No. 004

TEST REPORT

24-month Inhalation Carcinogenicity Study on Methanol in Fischer Rats

(Test No.: 5A-268)

September 30, 1985

Mitsubishi Kasei Institute of Toxicology and Environmental Sciences (currently identified as Mitsubishi Chemical Safety Institute, Ltd.)

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SUBMITTED TO: New Energy and Industrial Technology Development Organization

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Title: 24-month Inhalation carcinogenisity Study on Methanol in Fischer Rats

Purpose of the Study: study the carcinogenicity to methanol gas in rats after long-term low level exposure.

Test Facility:	Company Name: Mitsubishi Kasei Institute for Toxicological and Environmental Sciences (currently identified as Mitsubishi Chemical Safety Institute, Ltd.	
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	Laboratory Location:	1000 Kamoshida-cho, Midori-ku, Yokohama-shi, Kanagawa
	Representative:	Director of Laboratory, Yoshiaki Suzuki
Test Period:	Test Initiation:	September 3, 1982
	Arrival of Rats:	September 3, 1982
	Exposure Initiation:	September 16, 1982
	Final Sacrifice:	September 18-21, 1984 – male rats
		September 25-28, 1984 – female rats
	Final Report Preparation:	September 30, 1985

Storage of Raw Data and Reports

Raw data and reports will be kept in the document storage room within the test facility.

Storage period is five years upon submission of the report, and subsequent storage to be determined separately upon consultation with the client.

The present study was performed under charge of the following personnel, and the results are correctly reflected in the present report.

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A. Animal care; administration of test substance; testing; and observations
B. Data reduction
C. Laboratory tests
D. Pathological tests

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1. Overview

(1) Continuous inhalation test was performed on rats using systemic exposure method to study 24-month (104-week) carcinogenicity of methanol. The test groups consisted of four groups including a control group kept inside an inhalation chamber, and each group contained 52 male and 52 female rats. Exposure levels are 0, 10, 100 and 1000 ppm by volume.

(2) The levels of methanol barely fluctuated from the set value and were controlled satisfactory. Therefore, the exposure is believed to be done almost at intended levels.

(3) The average duration of methanol exposure was 19.5 hours per day for both male and female rats.

(4) There was no microbiological contamination that may have influences the result of study.

(5) Toxic effect of methanol gas was investigated through observation of general conditions, body weight check, food consumption check, laboratory tests (urinalysis, hematological test, and serum biochemistry test), organ weight check and pathological tests (pathological autopsy and histopathological test), and the following results were obtained:

① The percentages of cases that were found dead or killed *in extremis* were 19.2 to 34.6 % in male rats and 32.7 to 40.4 % in female rats for all groups, and no tendency of dose-dependency on methanol gas exposure was observed.

② No abnormalities that seem attributable to exposure to methanol gas were identified through observation of general conditions.

③ With regards to body weight, the female rats in the high level exposure group (1000 ppm) experienced mild suppression of weight gain during Week 51 to 72 of exposure; however, the difference to the control group was only about 4 %, however, there was no significant difference observed in the weight gain.

④ The male rats in the high level exposure group (1000 ppm) consumed less amount of food during Week 30 to 52 of exposure; however, no weight loss or abnormality in other tests that may have been related to this reduction were noticed.

⑤ In urinalysis, the male rats in the high level exposure group (1000 ppm) showed a tendency of increased level of glucose and the female rats showed a tendency of decreased level of pH and increased level of bilirubin. Individual values were not abnormal in particular.

⑥ In hematological test, no variation that seemed to be influenced by exposure to methanol gas was identified.

⑦ In serum biochemistry test, no variation that seemed to be influenced by exposure to methanol gas was identified either.

⑧ The organ weight measurement did not show any significant variation that suggests influence of exposure to methanol gas.

⑨ All groups showed various types of nonneoplastic changes; however, the majority is naturally occurring changes associated with 24-month old of *Fischer* strain, and none were considered specific to methanol gas exposure.

(6) As a result of pathological studies, investigating mainly pathological studies, investigating the carcinogenicity of methanol, the following findings were obtained:

① The male rats in the high level exposure group (1000 ppm) showed a trend towards slightly high incidence of papillary adenoma in lungs; however, taking into consideration the incidence of adenomatosis with which transition to papillary adenoma is suspected, the present tendency could not be deemed attributable to the influence of exposure to methanol gas.

② The female rats in the high level exposure group (1000 ppm) indicated slightly higher incidence of phaeochromocytoma in adrenal gland compared to natural occurrence.

③ Metastatic tumors were seen only in the high level exposure group (1000 ppm) – three cases of male and five cases of female rats; however, there was no primary organ specificity, and thus the onset was considered unrelated to the exposure to methanol gas.

(7) The results above may be summarized that the majority of changes seen in the high level exposure group (1000 ppm) was all very subtle, and could not be determined as signs of overt toxicity caused by exposure to methanol gas. The incidence of phaeochromocytoma in adrenal glands of female rats in the high level exposure group (1000 ppm), however, seemed to have a tendency to be slightly higher than the spontaneous incidence in *Fischer* rats. Due to a small number of bibliographic findings on this particular lesion as well as relatively small number of cases in the present study, it was not possible to clearly conclude, based only on the present experiment, that the present lesion suggests influence of exposure to methanol gas. Definite conclusion may be achieved by performing further study on exposure to methanol gas using larger number of subjects.

(8) Conclusion

The result of carcinogenic and inhalation toxicological study on *Fischer* rats through 24-month (104-week) consecutive and systemic exposure did not indicate chronic toxicological effect of exposure to methanol gas at levels lower than 1000 ppm in either male or female. Furthermore, the incidence of phaeochromocytoma in adrenal gland of female rats in the high level exposure group (1000 ppm) was slightly higher than spontaneous incidence. Positive carcinogenic effect, however, could not be identified.

2. Test Purpose

To investigate carcinogenicity of long-term exposure to low level methanol gas in rats.

3. Test Method

3.1 Deviations from the Protocol

The present study was conducted in accordance with the protocol (NED/ 001) that was initially prepared in 1981 and submitted to "Environmental Safety Committee for Methanol," which consists of academic experts and was approved after minor revisions.

The points that deviated from the description of the protocol as well as those with which interpretation was stretched are shown below:

(1) Protocol B-3-(2): Environment inside the exposure chamber

4) The temperature of 24 ± 2 ºC was changed to 23 ± 3 ºC.

(2) Protocol B-3-(3): Diet

As diarrhea and soft stool were observed, refined diet (solid refined diet for rats by Funabashi Farms) was used as a countermeasure for the following period. Formula and certificate of analysis are attached as Support Document 1.

Refined diet was supplied between July 25 and August 23, 1983 (Week 44-49 of exposure; the low level exposure group and the control group received from July 26, however) as well as between October 31 and November 30, 1983 (Week 58-62 of exposure).

(3) Protocol C-3-(2): Body weight check

In the protocol, the body weight was supposed to be checked once a week from the beginning until Week 13 and then once a month thereafter. Taking into consideration the importance of body weight check as one of the indicators to observe general conditions during inhalation study, the body weight was kept checked once a week after Week 13, as a general rule, however.

(4) Protocol C-3-(3): Food consumption rate

After Week 24, the plan was to check the food consumption rate every three months; however, since increase in the rate was detected, it was kept checked once a month as a general rule after Week 24 as well.

(5) Protocol C-3-(5)-3: Serum or plasma biochemical tests

Triglyceride was measured using modified method of Bucolo and David.

(6) Protocol C-3-(7): Statistical analysis method

Mann-Whitney U test was supposed to be used to verify significant difference in urinalysis; however, Armitage's Chi-square test was used instead to investigate more closely the scale of difference between groups.

3.2 Test Substance

3.2.1 Name and Properties[1)]

Chemical name: Methanol, methyl alcohol
General name: Methanol, methyl alcohol
Chemical formula: CH_3OH
Molecular weight: 32.04
Physical properties:
- Appearance: Colorless, transparent liquid under normal temperature
- Odor: Unique aroma
- Specific gravity: d 0.7928
- Vapor density: 1.11 (air = 1.0)
- Boiling point: 64.65 ºC (760 mmHg)
- Melting point: -97.6 ºC
- Critical temperature: 240.0 ºC
- Critical pressure: 78.5 atm
- Flash point: 11 ºC (direct vent system)
- Ignition point: 470 ºC (in the air)
- Explosion limit: 6.72-36.5 v/v % (in the air)
- Vapor pressure: 60 mmHg (12.1 ºC)
- Combustion heat: 173.6 kcal/ mol (25 ºC)
- Evaporation heat: 8.43 kcal/ mol (boiling point)
- Heat of solution: -1.59 kcal/ mol (water 25 mol, 25 ºC)
- Conversion factor: 1 ppm = 1.310 mg/ m^2
  (760 mmHg, 25 ºC) 1 mg/ m^2 = 763 ppm

Physical and Chemical Properties
- Solubility in water: Miscible at any ratio
- Solubility in organic solvents, such as alcohol, ketone, ester, and halogenated hydrocarbon: Fully miscible
- Corrosivity: Noncorrosive under normal conditions (except for lead and aluminum)

3.2.2 Method of Acquisition and Standards

The methanol used in the study is Pure Grade 1 reagent methanol obtained from Junsei Chemical Co., Ltd.

For quality assurance, certificate of analysis was requested to be attached to every lot purchased. Standards were prescribed by reference to Japanese Industrial Standards, K8891 Methanol (Regent)[2)]. On the grounds that the methanol will be used for carcinogenicity study, analysis of vinyl chloride and formaldehyde, carcinogenic substances that are highly volatile under normal temperature and may possibly be incorporated in methanol, was requested as well.

Analytical method is shown in Support Document 2.
Analysis items and standards are shown below.

Analysis Items and Standards of Test Substance

Items		Standards
1. Solubility in water		Within limit
2. Specific gravity (20/ 20 ºC)		0.794 or less
3. Fraction (64-65 ºC)	v/ v %	95 or more
4. Water	%	0.3 or less
5. Non-volatile matter	%	0.003 or less
6. Acid	%	0.002 or less
7. Alkali	%	0.0003 or less
8. Ethanol		Within limit
9. Substances reducing permanganate		Within limit
10. Substances darkened by sulfate		Within limit
11. Content	%	99.6 or more
12. Vinyl chloride		1 ppm or less
13. Formaldehyde		3 ppm or less

Certificate of analysis for each lot are shown in Support Document 3.

3.2.3 Method of Storage and Handling

(1) Storage Method

One 14-kg can contain 18 liters of methanol and 10 cans were purchased every time. This amount lasts for about a month and a half. As soon as the cans arrived, they were stored in the special indoor storage space for hazardous materials, and were carried out to the inhalation laboratory within the Animal Wing, one at a time, on as needed basis.

In the inhalation laboratory, each chamber has its own special tank depending on the level of methanol exposure, and the liquid methanol was sent to the methanol carburetor via stainless steel piping. Therefore, there is no risk of methanol leakage.

(2) Handling Method

Methanol is colorless and transparent highly volatile liquid under normal temperature, has a unique odor and miscible to water, ethyl alcohol and so forth.

As for explosibility and flammability, the flammability is 11 ºC, which is low, and when the methanol steam mixes with the air, it forms explosive gas mixture within the range of 6.72-36.5 %.

Harmful effects of methanol on living body is that it stimulates eyes, nose as well as pharyngeal mucous, and repeated contact with the liquid develops dry skin, scaly skin and cracked skin.

Furthermore, when dense steam is inhaled, it causes acute poisoning. About 1000 ppm of methanol develops symptoms, such as inebriety, pain, headache, and blurred vision.

Besides by nose and mouth, methanol is absorbed through skin as well. Methanol gradually oxidizes inside the body and accumulates[1)].

The *American Conference of Governmental Industrial Hygienists* (ACGIH) (1978) advises allowable concentration of methanol to be 250 ppm for 15 minutes or less exposure (TLV-STEL)[*1] and 200 ppm as weighted average concentration (TLV-TWA)[*2] for 8-hour day labor.

The inhalation laboratory that deals with methanol is located within the Animal Wing, and as the ventilation frequency is 12-15 times/ hour, it is reasonable to believe that the level of methanol within the laboratory would most likely not exceed the abovementioned allowable concentration, let alone explosion limit. Due to the properties of methanol discussed thus far, however, when handling methanol, use of protective gloves and goggles was mandated in order to prevent exposure to methanol gas, and when replacing methanol or other occasions where direct exposure to methanol gas caused by leaking and so forth, even briefly, is suspected, a protective suit and an organic chemical cartridge respirator were used.

Notes:

*1: TLV-STEL: *Threshold Limit Values – Short Term Exposure Limit*

*2: TLV-TWA: *Threshold Limit Values – Time Weighted Average*

3.3 Test Animals

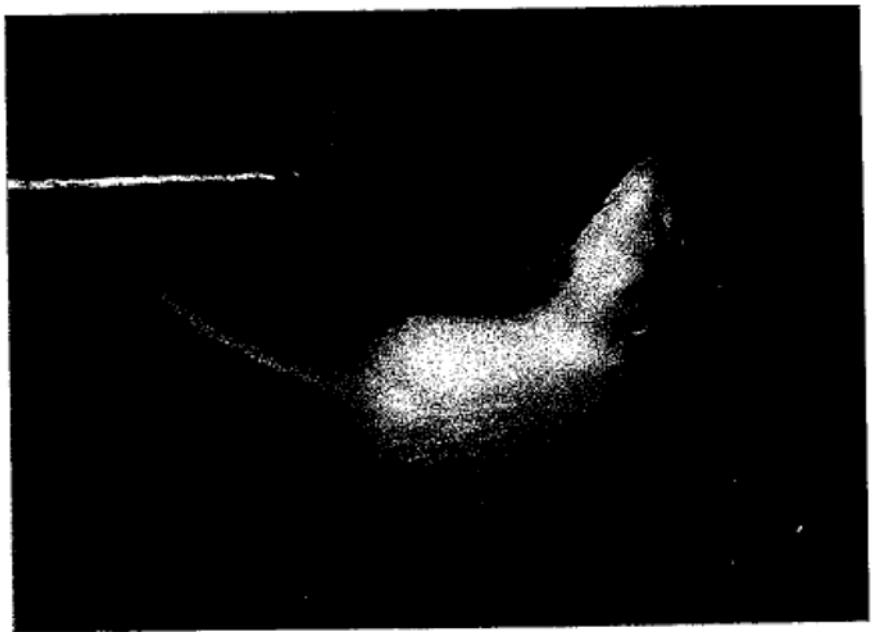
3.3.1 Type and Strain of Animals as well as a Reason for Selection

Fischer rats (F344/ DuCrj; microorganism level: SPF) were used for the study.

330 each of male and female four-week old (born between August 4-6, 1982) rats from the same lot were purchased from Charles River Japan, Inc. on September 3, 1982. In reality, 340 each of male and female rats were delivered.

At the time of shipping, the male rats weighed 55-70 grams and the female rats 50-65 grams.

This strain of rats was selected because of its frequent use in long-term toxicity studies, abundant background data, relatively low incidence of naturally occurring tumors, and recommendation in "*Guidelines for Carcinogen Bioassay in Small Rodents**" by *National Cancer Institute* (NCI) in the United States (see Photograph 1).



Photograph 1: Rat (F344/ DuCrj)

Note:

*: *"Guidelines for Carcinogen Bioassay in Small Rodents," Carcinogenesis Division of Cancer Cause & Prevention, National Cancer Institute (NIH), 1974*

3.3.2 Exposure Initiation Age in Week

In compliance with OECD guidelines*, the exposure was started within six weeks of birth.

3.3.3 Quarantine and Acclimation

After arrival, the rats were quarantined for one week in an animal room (Room No. B-214) within barrier system in the Animal Wing, and they were confirmed to be healthy through body weight check and observation of general conditions. During the quarantine period, the male rats weighed 74-99 grams and the female rats 58-75 grams. Having the body weights after one week of quarantine and acclimation as stratification factor, the rats were grouped using stratified random sampling method. All rats were further acclimated for six days in inhalation cages within chamber, and the exposure to methanol was initiated when the rats were 41-43 days old.

On the day of commencement of exposure, the male rats weighed 103-135 grams and the females 88-109 grams.

Note:

*: *OECD Guidelines for Testing of Chemicals* (October 1980)
Japanese translation: "OECD Guidelines for Toxicity Test on Chemicals," edited by Yoshihito Omori (The Chemical Daily)

3.4 Inhalation Test Facility

3.4.1 Inhalation Laboratory

Four inhalation chambers were set up in Room B-201 (8.1 x 6.6 meters) within the barrier system located on the second floor of the Animal Wing B to perform a long-term inhalation toxicological study on rats.

The air circulating the room was filtered through various types of filters and then the temperature and humidity of the air were controlled. The room was ventilated 12 to 15 times per hour. The pressure setting inside the room was positive by 1-2 mmAq compared to the hallway to prevent contamination from outside.

The room had no windows, and fluorescent lighting was artificially controlled: the fluorescent lamps were turned on and off in a 12-hour cycle. (ON: 7:00 am – 7:00 pm; OFF: 7:00 pm – 7:00 am)

The concrete floor of the room was coated by 5 millimeters of polyurethane resin coating to prevent contamination from cracks in concrete as well as facilitate disinfection of the floor.

The floor structure of the second floor of Animal Wing B (Barrier System) is shown in Figure 1 and the arrangements of the chamber and so forth in Room B-201 is shown in Figure 2.

The interior of the barrier system and Room B-201 are shown in Photographs 2 and 3, respectively.

