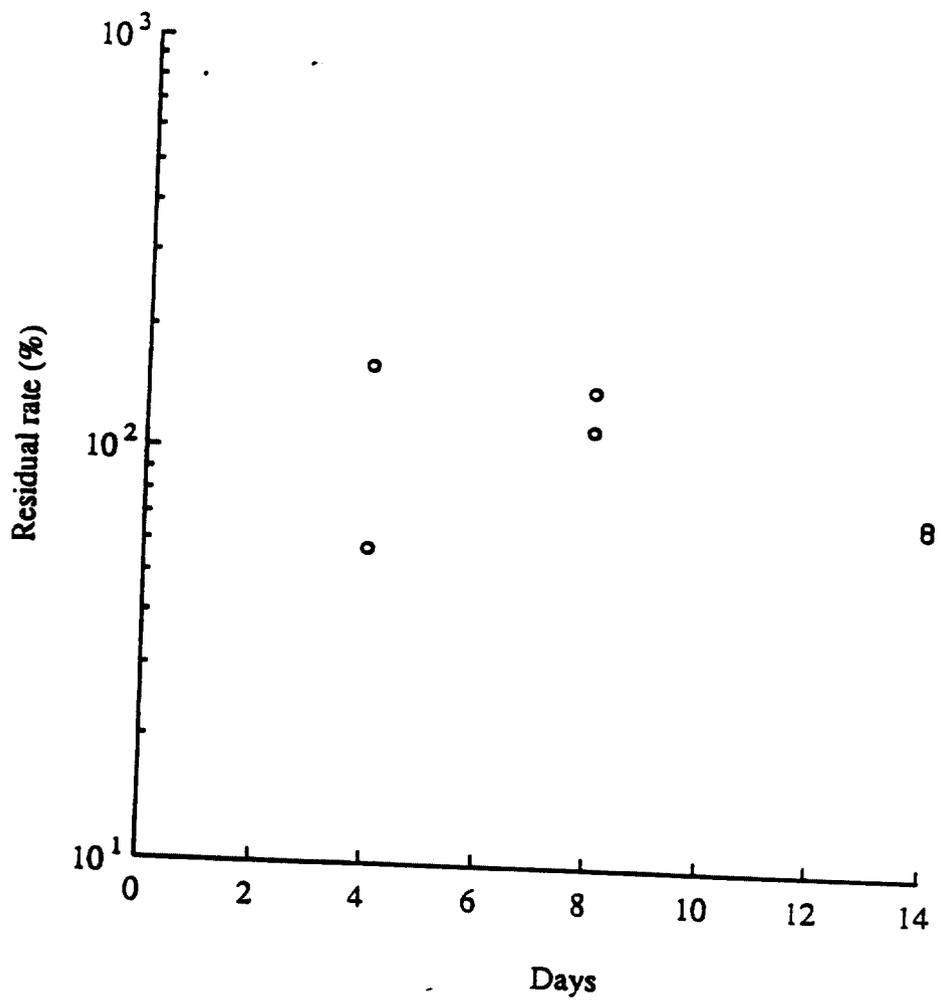


Fig. 4 Depuration curve (Level 2)

August 16, 1994

Dear Sirs,

Here is the translation of the document which you requested for your client.

This work is provided on a "best effort" basis. As you are aware translation is always an inexact procedure. As a simple example there is generally no distinction made between singular and plural in Japanese. The choice of which to use is, therefore, often arbitrary. In translating technical information questions of interpretation and specialized vocabulary also arise. Translators, like everyone else, also simply make errors. Therefore, while we have done our best to make the information in this document available to you in English, we cannot guarantee against errors due to typing, translation, interpretation or other causes.

Some special notes on this translation:

1) We were unable to satisfactorily interpret a reference on page 6 of the original document. We have used "sodium #2 mercury" in the translation. #2 may mean secondary, or this may be a chemical formula. ??

2) Japanese has a phonetic alphabet which is often used to translate foreign words into Japanese. Translating this alphabet back into English generally relies on making a connection between the Japanese pronunciation and the English pronunciation. For some words there is a well known correspondence, but for others the vocabulary is very specialized. In this document, the following cases were of particular concern:

* On page 6, reference is made to a "erubaajyuu" solution. We are unable to give a satisfactory interpretation of this term.

* On pages 12 and 17, the term "seppupakku" is used. This may refer to a "separation pack"?

* Also on pages 12 and 17, the term "memburan firutaa" appears on the original. We have translated this as "membrane filter".

RECEIVED

3) On page 8 of the original, there is a word which we were unable to translate. We have marked this with "XXX". This may be a specialized term we are unfamiliar with or a typographical error. From the context it seems to refer to a drying operation.

4) Names of companies, journals, societies, etc. can be translated either phonetically or in terms of their meaning. We have used both systems in the translation based on our judgment as to which would be most helpful.

When a translation based on the meaning is given, this may or may not correspond to the "official" name in English. For example, there is often some arbitrariness in the choice of words (society/association/institute etc.) and in the word order. Please be aware that the "official" English name is likely to be a permutation or variant of the translation given.

For phonetic translation, please also see the comments given in note 5).

5) For addresses and people's names a phonetic translation is, of course, appropriate. We have provided what we feel is the most probable the pronunciation, but the characters used to write names can have multiple pronunciations. In two cases (one address and one name) we have so far been unable to make a suitable translation. These are marked with "XXX". If this information is important to the client, please let us know and we will work further on it.

Names of individuals are given using the Japanese convention (i.e. family name first).

6) There are a number of references to rules, laws, codes, etc. which occur in a very stylized fashion. We are not familiar with the conventions used in these cases but have tried to provide a literal translation, while retaining as much as possible of the original format.

Please give us a call if there are any questions regarding this document. We are always willing to discuss whether alternate interpretations are possible, to correct errors, or clarify any uncertainties in the translation.

NOTE

The small, typed brackets in this report are used by the translator and they do not indicate confidential information. Each business confidential information is marked within a handwritten heavy bracket.

received number	S92-2274
examination number	42274

Final Report

Test for TPA Enrichment in Carp

March 23, 1993

Foundation

Chemical Product Examination Association
Chemical Product Safety Center, Kurume Research
Laboratory

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Summary

1. test title
Test for TPA Enrichment in Carp
2. test conditions
 - 2.1 acute toxicity test
 - (1) fish for test oryzias latipes
 - (2) exposure time 48 hours
 - (3) exposure method half cutoff (change water every 8 -16 hours)
 - 2.2 enrichment test
 - (1) fish for test carp
 - (2) test concentration
 - #1 concentration region 10 μ g/l
 - #2 concentration region 1 μ g/l
 - (3) exposure time 8 weeks
 - (4) exposure method continuous flowing water
 - (5) analysis method high speed liquid chromatography
3. test results
 - (1) tested material 48 hour LC50 value over 200mg/l
 - (2) enrichment ratio
 - #1 concentration region 3650~13900 times
 - #2 concentration region 6440~17800 times
4. stability of the tested material

The stability of the tested material was checked under storage and test conditions.

11. tested material

The TPA in this report has the following name and structural formula etc.

11.1 name *1 Tri (4-methyl phenyl) amine

11.2 structural formula etc. *1

structural formula

molecular formula $C_{21}H_{21}N$

molecular weight 287.40

11.3 purity *1 over 99.9 %

11.4 provider and lot number *1

(1) provider Fuji Xerox Co.

(2) lot number 920602

*1 provided by the test contractor's document

11.5 confirmation of the tested material

Agreement between the test contractor provided infrared absorption spectrum and the spectrum measured at this laboratory was confirmed (refer to figure 15 and reference 2).

11.6 Physical and chemical properties

appearance achromatic crystal

fusion point*1 116°C

solubility

water less than 10 mg/l

hexane 61 g/l

chloroform more than 100 g/l (visual observation)

ethyl acetate more than 100 g/l (visual observation)

methanol 490 mg/l

*1 based on documents provided by the test contractor

11.7 storage conditions and stability under the storage condition

(1) storage condition dark, cool place

(2) stability check The infrared absorption spectrum was measured before the start of the exposure and after completion of the test (see figure 15) . The fact that the 2 spectra were the same and the stability under the stored conditions was confirmed.

11.8 stability under the test conditions

Pre-examination was performed before the start of exposure and stability under the test conditions confirmed.

(6) full length average 3.2 cm

(7) verification

Based on Tabata Kenji^{*2}'s methods, fish of the same lot (TFO-921119) as those which passed a "sodium #2 mercury" test were provided.

*2 Water and waste water, 14, 1297-1303 (1972)

12.3 water for test

(1) type,

ground water pumped inside the Kurume Research Laboratory.

(2) analysis and water quality confirmation

The water temperature, pH, and dissolved oxygen were continuously measured at the laboratory.

Further total hardness, evaporation residue, chemical oxygen requirement, free chlorine, as well as harmful materials such as ammonia state nitrogen, organic phosphorous, cyanogen ion, heavy metal, etc. were routinely analyzed once every six months. When water was provided for a test, the total hardness and evaporation residue was confirmed based on [water quality standard based on city water law] (August 31, 1978, Ministry of Public Welfare # 56). With regard to the other items the fact that the concentration was below that written in [standard for fishery water] (Corporation Japan Fishery Source Protection Association, March, 1983) was confirmed (see reference 1).

12.4 test condition

- | | |
|------------------------------------|--|
| (1) test water tank | cylindrical glass water tank |
| (2) amount of test liquid | 4 l/ concentration region |
| (3) test water temperature | 25 ± 2 °C |
| (4) dissolved oxygen concentration | at exposure start 7.8 mg/l
at exposure end 6.1 mg/l |
| (5) pH | at exposure start 7.6
at exposure end 7.5 |
| (6) number of fish for test | 10 / concentration region |
| (7) exposure time | 48 hours |
| (8) exposure methods | half cutoff (change water every 8 ~16 hours) |
| (9) lighting | light shielded during test |

12.5 method for making solution

(1) dispersant

HCO - 40

(2) method of making

While applying ultrasound agitation the materials for test were dissolved in acetone. 50 times the amount of HCO-40 was added and well mixed. After XXX. A solution of 1000 mg/l was made by dissolving in ion exchanged water.

12.6 test implementation

(1) test place room 115LC50

(2) test date January 18 ~ 20, 1993

12.7 calculation of the 48 hour LC50 value
performed using Doudoroff method.

12.8 test results

48 hour LC50 value for the tested material

over 200 mg/l (see figure 3)

13. enrichment test implementation

13.1 fish for test

- (1) fish type Cyprinus carpio
- (2) source Sugishima Fishery
(address 123-2 Ichiban-cho, XXX,
Yatsushiro-shi, Kumamoto-ken 866)
date received November 13, 1992

(3) conditions for care of fish

time etc. Upon receipt, visual inspection was performed and abnormal specimens removed. After chemical bath, raised for 4 days in flowing water in fish care tank.

chemical bath Chemical bath was performed in standing water for 24 hours in a solution made with 50 mg/l terramycin powder for fisheries (made by the Taito Pfizer) solution and 7 g/l sodium chloride solution.

(4) purification condition Purification was done in a purification tank where any abnormal specimens were removed and fish were raised for 29 days in flowing water at 25 ± 2 °C. Then they were moved to the test water tank and raised for 7 days at the same temperature in flowing water.

date purification completed December 22, 1992

(5) exposure start weight, length, etc. *3
weight average 22.8 g
length average 9.6 cm
lipid percentage average 3.7 %

*3 measurement of lot (TFC-921113-I)

(6) fish food

type pellet mixed feed for carp
maker Japan Mixed Feed Company
feeding method About 2% of the weight of the test fish, divided into two portions and given during two feedings per day. Feeding stopped the day before fish were harvested.

13.2 water for test

Same as 12.3.

13.3 test and environment conditions

- (1) supply method for water for test
A flowing water type device assembled in the laboratory
- (2) test water tank 100 l glass water tank
- (3) amount of test water 1158 l/day water was supplied to the water tank with the proportions of 4 ml/min solution to 800 ml/min test water.
- (4) test temperature 25 ± 2 °C
- (5) dissolved oxygen concentration
 - # 1 concentration region 7.0 ~ 8.0 mg/l (see fig. 11)
 - # 2 concentration region 6.9 ~ 7.8 mg/l (see fig. 12)
 - contrast region 7.5 ~ 8.2 mg/l (see fig. 13)
- (6) number of fish for test
 - # 1 and #2 concentration regions 19 (at exposure start)
 - contrast region 5 (at exposure start)
- (7) exposure period 8 weeks
- (8) place of implementation 213 aquatron room

13.4 method for making solution

- (1) dispersant
same as 12.5 (1).
- (2) method of making
 - # 1 concentration region
Using the same method as in 12.5 (2), 2 mg/l solution was made.
 - # 2 concentration region
Using the same method as in 12.5 (2), 0.2 mg/l solution was made.
 - contrast region
A solution of 100 mg/l was made by dissolving HCO-40 in ion exchanged water.

The above was supplied from a 25 l glass solution tank to the water tank for the test.

13.5 test concentration

Considering the 48 hour LC50 preliminary value and the analytical sensitivity of the tested material, the tested material concentration was set,

1 concentration region 10 $\mu\text{g/l}$

2 concentration region 1 $\mu\text{g/l}$.

At the same time the contrast region was set as a blank test.

13.6 analysis of water and fish for test

13.6.1 number of analyses

Analysis of the water in both #1 and #2 concentration regions was performed twice every week for a total of 16 times during exposure. Each analysis used one sample point. And analysis of the fish in both #1 and #2 concentration regions was performed during the 2nd, 4th, 6th, and 8th weeks following exposure start for a total of 4 times. Each analysis used 2 fish. For the contrast region analysis was made before the exposure start and after the exposure finish. Each sample was 2 fish.

13.6.2 pretreatment of the analysis sample

(1) water for test

Take

#1 concentration region 50 ml

#2 concentration region 500 ml

from the water tank. The following flow scheme was used for the pretreatment operation of the sample for high speed liquid chromatography (HPLC).

flow scheme

analysis sample of water for test

<-- ion exchanged water 450 ml (graduated cylinder)
(only for #1 concentration region)

<-- dichloromethane 60 ml (graduated cylinder)

- shaking (5 minutes)

water
layer

dichloromethane layer

- filtration (No. 2 filter paper)
- drying (rotary evaporator, about 50°C, nitrogen purge)

<-- acetonitrile 2 ml (graduated pipette)

- ultrasound agitation (about 20 seconds)
- constant volume 5 ml (acetonitrile, graduated flask)

HPLC sample

(2) fish for test

Fish are taken from the water tank. The following flow scheme was used for the pretreatment operation of the sample for HPLC.

flow scheme

analysis sample of test fish

- measure the weight and length
- make small pieces
- <-- tetrahydrofuran 100 ml (graduated cylinder)
- homogenize (room temperature, about 1 minute)
- centrifuging (7000 x g, 5 minutes)
- filtration (cotton batten)

residue filtrate

- constant volume 150 ml (tetrahydrofuran, graduated flask)
- split sample 5 ml (whole pipette)
- column chromatography *4 (detail is described below)
- drying (rotary evaporator, about 40°C, nitrogen purge)
- <-- acetonitrile 5 ml (graduated pipette)
- ultrasound agitation (about 20 seconds)
- constant volume 10 ml (acetonitrile, graduated flask)
- filtration (membrane filter, 0.45 μ m, FN)

HPLC sample

*4 column chromatograph condition

"seppupakku" basic alumina

loading all sample liquid loaded

dissolution #1 solution tetrahydrofuran 5 ml

Test material was dissolved with the loading portion and the #1 solution.

13.6.3 quantitative analysis

Quantitative analysis was obtained from high speed liquid chromatography performed on the HPLC sample obtained from the 13.6.2 pretreatment, based on the following quantitative conditions. For fish quantitative analysis, HPLC samples were suitably diluted and the tested material concentration adjusted to be within the confirmed linear relationship region. The chromatograph peak area of a constant volume of the solution was compared to that of a standard of known concentration and a proportional calculation done for the tested material's concentration (see table - 4, 5, fig. - 6, table - 7, 8, 9, fig. - 8, 9, 10).

(1) quantitative conditions

machine	high speed liquid chromatograph
pump	made by Shimazu Manufacturing Company LC-6A
detector	made by Shimazu Manufacturing Company SPD-6AV
column	L-column ODS 15cm x 4.6 mm ϕ stainless
eluate	acetonitrile
flow rate	1.0 ml/min
measured wavelength	300 nm (see fig. 14)
fill amount	30 μ l
sensitivity	
detector	0.005 ABU/FS
recorder	range 1.2 mV

(2) method for making standard solution

To obtain the analysis sample's tested material concentration, a standard solution was made as shown next.

A precisely measured 0.1 g of the tested material was dissolved in tetrahydrofuran to make a 1000 mg/l standard solution. This was diluted with acetonitrile to make a 0.1 mg/l standard solution.

(3) making the calibration curve

0.05, 0.1 and 0.2 mg/l standard solutions were made using the same procedure as in (2). Following the quantitative conditions in (1), these were analyzed and the chromatogram peak areas from these concentrations used to make the calibration curve.

Considering the noise level, from the calibration curve the lower limit on detection of the tested material's peak area was $150 \mu\text{V}\cdot\text{sec}$ (tested material concentration is $4.9 \mu\text{g/l}$) (see fig. 4).

13.6.4 Recovery and blank tests

(1) methods

In order to obtain the recovery percentage of the test material for the previously described water and fish analysis operations, test material dispersant was added to the water and fish homogenate for the recovery test, and the recovery test was conducted based on the operations in 13.6.2 and 13.6.3. With water and fish homogenate to which no test material had been added for the recovery test, a blank test was performed using the same operations as in the case of the recovery test. For the recovery and blank tests two points were measured. In the blank test there was no peak on the chromatogram at the test material peak position. The recovery percentage and average recovery percentage for each analysis operation's two points are as shown below. The correction value for the test material concentration obtained from the analysis sample is set to the average recovery percentage. (see table - 3, 6, figure - 5, 7)

(2) results

recovery percentage for the analysis operation
water analysis (test material 0.5 μg added)
91.3%, 93.4%, average 92.3%
fish analysis (test material 30 μg added)
94.0%, 92.4%, average 93.2%

13.6.5 calculation and quantitative lower limit of the test material concentration in the analysis sample

- (1) calculation of the test material concentration in the analysis sample's water

Using the formula in table - 4, 5 for calculation, the results are shown rounded to three significant digits.

- (2) Quantitative lower limit concentration of the test material water

From the test material's detection lower limit obtained using the calibration curve from 13.6.3 (3), the quantitative lower limit concentrations ^{*5} of the test material water

#1 concentration region 0.53 $\mu\text{g/l}$

#2 concentration region 0.053 $\mu\text{g/l}$

were calculated.

(3) calculation of the test material concentration in the analysis sample's fish

Using the formula in table - 7, 8, 9 for calculation, the results are shown to rounded to three significant digits.

(4) Quantitative lower limit concentration of the test material fish

From the test material's detection lower limit obtained using the calibration curve from 13.6.3 (3), the quantitative lower limit concentration ^{*5} of the test material fish, was calculated as 53 ng/g for a 30g fish.

^{*5} test material quantitative lower limit concentration

$$\begin{array}{l} (\mu\text{g/l} \\ \text{or ng/g}) = \end{array} \frac{\text{A}}{\text{B}/100 \times \text{CxE}/\text{D}}$$

A: calibration curve's detection lower limit concentration ($\mu\text{g/l}$)

B: recovery percentage (%)

C: sample water amount (ml) or fish weight (g)

D: final liquid amount (ml)

E: sample split ratio

calculated results were rounded to two significant digits

13.7 concentration factor (BCF) calculation

Using the formula in table - 7, 8, 9 for calculation, the results are shown to rounded to three significant digits.

