A-97-03
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BEFORE THE
UNITED STATES ENVIRONMENTAL PROTECTION AGENCY

PETITION OF THE CHEMICAL MANUFACTURERS ASSOCIATION KETONES PANEL TO REMOVE METHYL ETHYL KETONE FROM THE LIST OF HAZARDOUS AIR POLLUTANTS UNDER SECTION 112(b) OF THE CLEAN AIR ACT

VOLUME IV OF IV

SUBMITTED BY

CHEMICAL MANUFACTURERS ASSOCIATION
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November 27, 1996

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2 Only the cover page and pages 70 and 87 have been submitted.

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U. S. TREASURY DEPARTMENT
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PUBLIC HEALTH SERVICE
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ACUTE RESPONSE OF GUINEA PIGS TO VAPORS OF SOME NEW COMMERCIAL ORGANIC COMPOUNDS

VIII. BUTANONE

BY
F. A. PATTY
H. H. SCHRENK
AND
W. P. YANT

REPRINT No. 1702
FROM THE
PUBLIC HEALTH REPORTS
VOL. 50, No. 36, SEPTEMBER 6, 1935
(Pages 1217-1228)

UNITED STATES
GOVERNMENT PRINTING OFFICE
WASHINGTON : 1935

For sale by the Superintendent of Documents, Washington, D. C. - - - - - - - - - Price 5 cents

INDEXED

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ACUTE RESPONSE OF GUINEA PIGS TO VAPORS OF SOME NEW COMMERCIAL ORGANIC COMPOUNDS

VIII. BUTANONE[1]

By F. A. Patty,[2] H. H. Schrenk,[3] and W. P. Yant[4]

This report on the acute response of guinea pigs to butanone (methyl ethyl ketone) vapor is the eighth of a series of similar reports [5] which deal with studies pertinent to establishing a criterion of the toxicity of some chemical products which have recently become commercially available for industrial application.

This investigation was undertaken at the request of Stanco, Inc., and was conducted jointly with the United States Bureau of Mines. The experiments were conducted at the Pittsburgh Experiment Station of the Bureau of Mines.

SCOPE OF WORK

The scope of the work included a study of the toxicity of, and the physiological response of guinea pigs exposed to, vapors of butanone (methyl ethyl ketone). Only acute effects as produced by a single exposure were studied. The experiments were planned to cover a range of concentrations which would produce slight or no response, moderate response, and serious response.

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[1] Contribution from the Pittsburgh Experiment Station, U. S. Bureau of Mines, Pittsburgh, Pa. Published by permission of the Director, U. S. Bureau of Mines. Work on manuscript completed Nov. 30, 1934.

[2] Associate chemist, health laboratory section, Pittsburgh Experiment Station, U. S. Bureau of Mines, Pittsburgh, Pa.

[3] Chemist in charge, toxicological and biochemical laboratory, health laboratory section, Pittsburgh Experiment Station, U. S. Bureau of Mines, Pittsburgh, Pa.

[4] Supervising chemist, health laboratory section, and supervising engineer, Pittsburgh Experiment Station, U. S. Bureau of Mines, Pittsburgh, Pa.

[5] Acute response of guinea pigs to vapors of some new commercial organic compounds:

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CHEMICAL AND PHYSICAL PROPERTIES

The butanone used in this study was a commercial grade of methyl ethyl ketone sold for industrial use. It was water clear and had an odor resembling that of acetone. An examination of the material gave the following physical properties:

Specific gravity

15.6°/15.6° C.... 0.8095
20°/15.6° C..... .8051

Boiling range

Distillate, percent	Temp., °C., corrected to 760 mm	Distillate, percent	Temp., °C., corrected to 760 mm
Initial boiling point	78.5	50.0	79.8
1.1	78.7	60.0	79.9
3.3	78.9	70.0	80.0
6.7	79.0	80.0	80.3
10.0	79.1	90.0	80.5
14.5	79.2	95.0	81.9
17.8	79.3	96.0	82.5
20.0	79.3	97.0	83.2
23.0	79.4	98.0	84.5
30.0	79.5	99.0	88.7
35.6	79.6	99.5	90.7
40.0	79.6		

0.2 percent residue; 0.3 percent lost

As determined by the Bureau of Mines, these physical properties agree closely with the specifications furnished by the manufacturer for the commercial product. The manufacturer also specified the product to be 92.3 percent ketone as determined by acetylation.

The boiling point of butanone as given in the International Critical Tables [6] is 79.6° C.

USE OF BUTANONE

Butanone is at present employed in the manufacture of pyroxylin solutions, chiefly in the artificial leather and lacquer industries, and is also used in paint removers. Although at present limited largely to these uses, butanone has possibilities as a solvent in other fields.

TEST APPARATUS

Figures 1 and 2 show an explosion chamber used for exposing animals to vapor-air mixtures which were near or within the flammable range. The chamber was made from a second-hand compressed-air receiver by cutting off one end. It was 41 inches in diameter, 40 inches long on the sides, and 46 inches long at the center, the closed end being convex. The walls and convex end were steel plate, approximately ¼-inch thick. The open end was closed by covering it with a sheet of clear cellophane, *a*, held in place by a large rubber band

[6] International Critical Tables, first edition, 1928, vol. 3, p. 218.

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made from the inner tube of an automobile tire. This cellophane served as a relief diaphragm in case an explosion occurred, and also admitted light. A 2-inch flanged hole, *b*, in the convex closed end of the cylinder, covered with a ¾-inch thick glass plate, served as an observation window. Animals in the chamber were observed without risk by means of a mirror, *c*, set in front of the transparent cellophane relief diaphragm and so inclined that a person at the protected end of the chamber could view the interior by reflection. A small opening at *d* was used for withdrawing samples of the chamber atmosphere for analysis. A stirring fan, *e*, was connected by a shaft through a close-fitting bushing to an externally located motor, *f*. The fan-shaft carried an externally located auxiliary fan for ventilating the space between the bushing and the motor (thus minimizing the possibility of a localized accumulation of flammable vapor-air mixture) and for drawing cooling air through the motor as required by the particular small commercial fan motor used for the apparatus. The internal stirring fan was used chiefly for keeping the vapor-air mixture homogeneous; in experiments in which an attempt was made to saturate the air with vapor it was also used to facilitate the evaporation of liquid from wicks, *g*, suspended in a reservoir of liquid. A wood floor, *h*, which had numerous 1-inch holes for circulation of air underneath, was placed in the bottom of the chamber. A wire screen, *i*, prevented the animals from rupturing the cellophane diaphragm. An opening about 4 inches in diameter at *j* was closed by a large removable cork, and through this opening guinea pigs were introduced into the chamber. The pigs were placed in a wire basket, *k*, suspended by a cord, and then lowered to the floor of the chamber. The pigs could thus be placed in the chamber after the desired vapor-air mixture had been created; and, by reversing the procedure, any animal or group could be removed at any time during the experiment without significant loss of the chamber atmosphere.

The chamber was mounted on a truck frame to facilitate moving. Owing to the danger of destruction of laboratory windows and other laboratory equipment by the explosion waves set up by the flammation of the comparatively large volume of flammable vapor-air mixture, all experiments with this apparatus were conducted outside the laboratory.

Figures 1 and 2 also show the apparatus for vaporizing the liquid and preparing the vapor-air mixtures. These mixtures were usually prepared by a dynamic method whereby a measured flow of liquid material was continuously vaporized in a measured stream of air. A constant supply of air was delivered from the blower, *o*, through the flow-meter, *p*, the rate being controlled by the pressure regulator, *q*. The liquid to be vaporized was delivered at a constant rate by proportioning pump, *r* (shown only diagrammatically in fig. 1), onto

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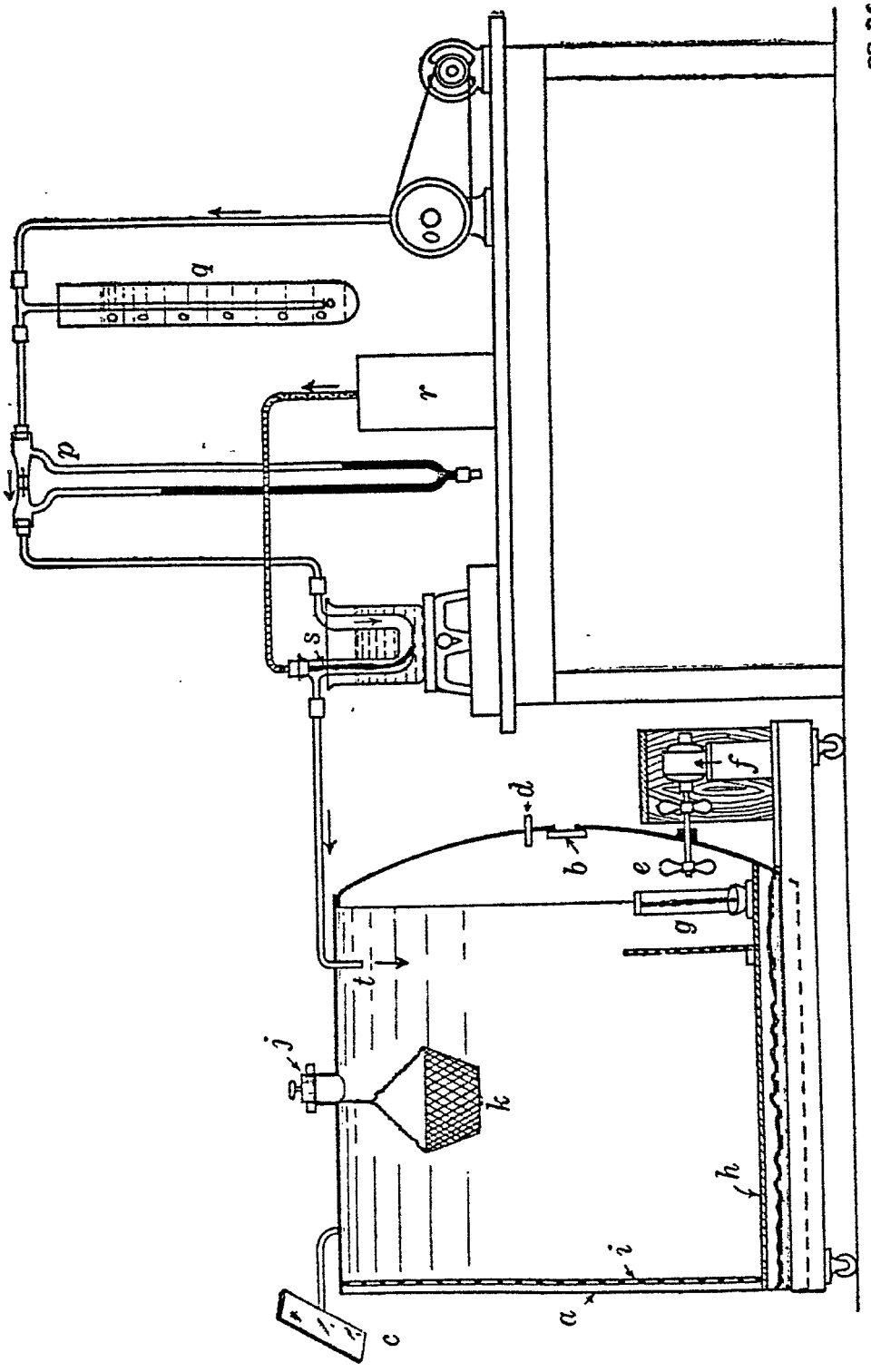

FIGURE 1.—Diagrammatic sketch of apparatus for making exposure to vapor-air mixtures close to or within the explosive limit.

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FIGURE 1.—Diagrammatic sketch of apparatus for making exposure to vapor-air mixtures close to or within the explosive limit.

PB-364

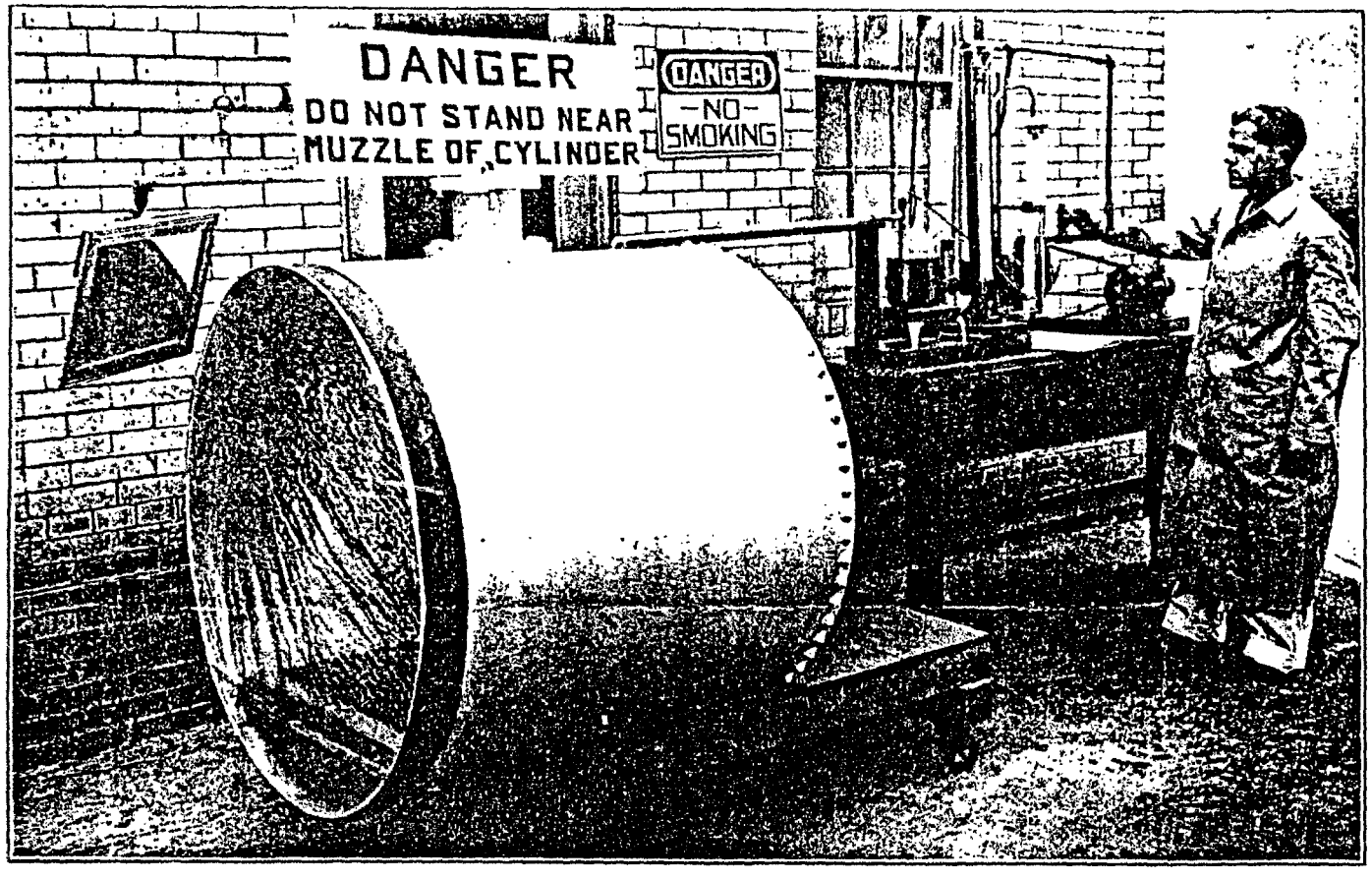

FIGURE 2.—PHOTOGRAPH OF APPARATUS FOR EXPOSING ANIMALS TO VAPOR-AIR MIXTURES CLOSE TO OR WITHIN THE EXPLOSIVE LIMIT.

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the wick, *s*, contained in a U-tube immersed in an oil bath heated by a hot plate to a temperature above the boiling point of the liquid to be vaporized. The air supply passed over the wick and carried the vapor into the chamber at *t*. The escape of the vapor-air mixture was mainly around the edge of the diaphragm and to a slight extent at the stuffing box of the fan shaft. A slight internal pressure prevented any inward leakage. The amounts of liquid and air were varied to produce the desired concentrations, but the total volume of air passed through the chamber always exceeded two air changes an hour, which was sufficient to prevent oxygen deficiency or accumulation of carbon dioxide from the respiration of the animals.

In preparing the highest concentration available on complete saturation of the air (approximately 10 percent vapor in air by volume), all the chamber openings were closed and air was blown by means of the stirring fan across a series of wicks suspended in a reservoir of butanone. The fan and saturator were operated several hours before the pigs were placed in the chamber and continued during the entire experiment, in order to create and maintain as nearly a saturated condition as could be attained practically at the prevailing temperature, approximately 30° C.

Experiments with 0.33 percent butanone vapor in air, which is well below the lower flammable limit, were performed in a larger chamber, which has been described in a previous publication.[7]

COMPUTATION AND ANALYSIS OF VAPOR-AIR MIXTURES

For control purposes in creating experimental conditions, the concentrations of vapor in air were estimated by computation from the quantity of air flowing through the meter and the quantity of liquid delivered to the vaporizer. This procedure was necessarily omitted in the experiments with air saturated with butanone vapor, in which a static method was employed. All concentrations were finally determined during the course of the experiments by chemical analysis, and in some cases by adsorption by air-equilibrated charcoal.

The chemical method of analysis consisted of pipetting 50 cm^3 of a solution of N/1 sodium hydroxide into a flask, which was closed by a rubber stopper fitted with a glass stopcock. The flask was connected in series with a mercury U-gage and partly evacuated; then the stopcock was closed. This sample receiver was then connected to a well-purged sampling tube and filled by vacuum displacement. The volume of vapor-air taken was calculated from the U-gage measurement at the end of the evacuation procedure and at the prevailing temperature and barometric pressure. The tube was shaken to absorb the

[7] Yant, W. P., Schrenk, H. H., and Sayers, R. R.; Methanol antifreeze and methanol poisoning. Ind. & Eng. Chem., vol. 23 (1931), pp. 551-555.

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butanone; an excess of N/10 iodine was slowly added. The sodium hydroxide was permitted to stand for 15 minutes, then was neutralized with 2N sulphuric acid, and a slight excess (about 0.3 to 0.5 cm^3) of acid was added. The excess iodine was determined by titration with N/20 sodium thiosulphate solution.

Table 1 gives the results of analyses of a standard solution of butanone in water made as a check on the accuracy of the method of analysis.

TABLE 1.—*Results of the analysis of portions of a standard aqueous solution of butanone*

Butanone taken by computation from standard	Butanone found by analysis	Recovery, percent
Mg	*Mg*	
16.1	17.1	106
32.2	34.9	108
32.2	34.8	108
32.2	34.9	108
32.2	34.6	107
48.3	51.7	107
48.3	51.4	106
48.3	51.3	106

The average recovery in 8 determinations was 107 percent. In a report on the determination of methyl ethyl ketone, Cassar[8] has shown that if the reaction of 6 moles of iodine per mole of ketone is taken as 100 percent, a recovery of 110.6 percent is obtained. This increase is due apparently to the influence of a secondary reaction in which 10 moles of iodine react with 1 mole of ketone, as shown in the following equations:

$$CH_3CH_2COCH_3+3I_2+3NaOH=CHI_3+CH_3CH_2COOH+3NaI+2H_2O \quad (1)$$

$$CH_3CH_2COCH_3+5I_2+4NaOH=2CHI_3+CH_3COOH+4NaI+3H_2O \quad (2)$$

On the basis of 110.6 being equivalent to 100 percent, the result, 107 percent, obtained by the Bureau of Mines for the commercial product used indicates it to be approximately 96.7 percent ketone. The commercial product was reported by the producers to contain 92.3 percent ketone, as determined by acetylization. The impurities in the ketone were also reported by the manufacturer to be primarily the corresponding secondary alcohol.

As an average recovery of 107 percent was obtained (table 1) for known amounts of the standard solution of commercial butanone, the values obtained for the amount of butanone in the vapor-air mixtures used in animal experiments (table 2) were corrected by multiplying the determined value by $\frac{100}{107}$, or 0.935.

[8] Cassar, H. A.: Determination of isopropyl alcohol in presence of acetone, and of methyl ethyl ketone in presence of secondary butyl alcohol. Ind. & Eng. Chem., vol. 19 (1927), pp. 1061–1062.

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Table 2 gives the concentrations computed from the volume of air and the amount of butanone vaporized, and the concentration found by chemical analysis for vapor-air mixtures used in animal experiments. The conversion from milligrams per liter, as determined chemically, to percent by volume is made on the basis that 1 gram molecular weight of butanone is equivalent to 22.4 liters of vapor at 0° C. and 760 mm Hg.

TABLE 2.—*Results of analysis of exposure atmospheres* [1]

Concentration by—		Concentration by—	
Computation	Analysis	Computation	Analysis
(2)	[3] 9.6	0.98	0.89
(2)	[3] 9.0	.92	.85
(2)	[3] 11.4	.96	1.18
(2)	8.7	.96	.96
4.2	3.6	1.02	.83
3.6	3.5	1.03	.91
3.6	3.2	.92	.86
3.2	3.6	.95	.95
3.2	2.9	.33	.30
3.3	2.9	.33	.32
1.04	1.05	.32	.35
1.01	1.22	.30	.32
.98	.89	.32	.28

[1] Concentration in percent by volume at 25° C. and 760 mm pressure. To convert to mg per liter, multiply by 29.5.
[2] Concentration obtained by recirculation of air (30° C., 740 mm pressure) over wicks wet with butanone. No computed composition.
[3] Determined by adsorption on air-equilibrated charcoal.

The maximum concentration attainable at 30° C. and 740 mm pressure averaged approximately 10 percent. The remainder of the results in table 2 represent experimental atmospheres prepared by continuously volatilizing a measured amount of butanone in a measured volume of air. For succeeding experiments this maximum of 10 percent was successively reduced by a factor of approximately one-third until no response was observed after 13.5 hours' exposure. By following this plan the general order of concentrations used was 10.0, 3.3, 1.0, and 0.33 percent by volume.

PROCEDURE FOR EXPOSING ANIMALS

In all experiments the desired concentration was created and analyzed before the guinea pigs were admitted. The pigs were exposed in groups of 6, several such groups being included in each experiment to permit study of the time-concentration effect. In the use of the explosion chamber, the animals to be exposed longest were admitted first. The second and other groups were added at a time which would permit the predetermined period of exposure for each group and allow the exposure of all to be terminated simultaneously. This procedure was usually reversed in the use of the chamber for nonflammable vapor-air mixtures. All the animals used for expo-

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sure to a particular vapor-air mixture were started at the same time, and groups were removed after predetermined intervals. Chemical analyses were continued during the course of exposure.

The description and information regarding the care of animals will be found in the report on ethylene dichloride.[9]

RESULTS OF TESTS

This report presents summarized results pertinent to signs or symptoms, fatality, and gross pathology.

OBJECTIVE SYMPTOMS

Control animals.—No signs or symptoms were exhibited by the 24 control guinea pigs taken at random from the stock animals used in these tests. No deaths occurred.

Exposed animals.—The signs or symptoms exhibited by animals exposed to butanone vapor in the order of their occurrence were as follows: Irritation of the nose and eyes manifested by rubbing nose with the forepaws and squinting; lacrimation; incoordination; narcosis; gasping type of respiration; and death. Table 3 gives the average time necessary to produce these symptoms by various concentrations of butanone vapor in air. The figures given indicate the average time for occurrence of the symptom, excepting those in parentheses, which indicate that the particular symptom did not occur in the maximum period of exposure as given.

TABLE 3.—*Signs and symptoms produced in guinea pigs exposed to vapors of butanone*

Type of symptom	Concentration of vapor in percent by volume			
	10.0	3.3	1.0	0.33
	Duration of exposure, minutes			
Nose irritation (rubbing nose)	(1)	1	2	[2] (810)
Eye irritation (squinting)	(1)	1	4	[2] (810)
Lacrimation	1	4	40	[2] (810)
Incoordination	3-5	18-30	90	[2] (810)
Narcosis (unconsciousness)	10-11	48-90	210-280	[2] (810)
Gasping-type respiration	20-30	180	[2] (810)	[2] (810)
Death	45-55	200-260	[2] (810)	[2] (810)

[1] Evident almost immediately after beginning exposure.
[2] Not observed during maximum exposure as given in parentheses.

No abnormal signs were observed during or following an exposure of 810 minutes to 0.33 percent butanone vapor in air by volume. Signs of irritation of the nose and eyes occurred in 2 and 4 minutes, lacrimation in 40 minutes, incoordination in 90 minutes, and uncon-

[9] See I, footnote 5.

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CAUSE OF DEATH DURING EXPOSURE

It is not clear whether death was due to irritation of the lungs or to a state of narcosis that terminated in death. It is noteworthy that without exception the animals either died during exposure or recovered.

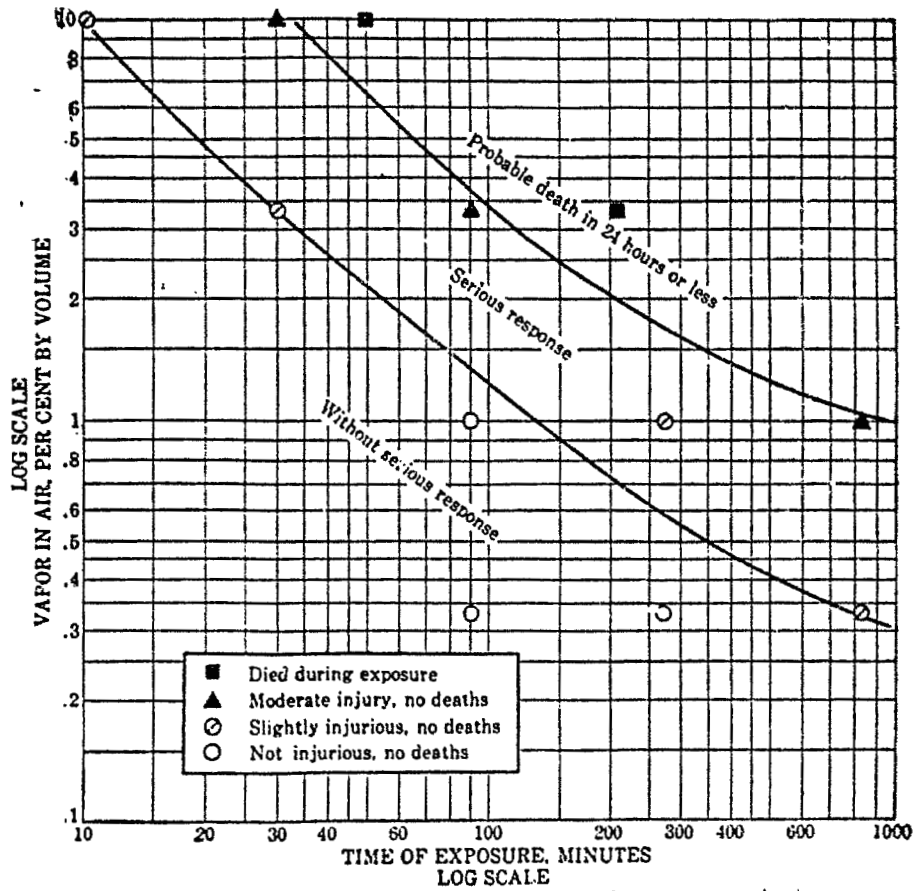

FIGURE 3.—Acute effects of exposure of guinea pigs to butanone vapor in air.

This indicates that the irritation of the respiratory center was secondary to the narcotic action. In some instances the animals were unconscious for several hours after exposure terminated (30 minutes' exposure to 10 percent, 90 minutes to 3.3 percent, and 810 minutes to

TABLE 4.—*Acute effects of exposure of guinea pigs to butanone vapor in air*

Acute effects after various periods of exposure	Concentration, percent by volume in air
Kills in a few minutes	[1]
Dangerous to life in 30 to 60 minutes	5.0-10.0
Maximum amount for 60 minutes without serious disturbance	1.0
Maximum amount for several hours without serious disturbance	0.3

[1] Not produced by 10 percent vapor in air, the highest concentration obtained in a closed chamber by extended recirculation of air (30° C., 710 mm pressure) over wicks wet with butanone.

1 percent), and in one instance (30 minutes to 10 percent) did not regain consciousness until 8 to 10 hours after exposure, but were approaching normal activity 24 hours after exposure and appeared normal in activity within 48 hours. Opacity of the cornea persisted in some cases for the 8-day period before the pigs were killed for autopsy.

WARNING PROPERTIES AND HAZARDS OF ACUTE POISONING

Men momentarily exposed to 3.3 and approximately 10 percent vapor in air pronounced the atmosphere intolerable because of irritation to the eyes and nasal passages. One percent vapor in air was found to have a strong odor and to be almost intolerable from irritation to the eyes and nose after several inhalations; 0.33 percent vapor had a moderate to strong odor and was moderately irritating to the eyes and nose.

Concentrations apparently harmless to guinea pigs after several hours' exposure have distinct warning properties of both odor and irritation, and concentrations which produced no serious effects in 1 hour were practically intolerable.

WARNING PROPERTIES AND EXPLOSION HAZARDS

The explosion hazard of butanone vapor is not to be ignored. The intensity of odor and irritating properties are high enough, however, to give distinct warning characteristics to vapor-air mixtures considerably below the flammable range, which may be considered to be about 2 percent. The upper limit probably is not greater than 12 percent.

SUMMARY AND CONCLUSIONS

The acute physiological response of guinea pigs to air containing butanone (methyl ethyl ketone) vapor was determined. The concentrations of the vapor ranged from those that produced death to those that produced no effect after several hours' exposure. The signs of response, fatality, and gross pathology are given. The warning properties as studied by the exposure of persons are described.

The symptoms are principally those of eye and nose irritation, and narcosis, the latter being apparently the most significant. Animals that did not die during exposure recovered.

The principal gross pathological findings immediately after exposure were congestion, edema, and hemorrhage of vital organs, death being due to a narcosis terminating in death.

At room temperature it was impossible to attain a concentration that would kill guinea pigs in a few minutes. Exposure to 5- to 10-percent vapor is considered dangerous to the life of guinea pigs after 30 to 60 minutes. One percent is considered the maximum amount for 60 minutes without serious disturbance and 0.3 percent the maximum amount for several hours without serious disturbances.

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E3-001 (4)

Toxicology Letters, 16 (1983) 69–75
Elsevier Biomedical Press

REF 43

TOXIC ACTIVITY OF SEVENTEEN INDUSTRIAL SOLVENTS AND HALOGENATED COMPOUNDS ON HUMAN LYMPHOCYTES CULTURED IN VITRO

(DNA damage; DNA synthesis inhibition; insecticides)

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(Received July 15th, 1982)
(Revision received September 30th, 1982)
(Accepted October 19th, 1982)

SUMMARY

Seventeen chemicals (solvents, insecticides and intermediates in the production of textiles and resins) were tested in a short-term in vitro system with human lymphocytes to determine their toxic action. The parameters studied were the tritiated thymidine uptake and cell viability in cultures grown with or without a rat liver metabolizing system (S-9 mix).

Data obtained showed that 1,3-dichlorobenzene, 1,2-dichlorobenzene, hexane, 1,2-diiodoethane, 1,4-dichlorobenzene, tetrachloroethylene, 2,3-dibromopropanol, chloromethyl methyl ether, 1,2- and 1,3-dibromopropane, in order, exerted the more toxic effects; ethyl acetate, cyclohexane, cyclohexanone and benzene showed lower toxic activity.

The chemicals lost their toxic power in the presence of the metabolizing system with the exception of 1,2- and 1,3-dichlorobenzene which maintained in some degree their toxicity even in the presence of the S-9 mix. Only chloromethyl methyl ether elicited unscheduled DNA synthesis acting as DNA damaging agent.

INTRODUCTION

In previous works we studied the effects exerted on human cells cultured in vitro by some pesticides and haloalkanes as toxic or DNA-damaging agents [1, 2]. In the present study we report data on a further seventeen chemicals widely used in industry and widespread in the environment, viz. solvents, insecticides and intermediates in the production of textiles and resins.

Some of the chemicals tested in this study have been previously studied in non-

Abbreviations: DMSO, dimethylsulfoxide; [^{3}H]TdR, tritiated thymidine.

0378-4274/83/0000–0000/$03.00 © Elsevier Biomedical Press

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human experimental systems to determine their toxic, mutagenic and/or carcinogenic activity [3–7]; epidemiological data are available for solvents [8–10].

This work was carried out in an in vitro experimental system with human lymphocytes and the toxic effects of the chemicals were measured determining cellular viability by trypan-blue staining and by the inhibition of the DNA synthesis.

Cultures were grown in the presence or in the absence of metabolic activation of the compounds and obtained data were compared to those of chloromethyl methyl ether taken as a positive control and of chloroform as a negative one.

In previous studies the former acted as toxic and DNA-damaging agent and the latter gave negative results [2].

MATERIALS AND METHODS

Chemicals. The following substances were tested (in parentheses purity grade): benzene (99%), 1,2-dichlorobenzene (99%), 1,3-dichlorobenzene (99%), 1,4-dichlorobenzene (99%), 1,2-diiodoethane (98%), 1,2-dibromopropane (97%), 1,3-dibromopropane (98%), chloromethyl methyl ether (90%), Merck-Schuchardt (Darmstadt, F.R.G.) and ethyl acetate (99.5%), methyl ethyl ketone (99%), *n*-hexane (99%), cyclohexane (99.8%), cyclohexanone (98.8%), chloroform (99.8%), 1,2-dichloropropane (–), 2,3-dibromopropanol (96%) and tetrachloroethylene (99%), Aldrich Europe (Beerse, Belgium). Three scalar doses of each chemical were tested and, taking into account the low water-solubility of most of them, they were dissolved in DMSO before their addition to the cell cultures at final concentrations which ranged from 10^{-2} M to 10^{-4} M and 1% DMSO.

Cell cultures. The in vitro test system utilized in this study was based on that previously described [1, 2] with modifications. Briefly, 50–60 ml of blood from healthy adult donors were collected and lymphocytes were separated according to Böyum [11].

Cells, $1–2 \cdot 10^5$/well of microtest plate 3040 (Falcon Plastics, Los Angeles, CA, USA), were cultured in a volume of 0.2 ml of RPMI 1640 medium supplemented with 2 mM glutamine, containing 1% DMSO with water-insoluble chemicals and 0.25 μCi of [^{3}H]TdR (20 Ci/mmol, The Radiochemical Centre, Amersham, U.K.).

Lymphocytes were cultured for 4 h at 37°C in a humidified atmosphere containing 5% CO_2 in the presence (treated) or in the absence (controls) of each dose of the chemicals in sextuplicate culture wells. The effects of the substances on the DNA synthesis were measured through cellular [^{3}H]TdR uptake. Short-term toxicity (4 h culture) was determined by the trypan-blue staining technique.

Cultures were grown in the absence or in the presence of a rat liver metabolizing system (S-9 mix) either to activate the chemicals to different forms [13, 14] or to transform toxic substances in inactive compounds. S-9 mix was obtained following the method of Ames et al. [12]: 1 ml of the metabolizing mixture contained 0.2 m

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of rat liver phenobarbital-induced S-9, 8 μmol $MgCl_2$, 33 μmol KCl, 5 μmol glucose-6-phosphate and 4 μmol NADP. At the end of the culture periods, [^{3}H]TdR uptake by lymphocytes, in sextuplicate wells for each experimental combination, was determined by a liquid scintillation spectrometer as previously described [1] and it was expressed in dpm ± S.E.

Data refer to several experiments; each of them included as much as possible chemically homogeneous substances and was performed with lymphocytes of the same donor. Repeated experiments with cells from different donors were performed to confirm obtained results.

RESULTS AND DISCUSSION

Table I gives the data on cellular viability and [^{3}H]TdR uptake by human lymphocytes cultured in vitro in the presence of the tested chemicals with or without metabolic activation.

As far as the toxic action is concerned, it appears that methyl ethyl ketone, chloroform and 1,2-dichloropropane do not cause any cytotoxic action. Both the [^{3}H]TdR uptake and the percentage of viable cells show values very similar to those of the corresponding controls. Data for chloroform, utilized as a negative control, confirm those previously obtained in vitro in our experimental conditions [2], even though this chemical has proved toxic for laboratory animals or in epidemiological studies [4, 8].

Chloromethyl methyl ether, *n*-hexane, 1,2- and 1,3-dibromopropane, 2,3-dibromopropanol, 1,2-diiodoethane, tetrachloroethylene and 1,4-dichlorobenzene are toxic when lymphocytes are grown in the absence of S-9 mix and, usually, with the highest doses used. Both the [^{3}H]TdR uptake and the viable cell percentage are reduced to a various extent by comparison with the corresponding controls.

Since this cytotoxicity disappears in the presence of the metabolizing system, it can be argued that the S-9 mix causes a detoxification of these chemicals as it is reported for other chemically related compounds [13, 15]. Alternatively, this protection could be explained through an unspecific absorption of the drugs by the S-9 mix so that their real concentrations are lowered. Such an action is hypothesized also by other authors [16]. Data for chloromethyl methyl ether-treated cultures utilized as a positive control, confirm those previously obtained [2]. 10^{-3} M 1,2- and 1,3-dichlorobenzene do not seem to be (completely) inactivated by the microsomal system because significant values of cellular death (50%) are still observable in S-9 mix-treated cultures also, while the corresponding [^{3}H]TdR uptake is unaffected in comparison with the control (230 and 208 dpm, respectively, vs. 218 dpm of the control). This finding suggests that the microsomal system partially reduces the toxic activity of these chemicals but it must be pointed out that no simple and direct correlation exists between cell viability and thymidine incorporation. This is to be expected since [^{3}H]TdR uptake could be affected

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without an alteration in viability. Moreover, it is possible that pools of cells are responding differently in that some lose viability while other cells continue to incorporate thymidine.

Ethyl acetate, benzene, cyclohexane and cyclohexanone cause a marked inh[illegible]tion of [^{3}H]TdR uptake in S-9 mix-lacking cultures while the corresponding [illegible]ular

TABLE I

CYTOTOXICITY AND GENOTOXICITY EXERTED ON HUMAN LYMPHOCYTES CULTURED IN VITRO FOR A 4 H PERIOD BY THREE SCALAR DOSES OF 17 CHEMICALS OF INDUSTRIAL UTILIZATION

Data are expressed as percent of viable cells/controls and as tritiated thymidine uptake for replicative and reparative DNA synthesis in presence or in absence of a rat liver metabolizing system. Data, in dpm, are the mean of sextuplicate samples ± S.E.

Compound	Dose (M)	Without S-9 mix		With S-9 mix	
		[^{3}H]TdR uptake	Viable cells/ control[b]	[^{3}H]TdR uptake	Viable cells/ control[b]
Ethyl acetate	10^{-2}	356 ± 13	100	287 ± 19	100
	10^{-3}	361 ± 18	100	264 ± 4	100
	10^{-4}	414 ± 8	100	264 ± 14	100
Methyl ethyl ketone	10^{-2}	583 ± 5	100	367 ± 24	10[illegible]
	10^{-3}	525 ± 14	100	400 ± 30	10[illegible]
	10^{-4}	501 ± 15	100	388 ± 16	100
Chloromethyl methyl ether	10^{-2}	249 ± 18	70	681 ± 35	100
	10^{-3}	310 ± 15	100	433 ± 9	100
	10^{-4}	606 ± 7	100	364 ± 16	100
Control	–	582 ± 14		332 ± 26	
n-Hexane[a]	10^{-2}	123 ± 11	40	307 ± 26	100
	10^{-3}	470 ± 10	70	285 ± 11	100
	10^{-4}	518 ± 25	100	268 ± 25	100
Cyclohexane[a]	10^{-2}	365 ± 12	100	254 ± 19	100
	10^{-3}	530 ± 27	100	258 ± 14	100
	10^{-4}	520 ± 19	100	306 ± 17	100
Cyclohexanone[a]	10^{-2}	327 ± 14	100	245 ± 17	100
	10^{-3}	550 ± 18	100	298 ± 28	100
	10^{-4}	555 ± 29	100	256 ± 16	100
Control[a]	–	517 ± 21		294 ± 10	
1,2-Dichloropropane[a]	10^{-2}	524 ± 17	100	388 ± 20	100
	10^{-3}	496 ± 10	100	396 ± 19	100
	10^{-4}	576 ± 15	100	370 ± 9	100

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Compound	Dose (M)	Without S-9 mix [^{3}H]TdR uptake	Without S-9 mix Viable cells/ control[b]	With S-9 mix [^{3}H]TdR uptake	With S-9 mix Viable cells/ control[b]
1,2-Dibromopropane[a]	10^{-2}	331 ± 6	25	354 ± 9	100
	10^{-3}	426 ± 13	100	400 ± 17	100
	10^{-4}	415 ± 18	100	428 ± 16	100
1,3-Dibromopropane[a]	10^{-2}	249 ± 18	70	352 ± 11	100
	10^{-3}	561 ± 27	100	435 ± 36	100
	10^{-4}	566 ± 19	100	392 ± 21	100
1,3-Dibromopropanol[a]	10^{-2}	186 ± 12	25	391 ± 17	100
	10^{-3}	516 ± 30	100	425 ± 23	100
	10^{-4}	493 ± 27	100	366 ± 20	100
Control[a]	–	521 ± 23		375 ± 10	
Chloroform[a]	10^{-2}	397 ± 38	100	293 ± 19	100
	10^{-3}	391 ± 27	100	312 ± 13	100
	10^{-4}	382 ± 16	100	285 ± 9	100
1,2-Diiodoethane[a]	10^{-3}	120 ± 22	30	242 ± 9	100
	10^{-4}	320 ± 28	85	299 ± 12	100
	10^{-5}	420 ± 19	100	299 ± 26	100
Tetrachloroethylene[a]	10^{-2}	157 ± 27	30	217 ± 18	100
	10^{-3}	379 ± 29	100	265 ± 17	100
	10^{-4}	417 ± 21	100	272 ± 24	100
Control[a]	–	380 ± 25		296 ± 9	
Benzene[a]	10^{-3}	271 ± 15	100	212 ± 20	100
	10^{-4}	413 ± 8	100	195 ± 12	100
	10^{-5}	408 ± 7	100	173 ± 3	100
1,2-Dichlorobenzene[a]	10^{-3}	116 ± 10	15	230 ± 7	50
	10^{-4}	300 ± 22	90	213 ± 7	100
	10^{-5}	292 ± 15	100	220 ± 21	100
1,3-Dichlorobenzene[a]	10^{-3}	98 ± 5	5	208 ± 12	50
	10^{-4}	290 ± 8	100	227 ± 3	100
	10^{-5}	293 ± 26	100	201 ± 3	100
1,4-Dichlorobenzene[a]	10^{-3}	151 ± 5	50	234 ± 18	100
	10^{-4}	322 ± 12	100	219 ± 19	100
	10^{-5}	315 ± 20	100	230 ± 10	100
Control[a]	–	425 ± 10		218 ± 15	

[a]Solubilized in DMSO (1% final concentration).
[b]In percent.

viabilities are unaffected (100%/controls). These data suggest that these chemicals exercise a cytotoxic action which is not immediately followed by cellular death, presumably because they inhibit some step of the DNA synthesis and/or interfere with the thymidine uptake processes.

Finally, [^{3}H]TdR uptake values significantly higher than that of the corresponding control are observable only in 10^{-2} and 10^{-3} M chloromethyl methyl ether-treated cultures grown in the presence of the metabolizing system (681 and 433 dpm vs. 332 dpm of the control). It can be hypothesized that the S-9 mix activates this chemical to a form which causes damage on DNA followed by [^{3}H]TdR uptake for the DNA reparative synthesis in accordance with previous observations [2].

Reported data are substantially in accordance with those of other authors obtained in different experimental conditions (for the literature see INTRODUCTION). However, it must be pointed out that controversial results may be obtained for a given substance utilizing different experimental schedules or different bacterial strains and animal species even in the same experimental conditions [17, 18]. Hence, it appears necessary that studies on the cytotoxic and genotoxic activities of a chemical widespread in the environment should be carried out utilizing several experimental schedules to obtain more reliable results.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. F. Cappi, director of the 'Centro Regionale per il Servizio Trasfusionale - U.S.L. 27 Bologna Ovest' for the human blood supply. This work was supported by grant 500.4/RSC/135/L/1208 from the Ministero della Sanità.

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Journal ~~of the~~ Water Pollution Control Federation 46, 1: 63-77, 1974

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Brine shrimp bioassay and seawater BOD of petrochemicals

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CONCERN over synthetic organic chemicals in the aquatic environment is primarily related to toxicity, biodegradation, and biomagnification. Among other uses, aquatic toxicity data are needed to aid in evaluating the hazards of transporting various organic chemicals by deep-water tanker, barge, train, and truck and to assess various means of disposing of the washing residue produced in cleaning these transport vehicles. Biodegradability data on specific materials may be used to estimate the impact of an organic spill on the oxygen resources of a stream; also, the rate and extent of biodegradation of an organic chemical can be used to evaluate the advisability of using a biological system for the treatment of a particular wastestream, captured spill, or washing residue. Biomagnification or food-chain buildup is of concern when relatively large amounts of a material that is both resistant to degradation and has a reasonable chance of entering microbial cells reaches the aquatic environment on a continuous basis.

Although several studies and compilations of biodegradability data are available in the literature,[1-7] many of the data are based on 5-day biochemical oxygen demand (BOD) tests or short-term activated sludge tests; virtually all tests have been performed in freshwater systems. The available data are often difficult to apply as the methods used are highly varied and not completely described. For example, BOD values are known to vary greatly depending on the state of acclimation of the microbial seed used. The results of activated sludge tests are influenced by air stripping, adsorption on biosolids, and the state of acclimation of the activated sludge. For many synthetic materials no biodegradability data are available for either freshwater or seawater systems. In general, aquatic toxicity data, particularly for marine organisms, are even more limited than is information on biodegradability.

Studies were conducted at the Union Carbide Technical Center, South Charleston, W. Va., to evaluate the biodegradability and toxicity of over 50 synthetic organic compounds of major commercial significance. Both fresh and seawater systems were used applying modifications of the standard BOD test. *Artemia salina* (brine shrimp) were used in toxicity bioassays.

TEST PROCEDURES AND RESULTS

Biodegradation tests were performed in three phases: initial screening of biodegradability in freshwater, acclimation of microbial systems to those materials that were resistant to biodegradation in the screening tests, and evaluation of biodegradability of most test materials in a high salinity environment simulating ocean water. Screening tests with brine shrimp were conducted before establishing median tolerance levels (TL_m).

Freshwater biodegradation. Testing of those chemicals that were readily water soluble followed the published BOD procedure.[8] A settled domestic wastewater was first filtered through glass wool and then added (3 ml/bottle) as seed material to clean BOD bottles. The bottles were half filled with aerated dilution water containing the specified minerals and buffer. Small aliquots of the test chemicals were added to these bottles from 0.1 percent stock solutions yielding concentrations of 3, 7, and 10 mg/l. At least two of these concentrations were tested in duplicate. These concentrations gave a potential oxy-

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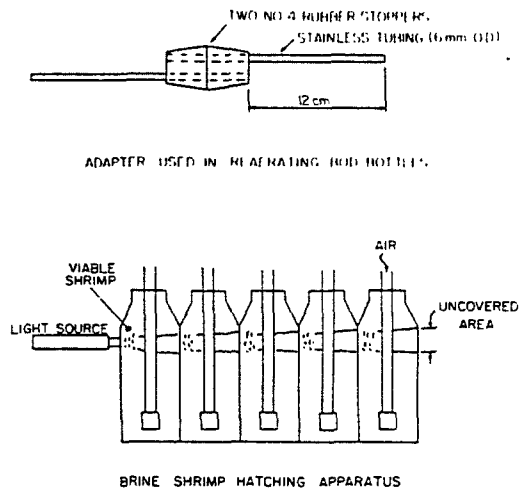

FIGURE 1.—Test apparatus.

gen demand of 3 to 30 mg/l over the 20-day test duration. Dissolved oxygen (DO) was monitored periodically in the individual bottles through the use of a commercial DO meter fitted with an agitated probe. The bottles were opened for sampling and DO measurements about five times during the course of the 20-day test.

When the DO in the test bottle dropped below 4.0 mg/l, the contents were reaerated through the use of the apparatus shown in Figure 1.[9] A clean, empty BOD bottle and the test bottle were connected with the adapter; by allowing about half of the liquid to pass into the new bottle, followed by moderate shaking, the DO level could be returned to about 7 mg/l. Once this reaeration was accomplished, all of the liquid was returned to the original bottle, and a new DO value was determined and recorded. Insertion of the DO probe displaced some water from the test bottle, and one or two marbles were dropped into the bottle to fill this void. This also was done when samples were withdrawn for nitrite-nitrate analyses.

Because ammonia nitrogen and organic nitrogen contained in the test system can be oxidized biochemically to nitrites and nitrates resulting in an oxygen demand, samples (2 ml) were analyzed routinely for these species during the course of the test. No attempt was made to inhibit nitrification because nitrogen analyses permit a nitrification correction to be calculated. Nitrification inhibitors were avoided because tests revealed that several of the published inhibitors could adversely affect the biodegradation of test chemicals.[9]

Each nitrification sample was initially screened by an ultraviolet absorption technique using a recording spectrophotometer*.[7] Samples showing an absorbance band in the wavelength range from 160 to 240 mμ were tested colorimetrically for nitrite-nitrate content using a modified brucine method described in "Standard Methods."[8] Principal modifications were the omission of sulfanilic acid from the color reagent so that both nitrite and nitrate would react with the brucine and adjustment of the procedures to permit smaller sample sizes could be made.

Results of the biodegradation tests were expressed in terms of percent bio-oxidation, defined as follows:

$$\text{percent bio-oxidized} = \frac{100(O'_s - O_b)}{C_x \cdot T_hOD}$$

where

O'_s = cumulative oxygen uptake for the oxidation of the carbonaceous material in the test sample bottle from day zero to the day of interest, mg/l.

O_b = cumulative oxygen uptake in a blank, containing the same amount and type of microbial seed as the test sample bottle, from day zero to the day of interest, mg/l.

C_x = initial concentration of compound being tested, mg/l.

T_hOD = theoretical oxygen demand or the weight ratio of oxygen required per milligram of compound for complete conversion of the compound to carbon dioxide and water. Organic nitrogen was assumed to be in the form of ammonia following oxidation of the carbonaceous portion of the material.

* DK-2-A Ratio Recording Spectrometer, Beckman Instruments, Fullerton, Calif.

TABLE I.—Summary of Biodegradation Data

Product Tested	Water Solubility (g/100 ml of solution)	Theoretical Oxygen Demand (mg/mg)	Measured COD (mg/mg)	Biodegradability (% Bio-Oxidation)											
				Fresh Water (BOD Dilution Water)								Salt Water (Synthetic)			
				Non-Acclimated (Days)				Acclimated (Days)				Non-Acclimated (Days)			
				5	10	15	20	5	10	15	20	5	10	15	20
Acetic acid (neutralized)	C	1.07	1.09	76	82	85	96				—	66	88	88	100
Acetone	C	2.20	2.00	56	76	83	84				—	38	67	69	76
Benzene	0.17	3.10	1.40	24	27	24	29	58	67	76	80	—	—		
n-Butanol	7.7	2.59	2.45	68	87	92	92					45	68	71	82
n-Butyl acetate	0.68	2.20	2.32	58	68	70	83	—	—	—	—	40	52	56	61
Cumene	0.05*	3.50	1.13	40	62	63	70	—	—	—	—	—	3	3	2
Di-decylphthalate	0.2	2.69	2.50	1	1	1	0	7	10	3	7	8	0	0	29
Diethanolamine	95	1.52	1.47	17	72	81	88					2	60	60	76
Diethylene glycol	C	1.50	1.49	5	8	13	30	43	55	61	67	3	7	7	14
Diethylene glycol monoethyl ether	C	1.90	1.74	17	71	75	87	—	—	—	—	11	44	57	70
Diethylenetriamine	C	1.55	1.57	4	4	4	0	23	46	51	70	—	—	—	—
Di-2-ethyl-hexyl phthalate	0.2	2.58	2.74	0	0	—	0	13	0	6	23	—	—	—	—
Diisobutyl ketone	0.05	2.93	2.88	4	39	57	88		—	—	—	—	4	9	18
Epoxidized soybean oil	0.03*	—	2.24	1	1	0	0	4	—	13	24	—	—	—	—
Ethanol	C	2.10	1.99	74	74	95	84	—	—	—	—	45	68	72	75
Ethyl acetate	8.5	1.82	1.69	62	62	69	69	—		—	—	47	54	55	60
Ethyl acrylate	2	1.92	1.71	28	32	32	35	66	74	76	79	11	53	53	53
Ethylenediamine	C	1.33	1.30	24	44	55	47	36	45	56	70	2	14	16	16
Ethylene dichloride	0.81	0.97	1.025	0	18		—		—	—	—	7	7	7	15
Ethylene glycol	C	1.30	1.29	34	86	92	100				—	20	60	65	77
Ethylene glycol monobutyl ether	C	2.30	2.25	26	74	82	88					29	64	70	75
Ethylene glycol monoethyl ether acetate	23	1.81	1.76	36	79	82	80					10	44	59	69
Ethylene glycol monoethyl ether	C	1.80	1.98	36	88	92	100	—	—	—	—	5	42	50	62
Ethylene glycol monomethyl ether	C	1.68	1.64	30	62	74	88	—	—	—	—	6	18	23	39
Ethyl hexanol	0.07	2.95	2.79	26	75	78	86	—	—	—	—	58	64	84	100
Ethyl hexyl acrylate	<0.01	2.60	2.48	0	24	29	30	9	15	23	40	9	14	14	35
Ethoxy triglycol	C	1.89	1.78	8	47	63	71	—	—	—	—	1	10	12	22
Formic acid (neutralized)	C	0.35	0.36	48	54	66	68	51	47	39	60	62	91	92	95
Hexylene glycol	C	2.30	2.11	2	29	47	48	55	85	88	90	0	7	9	63
Isobutanol	8.5	2.59	2.39	64	73	76	72	46	71	75	81	—	—	—	—
Isobutyl acetate	0.63	2.20	1.43	60	74	79	81	—	—	—	—	23	35	37	37
Isodecanol (mixed isomers)	0.014*	3.03	2.87	24	44	42	39	14	26	45	32	6	17	34	40
Isophorone	1.2	2.78	2.64	0	13	47	42	—	—	—	—	1	6	7	9
Isopropanol	C	2.40	2.30	28	77	80	78	—	—	—	—	13	42	60	72
Isopropyl acetate	2.9	2.04	1.67	61	72	74	76	47	53	54	62	14	39	43	49
Linear alkyl benzene	0.04*	3.18	2.31				—	16	5	5	11	0	3	6	6
Methanol	C	1.50	1.50	76	88	91	95				—	69	84	85	97
Methyl amyl acetate	0.13	2.44	2.36	20	87	62	69	—	—	—	—	11	22	52	67
Methyl amyl alcohol	0.62*	2.82	2.64	50	72	90	94	—	—	—	—	—	—	—	—
Methyl ethyl ketone	26.8	2.44	2.23	76	82	84	89	—	—	—	—	32	62	63	69
Methyl isobutyl ketone	1.9	2.70	2.40	56	66	69	69	—	—	—	—	15	46	50	53
Octyl epoxytallate	0.06*	—	2.25	—	—	—	—	1	1	3	6	4	19	57	24
Phenol	8.4	2.40	2.30	90	89	87	96	—	—	—	—	55	74	78	86
Primary amyl acetate	0.2	2.33	2.22	64	76	67	72	—	—	—	—	35	65	69	87
n-Propanol	C	2.40	2.18	64	76	81	75	—	—	—	—	43	64	67	73
n-Propyl acetate	1.03*	2.04	2.04	62	80	75	72	—	—	—	—	50	70	70	78
Propylene glycol	C	1.68	1.63	62	68	75	79	—	—	—	—	55	72	73	83
Styrene	0.03	3.07	2.88	65	65	78	87	—	—	—	—	8	12	21	80
Tetrahydronaphthalene	0.035*	3.15	2.50	—	—	—	—	—	—	—	—	3	3	2	3
Tetraethylene glycol	C	1.65	1.64	4	10	15	22	9	58	71	88	—	—	—	—
Toluene	0.08*	3.13	1.40	53	62	70	80	73	74	80	86	3	2	3	63
Triethanolamine	C	1.61	1.52	8	9	45	66	—	—	—	—	56	65	65	69
Triethylene glycol	C	1.60	1.60	4	10	17	24	32	64	77	86	4	6	6	23
Vinyl acetate	2	1.67	1.39	43	44	41	32	62	70	66	72	51	61	69	58

* Solubility measured at 25°C in synthetic seawater.

Example:

$$(CH_3CH_2)_2NH + 6O_2 \rightarrow 4CO_2 + NH_3 + 4H_2O$$

$$T_hOD = \frac{6 \times 32}{73} = \frac{2.63 \text{ mg } O_2}{\text{mg Diethylamine}}$$

Based on experimental experience, the total nitrite-nitrate measured by the technique was assumed to be principally nitrite. Thus, the oxygen consumed in the formation of nitrites was subtracted from the total sample oxygen uptake to obtain the net carbonaceous oxygen demand. This correction was necessary in only one case, ethylenediamine. Bio-oxidation data on all the test chemicals are summarized in Table I along with data on several other parameters. Bio-oxidation values in freshwater test systems are presented graphically in Figures 2 through 8, where differ-

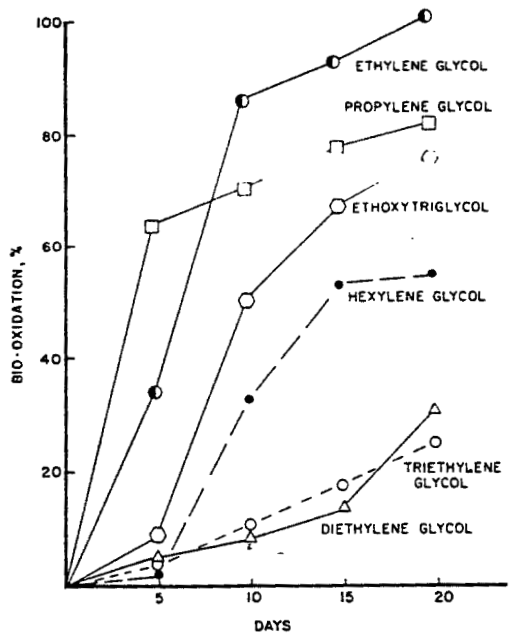

FIGURE 2.—Typical unacclimated biodegradation curves for glycols.

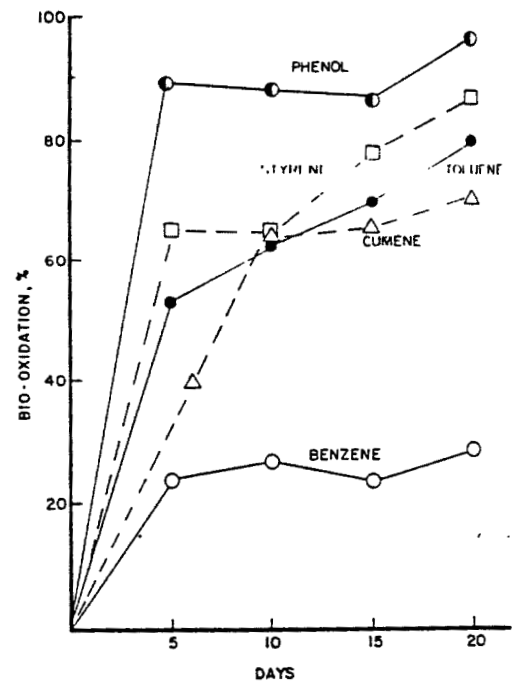

FIGURE 4.—Typical unacclimated biodegradation curves for aromatics.

ences in rate and extent of biodegradation can be seen within a chemical class as the molecular weight and structure vary.

Although the bio-oxidation values reported are based on calculated T_hOD, chemical oxygen demand (COD) measurements were made on these materials and are pre-

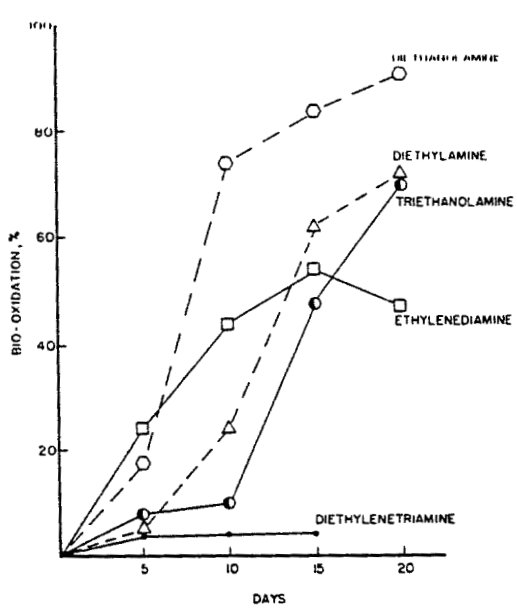

FIGURE 3.—Typical unacclimated biodegradation curves for amines.

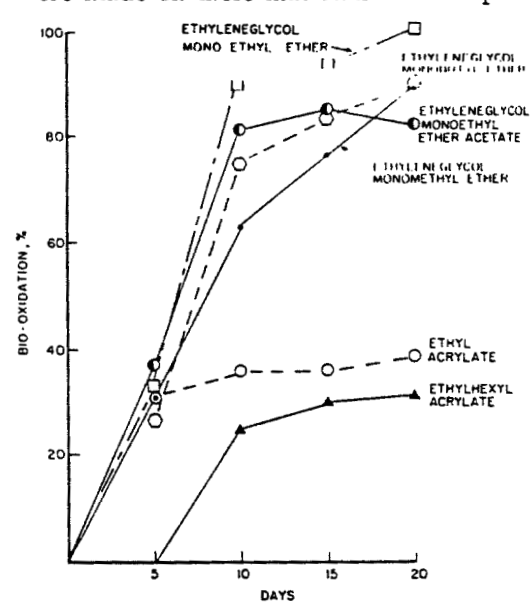

FIGURE 5.—Typical unacclimated biodegradation curves for glycol ethers and acrylates.

sented in Table I for comparison with the T_hOD. The chromic acid reflux procedure described in "Standard Methods"[8] was used for these determinations that, with the exception of a few aromatic hydrocarbons, show good agreement with the T_hOD.

Water solubility data are also presented in Table I. The BOD tests on compounds of low miscibility used a few significant modifications. The dilution water was sparged with pure oxygen to produce an available DO level of 30 to 35 mg/l. After the seed was added to the BOD bottle, the bottle was completely filled with the highly oxygenated dilution water. The pure chemical to be tested was drawn into a microhypodermic syringe; the volume used an all cases was 3.0 μl to provide an in-bottle concentration in the 300-ml BOD bottle of approximately 10 mg/l (corrections for specific gravity were made during calculations) or a maximum potential DO uptake of about 30 mg/l. The needle of the syringe was wiped clean of excess material and inserted into the BOD bottle well below the liquid surface. Experience indicated that the BOD bottle could be tilted approxi-

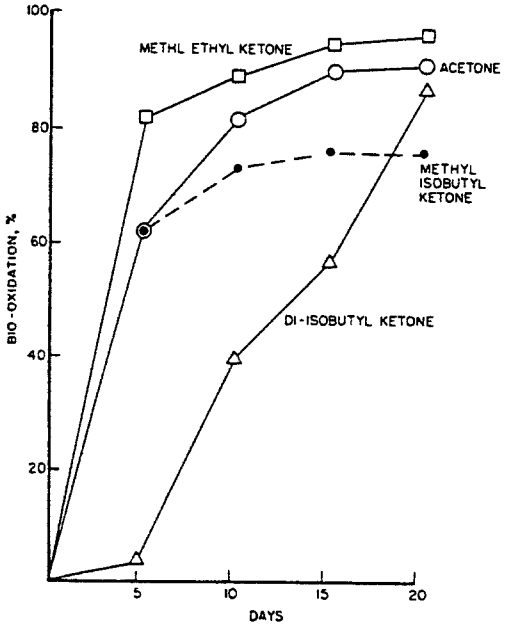

FIGURE 6.—Typical unacclimated biodegradation curves for ketones.

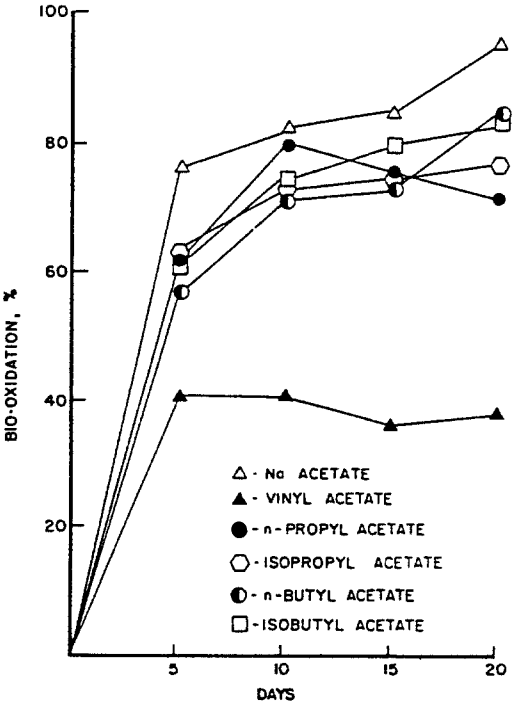

FIGURE 7.—Typical unacclimated biodegradation curves for acetate esters.

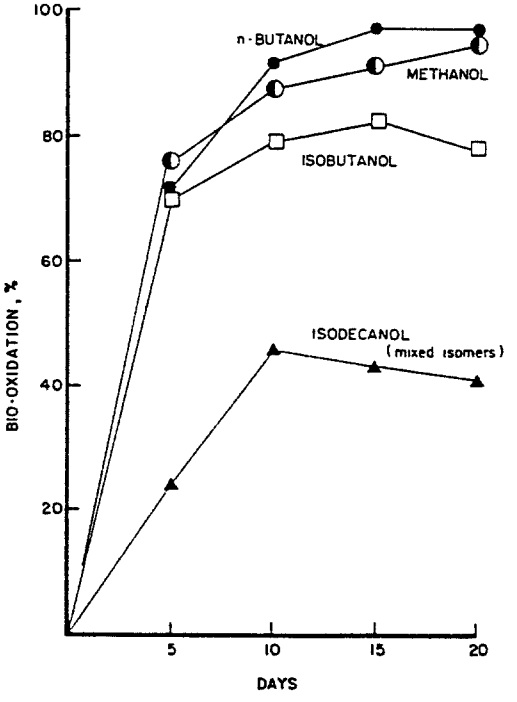

FIGURE 8.—Typical unacclimated biodegradation curves for alcohols.

TABLE II.—Effect of Acclimation

Material Tested	Bio-oxidation at 20 days (%)	
	Non-acclimated	Acclimated
Benzene	29	80
Diethylene glycol	30	67
Diethylenetriamine	0	70
Ethyl acrylate	35	79
Ethylenediamine	47	70
Hexylene glycol	48	90
Tetraethylene glycol	22	88
Triethylene glycol	24	86
Vinyl acetate	32	72

mately 30 deg from the vertical without spilling the dilution water while the injected sample rose and was trapped in the round "shoulder" of the BOD bottle. The glass stopper could then be inserted without any great haste.

Four bottles were prepared for each slightly soluble material being tested. Appropriately seeded blanks and glucose standards were prepared during each test run using the same dilution water used for the test samples. The high initial DO concentrations required the use of the Winkler wet-chemical method[8] at double reagent dosage for the determination of DO levels; the meter used in the earlier studies would read a maximum concentration of only 15 mg/l. Because the Winkler test destroys the test sample, one bottle from each compound test series was analyzed at 5-day intervals. Two seeded blanks were also analyzed at each 5-day interval; all glucose standards were analyzed on the fifth day of a test run. Samples were taken prior to the Winkler analysis for nitrite-nitrate analysis, using the methods previously described. Although ethylene glycol is highly soluble, it was evaluated by both the soluble test system and this high DO, pure-chemical-addition system as a check of the technique. The results agreed within the limits of the test procedure. Consequently, these data are presented in Table I without any method distinction.

Microbial acclimation studies. Materials that were not degraded at least 50 percent, as indicated by the ratio of observed oxygen demand to T_{∞}OD, by the twentieth day in the initial screening test were subjected to treatment designed to develop acclimated microbial cultures. Each 1-l acclimation cell was initially charged with 150 ml of an equal-volume mixture of: (*a*) two biologically treated petrochemical effluents, (*b*) settled domestic wastewater, (*c*) Kanawha River water taken from the Winfield Locks at Winfield, W. Va. (the Kanawha River receives the waste effluents from numerous industrial and domestic sources above this point), and (*d*) soil.

Each acclimator was filled to 800 ml with BOD dilution water. One pure compound was added to each of the aerated acclimation cells to provide an initial concentration of 40 mg/l; cells were re-fed at approximately 3-day intervals. The feeding process was accomplished as follows: shut-off air and allow solids to settle from the bulk liquid for 30 min, remove 250 ml of the supernatant from each cell and refill to the 800-ml mark with BOD dilution water, add test chemicals to respective acclimation cells to provide an increase in concentration of 40 mg/l, and turn on air. Acclimation cells were operated for a period of 45 to 60 days.

The effectiveness of the procedure was evaluated by using supernatant liquid from each acclimator as the seed for BOD tests with the respective compounds. There were 16 materials that showed improved rates of biodegradation using acclimated microbial seed as compared with domestic wastewater seed (Table I).

Following these tests, the mode of operation of the acclimation cells that did not produce sufficiently (degradation $\geq$ 50 percent) acclimated seed was modified. Instead of feeding the acclimation cells every 3 days, as previously described, the acclimators were reseeded with 25 ml of the previously described mixture of seed materials every 3 days. In addition, the cells were fed their respective chemicals about every 12 days. The effectiveness of this procedure was again evaluated by performing biodegradation tests with supernatant from the acclimators.

Through freshwater acclimation 9 of the

20 initially resistant materials reached greater than 50 percent bio-oxidation in the 20-day BOD test using the individually acclimated seed. These data are presented in Table II. Effectiveness of acclimation is presented graphically in Figure 9 for hexylene glycol and tetraethylene glycol.

Seawater biodegradation. Materials that were demonstrated to be biodegraded to an extent greater than 50 percent in 20 days in the freshwater screening tests were subjected to biodegradation tests in synthetic seawater (Table III). The nutrient salts and buffers for BOD dilution water recommended in "Standard Methods"[8] were added to the artificial seawater prior to use in biodegradation tests. With the exception of the seed source, the "seawater" biodegradation tests were performed in the same manner as were the freshwater tests. However, an oxygen meter that permitted correction for high salinity water was used for DO determinations where instrumental analysis was performed.

The seed used in the seawater biodegradation tests was developed in seawater taken from Lavaca Bay, Texas. This seed source was maintained by adding small amounts of settled raw wastewater about every 3 to 4 days as a source of substrate, seed bacteria, and growth factors. Evaporative losses were replaced with distilled water.

Table IV shows those materials that degraded well in freshwater with an unacclimated microbial seed but failed to degrade well when tested in seawater. Stanier *et al.*[10] indicated that halophilic bacteria frequently function more slowly than do freshwater microorganisms. Thimann[11] further stated that the biochemical capabilities of halophilic bacteria are similar to those of freshwater origin. Thus, biodegradation, though slower than in a freshwater system, would be anticipated for chemicals that degrade in freshwater. Four of the six materials listed in Table IV showed slow but rather constant rates of degradation through the 20-day test period in seawater. Cumene showed essentially no degradation in sea-

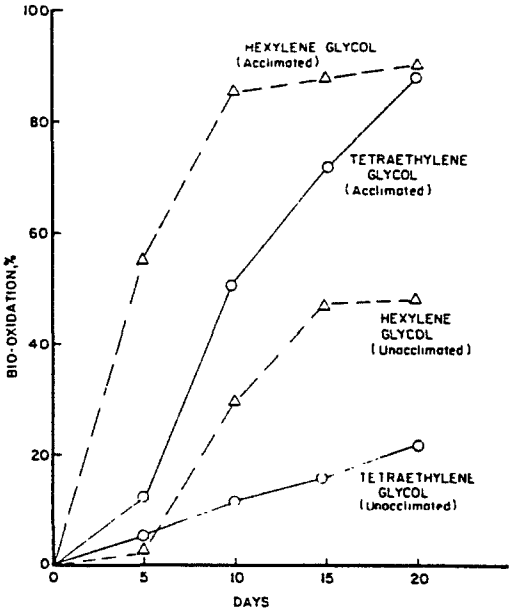

FIGURE 9.—Comparison of acclimated and unacclimated biodegradation rates.

TABLE III.—Von Flack's Formula for Artificial Seawater*

Constituent	Amount (gm)
Sodium chloride, NaCl	557.37
Calcium sulfate, $CaSO_4$	27.20
Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$	63.36
Magnesium chloride, $MgCl_2$	168.30
Potassium chloride, KCl	15.84
Magnesium bromide hexahydrate, $MgBr_2 \cdot 6H_2O$	3.14

* Dissolve in 20 l of distilled water in the order indicated.

TABLE IV.—Chemicals Exhibiting Low Degradation in Seawater

Chemical Tested	Bio-oxidation in 20 days (%)	
	Freshwater	Seawater
Cumene	70	2
Ethoxytriglycol	71	22
Isobutyl acetate	59	37
Diisobutyl ketone	88	18
Ethylene glycol monomethyl ether	88	39

water while isobutyl acetate reached a constant removal percentage of about 37 percent within 15 days and did not increase beyond that level.

Brine shrimp. The brine shrimp, *Artemia salina*, is a member of the class Crustacea, growing to about 12 mm in length, and having worldwide distribution. In North America the organism occurs naturally in Epsom Lake, Wash.; in natural salterns along the California coast from San Francisco southward; in Mono Lake and Little Soda Lake, San Luis Obispo, Calif.; and in Great Salt Lake, Utah.[12]

When a culture is established, it is continued by viviparous reproduction with the first batch of eggs from each female growing to the nauplius stage in a brood pouch. Subsequent batches of eggs, from the same female are resting eggs and must be dried before hatching. Dried eggs remain viable for several years and can be hatched readily within 40 to 48 hr providing large numbers of nauplii of the same age and history. This feature makes the brine shrimp particularly attractive as a toxicity bioassay organism for the saltwater environment.

The use of brine shrimp for toxicity bioassays has been reported by several investigators.[13-15] The possibility of standardizing a brine shrimp bioassay test for marine toxicity evaluation recently has been reported.[16]

Preliminary toxicity studies. The technique suggested by Hood[13] was used in the preliminary brine shrimp toxicity studies. Hood had shown that the seawater waste concentration that resulted in a decrease in oxygen uptake rate by the brine shrimp was similar to the TL_m for two species of marine fish. Therefore, the effect of the various pure compounds at several concentrations on the oxygen uptake rate of brine shrimp was envisioned as the method to be used for the evaluation of toxicities in this project.

Three rather serious difficulties were encountered with this technique. First, certain chemicals caused appreciable increases in the oxygen uptake rate of the brine shrimp. Materials such as ethanol evidently passed through the membranes of the filter-feeding brine shrimp, were oxidized in the shrimp digestive tract, and caused an increased respiration rate. Second, contamination of the shrimp suspension with yeast and possibly bacteria resulted in complete DO depletion, even in the controls, in two of the test runs. An attempt to wash the shrimp with fresh seawater prior to use was only partially successful. Finally, the differences in uptake rate between controls and dosed shrimp were relatively small. These problem areas made the results erratic and interpretation of the data very difficult. Therefore, this bioassay technique was not used further in this study.

Hatching procedure. The hatching technique used in these studies used five 1-l, wide-mouth bottles joined with black plastic tape with a small window left open at each end (Figure 1). Each bottle was equipped with a fritted-glass air diffuser and 1 g of eggs ‡ was placed in each bottle filled with synthetic seawater (Table III). The water was aerated until hatching was completed; aeration then was stopped to permit the unhatched eggs to settle out. A light source was placed at the window; the viable shrimp concentrated in the beam of light and were drawn off with a medicine dropper and placed in a separate container. The number of shrimp per milliliter was determined by filtering a known volume through a gridded membrane filter and counting the shrimp under a low-power microscope. Experience revealed that the shrimp could be pipetted reproducibly into test bottles provided that the shrimp suspension was kept thoroughly mixed with a magnetic stirrer. Dilution to attain the desired shrimp titer in the stock suspension also was found to be feasible.

Toxicity screening tests. Tarzwell[14] and others[12, 15] have reported on the determination of median tolerance limits, defined as the concentration at which 50 percent mortality is experienced in a set time period, for specific materials using brine shrimp. Tarzwell was principally concerned with

‡ Carolina Biological Supply Co., Burlington, N. C.

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determining the toxicity of oil dispersants, and, therefore, his methodology did not apply directly to the types of materials analyzed in this study. Where possible, however, his methods were used.

