FINAL REPORT

Biodegradation Study of Hexabromocyclododecane

Conducted with 1,2,5,6,9,10-Hexabromocyclododecane (Test Substance No. K-1035)

Chemicals Inspection and Testing Institute
Chemical Biotesting Center, Kurume Laboratory

Compliance with the GLP Standards

Kurume Laboratory
Chemical Biotesting Center
Chemical Inspection and Testing Institute

Sponsor	Ministry of International Trade and Industry
Study Title	Biodegradation study of hexabromocyclododecane conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)
Study No.	21035

The study described in this report was conducted in compliance with the "Standard concerning Testing Facility Provided for in Article 4 of the Ordinance prescribing Test Items etc. Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances" (Kankiken No.233, Eisei No.38, 63 Kikyoku No.823, November 18, 1988).

Laboratory Facility Manager

****

Sealed date: March 10, 1990

Quality Assurance Statement

Kurume Laboratory,
Chemical Biotesting Center
Chemical Inspection and Testing Institute

Sponsor Ministry of International Trade and Industry
Study Title Biodegradation study of hexabromocyclododecane conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)
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Above study was audited and inspected by the Quality Assurance Division of Kurume Laboratory, Chemical Biotesting Center, Chemical Inspection and Testing Institute.

The dates of study audit and inspection, and the dates of report of the results to the laboratory facility manager and study director are listed below.

Date of audit or inspection	Date of report to laboratory facility manager	Date of report to study director
January 16, 1990	January 16, 1990	January 16, 1990
January 16, 1990	January 18, 1990	January 17, 1990
January 30, 1990	February 19, 1990	February 19, 1990
February 13, 1990	February 19, 1990	February 19, 1990
February 19, 1990	February 19, 1990	February 19, 1990
February 20, 1990	February 20, 1990	February 20, 1990
March 10, 1990	March 10, 1990	March 10, 1990

I hereby certify that this report describes the precise methods of the study which was conducted in accordance with its protocol and with our standard operating procedures, and that the reported results reflect accurately the raw data of the study.

Quality Assurance Personnel

********** Sealed date: March 10, 1990

********** Sealed date: March 10, 1990

********** Sealed date: March 10, 1990

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1. Study title: Biodegradation study of hexabromocyclododecane conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

2. Biodegradation test

2.1 Test conditions

(1) Test concentration: 100 mg/L
(2) Activated sludge concentration: 30 mg/L (as suspended solid)
(3) Test suspension volume: 300 mL
(4) Test temperature: 25±1 ℃
(5) Incubation period: 28 days

2.2 Measurements and analyses

(1) Measurements of the biochemical oxygen demand (BOD) with closed system oxygen consumption measuring apparatus
(2) Analyses of the test substance with high performance liquid chromatograph (HPLC)

3. Test results

(1) Degradability based on the BOD		0%	0%	4%
(2) Degradability based on HPLC analyses	Component A*	11%	0%	4%
	Component B*	10%	4%	4%

* The test substance was separated by HPLC into 2 components which are referred to as components A and B according to the order of the peak appearance.

4. Stability of the test substance

Stability of the test substance under the storage conditions was confirmed.

FINAL REPORT

Study No. 21035

1. Study title

Biodegradation study of hexabromocyclododecane conducted with 1,2,5,6,9,10-hexabromocyclododecane (test substance No. K-1035)

2. Sponsor

Name: Ministry of International Trade and Industry

Address: 1-3-1 Kasumigaseki, Chiyoda-ku, Tokyo, Japan

3. Testing facility

Name: Chemical Inspection and Testing Institute
Chemical Biotesting Center, Kurume Laboratory
Address: 19-14 Chuo-cho, Kurume-shi, Fukuoka, Japan
Tel: +81-942-34-1500
Laboratory facility manager: **********

4. Purpose of the study

This study was conducted to evaluate biodegradability of the test substance (K-1035).

5. Test method

This study was conducted in accordance with the "Biodegradation Test of Chemical Substances" specified in the "Test Method Relating to New Chemical Substances (Kanpogyo No.5, Yakuhatsu No.615, 49 Kikyoku No.392, July 13, 1974)".