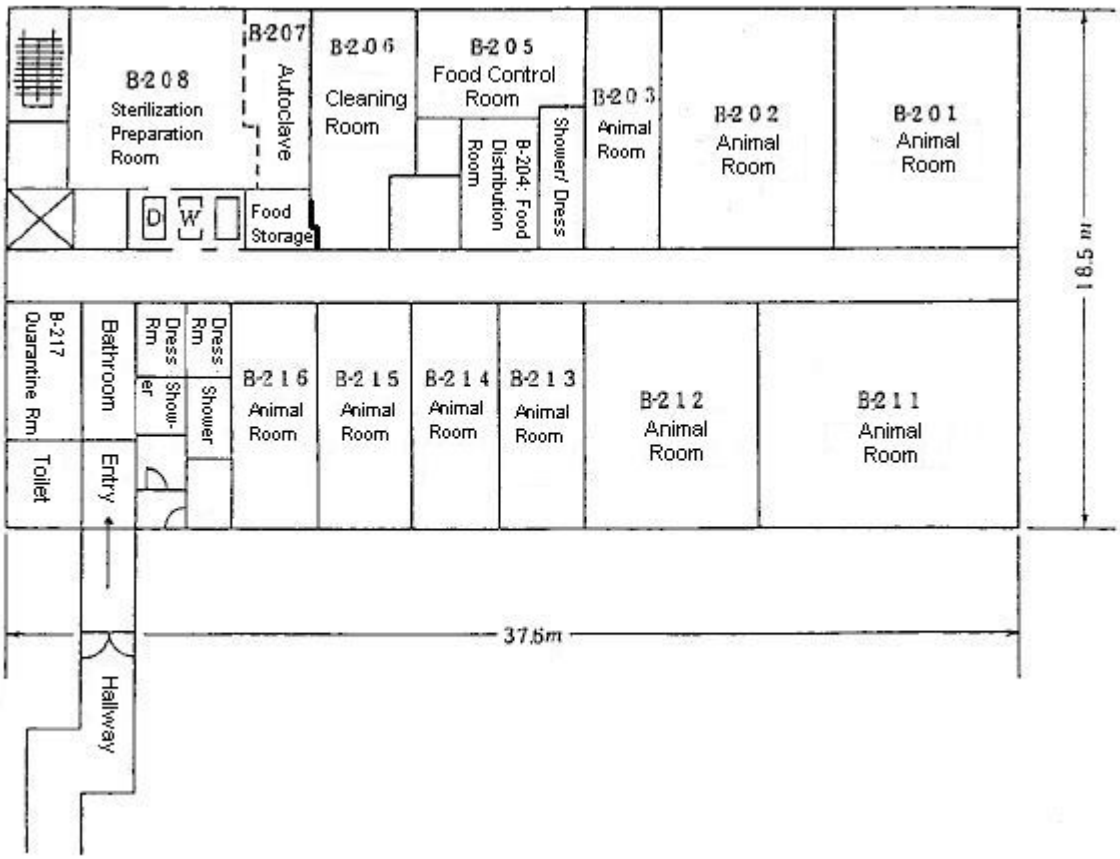


Figure 1: Animal Wing B, Second Floor (Barrier System) Floor Plan

Photograph 2: Animal Wing B, Second Floor (Barrier System) Interior

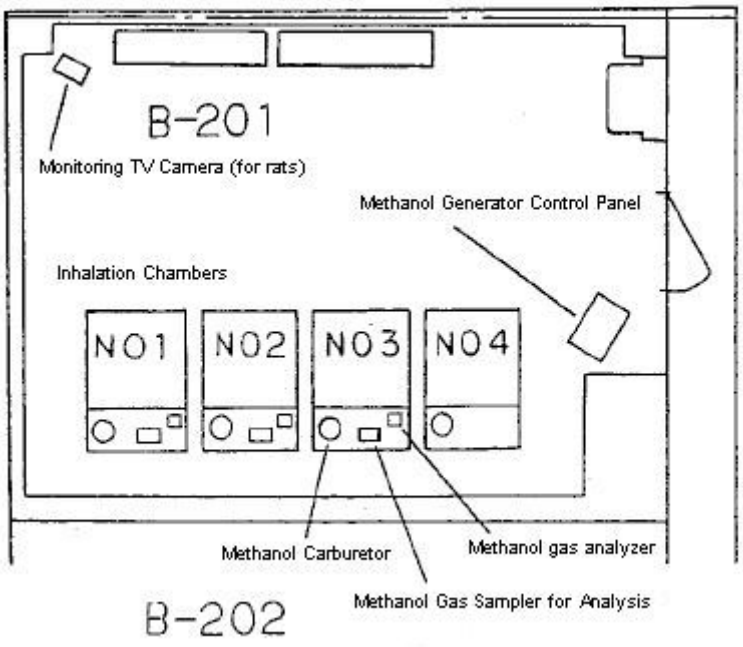
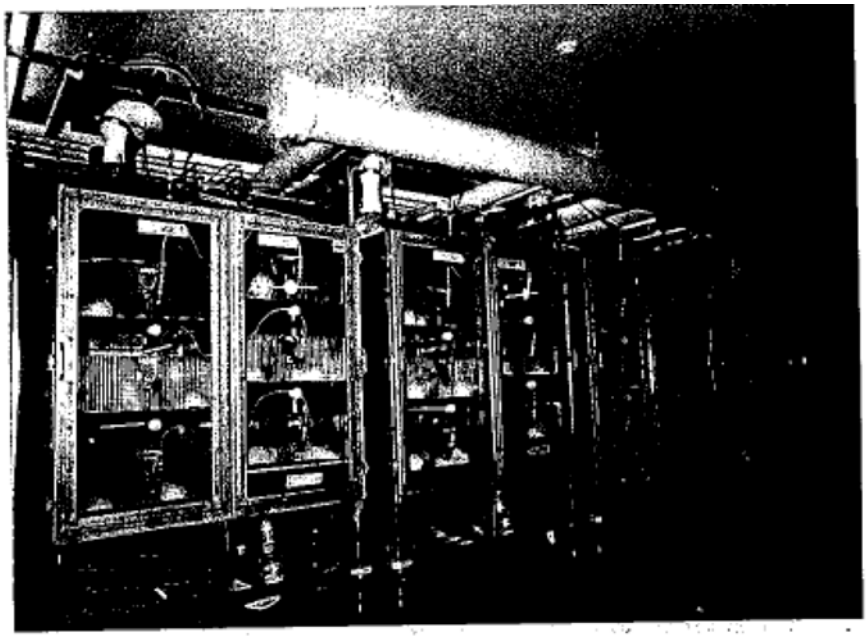


Figure 2: Inhalation Laboratory (Room B-201) Floor Plan



Photograph 3: Inhalation Laboratory Interior

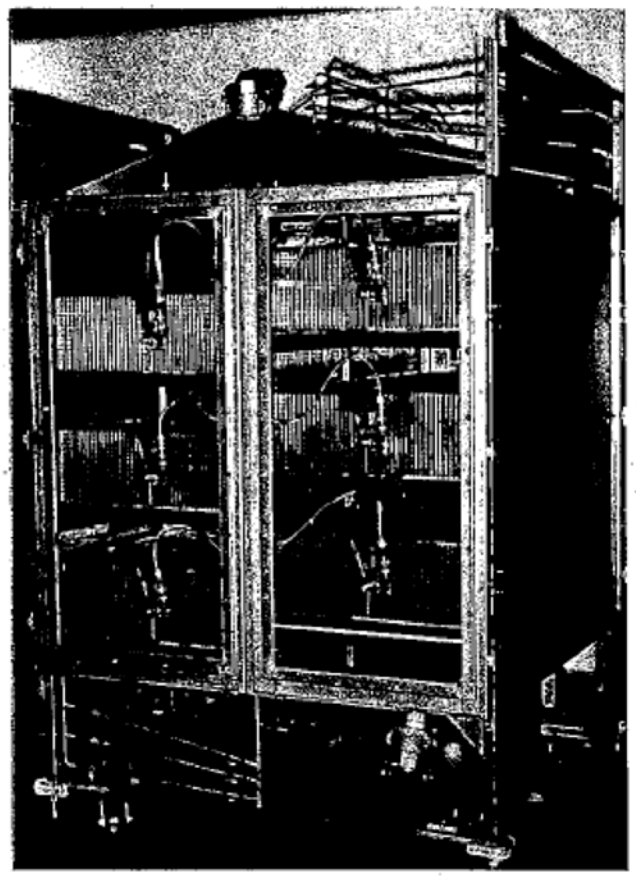
3.4.2 Exposure Chamber

Four units of systemic exposure chamber (Hazleton 1000 inhalation exposure chamber) made by Hazleton Systems, designed for inhalation study were used. The chamber is almost cubical, and the top and bottom are shaped like pyramid and connected to supply air piping and exhaust air piping, respectively. These ducts are equipped with quick joints and can be easily installed or removed. The frame of chamber is made of stainless steel. Two walls are also made of stainless steel, and the other two are glass doors. The glass doors are double doors, and are fit into the stainless steel frame and secured on to the chamber by a set of latches each attached to the top and bottom of the door. The rubber packing around the door is tightly sealed with silicon rubber filler to prevent the air inside the chamber from leaking out.

The specification of the exposure chamber is as follows:

Manufacturer:	Hazleton (USA)
Style:	Hazleton 1000 Inhalation Exposure Chamber
Size:	L132 x W127 x H206 cm
Capacity:	Approximately 2.5 m^3
Weight:	408 kg (chamber alone)
	544 kg (with inhalation cage and catch pan attached)
Material:	Stainless steel (with glass doors)
Sealing:	Silicon rubber filler
Air supply/ exhaust vent:	7.6 cm diameter
Drainage hole:	7.6 cm diameter

The exterior of the exposure chamber is shown in Photograph 4.



Photograph 4: Exposure Chamber Exterior

The following modifications were made, since it was not convenient to use the exposure chamber out of the box due to the fact that it was manufactured in a foreign country and was also missing specifications for accompanying air conditioning unit:

(a) Created a base for attaching thermohygrometer
(b) Installed an opening for thermometric (thermocouple) detection
(c) Installed an opening for detecting differential pressure measurement inside the chamber
(d) Closed the backup opening for sampling (silicon rubber stopper)
(e) Installed handles for moving the chamber
(f) Remodeled the drainage pipe
(g) Upgraded the strainers for the drainage hole and exhaust vent
(h) Installed a ball valve for automatic water supply
(i) Created an air supply dispersion plate (stainless steel) for inhalation chamber for mice (not used in this rat study)

3.4.3 Procedure to Generate Methanol Gas

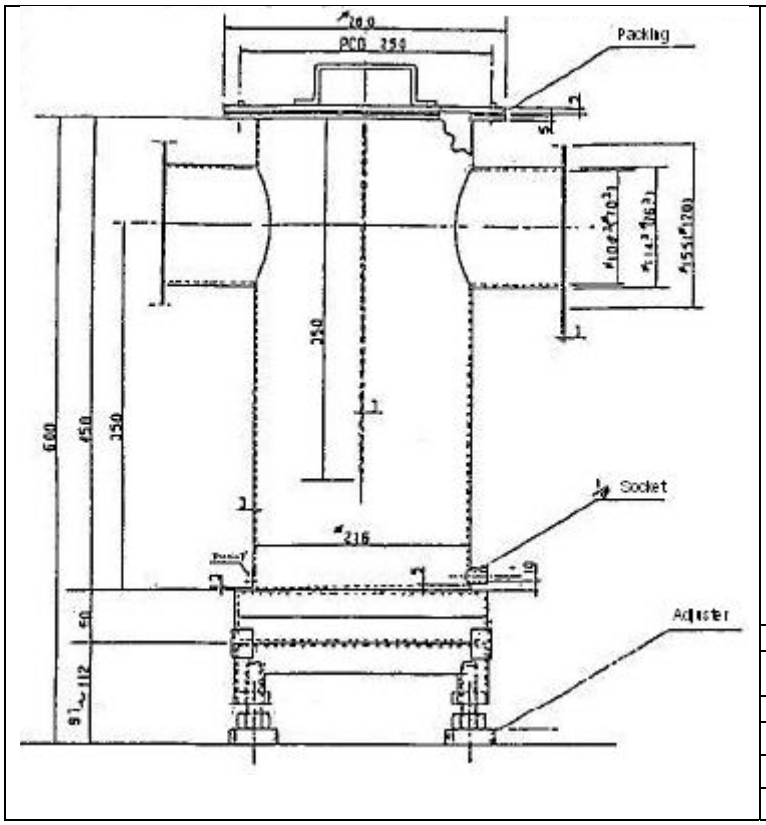
Methanol is highly volatile liquid having boiling point of 64.5 ºC and vapor pressure at 20 ºC of 96 mmHg. The weight of methanol in one cubic meter air having 1 ppm concentration is 1,310 mg at 25 ºC ,760 mmHg.

Based on above, when an inhalation chamber having approximately 2.5 m^3 capacity receives 1000 ppm of methanol gas and ventilation frequency is set to 30 times, 98.25 grams (124.2 ml based on 0.791 g/ mol density) of methanol gas will be emitted in one hour. The rate of methanol gas emission depends primarily on vapor pressure of methanol; and the air flow rate, the temperature of air flow, and the vapor area may possibly be the factors influencing the vapor pressure of methanol.

In order to understand the relationship between these factors, a model study was designed to obtain the methanol evaporation rate per unit vapor area (cm^3) and unit air flow rate (m^2), and the outcome was used as fundamental data to design a carburetor for methanol gas generation.

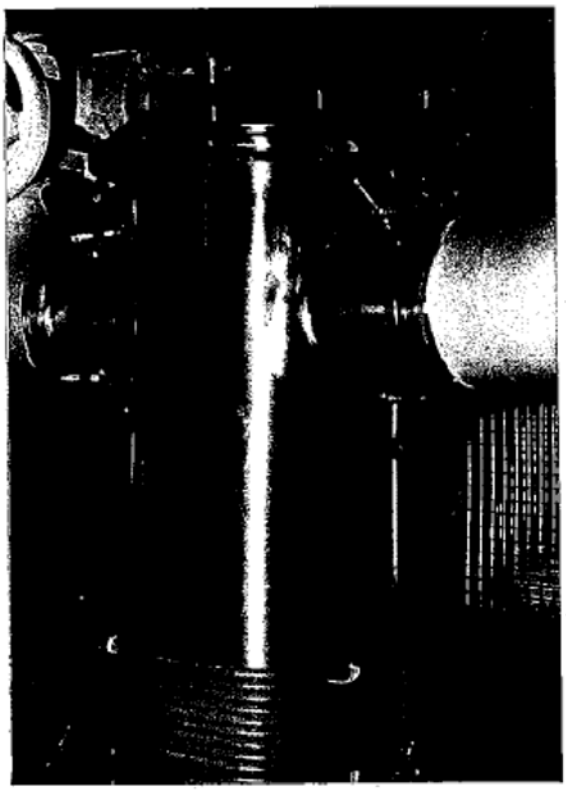
Initially, a horizontal model of methanol carburetor, which would sit parallel to the piping, was considered; however, a vertical model with more suitable features, such as a wider evaporation area and a simpler heating method was adopted. The final structure of carburetor is as shown in Figure 3 and Photograph 5.

The evaporation area is 366 cm^3 and the linear speed within the piping is 2.8 cm/ sec (when the flow rate is 80 m^2 /hr)



[illegible]	Name						
To: Mitsubishi Kasei Toxicological and Environmental Sciences Ltd.							
Product Name	Methanol - carburetor						
Unit	m/m	Scale	1/3				m.o
	S57.9			No.	TK-1047		
Tokiwa Kagaku Kikai K.K.							

Figure 3: Methanol Carburetor Blueprint



Photograph 5: Methanol Carburetor

3.4.4 Method to Control Methanol Gas

Figure 4 shows methanol gas generation control system.

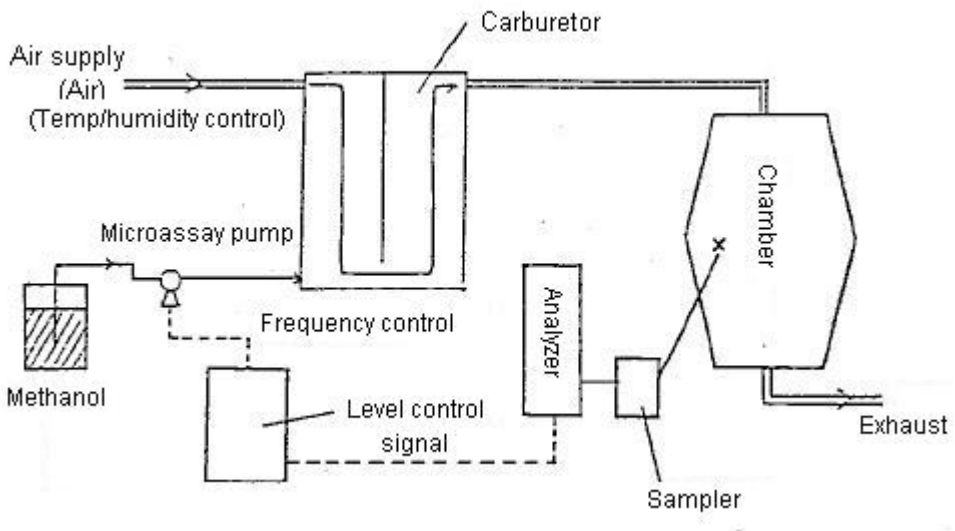
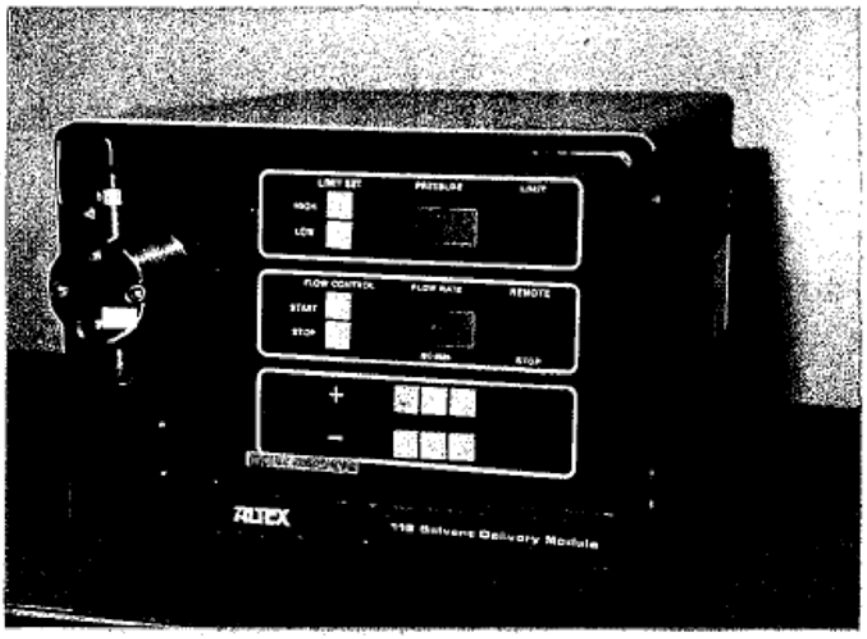


Figure 4: Methanol Gas Generation Control System

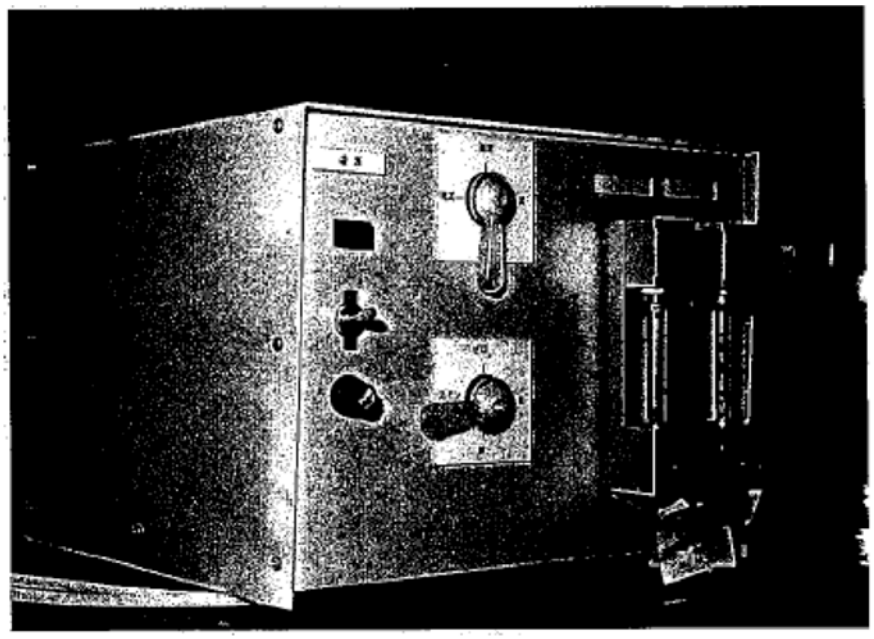
The outdoor air passes through a medium performance filter, a high performance filter, and an activated charcoal filter, and on the way to the chamber, it enters the carburetor (Photograph 5) after the temperature and humidity are adjusted. Liquid methanol is sent to the carburetor through a flow volume regulated micro-pump (Photograph 6), and is introduced to the chamber after being entirely vaporized by the outdoor air. The chamber has a Teflon pipe for air sampling (0.6/ 4 mm diameter) that extracts 1.0 liter per minute of air inside the chamber using the methanol gas sampler (Photograph 7) and sends to methanol gas analyzer.