Before determination of median tolerance limits, the range in which each material was toxic to brine shrimp was measured as follows:

1. Shrimp eggs were placed in the previously described hatching device approximately 48 hr in advance of the time that test shrimp would be required.
2. After shrimp hatching was complete, the shrimp were collected and diluted to provide a suspension of shrimp with a titer of 30 to 50 shrimp/ml.
3. Appropriate volumes of a 1 percent solution of each chemical were placed in 150-ml, wide-mouth bottles to provide concentrations of 100, 1,000, and 10,000 mg/l when diluted to 99 ml. Relatively insoluble materials were tested at saturation levels in seawater. Artificial seawater was added to provide a total volume of 100 ml.
4. The brine shrimp suspension (1 ml) was pipetted into each bottle containing test chemicals and into bottles containing only seawater (controls). The bottles were then loosely capped and incubated at ambient temperature (24.5°C) for 24 hr.‡
5. At the end of the 24-hr incubation period, the numbers of live and dead shrimp were noted by viewing the shrimp in the bottle aided by a colony counter.§

Brine shrimp have branch-like swimming appendages, phyllopodia, which also serve as gills. These appendages flutter very rapidly when the organism is healthy, and the flutter rate decreases when the shrimp are distressed. No movement of the phyllopodia indicates death. Another sign of distress at near lethal concentrations was the clinging together of two or more shrimp.

The results of the screening test are shown in Table V. These tests indicated that 14 materials were not lethal to brine shrimp at concentrations of 10,000 mg/l, 12 were lethal between 1,000 and 10,000 mg/l, 14 were lethal in the range from 100 to 1,000 mg/l, and 15 were lethal at less than 100 mg/l. Of the relatively insoluble materials, 12 were lethal at concentrations below their saturation level in seawater, and two phthalates were not toxic at saturation concentrations.

Median tolerance limits. The results obtained in the screening tests were used to establish the dosage concentrations to be used in determining the 24-hr TL_m:

Toxicity Range (mg/l)	Dosage Concentration for TL_m Determinations (mg/l)
>10,000	No TL_m determination
1,000–10,000	1,000, 1,800, 3,200, 5,600, 10,000
100–1,000	100, 180, 320, 560, 1,000
<100	10, 18, 32, 56, 100
<10	1, 1.8, 3.2, 5.6, 10
>saturation	No TL_m determination
<saturation	10, 25, 50, 75, 100% of saturation

The TL_m determinations were conducted on a dosage basis in the same manner as were the screening tests except that the relatively insoluble materials were tested at various percentages of saturation. By plotting the percent survival versus the test dosage concentration (logarithmic scale), a straight line plot is obtained (Figure 10). The concentration at which this line crosses 50 percent survival is reported as the TL_m.

The 48-hr TL_m for selected materials was determined for comparison with values reported in the literature for species of freshwater organisms (Table VI). Correlation on an order-of-magnitude basis for extension of the results to freshwater situations seems feasible (Figure 11). Recent bio-

‡ In brine shrimp bioassays conducted since the conclusion of this project, R. G. Gossett and J. R. Dawson of Union Carbide Corporation have developed a technique for achieving faster, more accurate counts of brine shrimp. After preparation of the desired test concentrations and addition of brine shrimp, the test solutions are transferred to and incubated in 150-mm × 25-mm gridded petri dishes with loosely fitting covers. Observation and organism counts are then accomplished by positioning each petri dish on an overhead projector and counting the projected images of the brine shrimp on a screen. This is done both before and after incubation.

§ Darkfield Quebec Colony Counter, American Optical Company, Buffalo, N. Y.

TABLE V.—Tolerance Limits for Brine Shrimp

Compound	24-Hr TL_m Dosage (mg/l)	Compound	24-Hr TL_m Dosage (mg/l)
Screening test, TL_m < 100 mg/l		n-Propyl acetate	820
Acetic acid	42	Screening test, TL_m 1,100–10,000 mg/l	
Amyl acetate (prim)	53	Acetone	2,100
Benzene	66	n-Butanol	2,950
Diisobutyl ketone	65	Diethanolamine	2,800
Ethylacrylate	12	Ethyl acetate	1,590
Ethylenediamine	14	Ethylene glycol monoethyl ether acetate	4,000
2-ethylhexanol	19	Hexylene glycol	5,900
2-ethylhexylacrylate	72	Isobutanol	1,400
Isodecanol	3.4	Isobutyl acetate	1,200
Linear alkylbenzene*	33	Methyl ethyl ketone	1,950
Sodium lauryl sulfate†	3.6	Methyl isobutyl ketone	1,230
Styrene	68	n-Propanol	4,200
Tetrahydronaphthalene	78	Triethanolamine	5,600
Toluene	33	Screening test, TL_m > 10,000 mg/l‡	
Vinyl acetate	45	Di-decyl phthalates	>saturation
Screening test, TL_m 100–1000 mg/l		Diethylene glycol	
n-Butyl acetate	150	Diethylene glycol monoethyl ether	
Cumene	110	Di-2-ethyl hexyl phthalate	>saturation
Diethylenetriamine	710	Ethanol	—
Ethylene dichloride	320	Ethoxytriglycol	—
Ethylene glycol monobutyl ether	1,000	Ethylene glycol	>20,000
Epoxidized soybean oil	240	Ethylene glycol monoethyl ether	
Formic acid	410	Ethylene glycol monomethyl ether	—
Isophorone	430	Isopropanol	—
Isopropyl acetate	810	Methanol	—
Methyl amyl acetate	230	Propylene glycol	—
Methyl amyl alcohol	370	Tetraethylene glycol	—
Octyl epoxytallate	300	Triethylene glycol	—
Phenol	160		

* 11 to 13-carbon alkyl group.
† Included in test series as a control.
‡ TL_m not precisely determined when screening tests indicated it was >10,000 mg/l.

assay tests with fathead minnows show even closer agreement.

DISCUSSION

Biodegradation. Many definitions of biodegradation have been presented and discussed in the literature. Perhaps the least important of these is the demonstration of some minor hydrolysis or oxidation that cause the compound of interest to lose its chemical identity. A more practical and useful definition of biodegradation is that point in destructive oxidation that results in removal of objectionable properties, such as color, foamability, or toxicity. Biodegradation as defined in this study is the ratio of biochemically consumed oxygen to the stoichiometric oxygen required to convert the carbonaceous portion of the material to carbon dioxide and water. This is perhaps the most rigorous definition of biodegradation.

There are several shortcomings in the test used, particularly as related to interpreting the results. First, that part of the compound incorporated into cellular material is not measured; the bio-oxidation percentages need to be increased appropriately to obtain a biodegradation value. Also, the time limitation of 20 days is based primarily on precedent and practicality. Incomplete oxidation might indicate only that a material is degraded very slowly under the test conditions.

Therefore, in interpreting results, not only bio-oxidation values at 20 days but also the shape of the oxygen uptake curve must be considered. A flat curve during

TABLE VI.—Brine

Compound
Acetic acid
Acetone
Benzene
Butanol
Isobutanol
n-Butyl acetate
Diethanolamine
Diethylene glycol
Ethanol
Ethylenediamine
Ethylene dichloride
Formic acid
Isopropyl acetate
Methanol
Methyl ethyl ketone
Phenol
n-Propanol
Sodium lauryl sulfate
Styrene
Vinyl acetate

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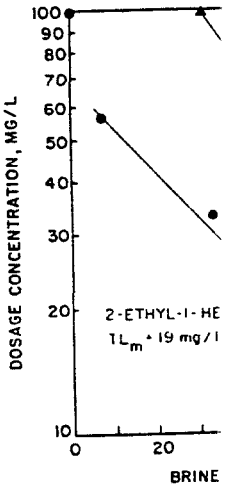

FIGURE 10.—T
limit graphi

TABLE VI.—Brine Shrimp Tolerance Compared with Freshwater Organism Literature Values

Compound	Brine Shrimp TL_m (mg/l)		Literature Value		
	24-hr	48-hr	TL_m (mg/l)	Time (hr)	Organism
Acetic acid	42	32	75	96	Bluegill
Acetone	2,100	-	13,000	48	Mosquitofish
Benzene	66	21	20	48	Bluegill
Butanol	3,000		15–90	48	Minnows
Isobutanol	1,000	600	10–20	24	Goldfish
			1,000–1,400	—	Gudgeon
n-Butyl acetate	150	32	44	48	Daphnia
Diethanolamine	2,800	—	1,850	48	Bluegill
Diethylene glycol	>10,000	—	>32,000	96	Mosquitofish
Ethanol	>10,000	—	>8,000	24	Gudgeon
Ethylenediamine	14	—	30–60	24	Creek Chub
Ethylene dichloride	320	—	160	—	Pinperch
Formic acid	410	—	120	48	Daphnia
Isopropyl acetate	810	110	—	—	—
Methanol	10,000	—	8,000	48	Trout
Methyl ethyl ketone	1,950	—	5,640	48	Bluegill
Phenol	157	56	22	48	Bluegill
n-Propanol	4,200	—	200–500	—	Gudgeon
Sodium lauryl sulfate	3.6	—	7	—	—
Styrene	68	52	63	24	Fathead
Vinyl acetate	45	—	38	24	Fathead

the 10- to 20-day period generally indicates a partial oxidation of all molecules, while a steadily rising curve indicates continued and possibly complete oxidation given sufficient time. For the purposes of this study oxidation greater than 50 percent in 20 days generally was taken to indicate that the material was biodegradable.

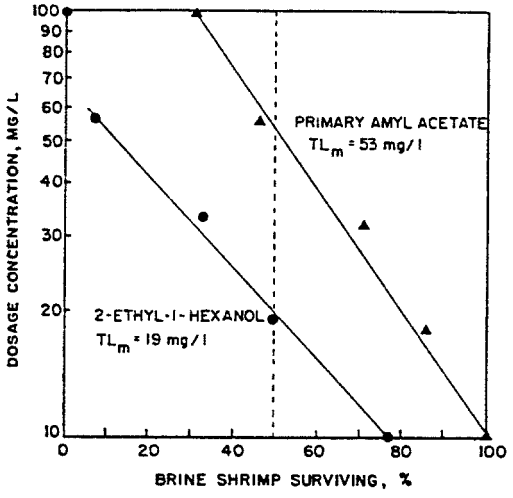

FIGURE 10.—Typical mean tolerance limit graphical determinations.

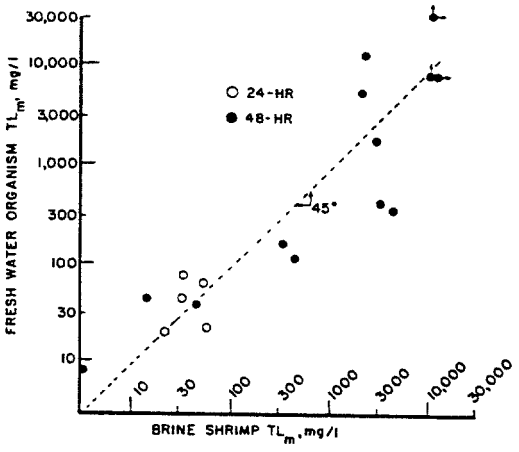

FIGURE 11.—Comparison of brine shrimp results with published data on various freshwater organisms.

Under the conditions imposed by the test procedure, a partial oxidation conceivably could result in an end-product that would not be degraded further by the organisms present in the initial seed but that would degrade in the environment. For example, the organisms capable of degrading some intermediate oxidation product might be so reduced in number during the early stage of the test (when the end-product concentration would be low) that substantial oxidation of the end product might not be observed under the 20-day time limitation. Evidence of the stepwise nature of the oxidation of some materials can be seen in the curves presented, such as that for styrene in Figure 4. A shortage of growth factors, which could be replenished in the natural environment, also could contribute to lower degradation levels.

The shapes of certain curves, such as that for ethylenediamine in Figure 3, raise a question regarding the validity of the direct application of a seed oxygen uptake correction. The percentage of oxidation going through a maximum is believed real and probably results from a difference in oxygen consumption rate by the seed material in the presence of a test chemical and alone in the seed blank.

The generally steep nature of the BOD exertion curves in Figures 2 through 8 in the region around 5 days points up the danger of relying on the 5-day test as an indicator of biodegradability. A small change in the state of acclimation (for example, relative numbers of species and presence or absence of adaptive enzymes) of the seed can cause extreme variations in the 5-day results.

Even though the seed material used in a test contains species capable of degrading a material, the 20-day time limitation may not be sufficient to permit adequate population shifts and development of adaptive enzymes. Prior acclimation of the seed is intended to enrich the culture with species that can degrade the test material. The necessary components of successful acclimation include providing proper environmental conditions with respect to pH, nutrients, growth factors, toxicant levels, and temperature as well as finding an inoculum that contains an organism capable of growth on the material to be tested. A wide variety of inocula were used in this study. Where unsuccessful, the results should not be construed to indicate that no organisms that could degrade the material existed in the environment. However, repeated unsuccessful attempts to develop an enriched culture certainly would indicate that the material should be classified as bioresistant for practical purposes.

The seawater biodegradation tests were not performed with the intent to determine the rate of degradation in the open ocean, but to determine the extent to which organisms capable of survival in the marine environment could degrade the materials. The bacterial content of the initial seed source would be expected to be higher than that of the open ocean. The potential for shift of microbial populations in seawater under laboratory conditions also was recognized. However, the biodegradation obtained using this seed was judged to provide strong evidence that marine species exist that could degrade the material.

In general, the extent of bio-oxidation during 20 days was less in the synthetic seawater than in comparable tests with freshwater. However, 11 materials showed slightly higher final bio-oxidation percentages. With respect to the five materials that had significantly lower bio-oxidation percentages, acclimation or enrichment of seawater cultures would need to be attempted prior to drawing any firm conclusions with respect to the biodegradability of these materials in the marine environment.

Brine shrimp bioassays. The brine shrimp bioassays performed in these studies were acute, static toxicity tests. The principal difficulty associated with this type of testing is the development of appropriate application factors to be used with the test result not only to avoid lethal doses of the material but also to preclude disruption of the reproductive cycle of marine organisms. Recognizing this inadequacy, the results obtained serve the purpose of identifying

problem groups of materials. In combination with biodegradation test results, acute toxicity test results can eliminate a material from serious consideration as an environment pollutant. For example, if a material is held well below its acute toxicity level and at the same time is rapidly degraded to carbon dioxide, water, and cellular materials, concern about chronic effects will be negligible.

APPLICATION OF RESULTS

The materials evaluated in this study are shipped in deep-water tankers.‖ One of the aims of this study was to evaluate the environmental impact of disposal of tanker washings in the open ocean. In making recommendations regarding ocean disposal of tanker washings the principal concern is whether or not the material is biodegradable, so that buildup of residues in the environment can be avoided. The rate of exertion of oxygen demand is certainly inconsequential in the open ocean, and the amount of time allowed for degradation would be very long compared with the 20 days used in the testing procedure. Discharge of materials identified as bioresistant and subject to biomagnification needs to be virtually eliminated.

Once a material is determined to be degradable, the other principal concern is that the local concentration be maintained below the threshold toxicity during dispersion operations. Data on the amount of material remaining in tanks, the wash water use rate, the initial dilution induced by pumping the wash water through a submerged discharge line, the dispersion rate resulting from turbulence imparted by the moving vessel, and the natural dispersion rate in the ocean are required in order to calculate the concentration of chemical in the ocean water at any time after the discharge of washing residues. This concentration must be compared with the acute toxicity level, adjusted by an appropriate application factor, in order to insure that localized toxic conditions are not developed. Dispersion practices in the tanker fleet have been reevaluated to insure that localized toxic levels of tanker washings are avoided.

In applying these data to freshwater problems, ultimate biodegradability is still a major concern, but the rate of degradation assumes a more important role. For example, in the case of an accidental spill of chemical into surface water, a damaging material would be one that degrades very rapidly, imposing a high oxygen demand, and possibly killing aquatic organisms through DO depletion. On the other hand, a material that degraded slowly would have, assuming similar toxicity levels, a lesser effect on the body of water. Along these lines, the Environmental Protection Agency has suggested tentatively that a material that degrades to 25 percent of theoretically complete oxidation within 5 days should be classified as a "hazardous and polluting substance."

In a case where an accidental spill could be routed through a biological waste treatment facility, the more readily degraded materials would be expected to present fewer problems than those materials that degrade only slowly or require prior acclimation, assuming the plant had sufficient hydraulic and oxygenation capacity to handle the spill.

Finally, in the case of materials discharged on a regular basis from processing or formulation activities to biological waste treatment facilities, only materials that are degraded very slowly or not at all with prior acclimation would be undesirable.

Biodegradability data such as those developed in these studies can be used to make judgments for each of the described situations and any of the other innumerable problems that can arise in the production, handling, and transport of petrochemicals and other materials.

The brine shrimp toxicity data are not in a strict sense applicable to freshwater systems. However, a comparison of brine shrimp data with toxicity values reported for freshwater organisms is in most cases quite close. Brine shrimp data still should be used with caution when applied to freshwater problems, but in the absence of any other data, the brine shrimp TL_m values

‖ Union Carbide, New York, N. Y.

should provide an order of magnitude estimate of toxicity.

CONCLUSIONS

With modifications in procedure including periodic reaeration, nitrogen analyses, direct pure compound injection, and pure oxygen sparging, the standard BOD test can be used as a relatively simple, yet reliable, technique for screening the biodegradability of soluble and marginally soluble petrochemicals in both fresh- and seawater environments.

A 20-day test period in the dilution-bottle BOD system is significantly more meaningful and reliable than the shorter 5-day test in evaluating the biodegradability of petrochemicals.

On an overall basis and within a class of compounds the rate and extent of degradation generally decreases with increasing molecular weight, particularly when the number of carbon atoms per molecule exceeds six; at carbon numbers less than six other factors such as substituent groups, branching, and types of linkages seem to exert dominant influence on the rate of degradation.

Acclimation of microbial seed to yield significantly greater than 50 percent bio-oxidation in 20 days was successful for benzene, ethyl acrylate, vinyl acetate, hexyl ene glycol, two ethyleneamines, and three polyglycols, all of which were relatively resistant to degradation using a domestic wastewater seed.

For most of the petrochemicals evaluated, the rate of degradation in the synthetic seawater environment was slower than the rate observed in the freshwater system.

Brine shrimp provide a rapid and precise means of evaluating the acute toxicity of petrochemicals in the seawater environment, for example the TL_m value for the standard sodium lauryl sulfate averaged 3.60 mg/l with a range of 3.30 to 4.15 mg/l for seven determinations.

Comparison of brine shrimp TL_m values with acute toxicity data reported for several freshwater organisms indicates that brine shrimp could be used to estimate order-of-magnitude values of acute toxicity to freshwate species.

The biodegradability and toxicity data developed in these studies can be used to evaluate operations for dispersing tanker washings in the open ocean, to estimate the effects of accidental spills on receiving waters, to project oxygen demand loads on biological treatment systems, and to judge the biological treatability of petrochemical wastes containing the test chemicals.

ACKNOWLEDGMENTS

Credits. The aid of J. R. Dawson in performing many of the laboratory tests and in offering several ideas regarding hatching of the brine shrimp is gratefully acknowledged.

This paper was presented at the 45th Annual Conference of the Water Pollution Control Federation, Atlanta, Ga., October 8–13, 1972.

Authors. At the time this paper was presented, Kenneth S. Price, Gene T. Waggy, and Richard A. Conway were, respectively, environmental engineer, chemist, and group leader, Research and Development Department, Chemicals and Plastics, Union Carbide Corporation, South Charleston, W. Va. K. S. Price is now associated with Clark, Dietz and Associates—Engineers, Inc., Urbana, Ill.

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OPTS-42026
E3-001 (4)

TOXICOLOGY AND APPLIED PHARMACOLOGY 81, 319–327 (1985)

Potentiation of 2,5-Hexanedione Neurotoxicity by Methyl Ethyl Ketone[1,2]

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Received April 11, 1985; accepted July 1, 1985

Potentiation of 2,5-Hexanedione Neurotoxicity by Methyl Ethyl Ketone. RALSTON, W. H., HILDERBRAND, R. L., UDDIN, D. E., ANDERSEN, M. E., AND GARDIER, R. W. (1985). *Toxicol. Appl. Pharmacol.* 81, 319–327. Chronic oral administration of a combination of 2.2 mmol methyl ethyl ketone (MEK) and 2.2 mmol 2,5-hexanedione (2,5-HD)/kg/day, 5 days/week resulted in more rapid onset of motor deficits than did chronic dosing with 2.2 mmol 2,5-HD/kg/day alone. In kinetic studies blood time courses of 2,5-HD were determined in rats in the presence and absence of MEK. Concomitant administration of MEK reduced blood 2,5-HD clearance and increased the area under the curve (AUC) for the blood 2,5-HD. In companion experiments with 2,5-[1,6-^{14}C]HD as a tracer, neural and nonneural tissues were examined 72 hr following the last treatment at Weeks 1, 2, and 3 of chronic administration of 2,5-HD alone or in combination with an equimolar dose of MEK. Rats treated with 2,5-[^{14}C]HD alone or in combination with MEK demonstrated no difference in total or trichloroacetic acid-precipitable radioactivity in blood, in liver homogenates, or in neurofilament-enriched fractions from sciatic nerve and spinal cord. The data support a suggestion that the potentiation of hexacarbon neurotoxicity by MEK is the result of the persistence of the neurotoxic metabolite in the blood and not the enhanced metabolism of parent hexacarbon to 2,5-HD. © 1985 Academic Press, Inc.

The central-peripheral distal axonopathy caused by chronic exposure to *n*-hexane and a number of its substituted analogs has been associated with biotransformation of these hexacarbons to a common neurotoxicant, 2,5-hexanedione (2,5-HD) (Couri and Milks, 1982). The neurotoxicity has such a rigid structure–activity relationship that the syndrome is often identified as "γ-diketone" neuropathy (Spencer *et al.*, 1980). Homologs of the neurotoxic ketones, that are not biotransformed to γ-diketones, are not neurotoxic.

Although the latter class of compounds does not cause "dying-back" neuropathy, at least

[1] A preliminary report of this work was presented at the 23rd Annual Meeting of the Society of Toxicology, March 12–16, 1984, Atlanta, Ga. The opinions and assertions contained herein are the personal ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The experiments conducted herein were performed according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council. This work was supported in part by the Naval Medical Research and Development Command Work Unit, Bethesda, Md.

[2] This paper is a part of a dissertation submitted by W. H. Ralston in partial fulfillment for the Ph.D. degree from Wright State University, Dayton, Ohio.

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0041-008X/85 $3.00
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one of these, methyl ethyl ketone (MEK), enhances the development and increases the severity of neurotoxic signs associated with γ-diketone precursors such as n-hexane (Altenkirch *et al.*, 1982; Takeuchi *et al.*, 1983), methyl n-butyl ketone (MnBK) (Couri *et al.*, 1978), and ethyl butyl ketone (O'Donoghue *et al.*, 1984). Similarly, the enhancement of neuropathic changes has been observed in organotypic cultures of fetal mouse spinal cord–dorsal root ganglion–muscle when these tissues were co-incubated with MEK and *n*-hexane (Veronesi *et al.*, 1984). One explanation is enhanced biotransformation of the precursor hexacarbons to 2,5-HD in the presence of MEK. A study which showed increased serum amounts of MnBK when coadministered with MEK (Abdel-Rahman *et al.*, 1976) could be an alternative explanation for the potentiation of toxicity.

The study reported here was conducted to determine what influence, if any, MEK would have on the relationship between the appearance of clinical neurotoxicity and the presence of 2,5-HD. The results are discussed relative to a presently proposed explanation of a cumulative incorporation of 2,5-HD into the affected axons with irreversible binding to neurofilament proteins (Graham *et al.*, 1982a,b).

METHODS

Male F-344 rats (Harlan Industries, Ind.) weighing between 200 and 320 g were used. Rats in any particular experiment were from the same stock shipment; however, this was not always true for interstudy comparisons. Rats received Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) and water *ad libitum* and were maintained between 20 and 26°C on a 12-hr light/12-hr dark cycle. Rats were dosed orally with a 3-in., 18-gauge curved feeding needle. They received doses of 1 ml/100 g body wt of either deionized water (DI water), MEK, 2,5-HD, or 2,5-HD + MEK in DI water.

2,5-HD (Eastman Kodak, Rochester, N.Y.) was analyzed with a 5985 Hewlett–Packard gas chromatography–mass spectrometer and found to be greater than 95% pure. Radiolabeled 2,5-[1,6-^{14}C]hexanedione (2,5-[^{14}C]HD) was custom synthesized by New England Nuclear (Boston, Mass.) and had a specific activity of 1.55 mCi/mmol. Working solutions of 2,5-[^{14}C]HD were made by diluting 2,5-[^{14}C]HD with unlabeled 2,5-HD. Microliter quantities were counted in a liquid scintillation counter, and the resultant specific activity was determined. MEK (Fisher Scientific Co.) was Fisher Certified grade. All other chemicals were reagent grade.

Neurobehavioral Tests

A sensorimotor behavioral battery consisting of two simple-reflex tests, hind-limb grasp and hind-limb place, and two more complex function tests, balance beam and accelerating rotorod performance, was used for early identification and quantification of neurological deficits leading up to the well-documented end-point of bilateral hind-limb drag (Spencer *et al.*, 1980). The tests were performed biweekly at least 20 hr following the last dose of test substances to avoid acute central nervous system effects.

Twenty rats were randomly assigned to four groups of five animals each and dosed 5 days/week by gavage. Controls were given DI water. The MEK, 2,5-HD + MEK, and the 2,5-HD-alone groups initially were given 4.4 mmol of the appropriate solvent per kilogram per day (4.4 mmol of each solvent in mixed group) for 8 days. At Day 9, the doses were reduced to 2.2 mmol/kg/day (due to rapid weight loss in the combined-treatment group) and continued at that concentration for the remainder of the study. Rats were weighed and evaluated biweekly. The animals were acclimated to the neurobehavioral tasks for 2 weeks prior to the initiation of the dosing regimen.

Hind-limb grasp, hind-limb place, and balance beam tasks were performed as described by Sterman and Sheppard (1982). The accelerating rotorod consisted of an open-top box, measuring 34 in. long × 12 in. wide × 18 in. high, with a plastic screen-covered 4-in.-diameter × 26-in.-long cylinder located with its longitudinal axis 13.5 in. above the floor of the box. The rod was surrounded with spacers 9 in. apart to give compartments for simultaneous evaluation of 3 rats. The linear acceleration of the rotorod was controlled by a programmable automatic motor speed control (PAMSC) linked to a 1/15-hp, 115-V, 13-A Dayton Gearmotor which turned the rotorod by a belt drive. The rats were placed on the rod having an initial rate of 10 rpm and then accelerated linearly to a maximum of 20 rpm over a period of 4 min. The scoring of the neurobehavioral tasks was as follows. For the hind-limb place and grasp reflexes, the number of successive positive responses at three trials/day resulted in a score of up to 3 points per test day. For the balance beam, the score was the average best two of three trials per test day, up to a maximum of 30-sec duration on the beam. A rest period of 10 min was used between trials to eliminate effects of fatigue. Score on the accelerating rotorod was the time reached when the rat fell from the rotorod, up to a maximum of 240 sec. Due to the length of the latter test, only one trial per animal per test day was conducted.

Blood Time Course of 2,5-HD

Two groups of 10 rats each were given either 2.2 mmol 2,5-HD or 2.2 mmol 2,5-HD plus 2.2 mmol MEK/kg/day. Subgroups of 5 animals each were designated as

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acutely or repeatedly treated rats. Repeatedly treated rats were dosed for 6 days prior to the testing. Twenty-two hr prior to 2,5-HD dosing, the rats were anesthetized with an ip dose of ketamine (49 mg/ml) and xylazine (1.8 mg/ml) in a dosing volume of 0.1 ml/100 g body wt. The left ventral portion of the neck was shaved and the external jugular vein isolated and nicked; a 22-gauge 8-in. cut-down catheter (Deseret Co., Sandy, Utah) was inserted with the tip resting just superior to the right atrium. The catheter (filled with 10% heparin in isotonic saline) was ligated in place, the incision closed, and a Velcro jacket fitted to protect the surgery. The following morning, rats were treated with either 2,5-HD or 2,5-HD + MEK. Serial blood samples of 150 μl were drawn at intervals over the next 9 hr. The catheter was refilled with 75 μl of 10% heparin following each sample withdrawal. Blood was placed in capped plastic vials (Cobas Sample Cups, Roche Analytic, Nutley, N.J.), and stored at −40°C until analysis. Loss of 2,5-HD from the blood due to storage at −40°C was examined by freezing aliquots of blood taken from a separate 2,5-HD-treated rat and analyzed over an 8-day period.

Analysis

Blood 2,5-HD was determined in a Hewlett–Packard 5840A gas chromatograph with a 1.8-m × 2-mm-id stainless-steel column packed with Carbopack C/0.1% SP-1000, 80/100 mesh (Supelco, Bellefonte, Pa.). The initial oven temperature of 150°C for 1 min was increased 20°C/min to 2[illegible] and held for 2 min. Flame ionization detector temperature was 250°C, injection port temperature was 225°C, and the carrier gas (N_2) was approximately 39 ml/min.

A 50-μl aliquot of a 0.2 ml methyl n-butyl ketone/1 ml water solution was added as an internal standard (IS) to a 50-μl blood sample from an exposed rat, and standard solutions of 2,5-HD were made from rat blood of unexposed animals spiked with 2,5-HD. A 1.0-μl sample of the blood mixture was drawn into a 10-μl syringe (Hamilton, Reno, Nev.) containing 1.0 μl DI water and injected into the gas chromatograph. Concentrations of 2,5-HD were determined by peak height ratios to standards (2,5-HD:IS) and are presented as milligrams of 2,5-HD per milliliter of blood. The area under the blood curve (AUC) was estimated by Simpson's rule.

Uptake of Radiolabel into Neural and Nonneural Tissues

Course of treatment. Rats, in groups of 15, were treated 5 days/week with either 2.2 mmol 2,5-[^{14}C]HD/kg/day alone or in combination with 2.2 mmol MEK/kg/day for up to 3 weeks. Following decapitation, neural and nonneural [illegible]er and blood serum) tissues were removed from 5 animals of each group 72 hr following the last treatment at Weeks 1, 2, and 3.

Enrichment of neurofilaments from sciatic nerve and spinal cord. Sciatic nerves, including the tibial nerve, extending from the iliac crest to the distal end of the tibia, were excised bilaterally. These were immediately placed in cold isotonic saline and desheathed of adhering muscle, fat, and perineural components by gentle proximal to distal retraction of the tissue with fine forceps and iris scissors. The nerve was blotted dry, weighed, and further processed by the methods of Schlaepfer and Freeman (1978) to obtain a crude homogenate (peripheral nerve I; PN I) and a neurofilament-enriched fraction (peripheral nerve II; PN II). This procedure required subjecting the nerve to osmotic shock by placement into 3 ml of hypotonic buffer [2.0 mM EDTA, 2.0 mM ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), 1.0 mM sodium phosphate buffer, pH 7.0] as described by Schlaepfer (1977). The final neurofilament-enriched pellet was resuspended in 350 μl of phosphate-buffered saline (PBS; 100 mM NaCl, 10 mM sodium phosphate buffer, pH 6.7) to maintain neurofilament integrity (Schlaepfer, 1978).

The vertebral column along with adhering musculature was removed from C2 to L7 and severed at L1. Cord segments, expelled by forcing cold isotonic saline from a 10-ml syringe through the vertebral foramenae, were stripped of membranes and vasculature, blotted dry, weighed, and processed just as the peripheral nerve to obtain a crude spinal cord homogenate (SC I) and neurofilament-enriched spinal cord fraction (SC II).

Blood serum. Serum was obtained from whole blood which was collected in a 12 × 75-mm culture tube, allowed to clot, centrifuged at 3000*g*/10 min, and transferred to a clean tube.

Liver homogenates. The livers were removed, blotted dry, and weighed. A 1-g sample was taken and placed into a 15 × 155-mm glass culture tube with 9 times the volume of cold hypotonic buffer and homogenized for 30 sec (Brinkmann Polytron, Lucerne, Switzerland).

Analysis. Aliquots of PN I, PN II, SC I, SC II, serum, and liver homogenate fractions were removed for protein determination and total and trichloroacetic acid (TCA)-precipitable ^{14}C activity. Protein in peripheral nerve and spinal cord fractions, serum, and liver homogenates was determined by the method of Lowry *et al.* (1951) with bovine serum albumin standards from 0.1 to 1.0 mg/ml.

Total ^{14}C activity of the various fractions was determined by solubilizing 100-μl (50 μl for serum) aliquots in 600 μl of tissue solubilizer (Protosol, New England Nuclear), allowing them to stand overnight with subsequent addition of scintillation fluid (Econofluor, New England Nuclear), and counting them in a Packard Tri-Carb 2660 liquid scintillation system with internal standardization.

TCA-precipitable ^{14}C activity was determined in the same manner except that 2.0 ml of 10% TCA was added to the samples in 12 × 75-mm glass culture tubes which were allowed to stand overnight. These were centrifuged at 3000*g* for 20 min, the supernatant fraction was decanted, and the tubes were drained over absorbent paper. The protein pellets were solubilized and counted as described for total activity.

Statistics

Analysis of variance (one-way ANOVA) and small sample *t* test for significant differences between treatment

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means were calculated with an EPSON QX-10 desk-top computer using Microstat (Ecosoft, Inc., Ind.) software.

RESULTS

Neurobehavioral Performance in Treated Rats

Rats treated with 2.2 mmol each of 2,5-HD + MEK/kg/day demonstrated earlier deficits in the simple reflex tasks, hind-limb place (Fig. 1A) and grasp (Fig. 1B), than those rats treated with 2.2 mmol 2,5-HD/kg alone. Animals given DI water (controls) or 2.2 mmol MEK/kg/day alone retained reflexes throughout the experiment. Similarly, animals receiving the combined treatment exhibited earlier deficits in performance on the balance beam (Fig. 2) and the accelerating rotorod (Fig. 3) as compared to rats receiving HD alone. Consistent with the earlier deficits in neurobehavioral performances, the 2,5-HD + MEK group developed bilateral hind-limb drag in about one-half the time as the rats treated with an equimolar dose of HD alone (Table 1).

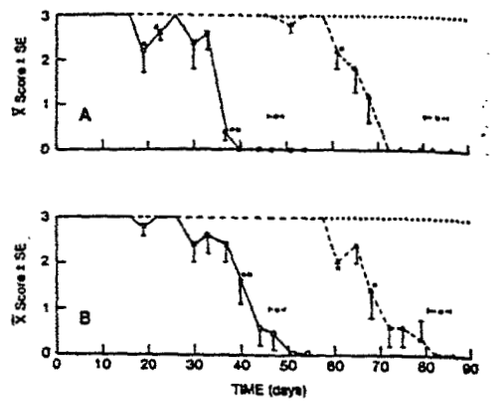

FIG. 1. Simple reflex scores: The average simple reflex scores are plotted against days of exposure to MEK, 2,5-HD (---), 2,5-HD + MEK (—), and water vehicle controls. (See Methods for doses and treatment schedule.) MEK and control animals are represented by the extended dotted line at the top of each graph. (A) Mean daily score ± SE (bars) for hind-limb place by treated and control rats. (B) Mean daily score ± SE (bars) for hind-limb grasp in treated and control rats. The initial statistically significant ($p < 0.05$.) differences between treated and control rats are indicated by a single asterisk. (It is to be understood that significant differences persisted thereafter.) The initial statistically significant ($p < 0.05$) difference between combined treatment and 2,5-HD alone is indicated by a double asterisk. The data represent the mean values for 5 animals ($n = 4$ after days 45 and 83 for 2,5-HD + MEK and 2,5-HD-alone groups, respectively). The average day to onset of bilateral hind-limb drag for each group is indicated by asterisks and standard error bars parallel to the abscissa (⊢ • ⊣).

Blood Concentration and Elimination of 2,5-HD

Blood clearance of 2,5-HD in rats treated once (acutely) or repeatedly for 7 days with 2,5-HD + MEK was slower than that in rats treated with an equimolar dose of 2,5-HD alone (Fig. 4). There were no significant differences in blood amounts of 2,5-HD between treatment groups up to 1 hr postadministration, indicating that the addition of MEK does not significantly alter the gastrointestinal absorption of 2,5-HD or its volume of distribution. From 2 to 9 hr postadministration, elimination of 2,5-HD was slower in the presence of MEK. Rats receiving 2,5-HD + MEK, either once or for 7 days, had a greater AUC than rats receiving 2,5-HD alone during that time period (Table 1).

A relative treatment ratio was calculated for each time to effect the parameter by dividing the days required to observe the effect with 2,5-HD + MEK by those required with 2,5-HD alone. The treatment ratio for blood 2,5-HD elimination curves was determined as the ratio of the reciprocals of the AUC measurements. The relative treatment ratios of neurobehavioral performances, ranging from 1.7 to 2.1 (Table 1), are consistent with the ratios for the time to onset of hind-limb drag. The ratios of the reciprocals of blood AUC are similar to those for hind-limb drag.

Retention of Radiolabel in Neural and Non-neural Tissues

Preliminary studies in this laboratory have indicated that 72 hr following cessation of chronic treatment with 2,5-[^{14}C]HD the majority of the total label detected in the neural

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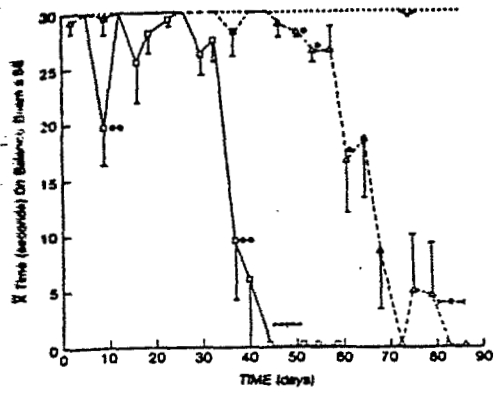

FIG. 2. Balance beam performance. The average balance beam performance scores (sec) of rats treated with vehicle (controls), MEK, 2,5-HD, or 2,5-HD + MEK are plotted against days of exposure. The data are presented as described in Fig. 1. ($n = 5$.)

tissues, blood serum, and liver was recovered in TCA-precipitable fractions. Less than 5% of the total radiolabel detected was recovered in the total lipid fractions of neural tissues. Th results were confirmed in the present stud., wherein 70 to 100% of the total label detected in these tissues was recovered in the TCA-precipitable fractions. Within each treatment group, there was a general increase in activity over time (Table 2). Weekly comparisons demonstrated a limited number of significant differences within tissue fractions between treatment groups (Table 2). By Week 3, the combined-treatment group had a lesser am : of radiolabel associated with the neurofilament-enriched fractions of peripheral nerve (PN II) and spinal cord (SC II), spinal cord crude homogenate (SC I), blood serum, and liver homogenate.

DISCUSSION

Rats treated with the combination of 2,5-HD d MEK developed neurobehavioral perf ance decrements and hind-limb drag earlier than rats treated with an equimolar dose of 2,5-HD. Enhancement of 2,5-HD-induced neurotoxicity by MEK could be correlated with higher tissue exposures of 2,5-HD in animals receiving the combined treatment. The AUC of 2,5-HD in the 2,5-HD + MEK group from 2 through 9 hr postadministration was greater than in those rats treated with the 2,5-HD alone. When a neurotoxic ratio was calculated (Table 1) comparing the AUC of 2,5-HD + MEK to 2,5-HD alone, the values of acutely and repeatedly treated rats were consistent with the neurotoxic ratios calculated for the neurobehavioral performances and onset of hind-limb drag. The relationship of blood AUC to neurotoxic endpoint has previously been demonstrated by Krasavage *et al.* (1980), who demonstrated that the AUC for blood 2,5-HD following exposure to equimolar amounts of hexane, 2-hexanol, MnBK, 2,5-hexanediol, 5-hydroxy-2-hexanone, and 2,5-HD was proportional to the days required to develop hind-limb drag.

Reduced blood clearance of 2,5-HD in the presence of MEK suggests that 2,5-HD elimination was reduced, presumably by competition between MEK and 2,5-HD for metabolic pathways. Following treatment with MnBK, 2,5-HD has been shown to be eliminated from rats and guinea pigs either in a conjugated form or as free 2,5-HD. Acid hy-

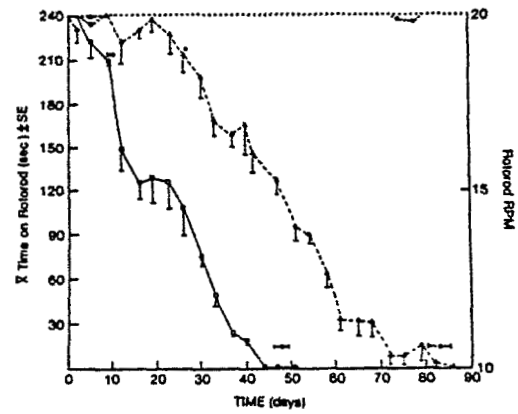

FIG. 3. Accelerating rotorod performance. The average accelerating rotorod performance scores (sec or rpm) of rats treated with vehicle (controls), MEK, 2,5-HD, or 2,5-HD + MEK are plotted against the days of exposure. The data are presented as described in the legend to Fig. 1. On Days 72 and 75, a slight performance deficit occurred in 1 rat treated with MEK. Each treatment group had an $n = 5$.

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TABLE I

COMPARISON OF NEUROBEHAVIORAL, CLINICAL, AND BLOOD CLEARANCE DATA FROM 2,5-HEXANEDIONE- AND 2,5-HEXANEDIONE PLUS METHYL ETHYL KETONE-TREATED RATS

Parameter	HD alone	HD + MEK	Relative treatment ratio[a]
Onset of hind-limb drag			
Total days[b]	82.8 ± 2.6	47.8 ± 1.5*	1.7
Days of dosing[c]	59.6 ± 2.7	34.8 ± 1.1*	1.7
Neurobehavioral performances at 50% of controls (days)[d]			
Grasp	66.7 ± 1.7	39.8 ± 1.0*	1.7
Place	66.0 ± 1.7	34.0 ± 1.0*	1.9
Balance beam	65.6 ± 1.8	36.6 ± 1.4*	1.8
Accelerating rotorod	47.6 ± 2.4	23.0 ± 2.1*	2.1
Area under the blood curve (2–9 hr)			
Acute [(μg/ml) × hr]	456	811	1.8
Repeatedly [(μg/ml)× hr]	549	860	1.6

[a] Ratio of days of HD alone to HD + MEK parameters *or* AUC ratios of HD + MEK blood to HD alone blood.
[b] Total number of days to the onset of bilateral hind-limb drag ± SE.
[c] Number of doses (5/week) to reach the average onset of hind-limb drag.
[d] $\bar{x}$ ± SE.
* Significant difference ($p < 0.05$) between treatment groups (ANOVA).

drolysis of the urine enhanced the recovery of 2,5-HD from rats exposed to n-hexane (Baker and Rickert, 1981), indicating that a substantial portion was conjugated. When guinea pigs were treated with 40 mg MnBK ip, 120 mg MEK ip, or a combination of the two, the amounts of nonhydrolyzed (0.3 mg) to hydrolyzed (2.0 mg) 2,5-HD recovered in the urine of MnBK treated animals shift to 2.9 and 1.8 mg, respectively, upon concomitant treatment with MEK (Couri *et al.*, 1978). Although the absolute amounts of 2,5-HD eliminated in the urine increased upon coadministration (in addition to the urine volumes), the ratio of conjugated to free 2,5-HD decreased. In addition, the ratio of conjugated to free MEK in the combined treatment decreased when compared to amounts recovered in the urine of animals treated with MEK alone.

The reduced 2,5-HD clearance in the presence of MEK suggest that MEK may be competitively inhibiting 2,5-HD metabolism. This is consistent with earlier evidence of Abdel-Rahman *et al.* (1976) who demonstrated that, upon exposure to MEK plus MnBK, blood MnBK amounts increased with continuous exposure (days) while MEK quantities decreased.

Most of the label in neural and nonneural tissues taken 72 hr following the last dose of 2,5-[^{14}C]HD was in the TCA-precipitable fraction, thus bound to protein as opposed to lipid. Measurement of radioactivity in these specimens does not differentiate between parent 2,5-[^{14}C]HD and its radiolabeled metabolites. Several possibilities exist concerning the resultant molecule(s) that have radiolabel as part of their molecular structure. The first possibility is that the label is still attached to 2,5-HD, which is covalently bound to an amino group, forming an imine or enamine. The second possibility is that following binding, rearrangement has occurred with the subsequent formation of 2,5-dimethylpyrrole adduct (DeCaprio and Weber, 1981). This latter reaction forms the basis for the hypothesis of adduct formation on neurofilaments with subsequent protein crosslinking (Graham *et al.*, 1982a,b). The third possibility is that 2,5-HD is metabolized (in the liver) prior to bind-

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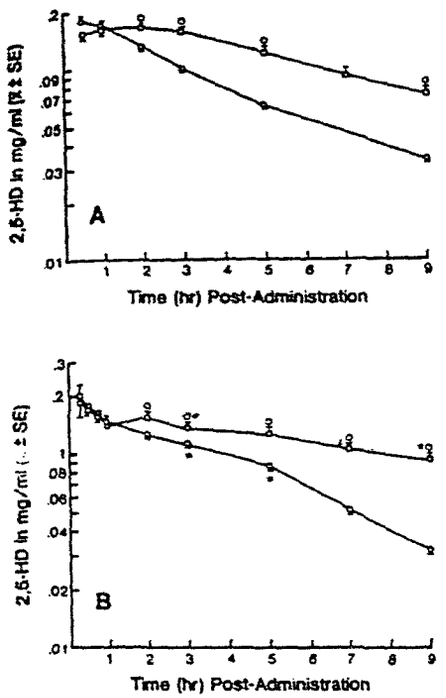

FIG. 4. Blood time course of 2,5-hexanedione. Concentrati[illegible] of 2,5-HD in the blood from rats administered eithe[illegible] -HD (O) or 2,5-HD + MEK (□) either (A) acutely or (B) for 7 days are plotted versus time after administration. Each point ± SE represents the average 2,5-HD concentration in 5 naive and 4 pretreated rats. Statistically significant ($p < 0.05$) differences between naive and pretreated rats given 2,5-HD alone are represented by asterisks. Statistically significant ($p < 0.05$) differences between naive and pretreated rats given 2,5-HD + MEK are represented by stars.

ing. [illegible] iolabel is then removed by decarboxylation, with the resultant $^{14}CO_2$ incorporated into the de novo synthesis of neural and nonneural proteins. The basis for the suggestion that 2,5-[1,6-^{14}C]HD may be metabolized to $^{14}CO_2$ comes from a report by Angelo (1981).

The enhancement of 2,5-HD-induced neuropathy by MEK was not reflected by an increased binding of radiolabel to the nervous tissues or their respective neurofilament-enriche[illegible] [illegible]ctions. This observation is consistent with [illegible] of White and Bus (1980), who showed that 2,5-HD quantities in the sciatic nerves of rats acutely exposed to the combination of n-hexane plus MEK were actually lower than in nerves taken from animals exposed to equivalent concentrations of n-hexane alone. The finding that MEK enhanced 2,5-HD-neurotoxicity provided a means to examine the hypothesis that hexacarbon neurotoxicity is dependent upon the cumulative formation of 2,5-HD-derived adducts on neurofilaments. Few differences in the amount of radiolabel associated with neural tissues were found whether expressed as equivalent nanomoles of 2,5-HD per milligram of protein (Table 2) or total activity per gram of tissue. Exceptions involved a comparatively lesser amount of activity in the combined-treatment group versus the 2,5-HD-alone group. These differences were most evident during the third week of administration. By that time performance scores on the accelerating rotorod of the combined-treatment group were approximately 50% of controls while rats treated with HD alone were not significantly different from controls. Therefore, rats treated with 2,5-[^{14}C]HD + MEK for termination and tissue analysis were probably experiencing a neurological deficit. In addition, nonneural tissues such as serum and liver showed a similar amount of recovered radiolabel. These responses were consistent with a study (DeCaprio *et al.*, 1983) that found pyrrole formation in hen serum peaked and then declined despite continuous 2,5-HD administration. These hens were dosed with 70 and 200 mg 2,5-HD/kg daily, with the high-dose group pyrrole amounts peaking and declining earlier than the low-dose group. This would be similar to our study in which the 2,5-HD + MEK group would be considered analogous to the high-dose group of DeCaprio *et al.* (1983).

MEK may compete with 2,5-HD for critical and noncritical binding sites at the target neurofilament proteins. Aliphatic ketones bind with primary amino groups of proteins and other model compounds *in vitro* (Damodaran and Kinsella, 1980; Anthony *et al.*, 1983; DeCaprio *et al.*, 1982). Although only the γ-diketones have been demonstrated to form substituted pyrroles, it is conceivable that the nonneurotoxic ketone MEK may compete for the binding sites with 2,5-HD. Unpublished

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TABLE 2

EQUIVALENT nmol 2,5-HD/mg OF TCA-PRECIPITABLE PROTEIN IN NEURAL AND NONNEURAL TISSUES OF RATS TREATED WITH 2,5-[^{14}C]HD ALONE OR IN COMBINATION WITH METHYL ETHYL KETONE[a]

Tissue[b]	Treatment	Weeks of treatment 1	2	3
PN I	HD	5.54 ± 0.54	9.25 ± 0.55	13.39 ± 0.91[c]
	HD + MEK	7.40 ± 0.72*	9.88 ± 0.59	14.64 ± 1.36
PN II	HD	6.85 ± 1.50	19.39 ± 1.72	22.72 ± 1.15[c]
	HD + MEK	14.62 ± 2.60*	16.70 ± 0.63	17.99 ± 1.52*
SC I	HD	4.84 ± 0.18	7.61 ± 0.20	10.63 ± 0.25
	HD + MEK	3.66 ± 0.13*	7.09 ± 0.25	9.05 ± 0.36*
SC II	HD	3.22 ± 0.26	4.93 ± 0.21	8.80 ± 0.36
	HD + MEK	3.78 ± 0.25	6.51 ± 0.23*	7.45 ± 0.57*
Blood serum	HD	5.38 ± 0.26	7.84 ± 0.27	9.62 ± 0.30
	HD + MEK	5.84 ± 0.23	6.30 ± 0.11*	7.43 ± 0.49*
Liver homogenate	HD	6.31 ± 0.20	8.20 ± 0.32	9.50 ± 0.50
	HD + MEK	5.25 ± 0.32*	7.67 ± 0.21	7.87 ± 0.54*

[a] Average activity (from 2,5-[^{14}C]HD) expressed as nmol 2,5-HD/mg protein ± SE; $n = 5$ except where noted.
[b] PN I, peripheral nerve crude homogenate; PN II, neurofilament-enriched fraction of peripheral nerve; SC I, spinal cord crude homogenate; SC II, neurofilament-enriched fraction of peripheral nerve.
[c] $n = 4$.
* Statistically significant ($p < 0.05$) differences between treatment groups (ANOVA).

studies from this laboratory have shown that when MEK, in varying amounts, was incubated with a mixture of 2,5-[^{14}C]HD and bovine serum albumin *in vitro*, the amount of covalently bound 2,5-[^{14}C]HD increased with time but varied inversely with the amounts of added MEK. This suggests that MEK competes with 2,5-HD for binding sites. The question then arises as to whether 2,5-HD and MEK compete for the same binding sites or if 2,5-HD binds to specific sites unavailable to MEK and also to general sites competing with MEK. Thus, MEK may compete with 2,5-[^{14}C]HD for noncritical sites thereby filling mainly critical sites with available 2,5-[^{14}C]HD, resulting in a lower total and TCA-precipitable activity in the mixed solvent *in vivo* study.

In summary, the potentiation of n-hexane and MnBK neurotoxicity by MEK cannot be totally based on enhanced biotransformation of parent compound to 2,5-HD. Reduced 2,5-HD clearance in the presence of MEK may be the primary mechanism. These studies neither prove nor disprove the adduct–neurofilament hypothesis. Unless there are a limited number of critical sites on the target neurofilament proteins, and these were masked by a relatively greater number of noncritical radiolabeled sites, the data do not support the hypothesis that the neurotoxicity of γ-diketones is dependent on the quantitative cumulative incorporation of 2,5-HD in target neural tissue neurofilaments.

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10/24/1996 EPA TSCA DOCKET

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Detoxification of specific organic substances by wet oxidation

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The discharge of toxic compounds into the environment has emerged as a topic of mounting concern in recent years. Numerous federal laws have been enacted to control the discharge of these compounds. These laws include the Federal Water Pollution Control Act (PL 92-500), the Toxic Substances Control Act (PL 94-469), the various manufacturing point-source effluent limitations of the Clean Water Act (PL 95-217), and others. The present status of the U. S. Environmental Protection Agency's (EPA) toxic material control strategy has been reviewed recently in the *Journal Water Pollution Control Federation*.[1] Of interest was the list of 65 compounds, or classes of compounds, published in the consent decree of *Natural Resources Defense Council* v. *Train* (8 ERC 2120), June 7, 1976, as having top priority for elimination from effluents. Indeed, final effluent standards for manufacturers of benzidine, aldrin/dieldrin, DDT, and related compounds, endrin and toxaphene, all contained in the list of 65, have been adopted by EPA. Based on chemical structure and function, a number of representative organic compounds from the list was selected for study.

OBJECTIVE

Wet oxidation, the Zimmermann Process,[2-5] is used worldwide to treat municipal and industrial wastes. Operating designs can be varied from low oxidations, for sludge conditioning, to high oxidations, for chemical and power recovery or destruction of highly toxic wastes. The objective of this study was to determine the ability of wet oxidation to achieve maximum destruction of the starting materials and thus detoxify the compounds selected for investigation. High-temperature (320°C) oxidation was investigated initially. More moderate temperatures of 275°C were also investigated, because they closely approximate normal operating conditions of most installations. At the lower temperature a catalyst may be required to bring about the desired detoxification of materials. The results of these laboratory batch studies can be correlated with actual continuous reactor plant operation. These studies are a convenient method of observing effects that can be achieved with commercially designed plants.

METHOD

A single, pure compound in distilled water was used as starting material to facilitate analysis of the compound following wet oxidation. Concentrations of starting material at 5 to 12 g/l were used for the study. Oxidations were carried out in a titanium 3.8-l (1-gallon) magna-drive stirred autoclave. The autoclave was fitted with a rupture disk rated for 3 000 psig at 315°C. Temperature regulation was achieved with a controller-recorder that regulated the external heating elements. Operation was in a batch-wise mode. Generally, 1 l of material was charged into the autoclave, which was sealed and pressurized to the desired pressure with compressed air. After heating to the desired temperature for 1 hour, the contents were cooled by an internal coil carrying cold water. The resulting gases were analyzed to determine oxygen use and the contents were removed for further testing.

In cases of starting material with low solubility in water, the oxidized material was extracted with an appropriate solvent and the extract analyzed for starting compound (Table I). Compounds were analyzed using a gas chromatograph with a flame-ionization detector or a high-performance liquid chromatograph with a reverse-phase column and a spectrophotometric detector. The methods of analyses are listed in Table I. Chloride analyses were performed on a Technicon Industrial AutoAnalyzer II employing Technicon Industrial Method #99-70W/B, a mercuric thio-

TABLE I. Analytical methods.

Compound	Method[b]	Conditions
Acenaphthene[a]	LC	65% CH_3OH in H_2O, 1.2 ml/min, 288 nm
Acrolein	GC	4′ × ⅛″ Tenex GC 40/60, Temp. program 50–200°C, 25 ml/min helium
Acrylonitrile	GC	8′ × ⅛″ Chromasorb 101, 80/100, 125°C, 25 ml/min helium
2-chlorophenol	LC	40% CH_3OH in H_2O, 1.2 ml/min, 277 nm
2,4-dimethylphenol	LC	50% CH_3OH in H_2O, 1.2 ml/min, 280 nm
2,4-dinitrotoluene[a]	LC	50% CH_3OH in H_2O, 1.2 ml/min, 242 nm
1,2-diphenylhydrazine[a]	LC	50% CH_3OH in H_2O, 1.2 ml/min, 245 nm
4-nitrophenol	LC	40% CH_3OH in H_2O, 1.2 ml/min, 312 nm
Pentachlorophenol[a]	LC	75% CH_3OH in H_2O pH 2, 1.2 ml/min, 303 nm
Phenol	LC	20% CH_3OH in H_2O, 1.2 ml/min, 272 nm
Acetic acid	GC	6′ × ⅛″ 10% SP-1200/1% H_3PO_4 on Chromasorb WAW 80/100, 125°C, 25 ml/min helium

[a] Compound extracted with methylene chloride and converted to methanol solvent prior to analysis.
[b] All LC analyses were performed on a 10-μm ODS (250 mm × 3.1 mm) column.
LC = high performance liquid chromatography.
GC = gas chromatography.
nm = nanometer.

cyanate/ferric nitrate colormetric method. The solutions from both the 275° and 320°C oxidations were used for toxicity testing, and the solutions from the 320°C oxidations were also analyzed for products of wet oxidations.

Copper catalyzed oxidations. These oxidations were performed by adding cupric ion at 500 mg/l in the form of copper sulfate ($CuSO_4 \cdot 5H_2O$). Prior to toxicity tests on the catalytically oxidized solutions, the copper was removed by precipitating it as the sulfide species, followed by filtering the solution through a 0.45-μm membrane. Excess sulfide addition was avoided by monitoring the soluble copper concentration as the cupric sulfide was precipitated. When a distilled water solution of 500 mg/l of Cu^{2+} was processed through the autoclave and then decoppered, the resulting solution was no more toxic than a distilled water blank processed through the autoclave. Thus the copper removal did not affect the toxicity tests of these solutions.

TABLE II. Spring water analysis (June 21, 1977–June 29, 1978).

Analysis	Average	Range
pH	7.7	7.0–8.2
Hardness (mg/l as $CaCO_3$)	154.5	89.5–180
Alkalinity (mg/l as $CaCO_3$)	137.7	95–156
Conductivity (μmohs/cm)	290.4	225–325
Ca (mg/l)	32.7	15.4–62
Mg (mg/l)	19.7	12.4–41
Na (mg/l)	2.4	1.6–3.6
K (mg/l)	1.3	0.9–2.5

Toxicity methods. Tests were conducted on the invertebrate cladoceran *Daphnia magna*, which were cultured according to the method of Beisinger.[6] The original stock of *daphnia* was obtained from the EPA laboratory in Duluth, Minn. Cultures of these animals have been maintained in our laboratory for about 2 years. Dilution water from a local spring-fed pond was used as culture media and for toxicity tests. The water was relatively hard, averaging 154.5 mg/l of hardness measured as $CaCO_3$ over the period of use. Other analyses are listed in Table II. The acute static tests were conducted as described in "Methods for Acute Toxicity Tests with Fish, Macroinvertebrates and Amphibians."[7] First instar *daphnia* (12 ± 12 hours old) were used for all tests. The tests were conducted in duplicate for 48 hours at 22°C in a constant-temperature chamber. All compound concentrations were nominal. The 48-hour TC_{50} values, or toxic substance concentrations that produces the effect of immobilization on 50% of the test population after 48 hours, and their 95% confidence limits were estimated by the method of Litchfield and Wilcoxon[8] unless otherwise noted.

Oxidation results. Tests at temperatures from 150 to 320°C for 1 hour were completed first. Figures 1 and 2 plot the percentage of starting material remaining after 1-hour oxidation versus reaction temperature

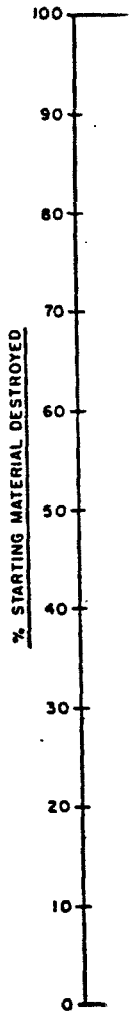

FIGURE 1. Percent

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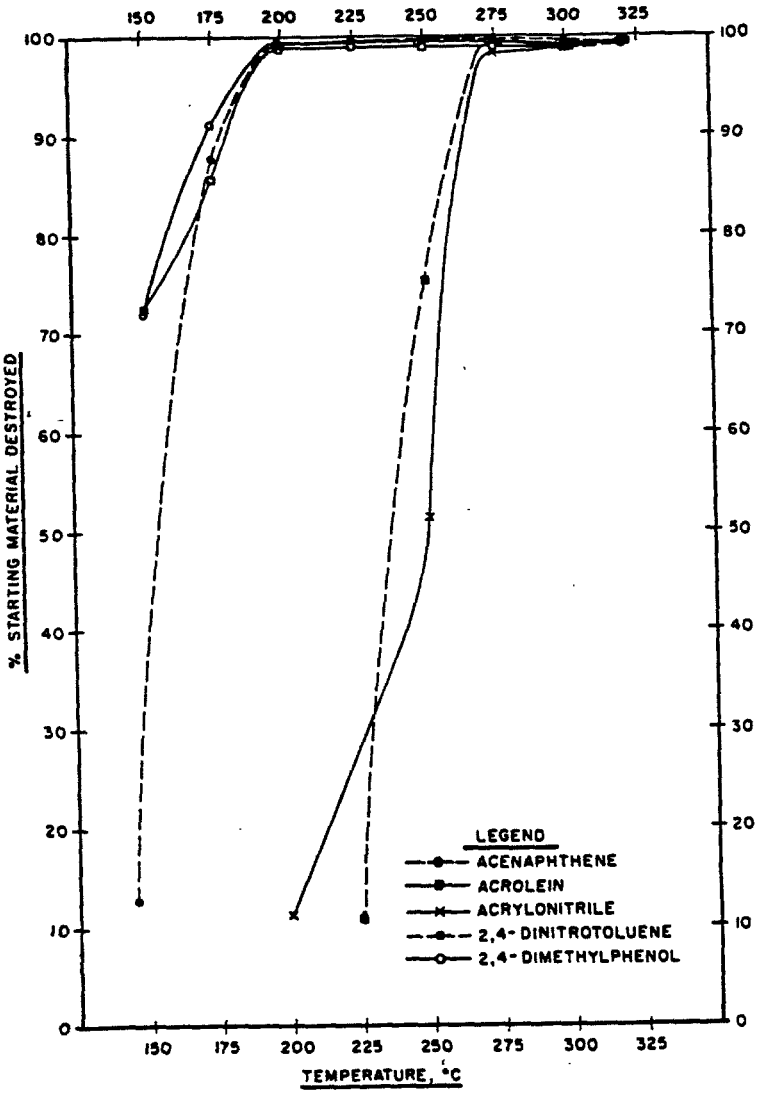

FIGURE 1. Percentage destroyed versus temperature.

for the compounds studied. Some compounds were easily destroyed at 200°C, whereas others required higher temperatures for destruction. More than 99.8% of the starting compound was destroyed after oxidation at 320°C for 1 hour (Table III). The 275°C oxidations also produced a high degree of starting material destruction for most compounds (Table III). In the case of several slow-reacting compounds at 275°C, addition of a copper catalyst caused a high degree of starting compound destruction in 1 hour.

Toxicity results. An evaluation of the use of *daphnia* as a toxicity test organism has been reviewed by Scott *et al.*[9] Most testing using *daphnia* has concentrated on metals [10-13] and pesticides or herbicides.[14-17] Anderson [10-11] studied the toxicity of numerous inorganic and organic compounds to *daphnia* as early as the 1940s. Sanders [16] found that of the 6 species of freshwater crusteceans tested, *daphnia* were generally the most sensitive to any of 16 specific herbicides. He observed 48-hour EC_{50} values for *daphnia* ranging from 0.025 mg/l

TABLE III. 1-hour wet oxidations.

Compound	Starting Concentration (g/l)	% Starting Material Destroyed		
		320°C	275°C	275°C/Cu^{++}
Acenaphthene	7.0	99.96	99.99	—
Acrolein	8.41	>99.96[a]	>99.05	—
Acrylonitrile	8.06	99.91	99.00	99.50
2-chlorophenol	12.41	99.86	94.96	99.88
2,4-dimethylphenol	8.22	99.99	99.99	—
2-4-dinitrotoluene	10.0	99.88	99.74	—
1,2-diphenylhydrazine	5.0	99.98	99.98	—
4-nitrophenol	10.0	99.96	99.60	—
Pentachlorophenol	5.0	99.88	81.96	97.30
Phenol	10.0	99.97	99.77	—

[a] The concentration remaining was less than the detection limit of 3 mg/l.

for Dichlone to greater than 100 mg/l for Dicamba and 2,4-D (acid). Recently Kopperman *et al.*,[18] determined the 48-hour EC_{50} values for a series of phenols on *daphnia* in an attempt to correlate physicochemical parameters with toxicity. They observed slightly lower toxic levels for phenol (10.6 mg/l) and 4-nitrophenol (8.4 mg/l) than our values (Table IV), but nearly the same value for 2-chlorophenol (7.43 mg/l). These previous tests, unlike this study, were conducted with food added to the dilution water. Adema[19] observed a 48-hour EC_{50} value of 0.6 mg/l for pentachlorophenol compared with our 0.95 mg/l value. Bringman and Kuhn[20] determined the 24-hour EC_{50} value on *daphnia* for 173 hazardous substances. Most organic materials exhibited toxic levels of 10 to 1 000 mg/l. These higher levels are probably because of the shorter period of exposure. The

TABLE IV. Toxicity comparisons (95% confidence limits in parenthesis).

Material	Starting Compound 48-Hr EC_{50} (mg/l)	320°C Oxidized Compound 48-Hr EC_{50} (vol. %)	320°C Dilution Factors Ratio, Ds:Do	275°C Oxidized Compound 48-Hr EC_{50} (vol. %)	275°C Dilution Factors Ratio, Ds:Do
Acenaphthene	3.45 (2.95–4.03)	7.10 (6.51–7.74)	144	6.05 (5.52–6.63)	1[illegible] 8
Acrolein	0.093 (0.085–0.101)	4.45 (3.82–5.19)	4 024	4.65 (4.40–4.91)	4 2[illegible]
Acrylonitrile	10.95 (9.54–12.56)	4.80 (4.09–5.63)	35	1.70 (1.45–1.99)	1 5
2-chlorophenol	6.20 (5.56–6.92)	2.60 (2.35–2.88)	52	0.87 (0.625–1.21)	1 4
2,4-dimethylphenol	2.37 (1.77–3.17)	6.50 (4.84–8.72)	225	7.70 (7.04–8.42)	2[illegible]
2,4-dinitrotoluene	26.2 (22.5–30.5)	4.00 (2.89–5.54)	15	3.35 (2.72–4.12)	1 3
1,2-diphenylhydrazine	2.18 (1.86–2.55)	7.20 (5.60–9.26)	165	6.60 (6.28–6.93)	15 4
Phenol	23.5 (21.0–26.3)	10.60 (9.72–11.56)	45	6.07 (5.56–6.63)	2 8
4-nitrophenol	20.0 (16.7–24.0)	7.70 (6.34–9.35)	38.5	6.40 (5.80–7.05)	3 0
Pentachlorophenol	0.95 (0.80–1.12)	0.28 (0.235–0.334)	14.75	1.13 (1.02–1.25)	5 5

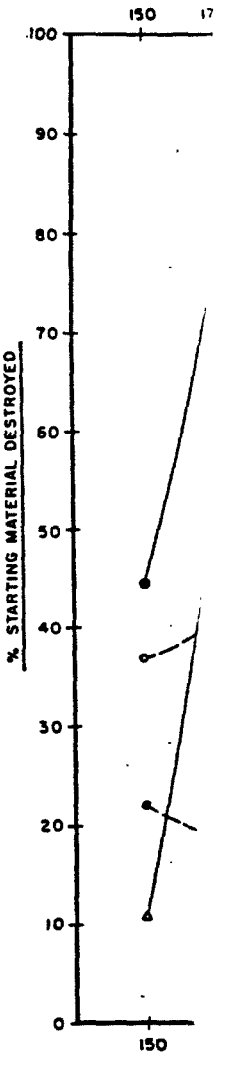

FIGURE 2. Percentage dest

TABLE V. Catalytic oxidatio

Material	O
Acrylonitrile	
2-chlorophenol	
Pentachlorophenol	

[a] Calculated by the Binomial for 10 test organisms.

:arting Material Destroyed	
275°C	275°C/Cu++
99.99	—
>99.05	—
99.00	99.50
94.96	99.88
99.99	—
99.74	—
99.98	—
99.60	—
81.96	97.30
99.77	—

g/L

study, were conducted with
he dilution water. Adema[19]
ur EC_{50} value of 0.6 mg/l for
l compared with our 0.95-
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ir EC_{50} value on *daphnia* for
ibstances. Most organic ma-
toxic levels of 10 to 1 000
gher levels are probably be-
ter period of exposure. The

C on rs o, Do	275°C Oxidized Compound 48-Hr EC_{50} (vol. %)	275°C Dilution Factors Ratio, Ds:Do
	6.05 (5.52–6.63)	122.8
	4.65 (4.40–4.91)	4 205
	1.70 (1.45–1.99)	12.5
	0.87 (0.625–1.21)	17.4
	7.70 (7.04–8.42)	267
	3.35 (2.72–4.12)	12.8
	6.60 (6.28–6.93)	151.4
	6.07 (5.56–6.63)	25.8
	6.40 (5.80–7.05)	32.0
5	1.13 (1.02–1.25)	59.5

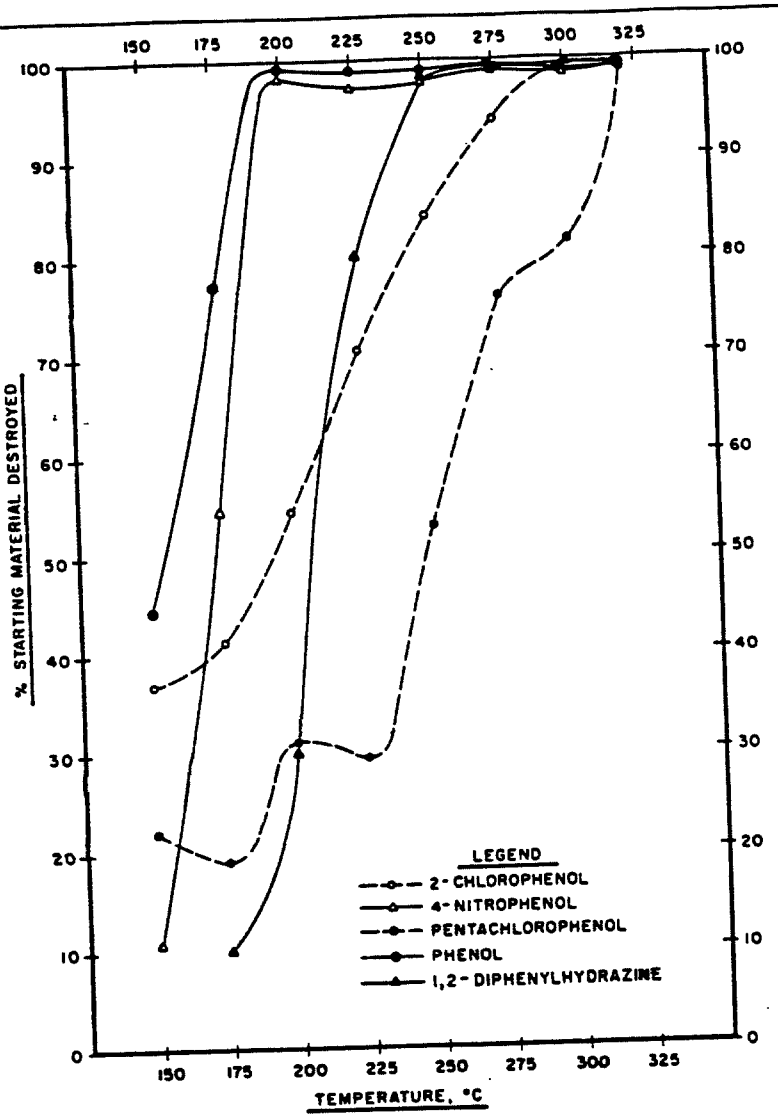


FIGURE 2. Percentage destroyed versus temperature.

TABLE V. Catalytic oxidations at 275°C.

Material	275°C Oxidized Compound 48-Hr EC_{50}	275°C Ds:Do	Catalytic 275°C Oxidized Compound 48-Hr EC_{50}	Catalytic 275°C Ds/Do
Acrylonitrile	1.70 vol.% (1.45–1.99)	12.5	1.58 vol.% (1.34–1.88)	11.6
2-chlorophenol	0.87 vol.% (0.625–1.21)	17.4	3.46 vol.%[a] (3.0–4.0)[a]	69.2
Pentachlorophenol	1.13 vol.% (1.02–1.25)	59.5	3.85 vol.% (3.46–4.28)	202.6

[a] Calculated by the Binomial Test [22], $EC_{50} = (AB)^{\frac{1}{2}}$ with confidence limits A-B, 99.8% confidence limit for 10 test organisms.

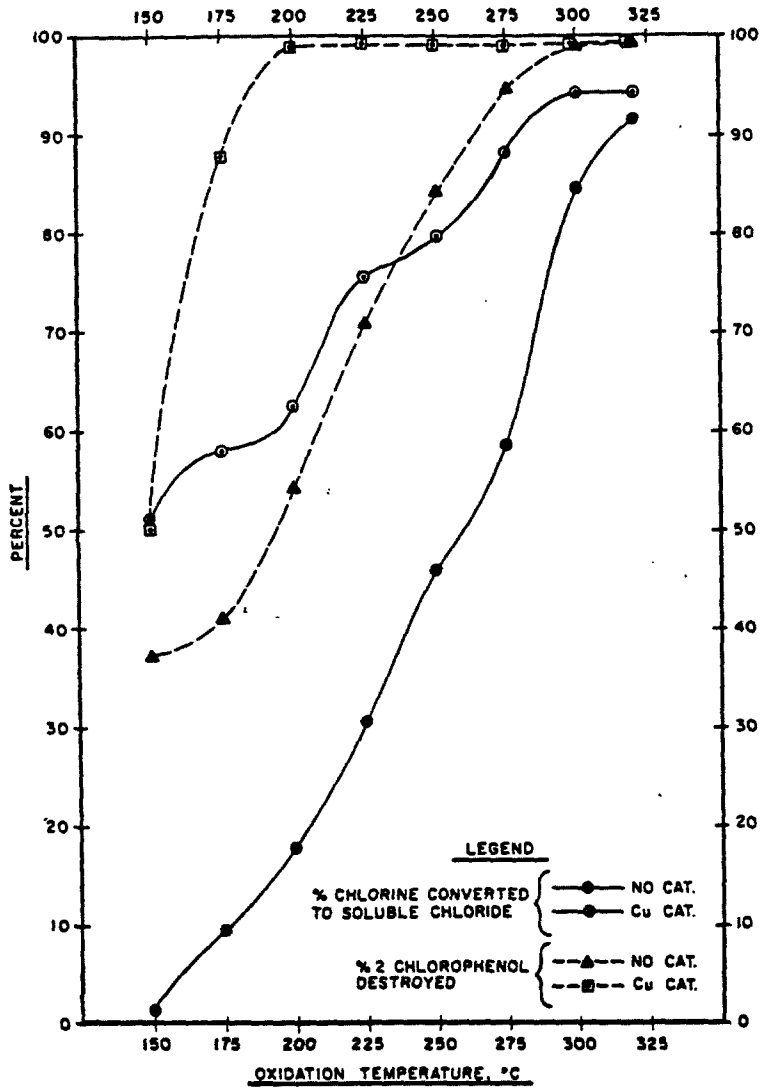


FIGURE 3. 2-chlorophenol oxidations for 1 hour.

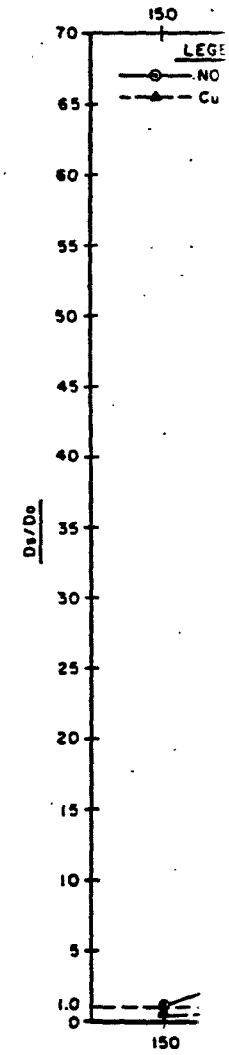
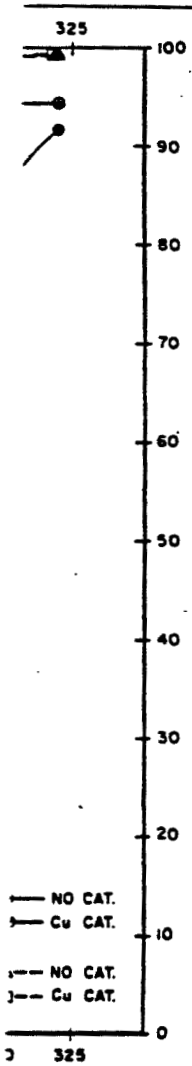


FIGURE 4. 2-chlorophenol

toxic levels for the compounds in this study ranged from 0.093 mg/l for acrolein to 26.2 mg/l for 2,4-dinitrotoluene.