6. Test period

(1) Start of the test: January 16, 1990

(2) Study period

Start of the use of activated sludge: November 13, 1989

Start of incubation: January 16, 1990

End of incubation: February 13, 1990

(3) End of the study: March 6, 1990

7. Testing personnel

Study director: **********

Experimental scientists: **********

****

Activated sludge supervisor: **********

Storage division manager: **********

8. Completion of the final report

March 6, 1990
Author: **********

9. Approval of the final report

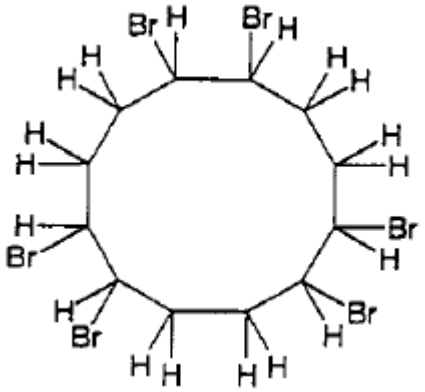
March 6, 1990
Study director: **********

10. Test Substance

The test substance (K-1035) described herein is identified by following name, structure, etc.

10.1 Name
1,2,5,6,9,10-hexabromocyclododecane

10.2 Structure, etc.
(1) Structural formula:



(2) Molecular formula: $C_{12}H_{18}Br_6$

(3) Molecular weight: 641.70

10.3 Purity[*1]
98.4%

The test substance was separated by high performance liquid chromatography (HPLC) into 2 components which are referred to as components A and B according to the order of the peak appearance. Assuming that ultraviolet absorption coefficient of each component is equal, relative proportion of each component was calculated from its peak area relative to the total peak area (See Fig.9).

Component	Relative proportion (%)
A	24.7
B	75.3

Concentration of each component was calculated by multiplying concentration of the test substance with respective proportion of the component.

*1: Calculated from the bromine content (73.5%) as provided in the attached information ******.

10.4 Supplier, grade and lot number of the test substance
(1) Supplier: **********
(2) Grade: **********

(4) Lot No.: AN01

10.5 Identification of the test substance

Structure of the test substance was identified by infrared spectroscopy (see Fig. 5), mass spectrometry (see Fig. 6) and nuclear magnetic resonance spectroscopy (see Fig. 7).

10.6 Storage conditions and stability under those conditions

(1) Storage conditions: Cool and dark place

(2) Stability: Stability of the test substance was confirmed by infrared spectra measured before and after incubation (see Fig.5).

11. Preparation of the activated sludge

11.1 Sludge sampling sites and period

(1) Sites: Sludge samples were taken at following 10 sites around Japan:

- Fushikogawa Sewage Treatment Plant (Sapporo-shi, Hokkaido)
- Fukashiba Sewage Treatment Plant (Kashima-gun, Ibaraki)
- Nakahama Sewage Treatment Plant (Osaka-shi, Osaka)
- Ochiai Sewage Treatment Plant (Shinjuku-ku, Tokyo)
- Kitakami River (Ishinomaki-shi, Miyagi)
- Shinano River (Nishikanbara-gun, Nigata)
- Yoshino River (Tokushima-shi, Tokushima)
- Lake Biwa (Otsu-shi, Shiga)
- Hiroshima Bay (Hiroshima-shi, Hiroshima)
- Dokai Bay (Kitakyushu-shi, Fukuoka)

(2) Period: September, 1989

11.2 Collection of samples

(1) Municipal wastewater: returned sludge of the sewage treatment plants

(2) River, lake and sea: surface water and surface soil at water's edge having contact with the atmosphere

11.3 Mixing of old and new sludge

Portions of 500 mL each taken from filtrates of sludge samples collected at above sites were mixed with 5 L of filtrate of the old sludge which had been used for testing to make 10 L of a new sludge suspension. This suspension was adjusted to pH of 7.0 ± 1.0 and aerated in an incubation tank[*2].

*2: Filtered outdoor air was used for aeration.

11.4 Culturing

After stopping the aeration of the incubation tank to let the sludge settle for approximately 30 minutes, about one-third of the supernatant was replaced by equal amount of 0.1% synthetic sewage water[*3] before resuming the aeration. This procedure was repeated daily to prepare the activated sludge culture. The incubation temperature was 25 ± 2℃.

*3: Synthetic sewage water (0.1%) was prepared by dissolving glucose, peptone and potassium dihydrogen phosphate into deionized water at concentrations of 0.1% each and adjusting to pH of 7.0 ±1.0.

11.5 Maintenance and use

Appearance of the supernatant and formation of the activated sludge flocks were observed. In addition, precipitability, pH, temperature and dissolved oxygen concentration of the activated sludge were recorded during incubation. Activated sludge used for the test was observed under an optical microscope as appropriate to confirm that no abnormalities were found in biota.

11.6 Activity of the sludge

Activity of the sludge was determined with the reference substance at the start of its use. Relevance with the old sludge was also noted.