Further, from the quantitative lower limit concentration of the test material in the fish obtained using 13.6.5 (4), concentration factor calculation is possible when the concentration exceeds the following multipliers:

#1 concentration region	6.0 times
#2 concentration region	65 times

13.8 handling of number

Number rounding follows JIS Z 8202-1985 reference 3 rule B.

14. test results

14.1 test material concentration in water

test material concentration in water is shown in table - 1.

Table 1 test material concentration in water (average measured value from exposure start)

(unit $\mu\text{g/l}$)

	week 2	week 4	week 6	week 8	attached table	attached figure
#1 conc. region	8.21	8.39	8.69	8.80	table - 4	fig. - 6
#2 conc. region	0.781	0.779	0.796	0.810	table - 5	

14.2 concentration factor

concentration factor is shown in table - 2

Table - 2 concentration factor

	week 2	week 4	week 6	week 8	attached table	attached figure
#1 conc. region	3650 4290	4910 6830	10100 9820	10800 13900	table - 7	fig. - 8
#2 conc. region	7450 6440	12900 11300	15100 17700	17800 13400		

The correlation between the concentration factor in table - 2 and the exposure period was shown in figure 1 and figure 2. For the test material in carp, the enrichment level is from 3650 to 13900 times for the number one concentration region and from 6440 to 17800 times for the number two concentration region.

In the results from observation of the fish, no abnormalities were noted.

And, the average test material concentration in the water had a value of around 80% of the set value as shown in table -1.

15. Storage of samples and documents

15.1 Test material

Approximately 20g of the test material was put in a container, sealed tightly and then stored in the research lab sample storage room during the period provided by the 32nd rule of [GLP standard].

15.2 Raw data, documents etc.

The analysis results from the test, measured results, observed results, raw data such as test notes etc. which were used for making final report, test plan, requests, investigation tables, documents etc., were stored together with the final report in the research lab document storage room during the period provided by the 32nd rule of [GLP standard].

16. Remarks

16.1 Key devices • machines, special tools, reagents etc. used in test

(1) Devices for the test (fish farming facilities)

micro constant-quantity pump for solution supply :

Tokyo Rika Kikai type GMW

dissolved oxygen measuring device :

Iijima Seimitsu Kogyo type 552

(2) Devices • machines, special tools, reagents used in analysis and solution making

Devices • machines

high speed liquid chromatograph :

see page 13

rotary evaporator :

Tokyo Rika Kikai type N-1

shaking machine :

Irie Shokai TS type

Taiyo Kagaku Kogyo type SR-IIW

homogenizer :

Kinemachika Company

centrifuging machine :

Hitachi Seisakusho type 20PR-52

Special tools

"seppupakku" basic alumina :

Nihon Millipore • Limited

membrane filter :

Nomura Micro Science type FN

Reagent

dichloromethane :

Kishida Kagaku first grade reagent

acetonitrile

Wakou Jyunyaku Kogyo for HPLC

tetrahydrofuran :

Nakaraitesuku first grade reagent

acetone :

Wakou Jyunyaky Kogyo first grade reagent

HCO - 40 :

Nikkou Chemicals

17. Contents of tables and figures

Table

Table - 1 Concentration of the tested material in the testing water (measured value) [described in this report]

Table -2 Concentration factor [described in this report]

Table -3 Calculation for recovery and blank tests (water analysis)

Table - 4 Calculation for #1 concentration region water analysis

Table - 5 Calculation for #2 concentration region water analysis

Table - 6 Calculation for recovery and blank tests (fish analysis)

Table - 7 Calculation for #1 concentration region fish analysis

Table - 8 Calculation for #2 concentration region fish analysis

Table - 9 Calculation for the contrast region fish analysis

Reference 1 Measurement for the test water quality

Figure

Figure - 1 Correlation between the exposure period - concentration factor (#1 concentration region)

Figure - 2 Correlation between the exposure period - concentration factor (#2 concentration region)

Figure - 3 test material concentration - mortality curve for the acute toxicity test

Figure - 4 calibration curve and its HPLC chart

Figure - 5 HPLC chart for recovery and blank test (water analysis)

Figure - 6 HPLC chart for water analysis

Figure - 7 HPLC chart for recovery and blank test (fish analysis)

Figure - 8 HPLC chart for #1 concentration region's fish analysis

Figure - 9 HPLC chart for #2 concentration region's fish analysis

Figure-10 HPLC chart for contrast region's fish analysis

Figure-11 Dissolved oxygen concentration for #1
concentration region's water

Figure-12 Dissolved oxygen concentration for #2 concentration
region's water

Figure-13 Dissolved oxygen concentration for contrast region's
water

Figure-14 Test material's ultraviolet absorption spectrum

Figure -15-1 Test material's infrared absorption spectrum
before exposure start

Figure -15-2 Test material's infrared absorption spectrum
after exposure completion

Reference 2 Infrared absorption spectrum provided by test
contractor

Huntingdon Research Centre Ltd.
Huntingdon
Cambridgeshire PE18 6ES
England

Telephone

Huntingdon (0480) 890431
International +44 480 890 431

Telex

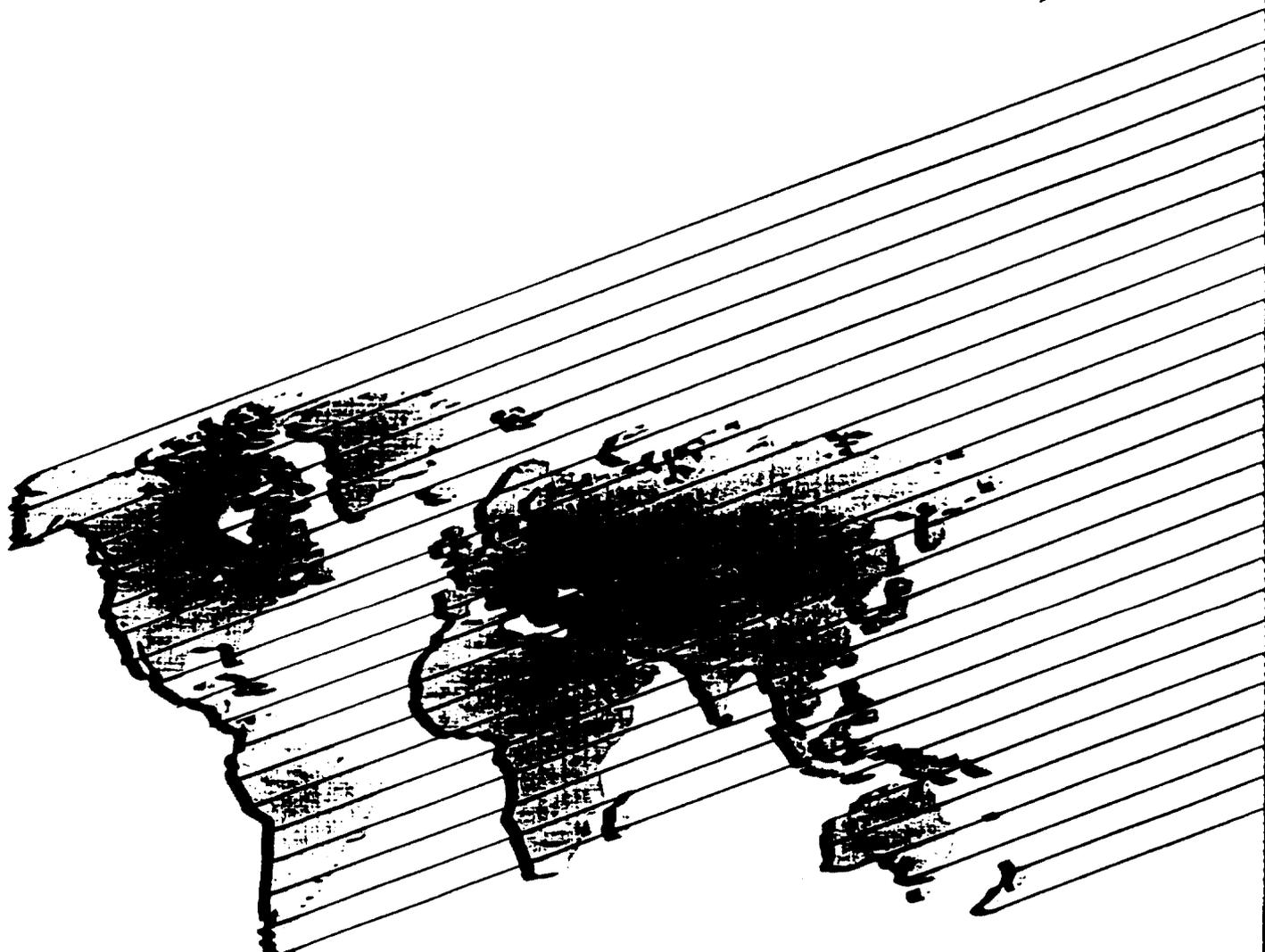
32100 HRCUK G

Cable

Research Huntingdon

Fax

(0480) 890693 Gp 2 & Gp 3
International +44 480 890 693





921037D/FTX 183/AC

20555

**ACUTE ORAL TOXICITY
TO THE RAT**

Addressee:



Testing facility:

Huntingdon Research Centre Ltd.,
P.O. Box 2,
Huntingdon,
Cambridgeshire,
PE18 6ES,
ENGLAND.

Report issued: 30 September 1993.

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

To the best of my knowledge and belief the study described in this report was conducted in compliance with the following Good Laboratory Practice Standards.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health & Social Security 1986 and subsequent revision, Department of Health, 1989.

United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, Federal Register, 22 December 1978, and subsequent Amendments.

United States Environmental Protection Agency, (FIFRA), Title 40 Code of Federal Regulations Part 160, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

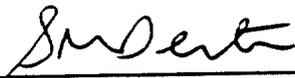
United States Environmental Protection Agency, (TSCA), Title 40 Code of Federal Regulations Part 792, Federal Register, 29 November 1983 and subsequent amendment Federal Register 17 August 1989.

Japanese Ministry of Health and Welfare, Notification No. Yakuhatsu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatsu 870, Pharmaceutical Affairs Bureau, 5 October 1988.

Japanese Ministry of Agriculture, Forestry and Fisheries, 59 NohSan, Notification No. 3850, Agricultural Production Bureau, 10 August 1984.

Japanese Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITT).

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.



Stuart M. Denton, B.Sc.,
Study Director,
Huntingdon Research Centre Ltd.

30.9.93

Date

RESPONSIBLE PERSONNEL

I the undersigned, hereby declare that the work was performed under my supervision according to the procedures herein described, and that this report provides a correct and faithful record of the results obtained.



Stuart M. Denton, B.Sc.,
Study Director,
Department of Industrial Toxicology.

SUMMARY

A study was performed to assess the acute oral toxicity of 20555 to the rat. The method followed was that described in EEC Methods for the determination of toxicity, Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B.1. Acute toxicity (oral).

A group of ten fasted rats (five males and five females) was given a single dose by gavage of the test substance, formulated in 1% w/v aqueous methylcellulose, at a dose level of 5.0 g/kg bodyweight. All animals were killed and examined macroscopically on Day 15, the end of the observation period.

There were no deaths. Clinical signs of reaction to treatment were limited to pilo-erection, recovery was complete by Day 2.

All rats achieved anticipated bodyweight gains throughout the study.

No abnormalities were recorded at the macroscopic examination on Day 15.

The acute lethal oral dose to rats of 20555 was found to be greater than 5.0 g/kg bodyweight.

20555 does not require labelling with the risk phrases R22 "Harmful if swallowed" in accordance with Council Directive 79/831/EEC Annex VI, Part II(D) as described in Commission Directive 91/325/EEC.

INTRODUCTION

The study was designed to assess the toxicity of 20555 following a single oral dose to the rat. The rats were dosed by oral gavage as the test substance may be ingested accidentally.

The study was conducted in compliance with EEC Methods for the determination of toxicity, Directive 84/449/EEC (OJ No. L251, 19.9.84), Part B, Method B.1. Acute toxicity (oral).

The rat was chosen as it has been shown to be a suitable model for this type of study and is the animal recommended in the test guideline.

The dose level for the study was chosen in compliance with the guideline.

The protocol was approved by the Study Director and HRC Management on 3 November 1992 and by the Sponsor on 9 November 1992.

The experimental phase of the study was undertaken between 13 and 27 November 1992.

TEST SUBSTANCE

Identity: 20555

Alternative name: Tri-p-tolylamine

Chemical name: Tri(4-methylphenyl)amine

Lot number : 920911

Expiry: 2 June 1993

Purity : >99.9%

Appearance: White crystalline powder

Storage conditions: Room temperature in the dark

Date received: 7 October 1992

EXPERIMENTAL PROCEDURE

ANIMAL MANAGEMENT

Equal numbers of healthy male and female CD rats of Sprague-Dawley origin (Hsd/Ola:Sprague-Dawley(CD)) were obtained from Harlan Olac Ltd., Bicester, Oxon, England.

They were in the weight range of 114 to 133 g and approximately four to seven weeks of age prior to dosing (Day 1). All the rats were acclimatised to the experimental environment for a period of fifteen days prior to the start of the study.

The rats were allocated without conscious bias to cages within the treatment group. They were housed in groups of five rats of the same sex in metal cages with wire mesh floors in Building R14 Room 6.

A standard laboratory rodent diet (Biosure LAD 1) and drinking water were provided *ad libitum*. Access to food only was prevented overnight prior to and approximately 4 hours after dosing.

The batch(es) of diet used for the study was analysed for certain nutrients, possible contaminants and micro-organisms (Appendix 1).

Results of routine physical and chemical examination of drinking water at source, as conducted, usually weekly by the supplier, are made available to Huntingdon Research Centre Ltd. (as quarterly summaries (Appendix 2)).

The mean daily minimum and maximum temperatures of the animal room were 20°C and 22°C respectively and the mean daily relative humidity value was 48% R.H. Air exchange was maintained at 10 to 15 air changes per hour and lighting was controlled by means of a time switch to provide 12 hours of artificial light (0700 - 1900 hours) in each 24-hour period.

Each animal was identified by cage number and ear punching. Each cage was identified by a coloured label displaying the dose level, study schedule number, animal mark and the initials of the Study Director and Home Office licensee.

TEST SUBSTANCE PREPARATION

20555 was prepared at a concentration of 50% w/v in 1% w/v aqueous methylcellulose and administered at a volume of 10 ml/kg bodyweight.

The test substance was prepared on the day of dosing.

The absorption of the test substance was not determined.

The homogeneity, stability and purity of the test substance were the responsibility of the Sponsor.

TREATMENT PROCEDURE

A group of ten rats (five males and five females) was treated at 5.0 g/kg bodyweight.

Control animals

No control animals were included in this study.

ADMINISTRATION OF TEST SUBSTANCE

The appropriate dose volume of the test substance was administered to each rat by oral gavage using a syringe and plastic catheter (10 choke).

The day of dosing was designated Day 1.

OBSERVATIONS

Mortality

Cages of rats were checked at least twice daily for any mortalities.

Clinical signs

Animals were observed soon after dosing and at frequent intervals for the remainder of Day 1 (a period of five hours). On subsequent days animals were observed once in the morning and again at the end of the experimental day. This latter observation was at approximately 16.30 hours on week days or 11.30 hours on Saturdays, Sundays and public holidays. The nature and severity of the clinical signs and time were recorded at each observation.

All animals were observed for 14 days after dosing.

Bodyweight

The bodyweight of each rat was recorded on Days 1 (prior to dosing), 8 and 15. Individual weekly bodyweight changes were calculated.

TERMINAL STUDIES

Termination

All animals were killed on Day 15 by cervical dislocation.

Macroscopic pathology

All animals were subjected to a macroscopic examination which consisted of opening the abdominal and thoracic cavities. The macroscopic appearance of all tissues was recorded.

ARCHIVES

All raw data and other documents generated at HRC during the course of this study, together with a copy of this Final Report, have been lodged in the Huntingdon Research Centre Ltd. Archives, Huntingdon, England.

RESULTS

MORTALITY

There were no deaths following a single oral dose of 20555 at 5.0 g/kg bodyweight.

CLINICAL SIGNS (Table 1)

Pilo-erection was observed in all rats within five minutes of dosing and throughout the remainder of Day 1. There were no other clinical signs and recovery, as judged by external appearance and behaviour, was complete by Day 2.

BODYWEIGHT (Tables 2 and 3)

All rats achieved anticipated bodyweight gains throughout the study.

MACROSCOPIC EXAMINATION

No macroscopic abnormalities were observed for animals killed on Day 15.

CONCLUSION

The acute lethal oral dose to rats of 20555 was found to be greater than 5.0 g/kg bodyweight.

TABLE 1

Signs of reaction to treatment observed in rats dosed orally with 20555

Signs	No. of rats in group of showing signs	
	Dose (g/kg)	
	5.0	
	♂	♀
Pilo-erection	5	5

TABLE 2

Individual bodyweights (g) of rats dosed orally with 20555

Sex	Dose (g/kg)	Animal number & ear mark	Bodyweight (g) at		
			Day 1	Day 8	Day 15
♂	5.0	1 RP	131	193	248
		2 LP	127	198	248
		3 RPLP	133	219	286
		4 RIRO	129	202	257
		5 LILO	128	203	255
♀	5.0	6 RP	122	169	188
		7 LP	118	164	190
		8 RPLP	119	162	178
		9 RIRO	114	157	183
		10 LILO	117	156	173

TABLE 3

Individual bodyweight changes (g) of rats dosed orally with 20555

Sex	Dose (g/kg)	Animal number & ear mark	Bodyweight gains (g) at	
			Week 1	Week 2
♂	5.0	1 RP	62	55
		2 LP	71	50
		3 RPLP	86	67
		4 RIRO	73	55
		5 LILO	75	52
♀	5.0	6 RP	47	19
		7 LP	46	26
		8 RPLP	43	16
		9 RIRO	43	26
		10 LILO	39	17

APPENDIX 1

Biosure Laboratory Animal Diet No. 1

Composition and quality assurance aspects of diet

Biosure LAD is a fixed formula diet suitable for normal health, growth and reproduction of laboratory rats and mice. Each batch of diet is analysed for nutrients, possible contaminants and micro-organisms, likely to be present in the diet, and which, if in excess, may have an undesirable effect on the test system.

Prior to release of diet for use HRC Quality Assurance Department checks each certificate of analysis for conformity with the specification detailed below. Occasional slight deviations to this specification may be permitted.

Nutrients	Target level	Tolerance %	Acceptable range		
Moisture	9.5	+10		10.5	% max
Crude fat	3.7	±15	3.1	- 4.3	%
Crude protein	21.5	±10	19.4	- 23.7	%
Crude fibre	2.0	±40	1.2	- 2.8	%
Ash	5.5	±15	4.7	- 6.3	%
Calcium	1.0	±20	0.8	- 1.2	%
Phosphorus	0.9	±20	0.7	- 1.1	%
Sodium	0.3	+100-50	0.15	- 0.60	%
Chloride	0.5	+100-50	0.25	- 1.0	%
Potassium	0.8	+100-50	0.4	- 1.6	%
Magnesium	0.15	±50	0.08	- 0.23	%
Iron	220	±50	110.0	- 330	mg/kg
Copper	15	±50	8.0	- 23	mg/kg
Manganese	70	±50	35.0	- 105	mg/kg
Zinc	60	±50	30.0	- 90	mg/kg
Vitamin A	12	+50-20	9.5	- 18	iu/g
Vitamin E	35	+150-20	28	- 88	mg/kg

Contaminants

Maximum concentration

Fluoride	40	mg/kg
Nitrate (as NaNO ₃)	200	mg/kg
Nitrite (as NaNO ₂)	10	mg/kg
Lead	2.5	mg/kg
Arsenic	1.5	mg/kg
Cadmium	0.5	mg/kg
Mercury	0.1	mg/kg
Selenium	0.6	mg/kg
Total Aflatoxins	5	mcg/kg
Total P.C.B.	50	mcg/kg
Total D.D.T.	150	mcg/kg
Dieldrin	50	mcg/kg
Lindane	150	mcg/kg
Heptachlor	50	mcg/kg
Malathion	5000	mcg/kg

APPENDIX 1

(continued)

Microbiological contents

Maximum concentration

	LAD 1 (nuts)
Total viable organisms	10,000 per g diet
Mesophilic spores	30,000 per g diet
<i>Salmonellae</i> species	0 per g diet
Presumptive <i>Escherichia coli</i>	0 per g diet
<i>E. coli</i> type 1	0 per g diet
Fungal units	1,000 per g diet
Antibiotic activity	0 per g diet

APPENDIX 2

Quality assurance aspects of drinking water

The water supplied to HRC, by Anglian Water, is potable water for human consumption. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, viz: Council Directive 80/778/EEC.