Toxicity comparisons. The following method was devised to compare the toxicity of the various solutions before and after wet oxidation. The 48-hour EC_{50} value was set as the point of comparison. Acrolein will be used as an illustration. Prior to wet oxidation, the starting concentration of acrolein, 8 410 mg/l, (Table IV) would have to be diluted by a factor of 90 430 to obtain the 48-hour EC_{50} value of 0.093 mg/l (8 410 mg/l/0.093 mg/l = 90 430 = Ds, the dilution factor the starting material). After oxidation a :0°C, the solution had a 48-hour EC_{50} valu 4.45 vol%, or 4.45 ml diluted to 100 m :able IV). The dilution factor now is 100 '4.45 ml = 22.47 = Do, the dilution factor : the oxidized material. (The smaller the .ution factor, the less toxic the substances.) rato of the dilution factors, Ds:Do = 90 4 22.47 = 4 024, is the reduction in dilutio factor required to reach the 48-hour EC value brought about by wet oxidation. T larger the Ds:Do ratio, the greater the redu ion i

toxicity. Results of this com in Table IV. The solution c are less toxic than the starti factor of 15 to 4 000, depe ticular compound. In ge from the toxicity tests of the are quite similar to those fr dilations. A decrease in oxic from 320 to 275°C results crease in toxicity reduction. paring the ratios of Ds:Do both temperatures. In catal acrylonitrile, 2-chlorophenol



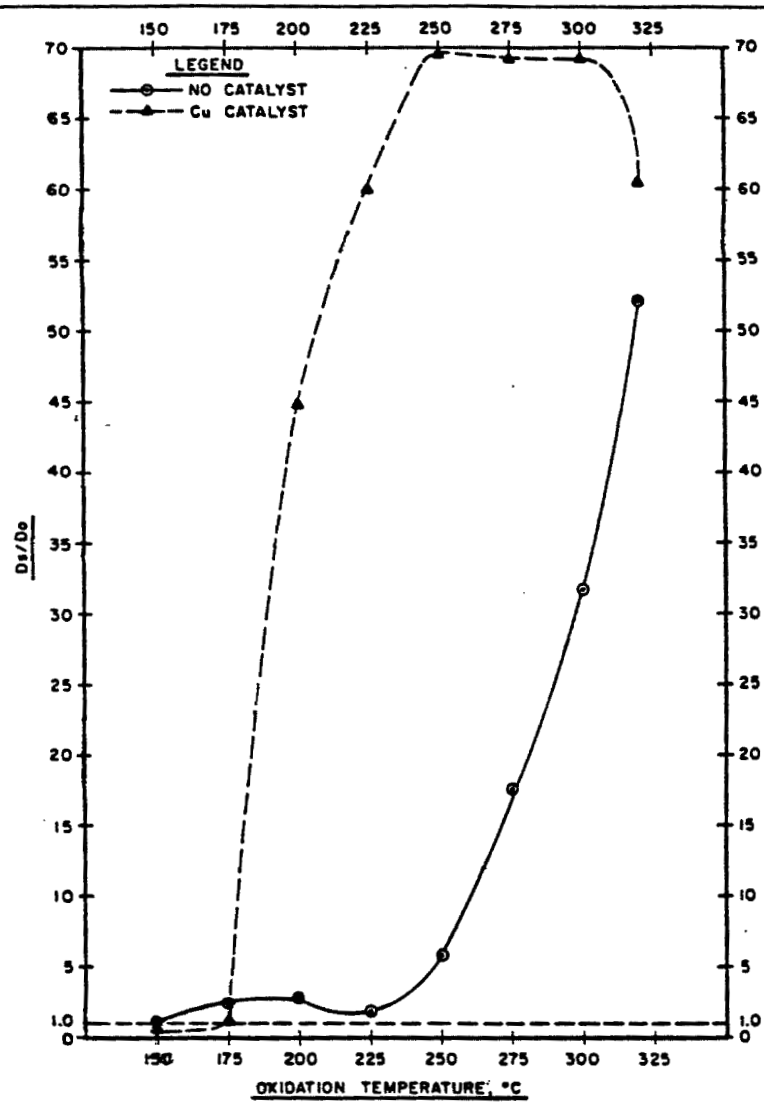


FIGURE 4. 2-chlorophenol Ds/Do versus oxidation temperature.

he dilution factor for the . After oxidation at 320°C, 48-hour EC_{50} value of 4.45 l diluted to 100 ml (Table n factor now is 100 ml/4.45 the dilution factor for the . (The smaller the dilution ic the substances.) The ratio ctors, Ds:Do = 90 430/22.47 reduction in dilution factor h the 48-hour EC_{50} value wet oxidation. The larger the greater the reduction in toxicity. Results of this comparison are given in Table IV. The solution oxidized at 320°C are less toxic than the starting solutions by a factor of 15 to 4 000, depending on the particular compound. In general, the results from the toxicity tests of the 275°C oxidations are quite similar to those from the 320°C oxidations. A decrease in oxidation temperature from 320 to 275°C results in very little decrease in toxicity reduction, as seen by comparing the ratios of Ds:Do for a compound at both temperatures. In catalyzed oxidations of acrylonitrile, 2-chlorophenol, and pentachlorophenol at 275°C, the catalyst had little effect on the Ds:Do for acrylonitrile, but almost a four-fold Ds:Do increase for 2-chlorophenol and pentachlorophenol (Table V).

2-chlorophenol oxidation study. Because nearly half of the materials on the priority pollutant list contain chlorine, and 2-chlorophenol was named specifically, a more detailed study of the wet oxidation of 2-chlorophenol was performed. Figure 3 plots the 2-chlorophenol destroyed versus reaction temperature for both the copper catalyzed and noncatalyzed oxidations. The percentage of chlorine converted

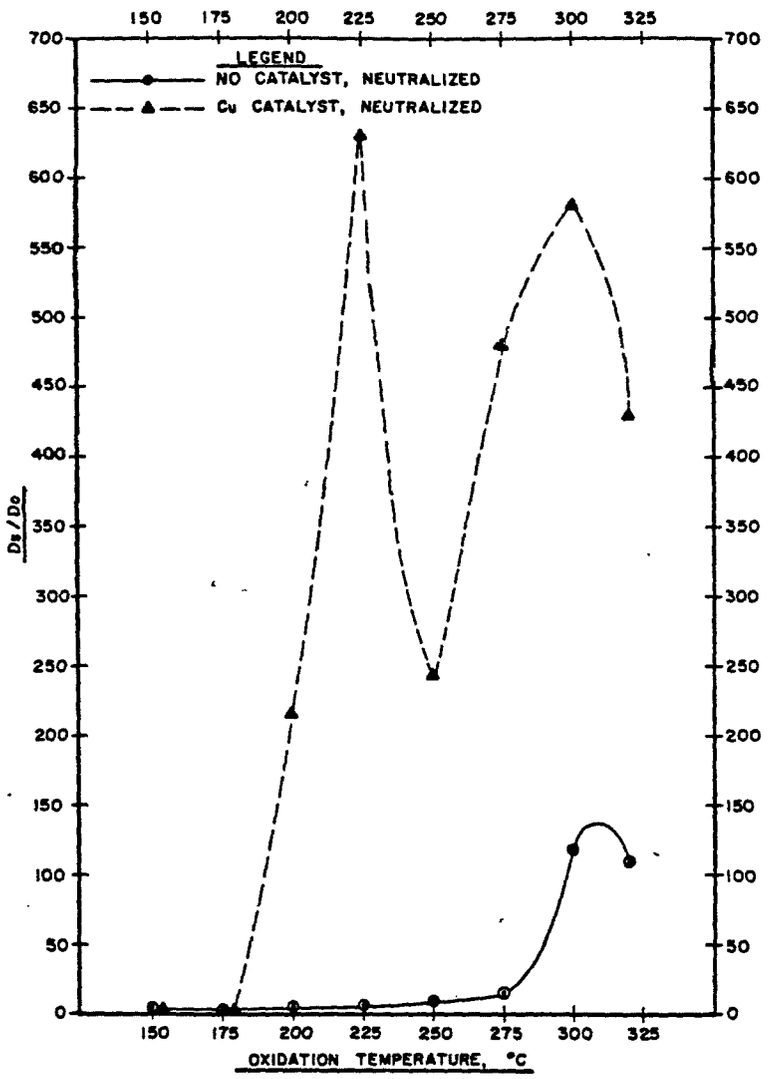


FIGURE 5. 2-chlorophenol Ds : Do versus oxidation temperature.

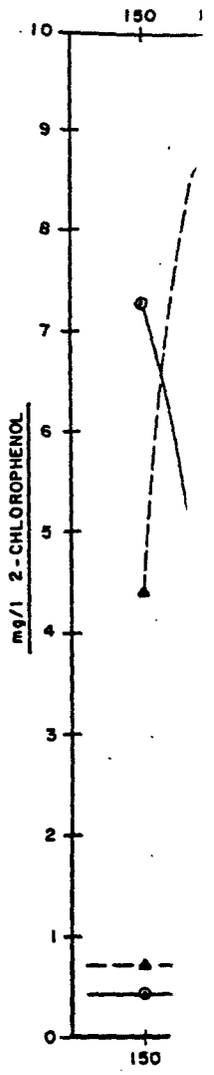


FIGURE 6. Concentratio
perature.

to soluble chloride at each oxidation temperature is also plotted for both the catalytic and noncatalytic oxidations.

In the noncatalytic oxidations, chloride ion production lags about 40% behind starting material destruction up to the 300°C oxidation, and reaches over 90% of the total at 320°C. The catalytic oxidations are quite similar, although more than 99% of the starting compound is destroyed at 200°C. The chloride ion produced attains more than 95% of the total at 300°C and above.

Toxicity measurements were performed on the two sets of solutions generated at th 25°C intervals studied. Ds:Do was ca culated at each temperature, and these va ue are plotted for the catalytic and noncatal ti oxidations in Figure 4. The noncatalytic Ds Do values remain close to 1.0 through th 225°C oxidation, then rise to a high of 52 fo the 320°C oxidation. The catalytic Ds D values rise quickly above the 175°C oxida io and are in the 60 to 70 range above 200°C

Because of the acidity of the oxidized s lu tions, the pH of the toxicity test solut on dropped to 4 or less for concentrations at o

above the 48-hour EC_{50} val
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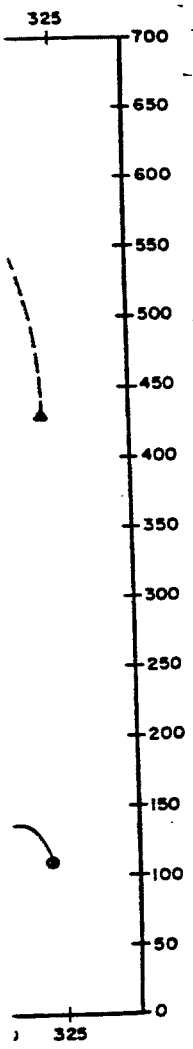

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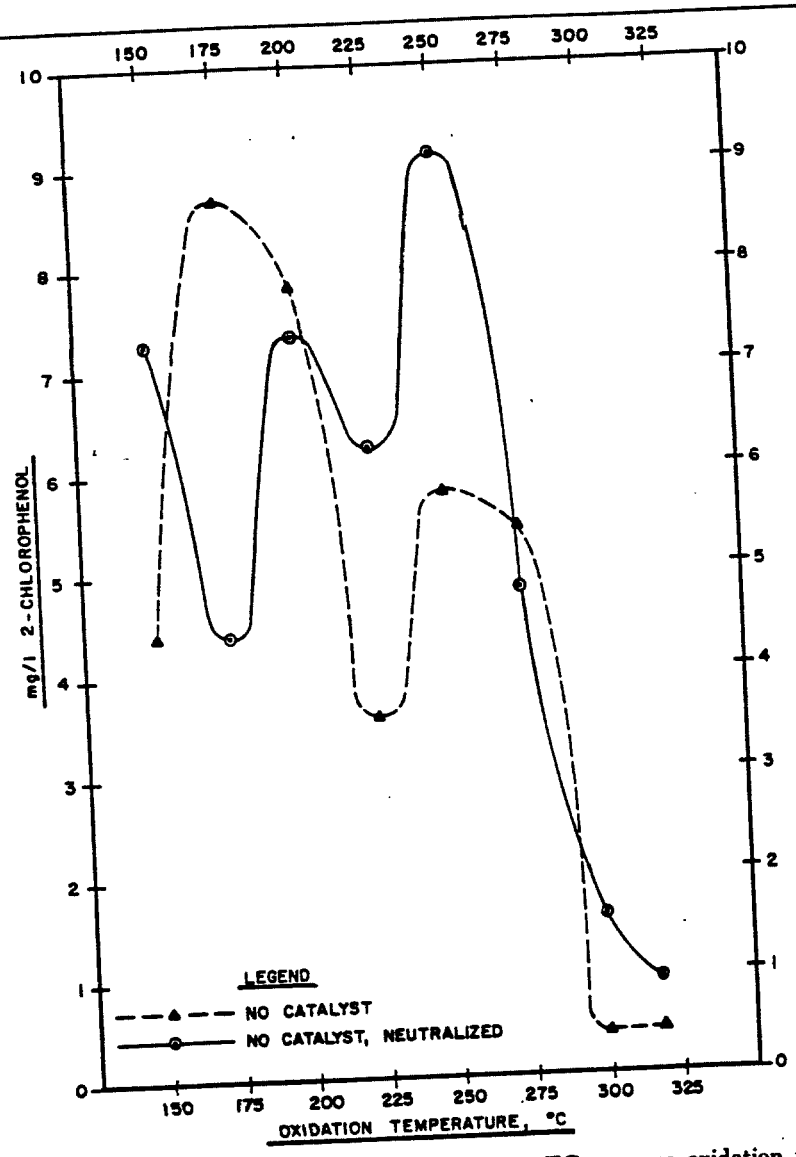


FIGURE 6. Concentration of 2-chlorophenol at 48-hour EC_{50} versus oxidation temperature.

above the 48-hour EC_{50} values for the catalytic oxidations. A smaller pH drop occurred at these concentrations for the noncatalytic oxidations. The sensitivity of *daphnia* to low pH has been observed in the work of Anderson,[10] who noted that many acids "were nontoxic as long as they were added in quantities that did not reduce the pH of the water to less than 6." To remove the effect of low pH, all oxidized solutions were adjusted to pH 7 and the 48-hour EC_{50} values again determined for both groups. The Ds:Do values versus temperature obtained for the neutralized solutions are plotted in Figure 5. Little change was noted on neutralization of the noncatalytic oxidations. A large increase in the Ds:Do was found on neutralization of the catalytic oxidations. This would seem to indicate that the acidity (HCl) of the catalytic oxidations was the major toxic factor, whereas in the noncatalytic oxidations, the acidity was not exerting much of a toxic effect.

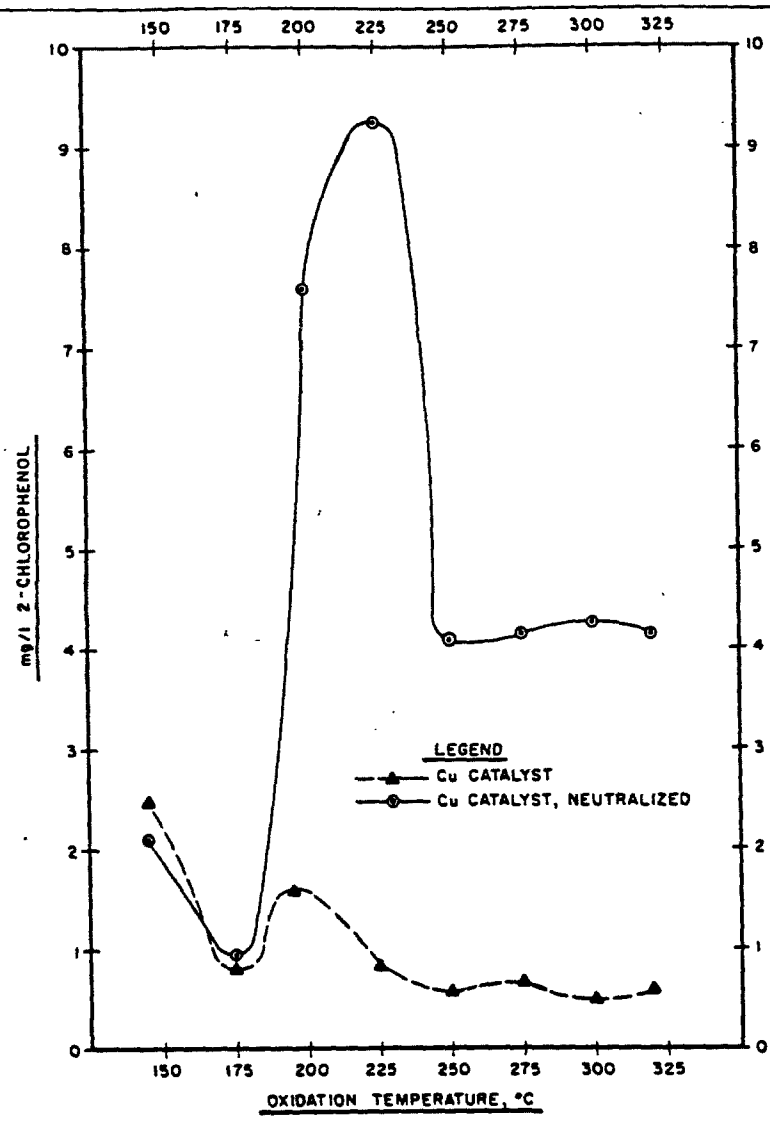


FIGURE 7. Concentration of 2-chlorophenol at 48-hour EC_{50} versus oxidation temperature.

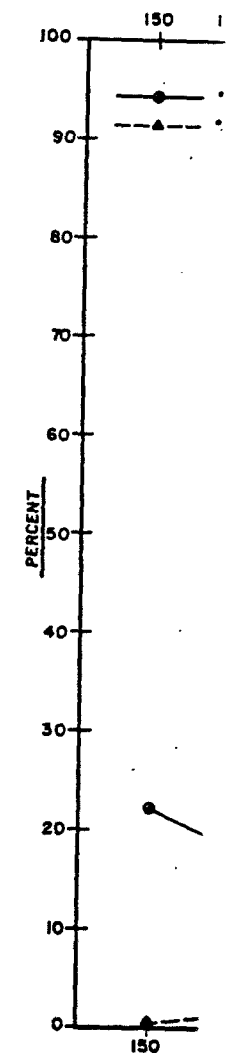
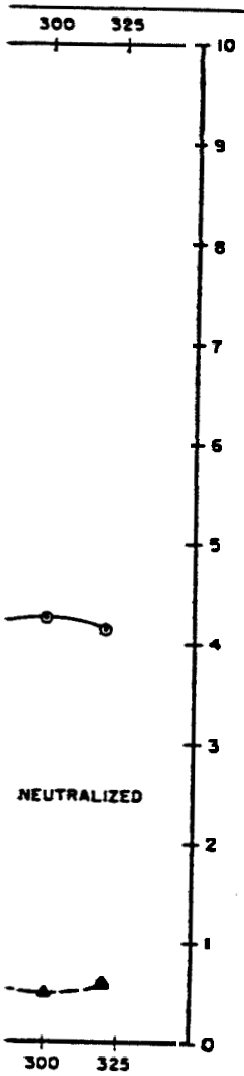


FIGURE 8. Pentachlorophe

The concentration of 2-chlorophenol at the 48-hour EC_{50} value was calculated for both sets of oxidations. Figure 6 plots these values for the noncatalytic oxidations before and after neutralization. The concentration of 2-chlorophenol stays close to the 48-hour EC_{50} value of 6.2 mg/l for both curves up to 275°C. At 300°C and above the concentration drops off. This indicates 2-chlorophenol could be exerting the toxic effect at the lower oxidation temperature, but probably not at 300°C and above. Catalytic oxidations are plotte Figure 7. The non-neutralized oxidized tions have low 2-chlorophenol concentra at the 48-hour EC_{50} values at all temperat The starting compound probably is not pr in sufficient concentration to cause tox under these conditions. When neutralized 2-chlorophenol concentration at the 48- EC_{50} value at 200°C and above is close to toxic 6.2-mg/l level. This seems to be toxic material under these test condit

The 2-chlorophenol concentra hour EC_{50} value for the ca at 150 and 175°C remained neutralization of the solutions ity was not causing the toxi chlorophenol was present at below the toxic 6.2-mg/l some other organic material, tion intermediate, was prese the toxicity. No attempt was other organic compounds that in these two oxidized sample



, versus oxidation tem-

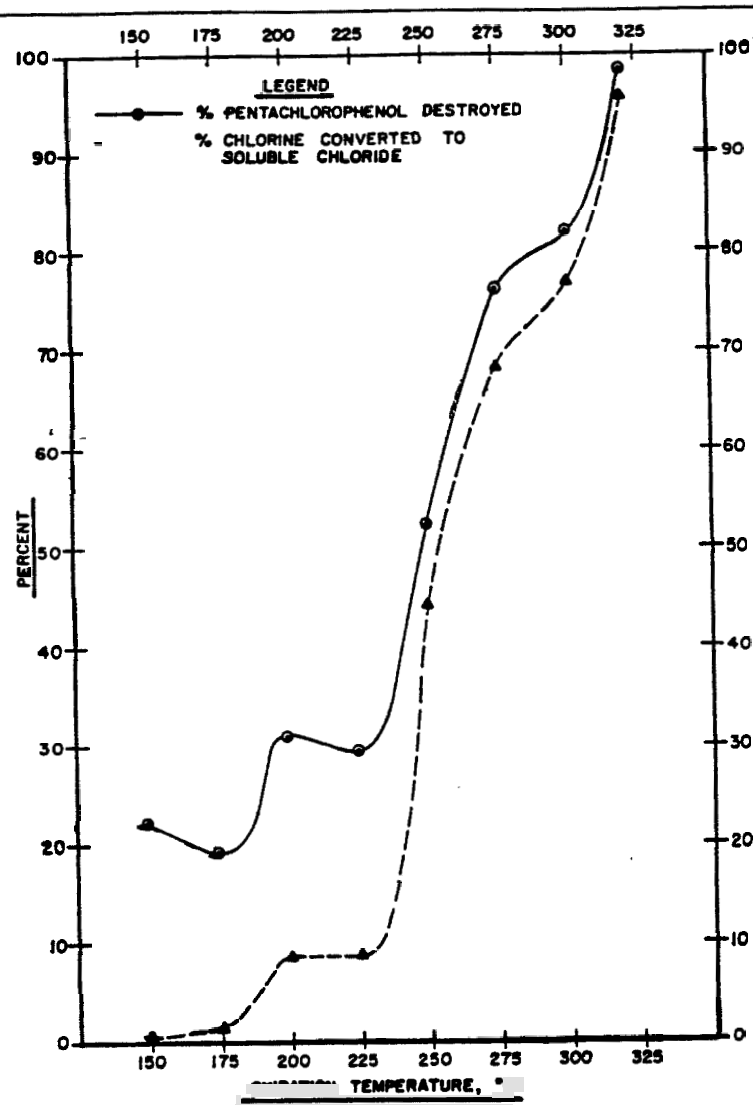


FIGURE 8. Pentachlorophenol oxidations for 1 hour.

·tic oxidations are plotted in · non-neutralized oxidized solu- 2-chlorophenol concentrations EC_{50} values at all temperatures. mpound probably is not present ncentration to cause toxicity ditions. When neutralized, the concentration at the 48-hour 00°C and above is close to the level. This seems to be the under these test conditions.

The 2-chlorophenol concentrations at the 48-hour EC_{50} value for the catalytic oxidations at 150 and 175°C remained unchanged upon neutralization of the solutions. Thus the acidity was not causing the toxicity, and the 2-chlorophenol was present at a concentration below the toxic 6.2-mg/l level. Evidently, some other organic material, possibly an oxidation intermediate, was present and producing the toxicity. No attempt was made to identify other organic compounds that might be present in these two oxidized samples.

The formation of chloride ion was also monitored for the pentachlorophenol oxidations. Figure 8 plots the percentage of pentachlorophenol destroyed versus temperature, and the percentage of chlorine converted to chloride versus temperature. Above 225°C, the chloride formed nearly matches the percentage destruction of starting material. Once the original structure of the pentachlorophenol molecules is disrupted, nearly all of the chlorine is converted to chloride ion.

Oxidation products. In addition to the

TABLE VI. Products identified from 320°C. 1-hr oxidations.

Starting Compounds	Formic Acid (mg/l)	Acetic Acid (mg/l)
Acenaphthene	6	443
Acrolein	81	852
Acrylonitrile	48	921
2-chlorophenol	52	442
2,4-dimethylphenol	75	1 527
2,4-dinitrotoluene	134	213
1,2-diphenylhydrazine	37	526
4-nitrophenol	793	500
Pentachlorophenol	0	101
Phenol	13	1 034

starting material, the solutions from the 320°C oxidations were analyzed for products of oxidation. The oxidation solution was subjected to fractional distillation to concentrate the volatile compounds in the distillate. This fraction was scanned by gas chromatography in an attempt to identify compounds. No identification could be made. The pot solution from the fractional distillation was then subjected to exhaustive steam distillation. The steam distillate was analyzed for volatile acids by gas chromatography (Table I) while formic acid content was determined via a mercuric chloride gravametric procedure.[21] Thus far, only formic acid and acetic acid have been identified in the oxidized solutions in the amounts shown in Table VI. Based on some earlier work, the 48-hour EC_{50} value of some low molecular weight compounds that might have been produced by wet oxidation was determined. These compounds are listed in Table VII. All of the tests with acids showed a sharp drop in pH to 5 or less at their respective 48-hour EC_{50} values. The non-ionizable compounds were generally of low toxicity with the exception of acetaldehyde. Table VIII lists the concentrations of formic acid and acetic acid present at the 48-hour EC_{50} value for the solutions resulting from the 320°C oxidations. In the case of phenol and 2,4-dimethylphenol, the acetic acid concentration was about 50% of the 48-hour EC_{50} value determined for pure acetic acid. Oxidized 4-nitrophenol gave the only high concentration of formic acid at the 48-hour EC_{50} value. This value was only 40% of the 48-hour EC_{50} concentration for pure formic acid. The solutions from the 320°C oxidations showed no pH drop to 5 or less at the 48-hour EC_{50} dilutions. Thus acidity from the organic or inorganic acids present was not the single toxic factor for these solutions.

CONCLUSIONS

Wet oxidation is well suited for the treatment of dilute aqueous wastes which are uneconomical to incinerate and too toxic for direct biological treatment. Although these wet oxidation studies were conducted on relatively concentrated solutions, it was demonstrated that 99+% destruction of contaminants in more dilute wastes would also occur. This would produce effluents with low levels of pollutants, in the parts per million range.

The products of wet oxidation are generally low molecular weight compounds such as formic and acetic acid, which are readily biodegradable by conventional treatment methods

TABLE VII. Toxicity of some simple organic compounds.

Compound	48-Hr EC_{50} (mg/l)	95% Confidence Limits (mg/l)	pH 48-
Formic acid	151.2[a]	138–165.6[a]	
Acetic acid	190	179–202	
Oxalic acid	136.9[a]	125–150[a]	
Malonic acid	275	233–324	
Maleic acid	316.2[a]	250–400[a]	
Fumaric acid	212	204–220	
Succinic acid	374.2[a]	350–400[a]	
Methanol	24 500	20 450–29 350	
Acetaldehyde	48.25[a]	39.4–59.1[a]	
Acetone	13 500	10 294–17 704	
2-butanone (MEK)[b]	5 091[a]	4 025–6 440[a]	

[a] Calculated by the Binomial Test.[22]
[b] MEK = methyl ethyl ketone.

TABLE VIII. Concentrations

Compounds
Acenaphthene
Acrolein
Acrylonitrile
2-chlorophenol
2,4-dimethylphenol
2,4-dinitrotoluene
1,2-diphenylhydrazine
4-nitrophenol
Pentachlorophenol
Phenol

NOTE: Formic acid 48-hr EC_{50}

Thus wet oxidation of a toxic
by biotreatment would yield
able for discharge into a public
ment plant.

Summary. Wet oxidation
pounds has been demonstrate
cellent method to detoxify t
Destruction of 99.8+% of the
dals was achieved by wet oxid
for 1 hour. Oxidation at 27
produced over 99% destructio
pounds. A copper catalyst w
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of the oxidized materials was
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Oxidation at 275°C was nearly
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TABLE VIII. Concentrations of organic acids at 48-hour EC_{50}.

Compounds	320°C 48-Hr EC_{50} (vol.%)	Conc. Acetic @ 48-Hr EC_{50} (mg/l)	Conc. Formic @ 48-Hr EC_{50} (mg/l)
Acenaphthene	7.10	31.4	0.4
Acrolein	4.45	37.9	3.6
Acrylonitrile	4.80	44.2	2.3
2-chlorophenol	2.60	11.5	1.4
2,4-dimethylphenol	6.50	99.3	4.9
2,4-dinitrotoluene	4.00	8.5	5.4
1,2-diphenylhydrazine	7.20	37.9	2.7
4-nitrophenol	7.70	38.5	61.1
Pentachlorophenol	0.28	0.3	0
Phenol	10.6	109.0	1.4

NOTE: Formic acid 48-hr EC_{50} = 151.2 mg/l. Acetic acid 48-hr EC_{50} = 190.0 mg/l.

Thus wet oxidation of a toxic waste followed by biotreatment would yield an effluent suitable for discharge into a publicly owned treatment plant.

Summary. Wet oxidation of toxic compounds has been demonstrated to be an excellent method to detoxify these materials. Destruction of 99.8+% of the starting material was achieved by wet oxidation at 320°C for 1 hour. Oxidation at 275°C for 1 hour produced over 99% destruction of most compounds. A copper catalyst was effective in bringing about the destruction of several of the more slowly reacting compounds at the 275°C temperature. In general, the toxicity of the oxidized materials was greatly reduced compared with that of the starting materials. Oxidation at 275°C was nearly as effective in reducing toxicity as 320°C oxidation. When the 320°C oxidation solutions were diluted to their respective 48-hour EC_{50} concentrations, the concentrations of the identified oxidation products, acetic and formic acid, were below the 48-hour EC_{50} value determined for the individual compound alone. A more detailed study of oxidations of 2-chlorophenol indicated that the acute toxicity of the catalytic oxidation solutions was from acidity, probably hydrochloric acid, whereas the toxicity of the noncatalytic oxidation solutions was because of the organic constituents present in them. The toxicity tests were short term and designed to provide only acute static toxicity measurements. Although all of the products of wet oxidation may not be identified, the toxicity data would indicate whether some highly toxic material were produced in significant amounts.

ACKNOWLEDGMENTS

Credits. This paper was presented at the 51st Annual Conference of the Water Pollution Control Federation, Anaheim, Calif., October 1–6, 1978.

Authors. Tipton L. Randall is Research Chemist, and Paul V. Knopp, formerly Vice-President and Technical Director, now Vice-President of Marketing, Zimpro Inc., Rothschild, Wis.

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JOURNAL OF NEUROPATHOLOGY & EXPERIMENTAL NEUROLOGY

VOLUME XXXV May/June 1976 NUMBER 3

(46)

~~OPTS-400026~~
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PERIPHERAL NERVE CHANGES INDUCED BY METHYL n-BUTYL KETONE AND POTENTIATION BY METHYL ETHYL KETONE

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JERRY R. MENDELL, M.D.,
AND
HAROLD S. WEISS, PH.D.
(Columbus, Ohio)

ABSTRACT

A study of the sequential morphological changes in the peripheral nerve induced by experimental inhalation exposure of methyl n-butyl ketone (MBK) revealed that the earliest change was an increase in the number of neurofilaments in the large myelinated nerve fibers. This change occurred prior to axonal swelling or myelin thinning. As the duration of exposure lengthened the number of neurofilaments gradually increased and ultimately produced axonal swelling with secondary thinning of the myelin sheath. This appears to be the pathogenesis of the "giant axonal" neuropathy. Another change observed early in this neuropathy was the presence of inpouchings of the myelin sheath, which also increased in number in parallel to the duration of exposure.

A careful study of the sequential changes in the entire motor unit did not show a predilection for early morphological changes at the axon terminal. Abnormalities at the neuromuscular junction occurred only after a full spectrum of changes were seen in the main nerve trunk, nerve roots and intramuscular nerves.

An important observation was the marked potentiation of peripheral neurotoxicity observed when animals were exposed to MBK in combination with methyl ethyl ketone (MEK) at a ratio of 1:5, MBK:MEK. The latter solvent showed no neurotoxic effect alone. This might help explain a recent outbreak of a polyneuropathy affecting many workers.

One further observation was that the sural nerve of a patient with prolonged exposure to MBK showed changes similar to those induced experimentally.

INTRODUCTION

Methyl n-butyl ketone (MBK) was recently observed to cause an industrial outbreak of polyneuropathy in a color print and plastic coated fabric plant (2,

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4). An experimental neuropathy was also produced by MBK in several animal species including mice, rats, chickens and cats (17, 24). The nerve changes observed had the characteristics of a giant axonal neuropathy.

Our continued studies of this neurotoxic agent have been relevant from several points of view. First, we have studied by serial examination of exposed animals, the sequential morphological changes in the nerve fiber induced by MBK. These studies have revealed the earliest change in the nerve fiber to be a gradual increase in neurofilaments which ultimately causes axonal swelling and secondary thinning of the myelin sheath resulting in the giant axonal neuropathy. Secondly, our studies have particular significance with regard to human exposure to MBK, as well as another commonly used industrial solvent, methyl ethyl ketone (MEK). We have been able to produce a neuropathy in rats with lower levels of exposure to MBK than has been previously reported (17, 24), and we found a marked potentiation of neurotoxicity when animals are exposed to a combination of MBK and MEK. In addition, we have been able to identify changes in a sural nerve biopsy from a patient with a prolonged exposure to MBK, that are identical to those induced experimentally.

MATERIALS AND METHODS

Sprague-Dawley rats were held in environmental chambers as previously described (17). Two groups of animals were studied: 1) The first group consisted of a dozen rats weighing 190–210 gm exposed to MBK 400 ppm, continuously, for 24 hours per day. 2) The second group was designed to study the interaction of MBK and MEK. Three dozen rats weighing 160–180 gm were continuously exposed for 24 hours per day to either MBK at 225 ppm, MEK at 1125 ppm or to the combination of MBK:MEK at 225:1125 ppm. Control animals were housed under similar environmental conditions without solvent exposure. In addition a cat was studied which had been held from our initial study (17). This cat had been exposed to MBK at 400 ppm for 7 weeks and was sacrificed after being returned to a normal environment for 4½ months.

24 hr day to 1125 ppm up to 42 days without effect.

At the time of sacrifice all animals were perfused through the abdominal aorta with 4% paraformaldehyde followed immediately by 5% glutaraldehyde both in 0.1 M phosphate buffer, pH 7.4. Sciatic nerve and intrinsic foot muscles were excised, blocked into 1 mm cubes and kept in glutaraldehyde for 2 hours. The tissue was post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 2 hours and dehydrated in graded ethanol solutions (25%, 35%, 50%, 70%, 80% and 95% for 15 minutes each), followed by 30 minutes in 100% alcohol and 30 minutes in propylene oxide. The tissue was embedded in Spurr low-viscosity embedding media. Some animals were perfused through the heart to obtain spinal cord and dorsal root ganglion specimens. For nerve fiber teasing, portions of the perfused nerve were kept in osmium tetroxide for 4–6 hours and then placed in a 60% glycerin solution for several days prior to teasing.

Quantitative histological studies were performed in the following manner: The number of neurofilaments and neurotubules per μm^2 was determined on electron micrographs of cross sections of random sampled nerve fibers at 20,000 magnification, enlarged to a final magnification of 300,000 under an American Optical dissecting microscope. The axon was divided into unit areas of 0.933 μm^2 and the number of neurofilaments and neurotubules were counted in these areas. The axonal area included for quantitative studies represented 70 to 90% of the total axon depending upon the number of unit areas (0.933 μm^2) the nerve could be divided into without overlap. The axon caliber myelin thickness ratio was also determined. For this measurement the orthogonal minor axis diameter of the axon and adjacent myelin were measured on electron micrographs of 20,000 magnification.

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To determine the frequency of inpouchings of the myelin sheath and denuded axons per mm^2, the number of nerve fibers showing these changes were counted by direct examination in the light microscope in 1 μm cross sections of epoxy resin embedded nerve. The nerve fascicles were then photographed, enlarged to a final magnification of 250, and cut out and weighed. The area was determined by comparison to a reference weight of known area. Only inpouchings of the myelin sheath with a concentric ring of myelin in the axon, separated from the outside myelin sheath (11), were counted. The inpouchings were of a thickness approximately equal to that of the myelin sheath. Paranodal or nodal regions of the axon were carefully ruled out in counting denuded axons.

To obtain the frequency of swollen nerve fibers, photographs of the above fascicles were randomly chosen and magnified 1000 times. The orthogonal minor axis diameter was measured on 300 random nerve fibers and the number of swollen axons per 300 fibers was determined.

RESULTS

Sequence of Structural Alterations in Animals Exposed to MBK

Rats exposed to MBK at 400 ppm and at 225 ppm were sacrificed serially to study the sequential morphological changes. Those exposed to 400 ppm were sacrificed at 16, 28 and 42 days and those exposed to 225 ppm were sacrificed at 16, 25, 35, 55 and 66 days. Clinically observable paralysis occurred at 42 days using MBK 400 ppm and at 66 days with MBK 225 ppm. Neuropathological changes preceded the clinical signs by many weeks. Two distinct changes occurred quite early and close to the same time. The first to appear was an increase in the number of neurofilaments and the other was an inpouching of the myelin sheath.

Quantitative studies of neurofilaments: The number of neurofilaments was determined in a control 200 gm rat using the identical fixation and embedding procedure (detailed under *Materials and Methods*) as animals exposed to MBK. The number of neurofilaments per unit area (0.933 μm^2) was 20.3 ± 0.96, illustrated by the regression coefficient in Fig. 1. There was a highly significant linear relationship between the number of neurofilaments and size of area counted with a correlation coefficient of 0.996. When this count per unit area was applied to the study of axon caliber the number of neurofilaments showed a significant linear regression as the axon caliber increased, 10.7 ± 2.45 neurofilaments/μm diameter (Fig. 2a). Another measurement we established in control nerve fibers was the ratio of axon caliber to myelin thickness. This proved useful in evaluating the degree of axon swelling and/or myelin thinning. In the control nerve fibers the axon caliber/myelin thickness (AC/MT) ratio ranged from 1.3 to 7.3 with the mean ratio of 4.6 ± 0.3 (n:24).

In animals exposed to MBK 400 ppm, the first observable change at 16 days was the presence in larger diameter nerve fibers of a 2–3 fold increase in neurofilaments (Fig. 2b). For the 16 day exposure the regression coefficient of 37.3 neurofilaments/μm diameter was significantly higher than the control value. This abnormality was seen at a time when there was preservation of a normal AC/MT ratio in most fibers, mean AC/MT:5.0 ± 0.3 (n:30). With continued exposure many additional changes occurred as outlined below but

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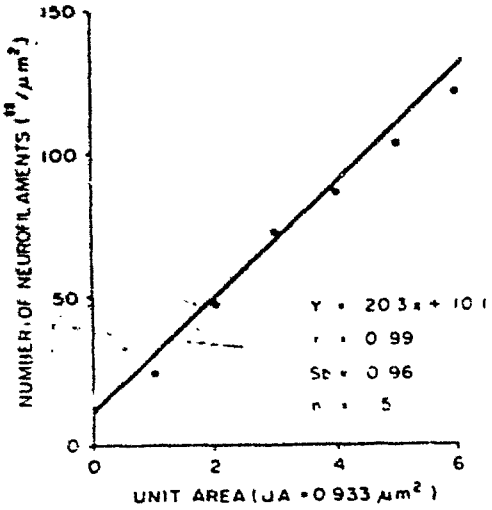

FIG. 1. Relation between number of neurofilaments and unit area (U.A. = 0.933 μm^2). Y: predicted number of neurofilaments; X: measured number of U.A.; r: correlation coefficient; b: regression coefficient; S_b: standard error of regression coefficient; n: number of plotted points.

even after 42 days of exposure when there was a definite increase in the number of neurofilaments, 41.8 neurofilaments/μm diameter (Fig. 2c), most nerve fibers still retained their normal AC/MT ratio with a mean of 4.4 ± 0.5 (n:32). In addition to an increase in neurofilaments some nerve fibers also showed a decrease in the number of neurotubules compared to controls. The control neurotubule count ranged from 12/μm^2 to 43/μm^2 (Fig. 3). As the number of neurofilaments increased there appeared to be a decrease in neurotubules in these areas. This was especially well illustrated after 42 days of MBK, 400 ppm exposure ($P < 0.01$) (Fig. 3).

These quantitative studies showed that in the early stages of this neuropathy, prior to significant axonal swelling, the neurofilaments gradually increased in number at a time when there was a normal relationship of axon caliber to myelin thickness (Fig. 4). The individual neurofilaments accumulating in these axons showed no abnormalities at high magnification (Fig. 5). They were of approximately 100 Å diameter with an electron lucent core and had normal side-arms.

Quantitative studies of myelin inpouchings: In individual teased nerve fibers, inpouchings of the myelin sheath could be seen clearly (Fig. 6). These occurred anywhere along the internodal segment and the direction of inpouched myelin could be either proximal or distal. Although similar inpouchings could be observed in normal animals, our quantitative studies demonstrated that the number of these myelin intrusions showed a strong correlation with duration of exposure. Normal control nerves were found to show up to 22 fibers with inpouchings per mm^2 of nerve fascicle. At MBK 400 ppm, the numbers increased as follows: 16 days, 6/mm^2; 28 days, 142/mm^2; and 42 days, 499/mm^2 (Table 1). At MBK 225 ppm exposure, the incidence increased progressively: 16 days, 23/mm^2; 25 days, 46/mm^2; 35 days, 92/mm^2; 55 days, 86/mm^2 (Table 1), and 66 days, 645/mm^2. Under the electron microscope the

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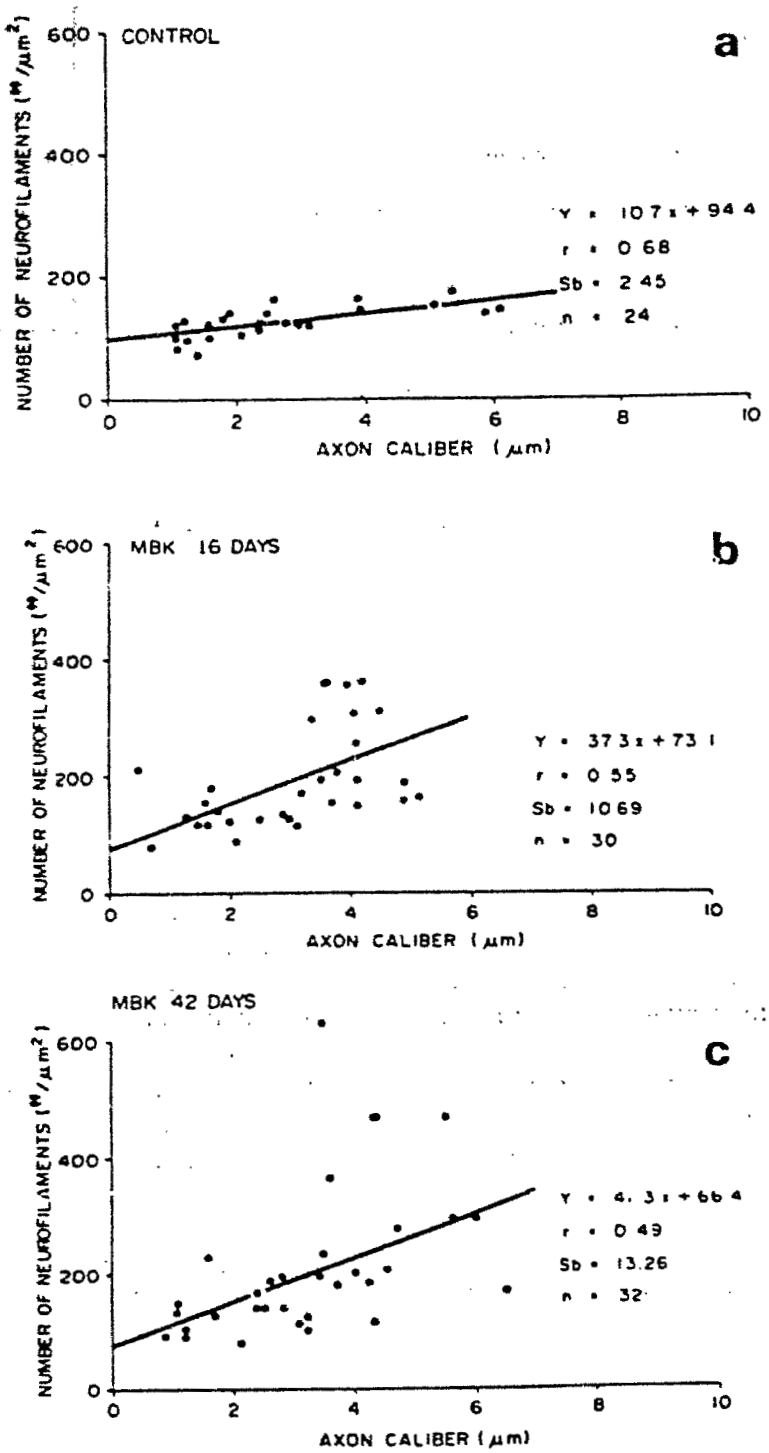

FIG. 2. Relation between number of neurofilaments and axon caliber. 2a (Top): control. 2b (Middle): MBK 16 days exposure. 2c (Bottom): 42 days exposure.

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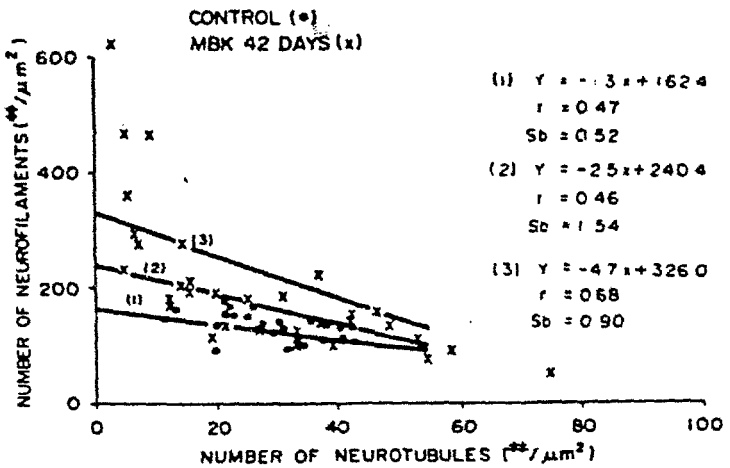

FIG. 3. Relation between number of neurofilaments and number of neurotubules. The control values are plotted by solid circles and its regression curve and equation are shown as (1). The regression curve and equation of MBK 16 days exposure are shown as (2), and the values of MBK 42 days exposure are plotted by cross marks with the regression equation (3).

myelin contained within these infoldings showed no abnormalities (Fig. 7). In addition no axonal changes were observed adjacent to these inpouchings and the adaxonal membranes were normal.

Interrelated morphological changes: As the duration of exposure lengthened and the number of neurofilaments increased several interrelated morphological observations were seen. In teased nerve fiber preparations, swellings of the axon could be seen frequently in the paranodal area and less often at focal sites along the internodal segment (Fig. 8). Increased numbers of neurofilaments were easily observable in the electron microscope without quantitative determinations in these areas of obvious axonal swelling. At times these areas of neurofilamentous increase would extend for very long distances along the internodal segment (Fig. 9). In some areas bundles of neurofilaments appeared to run parallel to a bundle of aggregated neurotubules and mitochondria which appeared to be pushed aside by the neurofilaments (Fig. 10). Other axoplasmic organelles were usually well preserved except for occasional degenerative changes in mitochondria and smooth endoplasmic reticulum. Swelling of axons and increase in neurofilaments were also found in unmyelinated nerve fibers (Fig. 11) but much less frequently than in myelinated nerve fibers. The swollen axons were usually associated with thinning of the myelin sheath (Fig. 12). In these areas the myelin sheath showed a normal configuration but was composed of a reduced number of myelin lamellae as described in myelin slippage (10) (Fig. 13). The myelin thinning appeared initially in the paranodal region but later extended for varying distances along the axis cylinder. As the axon diameter continued to enlarge some axons were found to be completely denuded of myelin (Fig. 14). The relationship between the duration of exposure and the numbers of nerve fibers showing axonal swelling and denudation of myelin increased with time. This can be seen in Table 1 showing a random quantitation of these

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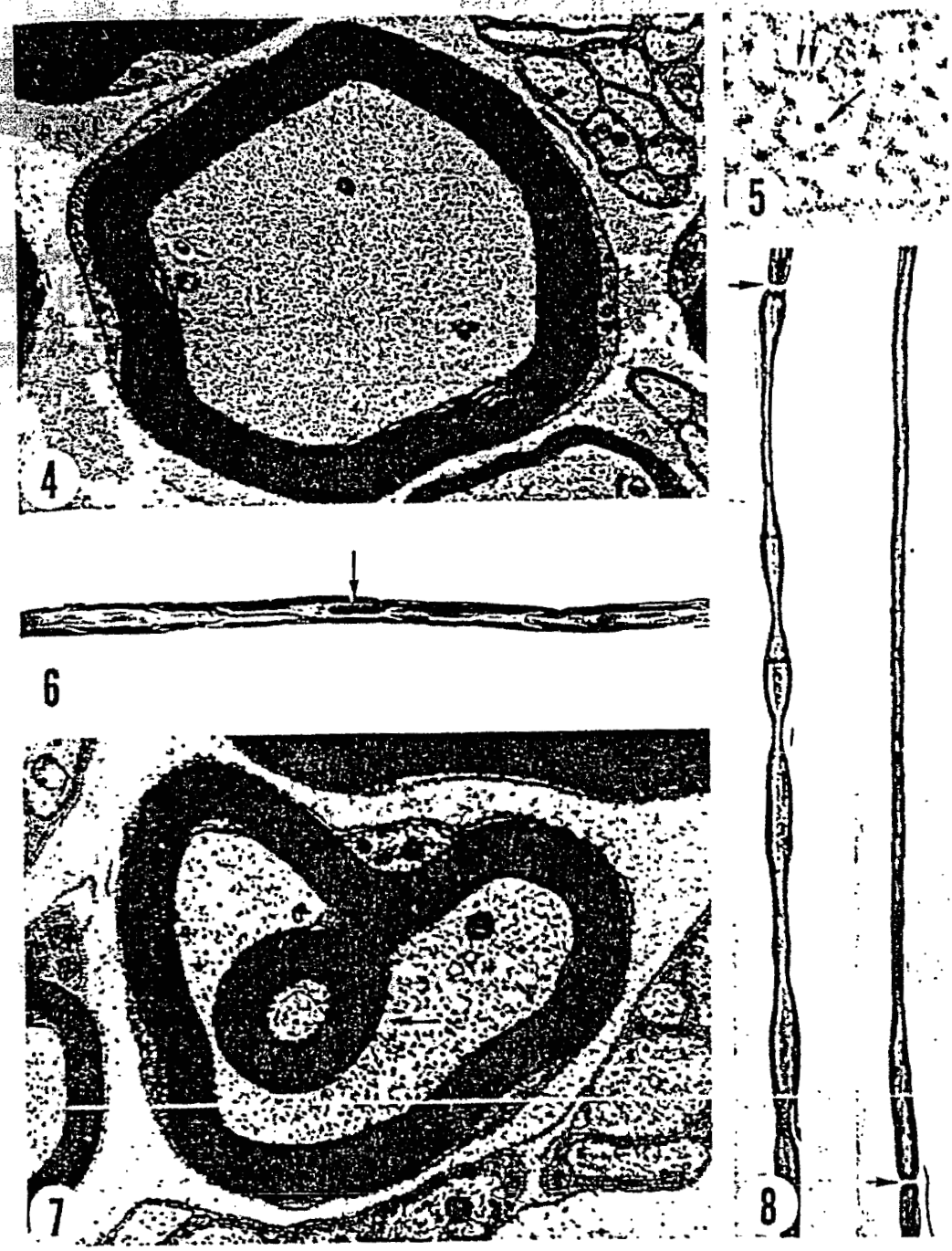

FIG. 4. Proliferation of neurofilaments in a nerve fiber with normal axon caliber-myelin thickness ratio, 5.3. × 13,000.

FIG. 5. Higher magnification of neurofilaments from a fiber with proliferation of neurofilaments. Neurofilament of 100 Å caliber with central electron lucent area (arrow) and side arms (double arrows) is seen. × 100,000.

FIG. 6. Inpouching of myelin sheath (arrow) protruding into the axoplasm of a teased nerve fiber. × 120.

FIG. 7. Inpouching of myelin sheath showing normal thickness of myelin (arrow). Axoplasm shows no significant changes. × 11,800.

FIG. 8. One internodal segment (between arrows) showing paranodal and internodal swellings of axon in a teased nerve fiber. × 63.

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TABLE 1

Comparative Sequential Clinical and Pathological Observations

Rats were exposed to MBK at 400 and 225 ppm alone, combination of MBK 225 ppm:MEK 1125 ppm and MEK 1125 ppm alone at day 16, 25, 35, and 55.

	MBK 400 ppm			MBK 225 ppm				MBK 225 ppm MEK 1125 ppm				MEK 1125 ppm			
Days exposed	16	28	42	16	25	35	55	16	25	35	55	16	25	35	55
Clinical findings	N	N	P	N	N	N	N	N	P	P	P	N	N	N	N
Inpouchings (# mm²)	6	142	499	23	46	92	86	70	216	336	435	8	11	6	8
Denuded fibers (# mm²)	0	4	11	0	0	1	2	0	0	7	13	0	0	0	0
Swollen axons >11 μm (# 300 fibers)	0	1	3	0	0	0	0	0	0	2	8	0	0	0	0

N: Normal. P: Paralysed.

observations in 1 μm cross-sections of epoxy resin embedded nerve. The total area counted in each animal averaged 0.75 mm². The incidence of swollen axons in this table is artifactually decreased since 11 μm had to be used as the upper limits of normal for the cross sectional diameter. Our control animals had normal fibers up to 10 μm in diameter. Actually fibers of smaller diameter with focal swellings were observed frequently in teased preparations, but they would not be included here if their diameter did not exceed 11 μm.

In the very late stages of the neuropathy, only after clinical paralysis occurred, did we observe occasional nerve fibers undergoing Wallerian degeneration. These were typical examples consisting of degeneration of the axon with numerous dense membranous bodies and myelin debris in the cytoplasm of Schwann cells.

A number of less frequent changes were observed while examining the sequential abnormalities. Generally Schwann cells appeared active with increased Golgi apparatus and increased rough endoplasmic reticulum. Rarely very large mitochondria were seen in the Schwann cell cytoplasm. In some nerve fibers, especially around Schmidt-Lanterman clefts or nodal areas, adaxonal membranes invaded the axoplasm and surrounded it (Fig. 15). These changes were similar to those observed by Prineas (20). In addition "islands" of Schwann cell processes were occasionally seen underneath the basement membrane and thin whorls of myelin were found in the Schwann cell cytoplasm (Fig. 16). Another abnormality of the myelin, often seen in the late stages, was a denudation of the myelin sheath which occurred anywhere along the internodal segment in areas unrelated to swollen axons (Fig. 17). This type of denudation was accompanied by numerous inpouchings of the myelin sheath.

Recovery phase: We had the opportunity to examine one cat in the recovery phase. This animal had been exposed continuously to MBK 400 ppm, for 7 weeks after which time he became paralyzed. He was then removed from the exposure chamber and kept in a normal environment for 4½ months. During

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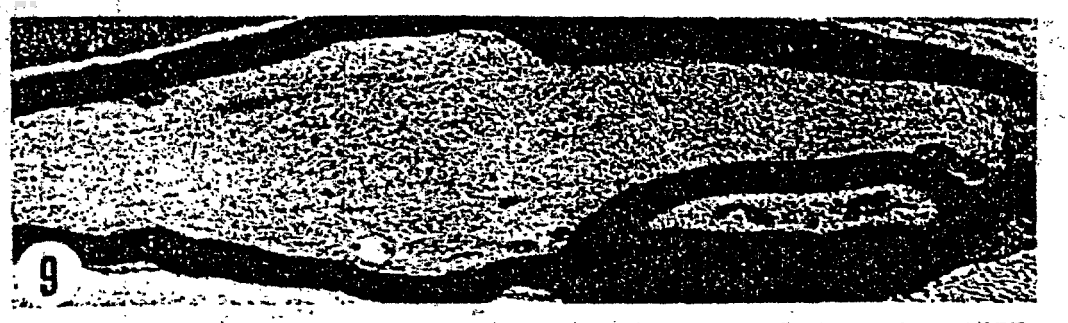

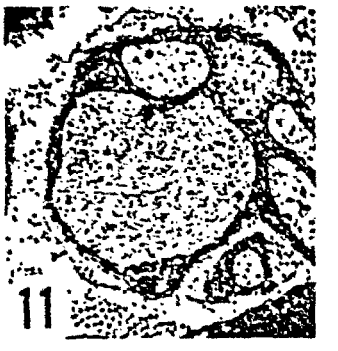

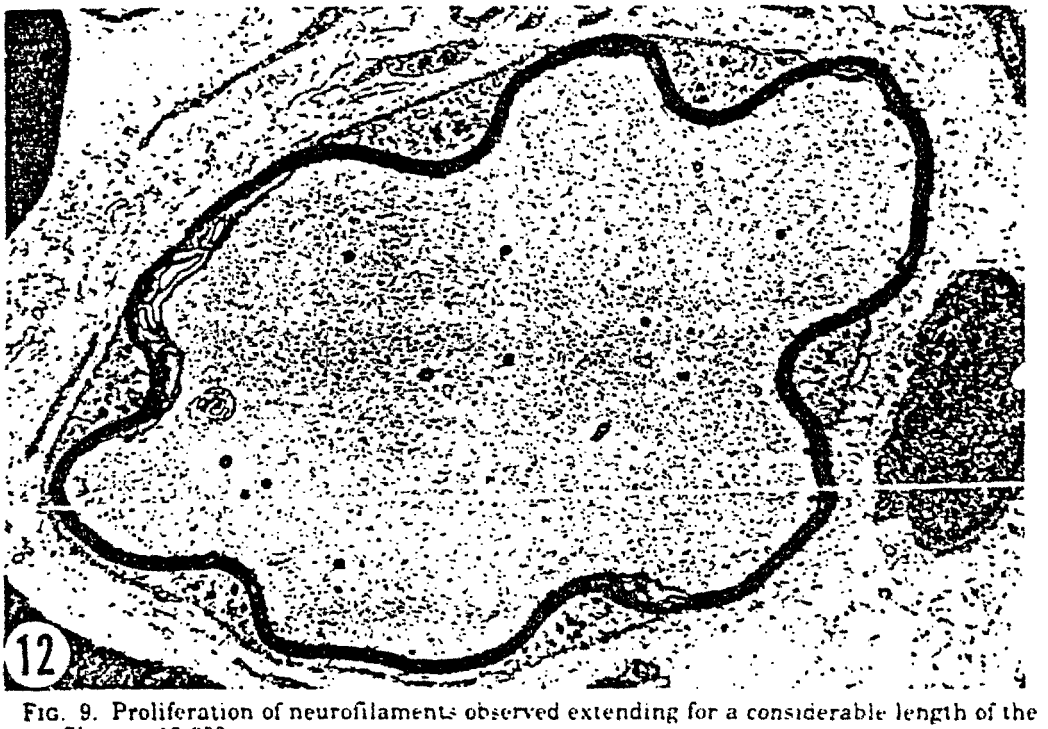

FIG. 9. Proliferation of neurofilaments observed extending for a considerable length of the nerve fiber. × 13,000.

FIG. 10. Bundle of neurotubules and mitochondria seen in upper left portion of picture along the edge of the axon in a fiber with increased number of neurofilaments. × 19,900.

FIG. 11. Unmyelinated nerve fiber with proliferation of neurofilaments. × 25,500.

FIG. 12. Thinning of myelin sheath of the axon with the proliferation of neurofilaments. Axon caliber-myelin thickness ratio (31.0) is greatly increased. × 11,800.

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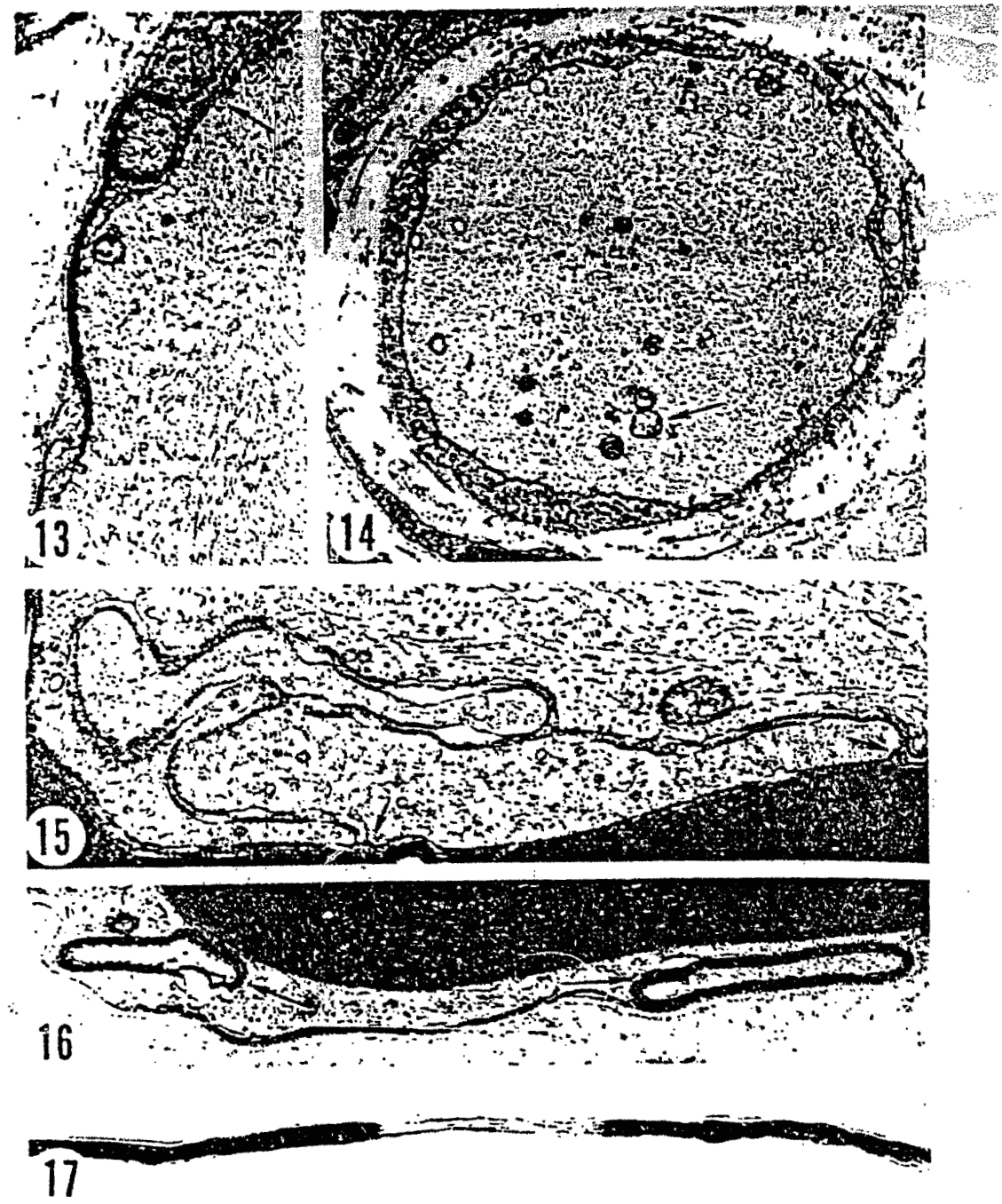

FIG. 13. Retraction of myelin sheath (arrow) in the paranodal area in a fiber with proliferation of neurofilaments. × 11,200.

FIG. 14. Denudation of myelin sheath in a fiber with an increase of neurofilaments. The degeneration of axoplasmic components is also seen (arrow). × 14,200.

FIG. 15. Adaxonal membranous intrusion originating at arrows and extending toward the axoplasm of the nerve fiber. × 32,500.

FIG. 16. Myelin whorls (arrows) in the cytoplasm of the Schwann cell. × 18,700.

FIG. 17. Denudation of myelin sheath in a teased nerve fiber without axonal swelling. × 140.

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that time he regained the ability to walk. In the sciatic nerve the most striking changes were a moderate loss of myelinated nerve fibers and an increase in connective tissue. At this stage the number of swollen axons was greatly reduced over that seen in the acute toxic stage and a larger proportion of fibers showed the Wallerian degeneration. In addition a large number of teased fibers had a marked shortening of the internodal segments and in the electron microscope, thinly myelinated nerve fibers were observed suggesting remyelination. These nerve fibers were surrounded by 2–3 Schwann cell processes (Fig. 18).

Site of Involvement

The anterior horn cells, nerve roots, nerve trunks, intramuscular nerves and motor end plates were studied sequentially to determine the earliest site of pathological involvement. In the animals exposed for 16 days to MBK, 225 ppm, no abnormalities were found in the motor end plates or intramuscular nerves of the intrinsic foot muscles. This was also true after 16 days of exposure to the MBK:MEK, 225:1125 ppm, combination. However, at this time the animals exposed to this combination were already showing the early changes of increased numbers of neurofilaments and inpouchings of the myelin sheath in the main trunk of the sciatic nerve. After 25 days of the MBK:MEK combination, when clinical paralysis was present and the main trunk of the sciatic nerve showed the full spectrum of neuropathological changes, similar changes were appearing in the nerve roots and intramuscular nerves. Even at this time no abnormalities were seen in the axon terminals or post-synaptic membrane at the neuromuscular junction. Only after extremely prolonged exposure, after 66 days of MBK, did we find typical changes of denervation in the motor end plates. These denervated end plates showed atrophic axon terminals with Schwann cell processes interposed between the nerve terminal and post-synaptic membrane (Fig. 19). There was also a loss of secondary synaptic clefts.

Anterior horn cells and dorsal root ganglion cells were also examined at various intervals of exposure. No changes were observed in these cell bodies even after typical changes were seen in the main trunk of the sciatic nerve. Specifically no abnormalities were seen which would suggest an increase in neurofilaments in these cell bodies and no cells were observed undergoing chromatolysis.

Interaction of MBK and MEK

We compared the effects of exposure to MBK 225 ppm, with MEK 1125 ppm, and the combination of MBK:MEK, 225:1125 ppm. Animals in these three groups were sacrificed serially for quantitative histologic studies. The differences were quite striking (Table 1). No peripheral neurotoxicity was seen with MEK alone at 1125 ppm, as shown in Table 1, up to 55 days. Additional studies were also carried out for as long as 5 months and no abnormalities

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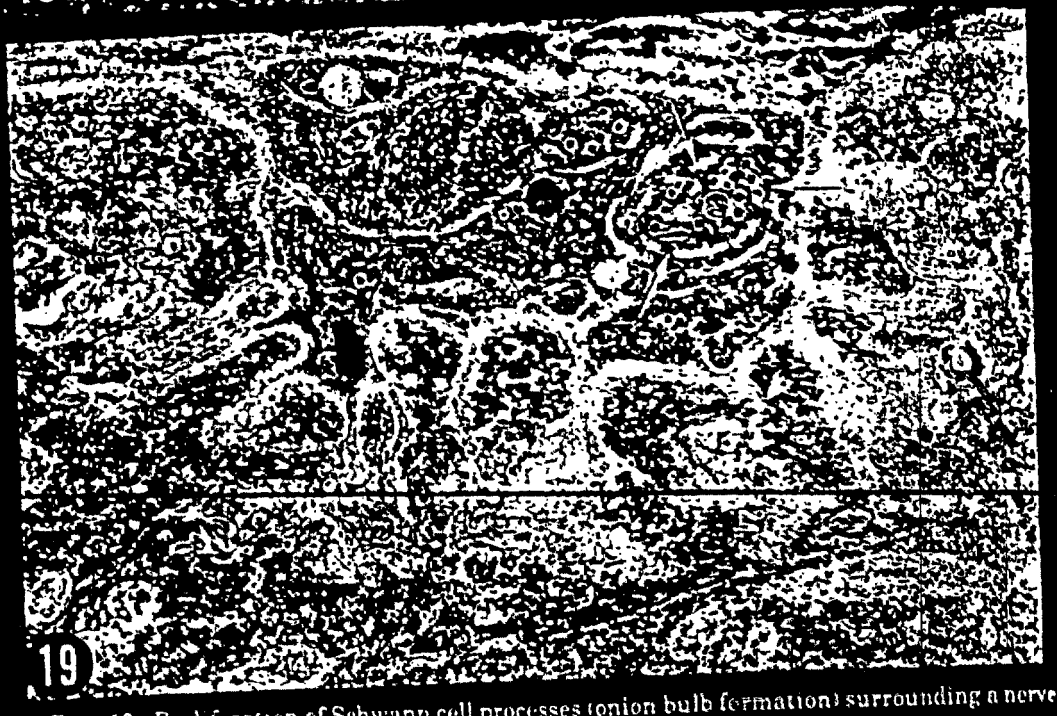

FIG. 18. Proliferation of Schwann cell processes (onion bulb formation) surrounding a nerve fiber of a cat recovered from paralysis. × 27,300.

FIG. 19. Neuromuscular junction of intrinsic foot muscle after prolonged MBK exposure. Shrunken axon terminal (arrows) with interposition of Schwann cell cytoplasm between terminal and primary synaptic cleft is seen.

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were seen in these animals. The combination of MBK and MEK was considerably more toxic than MBK alone. Clinically animals exposed to the combination failed to gain weight and showed a clinical paralysis by 25 days of exposure. Animals exposed to MBK alone did not develop paralysis until 66 days. Also the incidence of inpouchings of myelin sheaths, denudation of axons and axonal swellings was greatly accelerated with the combination as illustrated by the quantitative studies in Table 1. At 55 days of exposure random sampling of nerve fibers showed the incidence of inpouchings of the myelin sheaths to be 455/mm^2 compared to 86/mm^2 for MBK alone. The number of denuded axons was 13/mm^2 compared to 2/mm^2 for MBK alone and the number of axons with a diameter >11 μm was 8/300 fibers compared to 0 for MBK alone.

REPORT OF A CASE

A 41-year-old male worked as a painter using a paint thinner containing MBK from September, 1972 through July, 1973. He worked 8 hours per day, 5 days per week during that period. He used the paint thinner liberally, mixing it with the paint as necessary. Most of the work was done in a closed environment primarily applying the paint through a spray type applicator. He used a mask but he felt this was inadequate and the odor of the paint and solvent could be easily appreciated through the mask. In addition, at the end of each working period, that is for example, coffee breaks, lunch time and at the end of the working day, he and his colleagues would use the paint thinner to wash their hands and face to remove any residual paint on their skin.

He first developed leg weakness manifested by difficulty in climbing stairs in late July, 1973. His muscle weakness continued to progress for approximately three months until he was unable to walk and, later, unable to hold objects in either hand. These symptoms subsequently plateaued and remained the same for an additional two to three months and then gradually began to improve. The patient also complained of a feeling of a tightness of his skin below his knees down to his toes. The patient had lost 30 pounds in a two month period. At the time this work up was done two colleagues of this patient also complained of similar difficulties.

The patient had been a painter for fifteen years and had also been in contact with lead as evidenced by the laboratory data. There was no history of excessive alcohol intake and no family history of diabetes mellitus or inherited nervous system disease.

General physical examination on August 29, 1973, was described as within normal limits. The neurological examination showed a normal mental status and normal cranial nerve functions. The patient could walk only with assistance. There was bilateral foot drop as well as weakness of the quadriceps and hamstring muscles in the lower extremities. In the upper extremities the wrist flexor and extensor muscles were moderately weak and there was slight weakness of biceps and triceps muscles. The deep tendon reflexes were hypoactive in the upper extremities and absent at the knees and the ankles. The examination of the cerebellum was normal.

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Laboratory findings in August, 1973 were as follows: Hemoglobin 10.8, Hematocrit 34.2, MCV 79, WBC 8,900 with normal differential; positive basophilic stippling was observed; ESR 43, Serum Fe 76; TIBC 280 with 27.1% saturation; glucose 83; electrolytes normal; SGOT, LDH and total bilirubin were normal; uric acid 6.9; Vitamin B12 and folic acid were normal; L.E. cells negative × 3; ANA negative × 3. Spinal fluid was clear and colorless with normal opening pressure; glucose 70, protein 27, no cells; and negative VDRL. Electromyographic examination revealed positive waves, fibrillations and polyphasic motor unit potentials with an incomplete interference pattern; more in the lower extremities than in the upper extremities. The left median nerve motor conduction velocity was 36 m/sec with a latency of 6.5 msec. Chest X-ray and EKG were normal. G.I. series revealed normal functioning of the esophagus and stomach but a deformed duodenal bulb was present without any evidence of an acute ulcer. Gastroscopy showed a marked antral gastritis. Whole blood lead levels showed 85, 55, 60 and 66 mcg % on different occasions with normal values of 0–60 mcg %. Twenty-four hour urine examination for total lead showed values of 196 mcg (total urine volume 740 cc), 158 mcg (2360 cc) and 192 mcg (1165 cc) with normal 0–160. EDTA mobilization test revealed 24 hour excretion of lead in the urine to be 1,885 mcg compared with the normal value of less than 1,000 mcg.

When the patient was first admitted he was thought to have a lead neuropathy. The symptoms and the signs of the polyneuropathy were, however, felt to be somewhat unusual for a typical lead polyneuropathy (9, 13). The usual cases of lead neuropathy are associated with a higher concentration of lead in serum and urine (5, 6). This prompted the additional history which revealed the onset of symptoms which was clearly correlated with the use of MBK. In addition the outbreak of similar symptoms in two colleagues (whom we were not able to examine) also suggested MBK as the most likely cause. The patient was referred to us for a neurological examination and a sural nerve biopsy in May, 1974. The examination at that time showed mild bilateral foot drop with inability to walk on heels and toes. The patient's muscle strength was as follows (MRC Scale): finger extensors 4/5; finger flexors 4/5; dorsal and ventral interossei 4/5; opponens pollicis and opponens digiti quinti 4/5; anterior tibialis, posterior tibialis, peroneals and toe extensors 3/5; all other muscle groups were normal. Sensory examination in the lower extremities revealed normal position sense; minimal vibratory loss in toes, bilaterally; mild diminution of pin and temperature to the level of the tibial tuberosity, bilaterally; no loss of light touch; deep pain intact. In the upper extremities, there was a mild loss of superficial pain to wrists, bilaterally. Deep tendon reflexes in the biceps, triceps and brachioradialis muscles were 1+, bilaterally. Knee jerks were trace with reinforcement; and ankle jerks were absent bilaterally. Romberg's sign was negative and cerebellar examination was normal. Sural nerve biopsy showed the following results: the number of myelinated nerve fibers was moderately diminished with an increased connective tissue. The striking findings were the existence of denuded swollen axons with an enormous increase in neurofilaments (Fig.

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20). Mitochondria were degenerating in these fibers. Usually two or three nuclei of Schwann cells were noted around these denuded fibers. Active Wallerian degeneration with myelin debris in the Schwann cell cytoplasm and axonal degenerative changes were seen occasionally. Many small myelinated fibers were thought to be regenerating because of the relationship of the thickness of meylin sheath to axon caliber (23). Axonal sprouting in the residual basement membrane and remyelination in these sprouts was also encountered. Active myelin breakdown or demyelination was not found, but a slight degree of onion bulb formation was noted. These results were most compatible with a primary axonal degeneration. The existence of giant axons with proliferation of neurofilaments and occasional nerve fibers undergoing Wallerian degeneration was quite similar to those findings in the recovery phase of experimental MBK neuropathy. The clinical picture was also quite typical of the patients seen during the industrial outbreak of polyneuropathy secondary to MBK (2).

DISCUSSION

The sequential morphological studies of the changes induced by MBK revealed that the initial abnormality produced was a gradual increase of neurofilaments. Whether this was a proliferation of neurofilaments or represented focal accumulations cannot be definitely answered at the present time. Since we found no areas of the axon that had a decreased number of neurofilaments, our data appears to support a proliferation of neurofilaments to account for these increased numbers. It seems clear that as the neurofila-

FIG. 20. Swollen denuded axon from sural nerve biopsy. × 4,000. Inset: high magnification of a portion of the axon showing accumulation of neurofilaments. × 20,000.

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ments increased there was an enlargement of the axon diameter. The earliest swelling occurred in the paranodal regions but later they were seen anywhere along the internodal segment. The initial increase in the number of neurofilaments occurred at a time when the AC/MT ratio was unaltered. As this process continued there was a gradual thinning of the myelin sheath which resulted in an increase in the AC/MT ratio. The thinning of the myelin sheath at times proceeded to complete denudation of the swollen or "giant" axons. These alterations of the myelin sheath appeared to be secondary to changes in axon diameter as has been described by Friede and Martinez in Wallerian degeneration (10) and support the concept of myelin slippage. Since these changes so closely simulate the giant axonal neuropathy of childhood described by Asbury et al (3), and those reported occurring with n-hexane exposure (14), we believe a similar pathogenetic mechanism may account for the changes seen in the nerve fibers in these conditions. An additional change that occurred early was the presence of inpouchings of the myelin sheath. It seemed unlikely that this abnormality represented a primary effect of the solvent on the myelin sheath. A possible explanation could be that these myelin intrusions were the result of changes in axoplasmic transport. This concept is supported by the observation that in early Wallerian degeneration inpouchings of the myelin sheath are prominent in the distal portion of the transected nerve (11). As a matter of fact, it may be that both of the early changes observed in this neuropathy, accumulating neurofilaments and myelin inpouchings, could be secondary to changes in axoplasmic transport. Another possibility could be, however, that proliferating neurofilaments interrupted axoplasmic transport and resulted in secondary inpouchings of the myelin sheath.