12. Conduct of the biodegradation study

12.1 Prior procedures

(1) Determination of suspended solid concentration in the activated sludge

Method: Concentration of suspended solid was determined according to the method described in Japanese Industrial Standard "Testing methods for industrial waste water: suspended solid" (JIS K 0102-1986 14.1).

Date: January 16, 1990

Result: Concentration of suspended solid was 7200mg/L.

(2) Preparation of basal medium

Basal medium was prepared by mixing 3mL each of solutions A, B, C and D prescribed in Japanese Industrial Standard "Testing methods for industrial waste water: biochemical oxygen demand" (JIS K 0102-1986 21) and purified water to make a final volume of 1L and adjusting to pH of 7.0.

(3) Reference substance

Aniline was used as a reference substance.

12.2 Preparation of the test suspensions

Test suspensions were prepared in six separate bottles according to the procedures described below. Those bottles were incubated under the conditions described in section 12.3.

(1) Spiking of test substance or aniline

(a) Abiotic control (water and the test substance) [bottle No. 6]

Transfer 300 mL of basal medium into a culturing bottle and add the test substance at a concentration of 100mg/L

(b) Test cultures (activated sludge and the test substance) [bottles No.3-5]

Transfer 300 mL of basal medium into a culturing bottle and add the test substance at a concentration of 100 mg/L

(c) Positive control (activated sludge and aniline) [bottle No.1]

Transfer 300 mL of basal medium into a culturing bottle and add aniline at a concentration of 100 mg/L

(d) Inoculum blank [bottle No. 2]

Transfer 300 mL of basal medium into a culturing bottle

(2) Inoculation of the activated sludge

The activated sludge prepared as described in sections 11.1-6 was added to test cultures, positive control and inoculum blank bottles prepared above at a suspended solid concentration of 30mg/L each.

12.3 Incubation equipment and conditions

(1) Incubation equipment

Closed system oxygen consumption measuring apparatus (coulometer)

Test vessels:	300mL culturing bottles
CO_2 gas absorber:	soda lime, No.1
Stirring method:	rotary stirring with magnetic stirrer

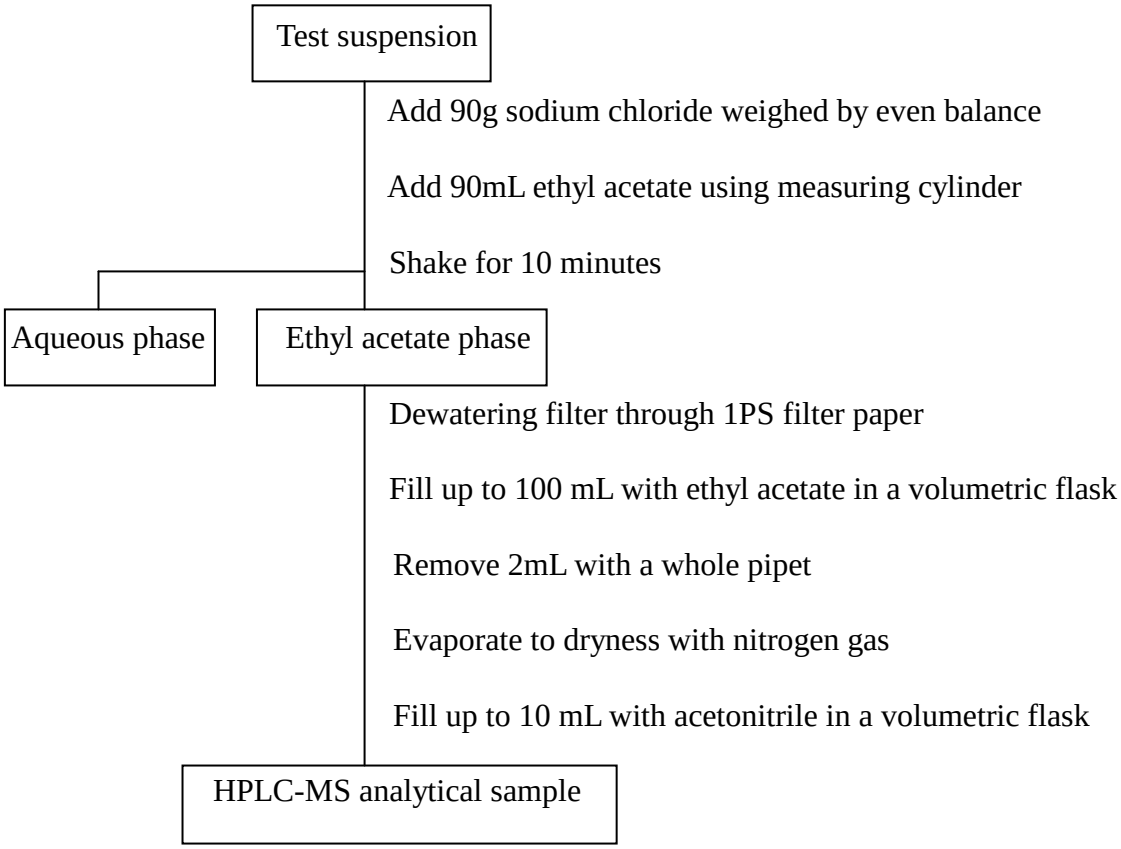
(2) Environmental conditions

Incubation temperature:	25±1℃
Incubation period:	28 days
Site:	Equipment room No.11