Results of routine physical and chemical examination of drinking water at source as conducted, usually weekly by the supplier, are made available to HRC as quarterly summaries.

These results include levels of:

Nitrites	Potassium	Chloride
Nitrates	Silicon	Iron
Calcium	Arsenic	Selenium
Magnesium	Barium	Silver
Sodium	Antimony	Phosphorus

as well as concentrations of pesticides, related products, polycyclic aromatic hydrocarbons, haloforms, chlorophenols and polychlorinated biphenyls.

COMPANY SANITIZED

Tri-p-tylgamine

20555



**FOUR-WEEK ORAL TOXICITY STUDY
IN THE RAT WITH TWO-WEEK
RECOVERY PERIOD**

NOTE

This report is considered by the Study Director to be the 'final draft' and has been submitted to the HRC Quality Assurance Department for Audit.

The sponsor is requested to review this document and communicate any comments to the Study Director as soon as possible. When these comments have been received and on completion of the QA audit, the FINAL REPORT containing Study Director and QA Statements will be issued.

PLEASE NOTE

In compliance with GLP any changes to the final report after the date of issue will be in the form of a separate amendment to the report.

Date: 2 June 1993.

V.1.

RECEIVED
SEP 10 1993

Address:

[]

Testing facility:

Huntingdon Research Centre Ltd.,
P.O. Box 2,
Huntingdon,
Cambridgeshire,
PE18 6ES,
ENGLAND.

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COMPLIANCE WITH GOOD LABORATORY PRACTICE STANDARDS

To the best of my knowledge and belief the study described in this report was conducted in compliance with the following Good Laboratory Practice Standards.

Japanese Ministry of Health and Welfare, Notification No. Yakuhatu 313 Pharmaceutical Affairs Bureau, 31 March 1982 and subsequent amendment Notification No. Yakuhatu 870, Pharmaceutical Affairs Bureau, 5 October 1988.

Japanese Ministry of International Trade and Industry, Directive 31 March 1984 (Kanpogyo No. 39 Environmental Agency, Kikyoku No. 85 MITI).

Organisation for Economic Co-operation and Development, ISBN 92-64-12367-9, Paris 1982.

Good Laboratory Practice, The United Kingdom Compliance Programme, Department of Health & Social Security 1986 and subsequent revision, Department of Health, 1989.

Sarah A. Allan, B.Sc.,
Study Director,
Huntingdon Research Centre Ltd.

Date

DRAFT

QUALITY ASSURANCE STATEMENT

This report has been audited by the Quality Assurance Department. It is considered to be an accurate description of the procedures and practices employed during the course of the study and an accurate presentation of the findings.

Date of reporting audit findings
to the Study Director and HRC Management

Huntingdon Research Centre Ltd.

DRAFT

QUALITY ASSURANCE STATEMENT

DATES OF STUDY INSPECTIONS

Inspections were made by the Quality Assurance Department of the various phases of the study described in this report. The dates on which the inspections were made and the dates on which the findings were reported to the Study Director and to HRC Management are given below.

Phase of Study	Date of Inspection	Date of Reporting
Protocol Review Pre-experimental Period Experimental Period		

Huntingdon Research Centre Ltd.

DRAFT

RESPONSIBLE PERSONNEL

We the undersigned, hereby declare that the work was performed under our supervision according to the procedures herein described, and that this report provides a correct and faithful record of the results obtained.

**Sarah A. Allan, B.Sc.,
Study Director,
Department of Industrial Toxicology.**

**Andrew D. Hawkins, B.Sc.,
Study Supervisor,
Department of Industrial Toxicology.**

**David Crook, B.Sc., Ph.D.,
Head of Department of Clinical Pathology.**

**William A. Gibson, B.Sc., (Medical Sciences),
Pathologist - Post Mortem Room,
Department of Pathology.**

**Harcharn Singh, B.V.Sc. & A.H., D.T.V.M., M.Sc., M.R.C.V.S.,
Senior Pathologist,
Department of Pathology.**

**Chirukandath Gopinath, B.V.Sc., M.V.Sc., Ph.D., F.R.C.Path.,
Director of Pathology.**

**Alan Anderson, B.Sc., C.Chem., F.R.S.C.,
Head, Department of Analytical Chemistry & Pharmacy.**

**I. Suzanne Dawe, M.Sc., C.Chem., M.R.S.C.,
Head of Formulation Analysis,
Department of Analytical Chemistry & Pharmacy.**

SUMMARY

This study was performed to assess the systemic toxicity of 20555 to the rat. The method followed was that outlined in:

Joint directive of the Japanese Environmental Protection Agency and the Ministries of Health and Welfare and International Trade and Industry, 5 December 1986.

EEC Methods for the determination of toxicity, Directive 84/449/EEC(OJ No. L251, 19.9.84), Part B, Method B.7. Sub-acute toxicity (oral).

OECD Guideline for Testing of Chemicals No. 407, "Repeated Dose Oral Toxicity - Rodent: 28-day or 14-day study". Adopted: 12 May 1981.

20555 was administered by oral gavage, once daily, to groups of at least five male and five female rats for a minimum of twenty-eight consecutive days, at fixed dosage levels of 10, 100 and 1000 mg/kg/day. The test material was prepared as suspensions in 0.1% w/v aqueous methylcellulose at concentrations of 0.1, 1.0 and 10% w/v. Control animals received the vehicle alone.

All rats of Groups 2 and 3 (10 and 100 mg/kg/day respectively) and five males and five females from each of Groups 1 and 4 (Control and 1000 mg/kg/day respectively) were killed following the four-week treatment period (Day 32). The remaining animals (five males and five females from Groups 1 and 4) were retained for a minimum two-week recovery period, following which they were also killed (Day 46).

Bodyweights, food consumption and clinical observations were recorded during the study. Blood and urine samples were taken from all rats shortly prior to termination following the four-week treatment and two-week recovery periods. All animals were killed and subsequently examined macroscopically; specified tissues were then prepared for histological examination.

There were no changes seen in the parameters measured in this study, namely clinical signs, bodyweight gains, food consumption, clinical pathology, organ weight analysis, macroscopic or microscopic pathology, that were considered to be related to treatment with 20555.

Conclusion

Based on the results of this study, it was concluded that 1000 mg/kg/day represents the no-observed effect level (NOEL) for 20555 in the rat.

INTRODUCTION

The study was designed to assess the systemic toxicity to the rat of 20555, lot number 920911, an industrial intermediate, when repeatedly administered orally for a period of at least 28 consecutive days. Additional animals from the control and high dosage groups were retained for a 2-week post treatment observation period.

The procedure used is described in this report. The procedure complied with that described in:

Joint directive of the Japanese Environmental Protection Agency and the Ministries of Health and Welfare and International Trade and Industry, 5 December 1986.

EEC Methods for the determination of toxicity, Directive 84/449/EEC(OJ No. L251, 19.9.84), Part B, Method B.7. Sub-acute toxicity (oral).

OECD Guideline for Testing of Chemicals No. 407, "Repeated Dose Oral Toxicity - Rodent: 28-day or 14-day study". Adopted: 12 May 1981.

The albino rat was chosen as the test species as it has been shown to be a suitable model for this type of study and is the species recommended in the test guidelines.

The rats were dosed orally as the test substance may be ingested accidentally.

Dosages of 0, 10, 100 and 1000 mg/kg/day were selected on the basis of available toxicity information, in particular rat acute toxicity data [LD_{50} (rat) > 5 g/kg bodyweight; HRC Report No.: 921037D/FIX 183/AC] and a 7-day preliminary oral toxicity study [HRC Report No.: 921080D/FIX 184/ST] conducted at HRC.

The study protocol was approved by the Study Director and HRC Management on 23 December 1992 and by the Sponsor on 6 January 1993.

The experimental phase of the study was conducted between 19 January 1993 and 16 April 1993.

TEST SUBSTANCE

Identity: 20555

Alternative name: Tri-p-tolyamine

Chemical name: Tri(4-methylphenyl)amine

Lot number: 920911

Expiry: 2 June 1993

Purity: >99.9%

Appearance: White crystalline powder

Storage conditions: Room temperature in the dark

Date received: 7 October 1992

EXPERIMENTAL PROCEDURE

ANIMAL MANAGEMENT

A total of 33 male and 33 female healthy CD rats of Sprague-Dawley origin (CrI: CD[®] BR VAF PLUS[™]), were received from Charles River (UK) Ltd., Margate, Kent, England on 6 January 1993.

The rats were 28 ± 1 days old, in a weight range of 67 to 94 g on arrival. A thirteen-day acclimatisation period was allowed between delivery of the animals and start of treatment.

All rats were initially caged, as far as possible, in groups of five according to sex in metal cages with wire mesh floors.

A standard laboratory rodent diet (Special Diet Services Rat and Mouse Maintenance Diet) and drinking water were provided *ad libitum*, except as noted under **CLINICAL PATHOLOGY**.

The batches of diet used for this study had been analysed for nutrients, possible contaminants and micro-organisms (Appendix 11).

Results of the routine physical and chemical examination of drinking water at source, as conducted usually weekly by the supplier, are made available to Huntingdon Research Centre Ltd. as quarterly summaries (Appendix 12).

The rats were housed in Building R17, Room 2. Animal room temperature was controlled in the range 18 to 21 °C and relative humidity was generally controlled in the range 40 to 59% RH although slightly low humidity values (<30%) were recorded between Day 24 and Day 30 of the treatment period. These low values were not considered to have affected the integrity of the study. These parameters were continuously monitored using a Kent Clearspan M105 7-day chart recorder. Air exchange was maintained at approximately 19 air changes per hour and lighting was controlled to provide 12 hours artificial light (0700 - 1900 hours) in each 24-hour period.

The health status of all animals was monitored, by daily observation throughout the acclimatisation period, to ensure that the rats selected for final assignment to the study were satisfactory.

Four days before the start of treatment, each animal was weighed and sixty rats were randomly allocated to four groups, two groups consisting of ten males and ten females and two groups consisting of five males and five females. This allocation was carried out using a computer program, so that the weight distribution within each group was similar and the initial group mean bodyweights were approximately equalised.

Each rat was identified within each cage by ear-punch and individually by tail mark (tattoo).

Following the commencement of treatment, spare animals were removed from the study. No further investigations were performed on these animals.

The cages (each containing five rats) constituting each group were distributed in batteries in such a manner that possible environmental influences arising from their spatial distribution were equilibrated, as far as possible, for all treatments (Figure 1).

Each cage was identified by a coloured label according to group. Each label displayed the study schedule number, cage number, sex, individual animal numbers and the initials of the Study Director and Home Office Licensees.

TEST SUBSTANCE PREPARATION

A 10% w/v suspension of 20555 was prepared freshly each day by grinding the test substance in a mortar with a small amount of 1% w/v aqueous methylcellulose (1% MC) until a smooth paste was formed. The formulation was then gradually made up to volume and mixed using a high shear homogeniser. Further concentrations (1.0% and 0.1% w/v) were prepared daily by direct dilution of the test substance.

The chemical stability and homogeneity of test substance formulations in 1% MC were assessed prior to the start of treatment by HRC Department of Analytical Chemistry and were found to be satisfactory (Appendix 13).

Concentration analyses of formulations prepared for administration on Days 1 and 22 were also performed by HRC Department of Analytical Chemistry. An additional sample of the high dose formulation (10% w/v) was taken on Day 24 due to an analytical problem with the Day 22 sample. Results of these analyses are presented in Appendix 13.

The absorption of the test substance was not determined in this study.

Data concerning the analytical purity and homogeneity of the test substance and its stability under the specified conditions of storage are the responsibility of the Sponsor.

TREATMENT PROCEDURE

Dosages were selected on the basis of available toxicity data and a preliminary oral toxicity investigation performed at this laboratory (HRC Report No.: 921080D/FIX 184/ST).

Groups of rats were dosed as follows:

Group	Cage label/ colour code	Treatment	Dosages (mg/kg/day)	Number of rats		Rat numbers	
				♂	♀	♂	♀
1	White	Control, 1% MC	-	10	10	1-10	31-40
2	Yellow	20555	10	5	5	11-15	41-45
3	Green	20555	100	5	5	16-20	46-50
4	Red	20555	1000	10	10	21-30	51-60

The test substance was administered by oral gavage to rats of Groups 2 to 4 inclusive, using a syringe and rubber catheter, at a dose volume of 10 ml/kg/day.

Control animals received the vehicle alone at the same dose volume.

Each animal received a constant dosage based on its most recently recorded bodyweight.

Animals were treated once daily for at least twenty-eight consecutive days. The last day of dosing was 18 February 1993.

Prior to dosing, the test substance formulations were mixed by inversion (x 20) and subsequently mixed using a magnetic stirrer for a period of at least ten minutes before dosing commenced. Dosing was completed within one hour of the commencement of stirring.

Shortly following the 4-week treatment period, five male and five female animals from Groups 1 and 4 (lowest animal numbers were selected) and all animals of Groups 2 and 3 were killed on Day 32 (post treatment sacrifice). These rats were dosed until the day prior to sacrifice.

The remaining animals of Groups 1 and 4 were retained for a 2-week post treatment observation period and were killed on Day 46 (recovery sacrifice); these rats received their last dose on Day 28.

OBSERVATIONS

Clinical signs

All animals were observed daily for signs of ill health, behavioural changes or toxicosis. Any observed changes were recorded.

All animals were checked early in each working day and again in the late afternoon to look for dead or moribund animals. On Saturdays and Sundays, a similar procedure was followed except that the final check was carried out at approximately mid-day. Animals were isolated if the signs or behavioural changes warranted such an action.

Bodyweight

All rats were weighed prior to dosing on Day 1 (Week 0) and subsequently at weekly intervals throughout the study.

Food consumption

The quantity of food consumed in each cage was measured at weekly intervals throughout the study.

Water consumption

Daily monitoring by visual appraisal was maintained throughout the dosing period.

CLINICAL PATHOLOGY**Removal of blood samples**

Food and water were withdrawn overnight prior to collection of samples. Overnight urine samples were collected and blood was withdrawn under light ether anaesthesia from the orbital sinus of the five male and five female rats selected from Groups 1 and 4 (see under **TREATMENT PROCEDURE**) and all rats of Groups 2 and 3. These samples were collected shortly after 4 weeks of treatment (Day 30).

Investigations were also carried out for remaining rats after the 2-week recovery period (Day 44), for all individual parameters significantly affected at Day 30. The blood sampling procedure in this instance was similar to that carried out on Day 30.

The collected blood samples were divided into tubes as follows:

EDTA anticoagulant tubes for haematological investigations
 Citrate anticoagulant tubes for coagulation test
 Heparin anticoagulant microtainer tubes* for biochemical tests.

* Microtainer, brand plasma separator tube, Becton Dickinson, Rutherford, New Jersey, U.S.A.

All the tubes were then mechanically agitated for at least five minutes and the microtainer tubes were subsequently centrifuged for a minimum period of two minutes (3000 'g').

The estimations performed have been listed below, together with an abbreviated title (for use in Tables and Appendices), the methods and the units of measurement applicable:

Haematology

The following parameters were analysed with an Ortho ELT-1500 Analyser, using standard Ortho methodology:

	Units
Packed cell volume (PCV)	%
Haemoglobin (Hb)	g/dl
Red blood cell count (RBC)	$\times 10^6/\text{mm}^3$
Absolute indices:	
Mean corpuscular haemoglobin concentration (MCHC) Calculated: $\text{Hb (g/dl)} \times 100 \div \text{PCV (\%)} $	%
Mean corpuscular volume (MCV) Calculated: $\text{PCV (\%)} \times 10 \div \text{RBC } (\times 10^6/\text{mm}^3)$	fl
Platelet count (Plts)	$\times 10^3/\text{mm}^3$
Total white blood cell count (WBC)	$\times 10^3/\text{mm}^3$

Units

The following estimations were measured using the appropriate methodology:

Thrombotest (TT) - Method of Owren, P.A. (Lancet, 1959, ii, 754)

s

Differential white blood cell count (Diff) - namely:

Neutrophils	(N)	}
Lymphocytes	(L)	
Eosinophils	(E)	
Basophils	(B)	
Monocytes	(M)	

x10³/mm³

The percentage distribution of each cell type was determined by standard microscopy of a blood smear stained with modified Wright's stain counting 100 cells. Percentage values were then converted to absolute values by computer, inevitably involving a "rounding off" in a proportion of the results. Hence, the measured total WBC may differ slightly from the calculated total for the differential count.

Additionally blood film slides were examined for morphological abnormalities (see below). The results of this examination are included in the haematology appendix.

P Polychromasia
 H Hypochromasia
 A Anisocytosis
 R Rouleaux formation
 S Separate film report (generated for additional abnormalities)

NAD No abnormality detected
 1 Slight
 2 Moderate
 3 Marked
 4 Gross

Biochemistry

The following parameters were analysed with a Hitachi 737 Clinical Chemistry Analyser:

Glucose - using BCL Test Kit (hexokinase mediated)	mg/dl
Total protein	g/dl
Albumin (Alb)	g/dl
Globulin (Glob)	
Calculated: Total protein (g/dl) minus Alb (g/dl)	g/dl

	Units
Albumin/Globulin ratio (A/G) Calculated from Total protein and Albumin concentrations	
Urea nitrogen (Urea Nitr)	mg/dl
Creatinine	mg/dl
Alkaline phosphatase (AP) - reaction temperature 30°C	mU/ml
Glutamic-pyruvic transaminase (GPT), also known as 'alanine aminotransferase (ALT)' - using BCL Test Kit, reaction temperature 30°C	mU/ml
Glutamic-oxaloacetic transaminase (GOT), also known as 'aspartate aminotransferase (AST)' - using BCL Test Kit, reaction temperature 30°C	mU/ml
Gamma-glutamyltransferase (γ GT) - using BCL Test Kit, reaction temperature 30°C	mU/ml
Total bilirubin (Bilirubin)	mg/dl
Sodium (Na)	mEq/l
Potassium (K)	mEq/l
Calcium (Ca)	mEq/l
Chloride (Cl)	mEq/l
Inorganic phosphorus (P)	mEq/l
Cholesterol (Chol)	mg/dl

The following parameter was analysed with a Roche Cobas Centrifugal Analyser:

Triglycerides (Tri-glyc) - using BCL Test Kit	mg/dl
---	-------

Urinalysis

The following estimations were measured using the appropriate methodology:

Volume (Vol)	ml
pH - using pH meter	

Units

Specific Gravity (SG) - using Atago UR-1 Refractometer,
sample compared with water (nominal value of 1000)

Protein - using Roche Cobas Centrifugal Analyser,
utilising modified method of Macart, M. and Gerbaut, L.
(Clin. Chim. Acta, 1984, **141**, 77)

mg/dl

The following tests were also performed using qualitative indicators (+) of analyte concentration:

Total reducing substances (TRS)
Glucose
Ketones
Bile pigments
Urobilinogen
Haem pigments*

+ Clinitest (total reducing substances) and Multistix (remaining parameters) are diagnostic reagents obtained from Ames Company, Stoke Poges, Berkshire, England.