Studies of the late changes and the recovery phase of this MBK neuropathy suggested that some of the abnormal fibers proceeded to Wallerian degeneration. Many, however, appeared to be able to recover with remyelination of the axon if further exposure was prevented. This may account for the clinical recovery observed in the industrial outbreak of the MBK neuropathy (2).

In comparison to other kinds of toxic neuropathies MBK exposure most closely simulates n-Hexane exposure (14). In considering this similarity it is of great interest that in certain mycobacteria, aliphatic alkanes are utilized as sources of carbon (16). For example, n-hexane is metabolized through hexanol to 2-hexanone (MBK). Furthermore, it has recently been observed that intraperitoneally injected MBK in guinea pigs is metabolized to n-hexanol (1). It is therefore conceivable that the neurotoxicity of MBK and n-hexane may be closely linked by a similar site of action or even by a common intermediary metabolite.

Accumulations of neurofilaments are also seen in other kinds of neuropathies (8, 18, 19, 22). They are especially prominent in acrylamide intoxication (20, 21, 25) where the earliest changes begin in the axon terminals. This is clearly different from MBK where we found changes in the axon terminal

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only as a very late finding, long after a full spectrum of changes were seen in the main trunk, nerve roots and intramuscular nerves of the sciatic nerve. The conclusions from our sequential morphological studies of MBK exposure differ from those of Spencer and Schaumberg (24) who considered this neuropathy an example of "dying-back" neuropathy as described by Cavanaugh (7). Our studies suggest that MBK induces changes in the axon from the root to the intramuscular nerve with no evidence of preferential involvement of the most distal portion of the axon as the earliest change. We attribute this difference in interpretation to the fact that we used much lower MBK exposure concentrations and studied the animals sequentially rather than later in the course of the disease process. In addition we found no significant changes in the cell bodies of the anterior horn of the spinal cord or dorsal root ganglion to support a "dying-back" neuropathy. These studies suggest that MBK does not produce a "dying-back" neuropathy as has been described with acrylamide intoxication (20, 21, 25).

These studies also have particular relevance to possible future human industrial solvent use. With regard to MBK, the neuropathy induced in these rats at 225 ppm exposure is the lowest level yet reported in mammals and strongly suggests that the margin of safety for MBK is extremely limited. No doubt this will seriously handicap its future use as a widespread industrial solvent. Furthermore the marked potentiation of neurotoxicity observed with simultaneous combined exposure to MBK and MEK may explain the cause for the widespread outbreak of polyneuropathy seen in 89 employees of one plant where these solvents were used together (2, 4). On the other hand, our very long continuous exposures (5 months) with MEK alone does suggest that this is a safe industrial solvent, apparently free of neurotoxicity, although potentiation of other neurotoxic agents may present a special problem and requires further studies.

In considering the clinical case reported of the patient with peripheral neuropathy and prolonged MBK exposure several parts warrent comment. There is no doubt that this patient had a chronic exposure to lead which resulted in slightly elevated urine and whole blood levels. It is very rare, however, for these levels to be associated with a clinical peripheral neuropathy (6, 13) and we believe that most of the evidence suggests that MBK caused this peripheral neuropathy. The clinical picture observed in this patient characterized by distal muscle weakness, as well as the sensory changes consisting of a distal loss of superficial pain, temperature, and vibration and the electrodiagnostic studies were identical to those seen in the industrial outbreak secondary to MBK (2). The onset of the patient's symptoms and signs was accompanied by similar complaints in two co-workers exposed to similar environmental working conditions. The changes observed in the sural nerve biopsy consisted of the striking presence of giant denuded axons and an overall appearance similar to the recovery phase of the MBK induced neuropathy. If chronic lead exposure did play a role in producing this neuropathy the pathological features are different from that described for experimental lead

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neuropathy (12, 15) and more closely simulate those seen with experimental MBK exposure. The possibility also exists that potentiation of effect between lead and MBK could have played a role.

Acknowledgements: This investigation was supported by Bremer Foundation, Ohio State University, Columbus, Ohio. The reported patient was originally examined and referred by I. O. Dein, M.D., the VA Hospital, Louisville, Kentucky.

Daily animal care was provided by Mr. Joseph F. Pitt and technical assistance was performed by Mr. Gregory L. Pittman.

The MBK and MEK used in this study was supplied by Tennessee Eastman Company, Kingsport, Tennessee.

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OPTS-400026
E3-001 (4)

(47)

REF 49

TOXICOLOGY AND APPLIED PHARMACOLOGY 28, 452–464 (1974)

Embryo- and Fetotoxicity of Inhaled Carbon Tetrachloride, 1,1-Dichloroethane and Methyl Ethyl Ketone in Rats

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Received September 14, 1973; accepted November 20, 1973

Embryo- and Fetotoxicity of Inhaled Carbon Tetrachloride, 1,1-Dichloroethane and Methyl Ethyl Ketone in Rats. SCHWETZ, B. A., LEONG, B. K. J. AND GEHRING, P. J. (1974). *Toxicol. Appl. Pharmacol.* 28, 452–464. These studies evaluated the effects of subanesthetic concentrations of carbon tetrachloride, 1,1-dichloroethane and methyl ethyl ketone on rat embryonal and fetal development. Groups of pregnant Sprague–Dawley rats were exposed to each solvent 7 hr/day on days 6 through 15 of gestation. All 3 solvents caused some degree of retarded fetal development, such as delayed ossification of sternebrae. In addition to this degree of embryotoxicity, exposure to methyl ethyl ketone, but not to carbon tetrachloride or 1,1-dichloroethane, also caused a low incidence of acaudia, imperforate anus and brachygnathia. These studies do not reveal a correlation between the toxicity incurred by the mother exposed to these solvents and that incurred by the embryos or fetuses.

Exposure to anesthetic concentrations (Schwetz and Becker, 1974) or subanesthe concentrations of chloroform (Schwetz *et al.*, 1974) during pregnancy has detrimen effects on the developing embryo and fetus of laboratory animals. In order to assess t hazard of exposure of the embryo and fetus to subanesthetic concentrations of ot solvents found in the working environment, pregnant rats have been exposed 7 hr da on days 6 through 15 of gestation to such concentrations of the vapors of carbon tet chloride, 1,1-dichloroethane and methyl ethyl ketone.

It was initially anticipated that if chloroform had an untoward effect on the emb or fetus, it might be correlated with the hepatotoxic activity of chloroform in the moth To evaluate the possible existence of such a correlation, carbon tetrachloride included in the study because of its high hepatotoxic potential and 1,1-dichloroeth was included because it has little or no hepatotoxic activity. Hepatotoxicity in pregn rats was monitored by the gross appearance of the liver, the total weight of the li the ratio of the liver weight to the body weight and the serum glutamic-pyruvic tra aminase (SGPT) activity. In addition, the SGPT activity of concurrently exposed n pregnant females was measured during and after the 10-day exposure period.

Methyl ethyl ketone was included in the study because it is an extensively used, n chlorinated solvent. More importantly, it was anticipated that the inclusion of met ethyl ketone might provide a means of differentiating embryo and fetal toxicity as ciated with chlorinated solvents from the effects of a nonchlorinated solvent.

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METHODS

Animals. Adult, Sprague–Dawley (Spartan) female rats weighing approximately 250 g were used. The day on which sperm were seen in a vaginal smear was considered day 0 of pregnancy. Between daily exposures, animals were housed in wire-bottom cages and were maintained on commerical laboratory rat chow[1] and water ad libitum in a room controlled for temperature, humidity and light cycle. Food or water were not provided during exposure. Food consumption of each animal was measured at 2-day intervals throughout the experimental period.

TABLE 1

NOMINAL AND ANALYTICAL CONCENTRATIONS OF SOLVENTS IN EXPOSURE ATMOSPHERES

Solvent	Nominal concentration[a] (ppm)	Analytical concentration[b] (ppm)
Carbon tetrachloride	300	334 ± 48
	1000	1004 ± 90
1,1-Dichloroethane	3800	3798 ± 462
	6000	5620 ± 39
Methyl ethyl ketone	1000	1126 ± 61
	3000	2618 ± 26

[a] Calculated from the ratio of the rate of liquid introduced to the rate of the dilution air flow.
[b] Determined by infrared spectrometry, mean ± SD of 3 measurements on each of 10 consecutive days of exposure.

Materials and exposure procedure. Reagent grades of carbon tetrachloride,[2] 1,1-dichloroethane[3] and methyl ethyl ketone[4] were used in these experiments. Exposures were conducted in 3.7 m^3 stainless steel, cubical dynamic exposure chambers. Vapors were generated by metering the liquid at known rates into a temperature controlled evaporating flask and were diluted with filtered room air at a rate calculated to give the desired concentration. The nominal concentration of each compound in the chamber atmosphere was calculated from the ratio of the material delivery rate to the rate of total air flow through the chamber. The analytical concentration was determined by analyzing the chamber atmosphere 3 times daily using a Beckman IR10 infrared spectrophotometer with a multipath gas cell having a variable path length of 1–10 m and a volume of 4.5 liters (Tables 1 and 2). In addition, the concentration in the chambers was continuously monitored using combustion conductivity analysis to assure the absence of significant deviations from the desired level. Because the concentrations determined by analyses were essentially the same as the nominal concentrations, the nominal concentrations will be referred to throughout this paper.

[1] Purina Rat Chow®, Ralston Purina Co., St. Louis, Missouri.
[2] Lot No. 9256, Burdick & Jackson Lab. Inc., Muskegon, Michigan.
[3] Lot No. 464992, The Dow Chemical Company, Midland, Michigan.
[4] Lot Nos. 8786, 9008, Burdick & Jackson Lab., Inc., Muskegon, Michigan.

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TABLE 2

DESCRIPTION OF TEST MATERIALS AND CALCULATED LEVELS OF EXPOSURE TO MINOR SAMPLE COMPONENTS

Sample and components	Volume % by weight in sample[a]	ppm in chamber[b]	
Carbon tetrachloride			
Carbon tetrachloride	99.9	334 ± 48	1004 ± 90
Unknown	0.1	0.3	1.0
1,1-Dichloroethane			
1,1-Dichloroethane	99.7	3798 ± 462	5620 ± 39
Ethyl chloride	0.02	1.1	1.7
Butylene oxide	0.08	4.2	6.2
Trichloroethylene	0.05	1.4	2.1
Ethylene dichloride	0.01	0.3	0.5
Unknown	0.14	5.3	7.8
Methyl ethyl ketone	99.7	1126 ± 61	2618 ± 26
Acetone	0.05	0.7	1.6
Unknown	0.25	2.8	6.5

[a] Determined by gas–liquid chromatography.
[b] Concentration of the major component was determined by infrared spectrometry. Concentration of minor components was calculated as a proportion of their concentration in the liquid sample relative to the major component.

Experimental design. In an initial experiment, groups of rats were exposed to 300 ppm carbon tetrachloride, 3800 ppm 1,1-dichloroethane or 1000 ppm methyl ethyl ketone In a second experiment, groups of rats were exposed to 1000 ppm carbon tetrachloride 6000 ppm 1,1-dichloroethane and 3000 ppm methyl ethyl ketone. Nonpregnant femal rats were exposed simultaneously with the pregnant rats and were used for evaluation o treatment-related changes in serum glutamic-pyruvic transaminase (SGPT) activit during and after exposure.

Control animals for each experiment were exposed concurrently to filtered room ai The data for the 2 control groups were combined for statistical comparison with th exposed animals for all parameters except the incidence of delayed ossification of sterne brae. The incidence of this anomaly differed between the air-control groups used in th first and second experiments. Therefore, the incidence of sternebral anomalies in ra exposed to the 2 levels of each solvent was compared to their respective controls. For a other statistical comparisons, the data for the 2 control groups were combined.

The following were observed or measured as described in the previous paper b Schwetz *et al.* (1974): maternal toxicity, examination of fetuses for anomalies, evalu tion of hepatotoxicity and statistical evaluation of body and liver weights, SGP activity and the frequency of anomalies and resorptions.

RESULTS

Carbon Tetrachloride

Embryo- and fetotoxicity. Exposure of pregnant rats to 300 or 1000 ppm CCl_4 o days 6 through 15 of gestation had no effect on the incidence of fetal resorptions (Tabl 3). At both concentrations, fetal body weight and crown–rump length were significantl

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TABLE 3

EFFECT OF INHALED SOLVENTS ON OBSERVATIONS MADE AT THE TIME OF CESAREAN SECTION OF RATS[a]

Statistic	Air control	Carbon tetrachloride		1,1-Dichloroethane		Methyl ethyl ketone	
Solvent concentration (ppm)	—	300	1000	3800	6000	1000	3000
Number of litters	43	22	23	16	19	23	21
Percent pregnancy (pregnant/bred)	91 (43/47)	92 (22/24)	82 (22/28)	80 (16/20)	95 (19/20)	79 (23/29)	100 (21/21)
Corpora lutea/dam[b]	14 ± 2	14 ± 2	14 ± 3	14 ± 3	16 ± 3[d]	14 ± 3	14 ± 3
Implantation sites/litter[b]	11 ± 3	13 ± 2	10 ± 4	11 ± 3	1[illegible] ± 2	12 ± 3	12 ± 3
Live fetuses/litter[b]	11 ± 3	12 ± 2	9 ± 4	10 ± 3	12 ± 2	11 ± 3	12 ± 3
% Resorptions/implantation sites	7 (34/492)	6 (16/284)	10 (25/239)	6 (10/172)	6 (14/245)	6 (15/268)	6 (15/257)
% Litters with resorption	44 (19/43)	41 (9/22)	48 (11/23)	38 (6/16)	47 (9/19)	52 (12/23)	48 (10/21)
Litters totally resorbed	0/43	0/22	1/23	0/16	0/19	0/23	0/21
Resorptions/litters with resorptions	1.8 (34/19)	1.8 (16/9)	2.3 (25/11)	1.6 (10/6)	1.6 (14/9)	1.2 (15/12)	1.5 (15/10)
Sex ratio, M:F	56:44	55:45	55:45	47:53	47:53	50:50	52:48
Fetal body weight (g)[c]	5.64 ± 0.34	5.29 ± 0.34[e]	4.96 ± 0.68[e]	5.42 ± 0.30	5.42 ± 0.30	5.34 ± 0.28[e]	5.45 ± 0.16
Fetal crown–rump length (mm)[c]	43.7 ± 1.0	42.2 ± 1.0[e]	41.8 ± 2.2[e]	42.8 ± 0.8	43.8 ± 0.8	42.3 ± 0.7[e]	43.1 ± 0.6

[a] Administered by inhalation 7 hr daily on days 6–15 of gestation. Starved controls were fed 3.7 g of food daily on days 6–15 of gestation.
[b] Mean ± SD.
[c] Mean of litter means ± SD.
[d] Significantly different from control by the Fisher Exact Probability test, $p < 0.05$.
[e] Significantly different from control by an analysis of variance and Dunnett's test, $p < 0.05$.

less than that of controls. No anomalies were seen upon gross examination of the fetuses (Table 4). A significant incidence of subcutaneous edema was observed at 300 ppm but not at 1000 ppm. The incidence of sternebral anomalies was significantly increased in the fetuses of rats exposed to 1000 ppm CCl_4.

Maternal toxicity. Food consumption of rats exposed to either 300 or 1000 ppm CCl_4 was reduced throughout the 10-day exposure period (Table 5). As expected, the maternal body weight of these rats was also significantly less than that of controls (Table 6). However, there was no effect on the conception rate or on the number of implantations or litter size (Table 3).

The hepatotoxicity associated with daily exposure to 300 or 1000 ppm CCl_4 is reflected by the significantly increased SGPT activity on all days on which it was measured (Table 7). By 6 days after the last exposure, the SGPT activity in pregnant and nonpregnant rats was not different from that of control animals. Gross changes in the appearance of the liver characteristic of CCl_4 intoxication (pale, mottled livers) were evident immediately following the last exposure but were not evident 6 days later. The relative liver weight of rats exposed to either concentration was significantly increased while the absolute liver weight was increased but not always significantly (Table 8). Gross observation of rats during exposure to 300 or 1000 ppm CCl_4 revealed no effects on demeanor or signs of toxicity.

1,1-Dichloroethane

Embryo- and fetotoxicity. Exposure of pregnant females to 3800 or 6000 ppm 1,1-dichloroethane on days 6 through 15 of gestation had no effect on the incidence of fetal resorptions, on fetal body measurements (Table 3) or on the incidence of gross or soft tissue anomalies (Table 4). A significantly increased incidence of delayed ossification of sternebrae was associated with exposure to 6000 ppm 1,1-dichloroethane. The incidence of vertebrae with bipartite centra was significantly *lower* in fetuses of rats exposed to 3800 ppm than in control fetuses.

Maternal toxicity. Maternal food consumption (Table 5) and weight gain (Table 6) were slightly but statistically significantly decreased among rats exposed to 3000 or 6000 ppm 1,1-dichloroethane. This exposure had no effect, however, on the conception rate, the number of implantations or litter size (Table 3), SGPT activity (Table 7) or the gross appearance of the liver. The relative liver weight of nonpregnant rats was increased 6 days after the 10th and last daily exposure (Table 8). Otherwise, no significant alterations in liver weight occurred in rats exposed to 1,1-dichloroethane. No alterations in demeanor or signs of toxicity were observed in the exposed rats.

Methyl Ethyl Ketone

Embryo- and fetotoxicity. Exposure of pregnant females to 1000 or 3000 ppm methyl ethyl ketone on days 6 through 15 of gestation had no effect on the incidence of fetal resorptions (Table 3). Although the fetal body measurements of rats exposed to 1000 ppm methyl ethyl ketone were decreased, these alterations were not seen in fetuses of rats exposed to 3000 ppm methyl ethyl ketone. There were no gross, soft tissue or specific skeletal anomalies which occurred at a significantly increased incidence among litters of dams exposed to 1000 ppm methyl ethyl ketone (Table 4). However, the total number of litters containing fetuses with anomalous skeletons was significantly increased compared to controls.

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TABLE 4

EFFECT OF INHALED SOLVENTS ON THE INCIDENCE OF FETAL ANOMALIES AMONG RAT LITTERS[a]

Solvent concentration (ppm)	Control	Carbon tetrachloride 300	Carbon tetrachloride 1000	1,1-Dichloroethane 3800	1,1-Dichloroethane 6000	Methyl ethyl ketone 1000	Methyl ethyl ketone 3000
Number of litters examined	43	22	22	16	19	23	21
		% of litters affected (No. of litters)					
Gross	(0)	(0)	(0)	6 (1)[b]	(0)	(0)	19 (4)[c,d]
Skeletal							
Skull anomalies (delayed ossification)	12 (5)	9 (2)	9 (2)	13 (2)	16 (3)	23 (5)	(0)
Lumbar ribs or spurs	24 (10)	41 (9)	27 (6)	25 (4)	47 (9)	27 (6)	38 (8)
Vertebral anomalies (bipartite centra)	21 (9)	27 (6)	14 (3)	(0)	16 (3)	18 (4)	33 (7)
Sternebral anomalies (bipartite; delayed ossification)	(a) 61 (14)	68 (15)		44 (7)		68 (15)	
	(b) 11 (2)		59 (13)[c]		42 (8)[c]		43 (9)[c]
Total skeletal	58 (25)	91 (20)[c]	68 (15)	69 (11)	74 (14)	95 (21)[c]	81 (17)
Soft tissue							
Subcutaneous edema	33 (14)	59 (13)[c]	50 (11)	31 (5)	37 (7)	9 (2)	48 (10)
Dilated ureters	12 (5)	14 (3)	5 (1)	13 (2)	26 (5)	30 (4)	24 (5)
Total soft tissue	51 (22)	68 (15)	59 (13)	44 (7)	58 (11)	70 (16)	76 (16)[c]

[a] Administered by inhalation 7 hr/day on days 6–15 of gestation.
[b] One acaudate fetus with an imperforate anus.
[c] Incidence significantly different from control by the Fisher Exact Probability test, $p < 0.05$.
[d] Two brachygnathous and 2 acaudate fetuses.

TABLE 5

EFFECT OF INHALED SOLVENTS ON RAT MATERNAL FOOD CONSUMPTION

Test material[a] and concentration	N	Food consumption[b] on gestation day number							
		4–5	6–7	8–9	10–11	12–13	14–15	16–17	18–19
Air control	43	20 ± 3	20 ± 3	20 ± 3	23 ± 2	23 ± 2	23 ± 3	25 ± 4	27 ± 3
Carbon tetrachloride									
300 ppm	22	18 ± 2	12 ± 3[c]	13 ± 3[c]	18 ± 3[c]	16 ± 4[c]	18 ± 4[c]	23 ± 3	ND[b]
1000 ppm	23	19 ± 3	9 ± 3[c]	13 ± 3[c]	14 ± 3[c]	12 ± 4[c]	12 ± 5[c]	23 ± 7	28 ± 7
1,1-Dichloroethane									
3800 ppm	16	19 ± 3	19 ± 6	16 ± 1[c]	16 ± 3[c]	16 ± 3[c]	17 ± 2[c]	23 ± 3	ND
6000 ppm	19	18 ± 3	12 ± 3[c]	14 ± 4[c]	13 ± 4[c]	15 ± 3[c]	16 ± 3[c]	24 ± 2	30 ± 3
Methyl ethyl ketone									
1000 ppm	23	21 ± 2	20 ± 2	22 ± 1	22 ± 2	21 ± 2	24 ± 2	25 ± 2	ND
3000 ppm	21	18 ± 5	18 ± 2	20 ± 2	20 ± 1[c]	21 ± 1	21 ± 1[c]	26 ± 3	29 ± 2

[a] Administered by inhalation 7 hr/day on days 6–15 of gestation.
[b] g/rat/day, mean ± SD. ND, not determined.
[c] Significantly different from control by Dunnett's test, $p < 0.05$.

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TABLE 6

EFFECT OF INHALED SOLVENTS ON MATERNAL BODY WEIGHT OF RATS DURING GESTATION[a]

	Air control	Carbon tetrachloride		1,1-Dichloroethane		Methyl ethyl ketone	
Solvent concentration (ppm)	—	300	1000	3800	6000	1000	3000
				Body Weight (g, mean ± SD)			
Number of dams	43	22	23	16	19	23	21
Gestation day 6	283 ± 17	290 ± 21	275 ± 19	278 ± 15	268 ± 13	289 ± 16	288 ± 17
13	317 ± 18	281 ± 25[b]	253 ± 22[b]	289 ± 24[b]	281 ± 24[b]	321 ± 18	311 ± 14
21	397 ± 24	368 ± 33[b]	336 ± 57[b]	353 ± 32[b]	381 ± 28	388 ± 26	400 ± 26

[a] Administered by inhalation 7 hr daily on days 6–15 of gestation.
[b] Significantly different from control by Dunnett's test, $p < 0.05$.

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TABLE 7

EFFECT OF INHALED SOLVENTS ON SGPT ACTIVITY IN RATS

			SGPT activity, Karmen units, mean ± SE					
			Carbon tetrachloride[a]		1,1-Dichloroethane[a]		Methyl ethyl ketone[a]	
Day of exposure	*N*	Air control[a]	300 ppm	1000 ppm	3800 ppm	6000 ppm	1000 ppm	3000 ppm
2nd	10	63 ± 3	159 ± 20[b]	ND[c]	69 ± 2	ND	68 ± 4	ND
4th	10	75 ± 2	145 ± 21[b]	81 ± 5[b]	75 ± 4	36 ± 3	64 ± 4	44 ± 1
7th	10	57 ± 5	218 ± 51[b]	ND	60 ± 3	ND	64 ± 3	ND
10th (last)	10	56 ± 2	241 ± 68[b]	154 ± 11[b]	54 ± 4	38 ± 2	60 ± 3	40 ± 2
Six days after last exposure								
Nonpregnant	6	57 ± 3	52 ± 5	46 ± 4	62 ± 4	57 ± 5	56 ± 2	51 ± 3
Pregnant	10	72 ± 3	62 ± 3	ND	70 ± 7	ND	66 ± 3	ND

[a] Administered by inhalation 7 hr/day for 10 consecutive days (days 6–15 of gestation for the pregnant rats). Blood samples were taken immediately upon removal from the chamber on the indicated days of exposure. Pregnant rats were bled at the time of cesarean section on day 21 of gestation. All other values are for nonpregnant rats.

[b] Significantly different from control by Tukey's test, $p < 0.05$.

[c] ND = not determined.

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TABLE 8

EFFECT OF INHALED SOLVENTS ON ABSOLUTE AND RELATIVE LIVER WEIGHT IN RATS[a]

Absolute liver weight (g, mean ± SD)	Air control	Carbon tetrachloride		1,1-Dichloroethane		Methyl ethyl ketone	
		300 ppm	1000 ppm	3800 ppm	6000 ppm	1000 ppm	3000 ppm
Following last exposure, nonpregnant ($N = 4$)	9.0 ± 0.6	10.0 ± 1.4	10.4 ± 1.5	8.6 ± 1.1	8.7 ± 0.5	9.5 ± 0.5	10.4 ± 0.2
Six days after last exposure, nonpregnant ($N = 6$)	9.3 ± 0.8	9.9 ± 1.9	11.4 ± 1.4	10.1 ± 1.3	10.7 ± 1.2	10.2 ± 1.5	10.6 ± 1.4
Pregnant ($N =$)	14.7 ± 1.2 (43)	14.9 ± 2.1 (22)	14.8 ± 2.4 (23)	13.4 ± 1.3 (16)	14.6 ± 1.6 (19)	13.5 ± 1.0 (23)	15.2 ± 1.2 (21)
Relative liver weight (mg liver/g body weight, mean + SD)							
Following last exposure, nonpregnant ($N = 4$)	34 ± 1	43 ± 3[b]	49 ± 4[b]	32 ± 2	36 ± 4	36 ± 3	39 ± 2
Six days after last exposure, nonpregnant ($N = 6$)	34 ± 3	39 ± 5	48 ± 3[b]	36 ± 3	42 ± 5[b]	36 ± 4	39 ± 4
Pregnant ($N =$)	37 ± 3 (43)	40 ± 3[b] (22)	45 ± 6[b] (23)	38 ± 3 (16)	38 ± 3 (19)	35 ± 2 (23)	38 ± 3 (21)

[a] Administered by inhalation 7 hr/day for 10 consecutive days (days 6–15 of gestation for pregnant rats).
[b] Significantly different from the air control value by Dunnett's test, $p < 0.05$.

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Among 4 litters of dams exposed to 3000 ppm methyl ethyl ketone, there were 2 acaudate fetuses with an imperforate anus and 2 brachygnathous fetuses. Considered collectively, these anomalous fetuses accounted for a significantly increased number of the fetal population and litters having gross anomalies compared to the incidence among the controls. Sternebral anomalies also occurred at an increased incidence among litters of dams exposed to 3000 ppm methyl ethyl ketone.

There was no single soft tissue anomaly which occurred at an increased incidence, but the total number of litters containing fetuses with soft tissue anomalies was significantly greater than in controls.

Maternal toxicity. Exposure of rats to 1000 or 3000 ppm of methyl ethyl ketone had very little effect on food consumption (Table 5) and had no effect on maternal weight gain (Table 6) during gestation. There was no effect on the conception rate, number of implantations or litter size (Table 3), SGPT activity (Table 7), absolute or relative liver weights (Table 8) or on the gross appearance of the liver. Changes in demeanor or other signs of toxicity were not observed in either group.

DISCUSSION

Embryo- and fetotoxicity. The results of this series of experiments, together with those conducted with chloroform (Schwetz *et al.*, 1974) show that exposure of pregnan rats to subanesthetic concentrations of certain solvents during gestation produced a variety of embryotoxic and fetotoxic responses.[5] These responses are summarized i Table 9. Exposure to CCl_4 at concentrations of 300 and 1000 ppm for 7 hr/day on days through 15 of gestation was not teratogenic to the developing embryo. The only anoma which may have been associated with exposure to CCl_4 was bipartite sternebrae amo fetuses of dams exposed to 1000 ppm.

Exposure of rats to 3800 or 6000 ppm of 1,1-dichloroethane caused very lit embryotoxicity. The single anomaly which occurred at an increased incidence, delay ossification of sternebrae, reflects retarded fetal development.

Exposure of rats to 3000 ppm of methyl ethyl ketone caused a low incidence of tr malformations (acaudia, imperforate anus and brachygnathia). In our laborato these anomalies have not been observed in more than 400 control litters of this strain rat. In addition to these terata, a significantly increased incidence of anomalies indi tive of retarded fetal development was observed among litters of dams exposed methyl ethyl ketone.

In a previous study, chloroform was found to be highly embryotoxic at a concent tion of just 4 times its Threshold Limit Value (TLV) of 25 ppm and was slightly embr toxic at 30 ppm (Schwetz *et al.*, 1974). A 7-hr daily exposure to a material at or below TLV is generally considered safe for humans. These values, which are recommendati of the American Conference of Governmental Industrial Hygienists (ACGIH, 197 were determined on the basis of the following criteria for the solvents used in th studies: CCl_4 and chloroform, liver and kidney damage; 1,1-dichloroethane, li damage and narcosis; methyl ethyl ketone, narcosis and irritant properties. In the p sent study, CCl_4 at concentrations up to 100 times its TLV of 10 ppm was not terat genic, and in fact, caused very little evidence of embryotoxicity. Exposure of rats

[5] A report by Thompson *et al.*, "Teratology studies on orally administered chloroform in the rat a rabbit" has been accepted for publication in a later issue of this journal.

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TABLE 9

SUMMARY OF THE TOXICITY OF INHALED SOLVENTS TO PREGNANT RATS

Exposure concentration ppm (×TLV)	Carbon tetrachloride[a]		Chloroform[a]				1,1-Dichloroethane[a]		Methyl ethyl ketone[a]	
	300 (30)	1000 (100)	30 (1.2)	100 (4)	300 (12)	Starvation	3800 (19)	6000 (30)	1000 (5)	3000 (15)
Embryo- and fetotoxicity										
Resorptions[c]	—[b]	—	—	—	INC	—	—	—	—	—
Fetal body measurements[c]	DEC	DEC	DEC	—	DEC	DEC	—	DEC	DEC	—
Incidence of:										
Gross anomalies[d]	—	—	—	INC	—	—	—	—	—	INC
Skeletal anomalies[d]	INC	INC	INC	INC	—	—	—	INC	INC	INC
Soft tissue anomalies[d]	INC	—	—	INC	—	—	—	—	—	INC
Maternal toxicity										
Weight gain during gestation[e]	DEC	DEC	DEC	DEC	DEC	DEC	DEC	DEC	—	—
Food consumption[f]	DEC	DEC	DEC	DEC	DEC	DEC	DEC	DEC	—	DEC
Conception rate[c]	—	—	—	—	DEC	—	—	—	—	—
Number of implantations[c]	—	—	—	—	—	—	—	—	—	—
Litter size[c]	—	—	—	—	DEC	—	—	—	—	—
SGPT activity[g]	INC	INC	—	—	—	ND	—	—	—	—
Absolute liver weight[h]	—	—	—	INC	DEC	DEC	—	—	—	—
Relative liver weight[h]	INC	INC	—	INC	INC	INC	—	—	—	—
Gross liver pathology	INC	INC	—	—	INC	—	—	—	—	—
Demeanor	—	—	—	—	—	—	—	—	—	—

[a] Administered by inhalation 7 hr/day on days 6 through 15 of gestation; food consumption by starvation controls matched that of rats exposed to 300 ppm of chloroform. Chloroform results are summarized from Schwetz *et al.*, 1974.

[b] —, not significantly different from air control; DEC, significantly decreased compared to air control; INC, significantly increased compared to air control; ND, value not determined.

[c] See Table 3 for specific data.

[d] See Table 4 for specific data.

[e] See Table 6 for specific data.

[f] See Table 5 for specific data.

[g] See Table 7 for specific data.

[h] See Table 8 for specific data.

1,1-dichloroethane at 19 or 30 times the TLV (TLV = 200 ppm) and methyl ethyl ketone at 5 or 15 times the TLV (TLV = 200 ppm) caused very little embryotoxicity. Thus, relative to their respective TLV, CCl_4, 1,1-dichloroethane and methyl ethyl ketone were not nearly as toxic to the developing embryo and fetus as chloroform.

Correlation between embryo or fetal toxicity and maternal toxicity. Of the solvents examined in these studies, CCl_4 caused the greatest degree of maternal toxicity. This included decreased weight gains and food consumption during pregnancy, increased SGPT activity, altered absolute and relative liver weight and gross appearance of the liver. In spite of this great degree of maternal toxicity, the embryo and fetal toxicity was limited to simple retardation of fetal development. Exposure to 1,1-dichloroethane caused little or no maternal toxicity as well as very little embryo and fetal toxicity. Fetal toxicity was limited to simple retardation of fetal development. Exposure to methyl ethyl ketone caused no measurable maternal toxicity. However, embryo and fetal toxicity included true terata as well as evidence of retarded fetal development. Exposure to chloroform caused some maternal toxicity but considerably greater embryo and fetal toxicity. The largest factor of the maternal toxicity, anorexia and its resultant weight loss, was demonstrated not to be responsible for the embryo and fetal toxicity associated with chloroform exposure.

These results suggest that there is no correlation between the toxicity incurred by the mother and that by the embryo or fetus with these solvents. The solvent which caused the least maternal toxicity, methyl ethyl ketone, showed a potential for producing terata. The compound which caused the greatest degree of maternal toxicity, CCl_4, did not produce terata. 1,1-Dichloroethane caused only very slight maternal toxicity and likewise only very slight fetotoxicity. It is interesting to recall that chloroform caused maternal toxicity and was highly toxic to the embryo and fetus.

Using the results of these studies in which pregnant rats were exposed to CCl_4, 1,1-dichloroethane or methyl ethyl ketone 7 hr/day on days 6 through 15 of gestation, the following conclusions are made: (a) Carbon tetrachloride at 300 or 1000 ppm is not highly embryotoxic; (b) 1,1-dichloroethane is only slightly embryotoxic at 3800 or 6000 ppm; and (c) methyl ethyl ketone at 1000 or 3000 ppm is embryotoxic, fetotoxic and potentially teratogenic. In addition, the results of these studies together with those on chloroform show that there is no correlation between the toxicity of these materials to the mother and to the embryo or fetus.

ACKNOWLEDGMENTS

The authors are grateful to P. A. Keeler and H. C. Pernell for their assistance in all aspects of this study.

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10/24/1996 EPA TSCA DOCKET

FUNDAMENTAL AND APPLIED TOXICOLOGY 16, 742–748 (1991)

Developmental Toxicity of Inhaled Methyl Ethyl Ketone in Swiss Mice

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Received July 25, 1990; accepted December 28, 1990

Developmental Toxicity of Inhaled Methyl Ethyl Ketone in Swiss Mice. SCHWETZ, B. A., MAST, T. J., WEIGEL, R. J., DILL, J. A., AND MORRISSEY, R. E. (1991). *Fundam. Appl. Toxicol.* **16**, 742–748. Methyl ethyl ketone (MEK) is a widely used industrial solvent to which there is considerable human exposure. To assess the potential for MEK to cause developmental toxicity in rodents, groups of Swiss (CD-1) mice were exposed to 0, 400, 1000, or 3000 ppm MEK vapors 7 hr/day on Days 6–15 of gestation. Groups consisted of about 30 bred females each. Exposure of pregnant mice to these concentrations of MEK did not result in overt maternal toxicity although there was a slight, treatment-related increase in relative liver weight which was statistically significant in the 3000 ppm group. Mild developmental toxicity was observed in the 3000 ppm group in the form of a reduction in mean fetal body weight. This reduction was statistically significant for the males only, although the relative decrease from the control values was the same for both sexes. There was no increase in the incidence of resorptions or the number of litters with resorptions among mice exposed to MEK. There was no significant increase in the incidence of any single malformation, but several malformations which were not observed in the concurrent control group or the controls of contemporary studies were present at a low incidence—cleft palate, fused ribs, missing vertebrae, and syndactyly. There was also a significant trend for increased incidence of misaligned sternebrae, a developmental variation. In summary, pregnant Swiss (CD-1) mice were relatively insensitive to the toxic effects of MEK at the inhaled concentrations used in this study. However, the offspring of the mice exhibited significant signs of developmental toxicity at the 3000 ppm exposure level. Neither maternal nor developmental toxicity was observed at 1000 ppm MEK or below. © 1991 Society of Toxicology.

Methyl ethyl ketone (MEK) is a widely used industrial solvent with a 1984 production figure of 556,000,000 lbs. (Yang, 1986, and references therein). Inhalation of MEK vapors and skin contact with liquid MEK are the major routes of human industrial exposure. The ACGIH (1988) has adopted a TLV of 200 ppm and a short-term exposure limit of 300 ppm for MEK.

The toxicology literature on MEK has been thoroughly reviewed by Yang (1986). Animal studies have shown MEK to cause relatively little toxicity from acute or short-term repeated exposures. For example, a 90-day inhalation study of MEK in male and female F344 rats exposed to 0, 1250, 2500, or 5000 ppm MEK 6 hr/day 5 days/week produced no adverse effects on the clinical health or growth of either sex (Cavender *et al.*, 1983). While this study showed no evidence of neurotoxicity, MEK is known to potentiate *n*-hexane-induced neu-

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0272-0590/91 $3.00
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rotoxicity as well as that caused by other hydrocarbons (Altenkirch *et al.*, 1978). MEK also potentiates the hepatotoxicity of chloroform. MEK has not been found to be mutagenic in mammalian or bacterial cell systems, but causes aneuploidy in yeast (Yang, 1986, and references therein).

The metabolism of MEK has been well characterized and is similar for all species examined—guinea pigs (Di Vincenzo *et al.*, 1976), rats (Dietz and Traiger, 1979), rabbits (Sanoyeshi, 1911), and humans (Perbellini *et al.*, 1984). The elimination half-life from serum was found to be 270 min following ip injection in guinea pigs (Di Vincenzo *et al.*, 1976). MEK is well absorbed through the skin; Munies and Wurster (1965) reported the presence of MEK in expired air within 2–3 min following application of the solvent to the skin of humans. The rate of elimination from the blood is independent of the route or mode of exposure. Induction of the cytochrome P450 systems by MEK and/or its metabolites has been demonstrated by Abdel-Rahman *et al.* (1976). Toftgard *et al.* (1981) also found increased liver weights and cytochrome P450 content of the liver of rats exposed to 800 ppm MEK for 6 hours/day 5 days/week for 4 weeks.

Developmental toxicity studies with MEK have been reported in rats. Schwetz *et al.* (1974) exposed timed-pregnant Sprague–Dawley rats to concentrations of 0, 1000, or 3000 ppm MEK on Days 6–15 of gestation. MEK exposure had no effect on resorptions, fetal crown–rump length, sex ratio, or on the maternal body weight or weight gain during gestation. Fetal body weight was slightly decreased for both exposure groups but the decrease was statistically significant only at the 1000 ppm level. Major malformations were observed only at 3000 ppm—one fetus in each of four litters. Two of these fetuses were acaudate and two were brachygnathic. There was a significant increase in the percentage of litters having fetuses with reduced ossification of bones and extra ribs at both concentrations of MEK. The incidence of soft tissue anomalies (subcutaneous edema and dilated ureters) was also greater in the MEK-exposed groups than the controls and this increase was statistically significant in the 3000 ppm group.

As a result of the findings of Schwetz *et al.* (1974), another study was conducted by Deacon *et al.* (1981), also in Sprague–Dawley rats. In this study, sperm-positive rats were exposed to 400, 1000, or 3000 ppm MEK, 7 hr/day on Days 6–15 of gestation. The dams did not exhibit any clinical signs of toxicity but decreased weight gain and increased water consumption were observed at 3000 ppm. As in the earlier study, there was no significant effect on the mean number of live fetuses or resorptions per dam. The percentage of litters with fetuses exhibiting delayed ossification of vertebral centra was increased in the highest exposure group, as in the earlier study. Also as previously reported, the percentage of litters with fetuses having reduced ossification of skull bones was lower in the 3000 ppm exposure group than in any of the other groups. While the reduction in the incidence of reduced ossification of the skull bones is of uncertain importance, both studies supported a conclusion that developmental toxicity was observed—in the presence of maternal toxicity in one study and in its absence in the other.

Epidemiologic studies have also supported that exposure may be associated with adverse effects on human development. Holmberg and Nurminen (1980) reported a statistically significant increase in the incidence of children born with central nervous system defects following maternal exposure to organic solvents during the first trimester of pregnancy when compared to children of unexposed mothers. MEK was among the organic solvents to which the affected cohort was exposed. These authors further reported that no significant difference in the incidence of children with such defects was found when occupational exposures to metals, pesticides, dyes, noise, temperature extremes, fumes, or radiation were used as the analysis variable. Thus, because of the large number of people exposed to MEK and because the results of two studies in rats suggested that MEK may be a developmental toxicant,

this study was conducted to evaluate the developmental toxicity of inhaled MEK in a second species of laboratory animal, Swiss (CD-1) mice. The concentrations of MEK selected for this study were chosen to duplicate those used in a previous developmental toxicity study in rats (Deacon *et al.*, 1981) and because 3000 ppm was anticipated to be an appropriately high concentration for a study in mice.

MATERIALS AND METHODS[3]

Animals and animal husbandry. Four groups of female Swiss mice [(COBS) (Crl:CD-1)(ICR)BR Swiss Albino mice][4] 12 weeks of age at the beginning of the studies, were exposed to either filtered air as the control group or 400, 1000, or 3000 ppm MEK vapors, 7 hr/day for 10 consecutive days. Each of the four exposure groups consisted of 10 virgin and 33 plug-positive female mice. All groups were randomly selected using body weight as a blocking variable. Virgin mice were included to assess maternal toxic effects which may result solely from the state of pregnancy. The day of plug detection in mice was designated as Gestation Day 0. Pregnant mice were killed on Gestation Day 18 and virgin females were killed 3 days after the last exposure. Control animals were housed in an exposure chamber in the same room and were handled in the same manner as the mice that were exposed to MEK. Mice were housed in quarantine rooms for 4 weeks prior to start of exposures. Males and females were housed separately on stainless-steel wire racks equipped with automatic waterers. During the quarantine period, six males and four females were killed and examined for gross and microscopic lesions. Nasal pharyngeal washes from these animals were cultured for bacterial pathogens and serum from each animal was tested for antibodies to selected pathogens. Another check for antibodies was performed on serum obtained at the final euthanization from five females in the control and highest exposure groups. All results were negative for significant pathogens and lesions. Study animals were observed daily for mortality, morbidity, and overt signs of toxicity.

Food[5] was provided free-choice during the entire time the animals were in the test facility except during the 7-hr daily exposure when it was removed to prevent contamination. Room lighting was maintained on a 12-hr cycle (0600–1800 for the light phase). Mean chamber temperatures during the study were maintained within the limits of 75 ± 3°F. Mean percentage relative humidity in all exposure chambers was within the specified limits of 55 ± 15%.

Test chemical and exposures. MEK (CAS No. 78-93-3) used for this study had an initial percentage purity greater than 99.9%, confirmed by infrared spectrometry. For inhalation exposures, 2.3 m^3 stainless-steel chambers were used which contained three levels of caging, each of which was split into two offset tiers.[6] Filtered air containing a uniform mixture of MEK flowed through the chamber at approximately 15 ft^3 per min to provide 15 air changes per hour. Within the chambers, air flow, vacuum, temperature, relative humidity, and test chemical concentrations were monitored at approximately 4-hr intervals. MEK vapor was generated by a rotary evaporation system which produced high purity MEK vapor. Nitrogen was introduced at low pressure to force MEK vapors out of the evaporator flask. The mixture of nitrogen and MEK were thoroughly blended with a conditioned air stream prior to entry into the chamber. Uniform distribution of the vapors in the chamber was confirmed. The concentrations of MEK in the chambers were monitored with a gas chromatographic system having an estimated precision of ±1% and detection limit of 0.5 ppm. MEK was not found in the control chamber air or in the air surrounding the chambers in the exposure room.

Observations. Female mice were weighed at intervals of 3–5 days throughout the study. On Gestation Day 18, bred mice were killed with CO_2, weighed, and examined grossly for signs of maternal toxicity. Maternal liver and kidney weights were recorded. Apparently nongravid uteri from mated females were stained with 10% ammonium sulfide to detect possible implantation sites. The number, position, and status of implants were recorded for each gravid uterus. Placentas were examined and discarded. Live fetuses were weighed and examined for gross defects, and their sex was determined by internal examination of the gonads after a lethal injection of Nembutal. Fifty percent of the live fetuses and any fetuses with gross external abnormalities were examined for visceral defects by dissection of fresh tissues. The heads of 50% of the live fetuses were removed and placed in Bouin's fixative. After fixation the heads were serial-sectioned with a razor blade and examined for soft tissue craniofacial abnormalities. All fetal carcasses were prepared for skeletal staining. Cartilage as well as ossified bone was visualized by double-staining with alcian blue and alizarin red S. The individual identity of each skeletal and head specimen was maintained throughout the study.

Statistical analyses. All means and standard deviations for animal data were analyzed using the SAS general linear models procedure (SAS, 1985) with an analysis of variance model for unbalanced data. Response variables, either body

[3] The NTP report of this study will be available through the National Technical Information Service. This study was conducted in accordance with FDA's Good Laboratory Practice, Regulations for Nonclinical Laboratory Studies (FDA, 1978).

[4] Charles River Breeding Laboratories, Raleigh, NC.

[5] NIH-07 diet, Ziegler Brothers, Inc., Gardner, PA.

[6] Harford System; Lab Products, Inc., Aberdeen, MD.

TABLE 1

SUMMARY OF THE METHYL ETHYL KETONE EXPOSURE CHAMBER CONCENTRATIONS (ppm) DURING A DEVELOPMENTAL TOXICITY STUDY WTIH MICE

	Exposure group (ppm)		
	400	1000	3000
Mean ± SD	398 ± 9	1010 ± 28	3020 ± 79
Maximum	439	1140	3340
Minimum	352	894	2490
% Samples ± 10% of target	100	98	99

weight or the arcsin transformations of proportional incidence data, were analyzed against the class variable "treatment" in a one-way analysis of variance model. A Tukey's t test (two-tailed) was used to assess statistically significant differences between control and exposed groups. If appropriate, the dose–response relationship was determined by means of an orthogonal trend test (Winer, 1971). In the case of proportional data, the t tests and trend analyses were performed on transformed variables. The litter was used as the basis for analysis of fetal variables.

RESULTS

The average concentrations of MEK in the chambers during this study are summarized in Table 1. The average purity of MEK in unoccupied chambers and the distribution line to the chambers was 99.4% during test generation and 99.9% in chambers occupied by animals during the exposures. Thus, the techniques used to generate MEK vapor for the inhalation exposures did not affect its stability.

There were no differences in MEK toxicity between the virgin and pregnant female mice (data not shown). Therefore, the adult toxicity data referred to in the rest of this report is for the pregnant females. The mean pregnancy rate of the bred females was 82% with no significant difference in pregnancy rates between the MEK-exposed and the control groups. Mean body weights and weight gains were not affected by exposure to MEK at any time during the study when compared to the 0 ppm group (Table 2). The relative liver weight of the 3000 ppm group was significantly higher than the corresponding control relative liver weight. The relative kidney weight for the 3000 ppm group was also significantly greater than the control value; however, since the mean relative kidney weight was essentially the same for the 400 and the 3000 ppm groups, and lower for the 1000 ppm group, this change was probably not biologically important.

TABLE 2

METHYL ETHYL KETONE MOUSE DEVELOPMENTAL TOXICITY STUDY: MEAN BODY AND ORGAN WEIGHTS, AND EXTRAGESTATIONAL WEIGHT GAINS

		Exposure group (ppm)			
		0	400	1000	3000
Body wt	n =	26	23	26	28
GD 6		28.8 ± 1.7[a]	28.4 ± 1.9	28.8 ± 1.8	28.7 ± 1.9
GD 9		31.5 ± 1.8	31.2 ± 2.1	31.4 ± 2.1	31.8 ± 2.0
GD 15		44.7 ± 3.4	44.2 ± 3.6	44.0 ± 3.3	45.0 ± 3.8
GD 18		53.3 ± 5.1	52.2 ± 5.1	51.9 ± 4.1	53.0 ± 4.9
Extragestational wt gain[b]		6.9 ± 1.4	6.5 ± 1.6	6.5 ± 1.9	7.6 ± 2.0
Uterine wt		20.1 ± 3.6	19.3 ± 3.7	18.8 ± 2.9	19.1 ± 2.7
Relative organ wt (g/100 g body wt)					
liver		5.28 ± 0.61	5.16 ± 0.62	5.41 ± 0.36	5.67 ± 0.41*
kidney		0.82 ± 0.07	0.87 ± 0.11	0.85 ± 0.05	0.87 ± 0.07*

[a] Grams, mean ± SD.
[b] Maternal weight gain during gestation corrected for gravid uterine weight.
* Significantly different from control, $p < 0.05$.

Exposure of pregnant mice to MEK vapors on Gestation Days 6–15 did not affect the number of implantations or the percentage of live fetuses or intrauterine deaths per litter as compared to the controls (Table 3). Reductions in body weight of both male and female fetuses were significantly correlated with increasing concentrations of MEK, with the difference from control being statistically significant at 3000 ppm for males but not at lower concentrations. However, both males and females body weights were lower than the control group by the same relative amount, about 4%. The number of live fetuses per litter that were malformed and the number of litters with one or more malformed fetuses was increased in the MEK-groups compared to the controls but the differences were not statistically significant on the basis of either the number of fetuses or litters affected or the mean number of malformed fetuses affected per litter. However, there were four malformations (Table 4) observed in exposed groups that were not present in either the current control or in contemporary control groups. These malformations consisted of three fetuses with cleft palate (two in the 400 ppm group and one in the 1000 ppm group), two with fused ribs (one in the 1000 ppm group and one in the 3000 ppm group), one with missing vertebrae in the 1000 ppm group, and five with syndactyly all from a single litter in the 3000 ppm group. Although there was not a significant trend for an increase of malformations with respect to increasing concentration of MEK, the 3000 ppm group did have a higher combined incidence of malformations than did either of the two lower exposure groups.

Gestational exposure to MEK resulted in a significant, treatment-related increase in the incidence of misaligned sternebrae with respect to the control group (Table 4). The increase (not statistically significant) in the mean percentage per litter of total variations was due mostly to the increase in misaligned sternebrae.

DISCUSSION

Exposure of pregnant Swiss (CD-1) mice to 0, 400, 1000, or 3000 ppm MEK did not result in apparent maternal toxicity, although an exposure-related increase in the relative liver-to-body-weight ratio was present and statistically significant at 3000 ppm MEK. This increase in liver weight, above the increase normally observed during pregnancy, may be related to

TABLE 3

METHYL ETHYL KETONE MOUSE DEVELOPMENTAL TOXICITY STUDY: *IN UTERO* DEVELOPMENT

	Exposure group (ppm)			
	0	400	1000	3000
Implantations/dam	12.4 ± 2.3[a]	12.2 ± 2.2	12.3 ± 1.8	12.3 ± 1.8
Live fetuses/litter	11.9 ± 2.3	11.3 ± 2.5	11.2 ± 1.6	11.5 ± 1.9
Resorptions/litter	0.5 ± 0.8	0.9 ± 1.0	1.1 ± 1.1	0.7 ± 0.8
Dead fetuses/litter	0	0.0 ± 0.2	0	0.1 ± 0.3
Litters with resorptions	9/26	12/23	16/26	16/28
Fetal wt, male (g)	1.38 ± 0.07	1.38 ± 0.06	1.34 ± 0.10	1.31 ± 0.08*
Fetal wt, female (g)	1.32 ± 0.08	1.33 ± 0.08	1.31 ± 0.06	1.27 ± 0.08
% males	50 ± 12	47 ± 13	43 ± 15	51 ± 18
No. live fetuses malformed/litter (%)	1 (0.3)	3 (1.2)	3 (1.0)	9 (2.8)
No. litters with malformed fetuses (%)	1 (3.8)	3 (13.0)	3 (11.5)	5 (17.9)

[a] Mean ± SD.

* Significantly different from control, $p < 0.05$.

TABLE 4

METHYL ETHYL KETONE MOUSE DEVELOPMENTAL TOXICITY STUDY: MALFORMATIONS AND VARIATIONS

	Exposure group (ppm)							
	0	400	1000	3000	0	400	1000	3000
	No. fetuses[a]				No. litters			
Malformations	310	260	291	323	26	23	26	28
Cleft palate	0	2	1	0	0	2	1	0
Fused ribs	0	0	1	1	0	0	1	1
Limb flexure	1	1	0	3	1	1	0	3
Missing vertebrae	0	0	1	0	0	0	1	0
Syndactyly	0	0	0	5	0	0	0	1
Variations								
Sternebrae								
misaligned	31[b]	27	49	58	18	14	18	21
fused	0	1	1	1	0	1	1	1
reduced ossification	6	6	16	14	5	4	11	7
Supernumerary ribs	56	44	58	65	17	13	15	22
Skull-reduced ossification	3	3	2	5	2	2	2	2

[a] All fetuses were examined for external and skeletal defects; one-half had head removed prior to skeletal staining for soft-tissue craniofacial evaluation. Visceral examinations were performed on approximately 50% of live fetuses and all fetuses with external defects.

[b] Significant trend, $p < 0.05$.

enzyme induction as reported for rats by Toftgard *et al.* (1981). Developmental toxicity was observed in the present study in the form of a significant decrease in fetal body weight at 3000 ppm MEK as well as a significant trend for an increase in the incidence of misaligned sternebrae, a skeletal variation. The incidence of fetal malformations in the MEK-exposed groups, although not statistically significantly different from control, is of some concern since several malformations were present which were not observed in concurrent or contemporary control groups of the same strain of mouse. The percentage of litters having fetuses with malformations was also greater for all MEK exposure groups than for the controls, although the differences were not statistically significant.

The results of this study in mice are reminiscent of those reported previously in two studies in Sprague–Dawley rats (Schwetz *et al.*, 1974; Deacon *et al.*, 1981) where major malformations were found in only the 3000 ppm group. The relative sensitivity of rats and mice for an effect on fetal body weight is unclear since in the present study fetal body weight was significantly reduced at 3000 ppm but not at lower concentrations; fetal rat weights were significantly reduced at the 1000 ppm level but not at 3000 ppm (Schwetz *et al.*, 1974). Considering these three studies collectively, MEK caused developmental toxicity in the presence of mild maternal toxicity in rats and mice, and, in one rat study, in the absence of maternal toxicity.

The developmental toxicity of 2-butanol, a known metabolite of MEK, was investigated by Brightwell *et al.* (1987) in Sprague–Dawley rats. Pregnant females were exposed by inhalation to 3500, 5000, or 7000 ppm 2-butanol, 7 hr/day, throughout gestation until they were killed on Day 20. Maternal toxicity was evident at the highest concentration, as manifested by a reduction of weight and food consumption. There was no increase in the incidence of fetal malformations at any

concentration of 2-butanol. A reduction in mean fetal body weight was observed at the 7000 ppm concentration. Thus, at least one metabolite of MEK was not developmentally toxic even when administered at concentrations above those used for the parent compound in this study.

In summary, the results of this developmental toxicity study of inhaled methyl ethyl ketone in mice are similar to those reported earlier for rats; in both species, developmental toxicity has been observed in the presence of mild maternal toxicity, and a low incidence of malformations along with other evidence of developmental toxicity. In the present study in mice, no maternal or developmental toxicity was observed at concentrations of 1000 ppm MEK or below.

ACKNOWLEDGMENTS

This study was conducted at the Battelle Pacific Northwest Laboratory, operated for the U.S. Department of Energy by Battelle Memorial Institute. The study was done under contract to the National Toxicology Program (a related services agreement under Contract DE-AC06-76RL0 1830). The authors express their appreciation to J. J. Evanoff, R. L. Romarine, and R. B. Westerberg for their expert assistance in the conduct of the study and to Mrs. Judy Bullard for her expertise in typing of the final manuscript for this publication.

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JOURNAL OF GEOPHYSICAL RESEARCH, VOL. 90, NO. D2, PAGES 3797-3805, APRIL 20, 1985

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TROPOSPHERIC LIGHT ALCOHOLS, CARBONYLS, AND ACETONITRILE: CONCENTRATIONS IN THE SOUTHWESTERN UNITED STATES AND HENRY'S LAW DATA

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Abstract. Aliphatic alcohols (C_1 - C_4), aldehydes (C_1 - C_2), and ketones (C_3 - C_4) have been determined at Tucson, Arizona, and at two rural sites about 40 km distant. Acetonitrile was also measured at the rural sites. The method involved condensation sampling, condensate preconcentration, and gas chromatography. Henry's law coefficients were required for all components and were determined. Mean concentrations in Tucson were higher than those in the rural areas by factors typically between 2 and 8; urban formaldehyde was only slightly elevated. Mean alcohol concentrations ranged from 7.9 ppb (C_1) to 0.12 ppb (C_4) within the city and from 2.6 ppb (C_1) to 0.06 ppb (C_4) at the rural sites. Acetone was found at 12 ppb in the city and 2.8 ppb at the rural sites. Concentrations of butanone were a factor of 5 lower. Acetaldehyde, at 23 ppb (city) and 6.9 ppb (rural), far exceeded formaldehyde concentrations (1.8 ppb in the city, and 1.5 ppb at the rural sites). Acetonitrile was found at the rural sites at a mean concentration of 60 ppt. A dimensionless Henry's law coefficient (mol L^{-1} of liquid/mol L^{-1} of vapor) was suprisingly similar for the alcohols at 0°C, ranging between 2×10^4 and 3.4×10^4 (900-1500 mol L^{-1} atm^{-1}); the ketones were a factor of 10 lower. For acetaldehyde the coefficient was 1.7×10^3 (76 mol L^{-1} atm^{-1}) and for acetonitrile 3.7×10^3 (165 mol L^{-1} atm^{-1}). Concentrations of oxygenated organics in the condensates and in precipitation were compared; it was tentatively concluded that concentration differences of the carbonyls were consistent with these species' being produced within the cloud, for example, by aqueous photochemistry.

Introduction

The degradation of atmospheric hydrocarbons by free radicals or ozone results in oxygenated derivatives; such derivatives may also have primary sources, both natural and biogenic [Graedel, 1978]. Their further oxidation can contribute to the production of formaldehyde, carbon monoxide, molecular hydrogen, etc. [Zimmerman et al., 1978; Hanst et al., 1980; Chameides and Cicerone, 1978; Aikin et al., 1982]. The extent to which nonmethane hydrocarbons affect ozone and HO_x radical concentrations is currently a matter of some discussion [e.g., Bottenheim and Strausz, 1980; Brewer et al., 1983].

Aldehydes, ketones, carboxylic acids, alcohols, organic peroxides, and a variety of multifunctionalized organic molecules have been identified in urban air [Graedel, 1978]. Most studies have examined the role of these compounds in the generation of photochemical smog; little is known about their gaseous concentration in clean air. Difficulties stem from the diversity of oxygenates, their low concentrations, especially in more pristine conditions, and the need for the sampling technique to discriminate between oxygenates and unfunctionalized organic vapors.

The condensation collection method [Farmer and Dawson, 1982] offered promise for such a discrimination. While most light oxygenated organics are not highly soluble (by the criterion of Farmer and Dawson), they are sufficiently more soluble than other atmospheric organics to give preferential incorporation into condensate. In this work, condensation sampling was followed by a preconcentration procedure to give readily detectable levels of the compounds of interest. Henry's law constants at the condensation collection temperature were required and were determined experimentally.

Theory

The theoretical basis of condensate collection has been presented by Farmer and Dawson [1982]; that paper should be consulted for details not given here.

A vertical surface cooled below the ambient dew point collects condensate. If the surface is clean, film condensation occurs; the film drains from the surface and can be analyzed for its aqueous components. A knowledge of the processes of condensation allows atmospheric concentrations to be inferred.

The ambient concentration, $\rho_{A\infty}$ (kg m^{-3}), of trace component A is related to the aqueous concentration, C_A (kg kg^{-1}), by

$$\rho_{A\infty} = C_A \left[\left(\frac{B_A D_V}{B_V D_A} \right) (\rho_{V\infty} - \rho_{VW}) + \frac{\delta}{H_A} \right] \qquad (1)$$

where subscript v denotes water vapor, D_A and D_V are molecular diffusion coefficients, B_A and B_V are related to convective boundary thicknesses (given in Farmer and Dawson [1982]), and ρ_{AW} and ρ_{VW} are vapor densities at the collector surface. H_A is a dimensionless Henry's law coefficient for A, defined as

$$H_A = \lim_{C_A \to 0} (C_A \delta / \rho_{AW}) \quad ;$$

δ is the density of water (1 kg m^{-3} at 4°C).

The form of (1) indicates that two factors relate the atmospheric concentration of A and its condensate concentration. The first product in (1) pertains to transport of vapor to the collector, while the second term is dependent on its aqueous solubility. For compounds of interest to this study, both terms are of comparable magnitude and must be retained. Condensate concentrations are quite low; C_A ranges from 10^{-8} to 10^{-10} (10 - 10^{-4} ppm) depending on atmospheric concentrations and the magnitude of H_A.

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Paper number 4D1304.
0148-0227/85/004D-1304$05.00

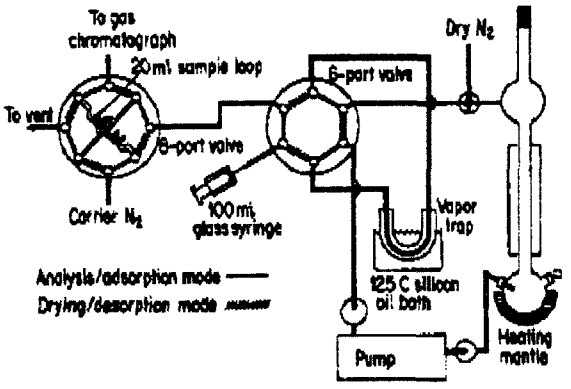

Fig. 1. Diagram of purging and trapping assembly.

Condensates

Method

Condensate was collected using the apparatus and techniques of Farmer and Dawson [1982] with one major difference. In the previous study, mostly highly soluble gases were collected, and for these, aqueous concentration is inversely proportional to collection rate. Farmer and Dawson therefore chose a relatively low rate of 10-15 mL/hour for each of the two collection plates. For the present study, where δ/H_A is not negligible, much higher collection rates can be used; they were typically 15 to 35 mL/hour for each plate when the temperature was kept between 273° and 278°K. Procedures were otherwise as in Farmer and Dawson [1982]. Sixty milliliters of sample were required for analysis of the volatile organics, while another 5 mL were used to determine formaldehyde. Ambient trace vapor levels are, therefore, approximately a 2-hour temporal average.

Collected samples were stored in glass and kept refrigerated prior to analysis. Formaldehyde analysis was performed within 20 hours of collection, while the other organics were analyzed within 1-3 days. Some rain and hail samples were collected, using a 28-cm diameter glass funnel draining into a 500-mL Erlenmeyer flask. These samples were stored and analyzed in the same manner as the condensate.

Analyses

A Hewlett-Packard 5710-A gas chromatograph, with flame ionization detector and heated 20-mL teflon-lined gas-sampling loop, was used in all work here. Areas under chromatograph peaks were integrated with a Hewlett-Packard 3390-A integrator. The integrator also registered retention times and calculated concentrations from standard runs. A 240 cm × 0.15 cm ID teflon-lined aluminum column packed with 160 cm of Porapack QS and 80 cm of Porapack S, mesh size 80/100, was used for analysis of the condensate and rain water. A similar column packed with Porapack S was used for some Henry's law determinations. The ultra-high purity nitrogen carrier gas was passed through a cartridge of molecular sieve (5A) and activated charcoal. Columns were conditioned for 2-3 days at 220°C under nitrogen. Optimum performance during analysis was obtained with a temperature program: 90°C for 0.3 min, 16°C min^{-1} for 6.2 min, and 190°C for 15 min.

Water used for standard solutions was doubly distilled, first from alkaline potassium permanganate; it was then degassed at 98°C for 5 hours with a stream of 99.9995% nitrogen that had passed through a bed of Porapack Q. Water treated in this manner was found to still contain detectable levels of some components present in condensate.

Condensates (and rain) were preconcentrated by microdistillation [Chian et al., 1977]; Figure 1 illustrates the purging and trapping assembly. Fifteen minutes prior to the start of purging, 60 mL of chilled condensate (5-10°C) were added

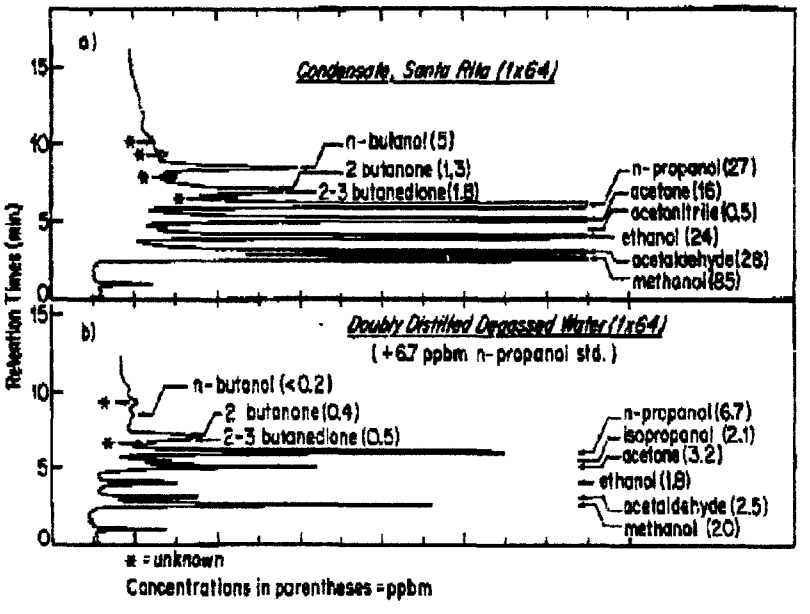

Fig. 2. Chromatograms of condensate sample and degassed distilled water.

TABLE 1. Mean Condensate Concentration, $\bar{C}$, Standard Deviation of the Concentrations, δC, and Number of Measurements, N, at Each Site

	Tucson Feb.-Sept. 1982		Santa Rita and Mt. Lemmon Aug.-Sept. 1982	
	$\bar{C}(\pm\delta C)$ppbm	N	$\bar{C}(\pm\delta C)$ppbm	N
		Alcohols		
CH_4O	200 (±70)	17	72 (±29)	18
C_2H_6O	150 (±120)	17	19 (±9)	18
n-C_3H_8O	1.0 (±0.9)	11		
n-$C_4H_{10}O$	5.7 (±2.5)	17	3.7 (±1.8)	18
tert-C_4H_{10}	3.7 (±1.8)	16		
		Ketones		
C_3H_6O	61 (±27)	17	18 (±5)	18
C_4H_8O	15 (±5)	17	3.4 (±1.0)	18
		Aldehydes		
CH_2O	200 (±80)	8	140 (±81)	27
C_2H_4O	56 (±28)	17	22 (±8)	18
		Nitrile		
C_2H_3N			0.35 (±0.1)	18

to the distillation pot, together with a 10-μL aliquot of the internal standard, n-propanol (160 ppm solution in purified water). The sample was then heated to boiling under nitrogen, while the vapor contents of the still were recirculated at 30 mL/min for 20 min through a valve and porous polymer trap at room temperature with a glass syringe pump. The trapped organics were thermally desorbed (125°C) into the 20-mL sample loop with 25 mL of nitrogen. Standard calibration solutions, of concentration similar to the condensates, underwent the same procedure. The overall efficiency of the trap-and-desorb procedure ranged from 10% for CH_3OH through 20% for CH_3CN, 30-40% for the propyl and butyl alcohols, to 40-45% for the carbonyls, depending on solubility, retention volume, and desorption efficiency.

A typical chromatogram obtained from a condensate sample and a background distilled-water blank are shown in Figure 2. Other than methanol, background impurities in the blank were present at or below 3 μg L^{-1}, while for methanol the level was larger than this (20 μg L^{-1}).

In the absence of interfering gas chromatographic peaks or background impurity in the water blank, the lower limit of detection is about 0.1 μg L^{-1}. At typical condensate concentrations (0.2 - 200 μg L^{-1}), the precision of this technique is 10-15% (depending on the particular analyte).

Formaldehyde was determined by the Nash [1953] method; bisulfite in large excess is a known interference, but in the low SO_2 conditions here should cause little problem [Klippel and Warneck, 1978]. Atmospheric SO_2 concentrations within Tucson and near the sampling sites here are usually less than 2 ppbv [Dawson and Farmer, 1984].

In Situ Production: Contamination

The film condensation technique is unable to distinguish between those chemical species which are actually collected from the atmosphere and those that result from reactions in the condensate, prior to or during analysis. Simple organic esters (methyl and ethyl acetate) have been shown to undergo no significant hydrolysis during storage and analysis. We have also investigated condensate stability; very little decomposition has been observed over a period of 1 week, provided the condensate is stored in glass and kept chilled (2-4°C). With the exception of the aldehydes, components are stable against oxidation by acidified dichromate and H_2O_2.

Aqueous Concentrations: Condensate and Rain

Table 1 contains a summary of mean organic levels found in condensate. Data were collected at three different sites: the Tucson site was located on the roof of the Physics and Atmospheric Sciences Building at the University of Arizona, 2 km from downtown Tucson. Mount Lemmon samples were obtained at the crest of the Catalina Mountains (elevation 2.9 km) located 27 km north of Tucson. The Santa Rita site is located in the foothills (elevation 1.3 km) of the Santa Rita Mountains, 52 km south of the University of Arizona campus. Both rural sites must be partially influenced by anthropogenic contamination from the greater Tucson metropolitan area; however, both locations are surrounded by forests of juniper and ponderosa

pine, both gave very similar results and have been lumped together in the tables.

Greater Tucson has a population of about 500,000, with no significant pollution sources other than automobiles. In addition, there are four isolated copper smelters in southern Arizona (the closest 54 km northeast of Tucson), which emit substantial quantities of SO_2. Data were collected during the spring and summer of 1982. Rain (and/or hail) was also collected at the Santa Rita site during condensate collection to allow a comparison between concentrations; this proved to be instructive and will be discussed later.

To convert condensate concentrations to atmospheric levels, Henry's law data under collection conditions are required. A review of the literature revealed that Henry's law coefficients do exist for most compounds of interest here, but they are usually at 298°K, rather than the collector temperature (273°K - 278°K). Calorimeteric data were inadequate to permit extrapolation for many components; therefore partition coefficients were determined experimentally.

Henry's Law Coefficients

Method

One-half liter of solution containing 1-4 compounds of interest was placed in a silanized 3-L Erlenmeyer flask equipped with stirbar. These samples were thermostatted in a constant temperature bath (25°C) or an ice/water slurry. Sixty milliliters of displacement gas, at 5 mL/min, displaced head-space gas into the 20-mL gas chromatograph sample loop. No decrease in component concentrations was apparent at displacement flow rates of less than 10 mL/min. Depletion of solute can be neglected in all cases here.

Calibration factors were established by static dilution in a similar container, followed by displacement into the sampling loop. In this case, allowance had to be made for the dilution effect during displacement.

Component concentrations in the vapor equilibrated above these test solutions and in the calibration were similar, but of necessity (since there was no preconcentration step) corresponded to aqueous concentrations much larger than those found in the condensate, or used for that calibration. Typical aqueous concentrations in the determinations of Henry's law constants were 300 mg L^{-1} for the aliphatic alcohols and 20 mg L^{-1} for the less soluble aldehydes, ketones, and nitrile. Other investigators [Friant and Suffet, 1979; Kieckbusch and King, 1979] have demonstrated adherence to Henry's law, for similar compounds, at aqueous concentrations below 1000 mg L^{-1}.

Results and Discussion

The partition coefficients are shown in Table 2. Literature values for 298°K are shown to the right of the present data. At 298°K, there is agreement, within experimental error, between the two sets of data.

Table 2 demonstrates that vapor solubilities of the alcohols at 298°K (H_A^{298}) decrease with increasing carbon number. However, the heat liberated on transfer of organic vapor to dilute solution (ΔH_{tr} = enthalpy of solution at infinite dilution - enthalpy of vaporization of pure organic liquid [Franks and Reid, 1973]) increases with chain length. The solubilities of the aliphatic alcohols (C_1 - C_4) are therefore quite similar at 273°K. Compounds such as acetone and acetonitrile have low intrinsic heats of transfer, and therefore only modestly increased solubility at 273°K. A finite difference form of the Van't Hoff relation can be used to calculate average heats of transfer ($\overline{\Delta H}_{tr}$) from our measurement of H_A at 298°K and 273°K.

$$\ln\left(\frac{H_A^{273}}{H_A^{298}}\right) = -\frac{\overline{\Delta H}_{tr}}{R}\left(\frac{1}{273} - \frac{1}{298}\right) \qquad (2)$$

These results are shown in Table 2 on the right. Comparisons of the calorimetrically determined values, obtained from the literature, with our values obtained from (2), show reasonable agreement in most cases. Discrepancies are undoubtedly due to the relatively small temperature interval investigated, plus experimental error in H_A.

The Henry's law coefficients and their temperature dependencies (via ΔH_{tr}) can now be used with the previously presented condensate concentrations to deduce atmospheric levels using (2).

Atmospheric Concentrations

Table 3 summarizes averaged atmospheric mixing ratios obtained here. Due to the relatively high concentrations of ethanol and acetone in some of the condensates (Table 1), a number of compounds were obscured; e.g., detection of acetonitrile was possible only at the two collection sites outside Tucson. On no occasion were we able to detect acrolein or isopropanol in the condensate samples; these two substances have retention times which are within 0.5 min of acetone. The technique used here is able to resolve all three compounds if present in solution at equal concentration. Glyoxal, $(HCO)_2$, was not found; it gave a very low response upon purging and chromatography with test solutions. Both 2,3-butanedione and 2-butanone have retention times which are within 0.2 min of each other. On many occasions, a broadening of the peak centered at 7.0 min indicated that the diketone may have been present in the condensate; however, the area of this peak is reported as resulting solely from 2-butanone. Tertiary butanol was identified and quantitated in Tucson, but was not detected outside town. Formaldehyde mixing ratios at or above 1 ppbv were found at all three sites but were highest in the city; urban and biogenic contributions cannot be separated. All other substances exhibited significantly lower mixing ratios when sampled outside Tucson.

Discussion

With the exception of formaldehyde, none of the organic vapors investigated in this study have been extensively studied in both urban and rural environments. Table 3 contains a summary of the measurements made by other investigators. Noteworthy are the sparse data on the aliphatic alcohols.

TABLE 2. Dimensionless Partition Coefficients (mol L^{-1} in liquid/mol L^{-1} in vapor) and Enthalpies of Transfer Found in This Study and by Others

	Partition Coefficients (Dimensionless)				Enthalpies of Transfer (kcal/mol)			
	Experimental This Study		Literature		This Study From Eq. (2)	Literature		
	H_A^{273} ($\times 10^3$)	H_A^{298} ($\times 10^3$)	H_A^{298} ($\times 10^3$)	Reference	$\overline{\Delta H}_{tr}$	ΔH_{tr}^{298}	ΔH_{tr}^{273}	Reference
Alcohols								
C_1H_4O	25 (±3)	5.5 (±0.5)	5.5	5	9.8	10.7	11.4	1, 2, 8
C_2H_6O	33 (±2)	4.7 (±0.2)	4.8	5	13	12.5	13.7	1, 2, 8
n-C_3H_8O	30 (±4)	3.3 (±0.1)	3.6	5	14	13.8	15.3	1, 2, 8
iso-C_3H_8O	28 (±3)	3.1 (±0.1)			14	14.0	15.5	1, 2, 8
n-$C_4H_{10}O$	26 (±1)	3.1 (±0.2)	2.9	4	14	14.8	16.6	1, 2, 8
sec-$C_4H_{10}O$	23 (±3)	2.7 (±0.1)			14	15.0	17.2	1, 2, 8
tert-$C_4H_{10}O$	20 (±3)	1.7 (±0.2)			16	15.4	17.9	1, 2, 8
iso-$C_4H_{10}O$		2.5 (±0.2)				14.4	16.2	1, 2, 8
Ketones								
C_3H_6O	2.5 (±0.1)	0.63 (±0.03)	0.67	4	8.9	9.7		6, 7
C_4H_8O	2.3 (±0.1)	0.43 (±0.02)	0.53	4	11			
$C_4H_6O_2$		1.4 (±0.2)						
Aldehydes								
C_2H_4O	1.7 (±0.1)	0.31 (±0.02)	0.38	4	11	10.3		6, 3
C_3H_6O	0.80(±0.09)	0.18 (±0.1)			9.7			
C_5H_6O			0.33	4				
Nitriles								
C_2H_3N	3.7 (±0.4)	1.2 (±0.1)			7.3			
C_3H_5N						7.5		6

1. Alexander and Hill [1969]
2. Arnett et al. [1969]
3. Bell and Clunie [1951]
4. Buttery et al. [1969]
5. Buttery et al. [1971]
6. National Research Council [1929]
7. Kister and Waldman [1958]
8. Polák and Benson [1971]

Alcohols

Cavanagh et al. [1969] found methanol, ethanol, and normal butanol at Barrow, Alaska, and concluded that they were produced by starch-fermenting bacteria in Arctic tundra. Photochemistry of methane and ethylene [Graedel, 1978; Meagher and Heicklen, 1976], combustion [Bellar and Sigsby, 1970; Hoff and Kapsalopoulou, 1964], anthropogenic emissions [Hoshika et al., 1981], and perhaps the oceans [Zafiriou, 1975] have also been suggested as potential sources of atmospheric alcohols. Dominant atmospheric sinks should be reaction with hydroxyl radical. Lifetimes against attack by OH (at a mean concentration of 10^6 cm^{-3}) range from 11 days for methanol [Overend and Paraskevopoulos, 1978] to 5 days for normal butanol [Graedel, 1978]. Wet removal, even for methanol, should be comparatively slow.