12.4 Analyses of the test suspensions

12.4.1 Pre-treatment of the test suspensions

After the end of the incubation period, test suspensions of abiotic control, test cultures and inoculum blank were pretreated according to the procedure described in the following flow chart to prepare samples for the HPLC analyses:



12.4.2 Quantitative analyses

Analyses of the test substance with HPLC

Samples prepared in the pretreatment procedures were analyzed for the test substance under the conditions shown below. Concentrations of each component in analytical samples were calculated from its peak height on the chromatogram relative to the peak height of 80.0 mg/L standard solution of the test substance (19.8 and 60.2 mg/L for components A and B, respectively) (see Table 2 and Fig. 2).

Limits of quantification (LOQ) of each component were determined to be 0.9 and 0.6 mg/L for components A and B, respectively by assuming the noise level on the chromatogram to be 2 mm (peak height).

(1) Analytical conditions

Instrument:	High performance liquid chromatograph
Column:	UNISIL PACK F3-100B
	10cm × 6mm ϕ stainless steel
Eluant:	acetonitrile/water (49:1,w/w)
Flow rate:	1.0mL/min.
Measured wave length:	220 nm (see Fig.8)
Injection volume:	50μL
Sensitivity	
Detector:	0.04ABU/FS
Recorder:	range 1mV

(2) Construction of a calibration curve

A 100mg portion of the test substance was accurately weighed, dissolved in acetonitrile to final volume of 100mL to give a 1000mg/L stock solution. This stock solution was diluted with acetonitrile to make 20.0, 40.0 and 80.0 mg/L standard solutions. These standard solutions were then analyzed by HPLC under the conditions described above. Peak heights of each component on chromatograms were plotted against concentrations in standard solutions to draw a calibration curve (see Fig. 4).

Concentrations (mg/L) of the components are as shown below.

Test substance	Components	
	A	B
20.0	4.94	15.1
40.0	9.88	30.1
80.0	19.8	60.2

12.4.3 Recovery test

Recovery rates of the spiked test substance in abiotic control and test culture suspensions prepared as described in section 12.2 were determined. Suspensions were pretreated according to the procedure described in section 12.4.1 and analyzed by HPLC under the analytical conditions described in section 12.4.2. Recovery rates of duplicate samples and average values are shown below. The average recovery rates were used to correct concentrations of the test substance in analytical samples (see Table 3 and Fig. 5).

Recovery rates in abiotic control

Component A	97.8	94.3	Av. 96.0
Component B	97.0	98.2	Av. 97.6

Recovery rates in test culture

Component A	94.3	94.3	Av. 94.3
Component B	100	98.6	Av. 99.3

12.5 Calculation of degradability

Degradabilities of the test substance were calculated by using formulas given below. Results were rounded at the first decimal place and presented in whole numbers.

(1) Degradability based on the BOD

$$\text{Degradability (\%)} = \frac{\text{BOD - B}}{\text{TOD}} \times 100$$

where

BOD: biochemical oxygen demand (mg) of the test culture (measured value)

B: biochemical oxygen demand (mg) of inoculum blank (measured value)

TOD[*4]: theoretical oxygen demand (mg) required for complete oxidation of the test substance (calculated value)

*4: TOD was calculated by assuming the purity of the test substance to be 100%.

(2) Degradability based on the HPLC analyses

$$\text{Degradability (\%)} = \frac{S_B - S_A}{S_B} \times 100$$

where

S_A: amount of residual test substance (mg) in test cultures (measured value)

S_B: amount of residual test substance (mg) in abiotic control (measured value)

12.6 Treatment of the values

Arithmetic mean was used for averaging of values. Figures were rounded according to the standard described in JIS Z 8401-1961.

13. Validity of the test

Validity of the test was confirmed by the degradabilities of aniline based on the BOD on days 7 and 14 being 75 and 85%, respectively.