The methanol gas analyzer (Photograph 8) output a signal that feeds back the deviation from the set value to automatically control the level as well as record the actual measurement of the level of methanol gas within the chamber. In response to the output signal, the methanol gas level controller (Photograph 9) sends the deviation from the set value as a control signal to the flow volume regulated micro-pump, and the pump adjusts the amount of liquid to be sent to maintain a constant level of methanol gas within the chamber.

The level of methanol gas within each chamber was recorded by a recorder incorporated into the methanol gas generator control panel (Photograph 10).

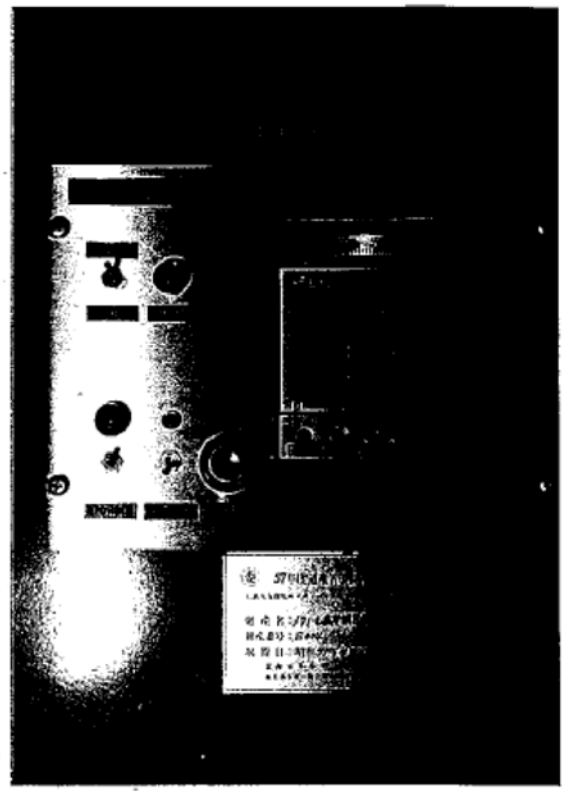


Photograph 6: Microassay Pump

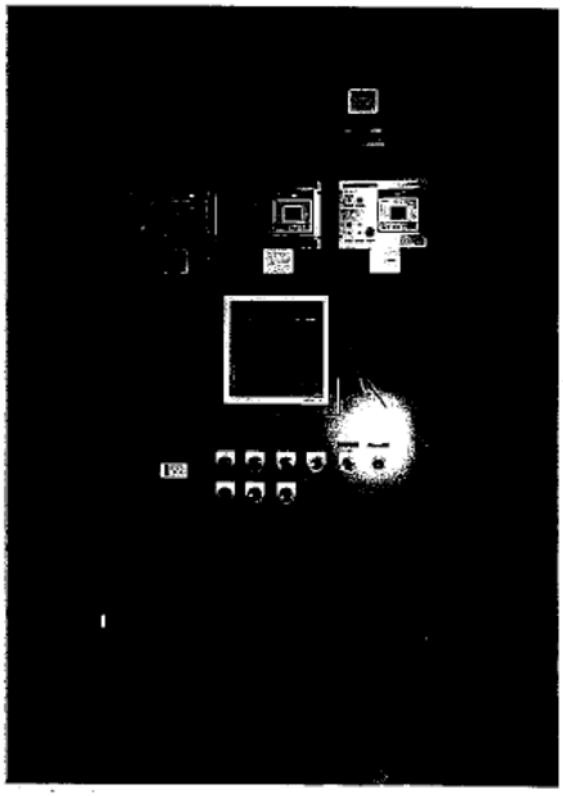


Photograph 7: Methanol Gas Sampler for Analysis

Photograph 8: Methanol Gas Analyzer



Photograph 9: Methanol Gas Level Controller



Photograph 10:
Methanol Gas Generator Control Panel

3.4.5 Distribution of the Level of Methanol Gas inside the Exposure Chamber

For all inhalation exposure chambers, the air inside the chamber was sampled from 23 locations per chamber prior to animal exposure and the level of methanol gas was checked. As a result, with or without inhalation cage or tray, all locations showed almost equal values. Therefore, the level of methanol gas within the chamber is believed to be evenly distributed.

3.4.6 Maintenance and Inspection of the Facility

In principle, the long-term inhalation study facility is operated 24 hours a day for two years, it is crucial to select a durable and reliable facility. At the same time, however, it is important to perform routine maintenance check to understand the contributing factor of failure and fix the problem in advance. For this reason, the following steps were taken:

(1) Daily inspection
Operating condition of long-term inhalation study facility (inside the inhalation laboratory) was recorded and daily inspection (outside the inhalation laboratory) was performed everyday, in principle, before and after operation in accordance with the inspection charts. (See Support Document 4)

(2) Periodically replace air blowers and exhausters
No. 1 and No. 2 air blowers and exhausters were exchanged every month.

(3) Clean exhaust cleaner fillers

(4) Calibrate methanol gas analyzer
As a general rule, calibration was performed once a month.

(5) Stock spares for instruments and equipments

(6) Conclude an annual contract with a contractor (Yamatake Corporation) to maintain the automatic controller for the air conditioning system
- Overall check ---------- Once a year before the summer term
- Simplified check -------- Once a year before the end of summer term
- Emergency call

3.4.7 Safety Measures

All possible measures must be taken to ensure that daily operational condition is recorded and surveillance system for emergency is maintained in order for the facility to be acceptable for long-term inhalation study of methanol in terms of successful experiment as well as health and safety. For this reason, the following steps were taken:

(1) TV monitor surveillance

The inhalation laboratory (Animal Wing B, Room B-201) is located within a barrier system, which requires relatively long time to prepare for entry, and emergency measures cannot be taken easily. For this reason, a TV camera for monitoring was installed in each room to quickly grasp the condition inside the barrier system from outside, in case of unexpected trouble.

(2) Emergency alarm/ notification system

Since consecutive exposure is the premise, the emergency system was prepared in a way that, in case of a trouble (such as increase in chamber temperature, air supply/ exhaust system failure, power distribution board failure) during night time or on holidays, the lamp would turn on and the buzzer would go off on the monitoring panel of the long-term methanol inhalation system, and at the same time, a monitoring panel with an alarm lamp and buzzer capability was installed in the guard station so that guards can immediately notify people in charge.

(3) Installation of automatic shutoff mechanism for inhalation study facility

If trouble occurs during night time or on holidays, it would take considerably long time for people in charge to arrive at the site to take actions, and elevation in methanol gas level may possibly invoke fire or explosion and interfere with the study.

For this reason, the automatic shutoff mechanism of the methanol gas generator and air supply/ exhaust system was set up to actuate, if necessary.

Automatic shutoff of methanol gas generator

① When the pressure inside the chamber becomes higher than that of inhalation laboratory

② When the air supply/ exhaust system shuts down

Automatic shutoff of air supply/ exhaust system

① Power distribution board failure

② Abnormal elevation of air supply temperature

(4) Installation of chemical cartridge respirators

Following two types of chemical cartridge respirators were placed near entry of inhalation laboratory to be used when supplying methanol into the methanol tank adjunct to the methanol gas generator, or to prevent inhalation of methanol gas in case of gas leakage.

- Chin-style, compact chemical cartridge respirators (for low level gas, 1000 ppm)

  Supplied to everyone, and the weight was checked when purchased as well as at the beginning of every month. It was replaced with a new one if the weight was 15 % more than the time of purchase, or if it has been used for three months.

  The cartridge is for organic gas, and the main ingredient is activated charcoal.
- Chin-style chemical cartridge respirators (for medium level gas, 10000 ppm)

  One respirator was supplied per room. This respirator was supplied to be used inside the room if the level of methanol became temporarily high due to leaking or other reasons.

3.5 Environmental Condition of Animal Care and Management

3.5.1 Inhalation Laboratory Environment

Laboratory:	Animal Wing B, Room B-201
Temperature:	22 ± 2 ºC
Humidity:	55 ± 15 %
Ventilation:	10-15 times per hour. All fresh air system using an activated charcoal filter and an absolute filter
Lighting:	7:00 am – 7:00 pm (light: 12 hours; dark: 12 hours)

The condition of Room B-214, which is used for acclimation and quarantine, is the same as above, and both rooms are within the barrier system.

3.5.2 Environment inside the Exposure Chamber

Temperature:	23 ± 3 ºC
Humidity:	50 ± 10 %
Ventilation:	30-32 times per hour. All fresh air system using an activated charcoal filter and an absolute filter
Air flow rate:	3-17 cm/ sec. (except for the section along the surface of chamber wall)
Odor:	Ammonium level of 3 ppm or less
Noise:	55 phons
Cleanliness:	13-90 pieces of 5 µm or smaller particles per cubic feet

The internal temperature and humidity of exposure chamber were continuously recorded. For the entire duration of the study, the temperature and humidity were well maintained almost within set values; however, the temperature and the humidity both exceed their upper limits (26 ºC and 60 %, respectively) for a short period of time. The length of time was too short to affect the study.

Support Document 5 shows an example of the case above.

3.5.3 Cages

(1) Polycarbonate resin cages

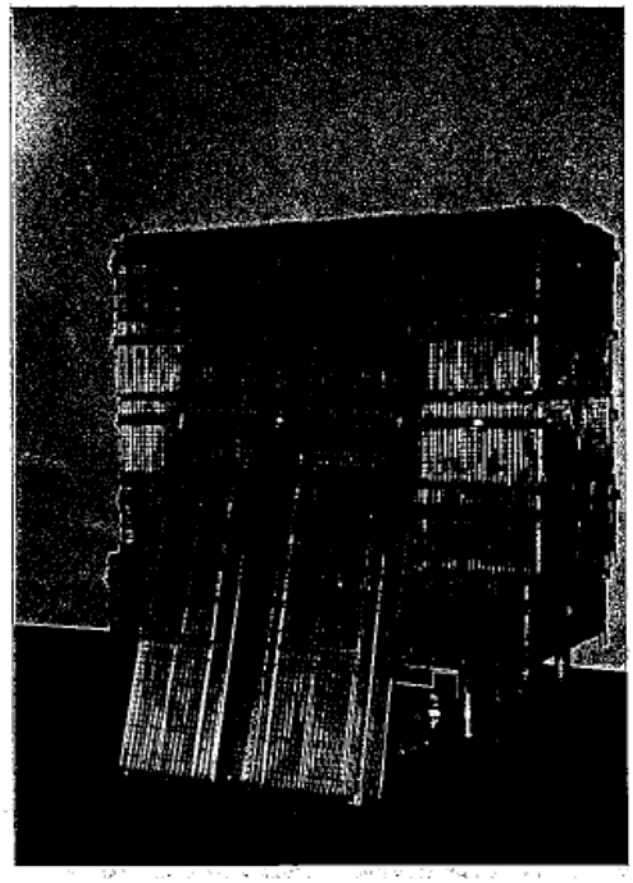
Utilized during quarantine and acclimation period (3 rats/ cage; substrate was used)

Model:	TR-PC-200
Size:	W280 x D 440 x H 200 mm
Manufacturer:	Tokiwa Kagaku Kikai K.K.
Substrate:	Beta-chip (Charles River Japan, Inc.)

(2) Inhalation cages

Wire mesh cages attached to exposure chamber were used.

Model:	F24
Size:	L1194 x W583 x H 198 mm (24 cages: one cage is L279 x W98 x H 198 mm)
Area:	6961 cm^3 (273 cm^3 per cage)
Material:	Stainless steel
Manufacturer:	Hazleton Systems (USA)



Photograph 11: Inhalation Exposure Cages

(3) Inhalation cage tray

The inhalation case tray was used as a catch tray while keeping the animals in the chamber.

Size:	L1194 x W583 x H 27 mm
Material:	Stainless steel

3.5.4 Animal Care and Management

Autoclave-sterilized diet for lab animals (solid diet for mice, rats and hamsters, CRF-1: Charles River Japan, Inc.) was freely available.

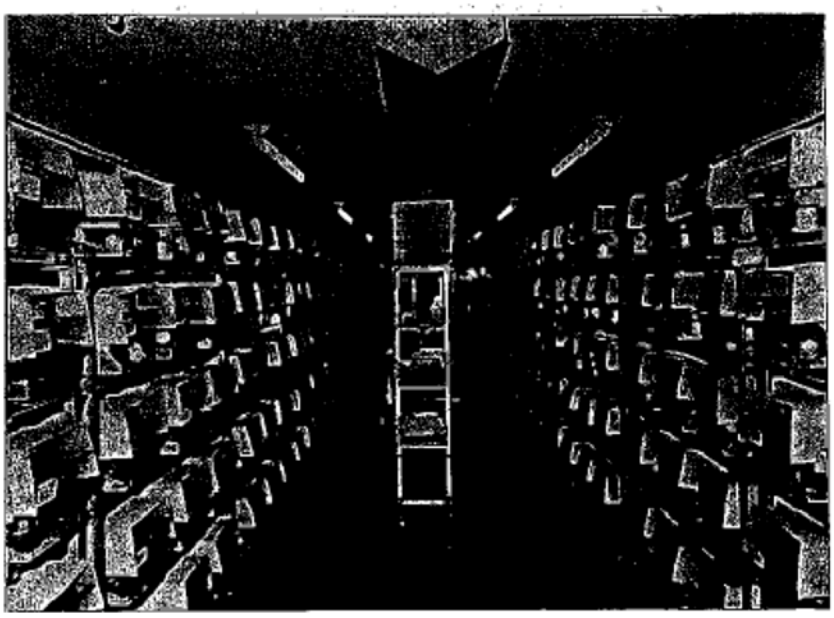
As for drinking water, tap water that has been filtered through a filter with pore size of 5 μ and then ran through ultra violet water sterilizer was freely available in an automatic feedwater feeder attached to the chamber.

All equipments used were disinfected and sterilized by either high-pressure steam sterilization, ethylene oxide gas, or chemical disinfection depending on the type of supply.

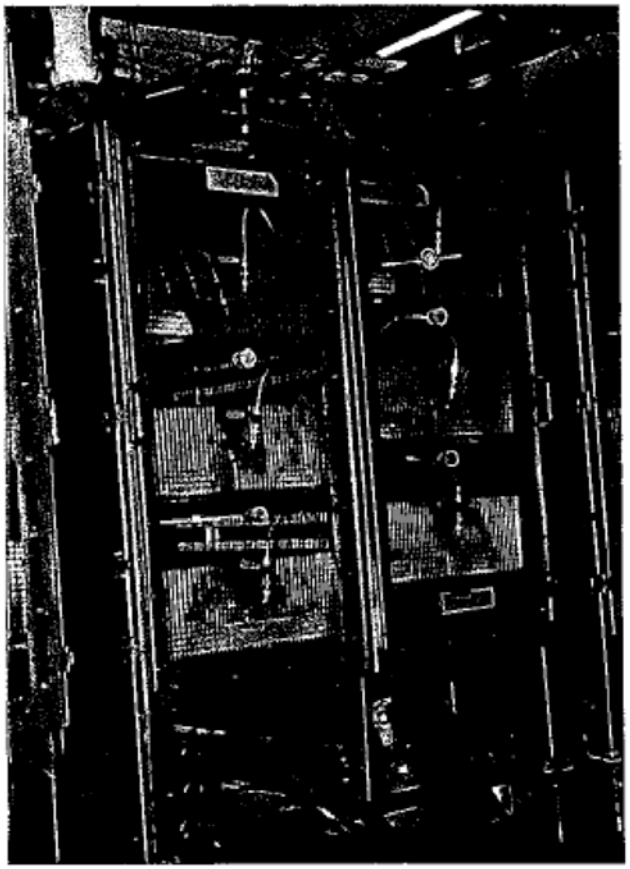
Replacement of cages, trays for inhalation cages, feeders and water feeders, as well rotation of cages were done carefully with sufficient regard to animal hygiene and with consideration to not interfere with the study.

Husbandry condition is shown in Photographs 12 and 13.

Details of animal care and management are shown in Support Document 6.



Photograph 12: Husbandry Condition during Quarantine and Acclimation



Photograph 13: Husbandry Condition inside the Inhalation Chamber

3.6 Methanol Gas Exposure Conditions

3.6.1 Levels of Exposure

Based on the result of four-week preliminary study on exposure, exposure level of high level exposure group was set at 1000 ppm as this level may slightly influence the subjects, but at the same time allows the subjects to withstanding the exposure for a long time. Using a common ratio of 10, the exposure level of the rest of the exposure groups were set to 100 ppm and 10 ppm, and another group with the exposure level of 0 ppm was created as a control group.

These levels include the acceptable TWA concentration of 200 ppm set by the *American Conference of Government Industrial Hygienists* (ACGIH) (1978).