In contrast to the results of Cavanagh et al. [1969], concentrations here decreased rapidly with carbon number, C_1 - C_4. Levels of n-propanol were surprisingly low; it and tertiary butanol could be detected only in the city. The city was clearly a source of methanol and ethanol, but not of n-butanol; the concentration ratio, urban to rural, was greater for ethanol than for all other species measured. The city source was apparently not vaporization of gasoline; none of the major suppliers in Tucson admitted to adding ethanol in 1982.

A statistical test was performed for difference between day and nighttime concentrations (see Table 4). Of the alcohols, only the butanols showed a significant difference, and then only for the Tucson data; nighttime values were higher. Since butanols are sometimes used as gasoline additives, these results suggest that gasoline evaporation after the evening "rush hour" and lower vertical mixing rates produce the larger nighttime values within the city. The absence of other measurable diurnal differences, city or rural, is not surprising for these long-lived species; it does not allow us to distinguish between primary emission or photochemical production. Both emissions and radical-mediated production and loss could easily have similar diurnal variation.

TABLE 3. Atmospheric Concentrations of Light Organics Observed in This Study and by Other Workers

	This Study						Other Investigators					
	Tucson Feb.-Sept.1982			Santa Rita and Mt. Lemmon Aug.-Sept.1982			Urban Ref.		Rural Ref.		Remote Ref.	
	C(±δC) ppbv		N	C(±δC) ppbv		N	ppbv		ppbv		ppbv	
Alcohols												
C_1H_4O	7.9	(±2.6)	17	2.6	(±1.1)	18					0.4-1.2	3
C_2H_6O	3.3	(±2.0)	17	0.40	(±0.17)	18					0.4-1.2	3
n-C_3H_8O	0.02	(±0.01)	11*									
n-$C_4H_{10}O$	0.12	(±0.06)	17	0.06	(±0.03)	18					50-130	3
tert-$C_4H_{10}O$	0.12	(±0.07)	16									
Ketones												
C_3H_6O	12	(±4)	17	2.8	(±0.8)	18	=20	10	1-3	7	0.1	8
C_4H_8O	2.8	(±1.3)	17	0.50	(±0.16)	18	0-13	4				
Aldehydes												
C_1H_2O	1.8	(±0.6)	8	1.5	(±1.0)	27	0.7-5	6	0.4-3.8	6,11	0.1-0.6	9,11
C_2H_4O	23	(±12)	17	6.9	(±2.5)	18	1-35	4	1-2	2	0.02	8
Nitrile												
C_2H_3N				0.06	(±0.02)	18			<7	1	>0.01	5

δC is the standard deviation of the concentration data, N the number of measurements.
*Toluene used as internal standard in early 1982
1. Becker and Ionescu [1982]
2. Breeding et al. [1973]
3. Cavanah et al. [1969]
4. Grosjean [1982]
5. Henschen and Arnold [1981]
6. Neitzert and Seiler [1981]
7. Robinson et al. [1973]
8. Singh and Hanst [1981]
9. Zafiriou et al. [1980]
10. Johansson [1978]
11. Lowe et al. [1981]

Carbonyls

The carbonyls also exhibited much higher concentrations in the city than at the rural locations. City concentration of acetaldehyde was particularly high, and one can safely assume that it resulted primarily from automobile emissions, while the rural levels reflected secondary photochemical production. The acetaldehyde/formaldehyde ratio was much larger than usually found for urban areas. No definitive explanation can be offered, and neither determination seems to be in error. The formaldehyde concentration in Tucson in 1982 is comparable to that found in 1980 using different techniques (55 measurements giving a mean of 1.2 ppb, unpublished). One must always consider the possibility of another compound coeluting with acetaldehyde, though the likelihood of this seems vanishingly small. The chromatographic identification follows condensation and distillation steps which closely define an acceptable range of solubility in water. No other light organics have similar retention time on Porapak and comparable water solubility. The large acetaldehyde/formaldehyde ratio must go unexplained.

As expected, acetone was present in fairly high concentration.

The carbonyls, other than acetone, showed significant diurnal differences at the rural sites; a daytime maximum was found. Only formaldehyde may have shown the same trend in Tucson (Table 4). The diurnal changes could result from photochemistry, precursor transport, or dilution by nighttime drainage. Since the alcohols did not show a similar trend, these observations argue that transport and drainage were not major factors. It seems likely, therefore, that the alcohol concentrations are dominated by primary emissions and the carbonyls by photochemical production.

TABLE 4. Parametric Test of Difference Between the Means of Daytime and Nighttime Mixing Ratios, $\bar{C}_d$ and $\bar{C}_n$

Compound	$\bar{C}_d \pm \delta C_d$	$\bar{C}_n \pm \delta C_n$	N_d	N_n	Probability of Difference Between $\bar{C}_d$ and $\bar{C}_n$
		Tucson			
tert-butanol	0.087 ± 0.073	0.152 ± 0.039	10	6	0.96
n-butanol	0.096 ± 0.053	0.160 ± 0.054	11	6	0.97
formaldehyde	2.25 ± 0.25	1.53 ± 0.56	5	3	0.91
		Nonurban Locations (Mt. Lemmon and Santa Rita Experimental Range)			
n-butanol	0.072 ± 0.033	0.057 ± 0.028	8	10	0.65
formaldehyde	2.0 ± 1.4	1.1 ± 0.5	13	14	0.96
acetaldehyde	8.3 ± 2.2	5.8 ± 2.2	8	10	0.96
2-butanone	0.59 ± 0.16	0.42 ± 0.13	8	10	0.97
acetone	3.2 ± 0.8	2.8 ± 0.7	8	10	0.70

δC is a standard deviation; N_d and N_n are the number of day and night measurements, respectively.

Acetonitrile

A brief note on the acetonitrile data has already been published [Snider and Dawson, 1984]. Expected surface concentrations [Brasseur et al., 1983] lie between 10 and 20 ppt. Our measurements are therefore compatible with this background concentration upon which are superimposed some contributions from Tucson itself.

Precipitation Samples

Thunderstorms occurred between September 9 and 11, 1982, at the Santa Rita site during the collection of condensate, allowing samples of rain and hail to be obtained. The processes of liquid water production and precipitation in clouds are vastly more complex than the processes of condensation on the collector surfaces used here. Nevertheless, there are similarities, since gas/liquid partitioning is involved at some stage in both cases. (We shall assume that vapor/liquid interactions, as opposed to vapor/ice interactions dominate.) Not surprisingly, the precipitation samples contained the same constituents as found in the condensates. The actual concentrations are not of great interest here since they depend, inter alia, on cloud liquid-water content, cloud base height, evaporation, and so on. Only ratios of concentrations, precipitation to condensate, will be discussed; the data are shown in Table 5. In general, carbonyl compounds exhibited enhanced levels in the precipitation, whereas alcohols and CH_3CN were greatly depleted. This difference appears to be based more on functionality than solubility; the alcohols, as a group, all have fairly large solubilities, but CH_3CN is a poorly soluble gas. Though all the carbonyls were enhanced, HCHO is more soluble than the alcohols, while the other carbonyls are less soluble than CH_3CN. Partition is not therefore the explanation. Similarly, different vertical distributions resulting from variations in solubility cannot be accepted either. Thunderstorms were occurring; the atmosphere was convectively unstable and should have been well mixed. None of the components is highly soluble; in a cloud, all should be partitioned primarily into the gas phase. As a result, the vertical distributions of all components are likely to have been similar. There is no evidence for some sort of special interaction between any of the compounds here and ice, and so we shall not suggest one. Rather, we shall suggest another explanation of these intriguing trends, which must, at this time, be considered highly speculative.

The data are most easily explained if either the alcohols and CH_3CN are being destroyed within the cloud, or carbonyls are being produced. Alcohols and CH_3CN are well-used solvents, all relatively stable against aqueous oxidation. The concentration ratio (precipitation/condensate) for these compounds is virtually identical within experimental error; it is surely highly unlikely that they would all be destroyed by a similar amount. Much more likely is that their concentration ratio is that of a substance not undergoing any chemical change within the cloud. If that is the case, then perhaps the carbonyl compounds, formaldehyde, acetaldehyde, acetone, and 2-butanone are produced by in-cloud processes. At this time, this appears to the authors to be the most likely, though speculative, explanation. Aqueous phase photochemical oxidations have recently been discussed by Chameides and Davis [1982] and Graedel and Weschler [1981].

Conclusions

This study was undertaken to examine the usefulness of the film condensation method for sampling moderately soluble organic vapors. Many of these substances are thought to be intermediates in the degradation of atmospheric methane and

TABLE 5. Mean Aqueous Concentrations of Precipitation and Condensate and Their Ration at Santa Rita

	Mean Condensate Concentration C_c ppbm	Mean Precipitation Concentration C_p ppbm	Number of Measurements	C_p/C_c
		Alcohols		
C_1H_4O	56	22	4	0.39 (±0.05)
C_2H_6O	15	4.6	4	0.31 (±0.03)
n-C_3H_8O				
n-$C_4H_{10}O$	3.2	0.89	4	0.28 (±0.09)
		Ketones		
C_3H_6O	16	23	4	1.4 (±0.4)
C_4H_8O	2.1	4.6	4	2.2 (±0.7)
		Aldehydes		
C_1H_2O	130	220	5	1.7 (±0.6)
C_2H_4O	15.5	66	4	4.3 (±1.7)
		Nitrile		
C_2H_3N	0.43	0.18	4	0.42 (±0.05)

nonmethane hydrocarbons, others originate directly from anthropogenic and biogenic emissions. This technique, when combined with a condensate preconcentration step, is capable of detecting these particular organic vapors at sub-ppb levels in the atmosphere. Henry's law coefficients at collector temperature are required and were determined.

Previous measurements of some of the components, e.g., the alcohols and acetonitrile are very sparse.

Sampling in Tucson, Arizona, and at two rural sites demonstrated that the light alcohols and carbonyls have some sources within that urban area. Alcohol levels showed little diurnal variation; carbonyls exhibited a definite daytime maximum at the nonurban sites.

Acetonitrile was detected at the two rural sites at concentrations which are consistent with those observed in the lower stratosphere by other workers. Acetonitrile is rather poorly soluble, and thus its wet removal from the atmosphere should be small.

Concentrations of oxygenated organics in precipitation and in condensates were compared. It was tentatively concluded that the light alcohols and acetonitrile remained relatively unreactive within cloud water, while concentrations of the carbonyls were consistent with their being produced within the cloud, for example, by aqueous photochemistry.

Acknowledgments. This work was supported by the National Science Foundation under grants ATM-77-24508 and ATM-82-15694 and by the Geophysics Branch of the Office of Naval Research under contract N00014-77-C-0099-P00005. The manuscript was edited by Margaret Sanderson Rae and typed by Elaine Segura.

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(Received December 19, 1983;
revised June 11, 1984;
accepted June 13, 1984.)

FEDERAL SECURITY AGENCY
U. S. PUBLIC HEALTH SERVICE

National Institute of Health Bulletin No. 176

ACUTE RESPONSE OF GUINEA PIGS TO THE INHALATION OF KETONE VAPORS

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From the Division of Industrial Hygiene
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Linden, N. J.

UNITED STATES
GOVERNMENT PRINTING OFFICE
WASHINGTON : 1940

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ACUTE RESPONSE OF GUINEA PIGS TO THE INHALATION OF KETONE VAPORS

I. INTRODUCTION

The study of the group of related ketones to be presented in this bulletin was undertaken by the Division of Industrial Hygiene of the National Institute of Health as a continuation of its basic plan for the investigation of the hazards of volatile solvents in industry. In order properly to plan and interpret subsequent studies on the subacute and chronic effects of volatile solvents it was found necessary to study first the responses of test animals to acute conditions. In the literature on this subject contradictory reports have been n[illegible] on the type and grade of response to volatile solvents; due, mai[illegible] to the fact that the experiments were made under widely differ[illegible] conditions and were judged by differing standards. Accordingly, it was found necessary to study the various substances by means of a uniform method incorporating the desirable features of preceding methods.

There are practical limitations to the extent to which such a study can be carried. It has been possible, however, to make quantitative measurements during the exposure of the pulse by palpation and the rectal temperature by thermocouple, whereas, heretofore only qualitative estimation could be made after the exposures had been concluded. It has also been possible to test equilibratory and other reflexes.

II. KETONES STUDIED

The ketones used in this study comprise the homologous series of straight-chain methyl ketones from dimethyl to methyl n-hexyl and four additional six-carbon ketones of different structures. The data concerning the grade of the compounds are presented in detail with the list of compounds in tables I and II. The boiling temperature ranges were obtained by the American Society for Testing Materials Method No. D86–30 but are presented in abbreviated form, showing the limits of the straight region of the boiling point curve. The purest grades of ketones were obtained wherever this was co[illegible]ible with reasonable cost of making the necessary exposure atm[illegible]s. In the case of acetone, where high concentrations were n[illegible] both c. p. and technical grades were used. From physical dat[illegible] they were found to be comparable and methanol was not detected in either grade.

[1] Inflammable mixtures were used only in few exposures because of the fire and explosion risk. Inflammability limits were based on the compilation of Coward and Jones (1938).

Table I.—*Physical data of acetone and its methyl homologs*

Ketone	Chemical formula	M. wt.[1]	B. P.[1]	M. P.[1]	Density [1]	V. P.[2]	Standard distillation	
			° C.	° C.	20° C.	mm. Hg.	Percent	Range in ° C.
thyl (Acetone)	CH_3COCH_3	58.05	56.1	−94.3	0.7915	185.0	95	56.0–56.5
ethyl (Butanone)	$CH_3COCH_2CH_3$	72.06	79.6	−86.4	0.805	76.0	95	78.5–80.0
yl propyl (Pentanone-2)	$CH_3COCH_2CH_2CH_3$	86.08	101.7	−77.8	0.812^{15}_{15}	30.6	85	100.0–102.0
yl n-butyl (Hexanone-2)	$CH_3COCH_2CH_2CH_2CH_3$	100.09	127.2	−56.9	0.830^{0}	10.0	80	126.0–128.0
yl n-amyl (Heptanone-2)	$CH_3COCH_2CH_2CH_2CH_2CH_3$	114.11	150.		0.822^{15}	4.2	90	150.0–152.0
yl n-hexyl (Octanone-2)	$CH_3COCH_2CH_2CH_2CH_2CH_2CH_3$	128.12	172.7	−21.6	0.818	1.7	80	173.0–174.0

[1] International Critical Tables.
[2] Doolittle (1935).

Table II.—*Physical data of six-carbon ketones of different constitution*

Ketone	Chemical formula	M. wt.[1]	B. P.[1]	M. P.[1]	Density [1]	V. P.[2]	Standard distillation	
			° C.	° C.	20° C.	mm. Hg.	Percent	Range in ° C.
yl n-butyl (Hexanone-2)	$CH_3COCH_2CH_2CH_2CH_3$	100.09	127.2	−56.9	0.830^{0}	10.0	80	126.0–128.0
yl iso-butyl (4-Me-Pentanone-2, "Hexone")	$(CH_3)_2CHCH_2COCH_3$	100.09	119.0	−84.7	0.803	15.5	80	114.0–116.0
yl iso-butenyl (4-methyl 3-pentenone-2, Mesityl de).	$(CH_3)_2C{:}CHCOCH_3$	98.08	135.	−59.0	0.863	5.8	80	128.0–132.5
etylethane (Hexanediene-2, 5, Acetonylacetone)	$CH_3CO(CH_2)_2COCH_3$	114.08	194.	−9.	0.970	1.3	80	191.0–192.8
ohexanone	$CH_2{<}^{CH_2-CH_2}_{CH_2-CH_2}{>}CO$	98.08	156.7		0.949	4.6	80	154.0–158.0

[1] International Critical Tables.
[2] Doolittle (1935).

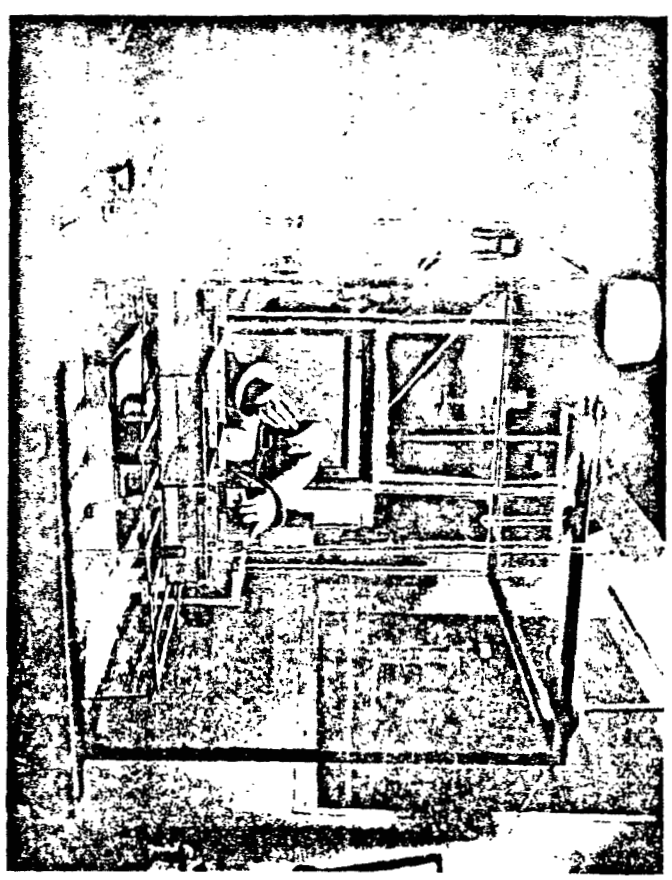

FIGURE 1.

One-cubic-meter exposure chamber showing door with gauntlets and vapor lock. To the right is a small circulating fan, in the center the wire mesh animal boxes, and in the foreground at the top of the chamber is the glass inlet tube from the larger chamber which is visible in part at the right. The sampling tube extends diagonally from the lot to the center of the chamber. Modifications in the chamber shown above were made in the circulating duct for use with the experiments reported in this bulletin. The size of the duct was increased and included a warming coil over which the vapor was forced by a strong type blower.

In addition to the numerous factors common to both groups of ketones mentioned above, the effect of the length of the carbon chain is studied in the former and the effect of diverse structure of the carbon chain in the latter.

III. METHODS OF EXPOSURE

A. ANIMALS USED.

Guinea pigs of mixed stock were used in these experiments after a period of quarantine during which time the individuals subject to infectious or other acute diseases were eliminated. The weight of the pigs varied between 400 and 600 grams in each group exposed together, thus reducing any weight factors between the different exposures. After exposure the surviving pigs from each group were kept together in one cage. Daily inspections were made of both exposed and non-exposed stocks. The pigs were handled frequently by the animal keeper and the experimenter to accustom them to this procedure and to reduce the adverse psychological effects of strange conditions on the various observable functions. It was found that with this training palpation of the heart and taking of rectal temperatures were easily carried out.

B. CHAMBER.

The exposures to the various ketones necessitated somewhat different procedures due to the differences in toxic properties and vapor pressures. Usually a large chamber of about 1000 cubic feet capacity served as a vapor reservoir and was connected by circulation ducts to a glass-walled observation chamber of one cubic meter capacity. (See fig. 1.) When the toxicity was high the small chamber was used alone with the circulation ducts shunted so that the vapor in the chamber was stirred. In addition to this forced circulation there was a small fan in the glass-walled chamber which helped keep the vapor in motion. The observation chamber was further fitted with flexible gauntlets extending into the center and a small vapor lock which permitted the guinea pigs to be introduced into the chamber after the vapor concentration had already been built up. The pigs were not allowed the complete freedom of the chamber but were placed two in a wire box which was open at the top. There were six such boxes, several inches above the floor of the chamber to allow circulation of the vapor.

C. CONDITIONING OF THE CHAMBER.

The exposures were made at 25° C. ± 1°, and regulated thermostatically when necessary by means of a warming coil. Cooling could not be done without condensing the vapor on the coil. In general the temperature of the laboratory-room air limited the temperature of the exposure chamber.

D. PRODUCTION OF VAPORS.

The desired concentration of ketone vapor was produced by interposing a large surface of filter paper in the air-stream of the circulating blower and allowing the requisite amount of ketone to drop upon this evaporating surface. In the same manner ketone was added, when necessary, to maintain the proper concentration in the chamber. Adjustments were made, if necessary, after periodic vapor analysis of samples from the center of the chamber.

E. VAPOR ANALYSIS.

The concentration of ketone vapor (except that of cyclohexanone) in the exposure chamber was determined by the iodine titration method, using the precautions recommended by Goodwin (1920). The procedure followed was that used by Patty, Schrenk, and Yant (1935) and modified by Reinhart (see Specht, 1938).

A 1,000 ml. flask fitted with a one-hole stopper and a glass stopcock was used for sampling and analysis. The volume of the flask was previously determined by calibration with water. The flask, containing 50 ml. normal sodium hydroxide, was connected to a vacuum pump and a mercury manometer, and was evacuated. The approximate concentration of the sample served as a guide to the degree of evacuation necessary to give the proper amount of ketone for titration within the accurate range of the method. The manometer reading was recorded and the stopcock closed. The flask was next connected to the sampling tube just after the latter had been flushed by withdrawing a portion of the vapor-air mixture from the chamber. The stopcock to the flask was then opened and the sample passed into the flask. The stopcock was closed and after disconnecting the sampling tube the flask was shaken so as to absorb the ketone vapor in the sodium hydroxide solution. The flask was next cooled in ice water to reduce the pressure of the contained gases and a measured excess of tenth-normal iodine was drawn in through the stopcock while the flask was shaken vigorously to insure rapid solution of the iodine.

After the reaction mixture had been allowed to stand in a dark place at room temperature for 30 minutes, 25 ml. of 2-normal sulfuric acid was added. In order that the resulting mixture should be slightly acid the concentration of 1-normal sodium hydroxide and 2-normal sulfuric acid were so adjusted that 50 ml. of sodium hydroxide was equivalent to 24.6 ml. sulfuric acid. The excess iodine was titrated with tenth-normal sodium thiosulfate.

The results obtained by this method are somewhat higher than theoretical, as has been shown by Haughton (1937) for acetone and by Cassar (1927) for butanone. The recovery factors for the other ketones were determined in the following manner in this laboratory.

Each ketone was dried over a suitable agent and distilled. Small [illegible] of the distillate were sealed into thin

glass bulbs and placed in flasks containing 50 ml. 1-normal sodium hydroxide. The bulbs were broken with a glass rod and the ketone determined in the manner described above. The recoveries thus made are shown in table III. The factors so obtained have not been applied to the analytical data as given in the following text since the variations in the exposure atmospheres as regards ketone concentration were of a comparable order of magnitude.

Table III.—*Data on recovery factors of ketones used in this study*

Ketone	Apparent recovery in percent	Ketone	Apparent recovery in percent
Di-methyl (acetone)	[1] 102.5	Methyl n-hexyl	
Methyl ethyl	[2] 110.6	Methyl iso-butyl	
Methyl propyl	110	Methyl iso-butenyl	
Methyl n-butyl	108	Acetonylacetone	
Methyl n-amyl	108	Cyclohexanone	

[1] Haughton, C. O. (1937).
[2] Cassar, H. A. (1927).

Cyclohexanone, because of its structure, cannot be determined by the iodine titration method. The oxime method of Marasco (1926) was used in this case. The sample was collected in the same manner as described above except that the sampling flask was charged with 100 ml. of a 0.2 percent solution of hydroxylamine-hydrochloride. The reaction proceeds as follows:

$$C_5H_{10}CO + NH_2OH.HCl \text{———} C_5H_{10}C{=}NOH + HCl + H_2O$$

The liberated hydrochloric acid is titrated with 0.05-normal sodium hydroxide, using as an indicator methyl orange-xylene cyanole as suggested by Hickman and Linstead (1922).

A comparison of the recoveries of butanone by the methods given above shows that the oxime method gives smaller values than the iodine titration method; 92.3 and 110.6 percent, respectively. Haughton (1937) got 97.1 and 102.5 percent, respectively, for acetone.

F. ANIMAL DATA COLLECTION.

A list of the observations made on the guinea pigs and the general symptoms with which they are related is given below. The criteria are listed in a rough order of appearance and thus define progressive stages of the response to vapor inhalation.

1. Irritation of the cornea and conjunctiva (squinting, lacrimation, rubbing of eyes). By direct observation.

2. Irritation of buccal, nasal, and pharyngeal passages (sneezing, coughing, salivation, retching, rubbing of nose and mouth). By direct observation.

3. Depression of the central nervous system (postural instability, loss of auditory and corneal reflexes, change in respiratory rate and

character). Change in the circulatory system: (pulse, hyperemia, cyanosis, rectal temperature). By direct observation, quantitative measurements with thermocouple, palpation and manual elicitation of reflexes.[2]

Autopsies were made where possible, and the tissues preserved in Orth's solution for further study of pathology.

G. GRAPHICAL PRESENTATION OF DATA.

Three types of graphical presentation are used in this bulletin. In the following paragraphs the construction and specific purpose of each is outlined:

1. Two-dimensional graphs, in which the abscissa may represent either time or concentration, and the ordinate may represent temperature, respiratory rate, or heart rate. Lines connecting the points and sloping down to the right indicate depression of function. Superposition of curves with common ordinates and abscissae affords direct comparison of the changes within each type of data, and the superposition of curves with common abscissae (for example, number of carbon atoms) but unlike ordinates is used in one instance to show similarity of data sequences not otherwise directly comparable.

2. Three-dimensional graphs, in which the base ordinate is concentration of vapor, the base abscissa is time, and the vertical ordinate is rectal temperature, respiratory rate, or heart rate. The construction of this graph follows the time course of the experiments, in that each experiment is represented by a plane erected vertically on the base parallel to the time axis at the particular vapor concentration for each experiment. The upper boundary of each plane is defined by a line drawn through the points on the vertical ordinates representing the data on rectal temperature, respiratory rate, or heart rate. In order that each plane may start at the same height the initial values for each type of data are averaged and this value is used as the beginning of each curve. Adjusting factors are then made for the remaining points of each curve by dividing its actual initial value by the general average initial value. The observed values for the experiment are then multiplied by this adjusting factor. In these three-dimensional graphs diminishing scales are usually applied to the axes by geometrical construction to represent the effect of perspective. In this case the vertical scale is arithmetic, while the base scales diminish to show proportionality. The time scale is, however, so adjusted to represent logarithmic changes in order that the early effects may be emphasized while including the gradual changes occurring with protracted exposure.

3. The qualitative data (time of appearance of irritation, neuromuscular weakness, coma, and death) are presented on a bilogarithmic chart. The ordinate is concentration of vapor and the abscissa is time. Points falling in the left lower quadrant indicate early response at low concentrations, in the left upper quadrant, early response to high concentrations, in the upper right quadrant, late response to high concentrations, and in the lower right quadrant, late response to low concentrations. Thus the position of the data on the chart is a general index by which comparisons between the response to different substances can be made. This type of presentation differs from that used by Patty, Schrenk, and Yant (1935, 1936 a, b,) by indicating points of positive response, while the latter show areas of different stages of response.

IV. INVESTIGATION OF ACETONE AND ITS HOMOLOGS

A. CONTROL EXPERIMENTS.

Control experiments were made by confining ten guinea pigs in the glass-walled chamber without connection to the large reservoir and taking the data as described in III.F. In addition, humidity and the concentration of oxygen and carbon dioxide were determined at intervals. Two such runs were made. The data are presented together in figure 2. From these records it is seen that the humidity reached only 70 percent even after nearly fourteen hours of confinement. The carbon dioxide concentration reached less than 4 percent in this period. The rectal temperature and heart rate are quite constant and the respiratory rate varies slightly. There is no increase in respiratory rate with increase in carbon dioxide concentration within the test period.

In view of these facts it is concluded, therefore, that no significant physiological changes are produced within fourteen hours by simple confinement during the experimental exposures to ketone vapors. Longer exposures were not interpretable beyond 14 hours but the additional data have in all instances been included for the sake of completeness.

B. RESULTS OF EXPERIMENTS WITH ACETONE AND ITS HOMOLOGS.

1. *Acetone.* In the paper of Specht, Miller, and Valaer (1939) the response of guinea pigs to the inhalation of 1.0, 2.0, and 5.0 percent acetone vapor has been presented in full. It will be sufficient to recapitulate here the protocol of an exposure at 2.0 percent acetone vapor, followed by a summary of all the findings and graphical data.

Two liters of acetone were evaporated in the large and small chamber and a concentration of 2.18 volumes percent was measured after the introduction of the animals. The chamber temperature maintained itself at 25.5±0.5° C. Two female guinea pigs were placed in the chamber via the air lock. Their normal rectal temperature, respiratory rate, and pulse were, respectively 38.4°C., 10 per minute, and 350 per minute. All values given subsequently are averages of

Up to 25 minutes.—Slight lacrimation, no signs of salivation, no rubbing, two pigs squinting.

240 minutes.—One pig has lost auditory response, others not very steady on their feet, somewhat restless, fur standing on end, very slight signs of lacrimation, no squint, rectal temperature 36.7° C., respiration 76 per minute, pulse 296 per minute, concentration of acetone, 1.99 volumes percent.

505 minutes.—All but two pigs are so weak they cannot stand, two are without auditory reflex, and two others are unconscious. Concentration of acetone 1.92 volumes percent.

540 minutes.—All pigs but one are in coma, all but two have auditory reflex, all have positive corneal reflex, righting reflex is very poor. Despite light coma there is occasional rousing and movement for a few seconds with a

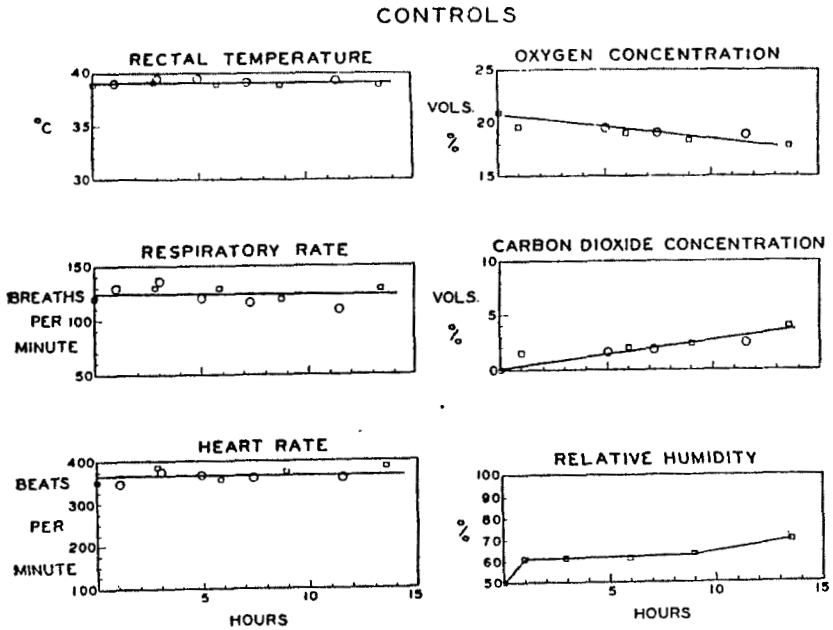

FIGURE 2.

The control data were collected under conditions identical with those for the vapor exposures. The results of two exposures to air without any added ketones are plotted here by means of square and round points. Relative humidity was measured in one exposure, carbon dioxide and oxygen measurements were made in both. The time scale is given in the lowest figures and is identical for all the figures. The chamber temperature was 25°C.

good deal of pawing or walking movements. Lacrimation is still not marked, but four pigs are beginning to salivate. Rectal temperature 33.8° C., respiratory rate 76 per minute, pulse 273 per minute.

865 minutes.—All on side and in moderately deep coma, two pigs have lost auditory reflex, and two others have lost corneal reflex, only one shows any righting reflex. All pigs are quiet, six are lacrimating, and seven salivating. Rectal temperature 31.2° C., respiratory rate 64 per minute, pulse 269 per

1,340 minutes.—All auditory reflexes gone, three pigs are dead, no further lacrimation or salivation, no righting reflex, no corneal reflex.

1,365 minutes.—Two more pigs dead, rectal temperature of survivors 26.7° C., respiratory rate 47 per minute, pulse 214 per minute.

1,405 minutes.—Two more pigs dead. Three survivors removed from chamber. All pigs observed to be in rigor even before death. Two pigs exsanguinated. Remaining pig died after several hours.

The quantitative data are presented in figures 3, 4, and 5 for rectal temperature, respiratory rate, and heart rate, respectively. It is seen in figure 3 that the rectal temperature falls off continually during the exposure to the several concentrations of acetone vapor. The rate at which this depression of the body temperature proceeds is proportional to the concentration of the vapor, and in the higher

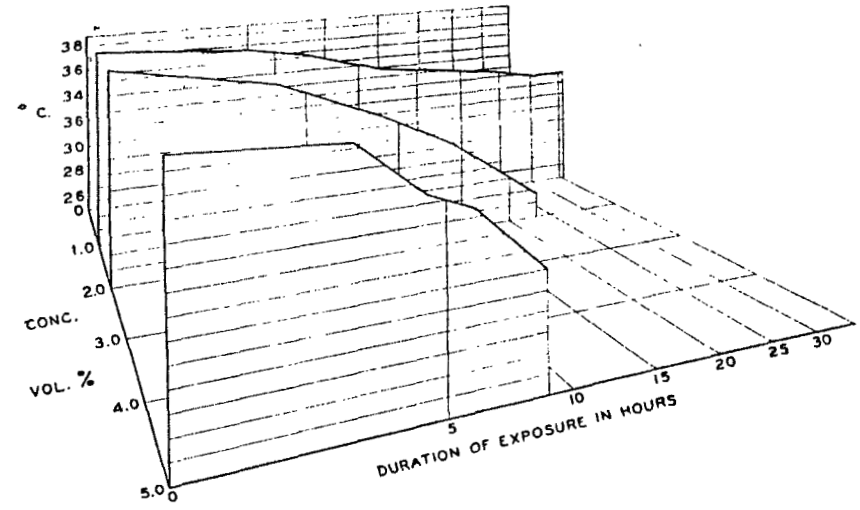

FIGURE 3.

Three-dimensional graph of the rectal temperature during exposure to 1.0, 2.0 and 5.0 percent acetone vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

concentrations becomes progressively greater as the exposure proceeds. In figure 4 it is seen that there is a somewhat variable depression of the respiration in the different concentrations of the vapor, and that the time course of this depression is also variable. At the highest concentration of vapor an immediate depression of the respiration is seen to occur, but after several hours of exposure the rate of respiration rises slightly and, at about 5 hours, falls again until terminal conditions set in. At the lower concentrations of vapor the respiratory rate is depressed more regularly, although slight tendencies to return toward the normal rate occur at 10 to 14 hours. In figure 5 somewhat similar conditions are apparent in

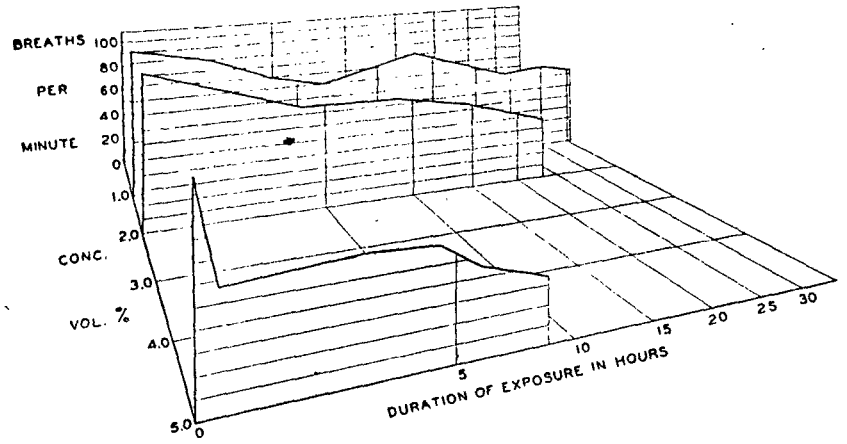

FIGURE 4.

Three-dimensional graph of the respiratory rate during exposure to 1.0, 2.0, and 5.0 percent acetone vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

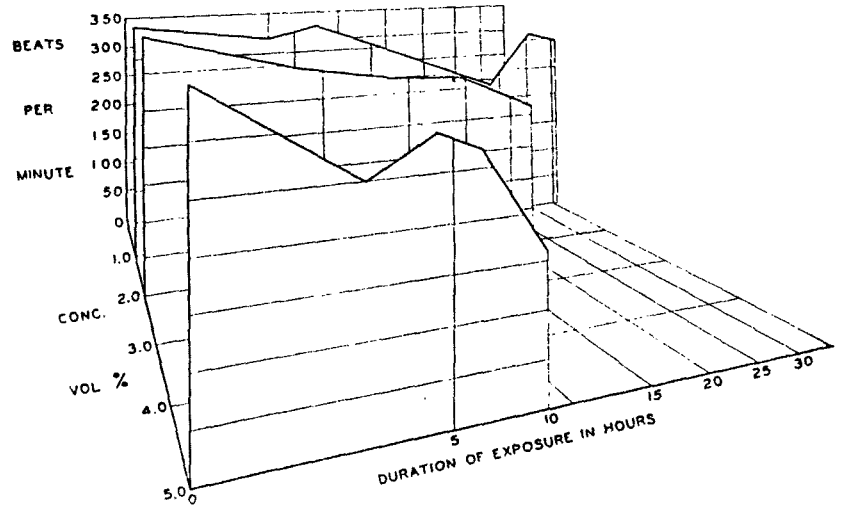

FIGURE 5.

Three-dimensional graph of the heart rate during exposure to 1.0, 2.0, and 5.0 percent acetone vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

the heart rate, although there is considerable irregularity. In any case the vapors of acetone are seen in both figure 3 and figure 4 to bring about a depression of the corresponding functions in rough proportion to the concentration and duration of exposure.

Figure 6 presents the data on the incidence of the qualitative symptoms and shows that the responses to acetone vapor are relatively delayed even at fairly high concentrations. Irritation seems to be immediate only at the highest concentration and is absent at one percent vapor as judged by these criteria.

The concentration of the acetone of the blood was found to increase continually during the exposures to each concentration of

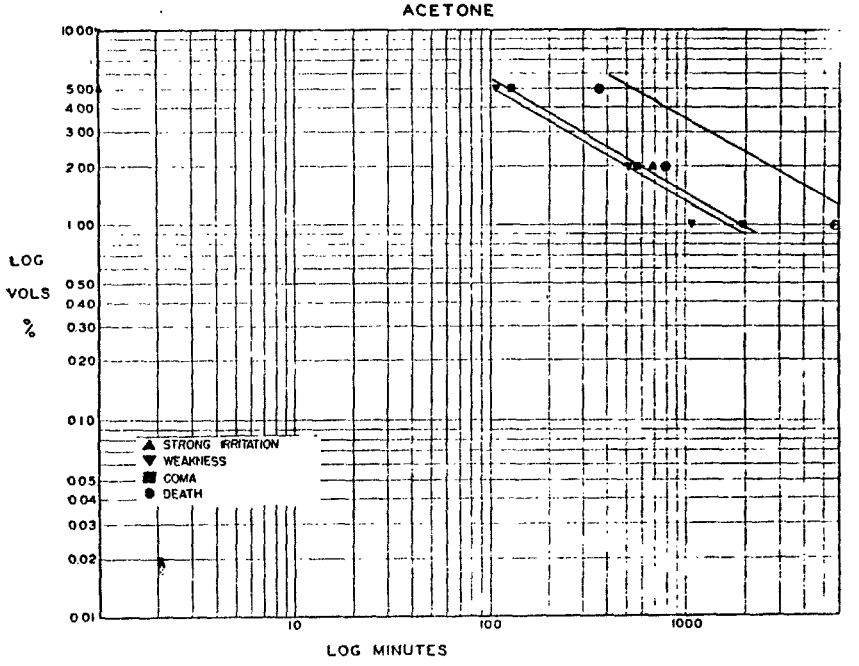

FIGURE 6.

Graph of the average time of onset of irritation, weakness, coma, and death at 1.0, 2.0, and 5.0 percent acetone vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete action of the animal sample.

vapor. There was no evidence of the attainment of an equilibrium with the external vapor even in the pigs that died on test.

The pathology of acetone inhalation is presented in a subsequent section.

b) Methyl ethyl ketone (butanone). Exposures were made to 1.0,

2.5 percent butanone is given here to illustrate the detailed behavior of the test animals:

The vapor of butanone was made up to 2.5 percent by volume in the 1-cubic-meter chamber which was maintained at 25°±0.5°C. Ten female guinea pigs were checked for normal rectal temperature, rate of respiration, and pulse rate and then introduced into the chamber via the air lock. The average values for the above functions were: 38.9°C., 103 per minute, and 342 per minute.

Up to 10 minutes—Marked salivation and moderate lacrimation with intermittent rubbing of muzzle. All pigs restless, many squinting.

30 minutes—All pigs down on their stomachs, one has lost response to auditory stimulus and is rolling. Lacrimation is ceasing but squinting is still prevalent. All pigs still salivating and all but two show a watery nasal discharge. Most pigs are quiet and very little rubbing is taking place. Average rectal temperature 38.5°C., respiratory rate 72 per minute, and heart rate 330 per minute.

55 minutes.—All pigs now on their sides; other symptoms show slight change.

75 minutes—Three pigs now without auditory reflex; no other changes.

90 minutes—Two pigs have very slight corneal reflexes; moderate rubbing and less salivation and lacrimation than previously. No righting reflex on manual rotation. Nasal discharge has stopped; seven pigs still squinting. Average rectal temperature 35.8°C., respiratory rate 50 per minute, and heart rate 286 per minute.

150 minutes—Only four pigs with positive corneal reflex, a few still rubbing muzzle occasionally, four lacrimating, three squinting. The average rectal temperature is 33.7°C., the respiratory rate is 50 per minute and the heart rate is 261 per minute.

180 minutes—First death.

210 minutes—Six pigs are now lacrimating, no salivation at all, only two pigs squinting. The average rectal temperature is 32.5°C., the respiratory rate is 20 per minute and the heart rate is 256 per minute.

Deaths at 245, 260, 265, and 290 minutes.

305 minutes—No positive auditory reflexes, no corneal reflexes. Four of the five survivors still lacrimating. The average rectal temperature is 30.6° C., the respiratory rate is 19 per minute and the heart rate is 239 per minute.

Deaths at 315 and 325 minutes. Three survivors taken out of the chamber. Two were dead the next day and the third survived till the day following but shivered continually and was otherwise incapacitated.

Figures 7, 8, and 9 illustrate graphically the relationship of rectal temperature, respiratory rate, and heart rate, respectively, to concentration of the vapor of and duration of exposure to methyl ethyl ketone. By comparing these figures with figures 3, 4, and 5, respectively, the differences in action between methyl ethyl ketone and acetone will be seen. In particular, rectal temperatures fall more precipitously in methyl ethyl ketone than in acetone and the duration of the experiments in the former where terminal conditions intervened is also comparatively shorter than in the latter. The respiratory rate shows much greater depression in butanone than in acetone and the rapid initial drop occurs at lower concentrations than in acetone. This is reflected to a lesser extent in the depression of the

heart rate. In the comparison of the qualitative data in figures 10 and 6, respectively, the points of incidence of the several criteria in butanone are shown to be to the left and below those for acetone. This indicates a greater effectiveness of butanone in depressing the observed functions.

These results are in good agreement with similar experiments performed in the Pittsburgh Experiment Station of the U. S. Bureau of Mines under somewhat different conditions. (Patty, Schrenk, & Yant, 1935)

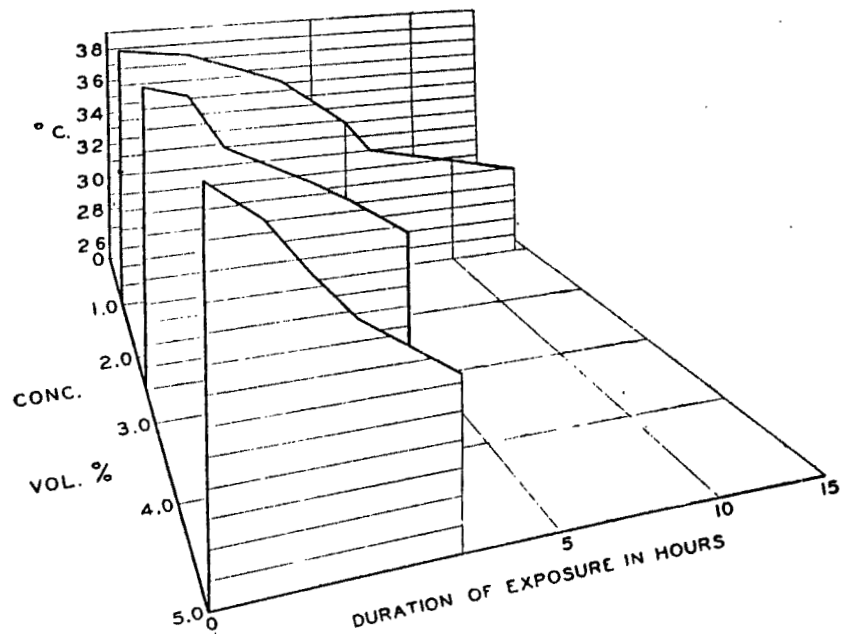

FIGURE 7.

Three-dimensional graph of the rectal temperature during exposure to 1.0, 2.5, and 5.0 percent methyl ethyl ketone (butanone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

3. *Methyl propyl ketone (pentanone-2)*.—Exposures were made to 4.0, 2.0, 1.0, 0.5, and 0.25 percent pentanone-2, respectively. The protocol of an exposure to 1.0 percent pentanone-2 vapor is presented here in order to illustrate the detailed behavior of the animals:

The vapor of pentanone-2 was made up to slightly over 1 volume percent in the glass-walled chamber. Ten female guinea pigs averaging 4.3° C. rectal temperature, 126 respirations per minute, and 350 pulse beats per minute were introduced into the chamber via the air lock. The vapor concentration was found to be 1.0 volume percent. The temperature was maintained at 24.5°±0.3° C.

Up to 10 minutes.—Loss of auditory reflex by one pig, coughing, copious lacrimation and salivation, a watery nasal discharge, and squinting in all pigs but no active rubbing of muzzle or eyes. All pigs fairly quiet.

35 minutes.—Two pigs are weak, one on side, copious salivation and lacrimation, average rectal temperature is 38.4° C, respiratory rate is 72.3 breaths per minute, pulse is 340 beats per minute.

80 minutes.—Five pigs weak and four on side, rest are unstable in stance.

125 minutes.—All pigs show weakness, all but three are on side.

140 minutes.—Vapor concentration equals 1.11 volumes percent, all but one pig on side, two have lost auditory reflexes, two pigs showing tremor of limbs when handled, (i. e., clonus) average rectal temperature is 34.8° C., respiratory rate is 72 breaths per minute, pulse is 285 beats per minute.

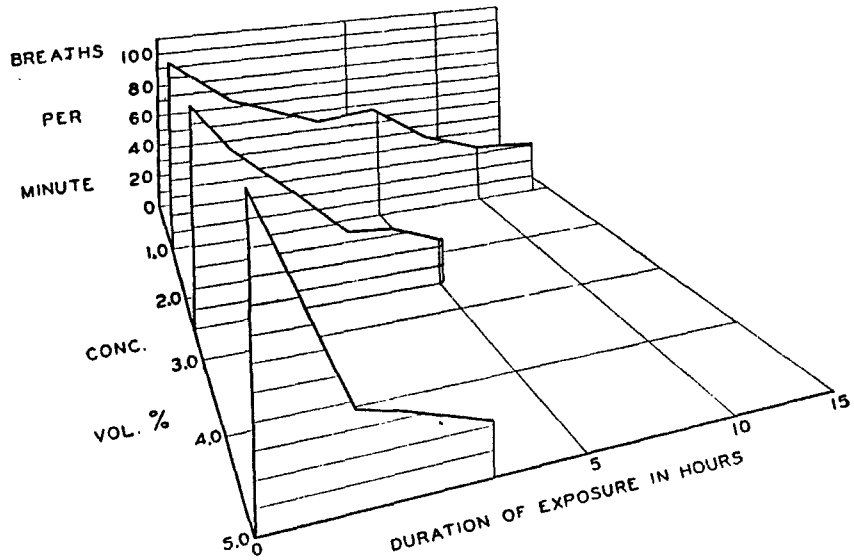

FIGURE 8.

Three-dimensional graph of the respiratory rate during exposure to 1.0, 2.5, and 5.0 percent methyl ethyl ketone (butanone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

160 minutes.—One pig has lost righting reflex, five are rubbing muzzle and eyes, one is coughing occasionally, five are lacrimating, seven are still salivating.

230 minutes.—All pigs are on side, none have auditory reflex, four have lost corneal reflex, six rubbing muzzle and eyes, three lacrimating, three salivating, all squinting, clonus has disappeared, average rectal temperature is 32.9° C., respiratory rate is 40 breaths per minute, pulse is 258 beats per minute, concentration of vapor is 0.99 volumes percent.

345 minutes.—Only four pigs with corneal reflex, none have any righting reflex, rubbing has stopped, one is making feeble pawing movements, only one is lacrimating, one salivating, only three are squinting, average rectal temperature is 30.9° C., respiratory rate is 24 breaths per minute, pulse is 236 beats per minute.

510 minutes.—Three pigs are dead, five others show watering of the eyes which is not copious but covers the surface of the cornea in a large drop, there is no salivation, one pig has severe asynchronous respiratory movements of diaphragm and chest, the average rectal temperature of the surviving pigs is 29.1° C., respiratory rate is 20 breaths per minute, pulse is 189 beats per minute. Another pig died at 525 minutes.

All survivors removed after 525 minutes. Next morning two more pigs were dead and rest still comatose but with positive corneal and righting reflex. At 10 a. m., the seventh pig died, and at 1:30 the eighth died.

At 2:30 p. m., two survivors were up and about but very groggy. They survived only until the next day.

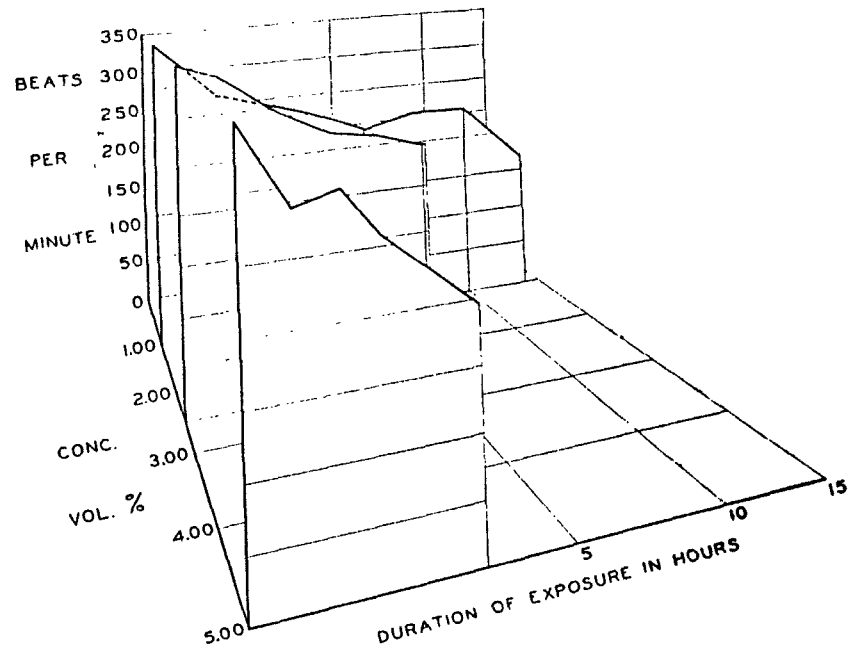

FIGURE 9.

Three-dimensional graph of the heart rate during exposure to 1.0, 2.5, and 5.0 percent methyl ethyl ketone (butanone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

Figures 11, 12 and 13 illustrate the general relation of rectal temperature, respiration, and heart rate, respectively, to duration of exposure and concentration of the vapor of methyl propyl ketone and that this vapor has a very similar action to that exhibited by methyl ethyl ketone in depressing the various functions listed above. Comparison of figures 10 and 14 which give the qualitative data for methyl ethyl and methyl propyl ketones, respectively, indicates that the incidence of qualitative symptoms is nearly alike. In regard to

irritation, in the case of methyl propyl ketone, however, there is an immediate effect down to concentrations of 0.25 percent, whereas the two preceding ketones are only irritating in much higher concentrations.

These data are generally corroborative of the observations made on pentanone-2 in the Pittsburgh Experimental Station of the U. S. Bureau of Mines (Yant, Patty, and Schrenk, 1936a), under somewhat different conditions.

4. *Methyl n-butyl ketone (hexanone-2)*.—Exposures to 0.13, 0.6, and 1.2 percent hexanone–2 vapor were made. The protocol of an

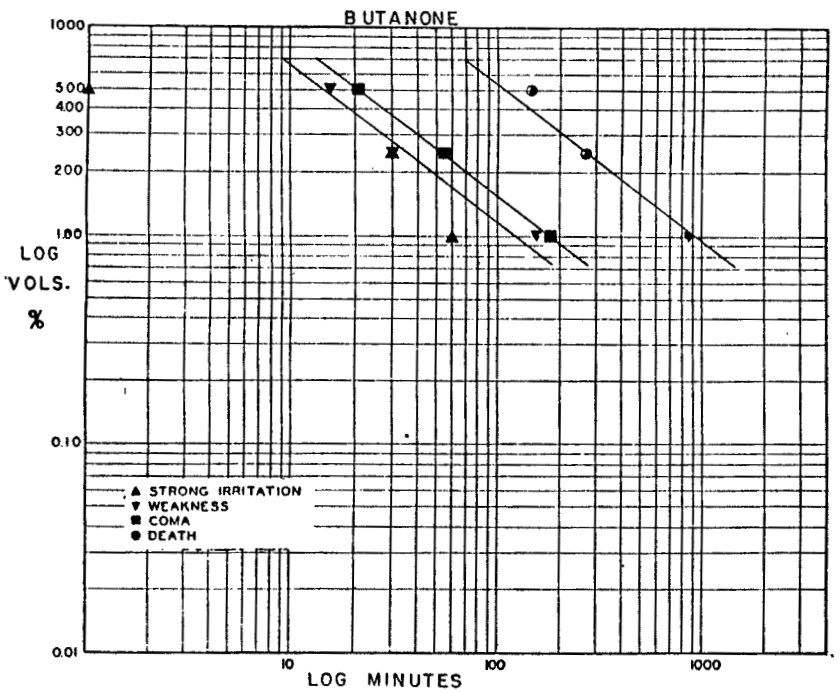

FIGURE 10.

Graph of the average time of onset of irritation, weakness, coma, and death at 1.0, 2.5, and 5.0 percent methyl ethyl ketone (butanone) vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete reaction of the animal sample.

exposure to 0.6 percent hexanone–2 is given here to illustrate the detailed behavior of the test animals.

The vapor of hexanone–2 was made up to 0.6 percent by volume in the one-cubic-meter chamber which was maintained at 25.5±0.3°C. Ten female guinea pigs were checked for normal rectal temperature, respiratory rate, and pulse. They were then introduced into the chamber via the lock. The average values for the above functions were: 39.0°C., 86 per minute, and 259 per minute, respec-

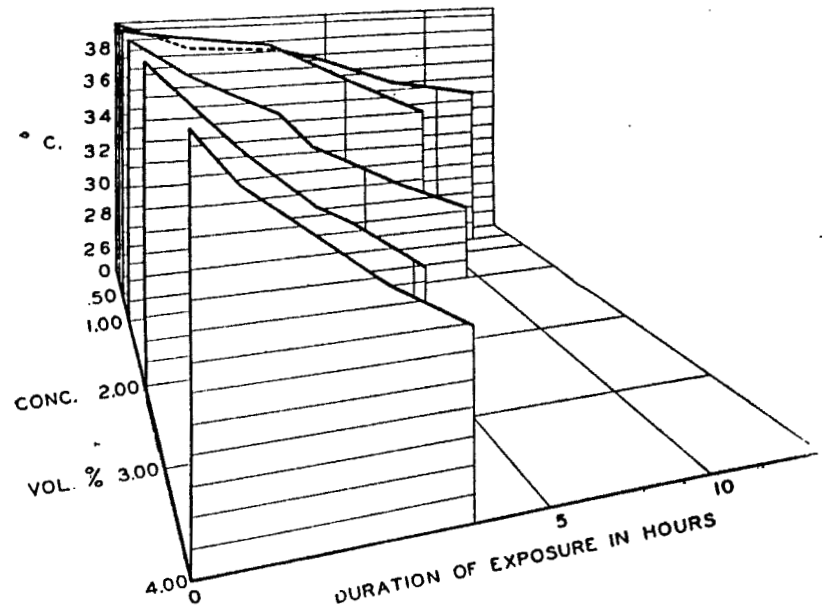

FIGURE 11.

Three-dimensional graph of the rectal temperature during exposure to 0.2, 0.5, 1.0, 2.0, and 4.0 percent methyl n-propyl ketone (pentanone–2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

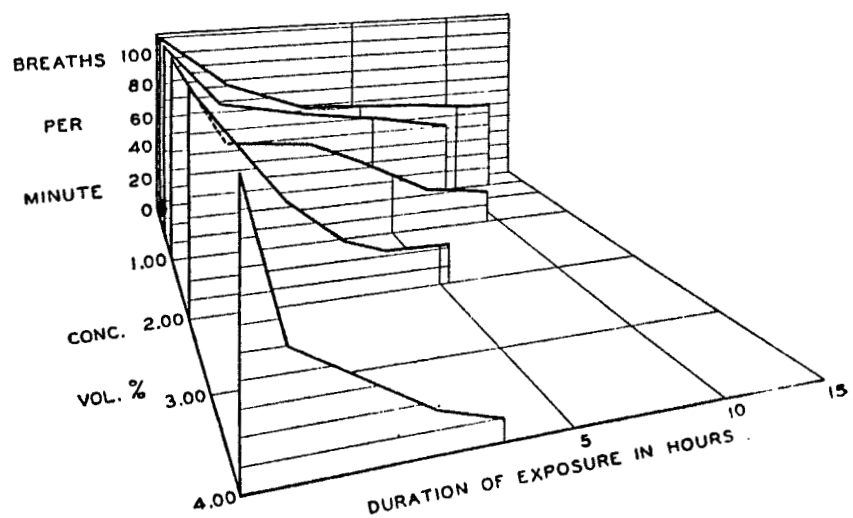

FIGURE 12.

Three-dimensional graph of the respiratory rate during exposure to 0.2, 0.5, 1.0, 2.0, and 4.0 percent methyl n-propyl ketone (pentanone–2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

Up to 15 minutes.—All pigs rubbing muzzle and moderately restless, four make a show of coughing; lacrimation and salivation are immediate and copious, There is a slight nasal discharge. All pigs squinting strongly. Coughing seems to have been very transient.

30 minutes.—Three pigs are down and three others do not respond to auditory stimulation. No other changes.

45 minutes.—Seven pigs are down, none on their sides yet. No other changes.

60 minutes.—All pigs down, four of them on their sides., Two pigs lost their corneal reflex. One pig does not respond well to righting stimulus. Two are still restless, four still rubbing occasionally. Lacrimation and salivation are stopping and in five or six pigs are absent. No nasal discharge now, squinting

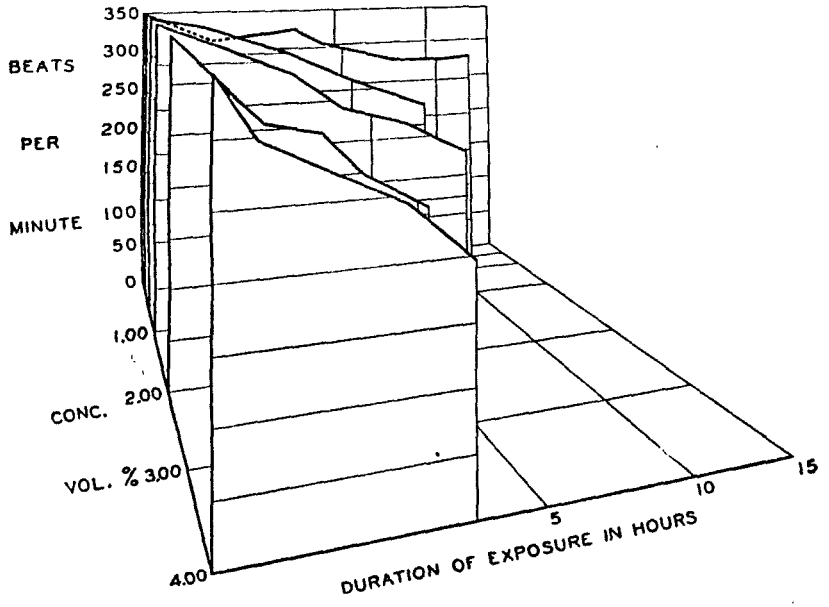

FIGURE 13.

Three-dimensional graph of the heart rate during exposure to 0.2, 0.5, 1.0, 2.0, and 4.0 percent methyl n-propyl ketone (pentanone–2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

is beginning to disappear. Average rectal temperature is 36.3°C., respiratory rate is 58 per minute, and heart rate is 250 per minute.

130 minutes.—All pigs on their sides and no auditory response. Six still give corneal reflex. Five still respond to righting stimulus. Two still rub occasionally. Four still lacrimating a little, none are salivating, eyes of several pigs still squinted (this may be due to swelling since the corneal reflex is not strong). The average rectal temperature is 33.5°C., the respiratory rate is 31 per minute, and the heart rate is 207 per minute.

180 minutes.—Only two pigs with corneal reflex patent. Two others show righting reflex, and two show some rubbing which is not very well directed. Lacrimation has nearly stopped, squinting is disappearing. Average rectal

temperature is 32.7°C., respiratory rate is 29 per minute and heart rate is 209 per minute.

240 minutes.—One pig attempting to get up but incapable of more than a rolling motion; it still has a positive corneal reflex and responds to the righting stimulus. No other pigs respond to manipulation but one makes feeble pawing movements. Lacrimation has stopped, average rectal temperature is 31.3°C., the respiratory rate is 20 per minute and the heart rate is 207 per minute.

400 minutes.—Two pigs are dead. No response in the others to manipulation. There seems to be a recurrence of lacrimation and salivation in about half the

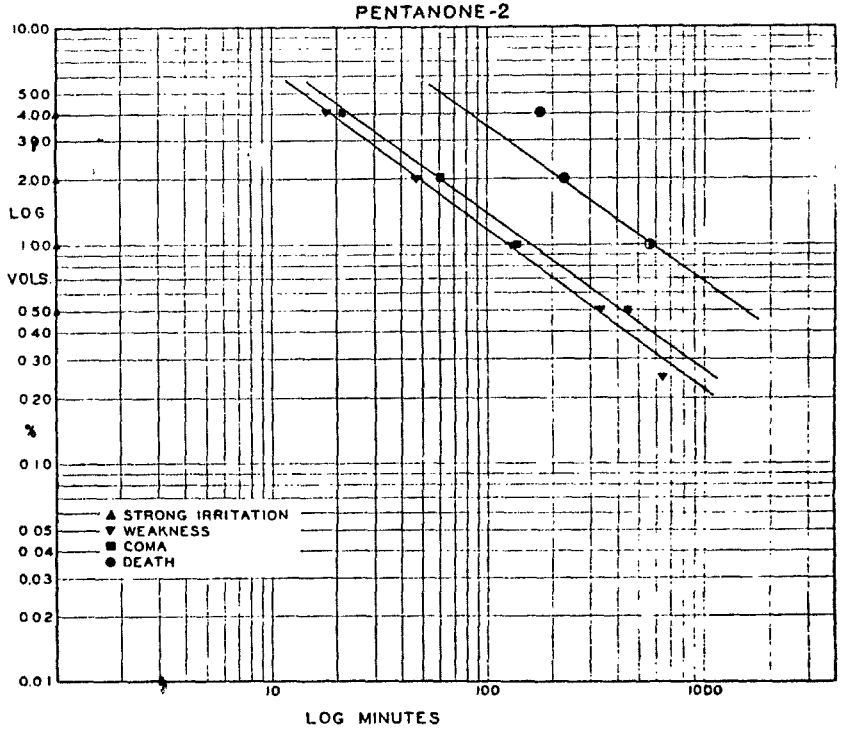

FIGURE 14.

Graph of the average time of onset of irritation, weakness, coma, and death at 0.2, 0.5, 1.0, 2.0, and 4.0 percent methyl n-propyl ketone (pentanone-2) vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete reaction of the animal sample.

pigs. The average rectal temperature of the survivors is 29.8°C., the respiratory rate is 16 per minute, and the heart rate is 168 per minute.

Deaths at 430, 440, 460, 485, and 525 minutes.

525 minutes.—Three survivors removed. One died soon after removal and the two others survived for two and three days, respectively, never recovering their ability to navigate properly and with obvious pilomotor activity during the whole period. The right cornea and aqueous of each pig was badly clouded

Figures 15, 16, and 17 present the general relations of rectal temperature, respiratory rate, and heart rate, respectively, to the duration of exposure to, and concentration of the vapor of methyl n-butyl ketone. They show that the depressant action of this ketone is greater than that of the preceding ketones. By comparing the appropriate figures; 15 with 11, 7, 8, and 16 with 12, 8, 4, and 17 with 13, 9, and 5, the relative effectiveness of methyl n-butyl ketone may be estimated.

In figure 18 the incidence of the several standard symptoms is seen to be earlier, by comparison with figures 14, 10, and 6, than for the preceding ketones. It is also evident that the vapor of this ketone is immediately irritating even at 0.12 volumes percent.

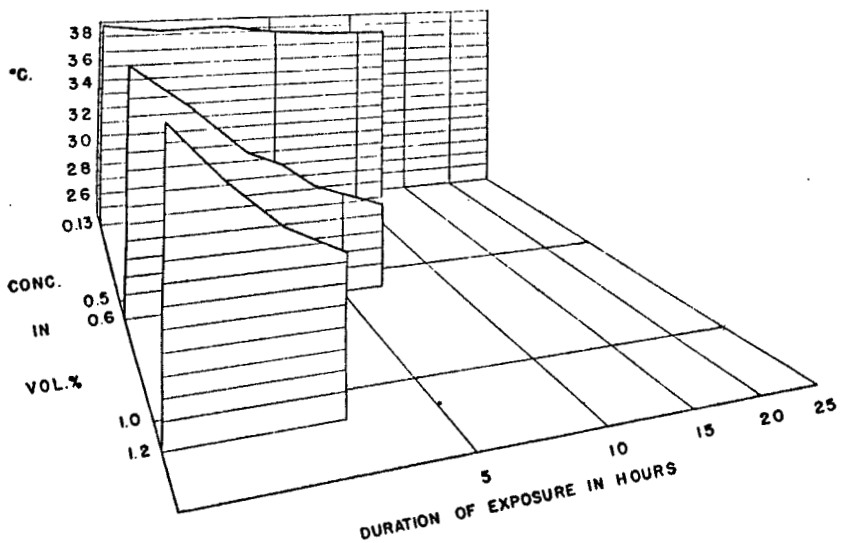

FIGURE 15.

Three-dimensional graph of the rectal temperature during exposure to 0.13, 0.6, and 1.2 percent methyl n-butyl ketone (hexanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

These data are generally corroborative of the observations made by Schrenk, Yant, and Patty (1936b).

5. *Methyl n-amyl ketone (heptanone-2)*.—Exposures were made to 0.15, 0.20, and 0.48 percent heptanone-2 vapor. A protocol of an exposure to 0.20 percent heptanone-2 vapor is presented here to illustrate the detailed behavior of the test animal.

The concentration of heptanone-2 vapor was adjusted to approximately 0.2 percent. Ten female guinea pigs were introduced into the chamber via the air lock. Their normal rectal temperature was 40.4° C., the respiratory rate was 116 per minute, and the pulse rate was 350 per minute. The temperature of

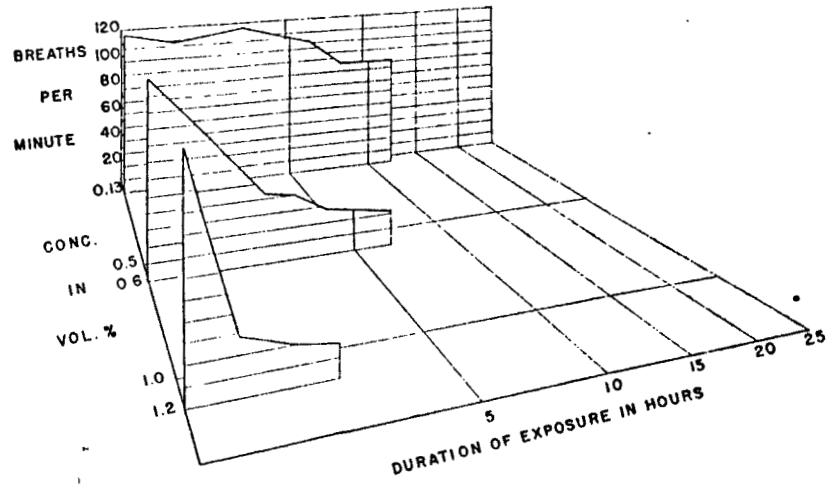

FIGURE 16.

Three-dimensional graph of the respiratory rate during exposure to 0.13, 0.6, and 1.2 percent methyl n-butyl ketone (hexanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

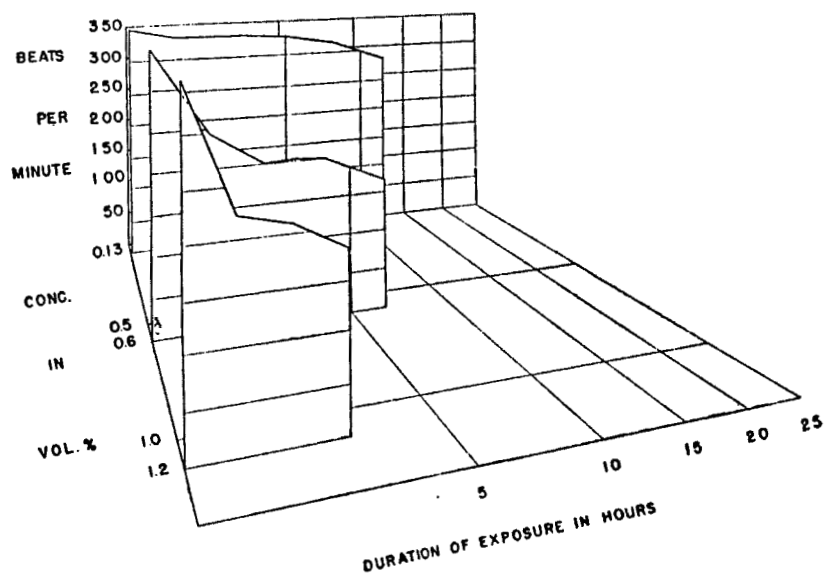

FIGURE 17.

Three-dimensional graph of the heart rate during exposure to 0.13, 0.6, and 1.2 percent methyl n-butyl ketone (hexanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

the chamber was maintained at 25.0 ± 0.5° C. throughout the experiment. One pig did not have an auditory reflex.

Up to 10 minutes.—Some rubbing immediately, a few coughing and all lacrimating, several starting to salivate, all squinting a little, concentration of vapor is 0.18 volumes percent.

45 minutes.—Lacrimation and salivation have stopped, average rectal temperature is 38.8° C., respiratory rate is 77 breaths per minutes, pulse is 327 beats per minute.

180 minutes.—Very little rubbing and no coughing, slight lacrimation in all pigs, three salivating, average rectal temperature is 38.4° C., respiratory rate is 65 breaths per minute, pulse is 322 beats per minute, concentration is 0.20 volumes percent.

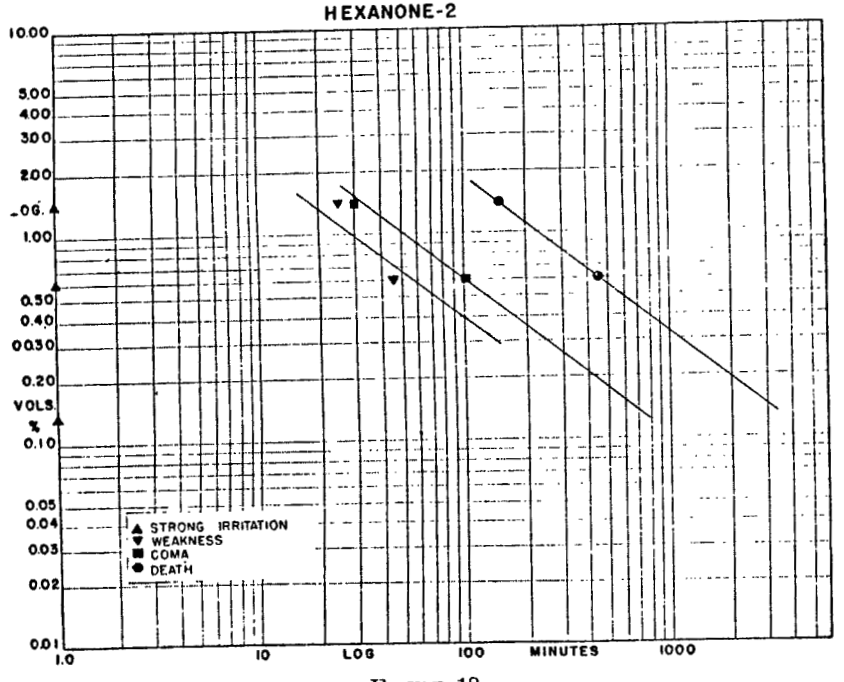

FIGURE 18.

Graph of the average time of onset of irritation, weakness, coma, and death at 0.13, 0.6, and 1.2 percent methyl n-butyl ketone (hexanone-2) vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete reaction of the animal sample.

290 minutes.—Four pigs are salivating, one pig has a watery nasal discharge, there are no other changes. Average rectal temperature is 37.6° C., respiratory rate is 57 breaths per minute, pulse is 314 beats per minute, concentration of vapor is 0.20 volumes percent.

860 minutes.—Three pigs are weak, two on side, three without auditory reflex, one is restless and rubs muzzle, there is no change in lacrimation but six pigs are now salivating, average rectal temperature is 37.2° C., respiratory

860 minutes.—Three pigs are weak, two on side, three without auditory reflex, one without corneal reflex, two without righting reflex, one rubbing muzzle, one making nondirected pawing movements, no change in salivation and lacrimation, average rectal temperature is 35.7° C., respiratory rate is 44 breaths per minute, pulse is 273 beats per minute, concentration of vapor is 0.25 volumes percent.

890 minutes.—All pigs removed from chamber and placed in cages, all recovered by next morning but were in poor condition for three days.

Figures 19, 20, and 21 present the general relations of rectal temperature, respiratory rate, and heart rate, respectively, to the duration of the exposure to, and the concentration of the vapor of methyl n-amyl ketone. As will be discussed in detail below, the depressant action of methyl n-amyl ketone vapor on these functions

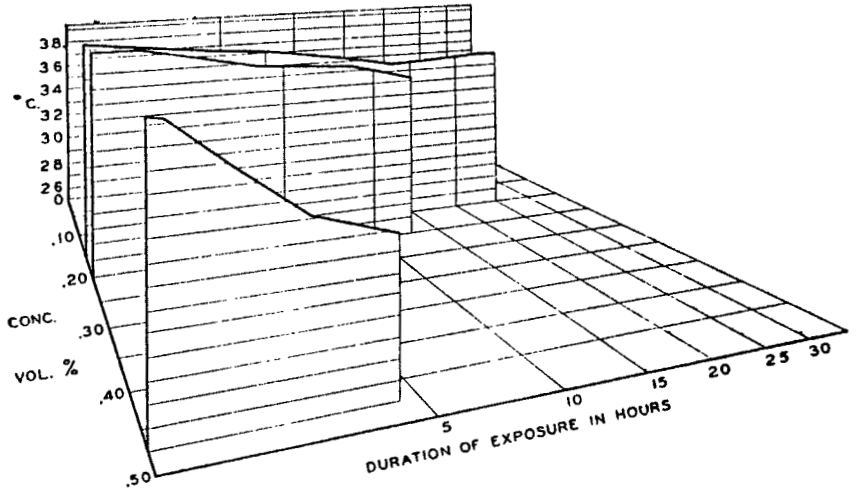

FIGURE 19.