* Positive or negative finding only

Results have been reported according to the following convention:

0 Negative
tr 'Trace' of analyte
+ 'Small amount' of analyte
++ 'Moderate amount' of analyte
+++ 'Large amount' of analyte

Microscopic examination of urine samples was carried out by centrifuging samples at approximately 1500 'g' for 10 minutes and spreading the resulting deposit on a microscope slide. The deposit was examined for the presence of the following:

Epithelial cells (E)
Polymorphonuclear leucocytes (P)
Mononuclear leucocytes (M)
Erythrocytes (R)
Organisms (O)
Renal tubule casts (C)
Sperm (SP)
Other abnormal constituents (A)

The frequency of the above parameters in the centrifugal deposit was as follows:

0 None found in any field examined
1 Few in some fields examined
2 Few in all fields examined
3 Many in all fields examined

TERMINAL STUDIES**Termination**

Following 4 weeks of treatment and clinical pathology investigations, animals selected for post treatment sacrifice (see under **TREATMENT PROCEDURE**) were killed on Day 32 by carbon dioxide asphyxiation and a complete autopsy undertaken. The order of sacrifice was determined using a pre-set cage sequence. Specified organs were weighed and relevant tissue samples were fixed for microscopic examination.

Remaining rats of Groups 1 and 4 were killed and examined in a similar manner on Day 46, following a 2-week post treatment observation period (recovery sacrifice).

Organ weight

The following organs from each animal killed after four or six weeks were dissected free of fat and weighed:

adrenals	ovaries
brain	spleen
kidneys	testes (with epididymides)
liver	

Macroscopic pathology

The macroscopic appearance of the tissues of all rats was recorded and samples of the following tissues were preserved in 10% buffered formalin:

adrenals* 副腎	ileum	skeletal muscle
aorta	jejunum	skin
brain (medullary, cerebellar and cortical sections)	kidneys*	spinal cord (cervical level)
caecum	larynx	spleen*
colon	liver*	sternum
duodenum	lungs	stomach
eyes (Davidson's fluid as fixative)	lymph nodes (cervical and mesenteric)	testes (including epididymis)*
femur (for bone and marrow sections) with joint	mammary glands	thymus (where present)
Harderian gland	oesophagus	thyroid (with parathyroid)
head (to preserve nasal cavity, paranasal sinuses, oral cavity, nasopharynx, middle ear, teeth, lachrymal gland and Zymbal's gland)	ovaries	tongue
heart*	pancreas	trachea
	pharynx	urinary bladder
	pituitary	uterus (with cervix)
	prostate	vagina
	rectum	
	salivary gland	any macroscopically abnormal tissue*
	sciatic nerve	
	seminal vesicles	

* Tissues required for histopathological examination for rats from Groups 1 and 4

Microscopic pathology

Fixed tissue samples required for microscopic examination were prepared by embedding in paraffin wax (m.p. 56°C); sections were then cut at 4 µm and stained with haematoxylin and eosin.

Microscopic examination of prepared slides (from tissues indicated under Macroscopic pathology) was carried out for all rats of Group 1 (Control group) and Group 4 (high dosage group) killed on Day 32 (19 February 1993).

Microscopic examinations were completed on 16 April 1993.

STATISTICAL ANALYSES

All statistical analyses were carried out separately for males and females using the individual animal as the basic experimental unit.

The following sequence of statistical tests was used for bodyweight gains, food consumption (using weekly cage totals), clinical pathology and organ weight data:

If the data consisted predominantly of one particular value (relative frequency of the mode exceeds 75%), the proportion of values different from the mode was analysed by Fisher's exact test (1) followed by Mantel's test for a trend in proportions (2). Otherwise:

Bartlett's test (3) was applied to test for heterogeneity of variance between treatments. If significant heterogeneity was found at the 1% level, a logarithmic transformation was tried to see if a more stable variance structure could be obtained.

If no significant heterogeneity was detected (or if a satisfactory transformation was found), a one-way analysis of variance was carried out followed by Student's 't' test and Williams' test (5) for a dose-related response (although only the one thought more appropriate for the response pattern observed was reported).

If significant heterogeneity of variance was present and could not be removed by a logarithmic transformation, the Kruskal-Wallis analysis of ranks (4) was used. This analysis was followed by the non-parametric equivalent of the 't' test and Williams' test (Shirley's test, (6)).

Covariate analysis of organ weight data (with final bodyweight as covariate), was also performed using adjusted weights for organs where a correlation between organ weight and bodyweight was established at the 10% level of significance (7).

Significant differences between control animals and those treated with the test substance have been expressed at the 5% (+ $P < 0.05$ for 't' test analysis or * $P < 0.05$ for Williams' test analysis) or 1% (++ $P < 0.01$ for 't' test analysis or ** $P < 0.01$ for Williams' test analysis) level.

References

1. FISHER, R.A. (1932) *Statistical methods for research workers*, 4th ed., Oliver and Boyd.
2. MANTEL, N. (1963) *J. Amer. Statist. Ass.*, **58**, 690.
3. BARTLETT, M.S. (1937) *Proc. Roy. Soc. A.*, **160**, 268.
4. KRUSKAL, W.H. and WALLIS, W.A. (1952/3) *J. Amer. Statist. Ass.*, **47**, 583 and **48**, 907.
5. WILLIAMS, D.A. (1971/2) *Biometrics*, **27**, 103 and **28**, 519.
6. SHIRLEY, E. (1977) *Biometrics*, **33**, 386.
7. ANGERVALL, L. and CARLSTROM, E. (1963) *J. Theoret. Biol.*, **4**, 254.

PROCEDURES

The procedures used during the study were those documented in HRC Procedure Manuals.

ARCHIVES

All specimens, raw data and study related documents generated during the course of the study at HRC, together with a copy of the final report, are lodged in the Huntingdon Research Centre Ltd. Archive.

Such specimens and records will be retained for a minimum period of five years from the date of issue of the final report. At the end of the five-year retention period the client will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the client's knowledge.

RESULTS

OBSERVATIONS

Clinical signs

There were no mortalities.

No clinical signs were seen for treated or control animals during the treatment or recovery periods.

Bodyweight (Figure 2, Table 1, Appendix 1)

Group mean bodyweights and cumulative bodyweight gains for treated animals were comparable to those of control animals throughout the treatment and recovery periods.

Food consumption (Figure 3, Table 2, Appendix 2)

No notable differences in weekly food consumption or cumulative consumption values were recorded for treated rats of either sex relative to controls throughout the treatment or recovery periods.

CLINICAL PATHOLOGY

Haematology (Tables 3 and 4, Appendices 3 and 4)

Week 5

Statistically significantly lower ($P < 0.05$) neutrophil, lymphocyte and total white blood cell counts were recorded for all male groups receiving 20555 whilst statistically significantly higher ($P < 0.01$ or $P < 0.05$) lymphocyte and total white blood cell counts were recorded for females receiving 1000 mg/kg/day relative to controls. These differences were not dosage-related and there was considerable variation between individual values. The variations in white blood cell counts for both sexes were therefore considered to have arisen by chance.

The group mean value for mean corpuscular volume (MCV) was statistically significantly higher ($P < 0.05$) for males treated at 1000 mg/kg/day than for controls. However, the magnitude of this difference from controls was minor and individual values overlapped between the groups, and considering that the packed cell volume and red blood cell count remained unaffected the difference was not considered to be treatment-related.

No other statistically significant differences between treated and control rats were recorded during the treatment period.

Week 7

The parameters examined at Week 7 as a consequence of statistical change at Week 5 showed no significant difference from control.

The occurrence of slight polychromasia and/or anisocytosis is not uncommon among young laboratory rats and at the incidence seen in this study (14/60 rats) was not considered to be related to treatment with 20555.

Biochemistry (Tables 5 and 6, Appendices 5 and 6)**Week 5**

Creatinine levels were statistically significantly lower ($P < 0.01$) for males treated at 1000 mg/kg/day in comparison with controls. Statistically significantly higher ($P < 0.01$) sodium ion levels were recorded for all male groups receiving 20555 relative to controls. Individual values for these parameters overlapped between the groups and the minor differences were not believed to be related to treatment with 20555.

No other statistically significant differences between treated and control rats were recorded during the treatment period.

Week 7

Parameters examined at Week 7 as a consequence of statistical change at Week 5 showed no significant difference from control.

Urinalysis (Table 7, Appendix 7)**Week 5**

No statistically significant differences between treated and control rats were found following the treatment period.

TERMINAL STUDIES

Organ weights (Tables 8 and 9, Appendices 8 and 9)

Week 5

Adjusted mean kidney weights were statistically significantly lower ($P > 0.01$ or $P > 0.05$) for all male groups treated with 20555 in comparison with controls. Individual values overlapped between groups and the differences from control were not dosage-related and there was no corroborative pathological change. Therefore, this finding was not considered to be attributable to treatment with 20555.

No other statistically significant differences were recorded between treated and control animals following the treatment period.

Week 7

No significant differences between previously-treated and control rats were found following the recovery period.

Macroscopic pathology (Tables 10 and 11, Appendix 10)

The macroscopic examinations performed at termination and following the recovery period revealed no changes attributable to treatment with 20555.

Microscopic pathology (Table 12, Appendix 10)

The following comments are made in summary:

No treatment-related changes were observed.

Histopathological findings observed only in male rats of 1000 mg/kg/day dose group included:

- | | |
|-----------------|---|
| Kidneys: | Focal unilateral medullary necrosis and a focus of cystic medullary tubule(s) containing necrotic detritus in 1 of 5 male rats. |
| Adrenal: | Unilateral, minimal, focal cortical hyperplasia in 1 of 5 male rats. |
| Testes: | Bilateral, minimal, focal reduced spermatogenesis in 1 of 5 male rats. |

In view of the low incidence and focal nature of these lesions, they were considered spontaneous in origin and, therefore, unrelated to treatment.

All other histopathological findings were considered of no toxicological importance.

DISCUSSION AND CONCLUSION

In this subacute toxicity study in the rat with 20555, no treatment-related changes were seen in any of the parameters measured within the treatment period, at post mortem or during microscopic examination of any dosage group.

Therefore, based on the results of this study, it was concluded that 1000 mg/kg/day represents the no-observed effect level (NOEL) for 20555 in the rat.

FIGURE 1

Group and cage arrangement in batteries

Group	Cage label/ colour code	Treatment	Dosages (mg/kg/day)	Number of rats		Rat numbers	
				♂	♀	♂	♀
1	White	Control, 1% MC	-	10	10	1-10	31-40
2	Yellow	20555	10	5	5	11-15	41-45
3	Green	20555	100	5	5	16-20	46-50
4	Red	20555	1000	10	10	21-30	51-60

Group arrangement

♂		♀	
1	2	1	2
3	4	3	4
4	1	4	1

Cage arrangement

♂		♀	
1	3	7	9
4	5	10	11
6	2	12	8

FIGURE 2
Bodyweights - group mean values

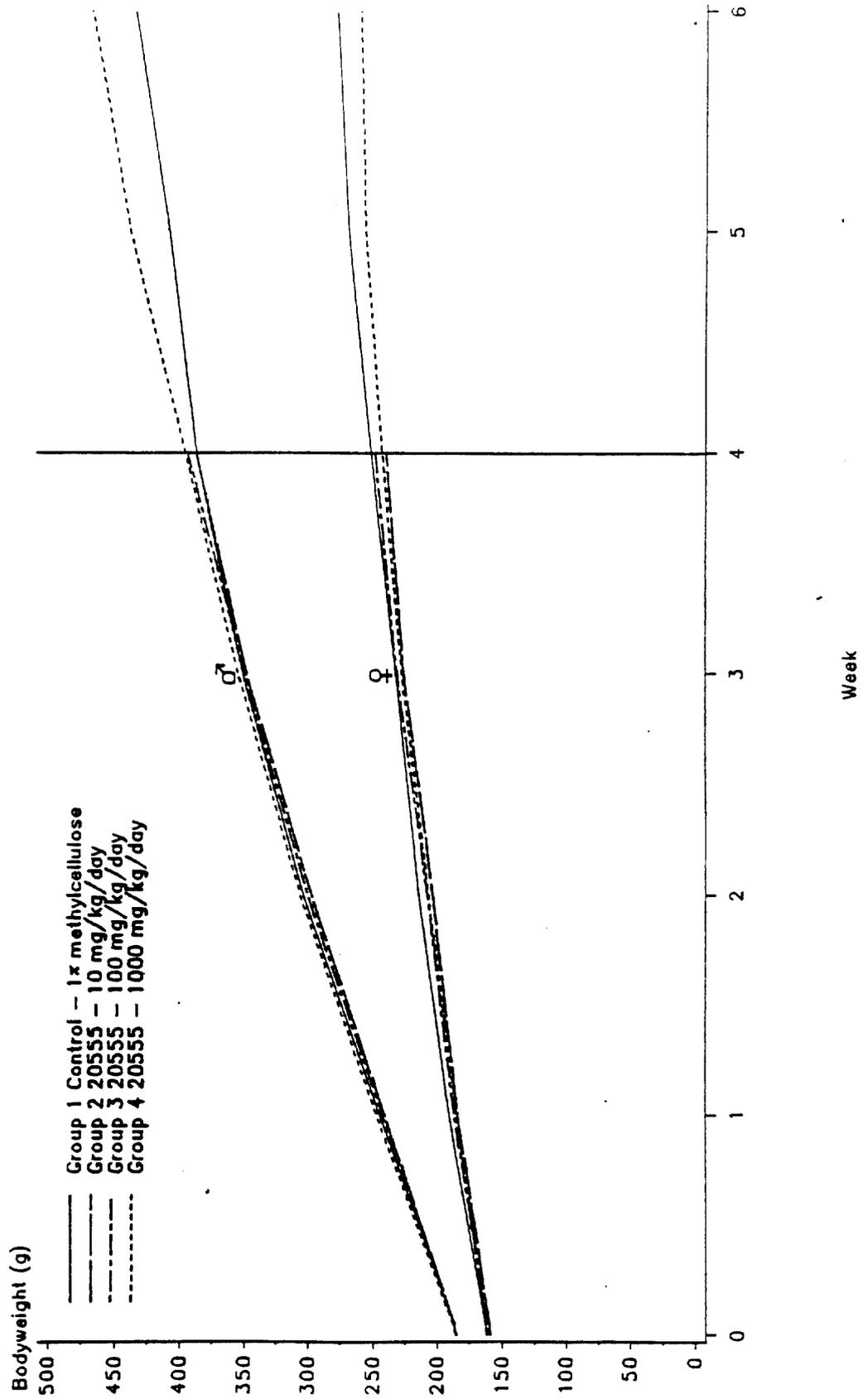


FIGURE 3

Food consumption - group mean values

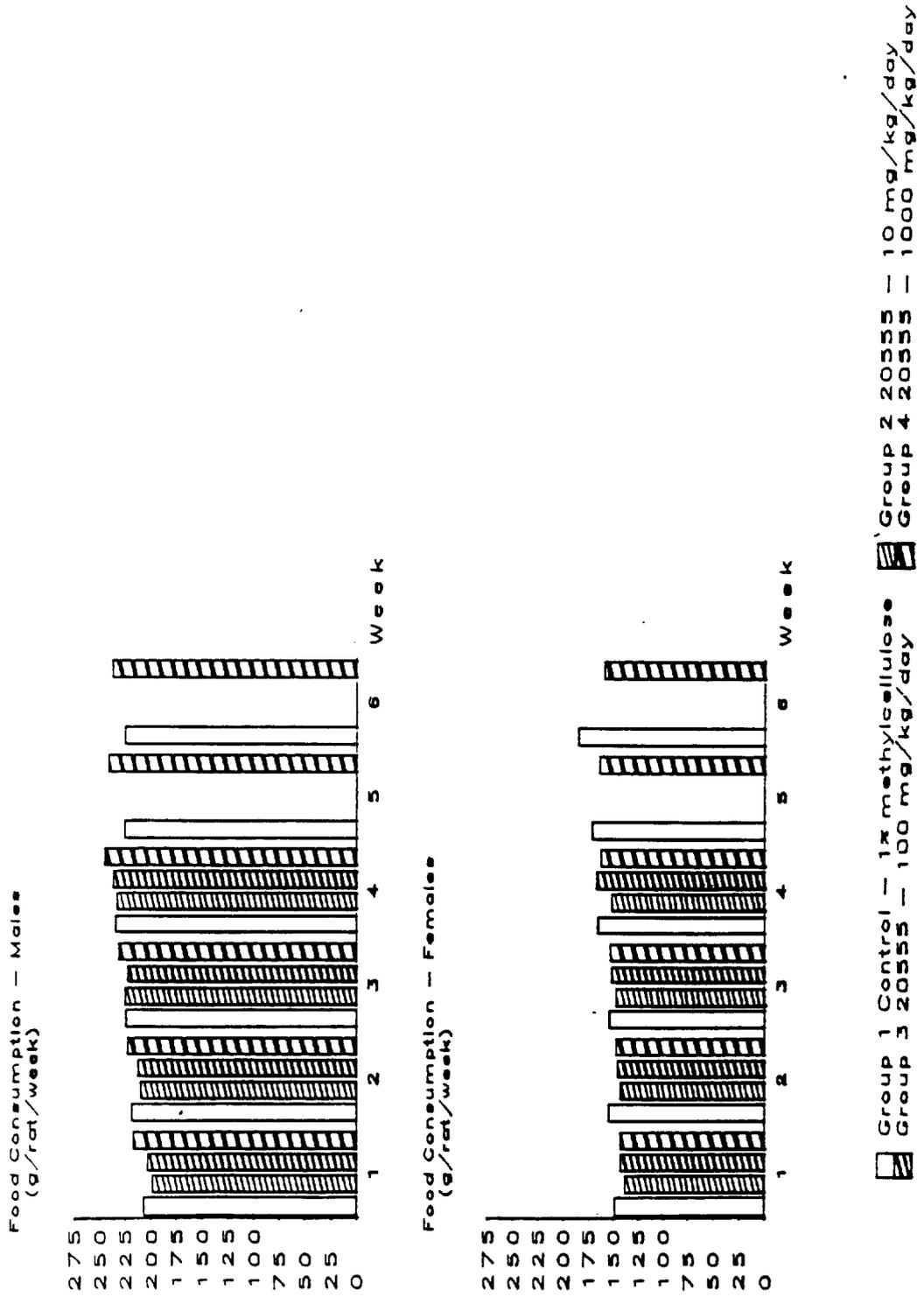


TABLE 1
Bodyweights - group mean values (g)

Week	Group and dosage (mg/kg/day)									
	1♂ Control 1% MC	2♂ 20555 10	3♂ 20555 100	4♂ 20555 1000	1♀ Control 1% MC	2♀ 20555 10	3♀ 20555 100	4♀ 20555 1000		
Dosing	0	Mean	184	184	184	185	162	158	160	161
		SD	10.3	10.8	5.2	12.1	7.4	6.8	5.7	7.1
1	Mean	245	241	242	247	190	184	187	185	
	SD	13.7	20.1	9.4	18.4	10.1	11.5	8.3	8.5	
2	Mean	303	297	300	306	214	203	208	207	
	SD	19.4	28.7	16.8	25.4	11.5	11.1	10.6	12.4	
3	Mean	350	347	348	353	231	225	230	227	
	SD	24.7	41.4	20.6	33.8	14.8	11.2	15.1	16.2	
4	Mean	386	386	392	394	251	239	247	242	
	SD	28.2	53.7	23.6	41.5	17.0	9.4	14.7	18.2	
Cumulative gains Week 0-4		202	202	208	209	89	81	87	81	
% control value		-	100	103	103	-	91	98	91	
Withdrawal	4	Mean	375		401	253			237	
		SD	29.9		28.2	20.9			21.5	
5	Mean	407		437	268			255		
	SD	33.9		35.4	22.7			26.8		
6	Mean	433		466	277			259		
	SD	38.8		43.6	25.0			26.1		
Cumulative gains Week 4-6		58		65	24			22		
% control value		-		112	-			92		

SD Standard deviation
Blank Non-significant

TABLE 2

Food consumption - group mean values (g/rat/week)