The methanol gas level of each chamber was continuously recorded by a multipoint recorder incorporated in the methanol gas generator control panel.

3.6.2 Period of Exposure

In accordance with the guidelines on carcinogenicity test recommended by OECD, the period of exposure was set to 24 months.

3.6.3 Method of Exposure

Four units of inhalation chamber for whole body exposure each having one group consisting of male and female rats were used and pre-grouped rats were individually put into an inhalation cage. Except for routine operations such as observation and checkup, the rats were continuously exposed to methanol gas.

Care was taken to maintain the duration of exposure to be almost equal across the groups including the control group.

The methanol gas analyzer was calibrated prior to the commencement of study using standard gas, and after the study has begun, it was calibrated once a month or more.

3.7 Group Constitution and Animal Identification

3.7.1 Group Constitution

Each group consisted of 52 male and 52 female rats. Since the present study is a concurrent study with chronic toxicological study, 72 male and 72 female rats, including the rats mentioned above, were placed within a chamber and exposed to methanol.

The group structure is as follows:

Name of Group (Abbreviation)	Methanol Level	Number of Rats	
		Male	Female
Control group (A)	0 ppm	52	52
Low level exposure group (B)	10 ppm	52	52
Medium level exposure group (C)	100 ppm	52	52
High level exposure group (D)	1000 ppm	52	52

Besides above, 14 male and 14 female rats from the same lot were kept separately for monitoring outside of inhalation chamber in a polycarbonate resin cage, and were subjected to various checkups, as necessary.

For convenience, these rats were called untreated rats and a part of data is cited within this report.

3.7.2 Grouping and Individual Identification Method

After completion of quarantine and acclimation, healthy rats, except for unusually heavy or light weight ones were grouped randomly using a stratified random sampling method having weight as the stratification factor.

Ear punch was used to identify group and individual ID number, and a tape that was color-coded by groups was applied to each cage with a cage number written on it.

3.8 Observation and Test Items

3.8.1 Observation of General Conditions

For the entire length of administration period, all the changes, including in appearance and behavior, were observed and recorded once a day or more, five to six days a week, in principle.

Detailed observation was done twice a week and the rest of the time was focused on checking for abnormality, necessity to kill *in extremis*, and whether the rats are surviving or not.

The observation was performed more often if the study director decided it is necessary.

Furthermore, autopsy was performed as soon as possible on dead or dying rats to investigate probable cause of death, and the internal organs were stored for histopathological examination.

3.8.2 Body Weight Check

Using an electronic balance (ED-4000-10 by Shimadzu Corporation), the body weight was checked on all cases once a week, as a general rule. Taking circadian change of body weight into consideration, the body weight check was performed almost at a regular time in the morning. Additionally, the amount of weight gain was calculated in reference to the body weight at the commencement of exposure.

3.8.3 Food Consumption Check

For 24 male and 24 female rats of each group, daily food consumption of one rat was calculate based on the weekly food consumption of two rats.

The consumption rate was checked every week up to Week 13 of the exposure and then once a month thereafter, in principle.

The electronic balance mentioned above was used for the measurement.

]

3.8.4 Laboratory Tests

The following tests were conducted on the final day of sacrifice or on a day close to the sacrifice after completion of exposure. For cases found dead or killed *in extremis*, the tests were performed in the same manner as much as possible.

(1) Urinalysis

Fresh urine was extracted from about 20 cases each of male and female rats from each group on a day close to the sacrifice, and subjected immediately to assay by an urinalysis paper (Clinitek-II by Miles Sankyo) to check the level of following components:

(a) pH
(b) Protein
(c) Glucose
(d) Ketone body
(e) Bilirubin
(f) Occult blood
(g) Urobilinogen

(2) Hematological Test

During autopsy, blood was collected from abdominal postcava under anesthesia induced by intraperitoneal administration of pentobarbital (Somnopentyl by Pitman-Moore Pharmaceuticals) for all cases, and a part of the blood was placed in a test tube that had been treated with anticoagulant (EDTA-2K) and mixed well and served for checking following components:

(a) Number of red blood cells: Automatic blood cell counter (ELT-8: Ortho Instruments Corporation)
(b) Number of white blood cells: Automatic blood cell counter (ELT-8: Ortho Instruments Corporation)
(c) Hemoglobin level: Automatic blood cell counter (ELT-8: Ortho Instruments Corporation)
(d) Number of platelets: Automatic blood cell counter (ELT-8: Ortho Instruments Corporation)
(e) Hematocrit level: Capillary centrifuge method (11000 rpm, 5-minute centrifuge: Kubota Corporation's centrifuge was used)

In addition, the following items were calculated based on the value of (a), (c), and (e) above:

(f) Mean corpuscular volume (MCV)
(g) Mean corpuscular hemoglobin (MCH)
(h) Mean corpuscular hemoglobin concentration (MCHC)

Furthermore, blood samples were created by smearing a part of the blood on a glass slide and stained by May-Giemsa staining method (Pappenheim method). 100 white blood cells were microscopically examined to obtain differential leukocyte count.

(3) Serum Biochemistry Test

For about 20 cases each of male and female rats from each group, the remaining blood from the hematological test was left in a room temperature (22 ± 2 ºC) for about 30 minutes to solidify it, and then placed in a centrifuge (3000 rpm for 10 minutes) to obtain serum. The serum was stored frozen (-20 ºC) until the test. The level of following items was checked within two weeks after collection:

(a) GOT: Modified *Henry*'s method
(b) GPT: *Wroblewski*'s method
(c) LDH: *Wacker* method
(d) γ-GTP: *Szasy* method
(e) ALP: Modified *Bessey-Lowery* method
(f) Total cholesterol: *Allain*'s method
(g) Triglyceride: Modified *Bucolo* and *David* method
(h) Free fatty acid: UV method (absorption wavelength: 340, 380 nm)
(i) Phosphatide: Molybdenum *blue* method

(j) Glucose: *Hexokinase-G6PDH* method
(k) Total protein: *Biuret* method
(l) A/ G ratio: Cellulose acetate electrophoresis
(m) Urea nitrogen: *Urease-GLDH* method
(n) Calcium: Modified *Connerty* and *Briggs*'s method
(o) Inorganic phosphorus: Molybdenum *blue* method
(p) Sodium: Flame photometer
(q) Potassium: Flame photometer
(r) Chloride: Silver coulometer

The following measuring instruments were used for the testing above:

(a)-(k) and (m)-(o):	Automatic serum analyzer (Abbott-VP by Abbott Laboratories)
(l):	Electrophoresis unit (Fuji-Riken) Densitometer (Densitron 20M b Jokoh)
(p), (q):	Fully automatic flame photometer, model-30C (Nippon Bunko Medical)
(r):	*Chloride Meter 920* (Corning)

3.8.5 Pathological Tests

(1) Pathological Autopsy

Autopsy was performed on all cases including the ones that were found dead or killed *in extremis* to search for gross lesion in each organ.

(2) Organ Weight Check

For the rats that were killed on schedule on Week 104, the wet weight of the following organs were checked using an electronic balance for weighing organ weight (AEL-160 by Shimadzu Corporation). Furthermore, the organ weight to body weight ratio was calculated based on the body weight checked at the time of sacrifice or on a day close to the sacrifice:

Brain, pituitary gland, thyroid gland, thymus gland, heart, lung, liver, kidneys, spleen, adrenal gland, testis, and ovaries

(3) Histopathological Study

For all cases, the organs below were isolated and fixed in 10 % neutral formalin with phosphate buffer. Following the routine procedure, the organs were embedded in paraffin, sliced thinly and dyed with hematoxylin-eosin (HE) stain to prepare slide, and subjected to histopathological study.

The histopathological test was performed on the cases killed on schedule on Week 104 among the control group and the high level exposure group (1000 ppm) as well as the cases that were found dead/ killed *in extremis* of all the groups. Furthermore, for male and female kidneys, male lungs as well as female adrenal glands in the high level exposure group (1000 ppm) that demonstrated significant difference in incidence of lesions as a result of the test above, the corresponding organs were tested for the low level exposure group (10 ppm) and the medium level exposure group (100 ppm).

Brain, pituitary gland, thyroid gland, heart, lung, bronchi, thymus gland, liver, kidneys, spleen, pancreas, adrenal gland, testis, epididymis, prostate, vesicular gland, preputial glands, ovaries, uterus, vagina, bladder, esophagus, stomach, duodenum, jejunum, ileum, appendix, colon, rectum, lymph nodes (submandibular, hilar, and mesenteric), salivary glands (subaural, submandibular, and sublinqual), perilacrimal gland, eyeballs, optic nerve, harderian gland, ischiadic nerve, muscles (femoral muscle), femur, bone marrow (femur), nasal cavity, laryngopharynx, spinal cord, mammary gland, skin, thoracic aorta, and other locations of lesion

3.8.6 Antibody Test on Pathogenic Microorganisms

As a general rule, blood was collected from caudal vein of one male and one female rat from each group every four months, and the blood was checked for serum antibody to search for infection.

The blood was tested for the following pathogenic microorganisms:

- Mouse hepatitis virus (*JHM*)
- *Sendai virus (HVJ)*
- *Mouse adenovirus*
- *Mycoplasma pulmonis*
- *Bacillus piliformis (Tyzzer)*
- *Salmonella typhimurium*
- *Corynebacterium kutscheri*
- *Ectromelia virus*

3.8.7 Blood Methanol Level Check

Methanol level of the blood that was collected during scheduled sacrifice on Week 104 was checked (test was outsourced to Industrial Research Institute of Japan). The analysis method and conditions are as follows:

Method of analysis

GC headspace method

GC-7A (Shimadzu Corporation)

HSS-1A (Shimadzu Corporation)

Conditions of analysis

Column:	*Polapak QS*, 40-80 mesh
Column temperature:	125 ºC
Carrier gas:	N_2, 50 ml/ min.
Detector:	FID

3.9 Data Assessment Method

3.9.1 Data Processing Method

The measured data recorded in floppy disk or paper tape were ultimately input and stored in the host computer, and data processing such as calculations of average values, standard deviations, and significant difference as well as tabulation and so forth was performed.

3.9.2 Statistical Analysis

The data, except for abnormal data due to clearly artificial mistake or erroneous operation of testers and so forth, was assessed for statistically significant difference between the control group and the inhalation exposure groups.

Student's t-test was conduced if no significant difference was observed in dispersion among the groups (if the result of F test is $P > 0.05$), and Welch's t-test when significant difference was observed.

Beside above, the following methods of assay were also used:

Armitage's Chi-square test:	Urinalysis Incidences of gross lesions as well as nonneoplastic lesions
Fisher's exact probability test:	Death rate and incidence of neoplastic lesions

4. Results

The rats were purchased on September 3, 1982, and after one week of quarantine and acclimation in polycarbonate resin cages, they were acclimated inside the chambers from September 10, and methanol exposure was initiated on September 16.

Two years later, the male rats were killed on schedule between September 18 and 21, 1984 and the female rats between September 25 and 28.

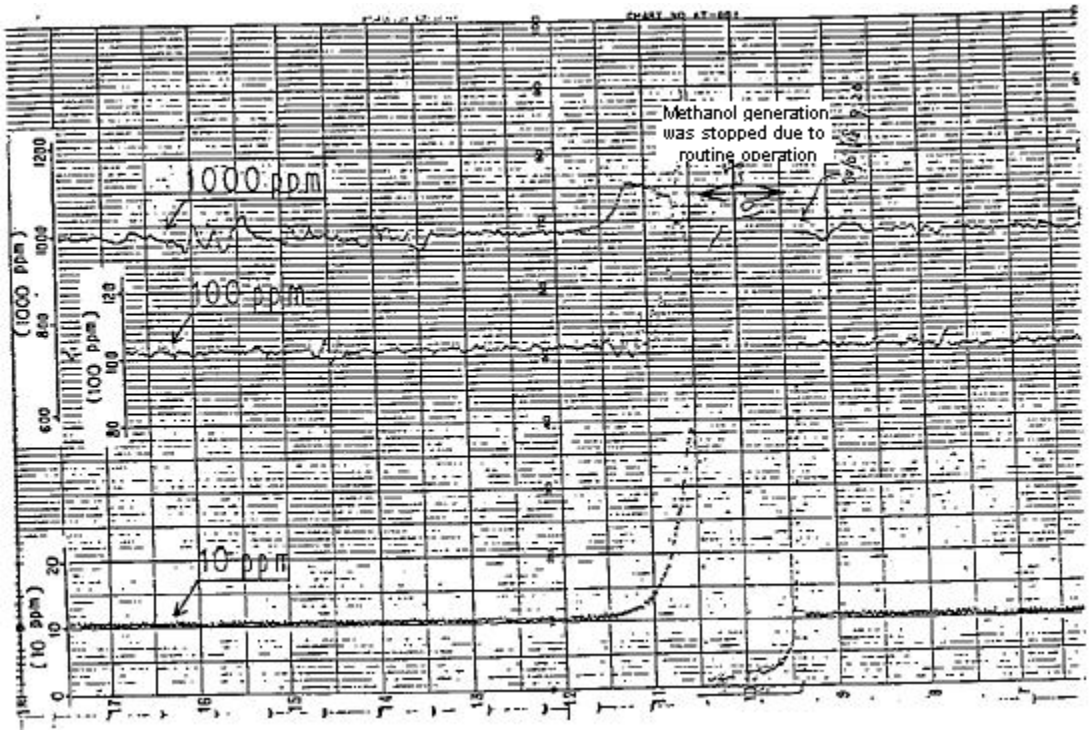
4.1 Conditions of Exposure to Methanol Gas

4.1.1 Levels of Methanol Gas Exposure

Figure 5 shows an example of controlling the level of exposure to methanol gas.

As shown in the graph, the fluctuation in the level of methanol gas was extremely small for all levels throughout the study, and the methanol level was controlled well.

On the other hand, according to the result of calibration performed once a month on methanol gas analyzer, each analyzer showed some level of variation; however, since the fluctuation in one month was less than 5 %, the methanol level was believed to be controlled well.



(Recorded on September 18, 1984)

Figure 5: Methanol Gas Generation Status

4.1.2 Duration of Methanol Gas Exposure

The duration of exposure was calculated based on the records of methanol gas levels as well as the start/ stop time of methanol generation recorded in the charts of operating conditions of inhalation laboratory facility, which is found in the inhalation laboratory.

The table below shows the total duration of exposure from the beginning of exposure in September 16, 1982 until the end of exposure, which is 24 months later. For reasons of autopsy processing capability, the female rats were dissected one week after the male rats. Furthermore, since autopsy on both male and female rats extended across several days, the duration of exposure differs slightly; however, in view of the whole duration of exposure, the difference is small enough to be ignored.

Average duration of exposure per day was 19.5 hours for both male and female rats.

Duration of Methanol Gas Exposure

	Male	Female
Total days of exposure	733 – 736 days	740 – 743 days
Total duration of exposure	14255 hrs 05 min – 14323 hrs 13 min	14407 hrs 10 min – 14468 hrs 16 min
Average daily exposure	19 hrs 27 min/ day – 19 hrs 28 min/ day	19 hrs 28 min./ day

24 month = 728 days

4.1.3 Blood Methanol Level

For five rats per group, the blood methanol level was measured on the blood collected during the scheduled sacrifice on Week 104. The results are as follows:

Blood Methanol Level

Gender	Group Name	Exposure Level (ppm)	Number of Rats	Methanol Level (ppm)		
				Average Value	Standard Deviation	C.V. (%)
Male	Control group	0	5	3.78	0.567	15.0
	Low level exposure group	10	5	3.32	0.492	14.8
	Medium level exposure group	100	5	3.32	0.217	6.5
	High level exposure group	1000	5	12.08	2.158	17.9
Female	Control group	0	5	3.60	1.122	31.2
	Low level exposure group	10	5	3.70	0.604	16.3
	Medium level exposure group	100	5	4.32	1.320	30.6
	High level exposure group	1000	5	8.50	1.290	15.2

C.V.: coefficient of variance

The blood methanol level of both male and female rats are noticeably higher in the high level exposure group (1000 ppm). The autopsy conduced at the time of scheduled sacrifice on Week 104, however, the focus was on pathological autopsy and hematological tests. Thus, it took time from the blood collection upon completion of exposure to methanol gas until the freeze preservation of serum and the blood alcohol level may very well not be reflected correctly due to the effect of alcohol dehydrogenase, loss of dissolved alcohol in blood and so forth.

For this reason, in order to measure the blood methanol level under the same conditions, the methanol level in the blood of rats exposed to methanol for four weeks was also checked. The blood level of methanol taken in through respiratory passage should come to equilibrium to a satisfactory extent in four weeks. The time required from the completion of exposure to methanol gas until the freeze preservation of serum was about 25 minutes, which is about one tenth of the time compared to before.

The results are shown in the table below:

Blood Methanol Level

Gender	Group Name	Exposure Level (ppm)	Number of Rats	Methanol Level (ppm)		
				Average Value	Standard Deviation	C.V. (%)
Male	Control group	0	7	4.01	0.662	16.5
	Low level exposure group	10	7	1.56	1.344	86.3
	Medium level exposure group	100	7	3.84	3.276	85.3
	High level exposure group	1000	9	53.59	14.256	26.6
Female	Control group	0	7	13.39	3.653	27.3
	Low level exposure group	10	7	6.73	2.870	42.7
	Medium level exposure group	100	7	4.34	1.115	25.7
	High level exposure group	1000	8	88.33	37.220	42.1

C.V.: coefficient of variance

Since the result of low level exposure group (10 ppm) and the medium level exposure group (100 ppm) is not much different from the control group, there is probably no methanol accumulation in the blood; however, the result of high level exposure group (1000 ppm) is significantly higher and is equivalent to blood concentration immediately after single intraperitoneal administration of about 50 mg/ kg.

4.2 Antibody Test on Pathogenic Microorganisms

The results of antibody tests performed on blood collected from preselected rats every four months were all negative. Reference should be made to attached Support Document 7 for test results.