14. Results

14.1 Appearance of the test suspensions

Appearance of the test suspensions were as follows:

	Test suspensions	Appearance
Start of the test	Abiotic control	Test substance was not dissolved
	Test cultures	Test substance was not dissolved
End of the test	Abiotic control	No change was observed
	Test cultures	No change was observed No growth of the sludge was observed

14.2 Results of the analyses

Results of the analyses after 28 days of incubation were as follows:

			Abiotic control	Test culture bottles			Theoretical amount	cf. Attached	
				3	4	5		Table	Fig.
BOD		mg	0.0	0.0	0.0	1.0	24.6	1	1
Residual test substance (HPLC analyses)	Component A	mg	7.2	6.4	7.4	6.9	7.4	2	2
		%[*5]	97	86	100	93			
	Component B	mg	22.1	19.9	21.2	21.3	22.6		
		%[*5]	98	88	94	94			

*5: Residual rates of the test substance were calculated by using formula below. Values were rounded at the first decimal place and presented in whole numbers.

$$\text{Residual rate (\%) } = \frac{\text{Residual amount (mg)}}{\text{Theoretical amount (mg)}} \times 100$$

14.3 Results of the biodegradation test

Degradabilities of the test substance after 28 days of incubation were as follows:

		Degradability (%) in bottles			cf. Attached Figure
		3	4	5	
Degradability based on the BOD		0	0	4	1
Degradability based on HPLC analyses	Component A	11	0	4	2
	Component B	10	4	4	

15. Storage and retention of the test substance and records

15.1 Test substance

A 5g portion of the test substance was placed in a storage container, tightly sealed and stored in the sample storage room of the Kurume Laboratory for the period specified in paragraph 32 of the “Standard concerning Testing Facility Provided for in Article 4 of the Ordinance prescribing Test Items etc. Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances (hereafter referred to as the “GLP standard”).

15.2 Raw data, records, etc.

Results of analyses, measurements and observations generated in the study and other raw data such as laboratory notebooks which were used for the development of final report, test protocol, records of inspection, reference materials, etc. are stored along with the final report in the archive of the Kurume Laboratory for the period specified in paragraph 32 of the above GLP standard.

16. Major apparatuses, instruments, reagents, etc. used in the test

16.1 Apparatuses and instruments

Coulometer:	Okura Electric Co. Ltd.
HPLC	
Pump:	Shimadzu Corporation SPD-2A
Detector:	Shimadzu Corporation LC-3A
Balance:	Sartorius 2007 MP6
UV-VIS spectrophotometer:	Hitachi, Ltd. 150-20

16.2 Reagents

Sodium chloride:	Manac Inc., reagent grade
Purified water:	Takasugi Pharmaceuticals Co., Ltd., Japanese Pharmacopeia
Acetonitrile:	Wako Pure Chemical Industries, Ltd., HPLC grade
Soda lime, No.1:	Wako Pure Chemical Industries, Ltd., reagent grade
Aniline:	Showa Chemical Co., Ltd., analytical reagent grade, Lot No. 292325
Ethyl acetate:	Kanto Chemical, Co., Inc., reagent grade

Fig.1

図 － 1　（1/5）　クーロメーター記録図

Test substance　K-1035

Apparatus　Coulometer No. CM-5
range　250 mg/l × 1
chart speed　2 mm/h

Cultivation condition
concentration
test substance　100 mg/l
reference substance (Aniline)　100 mg/l
activated sludge　30 mg/l
temperature　25 ± 1℃
period　1/16 ～ 2/13 (28 days) 1990