Week	Group and dosage (mg/kg/day)							
	1♂ Control 1% MC	2♂ 20555 10	3♂ 20555 100	4♂ 20555 1000	1♀ Control 1% MC	2♀ 20555 10	3♀ 20555 100	4♀ 20555 1000
Dosing								
1	207	199	204	218	149	138	142	143
2	219	211	213	224	154	143	146	147
3	225	226	223	232	153	147	151	153
4	235	234	238	246	165	151	166	162
Cumulative values Week 1 - 4	886	870	878	920	621	579	605	605
% control value	-	98	99	104	-	93	97	97
Withdrawal								
5	226			242	171			164
6	227			239	185			159
Cumulative values Week 5 and 6	688			731	525			486
% control value	-			106	-			93

Blank Non-significant

TABLE 3

Haematology - group mean values

Week 5 (17 February 1993)

Group/ dosage mg/kg/day		PCV %	Hb g/dl	RBC x10 ⁶ / mm ³	MCHC %	MCV fl	WBC + Diff x10 ³ /mm ³					Plts x10 ³ / mm ³	TT s	
							Total	N	L	E	B			M
1♂ Control 1% MC	Mean	56	16.0	7.3	28.6	77	11.6	2.12	9.32	0.08	0.00	0.04	942	23
	SD	1.2	0.32	0.31	1.01	1.5	1.95	0.80	1.68	0.12	0.00	0.10	51.7	1.0
2♂ 20555 10	Mean	55	15.9	7.0	29.0	78	*	*	*					
	SD	2.3	0.50	0.37	0.59	1.1	7.5	0.75	6.72	0.03	0.00	0.00	953	23
3♂ 20555 100	Mean	56	16.3	7.1	29.3	78	*	*	*					
	SD	0.8	0.17	0.05	0.29	1.0	7.4	1.34	6.06	0.01	0.00	0.03	778	23
4♂ 20555 1000	Mean	56	16.1	7.1	28.8	79	*	*	*					
	SD	2.2	0.41	0.34	0.47	1.1	9.1	1.78	7.22	0.05	0.00	0.02	958	22
1♀ Control 1% MC	Mean	55	15.9	7.0	28.8	79	6.1	1.31	4.76	0.07	0.00	0.00	1066	20
	SD	3.2	0.90	0.54	0.84	1.6	2.42	0.79	1.94	0.12	0.00	0.00	180.9	0.7
2♀ 20555 10	Mean	55	15.7	7.0	28.8	78	5.4	0.82	4.55	0.03	0.00	0.00	1090	20
	SD	2.1	0.73	0.36	0.42	1.6	1.12	0.13	1.14	0.03	0.00	0.00	160.2	1.2
3♀ 20555 100	Mean	54	15.4	6.8	28.5	79	6.6	1.19	5.36	0.06	0.00	0.02	1208	20
	SD	1.4	0.40	0.25	0.29	1.4	1.89	0.55	1.98	0.12	0.00	0.05	251.2	1.1
4♀ 20555 1000	Mean	55	15.4	7.0	28.2	78	**	*	*					
	SD	0.5	0.32	0.18	0.57	1.9	9.5	1.81	7.63	0.04	0.00	0.04	1001	19

SD Standard deviation
 Blank Non-significant
 * P<0.05
 ** P<0.01
 LT Data log-transformed
 K Kruskal-Wallis analysis
 F Frequency analysis

TABLE 4

Haematology - group mean values

Week 7 (3 March 1993)

Group/ dosage mg/kg/day		MCV‡	WBC + Diff x10 ³ /mm ³		
			f1	Total	N
1♂ Control 1% MC	Mean	74	13.7	1.80	11.75
	SD	2.3	1.69	0.49	1.46
4♂ 20555 1000	Mean	75	16.2	2.52	13.47
	SD	1.1	2.89	1.52	1.94
1♀ Control 1% MC	Mean	-	9.2	-	7.68
	SD	-	2.12	-	2.32
4♀ 20555 1000	Mean	-	8.7	-	7.59
	SD	-	2.75	-	2.25

Blank Non-significant

‡ The packed cell volume (PCV) and red blood cell count (RBC) were estimated in order to calculate the mean corpuscular volume (MCV) but have not been reported as no significant differences from control were seen for either PCV or RBC at Week 5

TABLE 5

Biochemistry - group mean values

Week 5 (17 February 1993)

Group/ dosage mg/kg/day		Glu- cose mg/dl	Protein g/dl			A/G	Urea Nitr mg/dl	Creat- inine mg/dl	AP mU/ ml	GPT mU/ ml	GOT mU/ ml
			Total	Alb	Glob						
1♂ Control 1% MC	Mean	147	6.8	3.0	3.8	0.79	13	0.6	380	29	65
	SD	18.6	0.13	0.12	0.11	0.05	2.6	0.10	30.3	1.1	13.8
2♂ 20555 10	Mean	129	6.6	2.9	3.6	0.81	13	0.5	405	33	64
	SD	17.4	0.32	0.08	0.29	0.07	2.2	0.04	45.7	8.0	16.4
3♂ 20555 100	Mean	131	6.5	2.9	3.6	0.80	13	0.5	407	28	52
	SD	7.5	0.35	0.12	0.25	0.04	1.5	0.04	48.0	3.5	6.4
4♂ 20555 1000	Mean	128	6.6	2.9	3.6	0.81	11	0.5	379	27	53
	SD	11.8	0.30	0.08	0.25	0.05	1.9	0.04	52.2	5.9	7.7
1♀ Control 1% MC	Mean	130	6.7	3.0	3.7	0.80	13	0.5	207	24	49
	SD	5.1	0.41	0.18	0.33	0.08	1.9	0.07	36.4	8.4	3.8
2♀ 20555 10	Mean	124	6.7	3.1	3.7	0.84	13	0.5	224	22	47
	SD	4.8	0.16	0.15	0.11	0.06	1.8	0.05	62.4	3.1	4.7
3♀ 20555 100	Mean	127	6.6	2.9	3.7	0.80	14	0.5	205	22	47
	SD	12.6	0.35	0.17	0.28	0.07	1.2	0.07	37.3	1.9	9.0
4♀ 20555 1000	Mean	125	6.6	3.0	3.7	0.81	13	0.5	236	21	48
	SD	5.4	0.19	0.18	0.29	0.10	2.3	0.04	36.6	4.3	3.7

SD Standard deviation

Blank Non-significant

** P<0.01

TABLE 5
(Biochemistry - continued)

Week 5 (17 February 1993)

Group/ dosage mg/kg/day		γ GT mU/ ml	Bili- rubin mg/dl	Na mEq/ l	K mEq/ l	Ca mEq/ l	P mEq/ l	Cl mEq/ l	Chol mg/dl	Tri- glyc mg/dl
1♂ Control 1% MC	Mean SD	<1	<0.1	144 0.9	4.0 0.43	5.4 0.08	5.3 0.47	97 0.5	70 21.9	94 15.2
2♂ 20555 10	Mean SD	<1	<0.1	** 145 0.0	4.0 0.14	5.4 0.18	5.1 0.46	98 1.5	78 6.2	96 17.3
3♂ 20555 100	Mean SD	<1	<0.1	** 145 0.4	3.9 0.26	5.4 0.13	5.6 0.24	98 1.2	70 7.9	108 35.2
4♂ 20555 1000	Mean SD	<1	<0.1	** 145 0.7	3.8 0.29	5.4 0.07	5.2 0.39	97 0.9	80 11.8	93 41.6
1♀ Control 1% MC	Mean SD	<2	<0.1	144 1.8	3.7 0.25	5.3 0.29	4.2 0.25	100 2.5	77 20.3	41 13.8
2♀ 20555 10	Mean SD	<1	<0.1	144 1.8	3.8 0.16	5.3 0.23	3.9 0.32	100 1.5	82 12.3	43 10.9
3♀ 20555 100	Mean SD	<1	<0.1	144 2.5	4.1 0.67	5.3 0.11	4.4 0.73	100 2.1	82 20.4	43 6.0
4♀ 20555 1000	Mean SD	<2	<0.1	144 1.8	3.5 0.42	5.3 0.17	4.2 0.41	100 1.1	85 26.1	38 6.0

SD Standard deviation

Blank Non-significant

** P<0.01

TABLE 6

Biochemistry - group mean values

Week 7 (3 March 1993)

Group/ dosage mg/kg/day		Creat- inine mg/dl	Na mEq/ l
1♂ Control	Mean	0.6	144
1% MC	SD	0.04	1.3
4♂ 20555	Mean	0.6	144
1000	SD	0.05	1.3
1♀ Control		-	-
1% MC			
4♀ 20555		-	-
1000			

SD Standard deviation

Blank Non-significant

TABLE 7

Urinalysis - group mean values

Week 5 (17 February 1993)

Group/ dosage mg/kg/day		Vol- ume ml	pH	SG	Pro- tein mg/dl
1♂ Control	Mean SD	7.1 2.05	6.6 0.25	1036 6.3	LT 229
1% MC					122.9
2♂ 20555	Mean SD	8.0 2.06	6.6 0.05	1035 4.2	195 114.8
10					
3♂ 20555	Mean SD	8.5 1.66	6.5 0.07	1032 1.3	173 67.4
100					
4♂ 20555	Mean SD	9.4 1.97	6.7 0.22	1030 3.5	134 9.7
1000					
1♀ Control	Mean SD	4.7 1.69	6.5 0.30	LT 1041 16.3	81 20.5
1% MC					
2♀ 20555	Mean SD	4.8 1.06	6.3 0.18	1038 5.2	75 6.4
10					
3♀ 20555	Mean SD	4.2 0.50	6.5 0.15	1042 2.9	81 12.6
100					
4♀ 20555	Mean SD	5.3 0.50	6.5 0.15	1037 2.9	87 12.6
1000					

SD Standard deviation
Blank Non-significant
LT Data log-transformed

TABLE 8

Organ weights - group mean values

Week 5 (19 February 1993)

Group/ dosage mg/kg/day		Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Testes+ Epidids g
1♂ Control	Mean	397	2.03	A	A	A	56.5	4.37
1% MC	SD	25.3	0.08	21.9 (21.4)	0.82 (0.81)	3.48 (3.44)	6.40	0.48
2♂ 20555	Mean	386	1.95	21.0	0.81	2.97	54.8	4.18
10	SD	54.1	0.10	3.89 (21.3)	0.18 (0.82)	0.30 (2.99)*	7.36	0.30
3♂ 20555	Mean	394	1.98	23.4	0.93	3.16	59.6	4.17
100	SD	26.6	0.09	1.75 (23.1)	0.05 (0.93)	0.21 (3.14)*	7.08	0.08
4♂ 20555	Mean	384	2.00	22.4	0.75	2.97	54.8	4.12
1000	SD	55.1	0.09	3.64 (22.8)	0.10 (0.76)	0.35 (3.01)**	9.84	0.62

SD Standard deviation

Blank Non-significant

* P<0.05

** P<0.01

A Values, adjusted for final bodyweight, given in parentheses

TABLE 8
(Organ weights - continued)

Week 5 (19 February 1993)

Group/ dosage mg/kg/day		Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Ovaries mg
1♀ Control 1% MC	Mean	248	1.89 ^A	11.2 ^A	0.65	2.25 ^A	72.9	100.7 ^A
	SD	12.0	0.05 (1.88)	0.53 (11.0)	0.13	0.18 (2.20)	10.6	17.91 (97.9)
2♀ 20555 10	Mean	237	1.81	11.0	0.57	2.08	62.3	85.8
	SD	11.3	0.04 (1.83)	0.49 (11.2)	0.10	0.17 (2.13)	6.90	10.17 (88.9)
3♀ 20555 100	Mean	244	1.86	11.5	0.61	2.25	65.3	102.0
	SD	13.5	0.06 (1.85)	1.21 (11.5)	0.08	0.17 (2.25)	5.07	17.55 (101.5)
4♀ 20555 1000	Mean	243	1.87	11.7	0.68	2.22	70.5	97.0
	SD	17.4	0.10 (1.87)	0.93 (11.8)	0.07	0.29 (2.22)	4.39	9.29 (97.1)

SD Standard deviation

Blank Non-significant

A Values, adjusted for final bodyweight, given in parentheses

TABLE 9

Organ weights - group mean values

Week 7 (5 March 1993)

Group/ dosage mg/kg/day		Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Testes+ Epidids g
1♂ Control 1% MC	Mean	435	2.01	22.3 ^A	0.83 ^A	3.53	69.5	4.26
	SD	39.4	0.08	2.68 (23.0)	0.13 (0.86)	0.30	17.04	0.20
4♂ 20555 1000	Mean	460	2.08	22.6	0.93	3.64	63.4	4.46
	SD	44.4	0.06	3.43 (21.9)	0.17 (0.90)	0.18	6.39	0.26

SD Standard deviation

Blank Non-significant

A Values, adjusted for final bodyweight, given in parentheses

TABLE 9
(Organ weights - continued)

Week 7 (5 March 1993)

Group/ dosage mg/kg/day		Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Ovaries mg
1 ♀ Control 1% MC	Mean	273	1.88	13.1 ^A	0.62	2.30	78.9	109.0
	SD	28.1	0.12	1.77 (12.8)	0.07	0.20	8.87	22.02
4 ♀ 20555 1000	Mean	258	1.90	12.2	0.63	2.30	75.3	105.9
	SD	24.6	0.03	1.45 (12.5)	0.04	0.21	7.82	18.98

SD Standard deviation

Blank Non-significant

A Values, adjusted for final bodyweight, given in parentheses

TABLE 10
Macroscopic pathology incidence summary (Terminal kill)

	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
Removal reason: Terminal	10	5	5	10	10	5	5	10
Animals on study	5	5	5	5	5	5	5	5
Animals completed	3	1	3	1	2	3	2	1
Lymph Nodes - Cervical Enlarged	0	0	1	0	0	1	2	0
Stomach Antrum Mucosa White nodule, near to limiting ridge								

TABLE II
Macroscopic pathology incidence summary (Recovery kill)

	Group	Group	Group	Group
Removal reason: Recovery	1	4	1	4
	--- Males	----	-- Females	--
Animals on study	10	10	10	10
Animals completed	5	5	5	5
Lymph Nodes - Cervical	3	1	1	0
Enlarged				
Spleen	0	1	0	0
Capsule thickened				
Stomach Antrum Mucosa	0	0	1	1
White nodule/s, near to limiting ridge				

TABLE 12
Microscopic pathology incidence summary

	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
	10	5	5	10	10	5	5	10
Heart								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected	5	5	5	5	5	5	5	5
Liver								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected	5	5	5	5	5	5	5	5
Centrilobular hepatocyte enlargement (Total)	1	1	1	1	0	0	0	0
Minimal	1	1	1	1	0	0	0	0
Centrilobular hepatocyte vacuolation (Total)	3	3	3	3	1	1	1	1
Minimal	3	3	3	3	1	1	1	1
Parenchymal inflammatory cells	2	2	2	2	1	1	1	1
Sinusoidal dilatation/congestion	5	5	5	5	1	1	1	1
Spleen								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected	5	5	5	5	5	5	5	5
Kidneys								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected	5	5	5	5	5	5	5	5
Tubular basophilis (Total)	4	4	4	4	5	5	5	5
Minimal	1	1	1	1	0	0	0	0
Medullary necrosis	1	1	1	1	0	0	0	0
Cystic medullary tubule(s) containing necrotic detritus	0	0	0	0	0	0	0	0
Pelvic inflammatory cell infiltration	0	0	0	0	0	0	0	0
Vacuolated epithelium of occasional cortical tubules (Total)	0	0	0	0	0	0	0	0
Minimal	0	0	0	0	0	0	0	0
Testes								
Examined	5	5	5	5	5	5	5	5
No abnormalities detected	5	5	5	5	5	5	5	5

TABLE 12
(Microscopic pathology incidence summary - continued)

	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
Animals on study	10	5	5	10	10	5	5	10
Animals completed	5	0	0	5	5	0	0	5
(Continued)								
Testes	0	0	0	1	0	0	0	0
Reduced spermatogenesis (Total)	0	0	0	1	0	0	0	0
Minimal								
Epididymides	5	0	0	5	0	0	0	0
Examined	5	0	0	5	0	0	0	0
No abnormalities detected								
Adrenals	5	0	0	5	5	0	0	5
Examined	4	0	0	4	5	0	0	5
No abnormalities detected	0	0	0	1	0	0	0	0
Cortical hyperplasia (Total)	0	0	0	1	0	0	0	0
Minimal	1	0	0	0	0	0	0	0
Vacuolated cortical cells (Total)	1	0	0	0	0	0	0	0
Minimal								
Lymph Nodes - Cervical	3	0	0	1	2	0	0	1
Examined	3	0	0	1	2	0	0	1
Lymphoid proliferation (Total)	3	0	0	1	2	0	0	1
Minimal								

APPENDIX 1

Bodyweights - individual values (g)

Group 1♂: Control 1% MC

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
1	1	189	249	307	360	395		
	2	204	271	332	382	417		
	3	189	254	323	376	417		
	4	168	224	277	320	357		
	5	187	254	311	361	397		
2	6	189	249	312	348	378	402	432
	7	178	242	310	368	416	456	491
	8	182	233	288	335	370	402	426
	9	181	239	300	347	378	412	436
	10	171	231	271	304	332	361	382

Group 2♂: 20555 10 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
3	11	180	229	276	317	346		
	12	171	217	267	306	335		
	13	194	269	341	412	471		
	14	196	251	301	346	381		
	15	178	237	299	353	396		

APPENDIX 1

(Bodyweights - continued)

Group 3♂: 20555 100 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
4	16	181	233	286	331	376		
	17	187	250	312	366	417		
	18	178	240	296	338	380		
	19	191	254	322	375	418		
	20	182	234	283	332	369		

Group 4♂: 20555 1000 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
5	21	205	280	354	422	481		
	22	181	234	288	336	372		
	23	167	219	267	308	341		
	24	187	235	282	330	368		
	25	183	243	299	336	371		
6	26	203	266	316	346	391	417	450
	27	183	256	328	396	447	496	542
	28	191	262	324	369	407	440	457
	29	177	238	295	338	374	406	430
	30	173	237	304	353	387	424	452

APPENDIX 1

(Bodyweights - continued)

Group 1♀: Control 1% MC

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
7	31	158	188	211	221	229		
	32	167	198	226	242	258		
	33	161	192	212	222	250		
	34	171	204	226	248	263		
	35	157	184	210	230	239		
8	36	166	192	216	235	262	277	291
	37	153	179	202	216	239	256	260
	38	166	190	208	231	253	264	269
	39	148	173	197	211	229	241	253
	40	168	204	234	258	283	301	314

Group 2♀: 20555 10 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
9	41	162	197	218	236	251		
	42	155	175	195	218	234		
	43	164	191	211	231	240		
	44	163	187	202	231	243		
	45	148	169	191	209	226		

APPENDIX 1

(Bodyweights - continued)

Group 3♀: 20555 100 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
10	46	156	182	199	223	238		
	47	166	199	226	252	273		
	48	155	177	205	228	243		
	49	156	186	203	212	238		
	50	166	190	205	237	244		

Group 4♀: 20555 1000 mg/kg/day

Cage number	Animal number	Week						
		0	1	2	3	4	5	6
11	51	158	183	212	229	246		
	52	168	187	209	238	256		
	53	151	174	194	216	228		
	54	165	189	203	216	238		
	55	170	197	224	249	266		
12	56	163	188	213	236	250	274	274
	57	151	175	196	209	219	241	246
	58	157	182	204	220	233	253	266
	59	170	197	225	249	267	287	288
	60	161	175	187	203	216	219	221

APPENDIX 2

Food consumption - cage mean values (g/rat/week)