Three-dimensional graph of the rectal temperature during exposure to 0.15, 0.20, and 0.48 percent methyl n-amyl ketone (heptanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

is markedly greater with comparable concentration than for the vapors of the preceding ketones. This may be seen by comparing the appropriate figures: 19 with 15, 11, 7, 3; 20 with 16, 12, 8, 4; and 21 with 17, 13, 9, and 5.

The time-concentration factors for the several standard reactions are smaller than is the case in any of the preceding ketones. Due to the very low vapor tension of this ketone, effective concentrations are not readily obtained. The lack of definite reactions precludes the construction of a bilogarithmic chart. The only qualitative reaction observed to be positive at all the concentrations studied is that

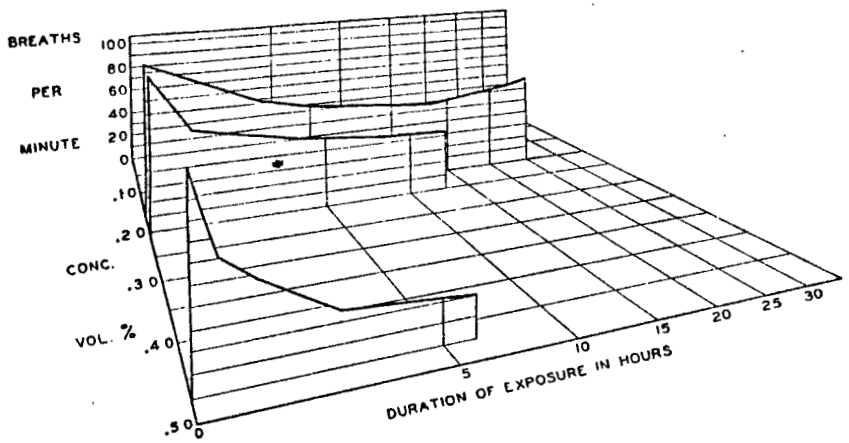

FIGURE 20.

Three-dimensional graph of the respiratory rate during exposure to 0.15, 0.20, and 0.48 percent methyl n-amyl ketone (heptanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

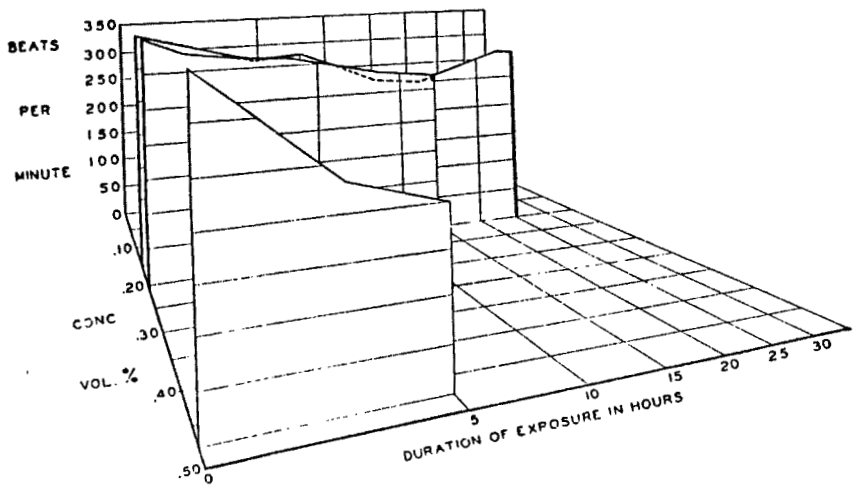

FIGURE 21.

Three-dimensional graph of the heart rate during exposure to 0.15, 0.20, and 0.48 percent methyl n-amyl ketone (heptanone-2) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

of immediate strong irritation. At 0.5 percent vapor weakness appeared at 47 minutes, and at 0.2 percent at 900 minutes. Coma and some deaths occurred at 0.5 percent vapor at 120 and 280 minutes, respectively. The preceding member of the series (methyl n-butyl ketone) is similarly effective as an irritant.

6. *Methyl n-hexyl ketone* (*octanone-2*).—Due to the extremely low vapor pressure of this ketone exposures were made only to saturated atmospheres. The protocol of the longer of two exposures to a saturated vapor, the data of which agree very closely, is given here:

A saturated atmosphere of octanone-2 was prepared by evaporating the ketone from a large surface in the circulating air stream. Concentration of the vapor was 0.13 percent by volume. The temperature remained at 25° C.±1°.

Ten female guinea pigs were placed in the chamber via the air lock aft[illegible] their normal rectal temperature, respiratory rate, and heart rate had b[illegible] recorded. These were, respectively, 38.8° C., 102 per minute, and 329 p[illegible] minute. One of these animals did not have an auditory response.

Up to five minutes.—Occasional rubbing of muzzle, all lacrimating and some are squinting.

60 minutes.—All pigs still up on their feet, no more rubbing, five have stopped lacrimating, four have started salivating, four others are showing a watery discharge from the nose, a slight squinting in all pigs. Average rectal temperature is 38.8° C., respiratory rate is 67 per minute, and heart rate is 313 per minute.

185 minutes.—Four pigs lacrimating, seven salivating, nasal discharge disappearing. Average rectal temperature is 38.4° C., respiratory rate is 62 per minute, heart rate is 293 per minute.

300 minutes.—Five pigs are lacrimating, five are salivating, nasal discharge has stopped, squinting is also not noticeable. Average rectal temperature is 37.9° C., respiratory rate is 49 per minute, heart rate is 300 per minute.

420 minutes.—Five pigs are down, a few are still rubbing, seven are lacrimating, and seven are salivating. Average rectal temperature is 37.4° C., respiratory rate is 57 per minute, heart rate is 272 per minute.

590 minutes.—Eight pigs are down, two are on their sides, one of these shows no response to manual righting, one pig is making pawing movements, one pig has no corneal reflex, lacrimation and salivation unchanged. Average rectal temperature is 36.3° C., respiratory rate is 55 per minute, heart rate is 266 per minute.

820 minutes.—All pigs are down and five of them are on their sides. Auditory reflex still patent in all but one, no corneal reflex in four pigs, one rubbing occasionally, only three pigs lacrimating and salivating. Average rec[illegible] temperature is 35.0° C., respiratory rate is 47 per minute, heart rate is [illegible] minute.

845 minutes.—All pigs removed from the chamber and placed in [illegible] cages. Recovery was slow.

Next morning all pigs but one recovered their usual equilibrium. The exception was found to have a large cyst on the pelvis and was killed after several days. The other pigs showed normal weight gain through the next few months.

Figure 22 presents the quantitative data of the two exposures made to octanone-2 vapor. The points of the two exposures are supple-

mentary since the concentrations were identical. Since only one concentration was used the three-dimensional type of presentation is not practical, and all three types of data are plotted on the same axes using different scales on the ordinate. The course of the exposure indicates a rather slow decline in all of the functions, but respiration shows an initial drop as is characteristic of irritative vapors in the preceding ketones.

The qualitative data obtained with octanone-2 indicate that this ketone has a depressant strength as great or greater than the preceding ketones. The irritation occurred immediately and muscular weakness and coma appeared at 620 and 720 minutes, respectively.

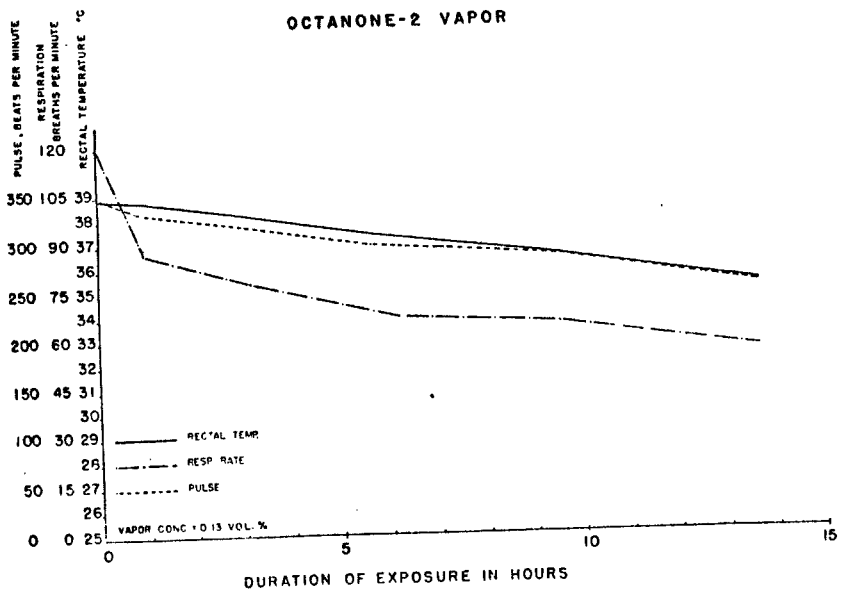

FIGURE 22.

Composite two-dimensional graph of the rectal temperature, respiratory rate, and heart rate during exposure to 0.13 percent methyl n-hexyl ketone (octanone-2) vapor. The time scale is in arithmetic units. Each curve starts from its own control level. The respective scale for each curve is found along the ordinate.

C. DISCUSSION OF ACUTE EFFECTS OF THE INHALATION OF THE VAPORS OF STRAIGHT-CHAIN METHYL KETONES

In the preceding section the depressant action of ketones on the various bodily functions of guinea pigs was demonstrated. It must be emphasized that these reactions are recorded only after a lapse of five or ten minutes, and that, consequently, immediate effects which cannot be observed without manipulating the animals may pass unnoticed.

Although, as was pointed out before, the ketones used were of the best commercial grade, there is a possibility that some of the reactions observed may have been influenced by impurities.[3] For this reason comparisons between experiments on the same substances are necessarily more strictly significant than those made between experiments with different ketones.

In the following section the observed relations of narcotic agent and function will be discussed with the findings of other workers.

By referring to the figures illustrating the behavior of the respiration (4, 8, 12, 16, 20, and 22) it will be seen that in several curves there is a tendency of the respiratory rate to return toward the normal value after the exposure has proceeded for at least one hour. In each case, however, the rate is finally depressed if the exposure is carried to the point of coma. This type of curve is coincident with strong irritation of the naso-pharyngeal area caused by the vapor. This effect can, however, not always be observed with high concentrations of vapor. diPrisco (1938) has shown that acetone inhalation results in apnea, caused by reflex stimulation in response to irritation of the nasal mucosa.[4]

There are, therefore, two mechanisms which may act to depress the respiratory rate: inhibition by reflexes and by direct depression of the respiratory center. Thus, with nonirritating narcotic vapors there would be a steady decline of the respiratory rate due to depression of the central nervous system;[5] with irritating nonnarcotic vapors there would be an abrupt initial depression, due to a nasal reflex; with moderately narcotic irritant vapors there would be an abrupt initial depression with a tendency to disappear as the reflex sensory endings became depressed; and with vapors that are strongly narcotic and irritant there would be an abrupt initial depression of the rate which is followed by a gradual decline from this level to the point of failure.

The strictly narcotic effect expected of the low concentrations and the purely irritant effect are not apparent in these experiments, presumably because there is always some narcosis and irritation. The effect of moderately narcotic irritant vapor is illustrated in figure 4, most markedly at 5 percent, in figure 8 at 1 percent, in figure 12 at

[3] The presence of such substances in the straight-chain methyl ketones is not indicated from the boiling point determinations. In the case of acetone no difference [illegible] could be detected between the effect of the purest product obtainable and a [illegible] grade which was free of methanol. See also Salant and Kleitman (1922 [illegible]

[4] Sercer (1936) found that there is a bilateral reflex arc from the nasal [illegible] the respiratory muscles by way of the ramifications of the second branch of the [illegible] nerve to the respiratory center, the intercostals, the phrenic, and the pneumogastric nerves. He states that bronchial contraction results from the irritation of the nasal mucosa.

[5] It is possible that a preliminary stage of excitation or "release" phenomena may occur, as has been found in other experimental intercessions in the central nervous system. diPrisco (1938) observed such a stage of hyperfunction.

1 percent, and in figure 16 at 0.13 percent. The characteristic of this condition is the hump in the record after the exposure has progressed for some time. The effect of predominantly narcotic irritant vapors is illustrated in the remaining figures and in the curves for the high vapor concentrations in the figures mentioned above. In these cases, when the nasal mucosa becomes anaesthetized, central narcosis follows the initial depression so rapidly that no opportunity for the rate to return toward the normal value for respiration occurs.

The literature on the narcotic action of ketones refers mainly to acetone. The following observations have the most direct bearing on our findings, although the conditions of the experiments often diverge widely from the procedure employed here.

The effect of the inhalation of ketones on the respiration has been observed by Albanese and Barabini (1892), Kagan (1924), Bourne (1926), Patty, Schrenk, and Yant (1935, 1936a, 1936b), and di Prisco (1938) to cause one or more of the responses to be listed, in the following order: apnea, polypnea, hyperpnea, dyspnea, and hypopnea. In general, these authors found that the effectiveness of ketones in producing these five types of response depended considerably on the concentration in which the administration was made and also on the types of ketones that were used. In comparison to other narcotic agents they judged the ketones to be relatively ineffective.

The mode of administration of the ketone alters to some extent the observed responses. That is, if ketone is given by oral (Albertoni 1884, and Salant and Kleitman 1922) or intravenous (Salant and Kleitman 1921–22, Schlomovitz and Seybold 1924, and Walton, Kehr, and Loevenhart 1928) administration the reflex depression and apnea seen in the inhalation experiments may not appear at all or be delayed until ketone is present in the exhaled air. Hypodermic administration (Rhamy 1912) or absorption through the skin (Cossman 1903) produces effects which are more marked than with other modes of administration, due in part to the drastic effects on the tissues at the site of injection and the high concentration of the ketone. Thus, there is observed twitching of the skin, face, and limbs which indicates secondary effects which may reflexly influence the respiration.

This résumé indicates that the different phases of the respiratory response to the vapors of the straight-chain methyl ketones have been observed by different experimenters. Although the data presented on the response of guinea pigs to vapors of straight-chain methyl ketones do not show any increase of the rate of respiration, its occurrence cannot be excluded because it may have preceded the period of observation. It is also possible that such effects could be averaged out when the observations on a number of animals are combined and it is [illegible] of these values may miss tran-

sient changes. Inspection of the individual records, however, of the guinea pigs does not afford any signs of increased respiratory rate.

The curves of the reaction of the respiration to irritant ketone vapors afford an index which may prove to be of practical value in defining safe limits of vapor concentration. It is apparent that those points at which the hump occurs in the curves mark a concentration-time value at which the irritant action of the ketone has succumbed to its narcotic action on the nasal mucosa. These points, therefore, define the limits of concentration and exposure where warning properties are no longer adequate to protect the organism.

The figures illustrating the behavior of the heart rate (5, 9, 13, 17, 21, and 22) indicate that, as with the respiratory rate, there is a response in accord with that expected when there are two factors tending cause depression: First, a transitory depression from reflex stimula by way of the vagus, and second, a general decline due to cen narcosis.

This is in general agreement with the observations made by other investigators with different procedures. The effect of ketone inhalation on the behavior of the heart has been shown by Caccuri (1937) to be a reflexly induced bradycardia. He stated that the mode of administration modified this behavior since oral or intravenous administration had little effect on the heart rate. Nevertheless intravenous injection (Salant and Kleitman 1921–22, Schlomovitz and Seybold 1924, and Walton, Kehr, and Loevenhart 1928) has also been observed to depress the heart rate and oral administration (Albertoni 1884) to accelerate it. In each case there was observed a decrease in blood pressure. Most investigators have indicated that the effect on the heart rate is variable and find that it may vary with the phase of narcosis. Preparations of surviving heart tissue (dog) were shown by Bagoury (1935) to relax in direct proportion to the concentration of perfused acetone, but the rate of contraction was relatively little affected except during active dilatation.

The figures illustrating the behavior of the rectal temperature (3, 7, 11, 15, 19, and 22) indicate that the decline in body temperature during exposure to the vapors of ketones is regular and proportional to both concentration and duration of exposure. Relatively few investigators have taken temperature measurements, except incidentally, during such exposures. Such reports were made by Albertoni (1884), Cossman (1903), and Tenconi (1931) who noted that the body temperature was reduced slightly.

The figures illustrating the qualitative data (6, 10, 14, and 18) show that there is a general relation between weakness, coma, and death; and concentration and time of exposure. These data do not show that this is a constant relationship. It will be observed that the [illegible] various groups of

symptoms and having the same slope do not always follow the experimentally determined points. It is probable that each symptom complex has its own functional relation to concentration and time. The closely related signs of weakness and coma always fall close together, while points of death and of strong irritation are less regularly placed with respect to the two former reactions and each other. Strong irritation appears to bear no regular relation to any of the other signs, although it precedes them in every case but one.[6]

In the literature numerous references may be found to the narcotic action of ketones, particularly acetone. The reports on the narcotic strength of the latter vary considerably, although it is generally judged the least potent of all the ketones, see Walton, Kehr, and Loevenhart (1928), and Davis (1930), large quantities of which can be given without serious consequences. Albertoni (1884), Albanese and Barabini (1892), in studying the effect of a series of ketones as anesthetics, claimed that the narcotic or anesthetic property of symmetrical ketones increased from methyl to ethyl but fell from ethyl to propyl. With corresponding phenyl ketones they found the activity to decrease with the increase in the number of carbon atoms in the molecule, but it appears that the order varies with different species of animals. The depressant action of acetone on the central nervous system has also been investigated by Cossman (1903), Rhamy (1912), Schlomovitz and Seybold (1924), and Bourne (1926). Schulze (1933) tested the narcotic effect of acetone on mice and cats in a wide range of concentrations with methods and criteria differing from those used here. Comparison of the reactions of different species is difficult but it is apparent that his cats were affected sooner than the guinea pigs reported here. Patty, Schrenk, and Yant found in their several papers (1935, 1936a, 1936b) on ketones essentially the same conditions for narcosis in methyl ethyl, methyl propyl, and methyl butyl ketones as observed here.

In order to evaluate the relation between the physiological activity and the physio-chemical data of the ketones it appears necessary to adopt a unit of effectiveness. This "effectiveness coefficient" may be based either on time or concentration.

The time of onset of coma, the condition characterized by inactivity and a prone position with corneal and auditory reflexes just extinguished, furnishes one reference point for such evaluation. The reciprocal of the times of onset of coma in the several ketones forms a sequence of values (see table IV 1/T) which increases proportionately with the number of carbon atoms in the molecule. It is conversely possible to establish such a criterion by determining the

[6] In this instance the criterion was copious secretion from the conjunctival and nasal mucosae and this may not be strictly comparable to the condition in which active

Table IV.—*NARCOTIC EFFECTIVENESS compared to PHYSICAL DATA of Acetone and its methyl homologs.*

Ketone	Narcotic effectiveness				Partition coefficients, oil over water					Surface tension dynes per cm.[1]		Dipole moment x10 [18]†	Solubility	
	(Absolute)		(Relative)		Olive oil	Cotton-seed oil	Lard oil	Cedar-wood oil	L. Mineral oil	25° C.	20° C.[2]		Ket. H_2O[4]	H_2O Ket.[4]
	1/T	1/C	1/C	Depr.										
Di-methyl	0.0005	0.1723	1.0	1.0	0.11	0.16	0.16	0.12	0.03	22.78	23.70	2.71	99.9	99.9
Methyl ethyl	0.0055	0.6670	3.9	4.9	1.87	1.70	2.39	2.19	0.82	24.50	24.60	2.79	25.6	11.88
Methyl propyl	0.0065	0.7130	4.2	7.9	16.58	9.04	7.66	11.55	11.30	24.57	25.20	2.72	0.54	3.34
Methyl n-butyl	0.0244	1.6670	9.8	13.9	26.52	18.32	8.95	30.18	15.72	25.50	25.39	2.73	0.22	2.14
Methyl n-amyl	0.0713	2.0000	11.8	12.0	42.16	26.90	18.29	30.18	49.20	26.35	----------	2.57	0.40	1.40
Methyl n-hexyl	0.1250	3.3300	19.5	20.8	59.00	30.50	17.00	30.18	40.80	27.33	26.79	2.70	0.09	0.60

[1] The data on surface tension were obtained in this laboratory by comparing the number of drops of water to that of ketone which fell from a Traube stalagmometer in the vapor above the solvent. The value of surface tension for water was taken as 71.97 dynes per cm. The calculation was made as recommended by Howard and Sollmann (1924).
[2] International Critical Tables.
†Smyth, C. P. (1931).
[4] Doolittle, A. K. (1935).

concentration of vapor necessary to produce coma in a standard time interval (see table IV, 1/C). It is further possible to make such a sequence of narcotic values from the data on the depression of the rectal temperature, the respiratory rate, and the heart rate in the several ketones (see table IV, Depr.). Assuming that acetone has a

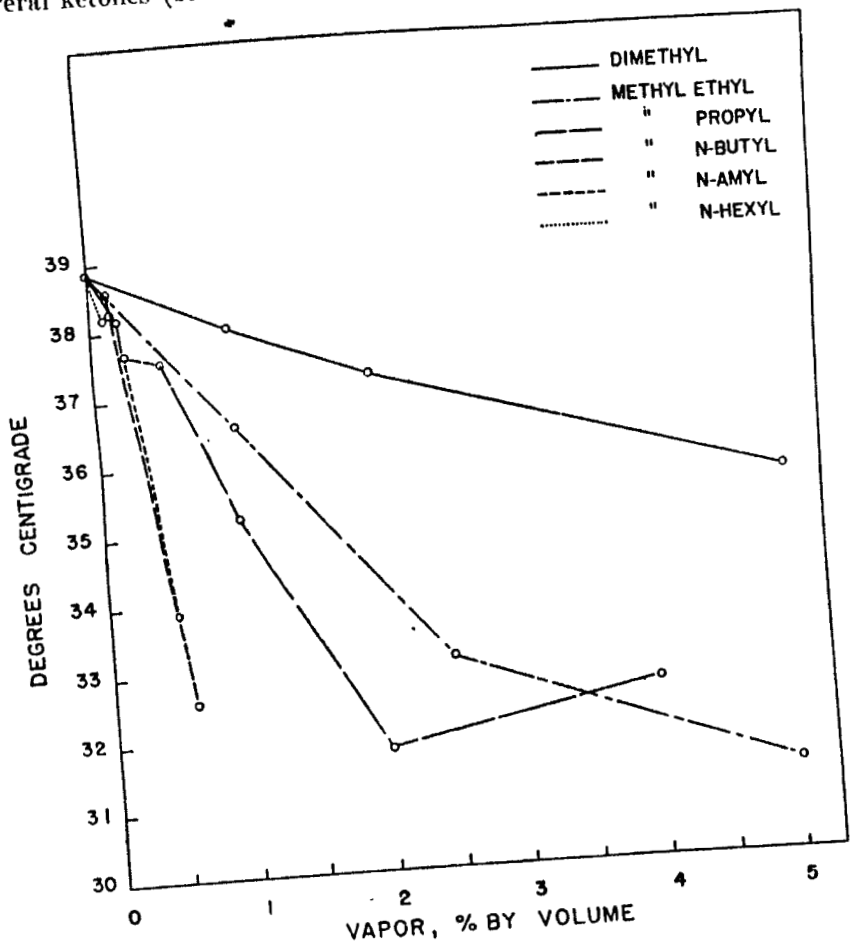

FIGURE 23.

Rectal temperature of guinea pigs after 3 hours' exposure to the different concentrations of dimethyl, methyl ethyl, methyl propyl, methyl n-butyl, methyl n-amyl, and methyl n-hexyl ketone vapor.

narcotic effectiveness equal to one, the relative effectiveness of the homologs may be obtained by dividing through the sequence with the acetone value. It can be seen (table IV, 1/C and Depr.) that a rough correspondence exists between the two methods.

Disregarding transient phenomena, the effect of the inhalation of [illegible] nervous system. This depres-

sion is characteristic of the ketone and increases from dimethyl to methyl hexyl ketone as is shown in table IV, narcotic effectiveness. In figure 23 this relation is expressed for the depression of the rectal temperature after three hours of exposure to various concentrations

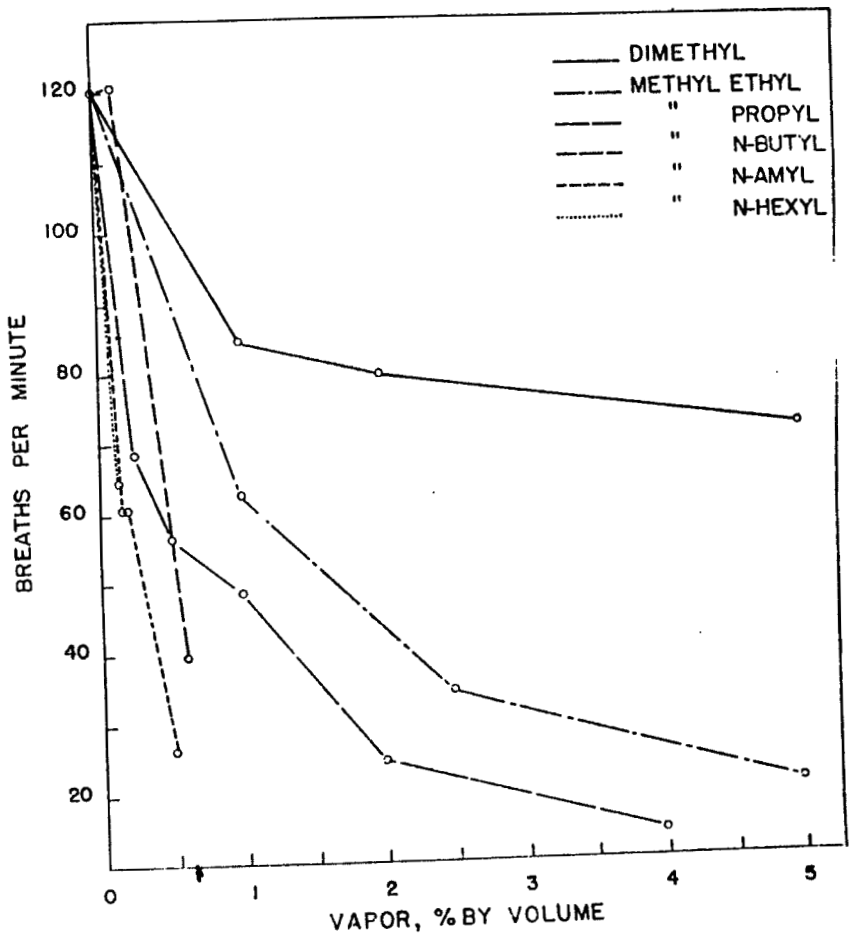

FIGURE 24.

Respiratory rate of guinea pigs after 3 hours' exposure to the different concentrations of dimethyl, methyl ethyl, methyl propyl, methyl n-butyl, methyl n-amyl, and methyl n-hexyl ketone vapor.

of the ketones. Figures 24 and 25 present similar material [illegible] data on respiratory rate and heart rate, respectively.[7]

From these figures it may be seen that the higher concentrations of the more volatile ketones have a smaller effect than expected from

[7] It is possible to make such a graph for any time interval. That at 3 hours was chosen as representative of the whole exposure.

a direct proportionality to the concentration of the ketones in the atmosphere. This is especially true of the data on the respiratory rate, where a considerable departure from straight-line relations is evident. This phenomenon may illustrate that the concentration of the ketone in the tissues is not in equilibrium with that of the inhaled a Cushny (1910), Widmark (1920), and Briggs and Schaffer

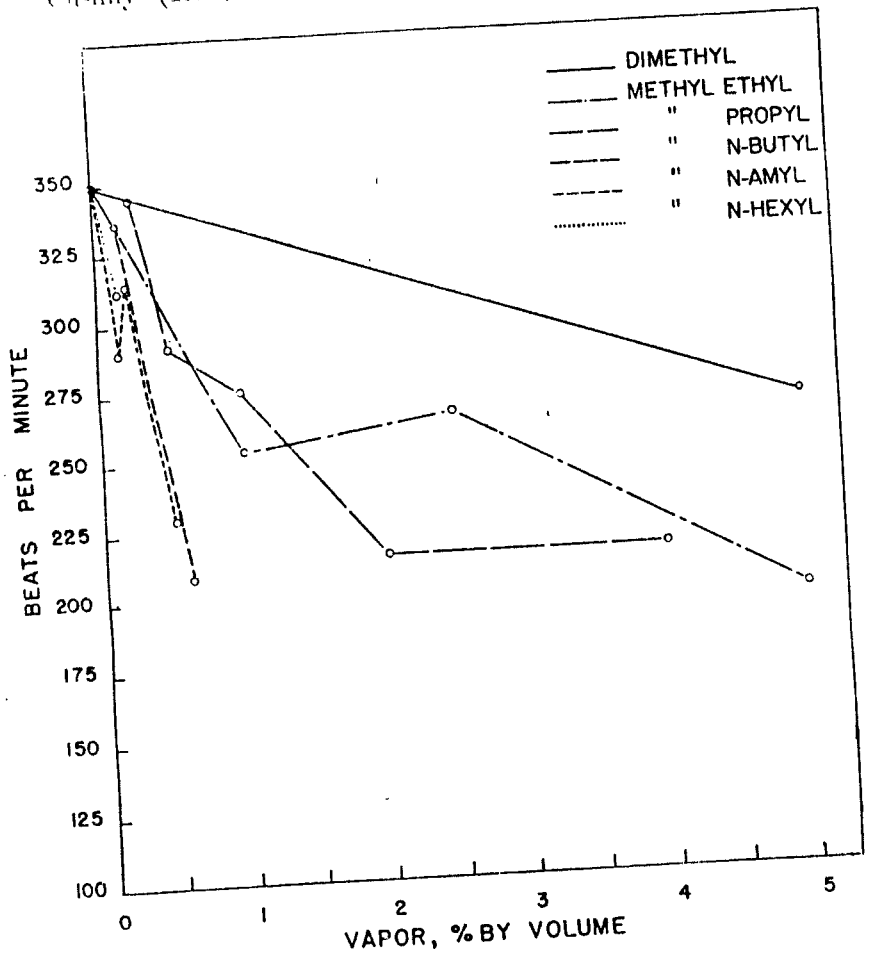

FIGURE 25.

Heart rate of guinea pigs after 3 hours' exposure to the different concentrations of dimethyl, methyl ethyl, methyl propyl, methyl n-butyl, methyl n-amyl, and methyl n-hexyl ketone vapor.

(1921) have shown that the rate at which these substances are absorbed in the living organism depends on simple diffusion. They find that the partition coefficient of acetone in blood over acetone in air is about 337 at body temperature. It is obvious that this coefficient favors the accumulation of ketone in the blood even when it is present in the inhaled atmosphere in low concentrations. The occurrence of such accumulation has been pointed out by Specht, Miller, and Valaer (1939). Archangelsky (1901) found that following the inhalation of acetone the blood of rabbits attains about half the concentration of acetone found in the brain tissue, the liver acetone concentration being intermediate. The quantity of ketone which is absorbed by the organism, however, may be limited by a number of secondary factors, some of which have been described in the foregoing experimental observations, i. e., changes in ventilation rate, changes in body temperature, changes in the rate of circulation of the blood, etc. For these reasons equilibrium is not readily attained in the organism and this fact alone limits seriously the analysis of the physiological data along physical lines.

Many investigators have attempted to correlate the physical properties of the narcotic agents with the observed narcotic effectiveness. Winterstein (1926) has set forth very extensively and clearly the various theories of the mechanism of narcosis and the empirical relations which have been found between narcotic strength and physical data. In the following section the assembled physical data for the ketones used in these studies are presented to acquaint the casual reader with the actual numerical sequences of these data as compared to the narcotic effectiveness.

The effectiveness coefficient (table IV 1/C) as determined by the concentration which induces coma in a standard interval (100 minutes) has been chosen as least arbitrary for comparisons with the physical properties because it obviates uncontrollable factors, such as lack of food and water which vary with time. It will be seen from table IV that the four- and five-carbon ketones are very similar in narcotic action, and, as shown in figure 26, this is concomitant with a similar relation of the surface tensions. It is not reflected in the solubility or the partition coefficients as seen in table IV.

This irregularity of the effect in surface tension and narcotic value is not necessarily that of the pure ketone, but may be characteristic of the particular grade of ketone used in these experiments, in spite of the fact that it is not reflected in the relation of the partition coefficients of the same products.

Winterstein (1926) states that surface energy seems to be the most important single factor which influences the narcotic activity of a compound. He feels that the physical properties assigned by earlier workers as measures of the narcotic action, such as solubility in oil over water, surface tension in air, etc., are only of indirect significance, except insofar as they parallel the adsorbability. Regular relationships will be observed only in limited conditions, such as in homologous series of compounds.

Observations made by Warburg (1921) on the inhibition of the *n vitro* oxidation of cystine by narcotics, including two ketones, eem to show that the adsorbability on charcoal is an index of the nhibiting power of the compound. His mathematical formulation of these relations would tend to make the adsorption coefficient and the molecular cross-section define the concentration for equivalent narcotic action of any compound. It is impossible to test this formulation strictly, because the coefficient of adsorption on the particular cell constituents that are responsible for narcosis must be known to make the calculations. The literature contains only the charcoal

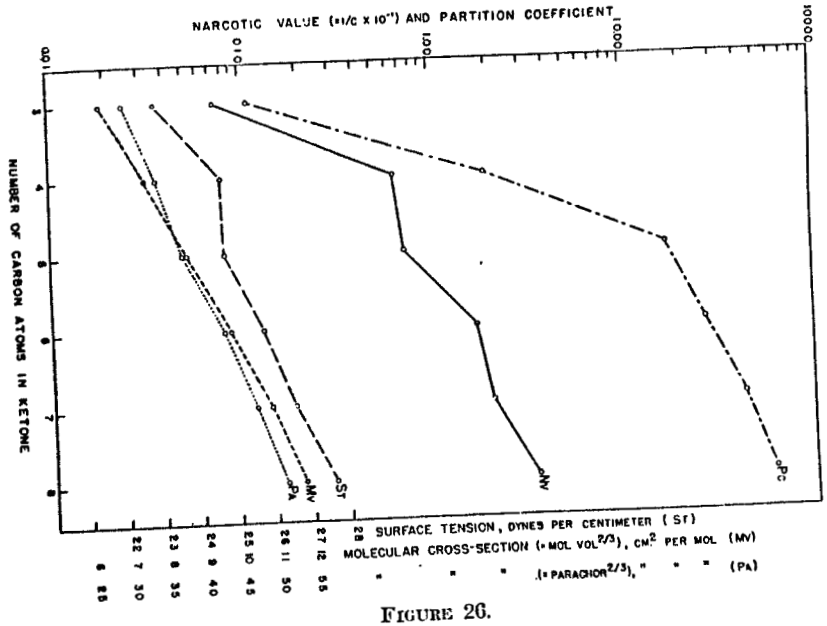

FIGURE 26.

Composite graph of narcotic value, partition coefficient, surface tension, and two relative measures of molecular cross-section; the ⅔ power of the molecular volume and the ⅔ power of the parachor of Sugden,[8] of the several straight-chain methyl ketones plotted in order of increasing number of carbon atoms contained in their molecules. The appropriate scales for the various physical data are given on the logarithmic and arithmetic (left and right, respectively) ordinates.

adsorption data for acetone and methyl ethyl ketone, and therefore the calculation of a constant through Warburg's formula must be deferred.

From Winterstein's (1926) review of the literature, it seems well established that, within homologous series of compounds, the narcotic

[8] The parachor is calculated from the formula of Sugden given in Lange (1934). It is slightly in error because the surface tensions were taken at 25° C., while the other data were taken at 20° C.

effectiveness increases with the length of the carbon chain. Thus, any other property which is based on dimensions of the molecule will tend to change in the same direction as the narcotic effectiveness. (See figure 27.) These relations are corroborated in tables I and IV in a longer sequence of ketones than has been tested heretofore. Albanese and Barabini (1892) indicate a lack of regularity in the narcotic properties of the three symmetrical ketones and phenyl homologs which they tested. With a small series, however, the sequential arrangement may be easily destroyed by variations which may be expected from biological material. In addition, physico-chemical factors may preclude a strict sequential regularity between the physical properties. Thus, Pearce and Hanson (1935) find that adsorption characteristics of ketones are straight-line functions only at fairly low pressures of the solvent, and above these pressures the behavior is relatively unpredictable.

D. SUMMARY OF THE ACUTE RESPONSE TO THE INHALATION OF VAPORS OF STRAIGHT-CHAIN METHYL KETONES

1. The inhalation of straight-chain methyl ketone vapors by guinea pigs produces a progressive general narcosis which is characterized by a depression of body temperature, respiratory rate, and heart rate, as well as the abolition of the corneal, auditory, and equilibratory reflexes.

2. The depression of the various bodily functions is proportional to the concentration of the inhaled vapor and the duration of the exposure, within certain limits.

3. This depression varies with the homologous methyl ketones, the number of carbon atoms in the molecule, the partition coefficient of the ketone between oil and water, the reciprocal of the vapor pressure, the reciprocal of the solubility in water, the surface tension, the molecular volume, and other related molecular constants. The best proportionality from the data at hand is with the partition coefficient of ketones between oil and water.

4. Reflex effects are observed on the respiration and the heart rate in irritating concentrations of these compounds. This phenomenon furnishes an index of the limits of adequate warning properties during inhalation of these vapors.

V. INVESTIGATION OF DIFFERENT SIX-CARBON KETONES

A. CONTROLS. See IV. A.

B. RESULTS OF EXPERIMENTS WITH DIFFERENT SIX-CARBON KETONES.

1. *Acetonylacetone.*—Exposures were made only to a saturated vapor of acetonylacetone because of its low vapor pressure. A protocol of the exposure to 0.04 percent acetonylacetone, the maximum concentration attainable at room temperature, is presented here for details of the animal reactions.

The vapor of acetonylacetone was prepared by recirculating the air in the glass observation chamber over the evaporating surface until a constant level was reached. The temperature was 25°±1° C. Ten female guinea pigs were checked for rectal temperature, respiratory rate, and pulse, and then introduced into the vapor-air mixture through the air lock. The average value for rectal temperature was 39.2° C., for respiratory rate was 87.7 breaths per minute, and for heart rate was 328 beats per minute.

Up [illegible] minutes.—All pigs are rubbing nose and eyes, all are lacrimating, none [illegible]ing. No signs of restlessness except as occasioned by rubbing. Some [illegible] are squinting.

90 minutes.—Infrequent rubbing. Lacrimation is stopping. A watery discharge from the nose of all but three pigs. Rectal temperature, 38.6° C., respiration is 78.4 per minute, and pulse is 325 per minute.

245 minutes.—No change in symptoms. Nasal discharge still evident. Rectal temperature is 38.5° C., respiration is 73.2 per minute, pulse is 330 per minute.

500 minutes.—No change in symptoms. Rectal temperature is 38.4° C., respiration is 64.4 per minute, and pulse is 319 per minute.

752 minutes.—All reflexes patent, all pigs quiet with occasional rubbing. One pig still has signs of lacrimation. No salivation. Three pigs no longer have the watery nasal discharge. Four pigs have lost the squint. Rectal temperature is 38.5° C., respiration is 62.6 per minute, and pulse is 327 per minute.

775 minutes.—All pigs taken out of chamber. No signs of even light narcosis, but animals are quiet when handled. No autopsies were made.

These animals all lived for at least 3 months after this exposure, eight were used for other experimental purposes after about 7 months.

Figure 27 illustrates, graphically, the data given in the preceding protocol and shows that although rectal temperature and heart rate were maintained at a level very near that of the normal value, the respiratory rate fell off continually with the duration of the exposure. Symptomatically only very slight signs of the effect of the vapor could be seen. The gradual cessation of signs of irritation are not indicative, necessarily, of narcosis of the sensory endings of the naso-pharyngeal mucosae since no marked reflex inhibition seems to be [illegible]sent.

The qualitative data were so negative that a time concentration [illegible] was not feasible in this instance. Irritation was the only sign [illegible] was elicited.

2. *Methyl iso-butyl ketone.* In a previous paper from this laboratory, Specht (1938), the details of acute effects on guinea pigs of the inhalation of the vapors of methyl iso-butyl ketone have been presented. A protocol at an exposure to a vapor concentration of 1.68 volume percent is given here:

One hundred and twenty-five cubic centimeters of methyl iso-butyl ketone were evaporated in the 1-cubic-meter chamber. The chamber temperature was 25.7° C., rising to 26.3° C. at the end of the exposure. Ten female guinea pigs were placed in the chamber through the air lock and a sample of the vapor air mixture withdrawn (1.81 volume percent). The normal rectal temperature taken immediately before exposure was 38.9° C. and the respiratory rate at rest was 117 breaths per minute.

Up to 1 minute.—Immediately upon introduction to the test atmosphere the pigs began to squint and rub their noses and eyes violently. Copious lacrimination occurred and sneezing and coughing was very frequent.

2 minutes.—Salivation was evident by the wet area about the mouth, neck, and brisket.

10 minutes.—All pigs were so weak that they could not remain erect; other symptoms in proportion.

15 minutes.—All pigs over on side making only feeble movements at most and usually quite inert. Corneal reflex still present. Pinnal flick in response to sharp click still present. (Auditory reflex.)

25 minutes.—Respiratory rate has dropped to 35 per minute, rectal temperature to 36.6° C.

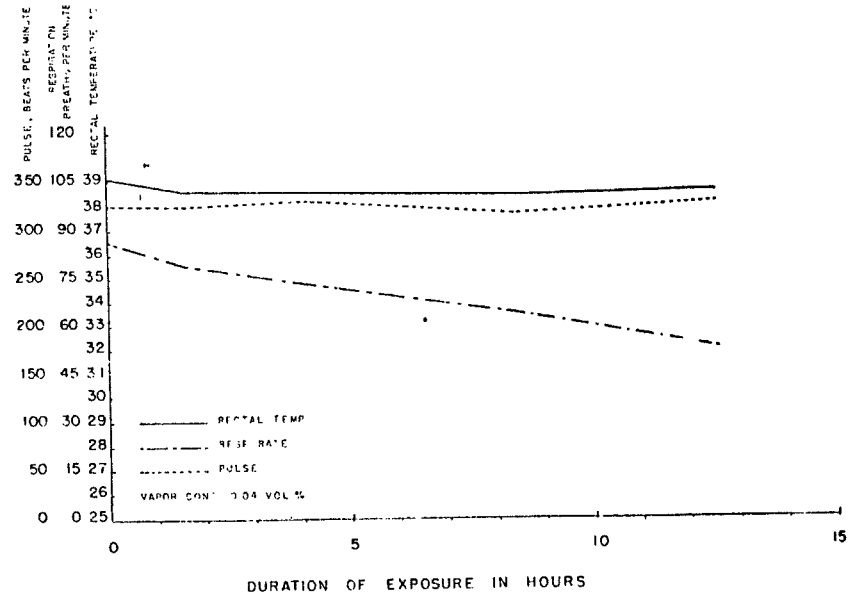

FIGURE 27.

Composite two-dimensional graph of the rectal temperature, respiratory rate, and heart rate during exposure to 0.04 percent acetonylacetone (hexandione-2,5) vapor. The time scale is in arithmetic units. Each curve starts from its own control level. The respective scales for each curve are found along the ordinate.

40 minutes.—Pulse by palpitation estimated at 240 beats per minute. Auditory and corneal reflexes gone. Respiration gasping and random pawing movements appear occasionally.

60 minutes.—Rectal temperature 34.7° C. Pulse 181 beats per minute. Concentration 1.70 volume percent.

85 minutes.—First death. Average temperature of survivors 33.4° C., pulse 143 beats per minute, respiratory rate 20 per minute.

Deaths (1) at 94, (1) at 108, and (2) at 125 minutes.

125 minutes.—Average rectal temperature of survivors 32° C., pulse 155 beats per minute, respiratory rate 85 per minute.

Deaths (2) at 131, and (1) at 134 minutes.

142 minutes.—Ninth death. Concentration 1.53 volume percent.

204 minutes—Chamber opened, one pig still gasping 100 breaths per minute, pulse 158 beats per minute, rectal temperature 30.4° C. Death occurred at least 2 hours after the end of the exposure.

It will be sufficient to recapitulate here the main findings as they relate to the other ketones and then to present the data in the three dimensional graphs as in the first section of this bulletin.

It was found that methyl iso-butyl ketone vapor inhalation caused irritation of the conjunctiva and nasal mucosa in man below concentrations of 0.1 percent, although this concentration was tolerated by guinea pigs. At higher concentrations marked irritation is exhibited by guinea pigs after about two hours or less, depending on the

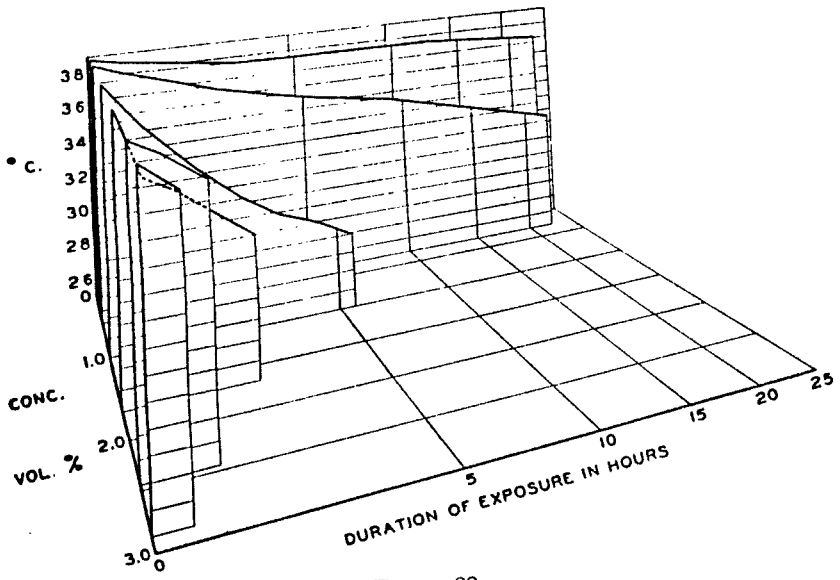

FIGURE 28.

Three-dimensional graph of the rectal temperature during exposure to 0.3, 1.0, 1.7, 2.5, and 3.0 percent 4-methyl pentanone-2 (methyl iso-butyl ketone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

concentration. Above 1.0 percent irritation is immediate for guinea pigs, as judged by the excessive lacrimation and salivation. A progressive narcosis occurs, causing depression of the body temperature, respiratory rate, and heart rate. A loss of static control, consciousness, and the deeper reflexes follows. Death finally ensues at 1.0 percent vapor in about 4 hours and in proportionately shorter periods at higher concentrations. Despite the very active effects of this solvent complete recovery was effected in those animals removed before terminal conditions had set in. The pathology is discussed in a later section.

In figures 28, 29, and 30 are presented the data on rectal temperature, respiratory rate, and heart rate by means of three-dimen-

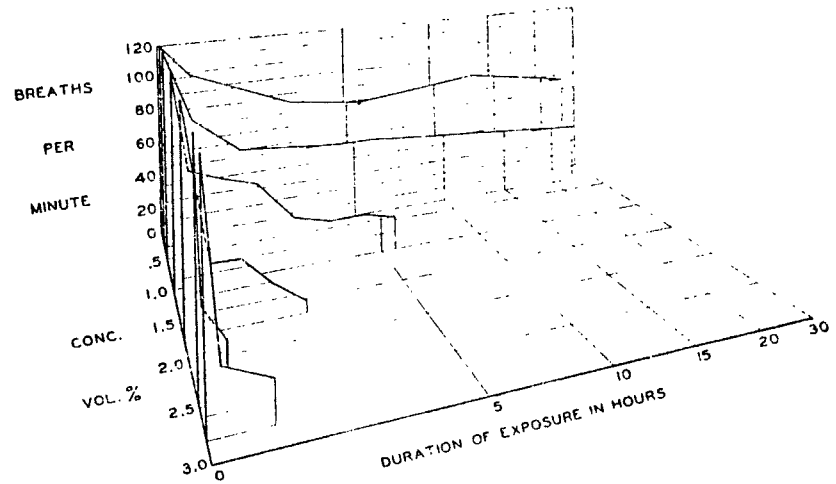

FIGURE 29.

Three-dimensional graph of the respiratory rate during exposure to 0.3, 1.0, 1.7, 2.5, and 3.0 percent 4-methyl pentanone-2 (methyl iso-butyl ketone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

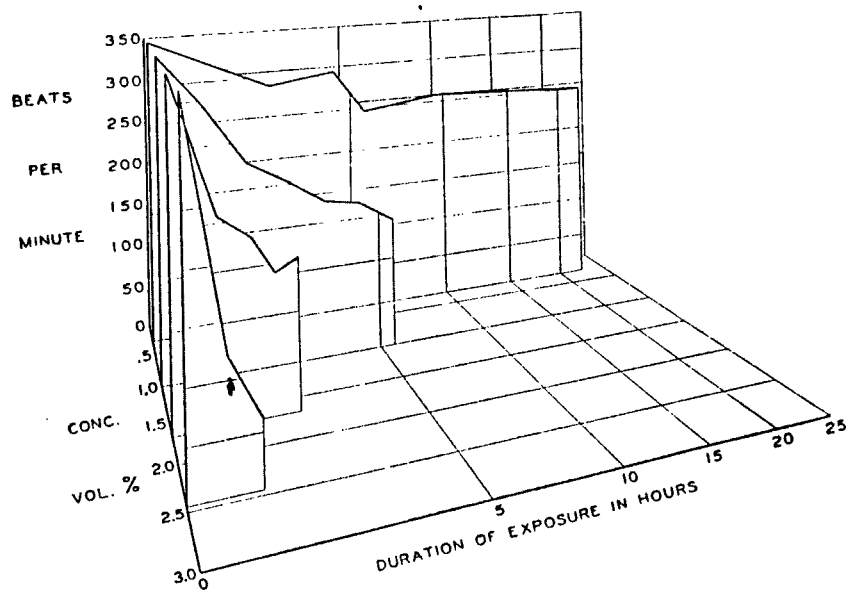

FIGURE 30.

Three-dimensional graph of the heart rate during exposure to 0.3, 1.0, 1.7, 2.5, and 3.0 percent 4-methyl pentanone-2 (methyl iso-butyl ketone) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

sional graphs. This type of presentation brings out in the two latter functions the reflex action of the irritant vapor as outlined in the section on the homologs of acetone. Figure 29 illustrates the respiratory rate and shows not only the marked depressant effect of this ketone vapor but also the tendency to return to the normal value when the sensory endings have become completely anaesthetized, i. e., abolition of the inhibition which resulted from irritation of the nasopharyngeal mucosa.

Figure 31 presents the qualitative data as recast on a slightly different scale than in the original publication. The data are from

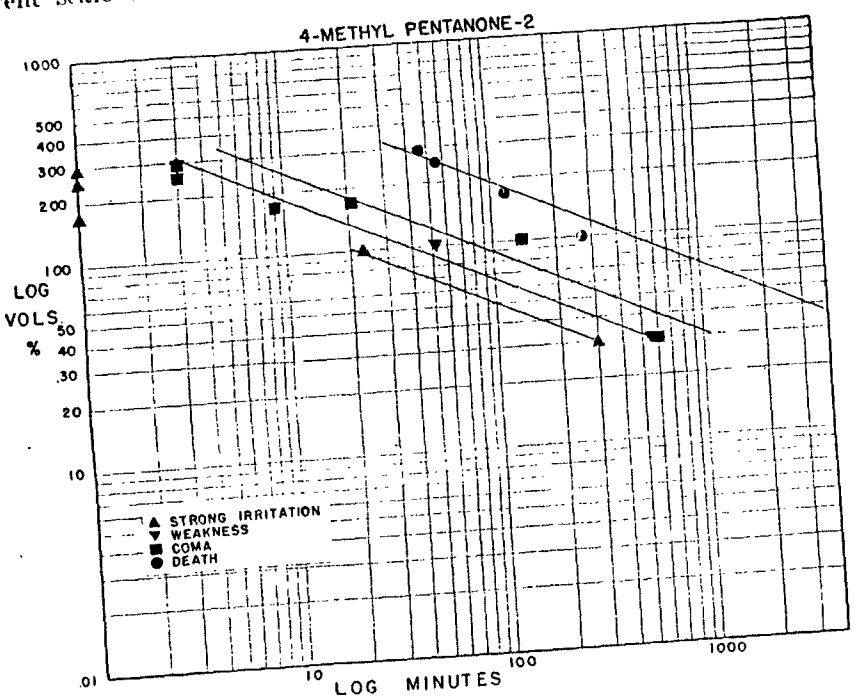

FIGURE 31.

Graph of the average time of onset of irritation, weakness, coma, and death at 0.3, 1.0, 1.7, 2.5, and 3.0 percent 4-methyl pentanone-2 (methyl iso-butyl ketone vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete reaction of the animal sample.

the same experiments. They indicate a rather effective vapor as judged by the action of acetone and particularly the isomer hexanone-2.

3. *Cyclohexanone.*—The exposure to cyclohexanone vapor was made in a similar manner to that of acetonylacetone due to the relatively low vapor pressure.

The protocol of the exposure to 0.4 percent cyclohexanone vapor is presented here for details of the animal reactions.

The vapor was prepared by recirculating the air in the glass chamber over an evaporating surface until a stable level was attained at 0.4 percent. Ten female guinea pigs were checked for normal rectal temperature, respiratory rate, and heart rate; 38.5° C., 90 per minute, and 321 per minute, respectively. They were then introduced into the vapor through the air lock of the glass chamber. The chamber temperature was maintained at 25° ± 1° C.

Up to 10 minutes.—All pigs are somewhat restless, no rubbing of nose or eyes. A slight nasal discharge (watery) in several of the pigs. All are lacrimating, salivating, and squinting.

30 minutes.—All pigs are still a little restless. There is occasional rubbing now. Nasal discharge in only two pigs. All are squinting and lacrimating, salivation has stopped in two pigs. Average rectal temperature is 38.3° C., respiratory rate is 46 per minute, and pulse is 348 per minute.

90 minutes.—Some restlessness, no rubbing, and nasal discharge in only one pig. All squinting and lacrimating. Salivation is disappearing. Average rectal temperature is 37.7° C., respiratory rate is 52 per minute, and the pulse is 319 per minute.

210 minutes.—Three pigs are down, and one of them is on its side. Four have lost the response to auditory stimuli. The righting reflex is disappearing in one pig. There is occasional rubbing. No more nasal discharge evident, squinting about the same. Lacrimation in only three pigs, salivation in six. Average rectal temperature is 36.5° C., respiratory rate is 44 per minute, and pulse is 291 per minute.

330 minutes.—All pigs are down and over on their sides. No auditory response in any pigs. Corneal reflexes still patent. Righting reflex poor in all. Occasional attempts at rubbing. One pig still lacrimating. No salivation. Average rectal temperature is 34.5° C., respiratory rate is 40 per minute, and pulse is 230 per minute.

355 minutes.—All pigs removed from the chamber. Recovery rather slow. Narcosis seems to be protracted despite removal from vapor.

Next day all pigs showed marked corneal opacity and very sluggish general movement. There was evidence of considerable pilomotor activity in many pigs. Considerable dyspnea appeared, persisted for several days. Three pigs died four days after exposure. The rest recovered in several weeks, the eyes clearing after other activity had become normal.

Figure 32 presents the data for respiratory rate, rectal temperature, and heart rate in a composite two-dimensional graph. It is apparent that the pulse was accelerated initially, although it later was depressed as were the rectal temperature and respiratory rate. This is the only instance in which such a marked acceleration has appeared in these exposures. The rectal temperature shows a rather rapid progressive decline for such a low concentration of vapor. The respiratory rate shows the reflex effect of the irritation of the nasal mucosa as described previously. It is interesting that the heart and respiration show opposite reactions in this case, since the reflex mechanism of both are closely allied. It is probable, judging from the accessory data on the reactions after the exposure, that there is considerable effect other than narcosis from this solvent.

The qualitative data taken at this one vapor concentration indicate that, although no deaths occurred during the exposure, it seems probable that besides the serious after effects this ketone has considerable narcotic effect.

4. *Mesityl oxide.*—Exposures to 0.2, 0.5, and 1.0 percent mesityl oxide vapor were made at 25° C. The protocol of the exposure to 0.5 percent vapor is given here to present the details of the animal reactions.

The concentration of mesityl oxide was adjusted in the chamber until it approximated 0.5 volumes percent. The temperature was maintained at 25° ±1° C. Ten female guinea pigs were placed in the chamber via the air lock. The

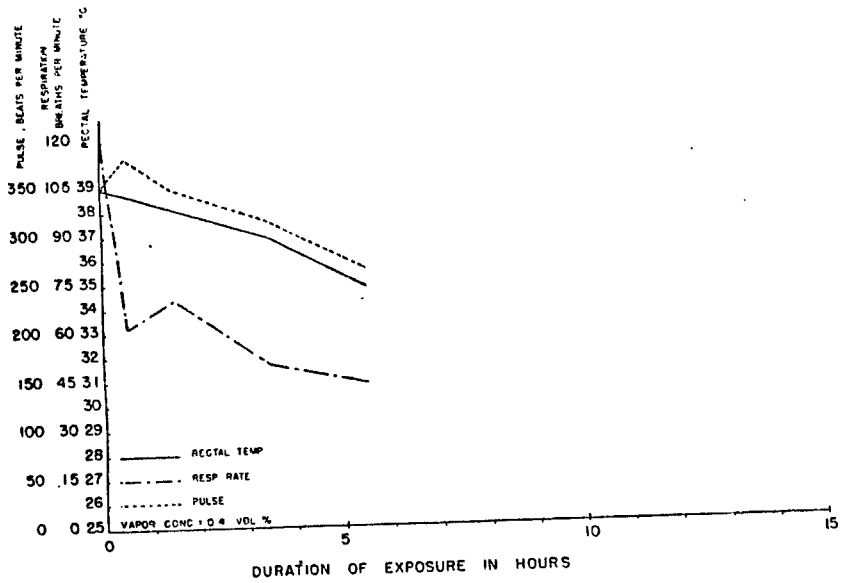

FIGURE 32.

Composite two-dimensional graph of rectal temperature, respiratory rate, and heart rate during exposure to 0.4 percent cyclohexanone vapor. The time scale is in arithmetic units. Each curve starts from its own control level. The respective scales for each curve are found along the ordinate.

average normal rectal temperature, respiratory rate, and pulse were, respectively: 39.5° C., 105 per minute, and 362 per minute.

Up to 5 minutes—Positive auditory, corneal, and righting reflexes, vigorous rubbing of nose and eyes, squinting and coughing. Active salivation and lacrimation began almost immediately.

30 minutes—Three pigs do not give auditory reflex response, all have positive corneal reflex and are able to maintain their balance, all very restless, and rubbing face and eyes, all squinting, some coughing, all lacrimating, all salivating, and four producing a foamy saliva not seen with other ketones, a nasal discharge of rather fluid mucous also appeared in all pigs. Average rectal temperature is 39.4° C., respiratory rate is 80 per minute, pulse is 367 per

60 minutes—No patent change in symptoms. Rectal temperature is 38.9° C., respiratory rate is 72 per minute, pulse is 344 per minute.

120 minutes—Two pigs are showing signs of weakness, and one is comatose, all show positive corneal reflex, almost all are now quiet, no rubbing except occasionally, lacrimation is stopping, but salivation continues, nasal discharge has ceased in all but one pig. Rectal temperature is 38.2° C., respiratory rate is 95 per minute, pulse is 346 per minute.

180 minutes—Two more pigs show weakness, no other changes except signs of cyanosis in the pigs with light-colored pelts.

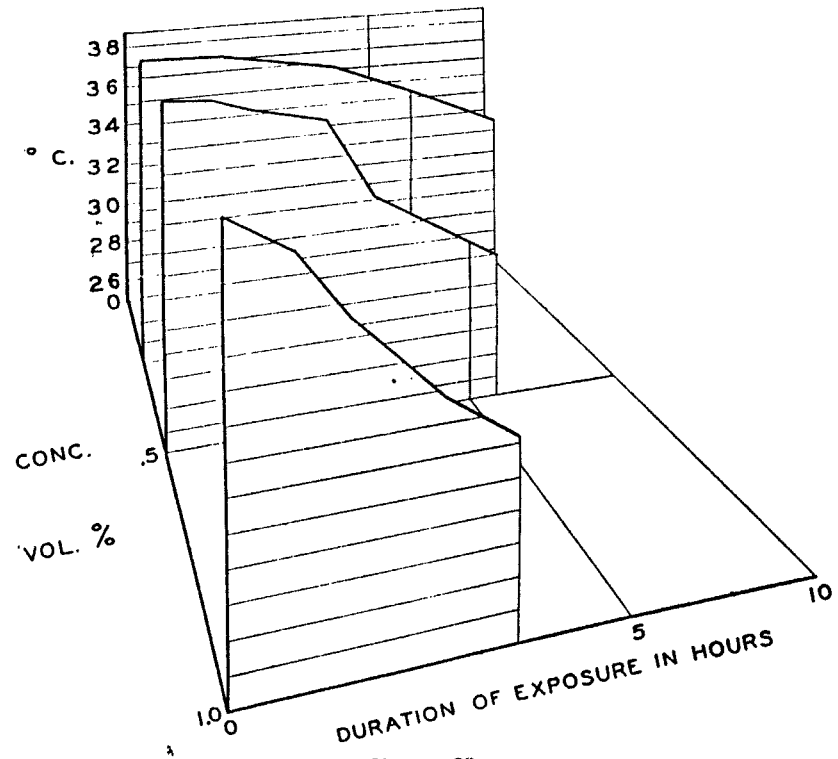

FIGURE 33.

Three-dimensional graph of the rectal temperature during exposure to 0.23, 0.5, and 1.0 percent methyl iso-butenyl ketone (mesityl oxide) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

240 minutes—All pigs are weak, and five are comatose, six have lost auditory reflex, two have lost corneal reflex, three have lost all indication of a righting reflex. Salivation and lacrimation have ceased, one pig shows a bleeding nose. Rectal temperature is 34.9° C., respiratory rate is 67 per minute, pulse is 298 per minute.

390 minutes—Three pigs dead, rest are comatose, four still have a positive auditory reflex, but none has a corneal reflex, no righting reflex evident. Rectal temperature is 31.7° C., respiratory rate is 28 per minute, pulse is 209 per minute.

422 minutes—Three more pigs dead, all others gasping. Removed from the chamber. (None survived for more than about 5 hours subsequent to the exposure.) Autopsies were made of pigs that died during the exposure. The pathology is reported in a subsequent section.

Figures 33, 34, and 35 illustrate the relations of rectal temperature, respiratory rate, and heart rate, respectively, to concentration of the vapor and duration of the exposure. The rectal temperature is seen in figure 33 to fall off progressively with duration of the exposure and proportionately with the increase of concentration, as is characteristic of all the solvent exposures presented in this bulle-

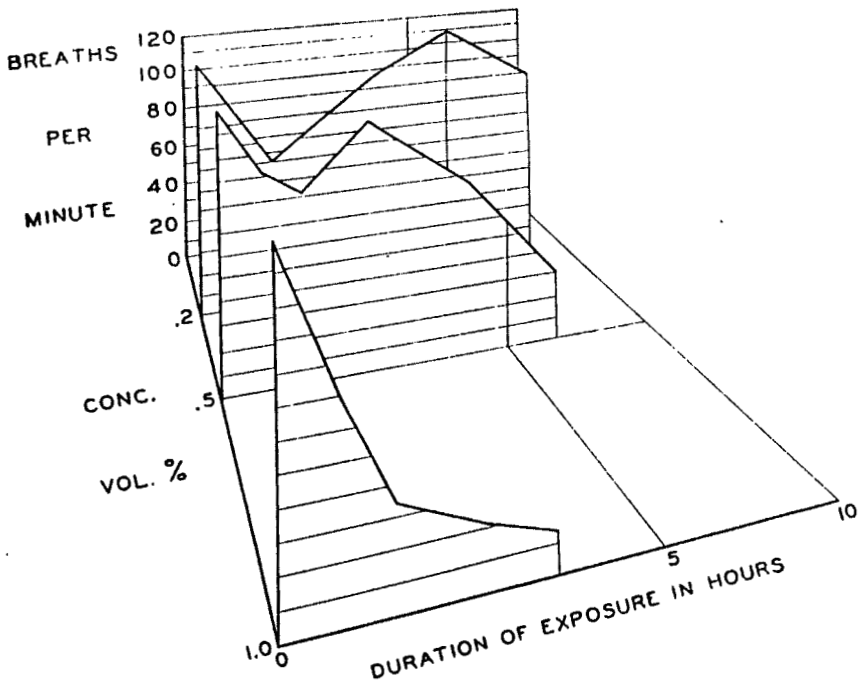

FIGURE 34.

Three-dimensional graph of the respiratory rate during exposure to 0.23, 0.5, and 1.0 percent methyl iso-butenylketone (mesityl oxide) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

tin. In figure 34, however, we have the most marked example of the effect of irritating vapors on the respiration. Here can be seen a transient return beyond the normal value after five hours' exposure to 0.2 percent vapor. At 0.5 percent the return is not as great and the subsequent decline greater than at the lower concentration, while at 1.0 percent the narcotic action is so great that no return to the normal value of respiration is possible. The heart rate, in figure 35,

does not show the response found in the respiration although the depression with the protraction of the exposure is as expected from central narcosis.

From accessory data following the exposures and the fact that in every experiment all the pigs died either on test or within the next 24 hours, it is probable that there are actions on the body tissues which are not necessarily characteristic of a narcotic. The polypnea at the long exposure to the lowest concentration indicates that there is a

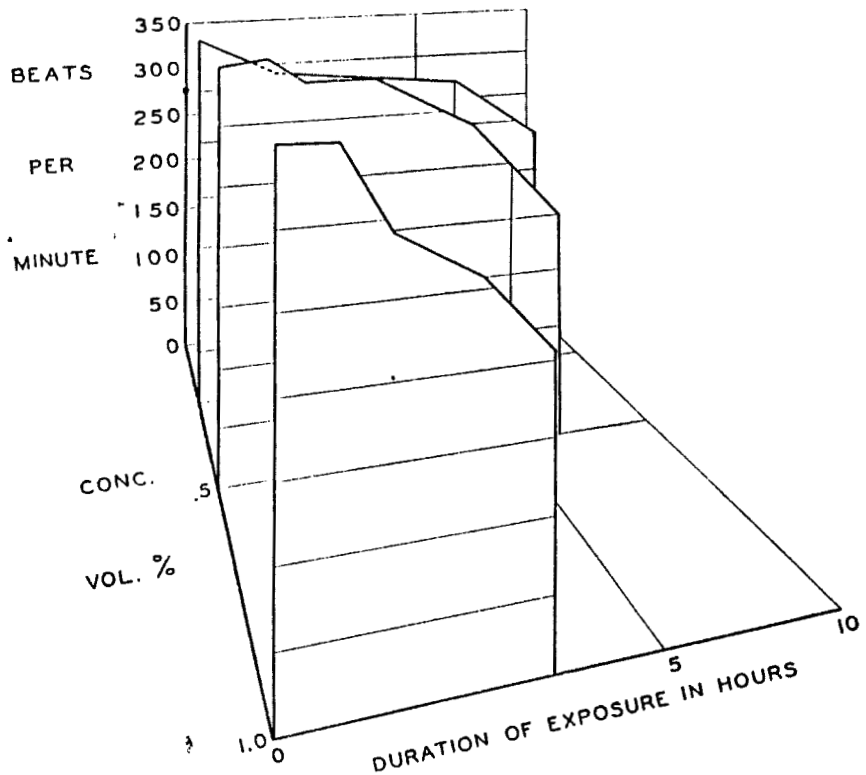

FIGURE 35.

Three-dimensional graph of the heart rate during exposure to 0.23, 0.5, and 1.0 percent methyl iso-butenyl ketone (mesityl oxide) vapor. The time scale is in logarithmically diminishing units. The last grid indicates the average control level.

resultant action of stimulation or release of stimuli and the expected central narcosis. The initially maintained elevation of the heart rate seems to corroborate this view. In any case, the rectal temperature indicates that despite the mixed effects on the respiration and heart there is a marked depression which is common to all the ketones. This supports the data from the qualitative signs, which may be seen

graphically in figure 36. This shows that irritation is immediate even at 0.2 percent mesityl oxide vapor, and that weakness and coma follow later in rapid sequence.

It was noted that, during manipulation of the animals through latex surgeon's gloves while the exposure was going on, considerable irritation of the hands of the operator resulted. The effect was transient. The vapors of mesityl oxide were also noted to attack iron surfaces in the exposure chamber. A rather vile smell resulted from the contact of the vapor with the bodies of the pigs. This odor was even more evident on autopsy. The bodies of pigs that died during

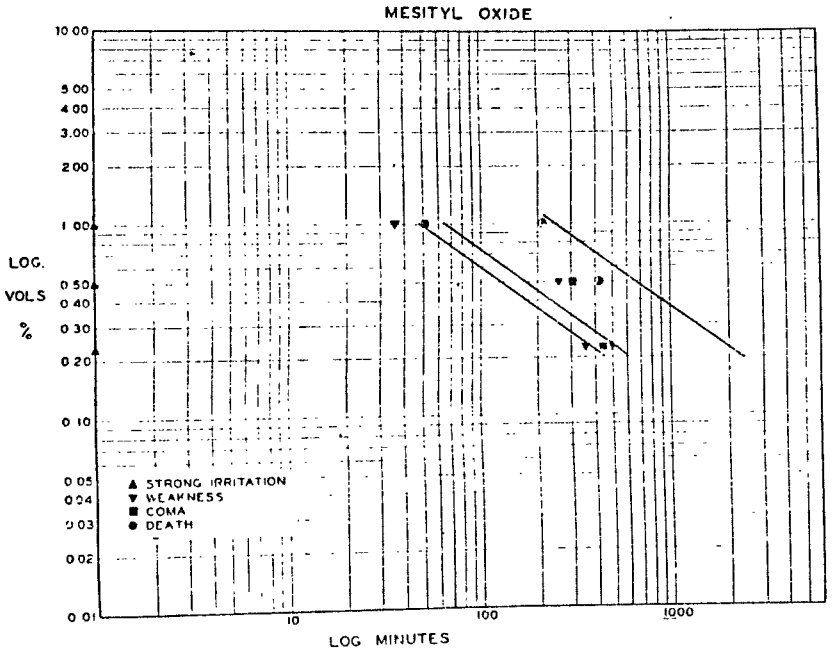

FIGURE 36.

Graph of the average time of onset of irritation, weakness, coma, and death at 0.23, 0.5, and 1.0 percent methyl iso-butenyl ketone (mesityl oxide) vapor. The units are plotted by their logarithms. The lines drawn on the graph indicate only an interpretation of the course of the data. Partially filled points indicate incomplete reaction of the animal sample.

the exposure were distended with gas and fluid which was in the alimentary tract, mainly in the stomach. The bodies of the pigs that died on test became rigid some time before death, resembling a condition seen before only in the case of high concentrations of acetone. Cyanosis was rather marked in the white-pelted guinea pigs but could also be seen in the dark-pelted pigs where the skin was unpigmented.

5. *Methyl n-butyl ketone.*—This six-carbon ketone has been fully discussed in the preceding section on straight chain ketones. The data are given in figures 15, 16, and 17 for rectal temperature, respiratory rate, and heart rate, respectively. Figure 18 gives the time-concentration chart for the qualitative signs of irritation, weakness, coma, and death. It is included here for the sake of comparison with the other six-carbon ketones, particularly its isomer, methyl iso-butyl ketone.

C. DISCUSSION OF THE ACUTE EFFECTS OF THE INHALATION OF VAPORS OF SIX-CARBON KETONES

In the preceding several accounts of the effects of the vapors of six-carbon ketones on the bodily functions of guinea pigs little attempt was made to relate the effect of one vapor to that of another. The assumption that such a relation is possible can only be based on the fact that these compounds have at least two common characteristics and properties: all contain a keto-group and all have the same number of carbon atoms in varying configurations. In view of the other radical differences between the several types of molecules (see table II) there seems to be no inherent reason for assuming any predictable relation between the intensity of the biological effects and their physical properties. It will be noted, however, that each vapor has the property of inducing narcosis with extended exposure, and that each has a depressant effect on the body temperature. The effects on the other functions are variable and will be discussed in the subsequent paragraphs.

The effect of the inhalation of vapors of the six-carbon ketones on the rectal temperatures of guinea pigs is one of progressive depression, as is shown in figures 27, 28, 32, 33, and 15. In the cases where several concentrations of vapor were tested it is seen that the decline in rectal temperature is proportional to the concentration, and in all cases except that of acetonylacetone the decline is proportional to the duration of the exposure. In the excepted case there seems to be a steady level which is about 0.5° C. below the normal rectal temperature. In the case of methyl iso-butyl ketone, figure 28, at high concentrations the rate of decline of rectal temperature appears to be less than at median concentrations of the vapor. This condition is related to the excessive disturbance of the circulatory system, such that the heat exchange between the interior of the body and the surface regions in strongly narcotized animals is very greatly retarded.[9] See figure 30 for the concomitant effects on the heart rate.

[9] Unpublished observations were made during the work on acetone (1939) comparing the decrease in body temperature of freshly killed pigs and those exposed to the various concentrations of vapor. In certain median stages of narcosis body temperature dropped even more rapidly in the surviving pigs than in the killed pigs. The inference seems to be that the circulating blood causes a greater heat exchange than simple conduction and radiation can attain alone.

The effect of the inhalation of vapors of six-carbon ketones on the respiratory rate is somewhat variable, but follows, in general, the course described in the section on the straight-chain ketones. In figure 27, acetonylacetone vapor is shown to depress the respiratory rate a little more rapidly at first than later, and the rate is seen to fall steadily throughout the exposure in contrast to the leveling off of the heart rate and rectal temperature. In figure 29 a striking series of curves illustrates the progressively enhanced reflex depression which occurs initially in response to the nasal irritation from methyl iso-butyl ketone vapor.

Similarly, in figure 32 is shown the reflex depression of the respiratory rate in cyclohexanone vapor. In figure 34 are shown the effects of the various concentrations of mesityl oxide vapor on the respiratory rate. This figure illustrates an unusual condition in that at the lowest concentration the rate rises from the initial depression to a level higher than normal. From general consideration of the accessory data it seems that mesityl oxide induces other changes than those resulting in narcosis.

The effect of the inhalation of vapors of six-carbon ketones on the heart rate is fully as variable as that on the respiratory rate. In figure 27 it can be seen that the vapor of acetonylacetone at saturation does not have any appreciable effect on the heart rate of guinea pigs. In figure 30, however, the vapor of methyl iso-butyl ketone at various concentrations is seen to depress the heart rate in proportion to the concentration and the duration of the exposure. There is no obvious reflex effect of bradycardia in these data, although some indications may be suggested by those at the lowest concentration. The heart rate fell so rapidly at the highest concentration tested that no heart rate counts could be made before the animals began to die. In figure 32 the heart rate in cyclohexanone vapor is seen to rise at first and then fall off. This initial tachycardia is rather unusual in our experiments, but a check on the individual rates shows that it is not a result of averaging in a few aberrant cases. In any case, the heart rate after extended exposure becomes depressed. In figure 35 the vapor of mesityl oxide is seen also to have a variable initial effect on the heart rate, followed by a progressive depression with the protraction of the exposure. At 0.5 percent there is evident a slight tachycardia which is not as great as in the case of cyclohexanone. It is possible that this is due to a few deviating cases. The rate of decline of the pulse is roughly proportional to the concentration of the vapor.

The narcotic properties of these vapors have been shown where feasible. (See figures 31 and 36.) In the case of acetonylacetone there was no evidence of some of the criteria used in making these [illegible] Estimation of the narcotic values as made in the manner

Table V.—*Comparison of the NARCOTIC EFFECTIVENESS and the PHYSICAL DATA of several six-carbon ketones.*

Ketone	Narcotic effectiveness				Partition coefficients, oil over water					Surface tension dynes per cm.[1]		Dipole moment $\times 10^{18}$ ‡	Solubility	
	(Absolute)		(Relative)											
	1/T	1/C [2]	1/C	Depr.	Olive oil	Cotton-seed oil	Lard oil	Cedar-wood oil	L. Min. oil	25° C.	20° C.[3]		Ket. H_2O [4]	H_2O Ket.[4]
Methyl n-butyl	0.0244	1.667	9.67	10.60	26.320	18.320	8.95	30.18	15.720	25.50	25.39	2.73	0.22	2.14
Methyl isobutyl	0.0077	0.910	5.28	14.20	20.640	24.000			26.900	23.64			1.91	2.61
Methyl isobutenyl	0.0200	1.423	8.29	6.93	4.550	4.670			3.060	28.95	27.0 28.30		2.89	2.85
Acetonylacetone	0.0057	0.667	3.87	23.09	0.056	0.050			0.025	35.62			99.99	99.99
Cyclohexanone	0.0100	0.714	4.15	5.23	24.090	10.410			7.660	34.55		2.75	0.13	8.68

[1] The data on surface tension were obtained in this laboratory by comparing the number of drops of water to that of ketone which fell from a Traube stalagmometer in the vapor above the solvent. The value of surface tension for water was taken as 71.97 dynes per cm. The calculation was made as recommended by Howard and Sollmann (1921).
[2] International Critical Tables.
‡ Smyth, C. P. (1931).
[4] Doolittle, A. K. (1935).

described in the discussion on the straight-chain methyl ketones shows that the order of increasing narcotic strength is, respectively, acetonylacetone, cyclohexanone, methyl iso-butyl ketone, mesityl oxide, and methyl n-butyl ketone. (See table V.)