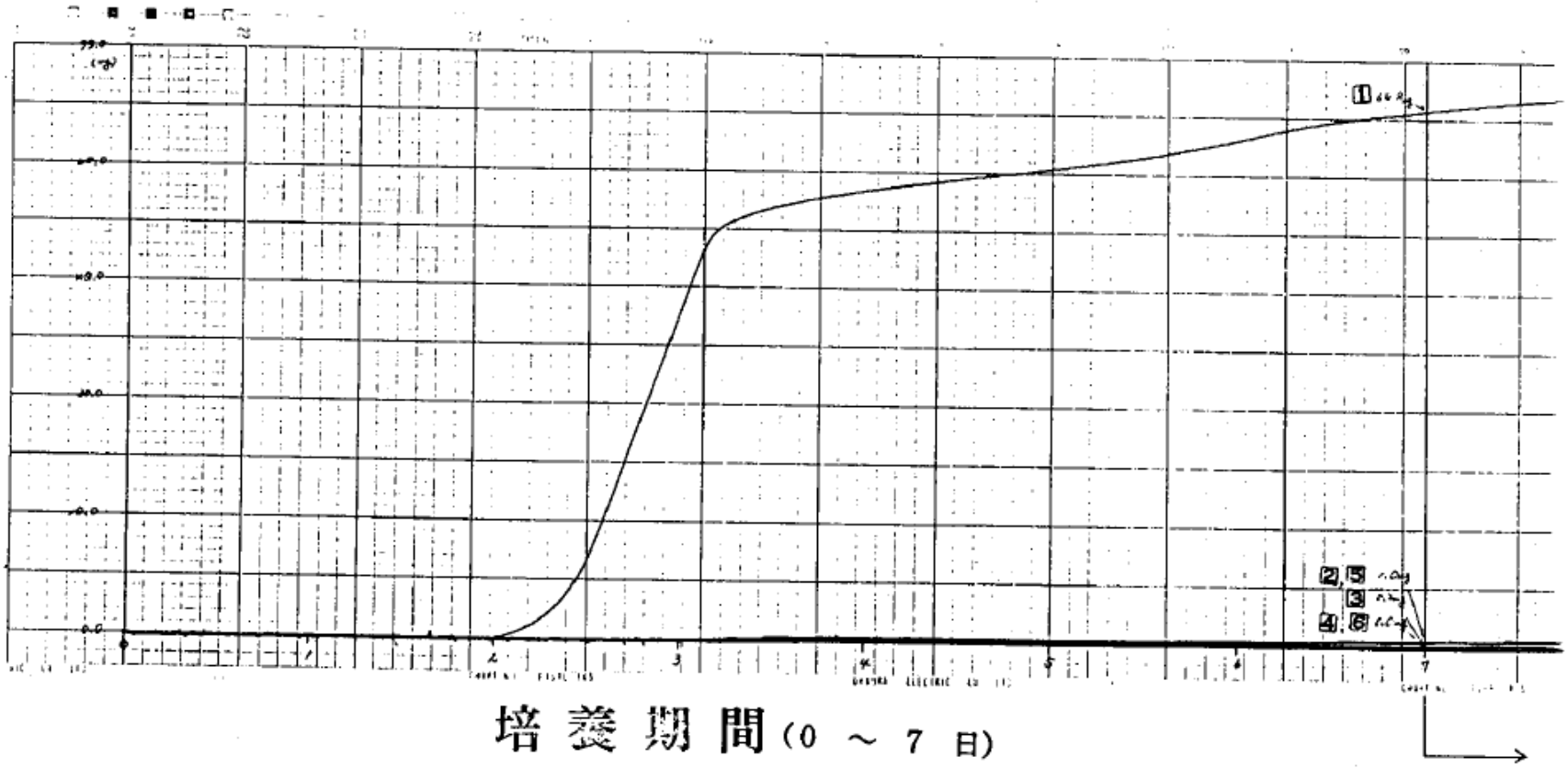
Bottle No.	Contents
①	汚泥＋アニリン
②	基礎呼吸
③	汚泥＋被験物質
④	汚泥＋被験物質
⑤	汚泥＋被験物質
⑥	水　＋被験物質

Note　：本試験

Operator [redacted]

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

図—1 (2/5)