Group 1♂:
Control
1% MC

Week	Cage	
	1	2
1	216	198
2	222	216
3	231	218
4	236	235
5		226
6		227

Group 2♂:
20555
10 mg/kg/day

Week	Cage
	3
1	199
2	211
3	226
4	234
5	
6	

Group 3♂:
20555
100 mg/kg/day

Week	Cage
	4
1	204
2	213
3	223
4	238
5	
6	

Group 4♂:
20555
1000 mg/kg/day

Week	Cage	
	5	6
1	206	229
2	211	236
3	226	239
4	243	250
5		242
6		239

APPENDIX 2

(Food consumption - continued)

Group 1♀:
Control
1% MC

Week	Cage	
	7	8
1	149	148
2	151	157
3	150	156
4	161	169
5		171
6		185

Group 2♀:
20555
10 mg/kg/day

Week	Cage
	9
1	138
2	143
3	147
4	151
5	
6	

Group 3♀:
20555
100 mg/kg/day

Week	Cage
	10
1	142
2	146
3	151
4	166
5	
6	

Group 4♀:
20555
1000 mg/kg/day

Week	Cage	
	11	12
1	143	142
2	145	148
3	149	157
4	162	163
5		164
6		159

APPENDIX 3

Haematology - individual values

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	PCV %	Hb g/dl	RBC $\times 10^6$ / mm^3	MCHC %	MCV fl	WBC + Diff $\times 10^3/\text{mm}^3$					Plts $\times 10^3$ / mm^3	TT s	
							Total	N	L	E	B			M
1♂ Control 1% MC	1PA	57	16.2	7.5	28.4	76	9.1	1.37	7.46	0.27	0.00	0.00	1031	22
	2	57	15.6	7.6	27.4	75	13.4	3.08	10.32	0.00	0.00	0.00	931	24
	3PA	54	16.2	6.8	30.0	79	13.7	2.06	11.51	0.14	0.00	0.00	895	22
	4	56	15.7	7.3	28.0	77	10.9	1.31	9.37	0.00	0.00	0.22	925	24
	5	56	16.3	7.4	29.1	76	10.7	2.78	7.92	0.00	0.00	0.00	930	23
	Mean SD	56 1.2	16.0 0.32	7.3 0.31	28.6 1.01	77 1.5	11.6 1.95	2.12 0.803	9.32 1.675	0.08 0.121	0.00 0.000	0.04 0.098	942 51.7	23 1.0
2♂ 20555 10	11	57	16.4	7.4	28.8	77	7.7	0.69	7.01	0.00	0.00	0.00	861	22
	12	57	16.4	7.3	28.8	78	7.4	0.67	6.66	0.07	0.00	0.00	977	23
	13P	53	15.8	6.8	29.8	78	10.1	0.81	9.29	0.00	0.00	0.00	1005	23
	14	56	15.8	7.1	28.2	79	6.3	0.76	5.54	0.00	0.00	0.00	983	24
	15PA	52	15.2	6.5	29.2	80	6.0	0.84	5.10	0.06	0.00	0.00	937	21
	Mean SD	55 2.3	15.9 0.50	7.0 0.37	29.0 0.59	78 1.1	7.5 1.62	0.75 0.074	6.72 1.636	0.03 0.036	0.00 0.000	0.00 0.000	953 56.8	23 1.1
3♂ 20555 100	16	57	16.6	7.2	29.1	79	6.7	2.08	4.56	0.00	0.00	0.07	96	23
	17P	56	16.2	7.1	28.9	79	6.8	1.22	5.51	0.00	0.00	0.07	970	23
	18	55	16.2	7.1	29.5	77	6.7	0.60	6.03	0.07	0.00	0.00	1090	23
	19	56	16.4	7.2	29.3	78	9.9	1.58	8.32	0.00	0.00	0.00	933	23
	20	55	16.3	7.1	29.6	77	7.1	1.21	5.89	0.00	0.00	0.00	803	22
	Mean SD	56 0.8	16.3 0.17	7.1 0.05	29.3 0.29	78 1.0	7.4 1.38	1.34 0.544	6.06 1.387	0.01 0.031	0.00 0.000	0.03 0.038	778 395.0	23 0.4
4♂ 20555 1000	21	55	16.0	6.9	29.1	80	11.5	1.73	9.66	0.12	0.00	0.00	893	22
	22PA	55	16.0	7.0	29.1	79	10.1	2.53	7.58	0.00	0.00	0.00	942	22
	23	58	16.6	7.4	28.6	78	8.0	1.36	6.56	0.00	0.00	0.08	1051	25
	24A	53	15.5	6.7	29.2	79	10.7	2.03	8.56	0.11	0.00	0.00	1028	21
	25	58	16.3	7.5	28.1	77	5.0	1.25	3.75	0.00	0.00	0.00	874	21
	Mean SD	56 2.2	16.1 0.41	7.1 0.34	28.8 0.47	79 1.1	9.1 2.61	1.78 0.521	7.22 2.256	0.05 0.063	0.00 0.000	0.02 0.036	958 79.2	22 1.6

SD Standard deviation
P Polychromasia
A Anisocytosis

APPENDIX 3

(Haematology - continued)

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	PCV %	Hb g/dl	RBC $\times 10^9$ mm ³	MCHC %	MCV fl	WBC + Diff $\times 10^3$ /mm ³					Plts $\times 10^9$ mm ³	TT s	
							Total	N	L	E	B			M
1♀ Control 1% MC	31	51	14.5	6.3	28.4	81	4.4	0.66	3.74	0.00	0.00	0.00	838	20
	32	53	15.4	6.7	29.1	79	7.6	2.58	4.94	0.08	0.00	0.00	1116	20
	33	55	16.6	6.9	30.2	80	6.7	1.47	5.23	0.00	0.00	0.00	1064	21
	34	57	16.2	7.3	28.4	78	9.0	1.17	7.56	0.27	0.00	0.00	984	20
	35	59	16.6	7.7	28.1	77	3.0	0.66	2.34	0.00	0.00	0.00	1330	19
	Mean SD	55 3.2	15.9 0.90	7.0 0.54	28.8 0.84	79 1.6	6.1 2.42	1.31 0.791	4.76 1.936	0.07 0.117	0.00 0.000	0.00 0.000	1066 180.9	20 0.7
2♀ 20555 10	41	55	15.6	7.2	28.4	76	4.5	0.68	3.78	0.05	0.00	0.00	1095	20
	42	56	16.0	7.3	28.6	77	6.0	0.96	4.98	0.06	0.00	0.00	999	21
	43	56	16.4	7.2	29.3	78	3.9	0.94	2.96	0.00	0.00	0.00	893	20
	44	55	16.0	7.0	29.1	79	6.4	0.77	5.63	0.00	0.00	0.00	1142	21
	45PA	51	14.5	6.4	28.4	80	6.2	0.74	5.39	0.06	0.00	0.00	1320	18
	Mean SD	55 2.1	15.7 0.73	7.0 0.36	28.8 0.42	78 1.6	5.4 1.12	0.82 0.125	4.55 1.138	0.03 0.031	0.00 0.000	0.00 0.000	1090 160.2	20 1.2
3♀ 20555 100	46	55	15.8	7.1	28.7	77	4.4	1.06	3.34	0.00	0.00	0.00	1076	20
	47	52	14.9	6.5	28.7	80	7.7	1.93	5.78	0.00	0.00	0.00	1044	19
	48PA	55	15.6	6.9	28.4	80	5.8	1.16	4.41	0.23	0.00	0.00	1131	21
	49	ctd	ctd	ctd	ctd	ctd	ctd	ctd	ctd	ctd	ctd	ctd	ctd	18
	50	54	15.2	6.8	28.1	79	8.6	0.60	7.91	0.00	0.00	0.09	1581	20
	Mean SD	54 1.4	15.4 0.40	6.8 0.25	28.5 0.29	79 1.4	6.6 1.89	1.19 0.552	5.36 1.972	0.06 0.115	0.00 0.000	0.02 0.045	1208 251.2	20 1.1
4♀ 20555 1000	51P	55	15.8	7.0	28.7	79	9.6	2.11	7.49	0.00	0.00	0.00	1107	20
	52	54	15.0	7.0	27.8	77	10.5	2.10	8.09	0.11	0.00	0.21	1126	19
	53P	55	15.4	7.2	28.0	76	10.9	1.96	8.94	0.00	0.00	0.00	928	19
	54	55	15.2	7.0	27.6	79	7.8	1.56	6.16	0.08	0.00	0.00	826	19
	55P	54	15.6	6.7	28.9	81	8.8	1.32	7.48	0.00	0.00	0.00	1017	19
	Mean SD	55 0.5	15.4 0.32	7.0 0.18	28.2 0.57	78 1.9	9.5 1.26	1.81 0.353	7.63 1.016	0.04 0.053	0.00 0.000	0.04 0.094	1001 125.6	19 0.4

SD Standard deviation

ctd Clotted sample

P Polychromasia

A Anisocytosis

APPENDIX 4

Haematology - individual values

Week 7 (3 March 1993)

Group/ dosage mg/kg/day	Animal no.	PCV# %	RBC# x10 ⁶ / mm ³	MCV fl	WBC+ Diff x10 ³ /mm ³					
					Total	N	L	E†	B†	M†
1♂ Control 1% MC	6	56	7.7	73	11.3	1.13	10.17	0.00	0.00	0.00
	7	53	6.8	78	14.8	1.48	13.17	0.00	0.00	0.15
	8	56	7.6	74	15.5	2.33	13.18	0.00	0.00	0.00
	9	54	7.4	73	14.3	2.15	11.87	0.14	0.00	0.14
	10	60	8.3	72	12.8	1.92	10.37	0.51	0.00	0.00
	Mean SD	56 2.7	7.6 0.54	74 2.3	13.7 1.69	1.80 0.492	11.75 1.456	0.13 0.221	0.00 0.000	0.06 0.079
4♂ 20555 1000	26PA	55	7.3	75	15.1	2.27	12.53	0.00	0.00	0.30
	27	56	7.5	75	21.1	5.06	16.04	0.00	0.00	0.00
	28	55	7.5	73	13.7	2.06	11.51	0.00	0.00	0.14
	29	55	7.2	76	14.8	2.22	12.28	0.15	0.00	0.15
	30P	55	7.4	74	16.3	0.98	15.00	0.00	0.00	0.33
	Mean SD	55 0.4	7.4 0.13	75 1.1	16.2 2.89	2.52 1.516	13.47 1.942	0.03 0.067	0.00 0.000	0.18 0.134

- SD Standard deviation
P Polychromasia
A Anisocytosis
† Data required for calculation of total WBC
Data required for calculation of MCV

APPENDIX 4

(Haematology - continued)

Week 7 (3 March 1993)

Group/ dosage mg/kg/day	Animal no.	PCV %	RBC $\times 10^9/mm^3$	MCV fl	WBC+ Diff $\times 10^3/mm^3$					
					Total	N#	L	E#	B#	M#
1♀ Control 1% MC	36	nr	nr	nr	12.1	0.97	11.01	0.00	0.00	0.12
	37	nr	nr	nr	6.5	1.82	4.68	0.00	0.00	0.00
	38	nr	nr	nr	8.9	1.07	7.65	0.09	0.00	0.09
	39	nr	nr	nr	8.2	1.39	6.72	0.08	0.00	0.00
	40	nr	nr	nr	10.3	1.75	8.34	0.10	0.00	0.10
	Mean SD					9.2 2.12	1.40 0.385	7.68 2.316	0.05 0.050	0.00 0.000
4♀ 20555 1000	56	nr	nr	nr	7.8	1.09	6.71	0.00	0.00	0.00
	57	nr	nr	nr	11.1	2.22	8.88	0.00	0.00	0.00
	58	nr	nr	nr	11.3	0.68	10.17	0.34	0.00	0.11
	59	nr	nr	nr	8.5	0.60	7.91	0.00	0.00	0.00
	60	nr	nr	nr	4.6	0.23	4.28	0.05	0.00	0.05
	Mean SD					8.7 2.75	0.96 0.766	7.59 2.245	0.08 0.148	0.00 0.000

Data required for calculation of total WBC

SD Standard deviation

nr Not recorded

APPENDIX 5

Biochemistry - individual values

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	Glu- cose mg/dl	Protein g/dl			A/G	Urea Nitr mg/dl	Creat- inine mg/dl	AP mU/ ml	GPT mU/ ml	GOT mU/ ml
			Total	Alb	Glob						
1♂ Control 1% MC	1	138	6.8	3.1	3.7	0.84	10	0.6	353	31	65
	2	127	6.7	2.8	3.9	0.72	14	0.5	370	28	65
	3	137	7.0	3.1	3.9	0.79	15	0.7	352	29	67
	4	166	6.7	3.0	3.7	0.81	11	0.5	411	30	84
	5	168	6.7	3.0	3.7	0.81	16	0.7	413	29	45
	Mean SD	147 18.6	6.8 0.13	3.0 0.12	3.8 0.11	0.79 0.045	13 2.6	0.6 0.10	380 30.3	29 1.1	65 13.8
2♂ 20555 10	11	140	6.6	3.0	3.6	0.83	15	0.6	448	30	83
	12	119	7.0	3.0	4.0	0.75	11	0.5	353	45	75
	13	155	6.6	2.9	3.7	0.78	10	0.5	365	34	46
	14	116	6.5	2.8	3.7	0.76	14	0.5	452	33	67
	15	117	6.1	2.9	3.2	0.91	14	0.5	408	23	48
	Mean SD	129 17.4	6.6 0.32	2.9 0.08	3.6 0.29	0.81 0.066	13 2.2	0.5 0.04	405 45.7	33 8.0	64 16.4
3♂ 20555 100	16	126	6.5	2.9	3.6	0.81	15	0.5	398	26	62
	17	137	6.7	3.0	3.7	0.81	11	0.5	452	34	51
	18	121	6.3	2.9	3.4	0.85	14	0.5	370	29	44
	19	130	7.0	3.0	4.0	0.75	13	0.5	354	26	53
	20	139	6.1	2.7	3.4	0.79	13	0.6	461	26	52
	Mean SD	131 7.5	6.5 0.35	2.9 0.12	3.6 0.25	0.80 0.036	13 1.5	0.5 0.04	407 48.0	28 3.5	52 6.4
4♂ 20555 1000	21	148	7.0	3.0	4.0	0.75	10	0.5	382	32	45
	22	128	6.7	3.0	3.7	0.81	10	0.5	456	32	54
	23	120	6.2	2.9	3.3	0.88	13	0.5	384	24	65
	24	119	6.4	2.8	3.6	0.78	13	0.5	361	18	48
	25	124	6.5	2.9	3.6	0.81	9	0.4	311	27	51
	Mean SD	128 11.8	6.6 0.30	2.9 0.08	3.6 0.25	0.81 0.048	11 1.9	0.5 0.04	379 52.2	27 5.9	53 7.7

SD Standard deviation

APPENDIX 5

(Biochemistry - continued)

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	γ GT mU/ ml	Bili- rubin mg/dl	Na mEq/ l	K mEq/ l	Ca mEq/ l	P mEq/ l	Cl mEq/ l	Chol mg/dl	Tri- glyc mg/dl
1 δ Control 1% MC	1	<1	<0.1	145	3.7	5.4	5.1	96	82	115
	2	<1	<0.1	143	3.7	5.3	5.0	97	53	76
	3	<1	0.1	143	4.2	5.5	5.0	96	102	93
	4	<1	<0.1	143	4.6	5.3	6.1	97	60	85
	5	<1	0.1	144	3.6	5.4	5.2	97	51	103
	Mean SD	<1	<0.1	144 0.9	4.0 0.43	5.4 0.08	5.3 0.47	97 0.5	70 21.9	94 15.2
2 δ 20555 10	11	<1	0.1	145	3.8	5.2	5.2	98	86	103
	12	1	0.1	145	4.1	5.6	5.1	98	76	74
	13	1	<0.1	145	4.1	5.5	5.4	97	82	120
	14	1	<0.1	145	4.1	5.3	5.6	98	70	87
	15	<1	<0.1	145	3.9	5.2	4.4	101	76	94
	Mean SD	<1	<0.1	145 0.0	4.0 0.14	5.4 0.18	5.1 0.46	98 1.5	78 6.2	96 17.3
3 δ 20555 100	16	1	0.1	145	4.1	5.4	5.9	98	83	87
	17	2	<0.1	145	4.0	5.5	5.3	97	66	146
	18	<1	<0.1	146	3.6	5.5	5.7	97	72	133
	19	<1	<0.1	145	3.7	5.5	5.4	98	65	115
	20	<1	0.1	145	4.2	5.2	5.5	100	64	59
	Mean SD	<1	<0.1	145 0.4	3.9 0.26	5.4 0.13	5.6 0.24	98 1.2	70 7.9	108 35.2
4 δ 20555 1000	21	<1	<0.1	144	3.8	5.4	4.9	98	99	167
	22	1	0.1	145	4.0	5.5	5.0	96	79	79
	23	1	<0.1	146	3.6	5.3	5.9	98	67	71
	24	<1	<0.1	145	4.2	5.4	5.2	98	79	69
	25	<1	<0.1	145	3.5	5.4	5.2	97	75	79
	Mean SD	<1	<0.1	145 0.7	3.8 0.29	5.4 0.07	5.2 0.39	97 0.9	80 11.8	93 41.6

SD Standard deviation

APPENDIX 5

(Biochemistry - continued)

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	Glu- cose mg/dl	Protein g/dl			A/G	Urea Nitr mg/dl	Creat- inine mg/dl	AP mU/ ml	GPT mU/ ml	GOT mU/ ml
			Total	Alb	Glob						
1♀ Control 1% MC	31	125	6.9	3.1	3.8	0.82	14	0.6	188	21	48
	32	137	6.8	2.8	4.0	0.70	13	0.5	161	16	44
	33	130	6.0	2.8	3.2	0.88	11	0.5	250	23	48
	34	134	7.0	3.0	4.0	0.75	12	0.5	237	38	53
	35	126	6.9	3.2	3.7	0.86	16	0.4	199	20	53
	Mean SD	130 5.1	6.7 0.41	3.0 0.18	3.7 0.33	0.80 0.076	13 1.9	0.5 0.07	207 36.4	24 8.4	49 3.8
2♀ 20555 10	41	120	6.6	3.1	3.5	0.89	12	0.6	146	19	50
	42	131	7.0	3.3	3.7	0.89	11	0.5	285	20	45
	43	120	6.6	3.0	3.6	0.83	14	0.6	173	21	53
	44	122	6.7	2.9	3.8	0.76	15	0.5	240	27	43
	45	127	6.7	3.0	3.7	0.81	15	0.5	278	21	42
	Mean SD	124 4.8	6.7 0.16	3.1 0.15	3.7 0.11	0.84 0.055	13 1.8	0.5 0.05	224 62.4	22 3.1	47 4.7
3♀ 20555 100	46	144	6.2	2.8	3.4	0.82	12	0.5	225	23	35
	47	123	7.0	2.9	4.1	0.71	15	0.4	194	20	54
	48	126	6.8	3.2	3.6	0.89	15	0.6	260	23	57
	49	133	6.8	3.0	3.8	0.79	14	0.5	177	20	49
	50	110	6.3	2.8	3.5	0.80	14	0.5	170	24	42
	Mean SD	127 12.6	6.6 0.35	2.9 0.17	3.7 0.28	0.80 0.065	14 1.2	0.5 0.07	205 37.3	22 1.9	47 9.0
4♀ 20555 1000	51	127	6.7	2.7	4.0	0.68	11	0.5	266	19	50
	52	119	6.7	3.2	3.5	0.91	13	0.5	196	26	47
	53	129	6.7	3.0	3.7	0.81	16	0.6	203	22	50
	54	131	6.3	3.0	3.3	0.91	15	0.5	236	15	51
	55	120	6.8	2.9	3.9	0.74	11	0.5	278	24	42
	Mean SD	125 5.4	6.6 0.19	3.0 0.18	3.7 0.29	0.81 0.102	13 2.3	0.5 0.04	236 36.6	21 4.3	48 3.7

SD Standard deviation

APPENDIX 5

(Biochemistry - continued)