4.3 Cases Found Dead or Killed *in Extremis*

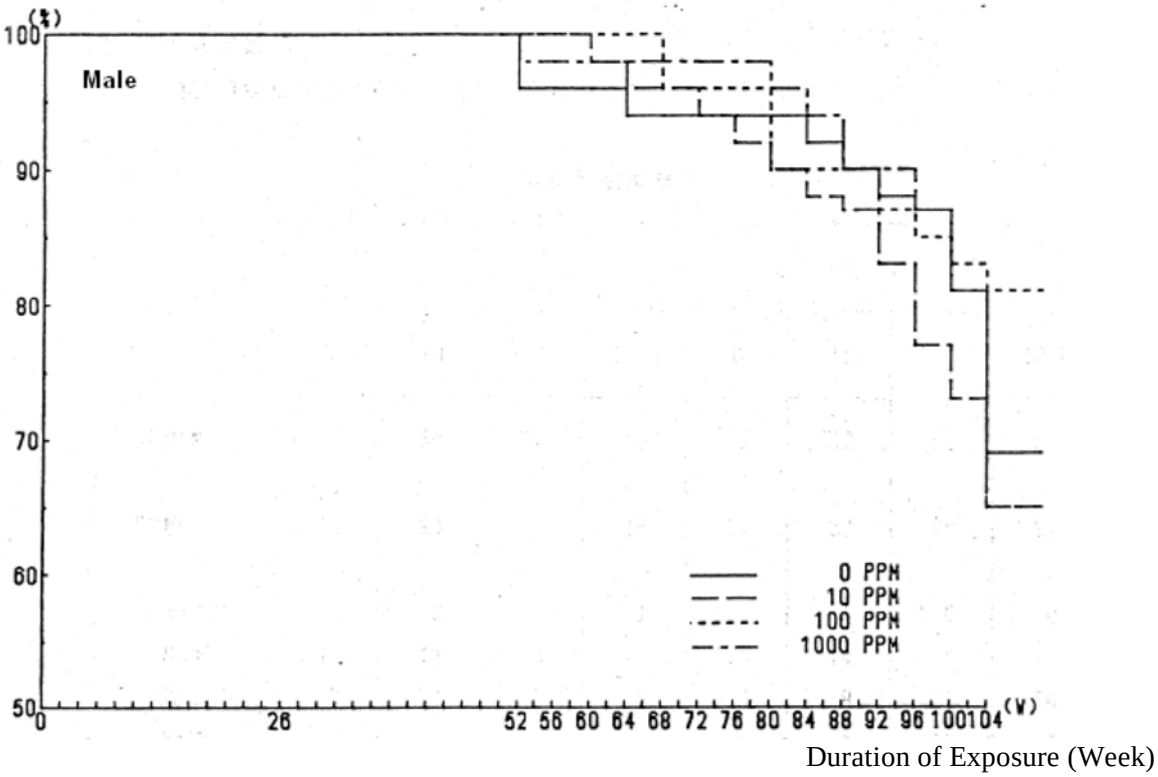
The transition of survival rate is shown in Figure 6 and *Table 1* and the mortality at the time of exposure termination is shown in the table below.

As shown in the table, the number of rats found dead or killed *in extremis*, either male or female, did not have a tendency to depend on the exposure level of methanol gas, and there was no difference between groups either.

For all groups, both male and female rats began to be found dead or killed *in extremis* about a year after the commencement of exposure; however, this period did not have a tendency to depend on the level of exposure and more than half the cases of death or sacrifice *in extremis* occurred approximately within three months following the week 90 of exposure.

Death Rate at the Time of Exposure Termination

Gender	Group Name	Exposure Level (ppm)	Death Rate (%)	Occurrence (Week)	
				Earliest Death	10 % Death
Male	Control group	0	16/52 (30.8)	49	89
	Low level exposure group	10	18/52 (34.6)	56	82
	Medium level exposure group	100	10/52 (19.2)	67	90
	High level exposure group	1000	18/52 (34.6)	49	92
Female	Control group	0	21/52 (40.4)	47	88
	Low level exposure group	10	19/52 (36.5)	78	94
	Medium level exposure group	100	21/52 (40.4)	61	87
	High level exposure group	1000	17/52 (32.7)	93	97



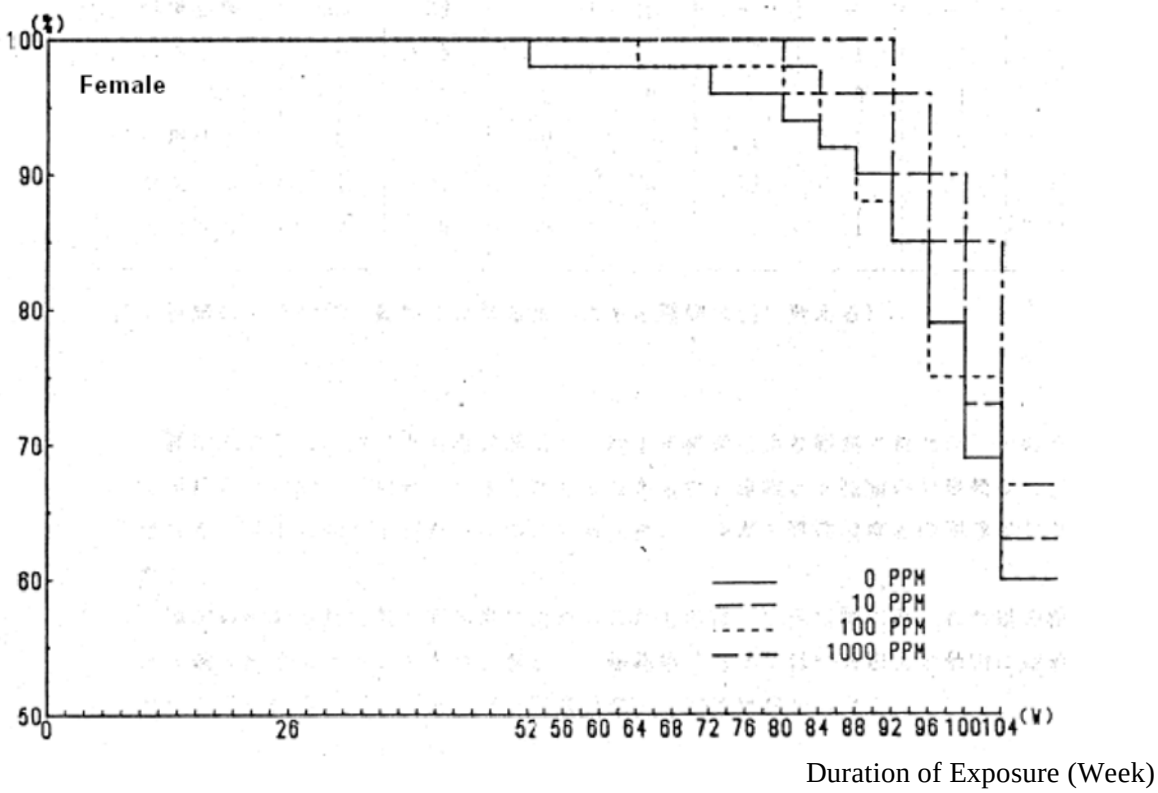


Figure 6: Survival Rate

4.4 Observation of General Conditions

Incidences of findings as a result of observation of general conditions are shown in the table below:

Result of Observation of General Conditions

Findings	Male				Female			
ppm	0	10	100	1000	0	10	100	1000
Number of cases	52	52	52	52	52	52	52	52
Tumor	16	24	20	16	17	22	26	21
Skin*	1	3	4	1	1	1	0	0
Subcutaneous	15	18	13	11	10	14	18	13
Intraperitoneal	3	4	5	5	9	9	11	10
Vaginal	-	-	-	-	0	1	0	1
Testicular atrophy	33	39	38	40	-	-	-	-
Cataract	2	5	1	1	2	1	4	5
Exophthalmos	1	0	0	0	0	0	1	1
Nanophthalmos	0	0	0	0	1	1	0	1
Hair loss	1	1	4	0	13	8	20	19
Paralysis of the hind leg	0	0	0	0	0	1	0	0

*: Location of tumor (if the same rat had multiple tumors in different locations, each tumor was counted)

The tumors developed in about a year after the beginning of exposure in both male and female rats, and more than a half of the cases developed after Week 84. Both male and female rats did not show difference in tumor development between groups, and period of incidence and location were not related to the level of exposure to methanol gas.

Most frequently observed finding besides tumor was testicular atrophy (by palpation) in male rats that were seen after Week 86, and more than 60 % of the rats in all groups showed this finding by the end of exposure period; however, the incidence had no relevance to the level of exposure to methanol gas.

From week 37, some male and female rats in all groups including the control group had soft stool or mild diarrhea. The medium level exposure group (100 ppm) and the high level exposure group (1000 ppm) developed the symptoms slight sooner than the other groups; however, since the incidences had no constant tendency in particular, the symptoms were clearly not caused by the exposure to methanol gas. The symptoms were improved by temporarily switching the mixed diet to refined diet (administered during Week 44 to 49 as well as Week 58 to 62).

Besides above, other findings that are shown in the table were seen occasionally; however, no specific findings suggest influence of exposure to methanol gas, and there was also no difference between the groups in the rate and period of incidence.

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4.5 Body Weight

The transition of average body weight is shown in Figure 7 as well as *Table 2*, and the amount of weight gain having the body weight of exposure initiation day as zero is shown in *Table 3*.

The body weight of the control group showed almost steady transition except for the measurements taken in two occasions during water supply accident: the male rats on Day 315 (Week 45) and the female rats on Day 329 (Week 47) lost an average of 4.0 to 6.4 g of body weight in one week, but they all recovered by the following week.

In the low level exposure group (10 ppm), the male rats temporarily lost an average of 7.3 g of body weight on Day 119 (Week 7) due to water leaking from the automatic water feeder. Besides this accident, the body weight of both male and female rats increased steadily, and the body weight of female rats from Day 574 thereon was about 10 g more than that of the control group, which indicated significant difference in weight gain.

The medium level exposure group (100 ppm) as well as the high level exposure group (1000 ppm) both showed a tendency of stagnant weight gain or slow weight loss from around Day 273 (Week 39) until Day 308 (Week 44), and a significant difference was observed compared to the control group; however, around that time, soft stool or mild diarrhea was observed in all groups including the control group, and the fact that the two groups had a tendency to show the symptoms slightly sooner may have caused the stagnancy in weight gain or the loss of weight. The body weight increased steadily again after changing the mixed diet to refined diet for a brief period of time. Beside above, both male and female rats of the medium level exposure group (100 ppm) showed a transition similar to that of the control group.

As for the high level exposure group (1000 ppm), 23 male rats (Animal No. 301-324) lost their body weight on Day 518 (Week 74) probably due to the water supply accident, and the group lost an average of 3.7 g of body weight. The body weight of the 23 cases recovered to about the same level as the rest of the group in two weeks. Except for the time mentioned above, the male rats showed a transition almost similar to that of the control group throughout the entire study.

The female rats showed a minor weight loss or stagnant weight gain from Day 357 (Week 51) until Day 490 (Week 70) and it was significantly different from the control group. The difference from the control group, however, was less than 4% in most cases, and the amount of weight gain did not show significant difference. The body weight and the amount of weight gain were no different from the control group from the subsequent time until the end of exposure.

For all groups including the control group, the average body weight was the heaviest in Week 70-77 of exposure in male rats and Week 96-100 in female rats. The heaviest body weight and that period did not have a tendency to depend on the level of exposure to methanol gas.

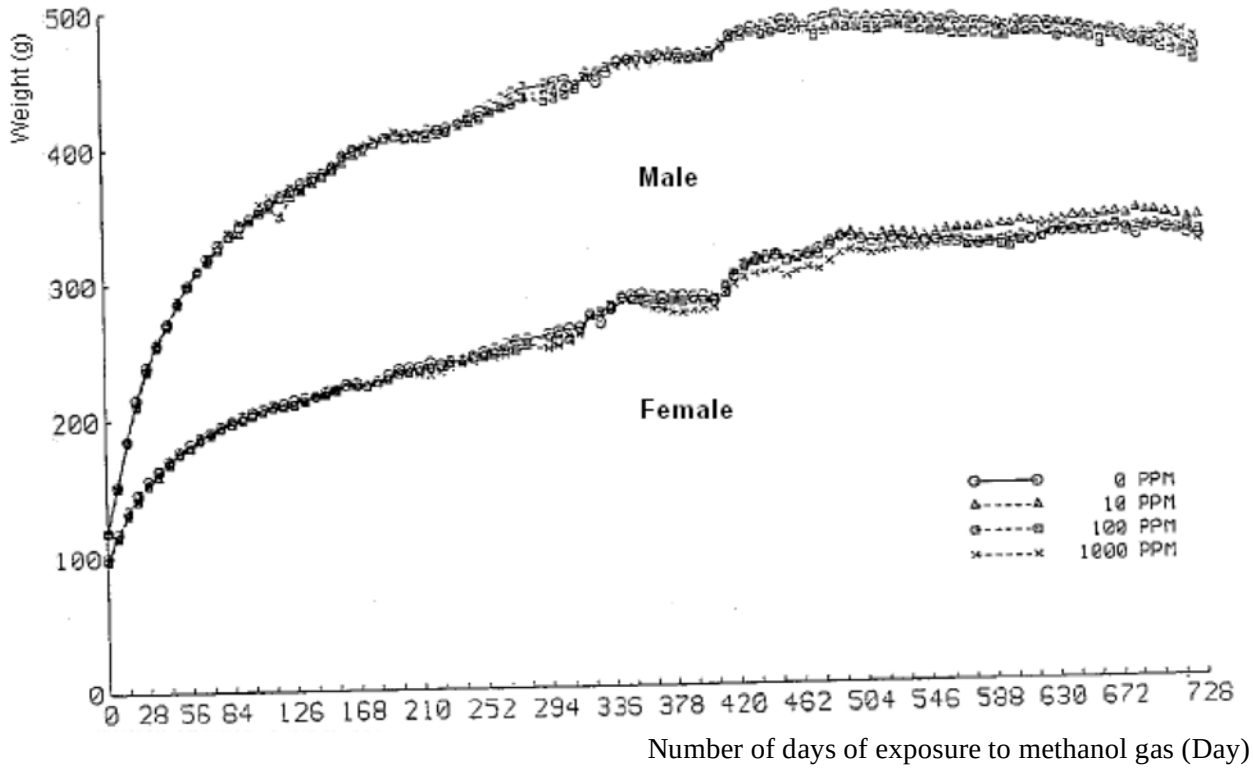

Figure 7: Transition of Average Body Weight

4.6 Food Consumption Rate

The transition of average food consumption rate per day per rat for each group is shown in Figure 8 and *Table 4*. A significant decline was observed between Day 210 (Week 30) until Day 365 (Week 52) in male rats in the high level exposure group (1000 ppm).

The male rats in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) as well as female rats in all groups exposed to methanol gas showed sporadic significant difference; however, in general, the transition was almost the same as the control group.

The consumption rate of Day 518 (Week 74) in male rats in the high level exposure group (1000 ppm) was abnormally low. Checking the corresponding body weight revealed that weight loss was observed only on cases that were subjected to consumption rate check (Animal No. 301-324; kept in the same inhalation cage), and it was speculated that only the cage in question had trouble with the automatic water feeder. Daily observation of general conditions as well as inspection of operating condition of inhalation facility, however, did not find any particular abnormality with rats or obvious accidents, and based on the status of weight loss and food consumption rate, the weight loss seemed to be temporary, which last around one day. Additionally, the consumption rate on Day 700 (Week 100) showed significant decline; however, it was due to significant decrease in consumption rate (around 11 g/ rat per day) caused by weakened cases (Animal No. 11, 21, and 19) in three cages of the high level exposure group (1000 ppm).

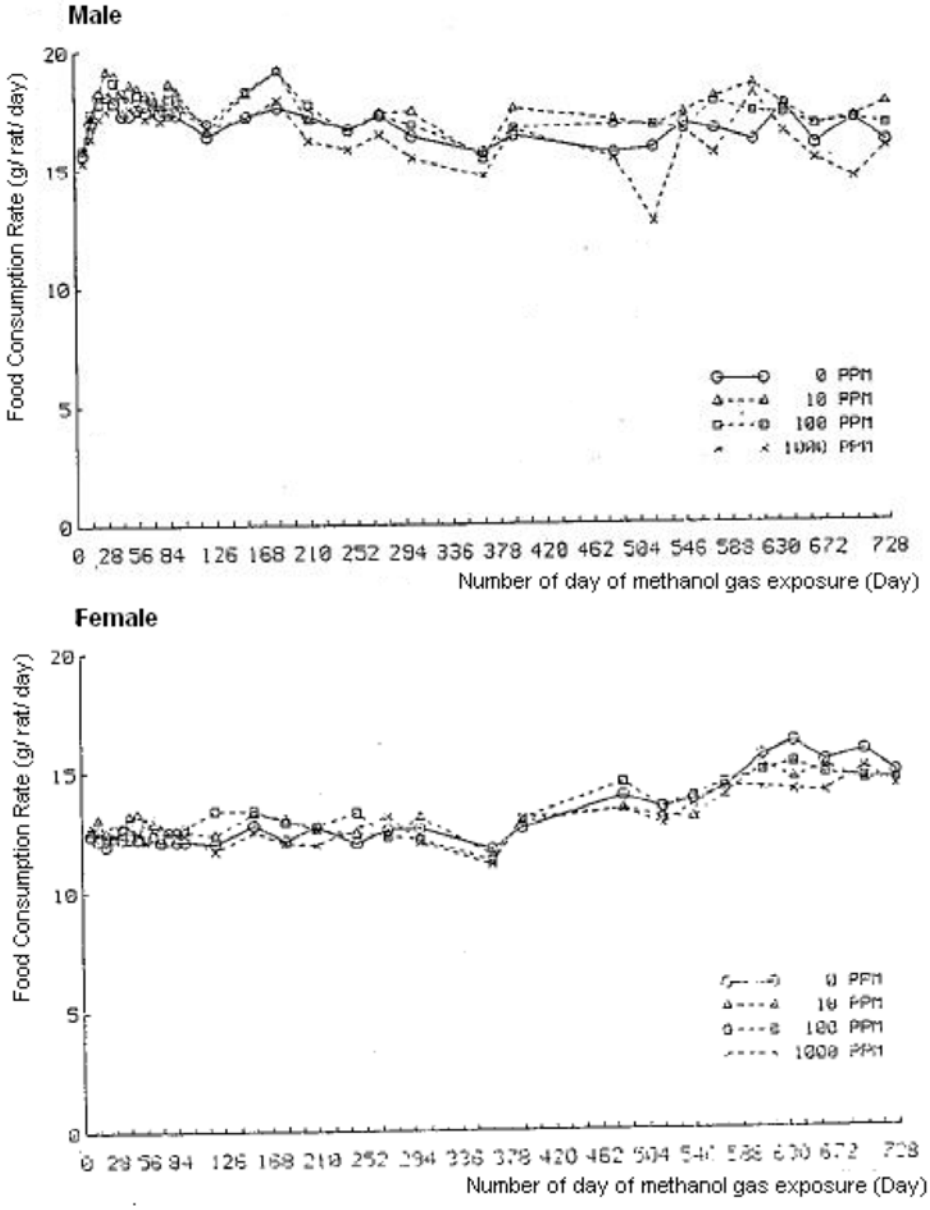

Figure 8: Average Food Consumption Rate

4.7 Urinalysis

The results are shown in *Table 5*.