It is apparent that the appreciable difference in narcotic strength between the two isomers, methyl n-butyl and methyl iso-butyl ketone accompanies a rather slight structural difference. Winterstein (1926) reports that the greater the amount of branching of a molecule the less its narcotic effect.

The lack of methyl groups, as in cyclohexanone, is accompanied by a lower narcotic strength, although not as low as going to the next shorter carbon chain, as in methyl propyl ketone.

Mesityl oxide, although it has a branched methyl structure, is nevertheless a stronger narcotic than methyl n-butyl ketone. The presence of the double bond as in ethylene compared to ethane has been shown by Winterstein (1926) to be accompanied by a greater narcotic effect than with single bond linkage. These effects may be attributed to changes in the adsorption coefficient, as defined by Langmuir (1917) and Freundlich (1926) to be dependent on the residual valencies, rather than on changes in the covering power or space dimensions of the molecule. Data on adsorption are not available to make this comparison.

The presence of two keto- groups, as in acetonylacetone, is accompanied by a relatively weak narcotic strength. It is comparable to that of the second lower straight-chain member, methyl ethyl ketone, but is not as low as that of acetone. The estimation of the narcotic value of this ketone is at best somewhat difficult through the fact that the effects are so slight and the working concentration so low that the establishment of a point on the concentration-time chart for qualificative signs is not feasible. The value finally arrived at is one which resulted from approximations taking into account all the data.

If the amount of depression of the different functions which were quantitatively measured be estimated at some particular concentration of vapor after a set period of exposure another order of effectiveness may be determined for each of the three types of data. It will be seen from a study of figures 37, 38, and 39 that the depression of rectal temperature, respiratory rate, and pulse, respectively, have no similarity and are not similar to the narcotic criterion of time of onset of coma. This is in distinct contrast to the condition of the straight-chain methyl ketones (figures 24, 25, and 26), where only minor irregularity exists.

It is seen, further, from figures 37, 38, and 39, that the amount of depression of the various functions is nearly linearly proportional to the concentration. This is true also of the comparable range of concentrations in the straight-chain methyl ketones. Comparison

at higher concentrations is, of course, impossible due to the low vapor tensions of these substances.

Attempts to relate the narcotic value to the various physical properties have not been satisfactory except in the case of the partition coefficient. In Tables II and V, it is seen that even this relation is not without irregularity, the value of mesityl oxide being aberrant in the

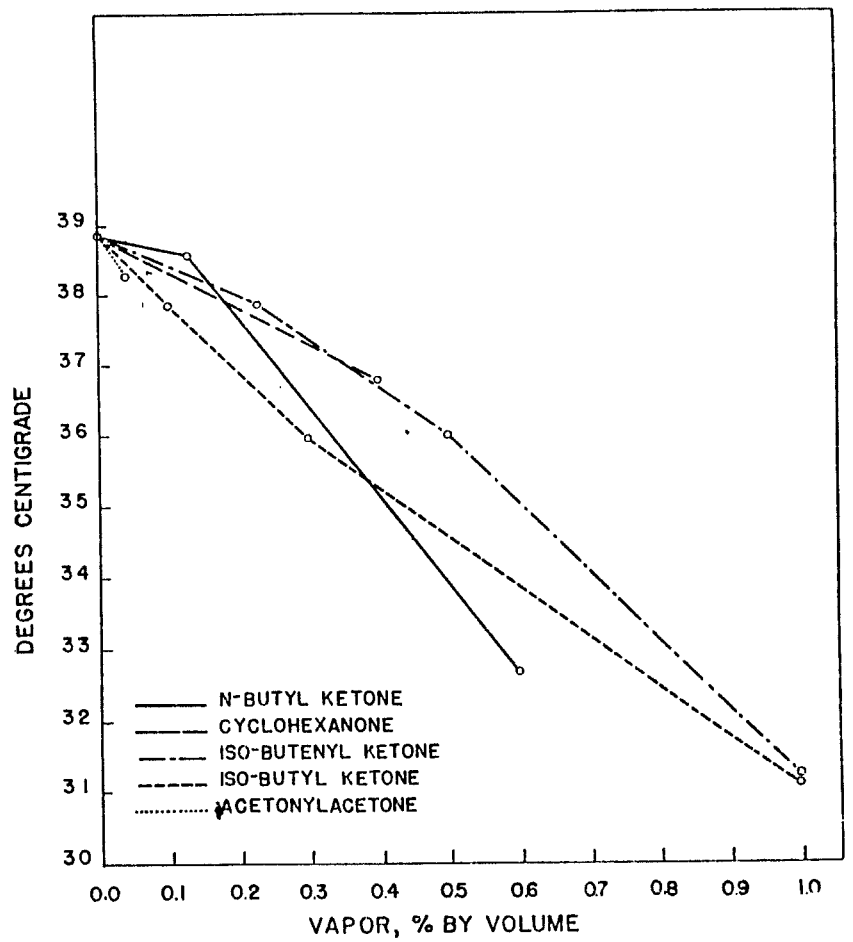

FIGURE 37.

Rectal temperature of guinea pigs after 3 hours' exposure to the different concentrations of acetonylacetone, methyl isobutyl ketone, cyclohexanone, methyl iso-butenyl ketone, and methyl n-butyl ketone vapor.

coefficient for olive oil over water, even less sequential similarity being shown by the other partition coefficients. Surface tension, as shown in Table V, does not seem to have any regular relation to the narcotic values or the solubility. No sequential relations seem to exist between these data and the values for the narcotic strength.

Thus, the closest index to the narcotic strength is the partition coefficient of olive oil over water with mesityl oxide showing a non-sequential position.

It is possible that the abnormal activity of mesityl oxide, in relation to its partition coefficient, may arise from its reaction products in the body. Schwarz (1898) has shown that the higher ketones of

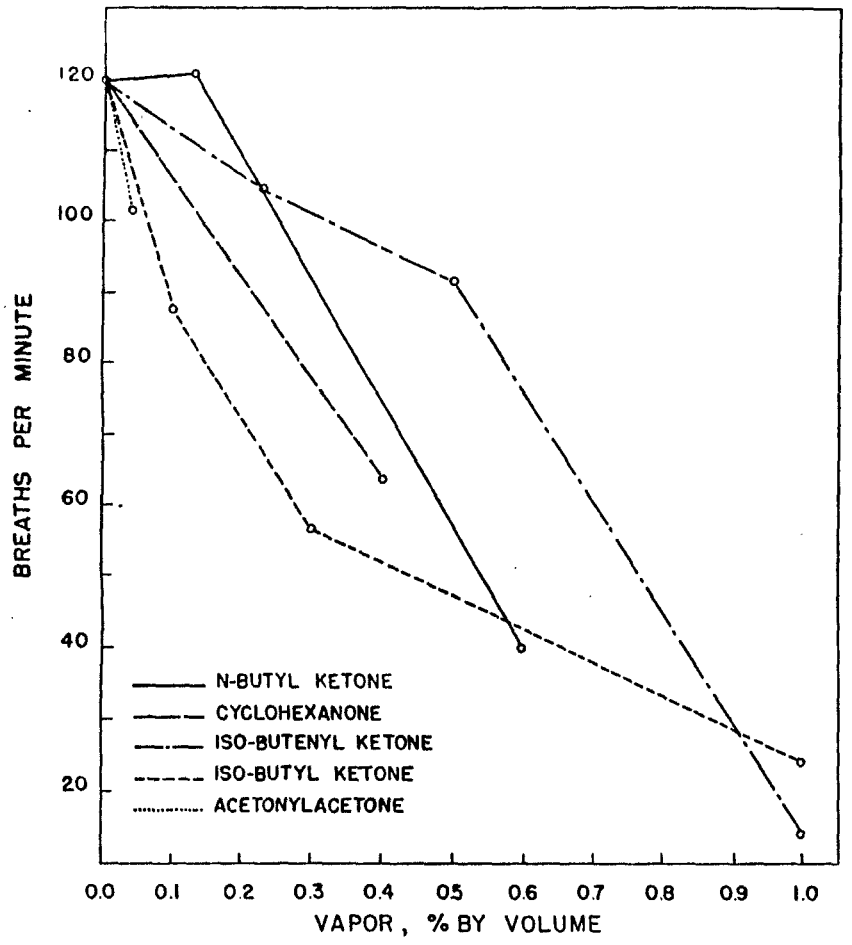

FIGURE 38.

Respiratory rate of guinea pigs after 3 hours' exposure to the different concentrations of acetonylacetone, methyl iso-butyl ketone, cyclohexanone, methyl iso-butenyl ketone, and methyl n-butyl ketone vapor.

the fatty acid series are relatively more rapidly oxidized in the body than acetone. Lewin (1906) found that mesityl oxide (methyl iso-butyryl ketone) and phorone (di-isobutyryl ketone) reacted with certain compounds in the body which contain sulfur, probably sulf-

hydryls, which produced, as *in vitro*, a vile-smelling product which he believed to be a sulfur-substituted ketone. Although he states that acetone also undergoes reaction with such sulfur compounds he finds that no such odorous products result. Lewin thought that these complex sulfur products were responsible for the ejection and char-

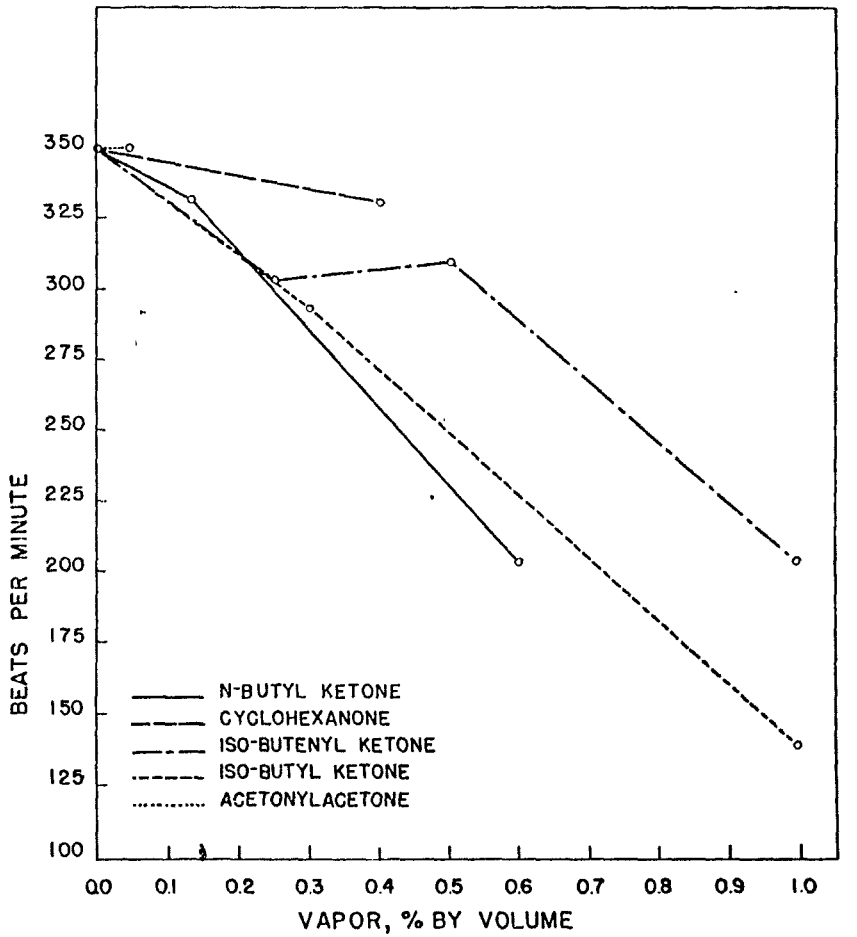

FIGURE 39.

Heart rate of guinea pigs after 3 hours' exposure to the different concentrations of acetonylacetone, methyl iso-butyl ketone, cyclohexanone, methyl iso-butenyl ketone, and methyl n-butyl ketone vapor.

acter of the soft fecal matter during the administration of mesityl oxide and phorone to animals. We are not aware of any further literature on the pharmacology of these particular chemical products. Woodward and Schroeder (1937) have shown that acetone forms readily hydrolyzable compounds with cysteine in vitro.

D. SUMMARY OF ACUTE RESPONSE TO THE INHALATION OF VAPORS OF SIX-CARBON KETONES

1. The inhalation of the vapors of the several six-carbon ketones produces a progressive general narcosis which is similar to that of the members of the homologous series of methyl ketones and characterized [illegible] depression of the body temperature, the respiratory rate, and [illegible] rate as well as an abolition of the corneal, auditory, and [illegible]atory reflexes.

2. In the case of methyl isobutyryl ketone, and a lesser extent cyclohexanone, the reactions were initially of an excitatory or "release" nature not observed in the other ketones.

3. In general, the depression of the various bodily functions is proportional to the concentration of the inhaled vapor and the duration of the exposure.

4. The depression of the various bodily functions is most readily correlated with the partition coefficient of ketone in oil over water. Methyl isobutyryl ketone is aberrant. No relation of the surface tension to the narcotic strength can be detected.

5. The inhalation of different six-carbon ketone vapors has differing effects on the reflex regulation of the heart and respiration. Methyl isobutyryl ketone has the most marked effect.

VI. PATHOLOGY

Tissue from groups of guinea pigs exposed to certain of the ketones was submitted to pathological examination. Table VI shows the compounds and exposure periods represented in the pathological study:

TABLE VI.

Ketone	Duration of exposure (hours)	Number of animals examined
[illegible]tone, 1 percent (Dimethyl ketone)	47 and 48	5
[illegible]ne, 2 percent	22 and 26	9
[illegible]tone, 5 percent	3, 4, 7¾, and 8¾	14
[illegible]tanone, 1 percent (Methyl propyl ketone)	8½ and 8¾	4
Pentanone, 2 percent	3 and 3½	7
Pentanone, 4 percent	2¾ and 3¼	8
Heptanone, 0.5 percent (Methyl n-amyl ketone)	3¼ and 4½	2
Mesityl oxide, 0.5 percent	6½ and 7	6
Hex[illegible]ne, 2.8 percent (Methyl isobutyl ketone)	1	6

All of the animals died during or shortly after exposure. So few of the animals exposed to heptanone were examined that evaluation of the findings on heptanone-exposed animals is inadequate.

Paraffin sections of all organs were stained with eosinated methylene blue (Lillie and Pasternack, 1936), and modified Gallego's connective tissue stain.[1] Sections of the spleens were treated with potassium ferrocyanide to demonstrate hemosiderin.

All of the ketones tested produced essentially the same systemic pathology with but few minor exceptions. The variations in concentrations and in the duration of exposure in most instances are not reflected in the pathological findings but this seems not unusual because practically all of the animals studied died during exposure.

Liver.—Occasional scattered cells containing fat droplets were observed in a few of the animals. These cells were generally in the subcapsular zone and seemed to be of no pathological significance. No fat was noted in the animals exposed to 2-percent pentanone and to heptanone. Areas of slightly congested capillaries were found in some animals, except those exposed to acetone and heptanone. In general, little or no significant changes were found in the liver.

Spleen.—Slight to moderately marked congestion of the cavernous veins was noted in all of the animals except those exposed to heptanone. Slight to marked hemorrhage in the pulp occurred in all animals and was usually perifollicular in those exposed to acetone. Slight to marked hemosiderosis, the pigment being both intra- and extracellular, was a fairly frequent finding. A slight to moderately marked increase in the reticuloendothelial tissue was present in a number of the animals but appeared to be of no distinguishing significance.

Heart.—No histopathological changes were observed.

Lungs.—Congestion of the interalveolar capillaries, varying from slight to marked, occurred in all of the animals. In those exposed to acetone and occasionally in those exposed to the other compounds this was accompanied by extravasation of red blood cells, both with and without serum, into the alveolar spaces. Slight to marked perivascular lymphocytic infiltration was usually present. Moderate to marked emphysema was present in the animals exposed to pentanone, heptanone, and hexone. No contraction of the bronchii or damage to the bronchial epithelium was noted.

Kidneys.—Slight to moderately marked congestion of the interstitial capillaries was present in almost all of the animals. This was least marked with acetone. Congestion of the glomeruli occurred infrequently. Moderate to marked distention of the straight and collecting tubules was common, but no distention of tubules and only slight congestion was found in the animals exposed to hexone. Distention of glomerular capsules was present in some of the animals exposed to acetone.

Adrenals.—With the exception of the animals exposed to acetone, slight to marked congestion of the capsular capillaries occurred. This was accompanied by extravasation of red blood cells in the animals exposed to hexone and to 4-percent pentanone. Moderate to marked numbers of medium and fine fat droplets were present in the [illegible] in all animals

Brain.—Congestion of the pia-arachnoid vessels and of the capillaries of the cerebral cortex was noted in the animals exposed to hexone. Brain tissue was not examined in the other animals.

Stomach and pancreas.—No histopathological changes were noted.

Interpretation.—No marked variations in pathology were noted in animals exposed to different ketones. The most consistent and marked change observed was congestion. The degree of congestion of the following organs descends in the order named: Lungs, kidneys, spleen, adrenals, and brain. Distention of the tubules of the kidney was another prominent finding. Congestion of the liver was so infrequent that it was considered negligible.

The pulmonary congestion has been attributed to the direct irritation of the pulmonary epithelium by acetone vapor (Specht, Miller and Valaer, 1939), but because of the lack of accompanying edema and the infrequency of hemorrhage into the alveolar spaces in the case of the other ketones studied it is thought the hyperemia is more likely due to the effects of the ketones on the vasomotor center. It is to be noted that the majority of animals exposed to acetone remained on test longer than those exposed to the other ketones. In all but 2 animals exposed over 8 hours extravasation of red blood cells into the alveolar spaces, with or without accompanying serum, was noted. No bronchial contraction was observed. According to Sĕrcer (1936) bronchial contraction is due to reflex action from irritation to the nasal mucosa. This condition would disappear at the death of the animal.

The elimination of ketones from the body by diffusion through the lungs and by excretion from the kidney has been demonstrated (Briggs and Schaffer, 1921; Widmark, 1920; Cushny, 1910). Elimination of ketone from the blood by way of the lungs in the animals studied here was impossible because of the constant presence of ketone in the atmosphere. In these exposures the surfaces of the alveolar tissue were in continual contact with the ketones. Possibly because of the mode of entrance of the ketones, the liver shows considerably less hyperemia than the kidney. The kidney derives its arterial supply directly from the renal artery in contrast to the liver which is supplied from the portal system. The distention of the renal tubules may indicate some selective action on the kidneys. The pathological picture in general is consistent with that produced by central action on the vascular system, not protracted long enough to cause secondary tissue changes.

VII. GENERAL SUMMARY OF THE ACUTE RESPONSES TO THE INHALATION OF KETONE VAPORS

1. The inhalation of vapors of dimethyl, methyl ethyl, methyl

n-hexyl ketone and acetonylacetone, cyclohexanone, and methyl iso-butenyl ketone by guinea pigs produces a progressive general narcosis characterized by the depression of the body temperature, the respiratory rate, and the heart rate, as well as the abolition of corneal, auditory, and equilibratory reflexes.

2. The depression of the various bodily functions is directly proportional to the concentration of the inhaled vapor, to the duration of the exposure, and, in the homologous series of straight-chain methyl ketones, to the number of carbon atoms in the chain. Respiratory and cardiac hyperfunction have been observed in cyclohexanone and methyl iso-butenyl ketone only during moderate and low concentrations in the stage following reflex depression.

3. The degree of depression of the various bodily functions is most closely paralleled by the partition coefficient of ketone in oil over water, methyl iso-butenyl ketone and acetonylacetone excepted.

Modifications in the molecular structure other than extension of the carbon chain are concomitant with relatively unpredictable narcotic strengths.

4. The inhalation of ketone vapors in concentrations that irritate the mucosae of the naso-pharyngeal region cause a transient reflex depression of the respiratory and heart rate. This phenomenon furnishes a limiting index for adequate warning properties during the inhalation of these ketones.

5. The pathology of acute exposure to ketone vapor by inhalation is characterized by a general congestion in guinea pigs due to central vasomotor disturbances. Long-continued exposures result in extravasations in the capillary beds, particularly in the lungs.

ACKNOWLEDGMENTS

During the planning of the experiments, the methods of making analyses, the choice of materials, and divers other prerequisites to the actual work of collecting the data the time of a number of the members of the laboratory staff was generously given. Dr. P. A. Neal, P. A. Surgeon, made the necessary arrangements for materials, test animals and their care, cleared the way for the prosecution of the experiments by the assignment of laboratory personnel, and assisted in criticizing the manuscript. Dr. Benjamin F. Jones, P. A. Surgeon, discussed the design of the preliminary work on methyl iso-butyl ketone and the setting up of further experiments. Dr. Lawrence T. Fairhall, principal industrial toxicologist, was consulted on critical points concerning the methods of chemical analysis of the ketone vapors. Dr. Frederick S. Brackett, principal physicist, discussed the construction of the thermocouples used for the measurement of rectal

temperatures. Mr. Laurence R. Crisp, instrument maker, made possible the construction of mechanical devices which were vital to the collection of the quantitative data.

In the preparation of the data for publication invaluable assistance was afforded by the discussion of the findings by Dr. W. F. von Oettingen, principal industrial toxicologist.

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TOXICOLOGY AND APPLIED PHARMACOLOGY 37, 301–311 (1976)

Feline Nervous System Response to Chronic Intoxication with Commercial Grades of Methyl *n*-Butyl Ketone, Methyl *iso*Butyl Ketone, and Methyl Ethyl Ketone[1]

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Received January 5, 1976; accepted March 23, 1976

Feline Nervous System Response to Chronic Intoxication with Commercial Grades of Methyl *n*-Butyl Ketone, Methyl *iso*Butyl Ketone, and Methyl Ethyl Ketone. SPENCER, P. S., AND SCHAUMBURG, H. H. (1976). *Toxicol. Appl. Pharmacol.* 37, 301–311. Twenty-eight 2–3 kg cats received sc injections twice daily, 5 days a week for up to 8.5 months, of the following commercial solvents: (1) methyl *n*-butyl ketone (MBK, 99.66% purity), (2) methyl ethyl ketone (MEK, 99.98% purity), (3) methyl *iso*butyl ketone (MiBK, 98.79% purity), (4) 9:1 mixtures of MEK/MBK, or (5) MEK/MiBK, respectively. Four control cats similarly received an equivalent volume of saline. Tissues removed at biopsy, or after systemic perfusion with fixatives, were examined by light and electron microscopy. The following tissues were studied: pacinian corpuscles, plantar nerves, and intrinsic muscles taken by biopsy from hindfeet of cats in groups 1–5 after 40 and 135 days of intoxication; posterior tibial nerves and pacinian corpuscles of cats in group 2 taken by biopsy after 8.5 months; hindlimb spinal roots and ganglia, nerves, and muscles, plus multiple levels of the spinal cord and medulla oblongata of group 1 animals sampled after perfusion following 2, 4, or 6 months of intoxication. Symmetrical, distal weakness developed progressively after 8 weeks of MBK treatment, first in the hindlimbs and later in all limbs. Nerve fiber pathological changes, characterized by multifocally swollen axons filled with neurofilaments, secondary changes of myelin sheaths and overt fiber breakdown, were seen concurrently in sampled areas of the peripheral and central nervous systems of MBK-treated animals with and without neurological signs. Degeneration first affected the distal parts of nerve tracts, spreading proximally at later stages. This spatial-temporal distribution of disease, a characteristic of several experimental toxic neuropathies, is designated *central-peripheral distal axonopathy*. In animals intoxicated with a mixture of MEK and MBK, subclinical pathological changes were seen in tibial nerves supplying calf muscles but not in pacinian corpuscles located in the hindfeet. These data indicate that MBK is neurotoxic to cats. Cats treated with MEK alone, MiBK alone, or 9:1 mixtures of these compounds, failed to develop detectable nervous system damage.

During the summer of 1973, an outbreak of peripheral neuropathy occurred among employees in a fabric manufacturing plant in Ohio (Billmaier *et al.*, 1974). The most

[1] Supported by a grant from Tennessee Eastman Company, a division of Eastman Kodak Company, and in part by PHS Grants Nos. OH 00535, NS 03356, and NS 08952.

[2] Recipient of a Joseph P. Kennedy, Jr., Fellowship in the Neurosciences.

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severely affected individuals worked in the printing department where coloring inks, dissolved in volatile solvents, were applied to the surfaces of plastic-coated fabrics. For several years, the printing process required the use of a solvent mixture containing 90% methyl ethyl ketone (MEK) and 10% methyl *iso*butyl ketone (M*i*BK). Methyl *n*-butyl ketone (MBK) was gradually introduced in the summer of 1972 to replace the M*i*BK. MBK was in its maximal use by December, 1972, and the first case of peripheral neuropathy occurred shortly thereafter (Allen *et al.*, 1975). The recent introduction of MBK into the printing department of the plant suggested the possibility that exposure to this compound was related to the neuropathy outbreak.

Peripheral neuropathy was induced in several species of experimental animal exposed to MBK by various routes of administration (Duckett *et al.*, 1974; Mendell *et al.*, 1974; Spencer *et al.*, 1975). These studies demonstrated that animals intoxicated by inhalation or sc injection slowly developed symmetrical weakness first in the hindlimbs and later in the forelimbs. The first signs of neuropathy developed after 4–12 weeks of continuous inhalation of 200–600 ppm of MBK and after 12–16 weeks of intermittent inhalation of 1300 ppm of MBK. The pathological basis for the clinical signs was shown to be degeneration of peripheral nerve fibers supplying the limbs. The distal portions of hindlimb nerves initially were involved and, with continued exposure, the degeneration ascended the affected nerves. This was associated with a reduction in sciatic nerve conduction velocity (Mendell *et al.*, 1974), an indication of nerve damage also found in rats and monkeys inhaling 1000 or 100 ppm of MBK intermittently for 3 and 8 months, respectively (Johnson, 1975). These peripheral nerve changes were accompanied by distal axonal degeneration of selected tracts of the spinal cord, medulla oblongata, and cerebellum (Spencer and Schaumburg, 1976).

Environmental examination of areas in the manufacturing plant with the greatest incidence of neuropathy revealed higher concentrations of MEK than of MBK. Air samples taken in proximity to the printing machines, where the greatest incidence of neuropathy was found, revealed 2.3–156 ppm of MBK and 85–763 ppm of MEK. In addition to atmospheric exposure, individuals working at the printing machines sustained skin exposure to MEK and MBK when handling cloths soaked in the solvent mixture (Billmaier *et al.*, 1974). The prominent role of MEK in the exposure history led to speculation that this substance might also be neurotoxic or might enhance any neurotoxic action resulting from exposure to MBK by modifying the absorption, metabolism or excretion of MBK. M*i*BK was also considered a possible neurotoxin because of its isomeric relationship to MBK. The present investigation was designed to address some of these issues. Cats were used in this study because of their susceptibility to MBK, the ease with which early clinical neuropathy could be identified and the possibility of sampling hindlimb distal nerves, muscles, and pacinian corpuscles repeatedly during the course of intoxication.

METHODS

A total of 35 2–3kg young adult cats were used for this study. All animals were housed singly or in pairs. Cages contained smooth floors to ensure that nerves in the cats' feet were not subjected to trauma during activity. Animals were provided with food and water *ad libitum*. Commercial grades of ketone solvents,[3] similar to those employed in

[3] Supplied by Eastman Chemical Products, Kingsport, Tennessee.

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the fabric-printing plant involved in the outbreak of neuropathy, were used in this study. The compositions of these solvents were determined by gas chromatography (Table 1).

Twenty-eight animals received twice daily subcutaneous injections each containing 150 mg/kg of either undiluted MiBK (4 cats), MBK (8), MEK (6), a 9:1 mixture of MEK and MiBK, respectively (6), or a 9:1 mixture of MEK and MBK (4), 5 days a

TABLE 1

GAS CHROMATOGRAPHIC ANALYSIS OF SOLVENTS

Methyl *n*-butyl ketone (MBK)	$CH_3 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH_2 \cdot CH_3$
methyl *n*-butyl ketone	99.66%
methyl *iso*butyl ketone	2.899%
mesityl oxide	0.36%
unknown "A"	0.01%
unknown "B"	0.04%
Methyl *iso*butyl ketone (MiBK)	$CH_3 \cdot CO \cdot CH_2 \cdot CH \cdot (CH_3)_2$
methyl *iso*butyl ketone	98.79%
methyl *n*-butyl ketone	0.94%
acetone	0.02%
other light impurities	0.14%
heavy impurities	0.11%
Methyl ethyl ketone (MEK)	$CH_3 \cdot CO \cdot CH_2 \cdot CH_3$
methyl ethyl ketone	98.98%
acetone	0.003%
secondary butyl alcohol	0.01
unidentified compounds	0.002%

week for periods up to 8.5 months (*vide infra*). Four cats received twice daily injections of an equivalent volume (0.2 ml/kg) of saline, 5 days a week for up to 5 months. Injection sites were rotated over the back. Three additional cats matched for age and weight were used to study normal tissue. Each animal was examined and weighed periodically.

Biopsies

Two series of 20 biopsies were performed, first on the right hindfeet, and on the second occasion, on the left hindfeet. Animals were biopsied after 45 and 135 days of intoxication. Biopsies were performed under sodium pentobarbital (25 mg/kg ip) anesthesia utilizing aseptic techniques. The tissues taken at biopsy included up to 10 pacinian corpuscles from the central plantar toepads, branches of the lateral plantar nerve, and portions of a superficial interosseous muscle. Two animals treated for 8.5 months with a 9:1 mixture of MEK and MBK also underwent biopsy of tibial nerve branches supplying calf muscles and a concurrent, third pacinian corpuscle biopsy from a previously unsampled region of one hindfoot. After tissue excision, the skin was closed with 4.0 nylon sutures and the animal allowed to recover before continuing ketone administration. The tissues were fixed by immersion in phosphate-buffered 5% glutaraldehyde (pH 7.4) for 3 hr.

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Perfusions

Three cats treated with MBK for 2, 4, or 6 months (*vide supra*) and three normal cats were sampled for extensive morphologic study after systemic perfusion with fixatives. At the time of sacrifice, each animal was anesthetized with sodium pentobarbital (50 mg/kg ip) containing heparin (1/10th total anesthetic volume), and perfused through the aortic arch with 4% paraformaldehyde (30 *s*) followed by 5% glutaraldehyde (15 min) each in a 0.1 M phosphate buffer, pH 7.4. After perfusion, the following tissues were sampled: pacinian corpuscles in hindfeet, sciatic, tibial, peroneal, sural and plantar nerves in the hindlimbs, proximal and distal hindleg muscles, lumbosacral dorsal root ganglia, corresponding dorsal and ventral roots, plus multiple levels of the spinal cord and medulla oblongata.

Tissue obtained at biopsy and after systemic perfusion was postfixed in 2% Dalton's chrome osmium, dehydrated, immersed in propylene oxide, and infiltrated with epoxy resin. Single nerve fibers were teased apart from hindlimb nerves, mounted on glass slides, and examined by light microscopy. Other tissues were embedded in epoxy resin, cut into 1 μm sections, stained with toluidine blue, and examined by light microscopy. Thin sections of selected areas were stained with uranyl acetate followed by lead citrate, and examined by electron microscopy.

RESULTS

Clinical Evaluation

There was a considerable variation in the ability of animals to tolerate the ketone injections. Narcosis and excessive salivation commonly commenced shortly after injection. Abscess formation and skin ulceration was seen in several animals. Generalized weakness was found in 10 animals treated with MEK or MEK/MBK mixtures and death occurred after 31–93 days of intoxication. Two animals treated with MBK alone died after 7 and 93 days, respectively. MiBK was tolerated well. No animals were lost and no infections occurred as a consequence of biopsy.

Neurological dysfunction was only detected in cats intoxicated with MBK alone. These animals first developed signs of a peripheral neuropathy after 8–10 weeks of intoxication. At this time, they displayed weakness of the hindquarters resulting in a crouching, unsteady gait. After 10–12 weeks, these cats were found to have a severe hindlimb footdrop. By 16 weeks, they were unable to walk but attempted to ambulate with their weakened forelimbs.

Animals in the remaining groups, including those intoxicated with a 9:1 mixture of MEK and MBK, displayed no neurologic dysfunction.

Microscopy of the Tissues Taken at Biopsy

Normal and saline-treated animals. All tissues appeared normal. Plantar nerves contained large and small myelinated fibers, and small unmyelinated fibers. Muscle tissue contained small nerve fascicles, occasional muscle spindles, and motor nerve terminals abuting neuromuscular junctions. Pacinian corpuscles were found singly or in small groups in the hindfoot pads. Each corpuscle was pierced by a single myelinated

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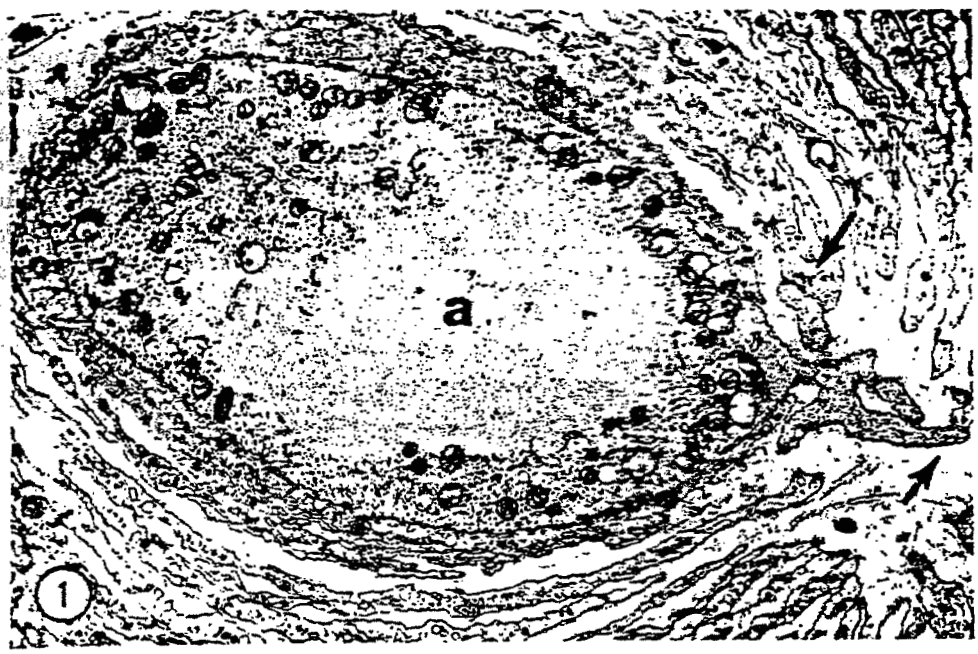

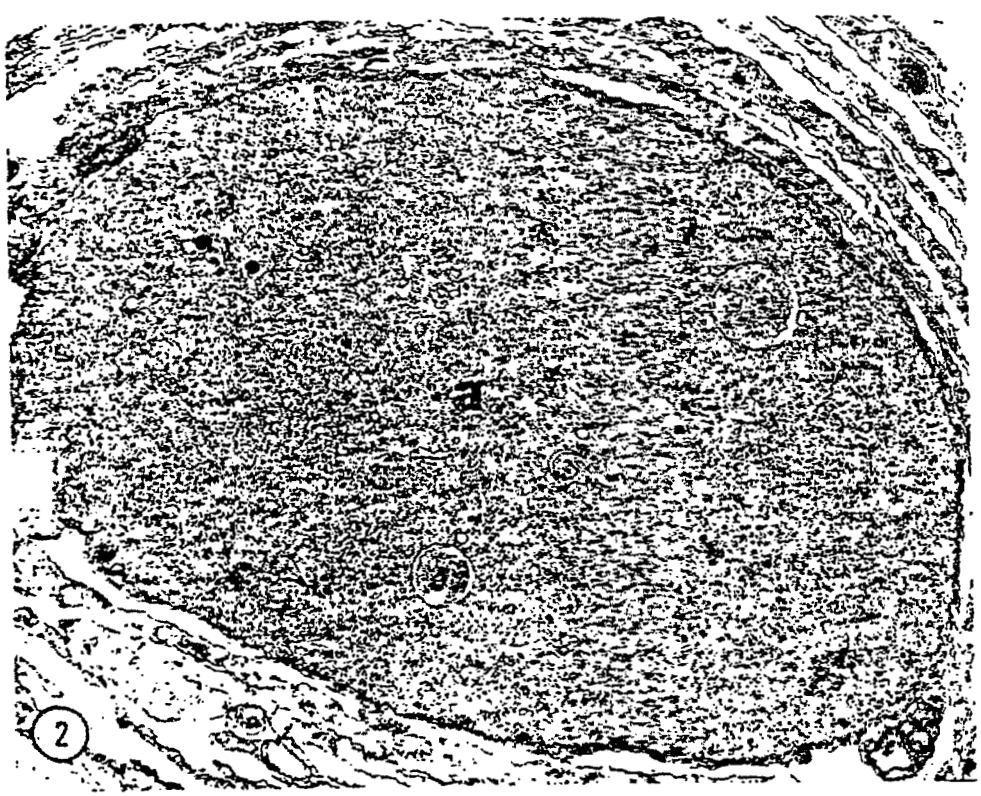

Fi[illegible] and 2. Electron micrographs of thin epoxy cross sections of corpuscles, taken from the hind-foot [illegible] cat intoxicated with MBK for 2 months, fixed by immersion in glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. FIG. 1. Surviving terminal of a pacinian corpuscle containing many axoplasmic neurofilaments (a). Note the normal arrangement of polar axon processes abuting the sides of the hemilamellar inner core cells (arrows). × 10,000. FIG. 2. Degenerating terminal containing a floccular axoplasm (a). × 10,000.

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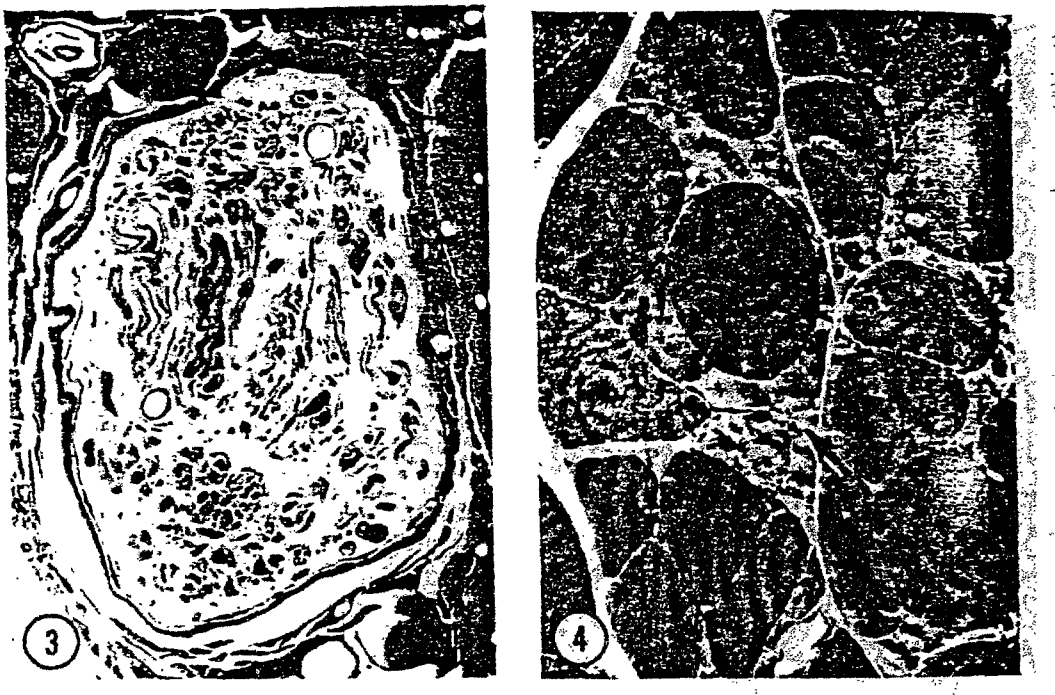
fiber which lost its myelin sheath after entry into the inner core of the corpuscle. The ultrastructure of the naked axon terminal has been described elsewhere (Spencer and Schaumburg, 1973).

Intoxicated animals. Nerve fiber damage was only found in tissue removed from animals treated with MBK or with the MEK/MBK 9:1 mixture. Tissue removed from all other animals appeared normal.

Cats treated with MBK for 45 days showed early nerve fiber damage in plantar nerves and in some pacinian corpuscles. The abnormality in pacinian corpuscles consisted of



FIGS. 3 and 4. Light micrographs of 1 μm epoxy cross sections of tissues, removed from a cat intoxicated with MBK for 4 months, fixed by systemic perfusion with glutaraldehyde and immersion in osmium tetroxide, and stained with 1% toluidine blue. FIG. 3. Degenerating nerve fascicle within the tenuissimus muscle. × 300. FIG. 4. Part of gastrocnemius muscle displaying early fiber atrophy (arrows). × 350.

a proliferation of axonal neurofilaments and subsequent degeneration of axoplasm (Figs. 1, 2) similar to the pattern seen in experimental acrylamide neuropathy (Schaumburg *et al.*, 1974; Spencer and Schaumburg, 1974). After 135 days, corpuscles either were denervated or displayed extensive axonal damage. In plantar nerves and interosseous miscles, nerve fiber degeneration was characterized by focal, giant axonal swellings containing masses of neurofilaments.

Cats treated with a 9:1 mixture of MEK and MBK, respectively, showed no changes in hindfoot tissue taken after 45 and 135 days of intoxication. Tibial nerve branches supplying the calf muscles, sampled after 8.5 months of intoxication, contained abnormal numbers of fibers displaying segmental remyelination. Pacinian corpuscles removed from the hindfeet at this time appeared normal.

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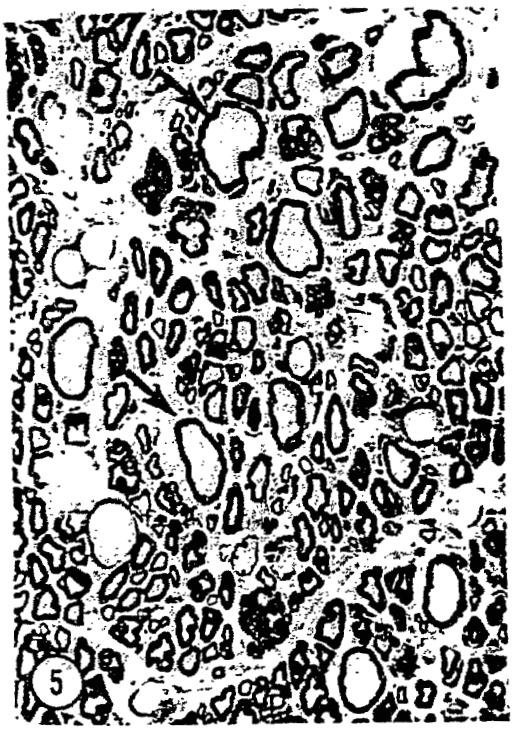
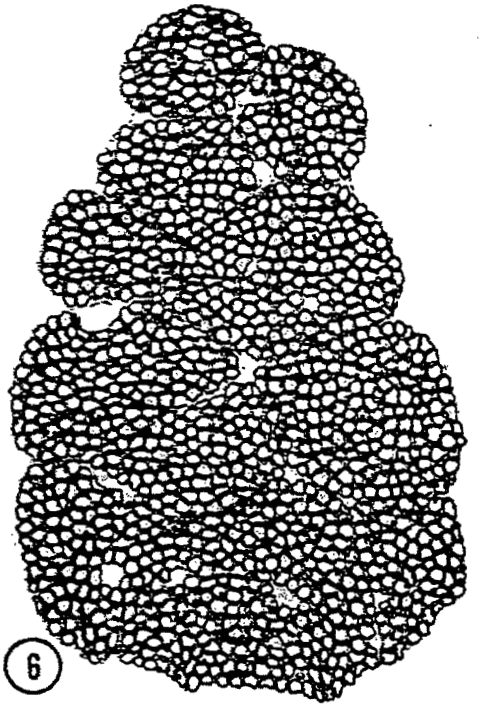
Microscopy of Perfused Tissues

The structure of peripheral and central nervous system tissue was studied in three normal cats and compared with similar tissue obtained from three animals intoxicated with MBK for 2, 4, or 6 months, respectively. One MBK-treated cat did not show any signs, but selected parts of the peripheral nervous system showed the changes characteristic of MBK neuropathy. For example, in the vulnerable tibial nerve branches to the calf muscles, fibers contained scattered giant axonal swellings, paranodal demyelination, an abnormally high incidence of remyelinated internodes, and myelin corrugation





FIGS. 5 and 6. Same tissue as stated in legend to Figs. 3 and 4. FIG. 5. Part of sciatic nerve in the upper thigh illustrating giant axonal swellings (arrows). × 300. FIG. 6. Lower lumbar ventral root of normal appearance. × 110.

within preserved internodes. Sparse changes were found in the plantar nerves of this animal and most of the fibers supplying hindfoot pacinian corpuscles were intact.

Two MBK-treated animals were perfused after development of overt clinical signs. Fiber degeneration was found in sampled nerves and proximal muscle fiber atrophy was also present (Figs. 3, 4). Giant axonal swelling in the sciatic nerve extended up to the level of the dorsal root ganglia but rarely involved the corresponding dorsal and ventral roots (Figs. 5–7). Extensive abnormalities were found in the central nervous system of these two animals. The pathological changes consisted of giant axonal swelling of myelinated fibers, enlargement of nerve terminals, and total fiber breakdown. These abnormalities were prominent in the distal regions of long ascending and descending pathways in the spinal cord and medulla oblongata, and preterminal and terminal axons located in gray matter adjacent to the aqueduct (Figs. 8–10).

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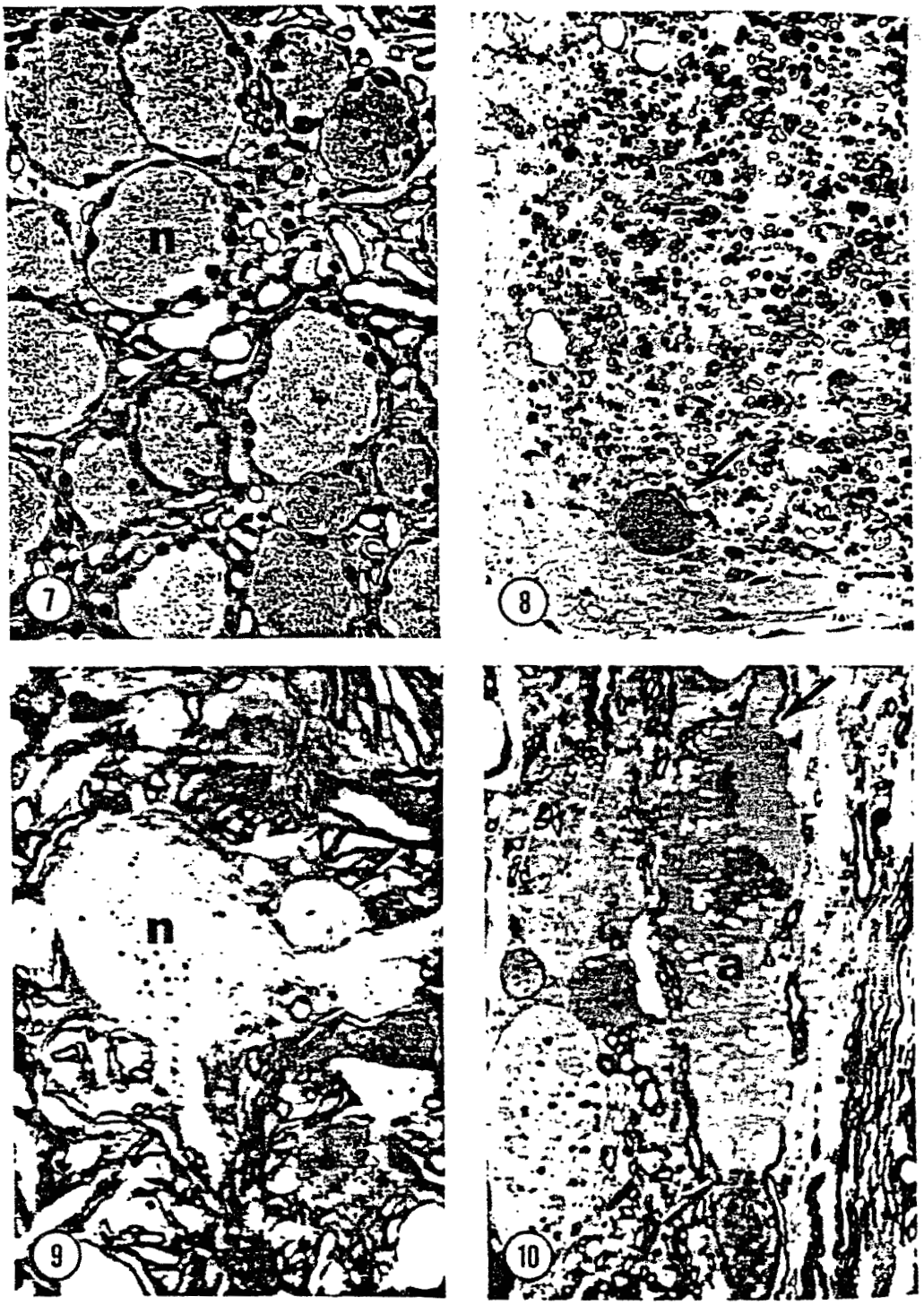

FIGS. 7–10. Same tissue as stated in legend to Figs. 3 and 4. FIG. 7. Lower lumbar spinal ganglion with preserved sensory neurons (n). Arrow points to a swollen axon. × 270. FIG. 8. Gracile tract, caudal to medulla oblongata, containing a giant axonal swelling (arrow) and many degenerating myelinated fibers. × 280. FIG. 9. Swollen axon terminals (arrows) abuting dendrite of a neuron located in sacral region of spinal cord. × 760. FIG. 10. Giant axonal swelling (a) displacing myelin sheaths (arrows) of a fiber located dorsal to the aqueduct in the lumbar spinal cord. × 1100.

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DISCUSSION

The present study revealed that cats chronically intoxicated with a commercial solvent containing 99.66% MBK developed a peripheral neuropathy and displayed concurrent distal nerve fiber degeneration in the peripheral nervous system and in ascending and descending spinal cord tracts of the central nervous system. These findings are consistent with earlier observations on rats chronically intoxicated with MBK in which concurrent peripheral and central nervous system degeneration was also noted (Spencer *et al.*, 1975).

The present study also determined that chronic intoxication with commercial grade MEK alone produced no clinical or pathologic evidence of neuropathy. This supports one of the findings of Saida *et al.* (1975) who observed no neuropathy in rats continuously exposed to 1125 ppm of MEK for 5 months. Similarly, the present study determined that commercial grade M*i*BK or 9:1 mixtures of MEK and M*i*BK produced no effect in the tissue sampled during the exposure period. The subclinical plantar nerve damage reported in rats exposed to 1500 ppm of commercial grade M*i*BK for up to 5 months (Spencer *et al.*, 1975), which may have been the consequence of trauma sustained during activity in cages with wire-mesh floors (Spencer and Schaumburg, 1977b), was not seen in this study. Animals intoxicated by sc injection with a 9:1 mixture of commercial grades of MEK and MBK, respectively, failed to develop clinical neuropathy although pathological evidence of nerve damage was present. The subclinical damage found in these cats appeared to be in proportion to the amount of MBK used in the solvent mixture, although enhancement of the neurotoxic action of MBK by the concurrent exposure to MEK is not excluded. Saida *et al.* (1975) have reported that the neurotoxic action of MBK is potentiated by simultaneous exposure to MEK: In their study, rats intoxicated by continuous exposure to air containing MEK and MBK vapor in a ratio of 1125:225 ppm, respectively, developed clinical evidence of neuropathy after 25 days, whereas rats inhaling 225 ppm of MBK alone exhibited neuropathy after 66 days.

The cardinal finding of this study was the presence of peripheral and central nervous system damage in MBK-treated cats. The distribution and spatial-temporal evolution of this damage, described in detail elsewhere (Spencer and Schaumburg, 1976a, b), indicates that MBK produces a primary axonal degeneration which first affects the distal regions of vulnerable nerve tracts and later progresses proximally. This distribution of peripheral and central nerve fiber degeneration, designated dying-back (Cavanagh, 1969), or *central-peripheral distal axonopathy* (Spencer and Schaumburg, 1976), is characteristic of many other experimental neuropathies produced by compounds such as acrylamide, tri*ortho*cresyl phosphate, *n*-hexane, 2,5-hexanedione, and carbon disulfide (Cavanagh, 1969, 1973; Prineas, 1969a, b; Schaumburg *et al.*, 1974; Schaumburg and Spencer, 1976; Spencer and Schaumburg, 1975, 1976b; Szendzikowski *et al.*, 19[illegible]) the latter three substances and MBK producing a pattern of multifocal, giant axonal swellings (Spencer and Schaumburg, 1976a). It is known that central-peripheral distal axonopathy occurs in humans intoxicated with tri-*ortho*cresyl phosphate (Aring, 1942) and it seems likely that each of the above list of compounds, including MBK, is capable of producing a similar incidence of disease in man.

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ACKNOWLEDGMENTS

The authors wish to thank for discussion W. Krasavage, and Drs. G. Di Vincenzo, A. N. M. Nasr, J. O'Donoghue, R. L. Raleigh, and C. J. Terhaar of the Health and Safety Laboratories of Eastman Kodak Co., Rochester, New York, Drs. R. D. Terry and C. S. Raine for their support, Monica Bischoff, Everett Swanson, Howard Finch, and Miriam Pakingan for technical assistance, and Elaine DelFavero for assistance in the preparation of the manuscript.

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OPTS-400026
E3-001 (4)

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British Journal of Industrial Medicine 1983;40:199–203

An experimental study of the combined effects of n-hexane and methyl ethyl ketone

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ABSTRACT This study was intended to determine whether or not methyl ethyl ketone (MEK) enhances the neurotoxicity of *n*-hexane at low concentration and after long term exposure. Separate groups of eight rats were exposed to 100 ppm *n*-hexane, 200 ppm MEK, 100 ppm *n*-hexane plus 200 ppm MEK, or fresh air in an exposure chamber for 12 hours a day for 24 weeks. The body weight, motor nerve conduction velocity (MCV), distal motor latency (DL), and mixed nerve conduction velocities (MNCVs) were measured before exposure and after four, eight, 12, 16, 20, and 24 weeks' exposure. One rat of each group was histopathologically examined after 24 weeks' exposure. Exposure of 100 ppm *n*-hexane did not significantly decrease the functions of the peripheral nerve throughout the experiment. Exposure to 200 ppm MEK significantly increased MCV and MNCVs and decreased DL after four weeks' exposure, but at the later stage no significant changes were found throughout the experiment by comparison with the controls. Mixed exposure to 100 ppm *n*-hexane plus 200 ppm MEK significantly decreased DL after four weeks' exposure and decreased MCV and MNCVs after 20 and 24 weeks' exposure by comparison with the controls. On histopathological examination of the tail nerve, however, no changes were found in any of the exposed groups or the controls. These results suggest that MEK might enhance the neurotoxicity of *n*-hexane at a low concentration, and mixed exposures to *n*-hexane and MEK should be avoided.

n-Hexane is widely used in industry and is well known to be neurotoxic. It is often used with other solvents; and, as a result, many workers are exposed to mixed organic solvents containing *n*-hexane.[1,2] The study of the combined effects of *n*-hexane and other solvents on the nervous system is important for the maintenance of the health of men exposed at work. Billmaire *et al*[3] and Allen *et al*[4] reported an outbreak of peripheral neuropathy in workers exposed to methyl *n*-butyl ketone (MBK) (a metabolite of *n*-hexane) and methyl ethyl ketone (MEK) in a coated fabrics plant. Saida *et al*[5] reported that MEK did not produce peripheral neuropathy in the rat, but the combination of MBK and MEK did. Altenkirch and Mager[6] reported a polyneuropathy in four youngsters who sniffed thinners containing *n*-hexane, MEK, and other solvents. Altenkirch *et al* also showed in an experimental study,[7] that exposure to 1000 ppm MEK plus 9000 ppm *n*-hexane shortened the onset of severe polyneuropathy compared with exposure to 10 000 ppm *n*-hexane. On the other hand, Takeuchi *et al*[8] showed that 1000 ppm toluene inhibited the neurotoxicity of 1000 ppm *n*-hexane. Ono *et al*[9] showed that even 200 ppm *n*-hexane could affect the peripheral nerve in rats. Therefore, in the present study we intended to clarify whether or not MEK could increase the neurotoxicity of *n*-hexane in the rat, even at the low concentrations used in industry, and whether 100 ppm *n*-hexane alone could affect the peripheral nerves.

Received 24 May 1982
Accepted 23 July 1982

Material and methods

Thirty two Wistar strain male rats (mean body weight 318 g, SD = 10 g) were divided into four groups of eight rats, and the separate groups were exposed to 100 ppm *n*-hexane, 200 ppm MEK, 100 ppm *n*-hexane plus 200 ppm MEK, or fresh air in an exposure chamber[10] for 12 hours a day, every day for 24 weeks. Food and water were provided freely.

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The temperature and the relative humidity in the chambers fluctuated from 23–24°C and 40–60%. The *n*-hexane and MEK used were more than 99% pure, and the concentrations of vapour in the exposure chamber were measured daily by gas chromatography. The measured concentrations were 106 ± 35 ppm of *n*-hexane, 200 ± 40 ppm of MEK, and 104 ± 26 ppm of *n*-hexane plus 203 ± 26 ppm of MEK (mean ± SD, for 24 weeks). The peripheral nerve conduction velocity was measured in the tail nerve of the rat by the methods previously reported.[8,11,12] The rat was wrapped in a towel to keep it immobilised without anaesthesia and laid on its back to allow electrodes to be inserted in the ventral part of the tail. The electrode was a stainless steel needle, 0·34 mm in diameter and about 15 mm long. Electrode A was inserted 3 cm distal from the anus, electrode C, 3–4 cm proximal to the end of the tail, and electrode B, 5 cm proximal to C. The references were inserted about 2 mm from each electrode, and the body earth was inserted between B and C. After insertion of the electrodes, the tail was immersed in a paraffin bath in which the temperature was maintained between 37° and 38°C. The conduction velocity of the tail nerve was measured more than four minutes after the immersion, and the measurement was finished within 20 minutes of the immersion. The tail nerve was stimulated by a square pulse of 0·3 msec duration, 2 c/s and supramaximal strength with an electrostimulator (SEN-7103, Nihon Koden), and the biopotentials were observed with an Addscope (ATAC-350, Nihon Koden). Points A and B were stimulated by a single stimulation and the muscle action potentials were observed at point C to obtain motor nerve conduction velocity (MCV) and the motor distal latency (DL). The tail nerve was stimulated 100 times at point C, and the action potentials at points A and B were summated to obtain mixed nerve conduction velocities (MNCVs). The formulae of these indices are as follows: MCV = distance (AB)/latency time (AC–BC), DL = latency time (BC), MNCV (CB) (distal) = distance (CB = 5 cm)/latency time (CB), MNCV (CA) (total) = distance (CA)/latency time (CA), and MNCV (BA) (proximal) = distance (BA)/latency time (CA–CB). The body weight, MCV, DL, and MNCVs were measured before exposure and every four weeks after exposure.

One rat of each exposed group and one control animal were killed after 24 weeks' exposure and pathohistologically studied. Under anaesthesia, the rat was perfused from the left ventricle with a fixative that contained paraformaldehyde and glutaraldehyde. About 1 cm of nerve tissue was dissected out from the proximal and distal portions of the dorsal tail nerve trunk. For light microscopic examination of the teased nerve, nerve tissues were postfixed in osmium tetroxide and dehydrated glycerin, and teased by a needle in glycerin under binocluar dissection microscope. For electron microscopic examination, tissues were fixed in the same fixative and then postfixed with osmium tetroxide. After staining en bloc with uranyl acetate, the tissues were dehydrated and embedded in epon. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with an electron microscope (JEOL 100 CX). The present electrophysiological results were compared with those from the previous 200 and 500 ppm *n*-hexane exposure.[9]

Results

The changes in body weight, MCV, and DL are shown in fig 1, and the changes in the MNCVs in fig 2. The body weight of the rats in the exposed groups showed no significant differences from the controls throughout the experiment. No abnormal behaviour was observed in either the exposed groups or the controls throughout the experiment. In the 200 ppm MEK group a significant increase in MCV

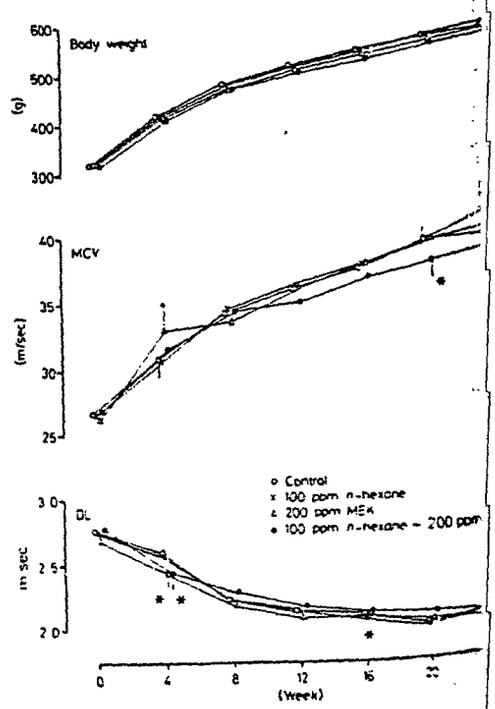

Fig 1 *Changes in body weight, MCV, and DL. Significance level: *p < 0·05.*

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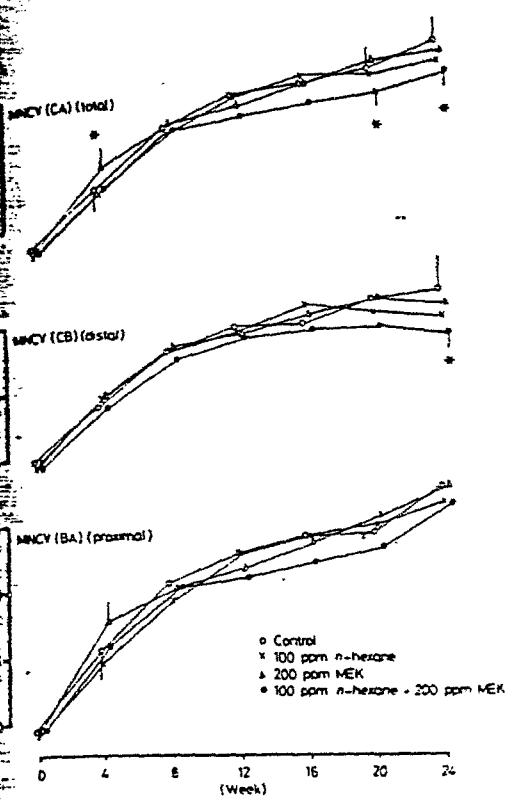

Fig 2 *Changes in MNCVs. Significance level: *(week) $p < 0.05$.*

MNCV (total), and a significant decrease in DL were shown by comparison with the controls after four weeks' exposure, but at the later stage no significant differences were found throughout the experiment. In the 100 ppm *n*-hexane group a significant decrease in DL after four weeks' exposure and a tendency for MNCV (total) to decrease after 24 weeks' exposure was shown, but no other significant differences were found in any electrophysiological index by comparison with the controls. In the 100 ppm *n*-hexane plus 200 ppm MEK group a significant decrease in MCV, and MNCVs (total and distal) after 20 and 24 weeks' exposure was shown by comparison with the controls. A significant increase in DL and a significant decrease in MNCVs (total and proximal) were found after 16 weeks' exposure in the mixed exposure group by comparison with the group of *n*-hexane alone; no other significant differences were found between the two groups. A significant increase in MNCV (total) was found in the mixed exposure group by comparison with the group of MEK alone after four weeks' exposure; no other significant differences were found. The results of the electrophysiological examinations are summarised in the table.

The changes in MCV of the present 100 ppm *n*-hexane, 200 ppm MEK, and 100 ppm *n*-hexane plus 200 ppm MEK, and the previous 200 ppm and 500 ppm *n*-hexane groups[9] are shown in fig 3. A dose-dependent effect of *n*-hexane on the function of the peripheral nerve is found in 100, 200, and 500 ppm *n*-hexane exposure, and the effects of 100 ppm *n*-hexane plus 200 ppm MEK are found between the

Summary of significant differences found in neurophysiological values in exposed and control animals

		n-Hexane 100 ppm							*MEK 200 ppm*							*n-Hexane 100 ppm + MEK 200 ppm*						
	Week	*0*	*4*	*8*	*12*	*16*	*20*	*24*	*0*	*4*	*8*	*12*	*16*	*20*	*24*	*0*	*4*	*8*	*12*	*16*	*20*	*24*
Control	BW																					
	MCV									↑											↑	↑
	DL					↓				↓							↓					
	MNCV (CA)									↓											↓	↓
	MNCV (CB)																					↓
	MNCV (BA)																					
n-Hexane 100 ppm	BW																					
	MCV									↑												
	DL																			↓		
	MNCV (CA)									↑										↓		
	MNCV (CB)																			↓		
	MNCV (BA)																					
MEK 200 ppm	BW																					
	MCV																					
	DL																↓					
	MNCV (CA)																					
	MNCV (CB)																					
	MNCV (BA)																					

Arrows show statistically significant level ($p < 0.05$) and directions.
BW = Body weight; MCV = Motor nerve conduction velocity; DL = Distal motor latency; MNCV = Mixed nerve conduction velocity.

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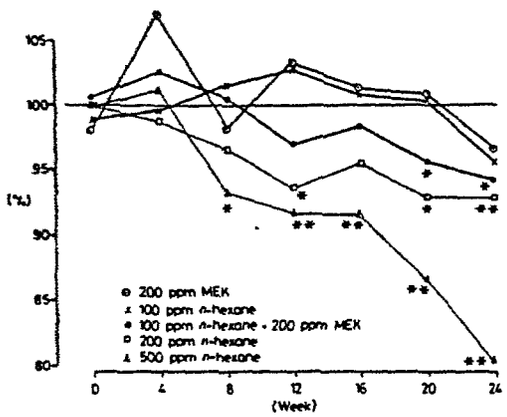

Fig 3 *Changes in MCV (percentage of the exposed groups in the controls). Significance level: *p < 0·05, **p < 0·01.*

exposure of 100 ppm and 200 ppm *n*-hexane. Microscopic examination of the teased nerves showed no changes in the tail nerves of rats in the present 100 ppm *n*-hexane, 200 ppm MEK, 100 ppm *n*-hexane plus 200 ppm MEK groups, or the controls.

Discussion

The present study showed that exposure to 100 ppm *n*-hexane plus 200 ppm MEK impaired the function of the peripheral nerve in the rat and that exposure to 100 ppm *n*-hexane alone did not. The differences between the mixed exposure group and the 100 ppm *n*-hexane group were not as clear as those that Altenkirch *et al*[7] showed at high concentrations. These results suggest that mixed exposure to *n*-hexane and MEK might be more toxic than *n*-hexane alone even at the low concentrations used in industry, and that exposure to this mixture should be avoided.

The present experiment showed no significant changes in the 100 ppm *n*-hexane group except for DL after 16 weeks' exposure by comparison with the controls throughout the experiment, although MNCV (CA) (total) showed a tendency to decrease after 24 weeks. On the other hand, Ono *et al*[9] and Kitoh *et al*[13] reported that exposure to 200 ppm *n*-hexane affected the peripheral nerve of the rat in conditions similar to those of the present experiment, and that changes could be detected by both electrophysiological and histopathological examination. The present histopathological examinations of the tail nerve trunks showed no changes in either the exposed groups or in the controls. From these results it might be inferred that the changes of the peripheral nerve in rats can be detected earlier by electrophysiological examination than by his topathological examination. And as the [illegible] ppm *n*-hexane for 12 hours a day every day is equivalent to that of 175 ppm for eight hours a day, [illegible] days a week, it might also be inferred that the present electrophysiological methods [illegible] impairment of the function of the peripheral nerve in rats exposed to the concentration between [illegible] 350 ppm of *n*-hexane for eight hours a day, six days a week for 24 weeks. Sanagi *et al*[14] reported that workers exposed to 58 ± 41 ppm (time weighted average for eight hours) *n*-hexane develop impairment of the nerve conduction velocities. And according to the previous review of the [illegible] response relationship of *n*-hexane,[1] it can be calculated that workers exposed to about 600 ppm (TWA for eight hours) *n*-hexane could develop polyneuropathy with muscle atrophy [illegible] turbance, whereas it would require a [illegible] about 4700 ppm (TWA for eight hours) *n*-hexane for rats to develop the same. Judging from these results, it may be inferred that man is several times as sensitive to *n*-hexane as rats at both low [illegible] concentrations.

In the 200 ppm MEK exposure group the MCV and MNCVs significantly increased and DL significantly decreased by comparison with the controls after four weeks' exposure, but these changes were not found at the later stage of the experiment. Savolainen and Seppäläinen[15] reported that a significant increase in MCV and in [illegible]onal membrane protein was noted in rats exposed to 300 ppm toluene for three weeks by comparison with the controls, and that these changes could not be found at the later stage. These results suggest that the [illegible] ppm MEK exposure might also affect the axonal membrane and transiently increase the nerve conduction velocity similar to 300 ppm toluene at the earlier stage of exposure, although the chronic mixed effects of MEK and *n*-hexane are opposite to those of toluene and *n*-hexane.[8] The mechanism by which MEK enhances the neurotoxicity of *n*-hexane is interesting but, as yet, not clear. Abdel-Rahman *et al*[16] reported that the combined exposure of [illegible] ppm MBK and 750 ppm MEK enhanced the neurotoxicity of MBK and increased MBK and 2,5-hexanedione (a common metabolite of *n*-hexane and MBK) in the blood of rats by comparison with 225 ppm MBK alone. Couri *et al*[17] reported that the combined administration of MBK/MEK (40/120 mg/kg ip) increased the urinary excretion of 2-hexanol and 2, 5-hexanedione compared with the administration of an equal dose of MBK alone. These results suggest that MEK itself is not toxic to the peripheral nerve but that it might modify the biotransformation of, and enhance the neurotoxicity of, *n*-hexane.

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THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 196, No. 2
Printed in U.S.A.
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THE PARTICIPATION OF 2-BUTANONE IN 2-BUTANOL-INDUCED POTENTIATION OF CARBON TETRACHLORIDE HEPATOTOXICITY[1, 2]

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Accepted for publication August 21, 1975

ABSTRACT

TRAIGER, GEORGE J. AND JAMES V. BRUCKNER: The participation of 2-butanone in 2-butanol-induced potentiation of carbon tetrachloride hepatotoxicity. J. Pharmacol. Exp. Ther. 196: 493–500.

The role of alcohol metabolism in 2-butanol-induced potentiation of carbon tetrachloride (CCl_4) hepatotoxicity was studied in rats. Animals were sacrificed at various times after the administration of 2-butanol (2.2 ml/kg p.o.) for the determination of blood 2-butanol and 2-butanone concentrations by gas chromatographic analysis. 2-Butanol exhibited an apparent elimination half-life of 2.5 hours. With the decline of 2-butanol concentrations, there was a rise in 2-butanone blood concentrations with 43 mg/100 ml detected at 1 hour and a maximum of 105 mg/100 ml detected 4 hours after the administration of the alcohol. A 16-hour pretreatment with either 2-butanol (2.2 ml/kg p.o.) or 2-butanone (1.87 ml/kg p.o.) markedly enhanced the hepatotoxic response of CCl_4 (0.1 ml/kg i.p.) as measured by serum glutamic pyruvic transaminase activity, hepatic glucose-6-phosphatase activity and triglyceride content. The enhanced hepatotoxicity produced by 2-butanol was not significantly different from that produced by 2-butanone. The potentiation of CCl_4 hepatotoxicity by both agents was substantiated morphologically. The results indicate that 2-butanone production *via* the oxidation of 2-butanol appears to contribute to the marked response of 2-butanol.

The potentiation of carbon tetrachloride (CCl_4) hepatotoxicity by ethanol has been recognized for four decades. Cornish and Adefuin (1967) have shown that several aliphatic alcohols can exert a similar effect. The extent to which the alcohols altered the hepatotoxicity of CCl_4 varied greatly among the alcohols tested, with isopropanol and 2-butanol possessing a greater potentiating effect than a homologous or isomeric primary alcohol. In a study of the relationship of alcohol metabolism to the production of a potentiated response, Traiger and Plaa (1972) have reported that the metabolic disposition of isopropanol contributes to the marked differences in potentiating capacity exhibited between ethanol and isopropanol. It was found that acetone derived during the *in vivo* oxidation of isopropanol contributes to the augmentation of isopropanol-potentiated CCl_4 hepatotoxicity. As 2-butanol shares with isopropanol a marked capacity to augment CCl_4 hepatoxicity, the present study was undertaken

Received for publication March 27, 1975.
[1] This investigation was supported by grants from the Pharmaceutical Manufacturers Association and the University of Kansas and by National Institutes of Health Grant DA-00890.
[2] Presented in part at the 1973 meeting of the American Society for Pharmacology and Experimental Therapeutics (Pharmacologist 16: 585, 1973).

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to investigate the relationship of 2-butanol metabolism *in vivo* with 2-butanol-induced potentiation of CCl_4 hepatotoxicity.

Methods

Male Sprague-Dawley rats weighing 180 to 250 g were used throughout. The animals were maintained on an *ad libitum* diet of commercial chow and water in a temperature-controlled room with 12-hour periods of alternating light and darkness. 2-Butanol (2.2 ml/kg, undiluted) or 2-butanone (1.87 ml/kg as an 18.7% aqueous solution) was administered orally. After 16 hours, the animals received an intraperitoneal injection of 0.1 ml/kg of CCl_4 in corn oil. The animals were sacrificed by decapitation 24 hours after CCl_4 administration. Blood and liver aliquots were taken for enzyme and triglyceride analyses.

Serum glutamic pyruvic transaminase (SGPT) activity was determined according to the method of Reitman and Frankel (1957) as described by Klaassen and Plaa (1966). Aliquots of liver were taken for the estimation of triglycerides by the method of Van Handel and Zilversmit (1957) as modified by Butler *et al.* (1961). Glucose-6-phosphatase activity was measured in homogenates of whole liver as previously described (Traiger and Plaa, 1971).

Liver specimens were rapidly excised upon sacrifice and placed into 10% formalin overnight for fixation. The tissues were then embedded in paraffin, sectioned and stained with hematoxylin and eosin for light microscopic examination. Frozen sections of each specimen were sectioned and stained for fat with Sudan IV.

Blood concentrations of 2-butanol and 2-butanone were determined by gas chromatography (Davis, 1966). In this procedure, 1,4-dioxane, to which an internal standard has been added, is used as a protein precipitant. The blood concentrations were estimated by the peak ratios of the unknown to the internal standard. A Hewlett Packard model 7620 gas chromatograph equipped with a flame ionization detector was used.

In the butanol elimination study, acetone at a concentration of 0.79 mg/1.0 ml of 1,4-dioxane was used as the internal standard for the simultaneous determination of 2-butanol and 2-butanone. The column used was 0.25 inch outside diameter × 6 feet containing 3% Carbowax 2 OM on 80/100 Chromosorb W HP. Injection port, column and detector temperatures were 150, 125 and 140°C, respectively. Nitrogen, hydrogen and air flow rates were 10, 25 and 330 ml/min, respectively.

In the 2-butanone elimination study, ethanol at a concentration of 0.79 mg/ml of 1,4-dioxane was used as the internal standard. The column used was 0.125 inch outside diameter × 10 feet containing 15% Halcomid M-18-OL on 70/80 Chromosorb W. Injection port, column and detector temperatures were 130, 117 and 145°C, repectively. Nitrogen, hydr and air flow rates were 30, 35 and 360 ml/ respectively.

The data were compared by an analysis of varia When the analysis indicated that a significant di ence existed, the means of selected groups compared by Student's *t* test (Steel and Torrie, 19

Results

Figure 1 depicts blood concentration c butanol and its metabolite 2-butanone at ous times after the oral administration of ml/kg of 2-butanol. The values for each estimated from the same blood sample. B alcohol concentrations of 82 mg/100 ml detected after 1 hour. These declined to than 5 mg/100 ml at 15 hours. The apparent half-life of the alcohol was about 2.5 hour the blood concentration of 2-butanol decl the concentration of 2-butanone rose mark to a maximum of 105 mg/100 ml at 4 h Thereafter, 2-butanone concentrations dec to a value of 4.9 mg/100 ml detected aft hours. The elimination profile exhibite 2-butanol and its metabolite 2-butanone i present study was comparable to that p ously found for the *in vivo* formation of ac after isopropanol administration (Traige Plaa, 1972).