Week 5 (17 February 1993)

Group/ dosage mg/kg/day	Animal no.	γ GT mU/ ml	Bili- rubin mg/dl	Na mEq/ l	K mEq/ l	Ca mEq/ l	P mEq/ l	Cl mEq/ l	Chol mg/dl	Tri- glyc mg/dl
1♀ Control 1% MC	31	<1	<0.1	144	3.6	5.3	4.0	101	79	30
	32	1	0.1	143	3.7	5.2	4.2	100	97	44
	33	<1	<0.1	145	3.6	5.0	4.3	102	58	33
	34	2	0.1	144	3.9	5.4	4.3	102	56	36
	35	1	0.1	143	3.9	5.5	4.4	96	94	62
	Mean SD		<2	<0.1	144	3.7	5.3	4.2	100	77
				1.8	0.25	0.29	0.25	2.5	20.3	13.8
2♀ 20555 10	41	1	<0.1	145	3.8	5.2	4.1	100	80	43
	42	1	<0.1	144	3.6	5.3	3.7	99	64	38
	43	1	<0.1	143	3.9	5.2	3.5	100	98	31
	44	<1	<0.1	144	4.0	5.5	4.3	99	83	58
	45	1	0.1	143	3.7	5.2	3.8	100	86	43
	Mean SD		<1	<0.1	144	3.8	5.3	3.9	100	82
				1.8	0.16	0.23	0.32	1.5	12.3	10.9
3♀ 20555 100	46	1	<0.1	146	4.2	5.2	4.1	102	66	37
	47	<1	<0.1	142	4.3	5.3	4.2	99	114	49
	48	<1	0.1	144	3.6	5.3	3.9	100	80	39
	49	<1	<0.1	145	3.5	5.3	4.2	101	84	45
	50	2	0.1	145	4.9	5.5	5.7	100	67	46
	Mean SD		<1	<0.1	144	4.1	5.3	4.4	100	82
				2.5	0.67	0.11	0.73	2.1	20.4	6.0
4♀ 20555 1000	51	<1	<0.1	145	3.5	5.4	4.2	100	66	38
	52	<1	<0.1	143	3.9	5.3	4.4	100	64	39
	53	1	0.1	144	3.1	5.2	3.7	100	110	43
	54	2	<0.1	145	3.4	5.0	4.5	101	69	28
	55	1	<0.1	144	3.8	5.4	4.3	98	114	42
	Mean SD		<2	<0.1	144	3.5	5.3	4.2	100	85
				1.8	0.42	0.17	0.41	1.1	26.1	6.0

SD Standard deviation

APPENDIX 6

Biochemistry - individual values

Week 7 (3 March 1993)

Group/ dosage mg/kg/day	Animal no.	Creat- inine mg/dl	Na mEq/ l
1♂ Control 1% MC	6	0.6	145
	7	0.5	146
	8	0.6	144
	9	0.6	143
	10	0.6	143
	Mean SD	0.6 0.04	144 1.3
4♂ 20555 1000	26	0.6	145
	27	0.5	143
	28	0.6	144
	29	0.6	143
	30	0.5	146
	Mean SD	0.6 0.05	144 1.3

SD Standard deviation



APPENDIX 8

Organ weights - individual values

Week 5 (19 February 1993)

Group/ dosage mg/kg/day	Animal no.	Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Testes+ Epidids g
1♂ Control 1% MC	1	388	1.97	20.4	0.71	3.06	48.6	4.35
	2	417	2.04	21.4	0.94	3.32	53.5	4.15
	3	427	2.14	24.7	0.89	3.94	57.9	5.13
	4	363	1.93	18.8	0.76	3.29	56.5	3.84
	5	391	2.08	23.9	0.82	3.78	66.0	4.37
	Mean SD	397 25.3	2.03 0.082	21.9 2.44	0.82 0.095	3.48 0.366	56.5 6.40	4.37 0.476
2♂ 20555 10	11	342	1.85	16.5	0.67	2.48	60.1	4.22
	12	333	1.87	17.5	0.66	2.96	49.3	3.83
	13	468	1.95	25.5	1.07	3.29	45.0	4.64
	14	385	2.10	22.0	0.91	3.09	62.2	4.02
	15	403	1.96	23.5	0.76	3.02	57.5	4.20
	Mean SD	386 54.1	1.95 0.099	21.0 3.89	0.81 0.176	2.97 0.299	54.8 7.36	4.18 0.299
3♂ 20555 100	16	375	1.95	21.7	0.98	2.86	64.2	4.19
	17	418	2.13	23.6	0.95	3.35	56.5	4.10
	18	390	1.99	24.4	0.88	3.06	53.2	4.10
	19	425	1.96	25.6	0.98	3.33	54.4	4.17
	20	364	1.90	21.6	0.87	3.18	69.7	4.28
	Mean SD	394 26.6	1.98 0.089	23.4 1.75	0.93 0.054	3.16 0.205	59.6 7.08	4.17 0.075
4♂ 20555 1000	21	479	1.99	28.1	0.74	3.52	56.3	4.13
	22	375	2.00	21.5	0.83	2.97	54.1	4.10
	23	336	2.12	18.1	0.59	2.54	46.4	4.85
	24	361	2.02	22.4	0.85	2.90	46.8	3.15
	25	371	1.86	21.7	0.76	2.93	70.6	4.37
	Mean SD	384 55.1	2.00 0.092	22.4 3.64	0.75 0.103	2.97 0.354	54.8 9.84	4.12 0.621

SD Standard deviation

APPENDIX 8

(Organ weights - continued)

Week 5 (19 February 1993)

Group/ dosage mg/kg/day	Animal no.	Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Ovaries mg
1 ♀ Control 1% MC	31	235	1.86	10.9	0.60	2.16	62.4	110.5
	32	258	1.89	12.0	0.70	2.42	73.7	91.8
	33	250	1.87	11.5	0.84	2.28	86.2	117.3
	34	261	1.97	11.0	0.63	2.40	79.8	110.3
	35	236	1.87	10.7	0.49	1.99	62.3	73.5
	Mean SD		248 12.0	1.89 0.047	11.2 0.53	0.65 0.130	2.25 0.177	72.9 10.58
2 ♀ 20555 10	41	252	1.82	11.7	0.54	2.36	70.5	98.6
	42	234	1.88	10.9	0.70	2.11	61.1	92.7
	43	238	1.78	11.2	0.55	1.96	68.3	78.7
	44	241	1.77	10.7	0.59	2.00	55.6	73.5
	45	220	1.81	10.5	0.44	1.94	55.9	85.3
	Mean SD		237 11.3	1.81 0.044	11.0 0.49	0.57 0.095	2.08 0.170	62.3 6.90
3 ♀ 20555 100	46	235	1.87	10.2	0.55	2.13	58.3	105.7
	47	266	1.94	13.3	0.62	2.35	63.5	129.5
	48	237	1.80	12.0	0.50	2.12	72.3	84.1
	49	235	1.87	11.3	0.70	2.51	65.7	100.3
	50	246	1.81	10.8	0.65	2.17	66.5	90.2
	Mean SD		244 13.5	1.86 0.057	11.5 1.21	0.61 0.080	2.25 0.169	65.3 5.07
4 ♀ 20555 1000	51	237	1.88	11.3	0.74	2.38	75.5	99.6
	52	253	1.85	11.1	0.66	2.00	68.7	108.4
	53	223	1.80	11.6	0.65	1.96	74.9	87.4
	54	233	1.77	11.4	0.58	2.10	66.2	87.5
	55	267	2.03	13.4	0.76	2.65	67.2	102.1
	Mean SD		243 17.4	1.87 0.103	11.7 0.93	0.68 0.074	2.22 0.293	70.5 4.39

SD Standard deviation

APPENDIX 9

Organ weights - individual values

Week 7 (5 March 1993)

Group/ dosage mg/kg/day	Animal no.	Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Testes+ Epidids g
1♂ Control 1% MC	6	431	1.98	17.9	0.79	3.01	70.5	4.07
	7	493	2.13	25.0	0.93	3.79	87.0	4.55
	8	434	1.92	22.5	0.95	3.65	46.8	4.10
	9	434	2.07	23.7	0.83	3.60	58.7	4.23
	10	382	1.97	22.5	0.64	3.61	84.4	4.35
	Mean SD	435 39.4	2.01 0.082	22.3 2.68	0.83 0.125	3.53 0.302	69.5 17.04	4.26 0.196
4♂ 20555 1000	26	444	2.15	20.8	1.05	3.72	67.3	4.77
	27	535	2.09	28.7	1.13	3.92	71.1	4.60
	28	451	1.99	21.4	0.69	3.54	60.9	4.08
	29	418	2.11	21.4	0.84	3.54	54.3	4.40
	30	450	2.08	20.6	0.95	3.48	63.3	4.47
	Mean SD	460 44.4	2.08 0.059	22.6 3.43	0.93 0.173	3.64 0.179	63.4 6.39	4.46 0.256

SD Standard deviation

APPENDIX 9
(Organ weights - continued)

Week 7 (5 March 1993)

Group/ dosage mg/kg/day	Animal no.	Body wt. g	Brain g	Liver g	Spleen g	Kidneys g	Adrenals mg	Ovaries mg
1 ♀ Control 1% MC	36	284	2.00	12.6	0.67	2.61	71.5	121.6
	37	252	1.76	11.4	0.70	2.23	85.3	95.3
	38	265	1.96	12.1	0.54	2.09	69.1	81.7
	39	247	1.75	13.6	0.57	2.21	78.6	138.1
	40	316	1.92	16.0	0.62	2.37	90.0	108.4
	Mean SD	273 28.1	1.88 0.117	13.1 1.77	0.62 0.068	2.30 0.199	78.9 8.87	109.0 22.02
4 ♀ 20555 1000	56	276	1.92	12.3	0.58	2.34	73.0	83.3
	57	242	1.86	11.7	0.62	2.09	66.0	122.3
	58	257	1.87	13.0	0.63	2.61	87.6	128.7
	59	289	1.93	13.9	0.64	2.35	74.2	97.4
	60	228	1.93	10.1	0.70	2.11	75.7	97.6
	Mean SD	258 24.6	1.90 0.033	12.2 1.45	0.63 0.044	2.30 0.211	75.3 7.82	105.9 18.98

SD Standard deviation

APPENDIX 10

**Clinical and pathological data relating to rats killed following
the 4-week treatment or 2-week recovery periods**

Group:	1	2	3	4
Compound:	Control		20555	
Level (mg/kg/day):	0	10	100	1000

In this appendix the clinical, macroscopic and microscopic findings relating to each animal are listed together. The macroscopic and microscopic findings are presented by an automated data collation system.



APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 1♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte enlargement: (Minimal)
Centrilobular hepatocyte vacuolation: (Minimal)
Sinusoidal dilatation/congestion

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: Control

Rat No/Sex: 2♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal)
Parenchymal inflammatory cells: (Focal)
Sinusoidal dilatation/congestion: (Areas)

Lymph Nodes - Cervical

Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: Control

Rat No/Sex: 3♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal)
Sinusoidal dilatation/congestion

Kidneys

Tubular basophilia: (Minimal , Focal)

Adrenals

Vacuolated cortical cells: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Testes; Epididymides

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 4♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Liver
Sinusoidal dilatation/congestion

Lymph Nodes - Cervical
Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 5♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Parenchymal inflammatory cells: (Focal)
Sinusoidal dilatation/congestion

Lymph Nodes - Cervical

Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: Control

Rat No/Sex: 6♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: Control

Rat No/Sex: 7♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: Control

Rat No/Sex: 8♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 9♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 10♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 11♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 12♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 13♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555

Dosage Level: 10 mg/kg/day

Rat No/Sex: 14♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 15♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 16♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 17♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 18♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 19♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

Stomach Antrum Mucosa
White nodule, near to limiting ridge: 1mm

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 20♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 21♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal , Some lobes)
Sinusoidal dilatation/congestion: (Some lobes)

Kidneys

Medullary necrosis: (Focal , Unilateral)
Cystic medullary tubule(s) containing necrotic detritus: (Focus)

The following tissues were considered normal:

Heart; Spleen; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 22♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Sinusoidal dilatation/congestion

Adrenals

Cortical hyperplasia: (Minimal , Focal , Unilateral)

Lymph Nodes - Cervical

Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 23♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Sinusoidal dilatation/congestion

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 24♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte enlargement: (Minimal)
Centrilobular hepatocyte vacuolation: (Minimal)
Sinusoidal dilatation/congestion

Testes

Reduced spermatogenesis: (Minimal , Focal , Bilateral)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 25♂ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal , Some lobes)
Sinusoidal dilatation/congestion

The following tissues were considered normal:

Heart; Spleen; Kidneys; Testes; Epididymides; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 26♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 27♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

Spleen
Capsule thickened

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 28♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 29♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 30♂ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 31 ♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal , Some lobes)
Sinusoidal dilatation/congestion

Lymph Nodes - Cervical

Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 32♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Lymph Nodes - Cervical
Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Liver; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 33♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Parenchymal inflammatory cells: (Focal)

The following tissues were considered normal:

Heart; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 34 ♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following tissues were considered normal:

Heart; Liver; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 35♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following tissues were considered normal:

Heart; Liver; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 36♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 37♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 38♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 39♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

Stomach Antrum Mucosa
White nodule, near to limiting ridge: 1mm

All the other organs and tissues appeared normal.

APPENDIX 10
(Pathology - continued)

Compound: 20555
Dosage Level: Control
Rat No/Sex: 40♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health or behavioural change were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 41 ♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 42♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 43♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 44♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Stomach Antrum Mucosa

White nodule, near to limiting ridge: 1mm

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 10 mg/kg/day
Rat No/Sex: 45♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 46♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 47♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Stomach Antrum Mucosa

White nodule, near to limiting ridge: 1mm

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 48♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 49♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Stomach Antrum Mucosa

White nodule, near to limiting ridge: 1mm

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 100 mg/kg/day
Rat No/Sex: 50♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 51 ♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following tissues were considered normal:

Heart; Liver; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 52♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Liver

Centrilobular hepatocyte vacuolation: (Minimal , Some lobes)
Sinusoidal dilatation/congestion

The following tissues were considered normal:

Heart; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 53 ♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following observations were noted:

Kidneys

Pelvic inflammatory cell infiltration: (Areas , Unilateral)

The following tissues were considered normal:

Heart; Liver; Spleen; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 54♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

MICROSCOPIC FINDINGS

The following tissues were considered normal:

Heart; Liver; Spleen; Kidneys; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 55♀ (Terminal)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed over the treatment period.

MACROSCOPIC FINDINGS

Lymph Nodes - Cervical
Enlarged

All the other organs and tissues appeared normal.

MICROSCOPIC FINDINGS

The following observations were noted:

Kidneys

Vacuolated epithelium of occasional cortical tubules: (Minimal)

Lymph Nodes - Cervical

Lymphoid proliferation: (Minimal)

The following tissues were considered normal:

Heart; Liver; Spleen; Adrenals

Pathologist: H.Singh

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 56♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 57♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 58♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 59 ♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

No abnormalities were seen in the animal

APPENDIX 10

(Pathology - continued)

Compound: 20555
Dosage Level: 1000 mg/kg/day
Rat No/Sex: 60♀ (Recovery)

CLINICAL FINDINGS

No signs of ill health, behavioural change or reaction to treatment were observed during the treatment or recovery periods.

MACROSCOPIC FINDINGS

Stomach Antrum Mucosa

White nodules, near to limiting ridge: (Two) 1mm

All the other organs and tissues appeared normal.

APPENDIX 11

Special Diets Services Rat and Mouse Maintenance Diet

Composition and quality assurance aspects of diet

SDS Rat and Mouse No. 1 SQC modified maintenance diet is a fixed formula diet. Each batch of diet is analysed for nutrients, possible contaminants and micro-organisms, likely to be present in the diet, and which, if in excess, may have an undesirable effect on the test system.

Prior to release of diet for use HRC Quality Assurance Department checks each certificate of analysis for conformity with the specification detailed below. Occasional slight deviations to this specification may be permitted.

Nutrients	Target level	Tolerance %	Acceptable range		
Moisture	10.0	+25		12.5	% max
Crude fat	3.0	±30	2.0	- 4.0	%
Crude protein	14.5	±15	12.0	- 16.5	%
Crude fibre	4.0	±50	2.0	- 6.0	%
Ash	5.0	±25	3.7	- 6.2	%
Calcium	0.9	±30	0.6	- 1.2	%
Phosphorus	0.6	±20	0.5	- 0.75	%
Sodium	0.25	±40	0.15	- 0.35	%
Chloride	0.5	±40	0.3	- 0.7	%
Potassium	0.9	±50	0.45	- 1.35	%
Magnesium	0.2	±50	0.1	- 0.3	%
Iron	200	±50	100	- 300	mg/kg
Copper	15	±60	6	- 24	mg/kg
Manganese	60	+60-40	36	- 100	mg/kg
Zinc	60	±50	30	- 90	mg/kg
Vitamin A	6	-50		3	iu/g min.
Vitamin E	70	-50		35	mg/kg min.
Contaminants					
			Maximum concentration		
Fluoride			20		mg/kg
Nitrate (as NaNO ₃)			30		mg/kg
Nitrite (as NaNO ₂)			10		mg/kg
Lead			2.0		mg/kg
Arsenic			1.0		mg/kg
Cadmium			0.7		mg/kg
Mercury			0.1		mg/kg
Selenium			0.6		mg/kg
Total Aflatoxins			5.0		mcg/kg
Total P.C.B.			50		mcg/kg
Total D.D.T.			250		mcg/kg
Dieldrin			50		mcg/kg
Lindane			300		mcg/kg
Heptachlor			20		mcg/kg
Malathion			5000		mcg/kg

APPENDIX 11

(Aspects of diet - continued)

Microbiological contents**Maximum concentration**

Total viable organisms	25000	per g diet
Mesophilic spores	25000	per g diet
Salmonellae species	0	per g diet
Presumptive E. coli	0	per g diet
E. coli type 1	0	per g diet
Fungal units	300	per g diet
Antibiotic activity	0	per g diet

APPENDIX 12

Quality assurance aspects of drinking water

The water supplied to HRC, by Anglian Water, is potable water for human consumption. Anglian Water takes its guidelines on water quality from the EEC directive relating to water for human consumption, viz: Council Directive 80/778/EEC.

Results of routine physical and chemical examination of drinking water at source as conducted, usually weekly by the supplier, are made available to HRC as quarterly summaries.

These results include levels of:

Nitrites	Potassium	Chloride
Nitrates	Silicon	Iron
Calcium	Arsenic	Selenium
Magnesium	Barium	Silver
Sodium	Antimony	Phosphorus

as well as concentrations of pesticides, related products, polycyclic aromatic hydrocarbons, haloforms, chlorophenols and polychlorinated biphenyls.

ANALYTICAL CHEMISTRY REPORT

**THE ANALYSIS OF 20555
IN 1% METHYLCELLULOSE
FORMULATIONS**

Authors:

**Alan Anderson,
I. Suzanne Dawe,
Sujit Patel.**

ANALYTICAL CHEMISTRY REPORT

(continued)

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ANALYTICAL CHEMISTRY REPORT

(continued)

INTRODUCTION

This appendix contains details of the analytical method used and the results obtained for:

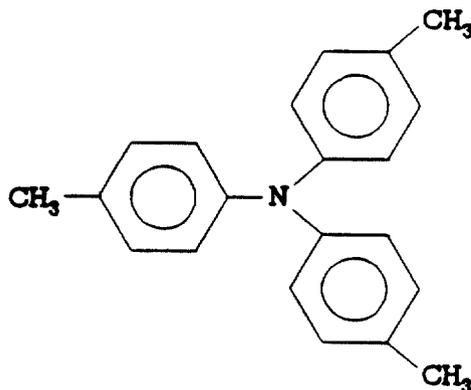
The determination of concentrations of 20555 in dose formulations¹ analysed during the study.