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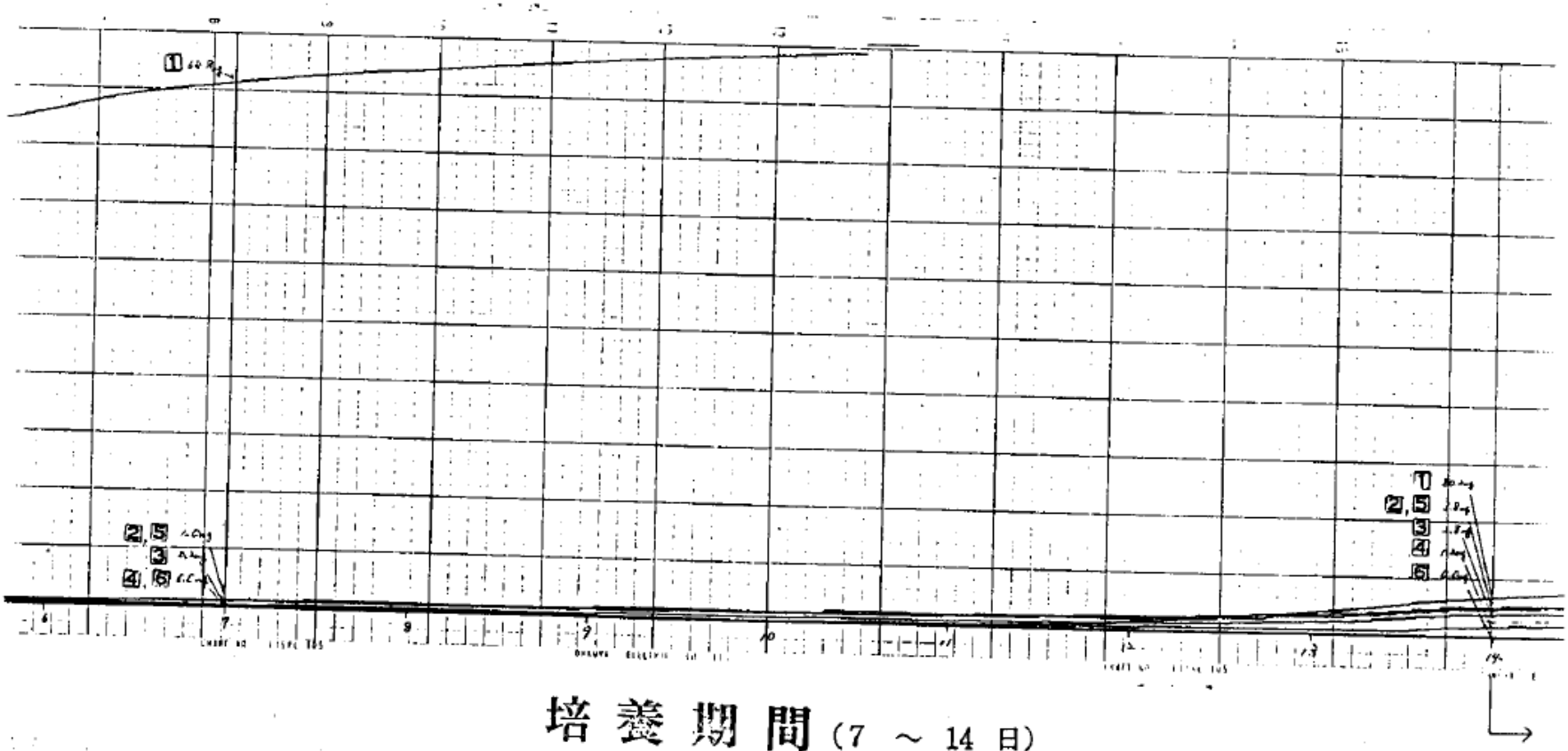