The male rats in the high level exposure group (1000 ppm) showed significant increase in glucose level ($P < 0.05$).

As for female rats, the pH level had a tendency to decrease dose-dependently and the high level exposure group (1000 ppm) showed significant difference ($P < 0.05$). The level of bilirubin in female rats also had a tendency to increase dose-dependently, and the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm) showed significant difference (both $P < 0.01$).

However, when the result of urinalysis on nine untreated male rats and ten untreated female rats kept outside of chamber was checked for reference, the glucose level in male rats was not very different from that of the high level exposure group (1000 ppm): one case of negative and eight cases of 0.1 g/ dl. Furthermore, the pH and bilirubin levels in female rats were also not very far from the high level exposure group (1000 ppm) either: two cases showed pH 6 and eight cases showed pH 7; and the bilirubin levels were seven cases of negative (-), two cases of 1+ and one case of 2+.

4.8 Hematological Tests

The results are shown in *Table 6*.

4.8.1 Blood Cell Count

Red blood cell count, leukocyte cell count, hematocrit level, hemoglobin level and platelet count of both male and female rats showed no significant difference between the control group and the groups exposed to methanol gas.

Leukocyte cell count increased significantly in one male in the medium level exposure group (100 ppm) and another in the high level exposure group (1000 ppm) (Animal No. 237 and 347) to the point where it exceeded the detection limit ($850 \times 10^2 / mm^3$) of the counter. Autopsy on these two cases revealed significantly enlarged spleen, and the Animal No. 347 was diagnosed as mononuclear cell leukemia as a result of histopathological study. The other case was not subjected to histopathological test, but was suspected to have leukemia as well.

The red blood cell index values of both male and female rats did not show difference between the groups.

4.8.2 Differential Leukocyte Count

The eosinophil ratio of female rats in the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm) was slightly higher, and was significantly different ($P > 0.05$), but no dose-dependency was observed.

There was no significant difference between the control group and the groups exposed to methanol for both male and female rats for other aspects.

4.9 Serum Biochemistry Test

The results are shown in *Table 7*.

(1) GOT, GPT, LDH, γ-GTP, and ALP

There was no noteworthy difference between the control group and the groups exposed to methanol for both male and female rats for these enzymatic activities.

(2) Total cholesterol, triglyceride, free fatty acid, and phosphatide

The amount of these lipids in female rats of the high level exposure group (1000 ppm) tended to be lower than that of the control group, and the level of free fatty acid was significantly different ($P < 0.05$). When individual values were checked, however, the control group had a tendency to show abnormally high valued more frequently, and the average value of free fatty acid in nine untreated rats was the lowest at 649.8 μEq/ liter.

(3) Glucose

There was no significant difference between the control group and the groups exposed to methanol for both male and female rats.

(4) Total protein, albumin, A/ G ratio, and urea nitrogen

There was no notable significant difference between the control group and the groups exposed to methanol for both male and female rats.

(5) Calcium, inorganic phosphorus, sodium, potassium, and chloride

The level of sodium in male rats in the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm) was significantly lower than the control group ($P < 0.01$ for both exposure groups).

As for female rats in the high level exposure group (1000 ppm), the level of inorganic phosphorus increased significantly and the potassium decreased significantly ($P < 0.01$ and $P < 0.05$, respectively).

The levels of sodium in male rats, however, were 144.0, 142.6 and 142.1 mEq/ liter for the control group, the medium level exposure group, and the high level exposure group, respectively, and the dose-dependency was not clear. The level of sodium in nine cases of untreated rats was 141.4 mEq/ liter, which was the lowest value. The level of inorganic phosphorus and potassium in female rats of untreated group were 4.10 and 3.63 mEq/ liter, respectively, and was not very different from the high level exposure group (1000 ppm).

4.10 Organ Weight

The average organ weight is shown in *Table 8* and the average organ weight to body weight ratio is shown in *Table 9*.

There was no noteworthy significant difference between the control group and the groups exposed to methanol gas in organ weight or the organ weight to body weight ratio of all measured male or female rat organs.

Some of the pituitary glands, thyroid glands, lungs, adrenal glands, spleens and ovaries had what seemed to be an apparent change, and the details are described below:

(1) Pituitary gland

The average weight of pituitary gland of both male and female rats in the high level exposure group (1000 ppm) is lighter than the other groups. This is due to fewer numbers of severely enlarged glands compared to other groups, and there was no significant difference between the control group and any of the exposure groups.

The histopathological test found pituitary adenoma in all cases with severely enlarged pituitary gland.

(2) Thyroid gland

The average weight of thyroid gland of male rats in the medium level exposure group (100 ppm) was heavier than the other groups, but it was because one case had extremely severely enlarged thyroid gland (Animal No. 238, 0.7417 g). There is no dose-dependency.

The case above was not subjected to histopathological test, but all the cases in the control group as well as the high level exposure group (1000 ppm) with severely enlarged thyroid gland had C-cell adenoma; therefore, the case in question was considered to have the same type of tumor as well.

The weight of thyroid gland was significantly lower ($P > 0.05$) in female rats in the low level exposure group (10 ppm), but no dose-dependency was observed.

(3) Lungs

The standard deviation of male rats in the low level exposure group (10 ppm) and the high level exposure group (1000 ppm) as well as female rats in the low level exposure group (10 ppm) is lower than the other groups, but it was due to fewer numbers of severely enlarged lungs. The average weight had no dose-dependency.

Mononuclear cell leukemia was present in all cases with severely enlarged lungs that underwent histopathological testing.

(4) Adrenal glands

The average weight and standard deviation of adrenal glands in male rats in the high level exposure group (1000 ppm) were significantly greater than other groups, but not significantly different from the control group. The greater values were caused by one case with extremely severely enlarged adrenal glands (Animal No. 351; 3.0345 g). Besides above, the control group and the low level exposure group (10 ppm) each had one case that showed 10 times the normal weight of adrenal glands (Animal No. 32 and 141; 0.5624g and 0.5246g, respectively), and the average weights of adrenal glands excluding the extreme cases above were 0.05904g, 0.05932g and 0.05792g for the control group, the low level exposure group (10 ppm) and the high level exposure group (1000 ppm), respectively.

As for female rats, one case in the low level exposure group (10 ppm) (Animal No. 1136; 0.4910g) and two cases in the high level exposure group (1000 ppm) (Animal No. 1339 and 1340; 0.1277g and 0.2481 g, respectively) showed severe enlargement of adrenal glands, and therefore the average weight was slightly greater than other groups; however, both groups were not significantly different from the control group.

Phaeochromocytomas were found in all cases with severely enlarged adrenal glands that underwent histopathological testing.

(5) Spleen

The average weight of spleen is lower in the male rats in the low level exposure group (10 ppm) and the high level exposure group (1000 ppm) as well as the female rats in the low level exposure group (10 ppm); however, it was due to fewer numbers of severely enlarged spleens and no dose-dependency was observed.

Mononuclear cell leukemia was present in all cases with severely enlarged spleen that went through histopathological testing.

(6) Ovaries

The average weight of ovaries is significantly greater in the female rats in the low level exposure group (10 ppm). This is due to one observed case with extremely severely enlarged ovaries (Animal No. 1126; 2.3622 g).

4.11 Pathological Studies

Table 10 shows the number of gross-pathologically studied cases. The cases that were subjected to the studied consisted of 52 male and 52 female rats from all groups, a total of 416 rats. Pathological autopsy was performed on all cases, and histo-pathological study was initially performed on rats killed on schedule on Week 104 of the control group and the high level exposure group (1000 ppm) as well as all the cases that were found dead or killed *in extremis*. As a result, incidence of reduced pigmentation of kidneys in male and female rats, increased neoplastic change in male lungs as well as increased neoplastic change in female adrenal glands in the high level exposure group (1000 ppm) was significantly different. Therefore, pathology examination was performed on on these organs for the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) as well.

Below, the findings are discussed in broad classification of visible changes found in pathological test, nonneoplastic changes, neoplastic changes as well as findings on cases found dead or killed *in extremis* observed during histopathological study.

4.11.1 Pathological Autopsy (Visible Changes)

Total incidences (incidences in cases killed on schedule on Week 104 + cases found dead/ killed *in extremis*) of visible changes observed in various organs are shown in *Table 11*, and the incidences by period (the incidences in cases killed on schedule on Week 104 as well as cases found dead/ killed *in extremis* were tallied up by three different terms: Week 1-51, 53-78 and 79-104) is shown in *Table 12*.

(1) Cases Killed on Schedule

The visible changes that showed statistically significant difference in the groups exposed to methanol compared with the control group are summarized in the following table:

Visible Changes (with Significant Difference)

Gender		Male						Female					
Period of Expression		Found in those killed on schedule on Week 104			Total			Found in those killed on schedule on Week 104			Total		
Group[1)]		L	M	H	L	M	H	L	M	H	L	M	H
Lung	Nodule			↑[2)]			↑↑						
Liver	Nodule										↓↓		
Pituitary gland	Enlargement										↑↑		
Thyroid gland	Enlargement							↓					
Brain	Depression					↓							

1) L: low level exposure group (10 ppm); M: medium level exposure group (100 ppm)
H: high level exposure group (1000 ppm)

2) ↑, ↓: P < 0.05; ↑↑, ↓↓: P < 0.01 (↑↑: high incidence; ↓↓: low incidence)

Significant difference was observed in pulmonary nodule and cerebral depression in male rats and liver nodule as well as enlarged pituitary gland and thyroid gland in female rats. Among these changes, the total incidences of pulmonary nodule in male rats were 2 out of 52 cases (3.8 %) in the control group, 4 out of 52 cases (7.7 %) in the low level exposure group (10 ppm), 5 out of 52 cases (9.6 %) in the medium level exposure group (100 ppm) and 10 out of 52 cases (19.2 %) in the high level exposure group (1000 ppm), which shows dose-dependency, and the high level exposure group (1000 ppm) was significantly high (P < 0.01). The cases killed on schedule on Week 104 also showed a similar tendency, and the incidence in the high level exposure group (1000 ppm) was significantly high (P < 0.05). These changes

were consistent with papillary adenoma or adenomatosis with suspected transition to papillary adenoma in histological studies.

As for other changes, the dose-dependency is unclear, and they were all considered incidental changes.

None of the changes without significant difference was deemed to have been caused by methanol exposure.

(2) Cases Found Dead/ Killed *in Extremis*

Autopsy result of cases found dead or killed *in extremis* will be discussed in 4.11.4 along with histological findings.

4.11.2 Histopathological Study (Nonneoplastic Changes)

Table 13 shows the total incidences and *Table 14* shows the total incidences by period.

Significantly expressed changes in the groups exposed to methanol are summarized and shown in the table below. Significantly different changes seen in organs other than kidneys of both male and female rats, lungs in male rats as well as adrenal glands in females rats in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) were considered to be superficial due to limited number of cases tested therefore, those changes were excluded from the result as well as object of discussion.

The changes are discussed below:

Histopathological Test (Significantly Different Nonneoplastic Changes)

Gender		Male						Female					
Period of Incidence		Found in those killed on schedule on Week 104			Total			Found in those killed on schedule on Week 104			Total		
Group[1)]		L	M	H	L	M	H	L	M	H	L	M	H
Kidneys	Pigmentation	↑[2)]		⬇	↑		↓		⬇			⬇	
	Nephropathy	↑	↑		↑			↓	⬇				
	Urine cast							↑	↑		↑	↑	
	Infiltration of leucocytes				↑					⬇			
	Calcification				↓	↓							
Adrenal gland	Extramedullary hematopoiesis	-[3)]	-		-	-							↑
	Vascular degeneration	-	-		-	-							↑
Thymus	Hyperplasia of epithelial reticulum cells	-	-		-	-	↑	-	-	↓	-	-	
Spleen	Congestion	-	-	↓	-	-	↓	-	-		-	-	
Bone marrow	Activation of hematopoiesis	-	-	↓	-	-		-	-		-	-	
Parotid gland	Regenerative changes	-	-		-	-		-	-		-	-	↓
Liver	Fatty degeneration	-	-		-	-		-	-	↓	-	-	
	Focal necrosis	-	-		-	-		-	-		-	-	↑
	Infiltration of leucocytes	-	-		-	-	⬇	-	-	↓	-	-	
	Small granuloma	-	-	↑	-	-		-	-		-	-	
Orchis	Hyperplasia of Leydig cells	-	-	↑	-	-		-	-		-	-	-
Brain	Pigmentation	-	-		-	-		-	-	↓	-	-	

1) L: low level exposure group (10 ppm); M: medium level exposure group (100 ppm)
H: high level exposure group (1000 ppm)

2) ↑, ↓: P < 0.05; ↑, ↓: P < 0.01; ⬇: P < 0.001 (↑↑: high incidence; ↓↓⬇: low incidence)

3) -: Not tested

1) Kidneys

a. Pigmentation (Photographs 1 and 2):

The total incidences of pigmentation in male were significantly high ($P < 0.01$) in the low level exposure group (10 ppm) and significantly low ($P < 0.05$) in the high level exposure group (1000 ppm). Additionally, the incidences in male rats killed on schedule on Week 104 was significantly high ($P < 0.01$) in the low level exposure group (10 ppm) and significantly low ($P < 0.01$) in the high level exposure group (1000 ppm). For female rats, the total incidences were significantly low ($P < 0.001$) in the medium level exposure group (100 ppm), and the incidences in ones killed on schedule on Week 104 were significantly low ($P < 0.001$) in the medium level exposure group (100 ppm). Dose-dependency was not observed in both male and female rats.

b. Chronic progressive glomerulonephropathy (Photographs 3 and 4):

The total incidences of chronic progressive glomerulonephropathy in male rats were significantly high ($P < 0.05$) in the low level exposure group (10 ppm), and the incidences in male rats killed on schedule on Week 104 were significantly high in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) (both $P < 0.01$). As for female rats, the incidences in cases killed on schedule on Week 104 were significantly low in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) ($P < 0.05$ and 0.01, respectively). Dose-dependency was not present in any of the cases.

c. Urine cast (Photograph 5) (Initial change in chronic progressive glomerulonephropathy not accompanied by basement membrane thickening):

The total incidences of urine cast in female rats was significantly high in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) (both $P < 0.01$). Similarly, the incidences in ones killed on schedule on Week 104 were significantly high in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) (both $P < 0.01$) as well. No dose-dependency was observed, however.

d. Infiltration of leucocytes (Photographs 4 and 6):

The total incidences of infiltration of leucocytes in kidneys were significantly high ($P < 0.05$) in male rats in the low level exposure group (10 ppm). With female rats killed on schedule on Week 104, the incidences were significantly low ($P < 0.01$) in the high level exposure group (1000 ppm). Since the present nonneoplastic change often occurs spontaneously, it was considered incidental.

e. Calcification (Photograph 7):

The total incidences of calcification in male rats were significantly low in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) (both $P < 0.05$). The incidences in cases killed on schedule, however, were not significantly different, and dose-dependency was not observed either. Therefore, the present nonneoplastic change was considered incidental.

2) Adrenal gland

a. Extramedullary hematopoiesis (Photograph 9):

The total incidences of extramedullary hematopoiesis were significantly high ($P < 0.05$) in female rats in the high level exposure group (1000 ppm). No significant difference was observed in incidences in cases killed on schedule, however.

b. Vacuolar degeneration (Photograph 9 and 10):

The total incidences of vacuolar degeneration were significantly high ($P < 0.05$) in female rats in the high level exposure group (1000 ppm).

3) Thymus

a. Hyperplasia of epithelial reticulum cells (Photographs 12 and 13):

The total incidences of hyperplasia of epithelial reticulum cells were significantly high ($P < 0.05$) in male rats in the high level exposure group (1000 ppm), and the incidences in female rats killed on schedule on Week 104 were significantly low ($P < 0.05$) in the high level exposure group (1000 ppm).

4) Spleen

a. Congestion (Photograph 14):

The total incidences of congestion and the incidences in cases killed on schedule on Week 104 were significantly low ($P < 0.05$) in male rats in the high level exposure group (1000 ppm). The present nonneoplastic change, however, is often observed with mononuclear cell leukemia in the present study, and therefore it is considered to be a secondary change.

5) Bone marrow

a. Activation of hematopoiesis (Photograph 16):

The incidences of activation of hematopoiesis in cases killed on schedule on Week 104 were significantly low ($P < 0.05$) in male rats in the high level exposure group (1000 ppm). Majority of the present nonneoplastic change is accompanied by mononuclear cell leukemia, and therefore it is considered a secondary change.

6) Parotid gland

a. Regenerative change (Photograph 18):

The total incidences of regenerative change were significantly low ($P < 0.05$) in female rats in the high level exposure group (1000 ppm). No significant difference, however, was observed in the incidences in cases killed on schedule on Week 104. The present nonneoplastic change is often observed as age-associated lesion in rats.

7) Liver

a. Fatty degeneration (Photograph 19):

The incidences of fatty degeneration in female rats killed on schedule on Week 104 were significantly low ($P < 0.05$) in the high level exposure group (1000 ppm). Severe fatty degeneration is often caused by lipid metabolic disorder, but mild degeneration is often associated with aging. During the current study, majority of fatty degeneration was mild; thus, it was considered to be age-associated lesion. The number of expression was 24 in the control group and 19 in the high level exposure group (1000 ppm).

b. Focal necrosis (Photograph 20):

The total incidences of focal necrosis were significantly high ($P < 0.05$) in female rats in the high level exposure group (1000 ppm). Majority of the present nonneoplastic change is accompanied by mononuclear cell leukemia, and therefore it is considered a secondary change.

c. Infiltration of leucocytes (Photographs 19 and 22):

The total incidences of infiltration of leucocytes in liver were significantly low ($P < 0.05$) in male rats in the high level exposure group (1000 ppm), and the incidences in female rats killed on schedule on Week 104 were significantly low ($P < 0.05$) in the high level exposure group (1000 ppm). The present nonneoplastic change is often seen in rats as nonspecific change in Glisson's capsule.

d. Small granuloma (Photograph 23):

The incidences of small granuloma in male rats killed on schedule on Week 104 were significantly high ($P < 0.05$) in the high level exposure group (1000 ppm). The present change, however, is a nonspecific, spontaneous lesion observed frequently, and the number of incidence was 18 in the control group and 27 in the high level exposure group (1000 ppm), which is only a mild difference between the groups. Therefore, the present change is considered incidental.