The determination of the physical stability (homogeneity and resuspendability) of 20555 in 1% MC formulations¹.

The determination of the chemical stability of 20555 in 1% MC formulations¹.

The validation of the method of analysis for the determination of 20555 in 1% MC formulations¹.

The formulations for this study were prepared as suspensions of 20555 in 1% methylcellulose by Formulation personnel at Huntingdon Research Centre Limited.



20555

¹ 1%MC: 1% w/v aqueous methylcellulose

ANALYTICAL CHEMISTRY REPORT

(continued)

EXPERIMENTAL PROCEDURE

ANALYTICAL PROCEDURE

Apparatus and instrumentation

High performance liquid
chromatograph (HPLC):

Pump:

Autosampler:

Detector:

Integrator:

As detailed below or suitable alternative.

Waters Associates model 510.

Waters Associates WISP 710A.

Spectra-Physics UV1000.

Spectra-Physics SP4270.

General laboratory glassware.

Reagents

Test material:

Supplier:

Batch no.:

Stated purity:

20555

Fuji Xerox Company Ltd.

920911

99.9%

Methanol:

Merck Ltd., HiPerSolv for HPLC.

Water:

Elgastat UHP-4, deionised reverse osmosis.

ANALYTICAL CHEMISTRY REPORT

(continued)

Sample extraction

A representative sample (approximately 1 ml) of test formulation was accurately weighed and dissolved in a suitable volume of methanol. The extract was appropriately diluted using methanol to provide a solution containing 20555 in the expected concentration range 2 - 4 $\mu\text{g/ml}$.

The final solution was filtered (Whatman PURADISC 25PP 0.45 μm) and the concentration of 20555 was quantified by high performance liquid chromatography using ultraviolet detection as detailed in the following section.

Typical chromatographic conditions

Analytical column:	LiChrospher 100 RP-18e, 5 μm , 125 x 4 mm ID., Merck Ltd..
Guard column:	LiChrospher 100 RP-18e, 5 μm , 4 x 4 mm ID., Merck Ltd..
Mobile phase:	Methanol/water (98/2 v/v).
Flow rate:	1.0 ml/minute.
Detector wavelength:	UV, 300 nm.
Injection volume:	15 μl .
Integrator attenuation:	64.
Retention volume:	3.5 ml.

Calibration

A primary **standard** solution was prepared for each analytical occasion by dissolving an accurately weighed quantity (50 mg) of 20555 in methanol (100 ml). Solutions for instrument calibration, containing 20555 in the concentration range 1 - 5 $\mu\text{g/ml}$, were prepared by appropriate dilution of the primary standard using methanol.

Calibration solutions were injected onto the HPLC, at the beginning and end of each sample analysis sequence, using the conditions detailed in the previous section.

ANALYTICAL CHEMISTRY REPORT

(continued)

Calculation

The peak response for 20555 in each calibration chromatogram was measured and calibration curves were constructed by linear regression of standard response versus standard concentration. The response of the peak observed at the characteristic retention volume for 20555 in sample and procedural recovery chromatograms was measured and the concentration of 20555 was determined using the following equation:

$$\text{Concentration, mg/ml} = \frac{Y-I}{S} \times \frac{V}{W} \times D \times 10^{-3}$$

Where Y = Peak response in test chromatogram
 I = Intercept derived from linear regression of calibration data
 S = Slope derived from linear regression of calibration data
 V = Dilution volume of sample (ml)
 W = Weight of sample (g)
 D = Density (g/ml), a value of 1 will be used where the formulation densities fall within the range 0.99 - 1.01 g/ml

Results were corrected for the appropriate mean procedural recovery value at analysis.

Limit of detection

The limit of detection, defined as the concentration of 20555 in control matrix producing a peak response equivalent to 3 × baseline noise, was determined as 0.075 mg/ml.

VALIDATION OF THE METHOD OF ANALYSIS

The analytical procedure was validated by fortifying a minimum of six samples (1 ml) of control vehicle with 20555, to concentrations of 1 mg/ml and 100 mg/ml, which were analysed in accordance with the analytical procedure. The test substance, 20555, was added either as a solution in methanol (inclusion levels < 20 mg/ml) or as solid test material (inclusion levels ≥ 20 mg/ml).

Procedural recoveries were determined for each inclusion level and analysed concurrently with test formulations.

ANALYTICAL CHEMISTRY REPORT

(continued)

DETERMINATION OF CONCENTRATIONS OF 20555 IN DOSE FORMULATIONS ANALYSED DURING THE STUDY

Representative samples (approximately 20 ml) of freshly prepared dose formulations were thoroughly mixed by vigorous shaking and duplicate sub-samples (1 ml) were analysed in accordance with the analytical procedure.

DETERMINATION OF THE PHYSICAL STABILITY OF 20555 IN 1% METHYLCELLULOSE FORMULATIONS

Freshly-prepared specimen formulations (approximately 200 ml), containing 20555 at nominal concentrations of 1 mg/ml and 100 mg/ml, were each thoroughly mixed by inversion and magnetically stirred. After magnetic stirring for 5 minutes (0 hour), 0.5 hour and 1 hour, samples (approximately 1 ml) were removed for analysis from points at approximately one-quarter, one-half and three-quarters the depth (representing the top, middle and bottom) of the formulation.

The magnetic stirring was discontinued and the remainder of each formulation was stored in the dark at ambient temperature. At a time point representing 4 hours after preparation, each formulation was re-mixed and sampled for analysis as above.

At each occasion, the three samples from each formulation were analysed in accordance with the analytical procedure.

DETERMINATION OF THE CHEMICAL STABILITY OF 20555 IN 1% METHYLCELLULOSE FORMULATIONS

Freshly-prepared specimen formulations (approximately 20 ml), containing 20555 at nominal concentrations of 1 mg/ml and 100 mg/ml, were each sub-sampled (6 replicates of approximately 1 ml) for analysis. Duplicate sub-samples were analysed immediately and the remaining samples were stored (ambient temperature during the day, refrigeration overnight) in the dark for 4 and 24 hours before analysis.

At each occasion, two sub-samples of each formulation were analysed in accordance with the analytical procedure.

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(continued)

RESULTS

The mean concentrations of 20555 in dose formulations analysed during the study and the deviation of mean results from nominal values are summarised in Table 1. Mean results for the formulations scheduled for analysis were within -9% of nominal concentrations, with the exception of one result (Day 22, 100 mg/ml) which was 12% above nominal. In view of this high result, the 100 mg/ml formulation prepared for dosing on Day 24 was also sampled and assayed at 19% above nominal. Individual analytical results and associated procedural recovery data are detailed in Table 2.

The results in Table 3 indicate that, at nominal concentrations of 1 mg/ml and 100 mg/ml, 20555 produces a homogeneous suspension in 1% methylcellulose formulation which can be maintained for up to 1 hour while magnetically stirred and successfully resuspended following storage at ambient temperature for 4 hours.

The results in Table 4 indicate that, at nominal concentrations of 1 mg/ml and 100 mg/ml, 20555 is chemically stable in the 1% methylcellulose formulation during storage (ambient temperature during the day, refrigeration overnight) for 24 hours.

Procedural recovery data obtained during method validation and the determination of stability are presented in Table 5. The data indicate that the analytical method is both precise and accurate: a mean procedural recovery value of 101.0% \pm 1.25 SD (n=8) was obtained for 1 mg/ml and 102.3% \pm 1.20 SD (n=8) for 100 mg/ml. Results for the analysis of test samples are corrected for the appropriate mean procedural recovery value at analysis.

A typical calibration standard graph is presented in Figure 1 confirming the linearity of detector response for 20555 over the concentration range 1-5 μ g/ml. Typical analytical chromatograms are presented in Figures 2 and 3. In Figure 2, the absence of a peak at the characteristic retention volume for 20555 in the control sample chromatogram demonstrates the specificity of the HPLC assay.

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(continued)

CONCLUSION

The analytical results indicate that the doses formulated during the study were within 20% of nominal. The data also indicate that the formulations were chemically and physically stable from the time of preparation to completion of dosing.

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(continued)

TABLE 1

Summary: mean concentrations of 20555 in dose formulations

Day of dosing	Group	Nominal inclusion (mg/ml)	Mean analysed concentration (mg/ml)	RME (%)
1	Control	0	ND	—
	2	1	0.915	-8.5
	3	10	9.71	-2.9
	4	100	99.8	-0.2
22	Control	0	ND	—
	2	1	0.962	-3.8
	3	10	10.0	0.0
	4	100	112	+12.0
24	4	100	119	+19.0

ND None detected (<0.075 mg/ml)

RME Relative mean error, representing the deviation from nominal

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(continued)

TABLE 2

Concentrations of 20555 in dose formulations
(individual values)

Day of dosing	Group	Nominal inclusion (mg/ml)	Analysed concentration (mg/ml)			Procedural recoveries (%)	
			Analysis 1	Analysis 2	Mean	At analysis	Mean ¹
1	Control	0	ND	ND	ND		
	2	1	0.903	0.927	0.915	100.0	
	3	10	9.67	9.75	9.71	98.9	101.4
	4	100	100	99.1	99.8	100.5	
22	Control	0	ND	ND	ND		
	2	1	0.957	0.968	0.962	98.5	
	3	10	10.0	10.0	10.0	99.7	100.9
	4	100	112	111	112	95.6	
24	4	100	115	123	119	103.0	101.0

ND None detected (<0.075 mg/ml)

¹ Represents the cumulative mean procedural recovery value and includes procedural recovery data from Table 5

Results are calculated using unrounded figures and are corrected for the appropriate mean procedural recovery value given in this Table

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(continued)

TABLE 3

Physical stability of 20555 in 1% MC formulations

Nominal inclusion (mg/ml)	Storage time (hours)	Analysed concentration (mg/ml)				CV (%)
		Top	Middle	Bottom	Mean	
1	0	0.963	0.984	0.972	0.973	1.11
	0.5	0.952	0.955	0.953	0.953	0.14
	1	0.927	0.922	0.946	0.931	1.34
	4	0.949	0.939	0.957	0.949	0.96
100	0	102	101	99.8	101	1.01
	0.5	98.6	96.9	95.1	96.9	1.81
	1	98.4	96.0	96.0	96.8	1.40
	4	99.3	97.5	100	99.1	1.47

CV Coefficient of variation

Results are calculated using unrounded figures and are corrected for the appropriate mean procedural recovery value in Table 5

ANALYTICAL CHEMISTRY REPORT

(continued)

TABLE 4

Chemical stability of 20555 in 1% MC formulations

Nominal inclusion (mg/ml)	Storage time (hours)	Analysed concentration (mg/ml)			RME (%)
		Analysis 1	Analysis 2	Mean	
1	0	0.968	0.960	0.964	-
	4	0.936	0.960	0.948	-1.7
	24	0.965	0.955	0.960	-0.4
100	0	101	101	101	-
	4	107	99.3	103	+2.0
	24	100	100	100	0.0

RME Relative mean error, representing the deviation from time zero

Results are calculated using unrounded figures and are corrected for the appropriate mean procedural recovery value in Table 5

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(continued)

TABLE 5

Procedural recovery data for 20555
in 1% MC formulations

(results are expressed as percent recovery)

Analytical phase	Nominal level of fortification (mg/ml)	
	1	100
Validation	100.4	102.6
	100.6	102.9
	101.1	103.1
	100.4	102.3
	100.7	103.5
	100.6	103.3
Stability	104.0	100.7
	100.1	100.3
Mean	101.0	102.3
SD (\pm)	1.25	1.20
Range	100.1-104.0	100.3-103.5
n	8	8

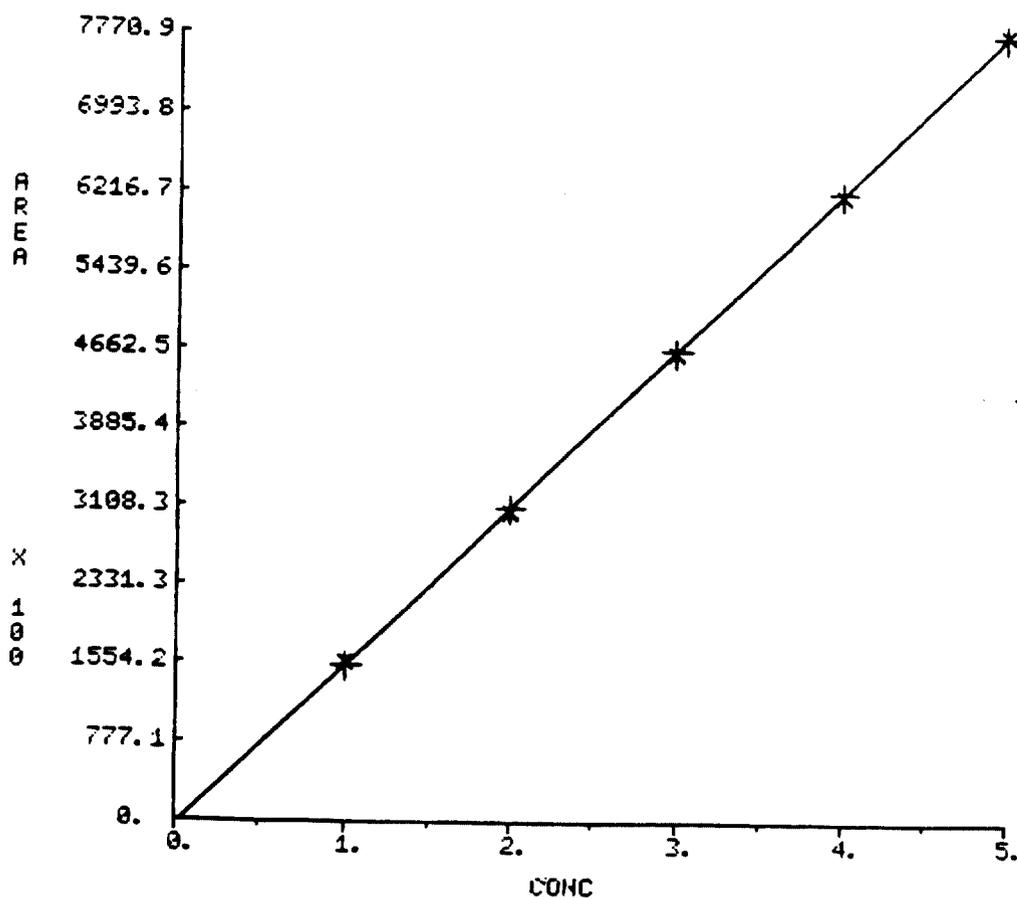
SD Standard deviation

n Number of determinations

ANALYTICAL CHEMISTRY REPORT

(continued)

FIGURE 1

Typical calibration standard graph
(Day 22)

COEFFICIENTS OF LEAST SQUARES FIT TO A LINEAR EQUATION
KA= 0. KB= 155142.45 KC=-4366.0984
CORRELATION COEFFICIENT OF X-Y PAIRS = 0.9999266
COEFFICIENT OF DETERMINATION = 0.9998533

ANALYTICAL CHEMISTRY REPORT

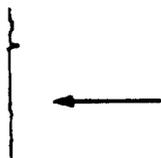
(continued)

FIGURE 2

Typical sample chromatograms
(Day 22)

Group 1, Control (1 g/500 ml)

CHANNEL A INJECT 10-02-93 16:57:08 STORED TO BIN # 32



Group 2, 1 mg/ml (1 g/500 ml)

CHANNEL A INJECT 10-02-93 14:49:49 STORED TO BIN # 13



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(continued)

FIGURE 2

(continued)

Group 3, 10 mg/ml (1 g/2500 ml)

CHANNEL A INJECT 10-02-93 15:30:21 STORED TO BIN # 19



Group 4, 100 mg/ml (1 g/25000 ml)

CHANNEL A INJECT 10-02-93 16:10:24 STORED TO BIN # 25



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(continued)

FIGURE 3

Typical procedural recovery chromatograms
(Day 22)

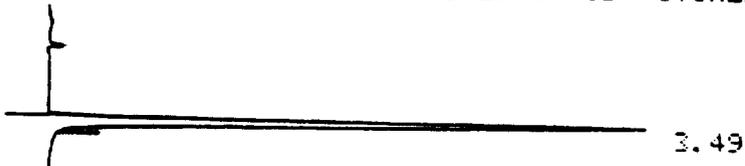
1 mg/ml, 98.5%

CHANNEL A INJECT 10-02-93 15:23:40 STORED TO BIN # 18



10 mg/ml, 99.7%

CHANNEL A INJECT 10-02-93 15:57:03 STORED TO BIN # 23



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(continued)

FIGURE 3

(continued)

100 mg/ml, 95.6%

CHANNEL A INJECT 18-02-93 16:43:47 STORED TO BIN # 30



Attachment 5
SUMMARY VERSION

MATERIAL SAFETY DATA SHEET

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For Emergency Health, Safety, and Environmental Information, call 716-722-5151

Date of Preparation: 11/16/87

Kodak Accession Number: 909313

SECTION I. IDENTIFICATION

- Product Name: Tri-p-tolylamine
- Synonym(s): 4-Methyl-N,N-bis(4-methylphenyl)benzenamine
- Formula: C21 H21 N
- Chem. No(s): 09313
- [] Internal Hazard Rating Codes: H: 1 S: 1 F: 1 C: 0

SECTION II. PRODUCT AND COMPONENT HAZARD DATA

COMPONENT(S):	Percent	ACGIH TLV(R)	CAS Reg. No.
Tri-p-tolylamine	ca. 100	---	1159-53-1

SECTION III. PHYSICAL DATA

- Appearance: White solid
- Melting Point: 112 C (234 F)
- Vapor Pressure: Negligible
- Evaporation Rate (n-butyl acetate = 1): Negligible
- Volatile Fraction by Weight: Negligible
- Specific Gravity (Water = 1): Not Available
- Solubility in Water: Negligible

SECTION IV. FIRE AND EXPLOSION HAZARD DATA

- Flash Point: Not Applicable
- Extinguishing Media: Water spray; Dry chemical; Carbon dioxide
- Special Fire Fighting Procedures: Wear self-contained breathing apparatus and protective clothing.
- Unusual Fire and Explosion Hazards: Fire or excessive heat may produce hazardous decomposition products. This material in sufficient quantity and reduced particle size is capable of creating a dust explosion.

SECTION V. REACTIVITY DATA

- Stability: Stable
- Incompatibility: Strong oxidizers
- Hazardous Decomposition Products: Combustion will produce carbon dioxide and probably carbon monoxide. Oxides of nitrogen may also be present.
- Hazardous Polymerization: Will not occur.

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SECTION VI. TOXICITY AND HEALTH HAZARD DATA

- A. EXPOSURE LIMITS: Not established.
B. EXPOSURE EFFECTS:
Inhalation: Low hazard for usual industrial handling.
Skin: Low hazard for usual industrial handling.
Eye: No specific hazard known. Contact may cause transient irritation.
Ingestion: Expected to be a low ingestion hazard.
C. FIRST AID:
Inhalation: Remove to fresh air following overexposure.
Skin: Wash after each contact.
Eye: Flush eyes with plenty of water.
Ingestion: Drink 1-2 glasses of water. Seek medical attention.

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SECTION VII. VENTILATION AND PERSONAL PROTECTION

- A. VENTILATION AND RESPIRATORY PROTECTION:
Good ventilation should be sufficient. Supplementary ventilation or respiratory protection may be needed in special circumstances.
B. SKIN AND EYE PROTECTION:
Safety glasses recommended in industrial operations involving chemicals. If prolonged or repeated skin contact is necessary, gloves or other protection may be required.

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SECTION VIII. SPECIAL STORAGE AND HANDLING PRECAUTIONS

Keep from contact with oxidizing materials.

=====
SECTION IX. SPILL, LEAK, AND DISPOSAL PROCEDURES

Sweep up material and package for safe feed to an incinerator. Dispose by incineration or contract with licensed chemical waste disposal agency. Discharge, treatment, or disposal may be subject to federal, state or local laws.



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