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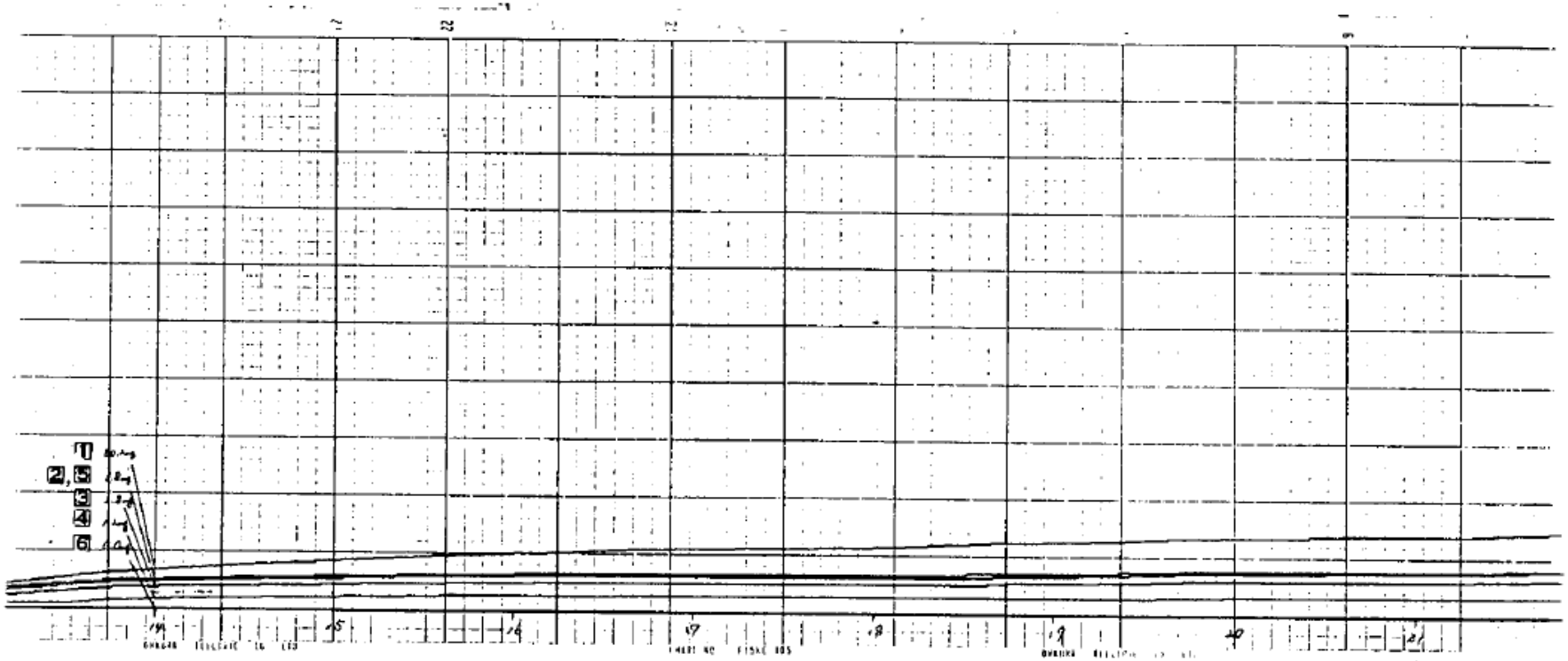
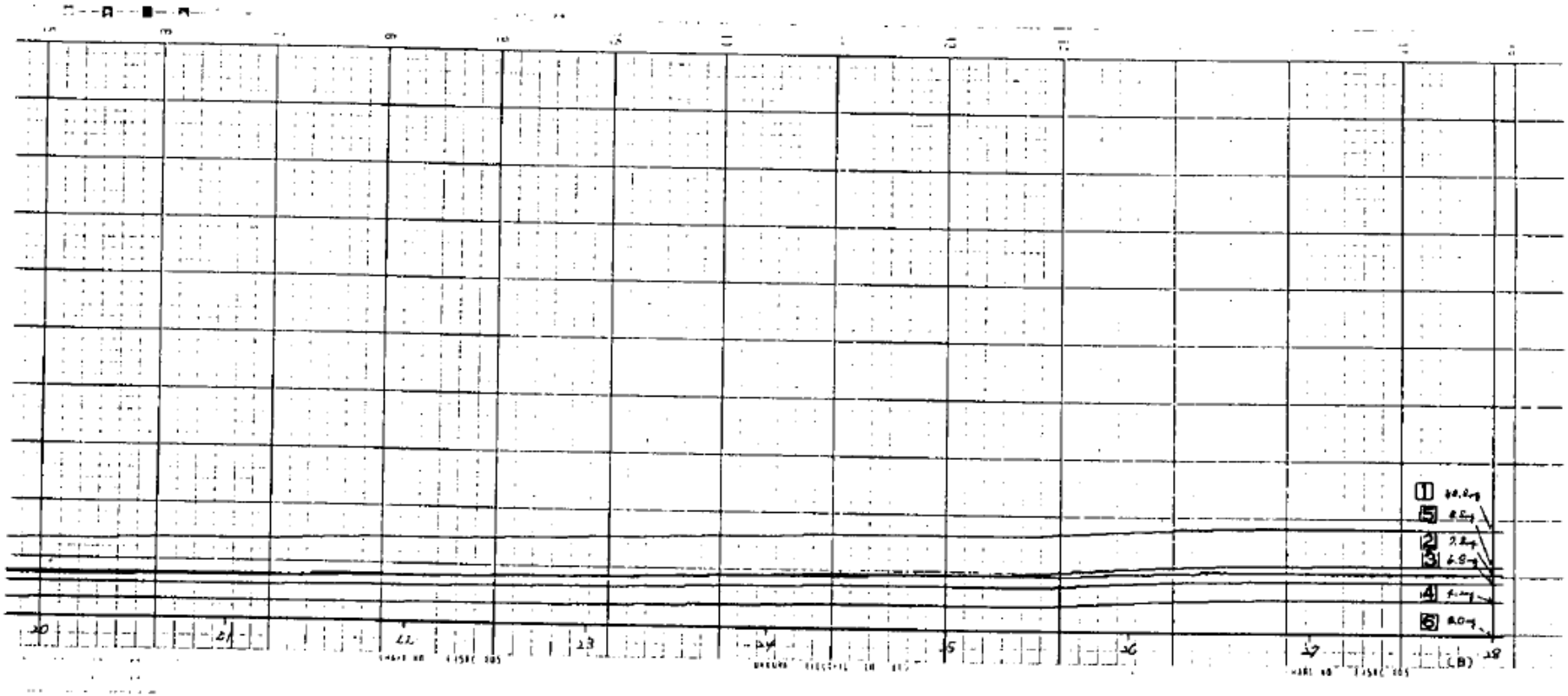


培養期間 (14 ～ 21 日)

図—1 (5/5)



培養期間 (21 ～ 28 日)