8) Testes

a. Hyperplasia of Leydig cells (Photograph 24):

The incidences of hyperplasia of Leydig cells in cases killed on schedule on Week 104 were significantly high ($P < 0.05$) in the high level exposure group (1000 ppm). Since the present nonneoplastic change is a precursor to Leydig cell tumor, details will be discussed in the section of neoplastic changes.

9) Brain

a. Pigmentation (Photograph 26):

The incidences of brain pigmentation in female rats killed on schedule on Week 104 were significantly low ($P < 0.05$) in the high level exposure group (1000 ppm). The pigment of the present nonneoplastic change is lipofuscin, and the present change is a nonspecific change observed in association with aging. Since the incidences were 28 in the control group and 24 in the high level exposure group (1000 ppm), which is a mild difference between the groups, the present nonneoplastic change is considered incidental.

10) Miscellaneous

Association with methanol exposure was not suspected in any of the nonneoplastic changes without significant difference.

4.11.3 Histopathological Study (Neoplastic Changes)

Table 15 shows the total incidences and *Table 16* shows the total incidences by period.

(1) Neoplastic expressions

Neoplastic changes with more than 5 % incidences in the total incidences in either the control group or the high level exposure group (1000 ppm) and hyperplasias with suspected transition to those neoplastic changes are described and summarized in the table below:

Neoplastic Changes with Incidences of More Than 5 %

Gender		Male				Female			
Group		C[1)]	L	M	H	C	L	M	H
No. of Tested Cases		52	52	52	52	52	52	52	52
Systemic	Mononuclear cell leukemia	10[2)] 19.2[3)]	-	-	10 (19.2)	13 (25.0)	-	-	12 (23.11)
Lung	Papillary adenoma	1 (1.9)	5 (10.0)	2 (3.8)	6 (11.5)	2 (3.8)	-	-	2 (3.8)
	Hyperplasia	4 (7.7)	1 (2.0)	5 (9.6)	4 (7.7)	3 (5.8)	-	-	1 (1.9)
Pancreas	Islet cell adenoma	2 (3.8)	-	-	3 (5.8)	1 (2.0)	-	-	3 (5.9)
Orchis	Leydig cell tumor	44 (84.6)	-	-	43 (82.7)				
	Hyperplasia	4 (7.7)	-	-	10 (19.2)				
Uterus	Polyp					7 (13.7)	-	-	6 (11.5)
Clitoral gland	Adenocarcinoma					2/2[4)]	-	-	4/5
Pituitary gland	Adenoma	12 (24.0)	-	-	17 (34.7)	17 (33.3)	-	-	18 (34.6)
	Hyperplasia	1 (2.0)	-	-	0 (0.0)	10 (19.6)	-	-	8 (15.7)
Thyroid gland	C-cell adenoma	4 (7.7)	-	-	5 (9.6)	9 (17.6)	-	-	5 (10.0)
	Hyperplasia	5 (9.6)	-	-	5 (9.6)	20 (39.2)	-	-	19 (38.0)
Adrenal gland	Phaeochromocytoma	7 (13.5)	-	-	4 (7.8)	2 (4.0)	3 (5.9)	2 (4.1)	7 (13.7)
	Hyperplasia	0 (0.0)	-	-	2 (3.9)	2 (4.0)	3 (5.9)	7 (14.3)	2 (3.9)
Subcutis	Fibroma	1 (1.9)	-	-	3 (5.8)	0 (0.0)	-	-	1 (1.9)
	Fibrosarcoma	6 (11.5)	-	-	2 (3.8)	0 (0.0)	-	-	0 (0.0)
Mammary gland	Fibroadenoma	0 (0.0)	-	-	0 (0.0)	5 (9.6)	-	-	6 (11.5)

1) C: control group; L: low level exposure group (10 ppm); M: medium level exposure group (100 ppm); H: high level exposure group (1000 ppm)
2) Number of cases
3) Incidences (%)
4) Number of cases/ number of tested organs

1) Systemic

Mononuclear cell leukemia (Photograph 27 and 28):

Mononuclear cell leukemia was observed in 10 out of 52 cases (19.2 %) of male rats in both the control group and the high level exposure group (1000 ppm), 13 out of 52 cases (25.0 %) in female rats in the control group and 12 out of 52 cases (23.1 %) in female rats in the high level exposure group (1000 ppm). The incidences, however, did not have significant difference between the groups.

2) Lungs

Papillary adenoma (Photographs 29 and 30):

In male rats, papillary adenoma was observed in 1 out of 52 cases (1.9 %) in the control group, 5 out of 50 cases (10.0 %) in the low level exposure group (10 ppm), 2 out of 52 cases (3.8 %) in the medium level exposure group (100 ppm) and 6 out of 52 cases (11.5 %) in the high level exposure group (1000 ppm). Although the incidences have no dose-dependency or significant difference between the groups, the high level exposure group (1000 ppm) showed the highest number. Furthermore, adenomatosis (Photographs 31 and 32), which is suspected to transform into papillary adenoma as a result of hyperplasia, was observed in 4 our of 52 cases (7.7 %), 1 out of 50 cases (2.0 %), 5 out of 52 cases (9.6 %) and 4 out of 52 cases (7.7 %) in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively.

In female rats, papillary adenoma was observed in 2 out of 52 cases (3.8 %) of the control group and 2 out of 52 cases (3.8 %) of the high level exposure group (1000 ppm). The incidences had no significant difference between the groups, however.

3) Pancreas

Islet cell adenoma (Photographs 33 and 34):

Islet cell adenoma was observed in 2 out of 52 male rats (3.8 %) and 3 out of 52 male rats (5.8 %) in the control group and the high level exposure group (1000 ppm), respectively and 1 out of 49 female rats (2.0 %) and 3 out of 51 female rats (5.9 %) in the control group and the high level exposure group (1000 ppm), respectively. The incidences had no significant difference between the groups, however.

4) Testes

Leydig cell tumor (Photograph 35):

Leydig cell tumor was observed in 44 out of 52 cases (84.6 %) in the control group and 43 out of 52 cases (82.7 %) in the high level exposure group (1000 ppm). In addition, hyperplasia of Leydig cells, which is suspected to transform into the tumor, was observed in 4 out of 52 cases (7.7 %) in the control group and 10 out of 52 cases (19.2 %) in the high level exposure group (1000 ppm). Since Leydig cell tumor and the hyperplasia of Leydig cells are considered to be almost an identical change, the incidences of cases having one of the neoplastic changes mentioned above were compared between the groups. As a result, there were 48 cases in the control group and 49 cases in the high level exposure group (1000 ppm). There was no difference between the groups.

5) Uterus

Polyp (Photograph 36):

The polyp was observed in 7 out of 51 cases (13.7 %) in the control group and 6 out of 52 cases (11.5 %) in the high level exposure group (1000 ppm). The incidences had no significant difference between the groups, however.

6) Clitoral gland

Adenocarcinoma (Photograph 37):

Adenocarcinoma of clitoral gland was observed in 2 out of 2 cases in the control group and 4 out of 5 cases in the high level exposure group (1000 ppm). The incidences had no significant difference between the groups.

7) Pituitary gland

Adenoma (Photographs 38 and 39):

Adenoma in pituitary gland was observed in 12 out 50 male rats (24.0 %) in the control group and 17 out of 49 male rats (34.7 %) in the high level exposure group (1000 ppm) as well as 17 out of 51 female rats in the control group and 18 out of 52 female rats (34.6 %) in the high level exposure group (1000 ppm). The hyperplasia of chromophobe cell (Photographs 40 and 41), which is suspected to transform into adenoma was observed only in the control group for male rats in 1 out 50 cases (2.0 %), and for female rats, 10 out of 51 cases (19.6 %) and 8 out of 51 cases (15.7 %) in the control group and the high level exposure group (1000 ppm), respectively. When the incidences of cases having either adenoma or hyperplasia were compared between the groups, there were 13 male rats in the control group and 17 in the high level exposure group (1000 ppm) as well as 27 female rats in the control group and 26 in the high level exposure group (1000 ppm). There was no significant difference between the groups.

8) Thyroid gland

C-cell adenoma (Photographs 42 and 43):

C-cell adenoma was observed in 4 out of 52 male rats (7.7 %) in the control group and 5 out of 52 male rats (9.6 %) in the high level exposure group (1000 ppm) as well as 9 out of 51 female rats (17.6 %) in the control group and 5 out of 50 female rats (10.0 %) in the high level exposure group (1000 ppm). The hyperplasia of C-cell (Photographs 44 and 45) that is suspected to change into the C-cell adenoma was observed in 5 out of 52 male rats (9.6 %) in both the control group and the high level exposure group (1000 ppm), and 20 out of 51 female rats (39.2 %) in the control group and 19 out of 50 female rats (38.0 %) in the high level exposure group (1000 ppm). When the incidences of cases having either the adenoma or the hyperplasia were compared between the groups, there were 9 male rats in the control group and 10 in the high level exposure group (1000 ppm) as well as 29 female rats in the control group and 24 in the high level exposure group (1000 ppm). There was no significant difference between the groups.

9) Adrenal gland

Phaeochromocytoma (Photographs 46 and 47):

Phaeochromocytoma was observed in 7 out of 52 male rats in the control group and 4 out of 51 male rats in the high level exposure group (1000 ppm). The incidences had no significant difference between the groups, however. As for female rats, it was observed in 2 out of 50 cases (4.0 %), 3 out of 51 cases (5.9 %), 2 out of 49 cases (4.1 %) and 7 out of 51 cases (13.7 %) of the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively. Although there was no significant difference between the groups, the high level exposure group (1000 ppm) showed the highest number.

As for hyperplasia of medullary cells (Photograph 48), which is suspected to be associated with phaeochromocytoma, it was observed in 2 out of 50 cases (4.0 %), 3 out of 51 cases (5.9 %), 7 out of 49 cases (14.3 %) and 2 out of 51 cases (3.9 %) of the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively. When the incidences of cases having either phaeochromocytoma or the hyperplasia were compared between the groups, there were 4 out of 50 cases (8.0 %), 6 out of 51 cases (11.8 %), 9 out of 49 cases (18.4 %) and 9 out of 51 cases (17.6 %) in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively.

10) Subcutis

Fibroma (Photographs 49 and 50):

Fibroma was observed in 1 out of 52 male rats (1.9 %) in the control group and 3 out of 52 (5.8 %) in the high level exposure group (1000 ppm), and only 1 out of 52 female rats (1.9 %) in the high level exposure group (1000 ppm). On the other hand, fibrosarcoma (Photograph 51) was observed only in male rats, and the incidences were 6 out of 52 cases (11.5 %) in the control group and 2 out of 52 cases (3.8 %) in the high level exposure group (1000 ppm): the control group showed slightly higher incidence. The incidences had no significant difference between the groups, however.

11) Mammary gland

Fibroadenoma (Photograph 52):

Fibroadenoma was observed only in female rats and the incidences were 5 out of 52 cases (9.6 %) in the control group and 6 out of 52 cases (11.5 %) in the high level exposure group (1000 ppm).

12) Miscellaneous

Neoplastic changes observed only in the groups exposed to methanol gas were: systemic malignant lymphoma (histiocytic sarcoma) (Photograph 53) and myelocytic leukemia (Photograph 54); malignant fibrous histiocytoma (Photograph 55) in fleura; mesenchymoma (Photograph 56), mesothelioma (Photograph 57), and sarcoma (Photograph 58) in peritoneum; thymoma (Photograph 59) in thymus; malignant lymphoma (Photograph 60) in spleen; papillary adenocarcinoma (Photograph 61) in lung; exocrine adenoma (Photograph 62) in pancreas; granulose-theca cell tumor (Photograph 63) in ovaries; endometrial stromal sarcoma (Photograph 64), fibroma (Photograph 65), as well as leiomyoma (Photograph 66) in uterus; mesenchymoma (Photograph 67) and polyp (Photograph 68) in vagina; squamous cell carcinoma (Photograph 69) in clitoral gland; chordoma (Photograph 70) in spine; rhabdomyosarcoma (Photograph 71) in muscle; sebaceous squamous cell carcinoma (Photograph 72) in skin; lipoma (Photographs 73 and 74), lipo-sarcoma (Photograph 75) as well as mesenchymoma (Photograph 76) in Subcutis; adenoma (Photograph 77), fibroadenocarcinoma (Photograph 78) as well as hamartoma (Photograph 79) in mammary gland; and astrocytoma (Photograph 80) in brain.

All of the above neoplastic changes, however, were found only in one to two cases, and therefore they were considered unrelated to the exposure to methanol gas.

(2) Summary of Tumor

Each tumor was divided into benign, malignant or uncertain, and the number of cases having (a) tumor(s) as well as the number of tumors are summarized and shown in the table below:

Summary of Tumor

Gender	Male		Female	
Group	Control Group	High Level Group (1000 ppm)	Control Group	High Level Group (1000 ppm)
Number of examined cases	52	52	52	52
Number of cases with a tumor	50	52	43	42
Number of cases with multiple tumors	30	37	23	27
Total number of tumors	101	105	73	84
<Benign tumors>				
Number of cases with a tumor	48	48	36	36
Total number of tumors	78	82	51	55
<Malignant tumors>				
Number of cases with primary tumor	21	22	21	23
Number of cases with metastatic tumor	0	3	0	5
Total number of tumors	23	23	22	28
Total number of primary tumors	23	20	22	23
Total number of metastatic tumors	0	3	0	5
<Uncertain tumors>				
Number of cases with a tumor	0	0	0	1
Total number of tumors	0	0	0	1

The tumors expressed in 96.2 % of male rats in the control group and 100 % in the high level exposure group as well as 82.7 % of female rats in the control group and 80.8 % in the high level exposure group; however, neither sex showed significant difference between groups. The number of cases with multiple tumors as well as the total number of tumors were slightly higher in the high level exposure group (1000 ppm) compared to the control group in both male and female rats. For male rats, slightly higher number of expressions of papillary adenoma in lung compared to the control group and three cases of metastatic tumor expressions were attributable to the result. For female rats, it was due to more frequent expression, although not significant, of phaeochromocytoma in adrenal gland in the high level exposure group (1000 ppm) compared to the control group as well as five cases of metastatic tumor expressions.

Next, the number of cases having the tumor that expressed in more than 5 % of cases is summarized by period.

The tumors found in the high level exposure group (1000 ppm) did not have a tendency of early incidence.

Number of Incidences of Cases with Tumor by Period

(Male)

Organs	Name of Tumor	Group	Control Group					High Level Exposure Group				
		Fate	Found Dead/ Killed *in Extremis*			Killed on Schedule	Total	Found Dead/ Killed *in Extremis*			Killed on Schedule	Total
		Week	0-52	53-78	79-104	104		0-52	53-78	79-104	104	
Systemic	Mononuclear cell leukemia		0[1)]	1	4	5	10	0	0	7	3	10
Lung	Papillary adenoma		0	0	1	0	1	0	0	0	6	6
	Hyperplasia		0	0	0	4	4	0	0	3	1	4
Pancreas	Islet cell adenoma		0	0	0	2	2	0	0	0	3	3
Orchis	Leydig cell tumor		0	0	10	34	44	0	0	11	32	43
	Hyperplasia		1	1	2	0	4	0	0	5	5	10
Pituitary gland	Adenoma		0	0	6	6	12	0	0	8	9	17
	Hyperplasia		1	0	0	0	1	0	0	0	0	0
Thyroid gland	C-cell adenoma		0	0	1	3	4	0	0	1	4	5
	Hyperplasia		0	0	0	5	5	0	0	1	4	5
Adrenal gland	Phaeochromocytoma		0	0	2	5	7	0	0	0	4	4
	Hyperplasia		0	0	0	0	0	0	0	0	2	2
Subcutis	Fibroma		0	0	0	1	1	0	0	0	3	3
	Fibrosarcoma		0	0	2	4	6	0	0	1	1	2

1): Number of cases

(Female)

Organs	Name of Tumor	Group	Control Group					High Level Exposure Group				
		Fate	Found Dead/ Killed *in Extremis*			Killed on Schedule	Total	Found Dead/ Killed *in Extremis*			Killed on Schedule	Total
		Week	0-52	53-78	79-104	104		0-52	53-78	79-104	104	
Systemic	Mononuclear cell leukemia		0[1)]	1	8	4	13	0	0	5	7	12
Lung	Papillary adenoma		0	0	0	2	2	0	0	2	0	2
	Hyperplasia		0	0	1	2	3	0	0	1	0	1
Pancreas	Islet cell adenoma		0	0	0	1	1	0	0	2	1	3
Uterus	Polyp		0	0	2	5	7	0	0	1	5	6
Clitoral gland	Adenocarcinoma		0	0	1	1	2	0	0	1	3	4
Pituitary gland	Adenoma		0	0	6	11	17	0	0	6	12	18
	Hyperplasia		0	0	4	6	10	0	0	3	5	8
Thyroid gland	C-cell adenoma		0	0	1	8	9	0	0	2	3	5
	Hyperplasia		0	0	6	14	20	0	0	4	15	19
Adrenal gland	Phaeochromocytoma		0	0	1	1	2	0	0	1	6	7
	Hyperplasia		0	0	1	1	2	0	0	1	1	2
Subcutis	Fibroma		0	0	1	4	5	0	0	5	1	6

1): Number of cases

4.11.4 Histopathological Study (Cases Found Dead/ Killed *in Extremis*)

The cases that were found dead or killed *in extremis* in the control group, low level exposure group (10 ppm), medium level exposure group (100 ppm) and the high level exposure group (1000 ppm) were 16, 18, 10 and 18 male rats, respectively, as well as 21, 19, 21 and 17 female rats, respectively, and the total number of cases was 140. Major histopathological changes that may be the probable cause of death or weakening are summarized in the table below.

The cases that were found dead or killed *in extremis* due to neoplastic changes were 57 male rats (91.9 %) and 72 female rats (92.3 %). Major neoplastic changes were: 21 cases of male rats (36.8 %) and 36 cases of female rats (50.0 %) having leukemia and malignant lymphoma; 16 cases of male rats (28.1 %) and 18 cases of female rats (25.0 %) having pituitary adenoma; 9 cases of male rats (15.8 %) and 5 cases of female rats (6.9 %) having primary cutaneous and appendicular tumor; 6 cases of male rats (10.5 %) and 2 cases of female rats (2.8 %) having primary peritoneal tumor; 3 cases of brain tumor; 3 cases each of female rats having primary uterus tumor and primary clitoral gland tumor; and 7 cases of other tumors. None of the cases, however, had a tendency to occur more frequency in the groups exposed to methanol. Therefore, all the histopathological changes were considered unrelated to the exposure to methanol gas.

The cases that were found dead or killed *in extremis* due to nonneoplastic changes were 11 male and female rats (7.9 %). The breakdown is one case (9.0 %) each of histiocytosis, pyostomatitis, nephropathy and encephalomyelitis as well as 7 cases (64.0 %) of other nonneoplastic changes. All the nonneoplastic changes were considered unrelated to the exposure to methanol gas.

Probable Cause of Cases Found Dead/ Killed *in Extremis*

Gender	Male					Female				
Group	C[1)]	L	M	H	Total	C	L	M	H	Total
No. of Cases Found Dead/ Killed *in Extremis*	16[2)]	18	10	18	62	21	19	21	17	78
<Neoplastic Change>										
Leukemia and malignant lymphoma	4	5	4	8	21	9	11	10	6	36
Pituitary adenoma	6	5	1	4	16	4	6	5	3	18
Primary cutaneous (including Subcutis) and appendicular tumor	2	5	1	1	9	3	0	1	1	5
Primary peritoneal tumor	1	0	3	2	6	1	0	1	0	2
Primary uterus tumor	-	-	-	-		0	0	1	2	3
Primary clitoral gland tumor	-	-	-	-		0	1	1	1	3
Brain tumor	0	0	0	1	1	1	1	0	0	2
Miscellaneous	1	2	1	0	4	0	0	0	3	3
Total	14	17	10	16	57	18	19	19	16	72
<Nonneoplastic Changes>										
Histiocytosis	0	0	0	0	0	1	0	0	0	1
Pyostomatitis	0	0	0	0	0	0	0	1	0	1
Nephropathy	0	0	0	0	0	0	0	1	0	1
Encephalomyelitis	1	0	0	0	1	0	0	0	0	0
Miscellaneous	1	1	0	2	4	2	0	0	1	3
Total	2	1	0	2	5	3	0	2	1	6

1): C: control group; L: low level exposure group (10 ppm); M: medium level exposure group (100 ppm); H: high level exposure group (1000 ppm)
2): Number of cases

5. Discussion and Evaluation

(1) Living environment and exposure to methanol gas

Based on the result of monitoring through antibody tests and observation of general conditions, such as appearance and behavior as well as measurements of living environment, there seemed to be no particular outbreak of infectious disease or deterioration of living condition that may have affected the evaluation of the result of study. Furthermore, since the transition of survival rate and body weight as well as the maximum body weight of the control group are roughly consistent with literatures[3)], the environment inside the present inhalation test facility seemed to be adequate as a long-term living environment for rats, and the result of present study may be considered sufficient for evaluation.

The analytical values of methanol gas as well as the measurements of methanol gas level inside each chamber suggest that the test substance was administered almost in accordance with the preset value. In addition, the condition for continuous exposure is considered satisfied since the average duration of exposure to methanol gas per day was 19.5 hours for both male and female rats.

The blood level of methanol in the low level exposure group (10 ppm) and the medium level exposure group (100 ppm) was not much different from the control group; however, in the high level exposure group (1000 ppm), the male rats had 54 ppm blood methanol level and the females 88 ppm, which suggests accumulation of methanol.

(2) Survival rate and the observational result of general conditions

The survival rate of both male and female rats upon completion of exposure to methanol gas as well as its transition do not have a tendency to depend on the level of exposure, and therefore the exposure to methanol gas does not seems to induce death.

Various findings, including tumors, were identified during observation of general conditions; however, none of the histopathological changes were specific enough to suggest influence of exposure to methanol gas, and all the changes were age-associated and naturally occurring.

(3) Body weight and food consumption rate

The body weight of female rats in the high level exposure group (1000 ppm) during Week 51 to 72 of exposure were slightly lighter than the control group and the difference was significant; however, the difference with the control group was only about 4 %, and there was no significant difference in the extent of weight gain. Furthermore, the food consumption rate of male rats during Week 30 to 52 of exposure decreased significantly; however, various tests, such as transition of body weight did not suggest any variation that might have been related to the decline in food consumption rate.

The variations above both only show a slight difference to the control group and no deterioration was observed over time either. Additionally, except for the periods mentioned above, the body weight and the food consumption rate were not much different from the control group; therefore, the variations are considered not determinable as signs of intoxication caused by exposure to methanol gas.

(4) Urinalysis

Significant difference was observed in the glucose level of male rats and pH as well as bilirubin levels of female rats in the high level exposure group (1000 ppm); however, none of the individual values were abnormal and other tests, including pathological tests, revealed no findings that would support the present variations. Therefore, it is highly likely that all of these variations

are incidental changes caused by fluctuation within the normal range, and the variations are considered not determinable as signs of intoxication caused by exposure to methanol gas.

(5) Serum biochemistry test

Significant difference was observed in sodium level of male rats, free fatty acid, inorganic phosphorus as well as potassium levels of female rats in the high level exposure group (1000 ppm); however, none of the individual values in particular suggest direct influence of exposure to methanol gas, and in addition, relationship to other tests, including pathological tests, is not clear. Therefore, the significant difference is considered to be incidental.

(6) Pathological studies

The pathological studies revealed incidences of tumors with suspected association with methanol exposure. The tumors in lungs and adrenal glands as well as metastatic tumors are discussed below:

① Nodule and papillary adenoma in lungs of male rats

The total incidences as well as incidences in cases killed on schedule on Week 104 of lung nodules in male rats were dose-dependent, and all cases showed significant difference in the high level exposure group (1000 ppm). As a result of histological testing, majority of nodules were consistent with adenomatosis with suspected transformation to papillary adenoma or adenoma.

The total incidences of papillary adenoma were 1 out of 52 cases, 5 out of 50 cases, 2 out of 52 cases and 6 out of 52 cases in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively, and the low level exposure group (10 ppm) and the high level exposure group (1000 ppm) showed slightly higher incidence, but no dose-dependency was observed. Furthermore, although not published yet, other study conducted on *Fischer* rats at our institution observed 7 out of 50 cases (14.0 %) of adenoma in the control group. Additionally, others reported[4)] 14 % incidence of papillary adenoma in 120- to 131-week old *Fischer* rats.

The incidences of adenomatosis were 4 our of 52 cases, 1 out of 50 cases, 5 out of 52 cases and 4 out of 52 cases in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively, and the incidences of cases having either adenoma or adenomatosis were 5 out of 52 cases, 6 out of 52 cases, 7 out of 52 cases and 10 out of 52 cases in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively. Dose-dependency was observed, but no significant difference was present. Given the facts, the present test results may be considered not attributable to methanol exposure.

② Phaeochromocytoma in adrenal glands of female rats

The incidences of phaeochromocytoma were 2 out of 50 cases (4.0 %), 3 out of 51 cases (5.9 %), 2 out of 49 cases (4.1 %) and 7 out of 51 cases (13.7 %) in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively. The high level exposure group (1000 ppm) showed a high value in two-sided *Fisher*'s exact probability test, and although insignificant, it was considered not ignorable.

Furthermore, the result of present experiment was analyzed using one-sided *Fisher*'s exact probability test: if the presumption is that there is no difference between the control group and the exposure groups, the probability (P) of the tumor incidence observed in the high level exposure group (1000 ppm) to be spontaneous would be like the numbers in the table below.

The focus of the one-sided test was on finding out how high the tumor expression would be in the exposure groups compared to the control group. As a result, the probability turned out to be 8.46 %, i.e., the tumor would express in 1 out of 12 cases, and was higher than the probability of 5 % that is accepted in general.

Result of One-sided *Fisher*'s Exact Probability Test

Group	Exposure Level	Tumor Incidence	P (One-sided)
Control group	0 ppm	2/ 50 (4.0 %)	--
Low level exposure group	10 ppm	3/ 51 (5.9 %)	50.90 %
Medium level exposure group	100 ppm	2/ 49 (4.1 %)	68.40 %
High level exposure group	1000 ppm	7/ 51 (13.7 %)	8.46 %

In general, incidence of naturally occurring tumors in rats is said to be associated with diet, environmental conditions, microbiological factors, hormones, genetic factors, and so forth, and the incidences fluctuate by different test facilities or per study even within a same test facility[6), 10)]. It has been reported[3), 5), 6), 7), 9)] that the incidence of naturally occurring phaeochromocytoma is 0.0-5.0 % in 110- to 116-week old female *Fischer* rats, and the tumor is one of the relatively low incidence tumors with minor fluctuations.

When analyzing data on low incidence tumors, the ordinary statistical processing is to compare administration groups and a *concurrent control* group using *Fisher*'s exact probability test. This process, however, may sometimes not detect biologically significant increase in the administration groups. This fact has already been discussed in detail through analyses of study results by CTP[*1] and NTP[*2] in addition to NCI[*3] in the United States.[15), 16), 17), 18)]

According to the discussion above, if the incidence of truly naturally occurring tumor is 3 %, the probability of *false negative* is 0.436 based on the assumption that the incidence of naturally occurring tumors in the administration groups is five times higher, i.e., 15 %. Similarly, if the incidence of truly naturally occurring tumor is 4 %, the probability of false negative is 0.259. In other words, the lower the incidence of spontaneous tumor, the higher the probability of *false negative*.[16)]

The incidence (in *historical control data*) of naturally occurring phaeochromocytoma in adrenal gland is 3.2-3.9 %[3), 6)] in female *Fischer* rats, and the value seems to be close to the limit of applicable scope of *Fisher*'s exact probability test, which, as mentioned above, compares to a *concurrent control* group for analysis.

Notes:

*1: CTP: *Carcinogenesis Testing Program*
*2: NTP: *National Toxicology Program*
*3: NCI: *National Cancer Institute*

In cases like this, a precise conclusion may sometimes be achieved if historical control data obtained from studies that were conducted at various research institutions in the past were employed as object of comparison, instead of results of concurrent control group obtained during the same study.

If the historical control data were used in the statistical processing, as Fears et al utilized in the analysis of renal tumor caused by chloroform[16], the incidence of phaeochromocytoma in the high level exposure group (1000 ppm) (13.7 %) and the probability of further incidence would become around 0.3 %. In which case, the phaeochromocytoma may be referred to as a low incidence phenomenon. For the calculation above, Solleveld el al[3]'s data (3.9 %; 92 out of 2370 cases) was used as the historical control data. Solleveld el al's data also fit well with data (not published) accumulated at our institution, which is 3.0 % (3 out of 100 cases).

As stated above, it is difficult to assess low incidence tumors solely based on statistical analysis, and the decision must be based further on a wide range of knowledge including pathological, toxicological as well as pharmacological understanding. For this reason, the following points are important in the current study:

(a) In the report of research[9] on tumor and hyperplasia in adrenal medullary cells, *Yeakel* suggested the link between the two lesions. In the present study, the incidences of medullary tumor including hyperplasia of medullary cells were 4 out of 50 cases (8.0 %), 6 out of 51 cases (11.8 %), 9 out of 49 cases (18.4 %) and 9 out of 51 cases (17.6 %) in the control group, the low level exposure group (10 ppm), the medium level exposure group (100 ppm) and the high level exposure group (1000 ppm), respectively, and significant difference was not observed.

(b) Although small number, some reports mentioned more than 13 % expression in control group.[6), 8)]

(c) The male rats did not show particularly high incidence in the high level exposure group (1000 ppm).

(d) Due to 10X difference between the exposure groups, the present study does not serve as a reference for the dose-dependency, which is an important indicator for evaluating carcinogenicity.

(e) Adrenal medullary tumor or hyperplasia of medullary cells induced by chemical substances or similar substances is not reported often, and until recently, the existing reports were limited to mild increase in incidences caused by long-term administration of nicotine[11], propylthiouracil[12], growth hormone[13], as well as 2, 5-dimetyl-4-aminoazobenzene[14] and the like. Recently, however, a study on definitive expression of tumors caused by retinol acetate was reported[19], and thus examples of chemical induction are not absent.

Even after the points above were taken into consideration, it was still difficult to find clear evidence that the incidence of phaeochromocytoma was influenced by methanol exposure based on the current result on the present tumor in adrenal glands observed in the female rats in the high level exposure group (1000 ppm).

③ Metastatic tumor expression

There were three cases of tumors that were identified to be metastatic in the male rats in high level exposure group (1000 ppm): one case of malignant fibrous histiocytoma in lungs that originated in thymus, one case of phaeochromocytoma in lungs that originated in adrenal gland, and one case of hamartoma in lungs that originated in mammary gland. As female rats, the breakdown of tumors identified to be metastatic were one case of malignant lymphoma in liver that originated in spleen, one case of endometrial cancer in vagina that originated in

uterus, one case of phaeochromocytoma in lungs that originated in adrenal gland, one case of cordoma in lungs that originated in spine, and one case of rhabdomyosarcoma in lymph nodes and lungs that originated in femoral muscle. For both male and female rats, the observed tumors, except for phaeochromocytoma, are generally easily transferable, and the incidence for all tumors was a single case; therefore, these tumors are considered unrelated to the methanol exposure.

(7) Summary

The results above may be summarized that the majority of changes seen in the high level exposure group (1000 ppm) was all very subtle, and could not be determined as signs of poisoning directly caused by exposure to methanol gas. The incidence of phaeochromocytoma in adrenal glands of female rats in the high level exposure group (1000 ppm), however, seemed to be slightly higher than the spontaneous incidence in *Fischer* rats. Due to a small number of bibliographic findings on this particular lesion as well as relatively small number of cases in the present study, it was not possible to clearly conclude, based only on the present experiment, that the present lesion suggests influence of exposure to methanol gas. Definite conclusion may be achieved by performing further study on exposure to methanol gas using larger number of subjects.

6. Conclusion

The result of carcinogenic and inhalation toxicological study on *Fischer* rats through 24-month (104-week) consecutive and systemic exposure did not indicate chronic toxicological effect of exposure to methanol gas at levels lower than 1000 ppm in either male or female. Furthermore, the incidence of phaeochromacytoma in adrenal gland of female rats in the high level exposure group (1000 ppm) was slightly higher than spontaneous incidence. Positive carcinogenic effect, however, could not be identified.

References

1. The Chemical Society of Japan. *Kagaku Bosai Shishin* [*Guidelines for Chemical Accident Prevention*] 7. Maruzen: Tokyo, 1980; 55.

2. Japanese Industrial Standards. *Methanol (Regent)* JIS K8891-1979.

3. Solleveld, H.A.; Haseman, J.K.; McConnell, E.E. Natural History of Body Weight Gain, Survival, and Neoplasia in the F344 Rats. *JNCI* **1984**, *72 (4)*, pp 929-940.

4. Onodera, H.; Tanigawa, H.; Maekawa, A. F344 ratto no choki shiiku ni okeru shizen hassei shuyo [Naturally Occurring Tumors in F344 Rats under Long-Term Care]. *Dai 2 kai Fischer ratto kenkyukai deeta shu* [*Journal of 2nd Research Conference on Fischer Rats*]. **1982**, pp 38-42.

5. Miki, K.; Sakai, T.; Okamiya, H.; Hanada, T. Rorei F344 ratto no shizen hassei shuyo [Naturally Occurring Tumors in Aging F344 Rats]. *Dai 5 kai Fischer ratto kenkyukai deeta shu* [*Journal of 5th Research Conference on Fischer Rats*]. **1985**, pp 23-28.

6. Tarone, R.E.; Chu, K.C.; Ward, J.M. Variability in the Rates of Some Common Naturally Occurring Tumors in Fisher 344 Rats and (C57BL/6N x C3H/HeN) F_1 ($B6C3F_1$) Mice. *JNCI* **1981**, *66 (6)*, pp 1175-1181.

7. Goodman, D.G.; Ward, J.M.; Squire, R.A.; Chu, K.C.; Linhard, M.S. Neoplastic and Nonneoplastic Lesions in Aging F344 Rats. *Toxicol. Appl. Pharmacol*. **1979**, *48*, pp 237-248.

8. Hazleton Laboratories America, Inc. *Representative Historical Control Data Part 6: Neoplasia in F344 Rats Untreated Controls.*

9. Yeakel, E.H. Medullary Hyperplasia of the Adrenal Gland in Aged Wistar Albino and Gray Norway Rats. *Arch. Pathol.* **1947**, *44*, pp 71-77.

10. Sher, S.P.; Jensen, R.D.; Bokelman, D.L. Spontaneous Tumors in Control F344 and Charles River-CD Rats and Charles River CD-1 and B6C3F1 Mice. *Toxicological Letters* **1982**, *11*, pp 103-110.

11. Staemmler, M. Die chronische Vergiftung mit Nicotin. *Arch. Pathol. Physiol.* **1935**, *295*, pp 366-393.

12. Marine, D.; Baumann, B.J. Hypertrophy of Adrenal Medulla of White Rats in Chronic Thiouracil Poisoning. *Am. J. Physiol.* **1945**, *144*, pp 69-73 and *Arch. Pathol.* **1947**, *44*, pp 71-77.

13. Moon, H.D.; Simpson, M.E., et al. Neoplasms in Rats Treated with Pituitary Growth Hormone. II. Adrenal Glands. *Cancer Res.* **1950**, *10*, pp 364-370 and *Toxicology Letters* **1982**, *11*, pp 103-110.

14. Nakamura, K. In *Dai 6 kai Kawaguchiko kanfaransu, Fukujin-seishoku naibunpitsu (I)* [*6th Kawaguchiko Conference, Adrenal Gland and Reproductive Endocrinology (I)*]; Miura, K., Shimizu, N., Osawa, N., Eds.; Ishiyaku Publishers: Tokyo, 1976; pp 29-41.

15. Chu, K.C.; Cueto, C.Jr.; Ward, J.M. Factors in the Evaluation of 200 National Cancer Institute Carcinogen Bioassays. *J. Toxicol. Environ. Health* **1981**, *8*, pp 251-280.

16. Fears, T.R.; Tarone, R.E.; Chu, K.C. False-Positive and False-Negative Rats for Carcinogenicity Screens. *Cancer Res*. **1977**, *37*, pp 1941-45.

17. Gart, J.J.; Chu, K.C.; Tarone, R.E. Statistical Issues in Interpretation of Chronic Bioassay Tests for Carcinogenicity. *JNCI*. **1979**, *62 (4)***,** pp 957-974.

18. Office of Science and Technology Policy. *Chemical Carcinogens: A Review of the Science and its Associated Principles.* Federal Resister **1985**, *50 (50),* pp 10372-10442.

19. Kurokawa, Y.; Hayashi, Y.; Maekawa, A.; Takahashi, M.; Kokubo, T. High Incidences of Pheochromocytomas after Long-Term Administration of Retinol Acetate to F344/ DuCrj Rats. *JNCI.* **1985**, *74 (3),* pp 715-721.