Before assessing the role of 2-butanone i potentiation of CCl_4 hepatotoxicity elicit 2-butanol, a study of 2-butanone elimin was carried out. As an estimate of an equiv dose of 2-butanone, it was assumed that 8 the alcohol is oxidized to the ketone *in* with the remaining 15% being eliminate changed and as a conjugated glucur (Kamil *et al.*, 1953). Animals which had fasted overnight were given 1.87 ml/kg butanone, and blood concentrations were mined for a period of 12 hours. As can be s figure 2, this ketone was slowly absorbed oral administration with a maximal conc tion of 83 mg/100 ml found 4 hours administration. The dose of 2-butanon lected approximated the 2-butanone conc tion detected in the blood after the admi tion of 2.2 ml/kg of the precursor al 2-butanol.

A time course study was carried determine whether either agent was capa producing hepatotoxicity when admini alone. Figure 3 depicts the effect of 2-b

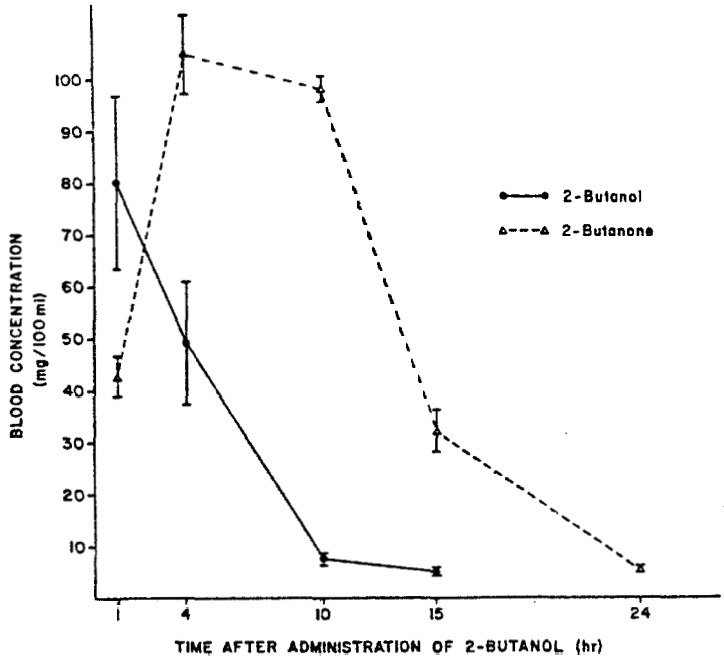

FIG. 1. 2-Butanol and 2-butanone concentrations detected after the administration of 2-butanol (2.2 ml/kg p.o.). Each point represents the mean for a group of four rats with vertical lines indicating ± 1 S.E.

(2.0 ml/kg p.o.) and 2-butanone (1.87 ml/kg p.o.) on SGPT activity for a period of up to 16 to 23 hours after their administration. As can be seen, 2-butanol or 2-butanone alone yielded values equivalent to those found for control animals. Hepatic glucose-6-phosphatase activities determined 1, 4, 8 and 16 hours after the administration of alcohol or ketone were not significantly different from the values found for the respective control groups. In agreement with the histopathologic findings of moderate reversible fatty infiltration of the liver after 2-butanol (Weese, 1928) and 2-butanone (DiVincenzo and Krasavage, 1974), hepatic triglyceride content was elevated 16 hours after treatment with either agent. 2-Butanol produced a 182% increase, whereas 2-butanone elicited a 163% increase in triglyceride levels. The values found for the treated animals were significantly different from control values ($P < .05$).

Table 1 depicts alterations of SGPT activity, hepatic triglyceride content and glucose-6-phosphatase activity in rats after administration of 2-butanol (2.2 ml/kg p.o.) or 2-butanone (1.87 ml/kg p.o.), 16 hours before receiving a challenging dose of CCl_4 (0.1 ml/kg i.p.). Twenty-four hours after the CCl_4 dosing, the animals were sacrificed. The dosage level of CCl_4 was selected to cause only a slight elevation in SGPT activity. It is apparent from table 1 that both 2-butanol and 2-butanone markedly enhanced the hepatotoxicity of CCl_4, as evidenced by the large increases in SGPT activity. As judged by these elevations in SGPT activity, 2-butanol and 2-butanone each potentiated CCl_4 hepatotoxicity to a similar degree.

The effect of alcohol or ketone pretreatment on the two other indices of CCl_4 hepatotoxicity also reflects an enhanced hepatotoxic response to the chlorinated hydrocarbon. It can be seen that either 2-butanol or 2-butanone administered 16 hours before CCl_4 produced an enhancement of hepatotoxicity as reflected by diminished glucose-6-phosphatase activity and an increase in hepatic triglyceride content. Similar to the findings at earlier time periods, no significant effect on SGPT or hepatic glucose-6-phosphatase activity was detected 40 hours after the administration of alcohol or ketone alone. Hepatic triglyceride concentrations had returned to normal at this time.

Examination of histopathologic specimens revealed manifestations of potentiation of CCl_4 hepatotoxicity analogous to alterations seen in

the biochemical assessment of hepatotoxicity. Control livers were unremarkable (fig. 4) in hematoxylin and eosin-stained and in fat-stained sections, as were livers from rat administered solely 2-butanol or 2-butanone. Livers from animals receiving only CCl_4, however, exhibited a very limited degree of patholo change (fig. 5). Occasional hepatocytes in centrolobular regions of such livers showed fa vacuolation. Both 2-butanol and 2-butan markedly enhanced CCl_4 hepatotoxicity, as denced by extensive centrolobular fatty vac lation and necrosis. Centrolobular fatty vac lation was more prominent in livers of butanol-pretreated rats (fig. 6) than in liver 2-butanone-pretreated rats, with centrolob necrosis commonly seen in the latter group (7). The degree of fatty deposition observe fat-stained liver sections paralleled hepatic glyceride levels measured for all groups animals.

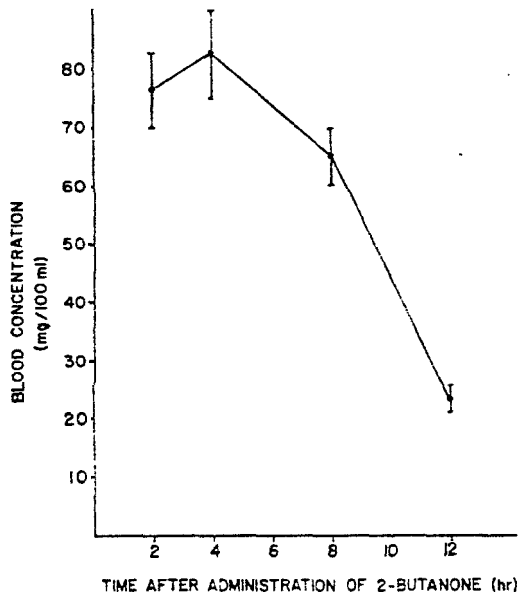

FIG. 2. Blood 2-butanone decay profile obtained after the administration of 2-butanone (1.87 ml/kg p.o.). Each point represents the mean for a group of four rats with vertical lines indicating ± 1 S.E.

Discussion

The present study confirms and exten previous report that 2-butanol pretreatm markedly enhances the necrotizing effec CCl_4 (Cornish and Adefuin, 1967). In addi to the elevation of serum transaminase acti other indices of hepatotoxicity such as hep triglyceride content and glucose-6-phospha activity similarly reflect an increased resp to CCl_4. The effect of combined treatment substantiated morphologically.

The data would indicate that the ma potentiation of CCl_4 hepatotoxicity produce 2-butanol pretreatment may not entirely attributed to the alcohol alone.

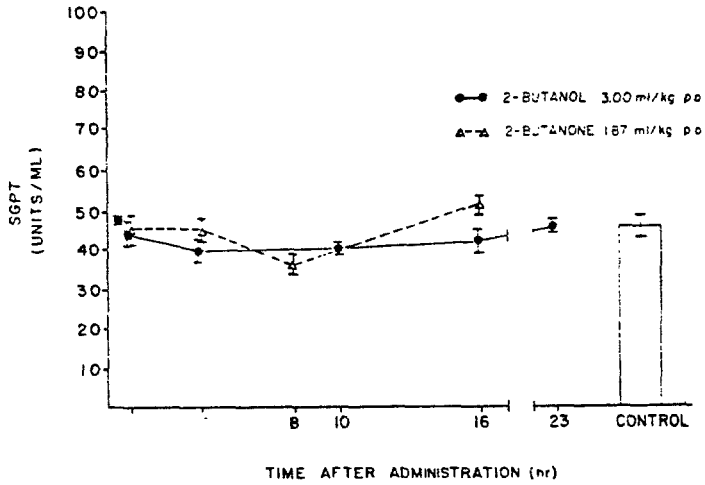

FIG. 3. SGPT activity at various times after the administration of 2-butanol (3.0 ml/kg p.o.) or 2-but (1.87 ml/kg p.o.). Each point represents the mean for a group of three to four treated rats with vertical indicating ± 1 S.E. The bar represents the SGPT activity of 20 untreated control animals with the vertica indicating ± 1 S.E.

TABLE 1

Effect of 2-butanol and 2-butanone on CCl_4 hepatotoxicity [a]

Treatment	SGPT	Glucose-6-Phosphatase	Triglyceride
	U/ml	*mgP/g liver/20 min*	*mg/g liver*
Control	52 ± 4	6.48 ± 0.27	6.3 ± 1.1
2-Butanol	59 ± 3	5.91 ± 0.62	7.6 ± 1.8
2-Butanone	64 ± 3	6.56 ± 0.40	8.34 ± 1.5
CCl_4	85 ± 10	5.60 ± 0.37	8.33 ± 1.06
2-Butanol + CCl_4	2638 ± 616[b, c]	2.47 ± 0.15[b, c]	24.4 ± 3.9[b, c]
2-Butanone + CCl_4	2490 ± 591[b, c]	2.70 ± 0.29[b, c]	17.6 ± 3.3[b, c]

[a] Each value represents the mean ± S.E. of five animals. 2-Butanone (1.87 ml/kg p.o.) or 2-butanol (2.2 ml/kg p.o.) was given 16 hours before CCl_4 (0.1 ml/kg i.p.); the rats were sacrificed 24 hours after CCl_4.

[b] $p < .05$ *vs.* control.

[c] $p < .05$ *vs.* CCl_4.

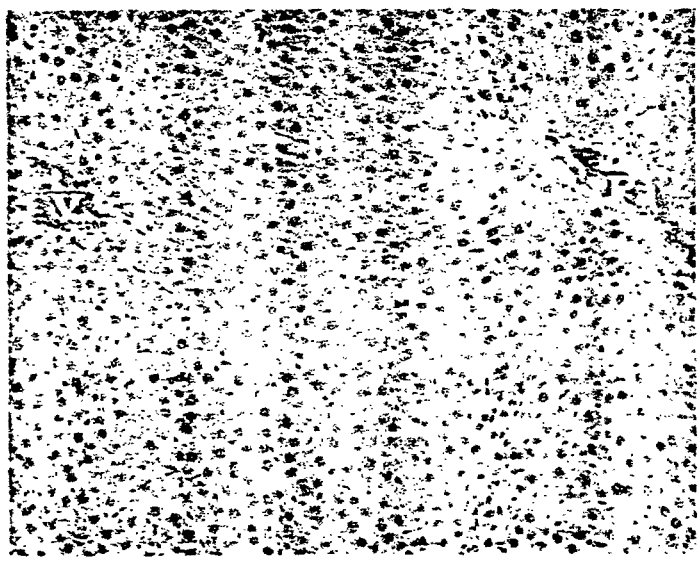

FIG. 4. Portion of a liver lobule of a control rat. The hepatic parenchyma encompassing both the central vein (V) and portal triad (P) is unremarkable. Hematoxylin and eosin stain: ×315.

In vivo, 2-butanol is oxidized to 2-butanone. The 2-butanone blood concentrations detected after 2-butanol can be approximated by the administration of 1.87 ml/kg of 2-butanone; this dose of 2-butanone when given alone is capable of augmenting CCl_4 hepatotoxicity. Based on the similarity of the hepatotoxic response seen upon pretreatment with either alcohol or ketone, it would appear that 2-butanone might possibly play a major role in the potentiation elicited after the precursor alcohol. Furthermore, the *in vivo* formation of this slowly eliminated ketone may also account for the more striking effect of 2-butanol, as compared to the homologous primary alcohols, on CCl_4-induced liver damage. These findings are similar to those reported for the participation of acetone in isopropanol-induced potentiation (Traiger and Plaa, 1972).

The mechanism by which 2-butanol and 2-butanone augment the necrogenic action of CCl_4 in unclear. There is considerable evidence that CCl_4 must be converted to an active intermediate in the hepatic endoplasmic reticulum to produce its acute hepatotoxic effect (Slater, 1966; Recknagel and Ghoshal, 1966; Reynolds and Yee, 1967; Gordis, 1969). Biotransformation has been proposed to involve the homolytic cleavage of the carbon-chlorine bond leading to the production of free radicals (But-

ler, 1961; Slater, 1966). The trichloromethyl and chlorine free radicals then react with tissue macromolecules to initiate a sequence of events culminating in necrosis. The acute administration of 2-butanol or 2-butanone has been reported to produce an increase in microso acetanilide hydroxylase and aminopyrine methylase activity (Traiger *et al.*, 1975). S an enhancement of microsomal drug-metab ing activity could possibly increase the

FIG. 5. Liver specimen from a rat given CCl_4 (0.1 ml/kg i.p.) and sacrificed 24 hours later. Occa hepatocytes (arrows) around the central vein (V) show marked vacuolation. Portal triad (P); hematoxyli eosin stain: ×481.

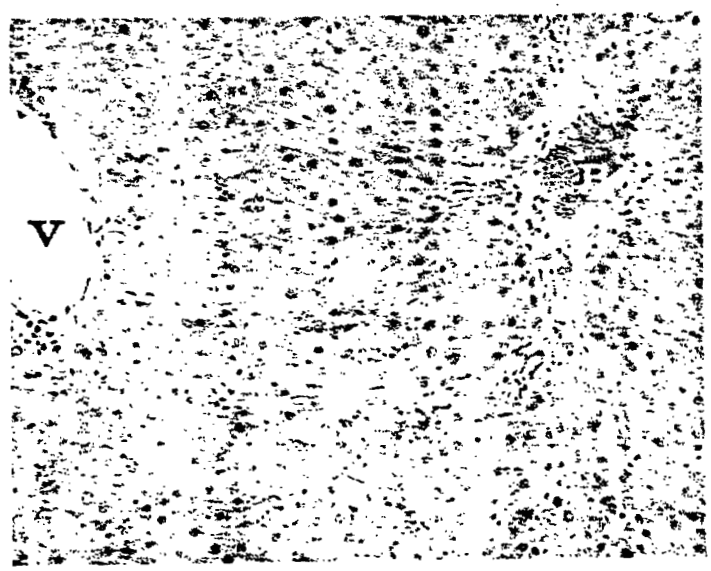

FIG. 6. Liver section from a rat administered 2-butanol (2.2 ml/kg p.o.) 16 hours before CCl_4 (0.1 ml/k sacrifice was carried out 24 hours after CCl_4. Note the extensive area of vacuolated parenchyma encomp the central vein (V), and the relatively unaffected parenchyma surrounding the portal triad (P). Hemat and eosin stain: ×315.

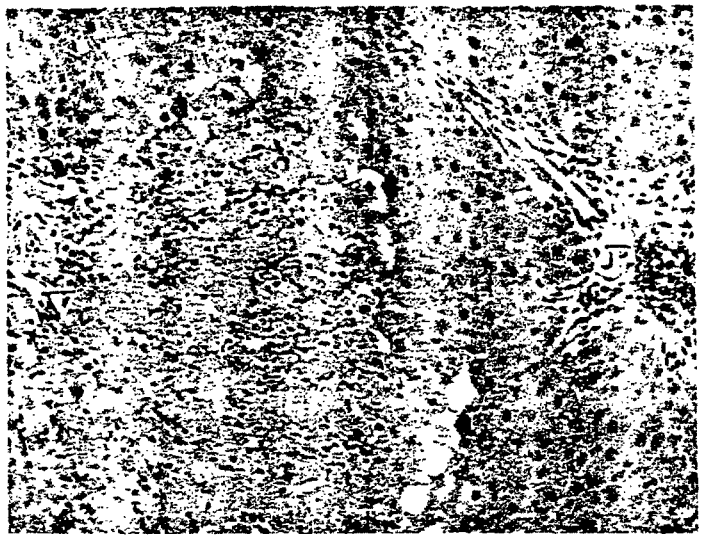

FIG. 7. Liver section from a rat administered 2-butanone (1.87 ml/kg p.o.) 16 hours before CCl_4 (0.1 ml/kg i.p.); sacrifice was carried out 24 hours after CCl_4. The necrotic centrolobular region is ringed with vacuolated hepatocytes (arrows), whereas the portal region appears unaffected. Central vein (V); portal triad (P) hematoxylin and eosin stain: ×373.

transformation of CCl_4, as has been postulated for the augmentation of CCl_4 hepatotoxicity by phenobarbital (Garner and McLean, 1969). Studies with isopropanol or acetone indicate that these agents can stimulate the drug-oxidase system of the endoplasmic reticulum. However, isopropanol and acetone produce rather selective changes which differ from the stimulatory effects produced by a classical inducer such as phenobarbital. When administered *in vivo*, this alcohol and this ketone produce an increase in microsomal aniline hydroxylase activity and enhanced N-demethylation of dimethylnitrosamine, however, the concentration of microsomal protein and cytochrome P-450 remains unchanged (Traiger and Plaa, 1973; Clark and Powis, 1974; Sipes *et al.*, 1973). Clearly, further work needs to be carried out on the mechanism by which these secondary alcohols and aliphatic ketones exert an action on the endoplasmic reticulum.

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enlarged hepatocytes. Many cells contained inclusions in their cytoplasm. Electron microscopic studies showed a slight increase in smooth endoplasmic reticulum, atypical mitochondria and some lipid vacuoles. The livers of all control rats were normal. Groups of ten male and female rats were exposed to 500, 100, 20, and 0 ppm of technical pentachlorophenol for 8 mo. The livers of the rats fed 500 ppm were also enlarged at autopsy.

24. *Bioactivation of 1-Nitronaphthalene.* D. JOHNSON and K. KHANNA, University of Michigan, Ann Arbor, Michigan. (R. Hartung)

Certain carcinogenic metabolites can be formed by oxidation of aromatic amines. The possibility exists that nitroreduction of analogous aromatic nitro compounds may produce the same active intermediates. To study the potential bioactivation of aromatic nitro compounds, 1-nitronaphthalene (1-NN) was chosen as a prototype since two possible intermediates in the metabolism of both 1-NN and 1-naphthylamine, 1-nitrosonaphthalene and N-hydroxy-1-naphthylamine, have been shown to be liver carcinogens in rats. It was thought that the occurrence of liver toxicity in acute studies, suggesting binding to liver macromolecules, correlated with urinary metabolite excretion patterns, might serve as an indicator of potential liver carcinogenesis and suggest the tentative identity of an active intermediate. Single ip injections of 1-NN (100 mg/kg) were given to male Sprague–Dawley rats maintained in individual metabolism cages. A majority of the rats developed severe pulmonary edema which was abolished by phenobarbital pretreatment. Histopathological studies showed that extensive liver necrosis occurred in both groups within 48 hr. The urinary excretion pattern indicates that nitroreduction and/or hydroxylation are the primary metabolic pathways. These findings suggest that certain aromatic nitro compounds may have carcinogenic potential if metabolized to active intermediates and binding to liver macromolecules occurs.

25. *Effect of 2-Butanol and 2-Butanone on Rat Hepatic Ultrastructure and Microsomal Drug Metabolizing Enzyme Activity.* G. J. TRAIGER, J. V. BRUCKNER and P. H. COOKE, Department of Pharmacology and Toxicology, School of Pharmacy, University of Kansas, Lawrence, Kansas. (D. Wenzel)

In the rat, CCl_4-induced hepatotoxicity can be markedly enhanced by a 16-hr pretreatment with either 2-butanol or 2-butanone (Traiger and Robert, *Pharmacologist* 15, 585, 1973). Since the hepatotoxic action of CCl_4 is known to be associated with the microsomal drug oxidase system, the present study was undertaken to determine the effect of 2-butanol (2.2 ml/kg po) and 2-butanone (1.87 ml/kg po) on hepatic ultrastructure and microsomal drug metabolizing activity. Rats were sacrificed 16, 28, and 40 hr after dosing for the in vitro determination of microsomal acetanilide hydroxylase and aminopyrine *N*-demethylase activity. A 50–97% increase in acetanilide hydroxylase activity was found in animals sacrificed 16, 28, and 40 hr after administration of 2-butanol or 2-butanone. Microsomal aminopyrine *N*-demethylase activity was significantly elevated only in those animals given a 40 hr pretreatment with 2-butanone. Less pronounced increases in *N*-demethylase activity were noted in rats sacrificed 16 or 28 hr after dosing with either agent. Electron microscopic examination of hepatocytes 16 hr after either 2-butanol or 2-butanone revealed a marginal increase in the amount of smooth endoplasmic reticulum. However, hepatocytes examined from animals that had been sacrificed 40 hr after receiving 2-butanol or 2-butanone exhibited a marked proliferation of the smooth endoplasmic reticulum. These results would indicate that the potentiation of CCl_4 hepatotoxicity by 2-butanol or 2-butanone may be related in part to their stimulatory effect on the drug metabolizing system of the endoplasmic reticulum. (Supported by a Pharmaceutical Manufacturers Association Foundation Research Starter Grant.)

26. *Hepatotoxicity Produced by the Lung-Toxic Furanoterpenoid, 4-Ipomeanol, after mixed-Function Oxidase Induction: Reversal of Target Organ for Toxicity and Covalent Binding in Rats Pretreated with 3-Methylcholanthrene.* M. R. BOYD, Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee. (B. J. Wilson)

Intraperitoneal administration of toxic doses (LD50, 24 mg/kg) of 4-ipomeanol (1-[β-furyl]-4-hydroxypentanone) to normal rats produces predominantly lung pathology, characterized by pulmonary edema, vascular congestion, and hemorrhage. No gross hepatotoxicity is seen.

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pended solids, oil, oxygen demand, and the usual mineral determinations does not present enough information.

The most probable use for treated refinery wastes is as make-up to recirculating cooling systems, although the value of the water for this purpose will depend upon the organic and inorganic material present in the water. The effects of organic acid salts on a cooling system must be carefully considered in view of the possibilities of their precipitation or their effect on the calcium sulfate equilibrium. In addition, slime control due to the organic material in the treated wastes must be carefully considered because of the normally high chlorine demand of the re-used water. The introduction of a high solids waste as part of the cooling system make-up can increase the necessary blowdown from the system to a point where little benefit would be obtained. Moreover, as the blowdown from a cooling system will contain essentially all of the impurities present in the waste added to the system, but at a higher concentration, it might be necessary to install a second treating system to purify the blowdown for disposal from the plant.

There will be cases where stream requirements necessitate two-stage treatment—for clarification and also for C.O.D. and phenol removal. The clarified water in these cases might be used in a cooling system, with the blowdown being treated biologically prior to disposal. This would not necessarily reduce the size of the treating facilities, but the refinery would have the benefit of the water which was re-used.

The economics of refinery wastes reclamation will depend to a large extent upon the measures that can be taken to reduce the contamination of the wastes which are being considered for re-use. The use of surface condensers instead of barometric condensers will minimize contamination of cooling water. The stripping of sulfur compounds and phenols and the segregation of waste caustics and spent acids will minimize contamination of the wastes and make it more practical to re-use the water economically.

Re-use of water may involve a considerably higher capital expense for the treating facilities. The installation of multiple collection systems to segregate sanitary and strong wastes from the "clean" wastes would be advisable, and the multiple piping systems would add appreciably to the cost. In addition, unless a convenient arrangement for re-using the treated water were possible—such as introduction to the cooling system—a multiple distribution system would be required. Chemically treated wastes could be used for clean-up purposes, but this might involve elaborate changes in the plant piping.

Moreover, the re-use of water would make it necessary to provide a competent operator, with technical supervision for the treating plant. The probability of spills, breakdowns in the plant, and inadvertent dumping of contaminants into the clean water system would require constant vigilance to prevent high solids wastes from being treated and introduced into systems where they might cause trouble.

It is doubtful whether re-use of refinery effluents can be justified in many cases because of the capital and operating costs. However, if the water is urgently needed or if pollution control requirements are very strict, reclamation of refinery effluents is technically feasible.

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Received for review April 15, 1953. Accepted August 28, 1953.

Toxicity of Various Refinery Materials to Fresh Water Fish

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ANTIPOLLUTION legislation has stimulated considerable interest in methods for evaluating toxicity of industrial wastes to fish, because most legislation specifically states that waste water discharges shall not be injurious to aquatic life. As the killing of fish is the most obvious evidence of injury to aquatic life, it was found advisable to develop methods for the evaluation of acute toxicity to fish (*2*). Such tests afford an opportunity, in the laboratory, to determine probable toxic effects and thereby make it possible to avoid the discharge of critical concentrations of wastes to natural waters. The research investigations in the laboratory aid in establishing basic data from which it may be possible to control the discharge of toxic ions or materials by simple chemical analysis. Such investigations also throw light on the effect of oxidation, precipitation, volatilization, and synergistic or inhibiting effects as may occur on dilution in natural waters. The bioassay provides a direct and satisfactory method for evaluating acute toxicity to fish.

Bioassay procedures using fish as test animals have been used in the Waste Control Laboratory of The Atlantic Refining Co. since 1935. This paper presents the results of some of the investigations and experiences with a bioassay procedure and discusses limitations of the test. No effort is made to provide a comprehensive discussion of the scientific principles and details of the test procedure. This has been done elsewhere (*1*, *2*).

SCOPE OF BIOASSAY PROCEDURE

It is necessary to delineate the scope of the bioassay procedure used in the evaluation of toxicity in order to avoid any arbitrary conception or misinterpretation of the results obtained.

Because the killing of fish is the most obvious evidence of injury to aquatic life, the toxicity of various chemicals and materials to the fresh water fish commonly known as bluegill sunfish was investigated. The practical use of the results obtained requires considerable caution because of the influence of local conditions on the toxicity of a specific material to fish. Nevertheless, data obtained from procedures developed afford the best means known at this time for determining probable toxic effects on fish. After permissible concentrations of toxic materials have been determined by bioassay procedures, the more simple and less costly chemical test may be used for control purposes.

The bioassay method used in this study is intended to obtain information on the relative acute toxicity to fresh water fish of the substance or waste tested under prescribed experimental conditions in which the duration of the test is necessarily limited. Acute toxicity is demonstrated by the death of fish in 24 to 96 hours. Chronic or cumulative toxicity is not evaluated by the test.

Cumulative toxicity can affect the growth and reproduction of organisms without killing them, but such effects will appear only after a prolonged time, and their detection is beyond the scope of the bioassay method used in these studies.

Another aspect to toxicity requires clarification. Many wastes exert a high oxygen demand which can cause a depletion of dissolved oxygen in the receiving body of water, resulting in the death of fish due to a lack of oxygen. It is necessary, therefore, to make a distinction between death due to a deficiency of dissolved oxygen and death due to acute toxicity. The bioassay test requires that adequate dissolved oxygen concentrations be maintained throughout the duration of the test in order to evaluate the lethality of the waste per se rather than the indirect or secondary effects such as oxygen depletion. [Special apparatus and techniques are described in the literature (*1*, *2*) for aerating tests so as to minimize loss of volatile toxic constituents.] The toxicity determined by the bioassay test has been defined (*1*) as any direct lethal action of pollutants, including both internal and external effects but excluding indirect action such as depletion of dissolved oxygen through chemical or biochemical oxidation of the waste.

TERMINOLOGY

The recommended index of relative acute toxicity is the median tolerance limit, TL_m, which is defined as the concentration of test material at which just 50% of the test animals survive for a specified period of exposure—24, 48, or 96 hours. At least the 24-hour and 48-hour median tolerance limits should be determined whenever the toxicity is sufficiently pronounced.

A median tolerance limit may be a concentration at which 50% survival actually was observed in a test, provided higher and lower percentages have been recorded for the next lower and next higher test concentrations, respectively; or it may be a value derived by graphical interpolation. In the second case, it is based on observed percentages of test animals surviving at concentrations which were lethal to more than half and to less than half of the animals used as test subjects (*1*, *2*).

If a waste at its maximum test concentration exhibits no toxicity toward test animals after 96 hours' exposure, it may be reported that the substance does not exhibit acute toxicity which is demonstrable and measurable by this test procedure.

SELECTION OF TEST ANIMALS

The results obtained from any toxicity test will depend upon the size and kind of test animal used in the experiment. No standard test animal has been accepted for this purpose, for several reasons. It would be difficult to recommend a standard test animal which would be readily accessible to experimenters in different sections of the country, and toxicities obtained with this standard fish might not be applicable to the fish indigenous to the region in which the test results are to be used. Consequently, it is desirable to utilize test specimens which are representative of the fish fauna of the region in which the test results are to be applied.

The test results reported herein were obtained using the fish known as bluegill sunfish (*Lepomis macrochirus*), which belong to the family *Centrarchidae*, the genus *Lepomis*, and the species *macrochirus*. The sources of the test animals were the Pennsylvania State Hatchery, Torresdale, Pa., and Hiram People's Hatchery, New Providence, Pa.

In order to facilitate the compilation and comparison of toxicity tests performed by various investigators, it is recommended that the choice of test animals be limited to those suggested by the Committee on Development and Standardization of Bio-Assay Methods of the Federation of Sewage and Industrial Wastes Associations (*1*). The selection of test animals from this list will enhance the value of the results obtained and facilitate comparisons with the results of other workers. In any case, the family, genus, and species to which the test animals belong should be reported with the toxicity data.

Although there is no limit to the size of test specimens which may be used, small fish are more desirable than large fish. They use less oxygen and can be maintained more easily in large numbers without overcrowding. Furthermore, they are generally more sensitive to harmful substances and are more adaptable to laboratory conditions than larger specimens of the same species. Uniformity of size and a distribution of sizes such that the average value reported will be representative of the test lot are more important than the sizes themselves. The test specimens used in these tests varied from 5 to 11 cm. in standard length (*2*); the average standard length was 7 cm. The average weight of the test specimens was about 5 grams.

It is desirable to have a stock supply of fish available in the laboratory. Test specimens should not be used until they have been acclimatized for 10 to 14 days.

INTERFERENCE

Bioassay procedures (*1*, *2*) are subject to certain limitations.

The chemical or solution under test is added to the test jar at the start of the test period. No subsequent additions are made during the course of the test. Therefore, the final test concentration is frequently considerably lower than the initial. This may be due to bacterial decomposition, volatilization, precipitation, absorption, and other phenomena.

When a strongly acidic waste is dissolved in the dilution water, it reacts with carbonates naturally present in water and releases carbon dioxide. This may be injurious either directly by its toxic action or by holding in solution other toxic components. Thus, the toxicity observed may be due to carbon dioxide rather than the acid being tested.

Strongly alkaline solutions may exhibit changes due to carbon dioxide produced by the test animals or absorbed from the air.

Relatively nonvolatile compounds may undergo hydrolysis to form volatile compounds—for example, hydrogen sulfide may be produced from solutions of sodium sulfide.

Figure 1. Test Bath and Oxygenation Assembly

Certain chemicals may be oxidized to less toxic forms during the bioassay test.

Some highly toxic metals form insoluble or only slightly soluble carbonates or hydroxides which will precipitate when their soluble salts are added to the dilution water in preparing test dilutions. The actual concentration of toxic metals in the test dilution will, therefore, depend upon the concentration and kind of ions in the dilution water. Under these conditions, different median tolerance limits may be obtained by investigators using different dilution waters.

Because the dilution water may influence the results obtained, it is highly desirable to include its composition with the report of the median tolerance limit.

In selecting a dilution water for determining the toxicity of a waste, it is preferable to obtain that water into which the waste is to be discharged, provided the water may be obtained from a point where there is no pollution from any other source. If uncontaminated dilution water cannot be obtained from the body of water under consideration, a water of similar dissolved mineral content from another source should be obtained or prepared. It is preferable to use a water which does not differ from the receiving body of water by more than 25% in calcium, magnesium, sulfate, and total alkalinity (*1*, *2*).

The temperature of the test may appreciably influence the results. In general, greater toxicity is exhibited at higher temperatures. Higher temperatures may contribute to fungus infections and other diseases which may alter the test results. If test temperatures above 25° C. are contemplated, the lethal temperature for the fish to be tested should be determined.

The same species of healthy fish from different localities may vary in their resistance to toxic materials. Obviously, diseased fish are less resistant than healthy fish.

TEST EQUIPMENT

The equipment required for fish testing included stock tanks, constant-temperature acclimatization tanks, constant-temperature test jars, used fish tanks, air supply, oxygen supply, and dechlorinated fresh water supply.

The test jars used in these investigations were 5-gallon battery jars. Constant temperature control was accomplished by placing the jars in a thermostatically controlled water bath.

The stock and acclimatization tanks, test baths and jars, and reoxygenation equipment are pictured in Figures 1 and 2.

The reoxygenation equipment used almost exclusively in these toxicity determinations has been described (*3*).

An interface contact between pure oxygen and the test sample is maintained inside an inverted funnel immersed in the test solution. The stem of the funnel is extended upward; inside the funnel the stem is extended downward to serve as a guide for the shaft of an agitator which rotates slowly to facilitate the absorption and diffusion of oxygen. In this way, violent bubbling resulting in loss of volatile toxic constituents is avoided, yet adequate dissolved oxygen concentrations can be maintained (Figure 1).

In an apparatus suggested by the Committee on Research, Sub-Committee on Toxicity, Section 3, Federation of Sewage and Industrial Wastes Associations (*1*), a 5-gallon wide-mouthed bottle is used as a test container. A three-hole rubber stopper is fitted with two glass tubes (7 mm. in outside diameter) extending almost to the bottom of the container. Compressed air and oxygen can be introduced through these tubes at a constant rate regulated by a suitable pressure-control valve without bringing about undue loss of volatile materials from the test solution. The third hole in the stopper serves as a vent. A more complete description of this apparatus may be obtained from the Environmental Health Center, U. S. Public Health Service, Cincinnati, Ohio.

CHARACTERISTICS OF DILUTION WATER

In determining the toxicity of industrial wastes, it will be necessary, in general, to prepare several dilutions of the waste in an appropriate dilution water. Philadelphia tap water, filtered through a carbon filter, was used in most of these toxicity determinations. The major characteristics of this water are given in Table I.

TABLE I. CHARACTERISTICS OF PHILADELPHIA TAP WATER

Characteristic	Range in Concentration
Total alkalinity as $CaCO_3$ (to methyl orange), p.p.m.	33.0–81.0
pH	6.9–7.5
Total hardness as $CaCO_3$, p.p.m.	84.0–163.0
Specific conductivity, micromhos	228–301
Dissolved solids, p.p.m.	154–210
Sulfates as SO_4, p.p.m.	56.0–98.0
Chlorides as Cl, p.p.m.	3.0–26.0

In a few cases, synthetic dilution waters were prepared in order to show the effect of dissolved mineral content on the toxicity evaluation. United States mean water (*4*) was used in these cases either in one half or double the concentration of U. S. mean water, unit concentration. The analyses of these synthetic waters are given in Table II. U. S. mean dilution water is recommended as a standard reference water for comparative studies.

BIOASSAY PROCEDURE

The bioassay method employed in this laboratory is in general agreement with procedures recommended elsewhere (*1*, *2*) with respect to basic principles and most details.

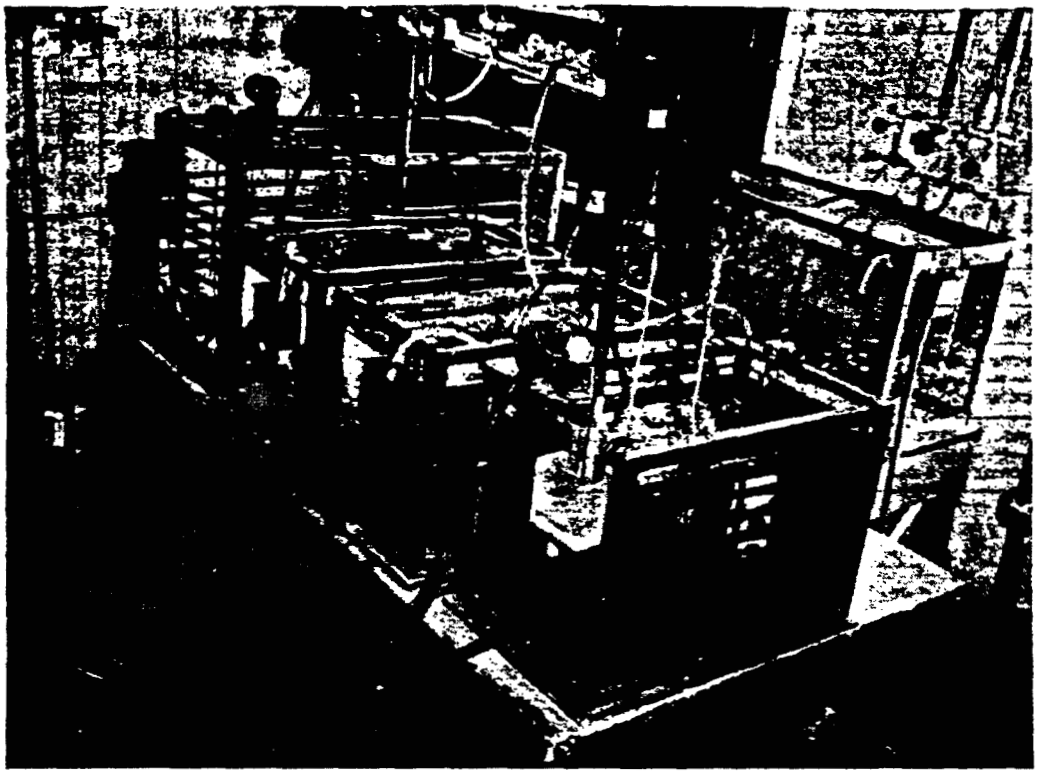

Figure 2. Stock and Acclimatization Tanks

The test jars are filled to the 15-liter mark with dilution water and placed in the (20° C.) constant-temperature bath.

An oxygenation apparatus is inserted in each test jar and the impellers and oxygen supply are turned on.

The chemical to be tested is added to the test jars so as to provide an adequate range of concentrations for testing.

Dissolved oxygen determinations are run on each test jar by siphoning off samples. The dissolved oxygen content is maintained at 5 p.p.m. or more during the entire test period.

Ten acclimatized fish are added to each jar as soon as possible, but not before the dissolved oxygen concentration equals or exceeds 5 p.p.m. In these tests the delay in adding the fish never exceeded 30 minutes.

Acclimatization is obtained by holding the fish for not less than 10 days (at the test temperature of 20° C. in this case) in water similar to that used in the test as dilution water. The fish were not fed during the 24-hour period just prior to the start of the test. The fish deaths in the acclimatization tank never exceeded 3% per day immediately prior to testing. A 5% loss is considered the maximum acceptable.

Twenty-four and 48 hours after the fish had been inserted into the test jars, the number of fish surviving in each jar and the dissolved oxygen content are recorded.

The percentages of fish surviving for 24 hours are plotted against their respective concentrations of chemical on semi-logarithmic paper and a smooth curve is drawn through these points. The intersection of this curve with the line representing 50% survival gives the 24-hour TL_m. A similar procedure is used for locating the 48-hour TL_m.

Table II. Major Constituents of U. S. Mean Water

(Varied mineral concentrations)

Characteristic	Concentration 0.5	1.0	2.0
Total alkalinity as $CaCO_3$ (to methyl orange), p.p.m.	43.0	72.0	144.0
pH	8.0	8.3	8.6
Total hardness as $CaCO_3$, p.p.m.	46.0	101.2	190.0
Specific conductivity, micromhos	142.0	255.0	452.0
Dissolved solids, p.p.m.	96.0	172.0	305.0
Sulfates as SO_4, p.p.m.	46.0	60.0	82.0
Chlorides as Cl, p.p.m.	5.9	9.9	24.0

The concentration of each constituent in U. S. mean water is the average value for that constituent found in the surface waters of the United States (4).

REPORTING TOXICITY RESULTS

Table III is a recommended outline for the data needed in reporting and interpreting toxicity investigations.

DISCUSSION OF OBSERVATIONS

The bioassay tests reported herein were made using C.P. chemicals, industrial products and wastes, and refinery wastes. The TL_m data using C.P. chemicals and industrial products and wastes are found in Table IV. The graphic summary of the 24-hour TL_m data for these materials is shown in Figures 3 and 4. The characteristics of refinery wastes and toxicity data for the same are found in Table V. Figure 5 shows the 24-hour TL_m for refinery wastes. Observations made during the course of some of the tests are reported below.

Chemicals, C.P. Ammonium Hydroxide. The gill region of the fish became covered with a gray mucous sheath. At concentrations of 5 to 10 p.p.m. as NH_3, the chemical apparently caused blindness. This was indicated by the lack of perception to avoid objects.

Sodium Hydroxide. At concentrations of 40 to 50 p.p.m. as OH, the reaction was similar to that of ammonium hydroxide.

Sulfuric Acid. The fish reacted violently immediately on being placed in the test jars at concentrations of 40 to 80 p.p.m. as SO_2. Their bodies were covered with a gray mucuslike sheath. The eyes were the first organs to become visibly affected.

Copper Sulfate. The precipitate formed on admixture of the copper sulfate with the dilution water adhered to the bodies of the fish. All fish became sluggish. The first death, using 0.5 U. S. mean dilution water, occurred in 2 hours at a copper sulfate concentration of 100 p.p.m. The first death, using 2.0 U. S. mean dilution water, occurred in 5 hours at a concentration of 180 p.p.m. In this case, the soluble copper content was 0.86 p.p.m.

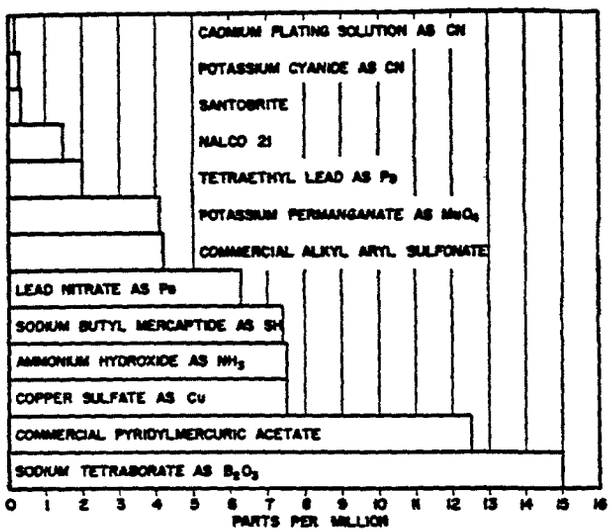

Figure 3. 24-Hour TL_m Values Less Than 15 P.P.M.

Potassium Permanganate. The first death occurred in 2 hours at a concentration of 10 p.p.m. At a concentration of 100 p.p.m., a dark brown film of coagulated mucus covered the body of the fish. When killed by potassium permanganate, the entire body of the fish is so affected.

Potassium Cyanide. A 1.0 p.p.m. concentration of cyanide caused violent activity after about 15 minutes of contact. A heavy discharge of feces occurred. All fish were dead in 1 hour. At 0.5 p.p.m. of cyanide all fish were living at the end of 1 hour, but all had lost equilibrium and appeared to be paralyzed. At 4 hours all fish were floating aimlessly with complete loss of equilibrium and occasional violent spasms. Discomfort was not observed at cyanide concentrations below 0.21 p.p.m.

Sodium Sulfide. Because of sulfide loss, it was difficult to obtain good reproducibility on duplicate runs. The data given are the average of three runs. During the course of the test, the fish appeared to be uncomfortable, but no violent action was observed. They were relatively inactive and remained near the bottom of the test jar. After several hours, loss of equilibrium was noted at 20 to 30 p.p.m. as sulfide ion.

Boron Trifluoride. A synthetic mix was prepared of boron trifluoride in sodium hydroxide solution. The boron trifluoride content was 2.7% and the alkalinity was 1750 p.p.m. The alkalinity of the solution may have contributed to the observed toxicity.

Diethanolamine. The fish became coated with coagulated mucus at concentrations between 1500 and 3800 p.p.m. of diethanolamine.

Furfural. The fish became violent as soon as they were placed in the test jars. Their activity was so great that many jumped out of the jars. They reacted violently in a concentration as low as 21.3 p.p.m.

Maleic Anhydride. The fish became coated with a coagulated mucus in the concentration range of 100 to 150 p.p.m. The mucus was particularly noticeable over the eyes of the fish.

Methyl Ethyl Ketone. The fish lost equilibrium at concentrations of 3380 p.p.m. At 4510 p.p.m. they became paralyzed, with little or no locomotion during the first 20 hours. After this time, equilibrium and locomotion were regained slowly. At concentrations ranging from 3380 p.p.m. to 7000 p.p.m. the fish lost equilibrium in 15 minutes.

Sodium Butyl Mercaptide. The fish lost equilibrium and remained close to the surface of the test solution between 4 and 12 p.p.m. as SH^-. The rate of respiration increased appreciably.

Phenol. The fish moved about violently immediately after being placed in the test jars at concentrations between 13 and 23 p.p.m.

Benzene. A saturated solution of benzene in tap water was prepared at 20° C. The benzene content of this solution was obtained by extracting a sample and measuring the absorption of the extract in the ultraviolet region. At a concentration of 60 p.p.m. all the fish died within 2 hours. At 34 p.p.m. all died within 24 hours. Between 20 and 34 p.p.m. the fish appeared to be in distress; intermittent violent movement was observed.

Industrial Products and Wastes. Nalco 21 (a chlorophenol type of compound). The fish became violent and slowly lost equilibrium with no power to control their direction of motion. The first death occurred in less than 1 hour at a concentration of 10 p.p.m.

TABLE III. REPORT OF TOXICITY INVESTIGATION

1. Substance investigated
2. Date testing began
3. Purpose of investigation
4. Test results at 20° C.
   - 24-hour TL_m
   - 48-hour TL_m
   - 96-hour TL_m
5. Test animals record
   - Species
   - Family
   - Common name
   - Identified by
   - Source
   - Mean size and size range
6. Characteristics of dilution water
   - pH
   - Hardness
   - Alkalinity
   - Sulfates
   - Chlorides
   - Dissolved solids
   - Specific conductivity
7. Properties of substance investigated
8. Test procedure (method of aeration), remarks
9. Observations during test
   - Chemical concentration
   - pH
   - Dissolved oxygen
   - First evidence of discomfort
   - First evidence of loss of equilibrium
   - First death
   - Per cent survival
   - Remarks
10. Post-mortem (external)
    - Fins
    - Scales
    - Operculum
    - Eyes
11. Post-mortem (internal)
    - Gills
    - Brain
    - Circulatory system
    - Liver
    - Intestines
12. Conclusions
13. Date tests completed
14. Investigator

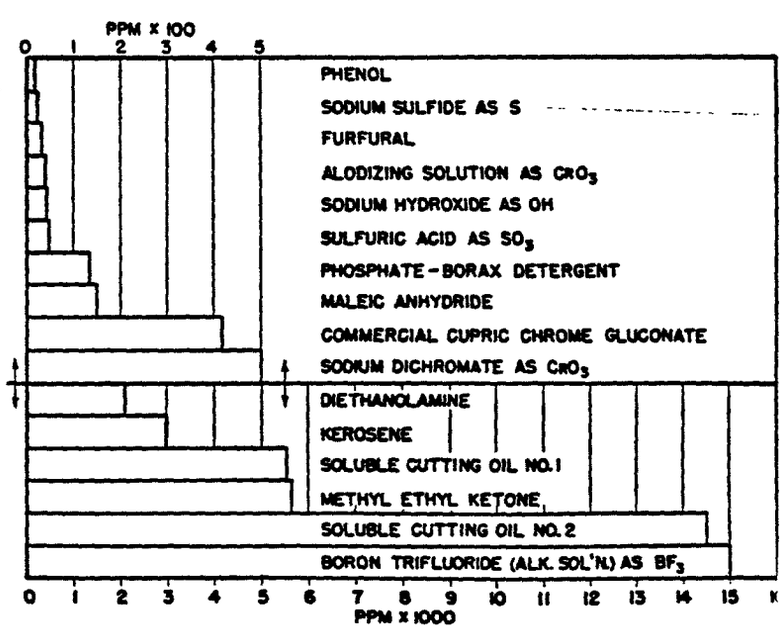

Figure 4. 24-Hour TL_m Values Ranging from 19 to 15,000 P.P.M.

TABLE IV. MEDIAN TOLERANCE LIMIT DATA

Substance Tested	Results Expressed as	Oxygenation Interface Method, P.P.M. 24-hour TL_m	48-hour TL_m	Safe concn.	Experimental Water
		C.P. CHEMICALS			
Ammonium hydroxide	NH_3	(10.0)[a] 7.5	(9.0)[a] 7.5	(2.2)[a] 2.5	Phila. tap
Sodium hydroxide	OH	43	42	12	Phila. tap
Sulfuric acid	SO_3	49	49	15	Phila. tap
Copper sulfate	Cu	3.2	3.0	0.8	0.5 U. S. mean
		7.5	7.0	1.8	1.0 U. S. mean
Copper sulfate[b]		44	44	13	2.0 U. S. mean
Lead nitrate	Pb	6.3	6.3	1.9	Distilled water
Potassium permanganate	MnO_4	4.1	3.9	1.1	1.0 U. S. mean
Sodium dichromate	CrO_3	500	410	76	Phila. tap
Sodium tetraborate	B_2O_3	15	...	...	Phila. tap
Potassium cyanide	CN	0.28	0.28	0.08	Phila. tap
Sodium sulfide	S	25	25	7.5	Phila. tap
Boron trifluoride (alkaline solution)	BF_3	15,000	...	...	Phila. tap
Diethanolamine	$NH(CH_2CH_2CH)_2$	2,100	1,850	430	Phila. tap
Furfural	C_4H_3OCHO	(25)[a] 32	(17)[a] 16	(2.4)[a] 1.2	Phila. tap
Maleic anhydride	$(CHCO)_2O$	(165)[a] 150	(145)[a] 138	(34)[a] 35	Phila. tap
Methyl ethyl ketone	$CH_3COC_2H_5$	5,640	5,640	1,690	Phila. tap
Sodium butyl mercaptide	SH	(4.1)[a] 7.4	(3.5)[a] 5.5	(0.77)[a] 0.92	Phila. tap
Phenol	C_6H_5OH)	19	19	5.7	Phila. tap
Benzene	C_6H_6	20	20	6	Phila. tap
Tetraethyllead	Pb	2.0	1.4	0.20	Phila. tap
		INDUSTRIAL PRODUCTS AND WASTES			
Santobrite	Commercial	0.35	0.35	0.10	Phila. tap
Nalco 21	Commercial	1.5	1.4	0.37	Phila. tap
Cupric chrome gluconate	Commercial	420	365	83	Phila. tap
Pyridyl mercuric acetate	Commercial	12.5	11.3	2.8	Phila. tap
Alkyl aryl sulfonate (commercial dodecabenzene sulfonate)	Commercial	4.2	3.7	0.86	Phila. tap
Phosphate-borax detergent	Commercial	135	112	23	Phila. tap
Soluble cutting oil					
No. 1	Commercial	5,540	...	...	Phila. tap
No. 2	Commercial	14,500	...	...	Phila. tap
Kerosine (dissolved and floating)	Commercial	2,990	2,820	750	Phila. tap
Truck washing soap solution	Commercial	0.28%/vol.	0.28%/vol.	0.084%/vol.	0.5 U. S. mean
	Commercial	0.25%/vol.	0.24%/vol.	0.067%/vol.	1.0 U. S. mean
	Commercial	0.34%/vol.	0.31%/vol.	0.077%/vol.	2.0 U. S. mean
Synthetic truck washing waste	Commercial	0.34%/vol.	0.32%/vol.	0.10%/vol.	0.5 U. S. mean
	Commercial	0.29%/vol.	0.27%/vol.	0.07%/vol.	1.0 U. S. mean
	Commercial	0.35%/vol.	0.32%/vol.	0.08%/vol.	2.0 U. S. mean
Treated synthetic truck washing waste	1:30 dilution	62.0%/vol.	62.0%/vol.	18.6%/vol.	1.0 U. S. mean
	1:7 dilution	15.6%/vol.	15.6%/vol.	4.7%/vol.	1.0 U. S. mean
Cadmium plating solution	CN	0.18	...	...	Phila. tap
Alodine plating solution	CrO_3	39	...	...	Phila. tap

[a] Reoxygenation method proposed by FSIWA Research Committee (*1*).
[b] Soluble copper concentration was 0.86 p.p.m.
All data obtained at 20° C.

Santobrite (a commercial sodium pentachlorophenate). The fish became violent immediately upon being placed in the test jars and lost equilibrium in a short period of time. The loss of equilibrium occurred only during the first 24 hours of the test. The first death occurred in about 17 hours at a concentration of 1 p.p.m.

Cupric Chrome Gluconate. On contact with the test solution, the respiration rate of the fish increased rapidly. Simultaneously, the operculum became distended from the body. The first death occurred within 21 hours at a concentration of 420 p.p.m.

Alkyl Aryl Sulfonate (commercial dodecabenzene sulfonate). During the course of the test, no abnormal reactions were observed prior to death.

Kerosine. The test solution was prepared by mixing a given weight of kerosine with dilution water to saturate the water with kerosine at 20° C. and to provide a very small amount of free-floating kerosine. The concentration of kerosine in solution was not determined. Consequently, the median tolerance limit reported includes both soluble and insoluble kerosine.

Truck Washing Soap Solution. The truck washing soap consisted of pine oil, linseed oil soap, and a commercial alkyl aryl sulfonate. The soap solution was made up from 1 pint of soap and 32 pints of water. The fish lost equilibrium almost immediately on being placed in the test jars. This caused the fish to linger and to force locomotion. The first death occurred in less than 20 minutes at a concentration of 10,000 p.p.m.

Synthetic Truck Washing Waste. Synthetic truck washing waste is the truck washing soap solution to which has been added 8 pints of No. 2 furnace oil. The synthetic truck washing waste using the soap mentioned above was oily and high in suspended solids. The fish neither lost equilibrium nor lingered in the test solutions. The first death occurred in approximately 2 hours at a concentration of 3200 p.p.m.

Treated Synthetic Truck Washing Wastes. Synthetic truck washing wastes that were flocculated with alum and filtered through sand were not so toxic as the untreated wastes. The fish reacted the same to the treated wastes as to the untreated wastes. The first death occurred in 10 minutes at a 2.6% concentration and in approximately 6 hours at a 1.4% concentration (*5*).

Refinery Wastes. The refinery wastes reported herein have been identified as general refinery, fractionation area, cracking area, lubricating oil treating area, paraffin treating area, acid plant area, naphtha treating area, fluid catalyst unit, sulfuric acid alkylation unit, combination unit, distillate tank draw-offs, phenolic waste, carbolate waste, and waste caustic.

The characteristics of these wastes and their toxicity to fish are shown in Table V and Figure 5.

General refinery composite wastes are composed of waste waters from fractionation, cracking, polymerization, lubricating oil treating, paraffin treating, and acid manufacturing units.

Fractionation area wastes are from fractionation, desalting, and polymerization units.

Cracking area wastes include waste water discharges from a fluid catalytic cracker, a combination unit, a desalting unit, and a sulfuric acid alkylation unit.

Lubricating oil treating wastes, discharges from the lubricating oil treating area, include wastes from vacuum distillation units and selective solvent refining units.

Paraffin treating area wastes include waste water emulsions, acidulous waters, and alkaline waters from the acid treating and neutralization of lubricating and paraffin stocks.

Acid plant area wastes are primarily acidulous waters and emulsions from a sulfuric acid sludge decomposition catalytic conversion type of acid plant.

Naphtha treating area wastes include acidulous and alkaline waste waters resulting from the acid treating of some naphtha stocks and the doctor sweetening and caustic washing of naphtha and furnace oil. Waste caustic discharges are not included in this waste.

Fluid catalyst unit wastes include cooling waters and a portion of the condensate draw-offs from a fluid catalytic cracking unit.

Sulfuric acid alkylation unit wastes include continuous acidulous water draw-offs, continuous alkaline water draw-offs, and intermittent leaks and spills from the sulfuric acid alkylation unit.

Table V. Analytical Data on Refinery Water Wastes

(All results expressed as p.p.m., except as noted)

Source of Waste Water	No. of Detns.	Oil R	Oil M	B.O.D. R	B.O.D. M	C.O.D. R	C.O.D. M	Sanitary Analysis: Phenol R	Phenol M	Sulfides R	Sulfides M	Organic Nitrogen R	Organic Nitrogen M	Ammonia as N R	Ammonia as N M	Nitrite as N R	Nitrite as N M	Nitrate as N R	Nitrate as N M	Phosphorus as P R	Phosphorus as P M	Chlorine Demand R	Chlorine Demand M
Fractionation area	21	6	...	17	...	...	...	0.10	...	0	...	...	...	5.0	...	...	...	...	...	...	...	...	...
		38	19.0	40	27	...	...	0.96	0.466	0.3	0.1	...	...	19.0	10.0	...	...	...	...	...	...	...	...
Cracking area	6	48	...	155	...	...	...	0.58	...	T	...	1.7	...	7.2	...	...	...	...	...	0	...	...	...
		200	129	450	232	...	...	12.7	6.6	0.1	0.08	7.4	4.6	31.9	20.5	...	...	...	...	0.65	0.3	...	...
Lube oil treating area	5	23	...	16.6	...	...	...	0.04	...	T	...	0	...	5.8	...	...	...	...	...	T	...	...	...
		73	39.4	53.0	27.5	...	...	0.09	0.05	0.1	0.08	8.4	2.0	11.0	8.1	...	...	...	...	0.14	0.08	...	...
Paraffin treating area	6	20	...	86	...	561	...	...	...	0	...	0.7	...	3.4	...	0.27	...	0.23	...	0.06	...	...	...
		444	182	272	148	2510	1133	0	0	T	0	2.4	1.5	10.6	5.3	0.36	0.3	0.35	0.28	0.73	0.44	...	...
Acid plant area	6	42	...	17	...	770	...	0	...	0	...	2.3	...	1.6	...	0.22	...	0.6	...	0	...	...	...
		5500	1001	272	116	1084	946	44.0	7.35	10.0	1.7	11.0	4.8	8.1	5.7	0.45	0.31	4.3	1.8	3.7	2.2	...	...
Naphtha treating area	12	0	...	19	...	69	...	1.2	...	0	...	0.4	...	0.28	...	0.013	...	0.58	...	0	...	20	...
		210	24	155	54	2690	426	9.4	5.2	0.2	0.017	2.3	1.5	1.84	1.2	0.210	0.058	1.27	1.02	0.15	0.042	80	42
Fluid catalyst unit	13	...	...	256	...	620	...	69	...	40	...	0	...	87	...	0.15	...	0.03	...	0.09	...	500	...
		...	...	1070	554	2333	1391	258	138	305	122	7.0	2.6	443	240	1.60	0.5	0.09	0.056	0.52	0.24	1100	731
Sulfuric acid alkylation unit	9	25	...	15	...	365	...	0	...	...	...	0	...	0.13	...	0.10	...	0.07	...	0.30	...	50	...
		295	139	61	32	2140	1278	0.06	0.025	0	0	4.2	2.3	2.5	1.1	0.26	0.16	0.83	0.35	0.56	0.45	590	303
Combination unit	9	15	...	109	...	496	...	1.2	...	0	...	0.7	...	0.84	...	0.04	...	0.04	...	T	...	8	...
		93	51	413	187	4585	1582	2.5	1.9	0.6	0.09	3.2	2.2	10.2	4.4	0.13	0.097	0.15	0.058	0.11	0.015	23	19
Distillate tank draw-offs	17	6	...	55	...	214	...	0	...	0	...	0	...	2.7	...	0.32	...	0.02	...	0	...	9	...
		230	63.0	7070	1465	16,255	3298	213	20	5000	550	44.0	8.9	8000	1352	0.86	0.5	1.00	0.18	6.06	0.88	5600	970
Phenolic waste 1	1	...	3	...	109	...	216	...	67	...	0	...	...	...	...	...	...	...	...	...	...	...	...
Phenolic waste 2	1	...	T	...	101	...	216	...	62	...	0	...	...	...	...	...	...	...	...	...	...	...	...
Carbolate waste diluted 1/2300	1	...	2	...	119	...	340	...	85	...	0	...	...	...	...	...	...	...	...	...	...	...	...
Waste caustic	1	...	...	...	77,000	...	151,000	...	14,000	...	18,000	...	...	...	...	...	...	...	...	0	...	...	...
Schuylkill River water	17	T	...	2.0	...	...	...	0	...	0	...	...	...	1.2	...	...	...	...	...	0.1	0.033	...	...
		27	6.9	37.1	8.6	...	...	0.14	0.047	0.1	T	...	...	3.2	2.23	...	...	...	...	0.0	...	...	...
General refinery composite	34	15	...	29	...	...	...	0.22	...	0	...	...	...	5.0	...	...	...	...	...	0.62	0.18	...	...
		170	74.6	303	95	...	...	14.5	4.2	18.0	0.62	...	...	17.7	10.2	...	...	...	...	...	...	2	...
General refinery comp. waste	14	67	...	84	...	384	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	364	58
		295	118	315	157	1130	692	...	...	...	...	...	...	...	...	...	...	...	...	...	...	4	...
Treated, alum + silica, 1	10	T	...	9	...	79	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	192	32
		33	12.2	133	48	435	236	...	...	...	...	...	...	...	...	...	...	...	...	...	...	3	...
Treated, alum + silica, 2	15	2	...	11	...	45	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	190	33
		70	20.0	135	51	495	248	...	...	...	...	...	...	...	...	...	...	...	...	...	...	25	...
General refinery comp. waste	14	97	...	92	...	404	...	0.9	...	...	...	...	...	...	...	...	...	...	...	...	...	84	46
		393	200	320	158	2016	898	5.3	2.2	...	...	...	...	...	...	...	...	...	...	...	...	14	...
Treated, alum, 1	14	7	...	21	...	92	...	0.4	...	...	...	...	...	...	...	...	...	...	...	...	...	30	22
		52	20	73	43	400	235	5.8	1.9	...	...	...	...	...	...	...	...	...	...	...	...	19	...
Treated, alum, 2	3	8	...	34	...	136	...	0.8	...	...	...	...	...	...	...	...	...	...	...	...	...	22	20
		59	32	60	43	354	234	1.6	1.3	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Source of Waste Water	Odor: Characteristic	Odor: Intensity		Odor: Threshold		Toxicity, % by Volume: Av. 24-hr. TL_m	Av. 48-hr. TL_m	Av. safe concn.	pH R	pH M	Turbidity R	Turbidity M	Flow, Cu. Ft./Sec. R	Flow, Cu. Ft./Sec. M
Fractionation area	Mercaptan	F	S	2-5	3.7	Nontoxic	Nontoxic	...	7.1	...	38	...	9.3	...
									9.0	7.8	430	129	15.6	12.2
Cracking area	Nitrobenzene	M	S	3-4	3.2	31	28	7.0	2.5	...	50	...	0.9	...
									7.9	5.2	155	90	4.8	2.6
Lube oil treating area	Nitrobenzene	F	M	2-3	2.8	Nontoxic	Nontoxic	...	7.5	...	53	...	...	...
									8.8	8.1	304	118	...	...
Paraffin treating area	SO_2	S	...	0	4	87	36	10.0	2.1	...	107	...	...	...
									3.0	2.4	810	287	...	...
Acid plant area	Oily SO_2	F	S	2-4	3.2	3.1	2.3	0.4	1.4	...	86	...	...	...
									9.1	3.4	248	130	...	...
Naphtha treating area	Nitrobenzene	F	M	1-3	1.9	75	...	...	5.8	...	121	...	2.4	...
									11.0	9.1	785	283	3.4	3.0
Fluid catalyst unit	Oily, phenolic	F	S	2-4	3.3	3.1	3	0.9	8.9	...	53	...	...	...
									9.5	9.1	376	144	...	...
Sulfuric acid alkylation unit	SO_2, gasoline	M	S	3-4	3.7	0.4	0.4	0.1	0.6	...	159	...	...	...
									1.9	1.2	333	276	...	...
Combination unit	Oily	F	M	2-3	2.8	29	25	6	6.4	...	61	...	...	...
									8.8	7.1	166	108	...	...
Distillate tank draw-offs	Oily, sulfide	M	S	3-4	3.6	12	7.1	0.7	6.2	...	80	...	...	...
									9.2	7.7	520	226	...	...

Distillate tank draw-offs	Oily, sulfuric	...	..	..	...	...	...	...	...	9.2	7.7	520	220	...	...
Phenolic waste 1	...	..	..	..	...	...	...	...	...	...	6.7	...	...	...	...
Phenolic waste 2	...	..	..	..	...	...	...	...	...	...	6.7	...	...	...	...
Carbolate waste diluted 1/2300	...	..	..	..	...	...	...	...	...	...	10.1	...	...	...	...
Waste caustic	Phenolic	S	..	..	..	5	0.039	...	...	...	13.0[a]	...	...	...	...
Schuylkill River water	Earthy	VF	M	1-3	1.6	Nontoxic	Nontoxic	...	...	7.2	...	44	...	921	...
General refinery composite	Oily-nitro	F	S	2-4	3.0	21	18	4.0	...	8.7	7.4	820	155	18,500	2376
General refinery comp. waste	Nitrobenzene	F	M	1-3	2.1	55	...	...	...	3.5	...	62	...	14.5	...
Treated, alum + silica, 1	Nitrobenzene	F	..	1-2	1.5	85	...	...	...	9.6	6.9	330	153	22.0	17.8
Treated, alum + silica, 2	Nitrobenzene	F	..	1-2	1.3	77	...	...	...	3.1	...	138	...	...	...
General refinery comp. waste	Oily, nitro	M	S	3-5	3.9	62	...	...	...	10.0	6.8	652	372	...	...
Treated, alum, 1	Oily, nitro	F	..	0	2	88	...	...	...	6.1	...	15	...	...	...
Treated, alum, 2	Oily, nitro	F	..	0	2	75	...	...	...	9.5	7.7	222	84	...	...
										3.5	...	5	...	...	...
										9.5	6.8	330	108	...	...
										3.2	...	345	...	...	...
										9.3	7.6	1157	679	...	...
										6.4	...	28	...	...	...
										7.4	7.1	142	84	..	...
										7.1	...	33	...	...	...
										7.3	7.2	153	92	...	...

Mineral Analysis

Source of Waste Water	Total Hardness $CaCO_3$ R	Total Hardness $CaCO_3$ M	Calcium Ca^{++} R	Calcium Ca^{++} M	Magnesium Mg^{++} R	Magnesium Mg^{++} M	Sodium and Potassium as Na^{+} R	Sodium and Potassium as Na^{+} M	Alkalinity $CaCO_3$ R	Alkalinity $CaCO_3$ M	Chlorides as Cl^{-} R	Chlorides as Cl^{-} M	Sulfate as SO_4^{--} R	Sulfate as SO_4^{--} M	Dissolved Solids R	Dissolved Solids M	Suspended Solids R	Suspended Solids M	Iron Fe R	Iron Fe M	Lead Pb R	Lead Pb M	Copper Cu R	Copper Cu M
Fractionation area	68	...	17	...	6	...	11	...	29	...	3	...	54	...	143	...	33	...	0.21	...	T	...	..	..
	165	120	41	30	15	11	61	39	89	56	34	21	148	106	382	274	542	127	0.98	0.51	0.5	0.09	..	..
Cracking area	134	...	34	...	12	...	65	...	51	...	T	...	213	...	390	...	46	...	0.49	...	0.03	...	T	...
	176	155	45	39	18	14	392	159	94	66	20	8	560	312	968	593	106	72	4.5	1.8	1.0	0.21	32	6.0
Lube oil treating area	60	...	18	...	3.4	...	20	...	53	...	2	...	35	...	152	...	55	...	0.19	...	0.03	...	..	..
	155	120	40	31	14	10	43	35	93	61	20	12.4	111	84	340	262	388	191	0.40	0.3	0.08	0.04	..	..
Paraffin treating area	183	...	45	...	16	...	19	...	220	...	25	...	660	...	1136	...	102	...	19.0	...	0.07	...	..	..
	262	226	73	58	23	20	477	225	710	550[a]	105	50	1665	1023	2893	1888	410	223	90.0	36.4	1.3	0.54	..	..
Acid plant area	173	...	68	...	15	...	26	...	120	66	24	...	755	...	1178	...	64	...	24	...	0.07	...	..	..
	1173	548	256	122	130	55	1250	465	1220-4500	2835[a]	92	41	4250	2469	8959	4816	2020	422	200	95.5	5.6	1.73	..	..
Naphtha treating area	41	...	12	...	4.5	...	4	...	26	...	1.0	...	28	...	128	...	27	...	0.2	...	...	...	..	..
	77	60	20	17	8.2	4.3	170	83	309	102	3.0	1.7	255	116	659	349	842	152	6.0	2.5	...	...	..	..
Fluid catalyst unit	83	...	24	...	6	...	346	...	515	...	7	...	142	...	1342	...	59	...	0.4	...	0.02	...	..	..
	187	152	50	41	16	12	936	575	1740	975	70	26	402	243	3240	2086	131	92	3.7	1.4	0.05	0.026	..	..
Sulfuric acid alkylation unit	92	...	33	...	2	...	437	...	1130	...	100	...	1920	...	3650	...	106	...	1.4	...	0.08	...	..	..
	147	113	47	39	11	4	1342	809	12,325	6879[a]	122	107	12,564	8096	25,468	14,824	239	199	8.3	4.4	1.0	0.78	..	..
Combination unit	125	...	32	...	9	...	19	...	7	...	12	...	134	...	230	...	114	...	0.6	...	0.03	...	..	..
	172	151	47	40	15	12	77	40	34	22	61	20	213	173	367	310	388	216	4.9	3.4	0.07	0.048	..	..
Distillation tank draw-offs	22	...	7	...	1	...	7.1	...	69	...	2	...	14	...	216	...	16	...	1.2	...	0.01	...	..	..
	297	157	68	37	37	16	9370	1458	11,725	1776	5000	733	1450	272	29,533	4666	336	88	69.0	16.6	0.12	0.03	..	..
Phenolic waste 1	...	...	...	...	...	...	...	...	...	...	...	14	...	...	...	...	...	16	...	...	...	...	..	..
Phenolic waste 2	...	...	...	...	...	...	...	...	...	...	...	14	...	...	...	...	...	16	...	...	...	...	..	..
Carbolate waste diluted 1/2300	...	...	...	...	...	...	...	...	...	...	...	4	...	...	...	...	...	26	...	...	...	...	..	..
Waste caustic	...	...	...	...	...	...	...	...	...	123,000	...	...	...	...	...	...	...	...	...	...	...	...	..	..
Schuylkill River water	65	...	16	...	5	...	T	...	23	...	3	...	41	...	91	...	64	...	0.14	...	0	...	..	..
	155	116	40	29	15	11	56	22	78	55	29	12	106	82	303	224	1455	253	1.66	0.59	0.28	0.04	..	..
General refinery comp. waste	47	...	17	...	1	...	8.3	...	7	...	T	...	94	...	184	...	60	...	0.25	...	0	...	..	..
	192	139	56	37	17	11	183	63	188	62	84	19	372	182	666	375	278	112	4.5	1.2	0.82	0.98	..	..
Treated, alum + silica, 1	...	...	...	...	...	...	...	...	14	...	...	...	...	...	...	...	98	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	135	54	...	...	...	...	...	...	304	177	...	...	...	...	..	..
Treated, alum + silica, 2	...	...	...	...	...	...	...	...	21	...	...	...	...	...	...	...	4	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	116	58	...	...	...	...	...	...	88	31	...	...	...	...	..	..
General refinery comp. waste	...	...	...	...	...	...	...	...	9	...	...	...	...	...	...	...	4	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	115	50	...	...	...	...	...	...	80	29	...	...	...	...	..	..
Treated, alum, 1	...	...	...	...	...	...	...	...	33	...	...	...	...	...	...	...	92	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	150	84	...	...	...	...	...	...	316	196	...	...	...	...	..	..
Treated, alum, 2	...	...	...	...	...	...	...	...	20	...	...	...	...	...	...	...	8	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	106	53	...	...	...	...	...	...	64	29	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	43	...	...	...	...	...	...	...	8	...	...	...	...	...	..	..
	...	...	...	...	...	...	...	...	150	114	...	...	...	...	...	...	44	26	...	...	...	...	..	..

F. Faint odor
M. Mean
R. Range
S. Slight odor
T. Trace
VF. Very faint
Odor threshold is log of reciprocal of lowest dilution with odor-free water which has no perceptible odor.
[a] Acidities.

Combination unit wastes include wastes from a desalting operation, condensate wastes, cooling waters, and miscellaneous leaks and spills.

Distillate tank draw-offs include waste waters from a running tank area in which ammonium polysulfide condensate waters accumulate as tank bottoms.

Phenolic wastes are synthetic wastes made from Schuylkill River water and pure phenol.

Carbolate wastes result from the caustic washing of catalytically cracked naphtha stocks. The waste is primarily phenolic in composition.

Waste caustic results from the doctor sweetening of naphtha stocks. The organic matter content is approximately evenly distributed between phenolates and naphthenates.

All of the waste waters referred to above are discharged into the Schuylkill River. The characteristics of Schuylkill River water are found in Table V. Investigations demonstrated that the river water was nontoxic to bluegill sunfish 100% of the time.

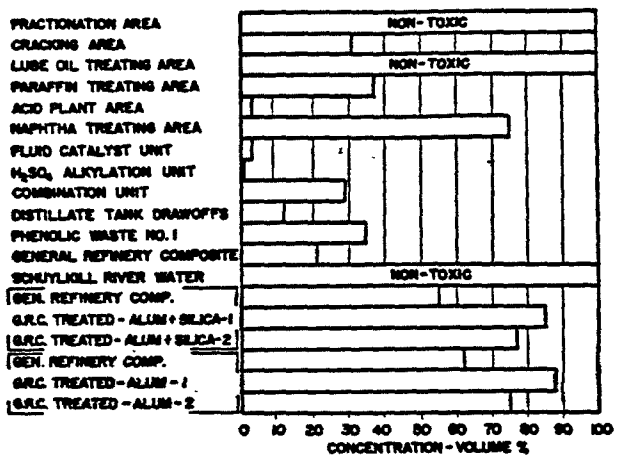

Figure 5. 24-Hour TL_m Values of Refinery Wastes

Most samples used for testing purposes from the general refinery and the various areas and units were 24-hour composite samples collected by an automatic sampler provided with a refrigerated storage compartment.

The general refinery composite wastes were treated by chemical flocculation procedures using pilot plant equipment. The average TL_m for one group of these wastes was 55% by volume. The average TL_m for these wastes, when treated with alum and activated silica, was 77% by volume for one experimental unit and 85% by volume for the other unit. There were minor differences in treatment procedure for the two treatment units, which resulted in a difference in efficiency of operation. The average TL_m for another group of refinery composite wastes receiving treatment was 62% by volume. The average TL_m of the alum-treated wastes from two duplicate units receiving slightly different treatment procedures were 75 and 88% by volume. In this case, also, there were minor differences in efficiency of operation due to differences in the treatment procedures.

During the completion of experimental investigations on phenolic wastes, it was not possible to obtain bluegill sunfish for bioassay purposes; therefore, goldfish were used. Comparative test data on the toxicity of materials to bluegill sunfish and goldfish indicate that the goldfish are considerably more tolerant to phenolic materials. The 24-hour TL_m of sunfish for a given phenolic waste was 35% by volume; using goldfish it was 94% by volume. A repeat test on the same type of material indicated a 49% by volume 24-hour TL_m for sunfish and a nontoxic waste for goldfish.

For comparing the effects of oxygenation procedure on the test results, comparative tests were run using the pure oxygen-water interface oxygenation equipment described by Hart, Weston, and DeMann (3), and the more simple controlled bubbling rate oxygenation equipment described by Doudoroff *et al.* (1). As shown in Table IV, there is but a minor difference in the results obtained by the two oxygenation procedures.

APPLICATION OF TEST DATA

The test procedure used in these investigations measures the acute toxicity of the test material to the test animals—i.e., it determines that concentration of test material which will cause death in a relatively short time. In order to obtain reproducible results and statistically significant data, it is advisable to use the concept of median tolerance limit, TL_m (1, 2), the concentration of material required to kill 50% of the test animals in a given period of time. The test results indicate the concentration that will kill fish rather than the concentration that will not kill fish. Therefore, in using the data for water pollution control it is necessary to apply a factor of safety in determining "safe concentrations." The safe concentration formulas try to account for the effect of time of contact on toxicity. In general, lower TL_m values are obtained as the test time increases. The combined 24-, 48-, and 96-hour data generally provide sufficient information to approximate TL_m values for infinite time. The adequacy of these data can be determined by observing the differences between successive values of TL_m as time increases. These differences should decrease as time increases, so that consecutive TL_m values approach a constant. As there is limited field experience in determining safe concentrations at this time, the formulations referred to below must be considered as strictly preliminary. They have not been formally sanctioned by any committee of experts. The use of these formulations may be unreliable in many applications. As used in this paper, the safe concentration is defined as follows (2):

$$\text{Safe concentration} = \frac{\text{24-hour } TL_m \times 0.3}{(\text{24-hour } TL_m/\text{48-hour } TL_m)^3} \quad (1)$$

or

$$\text{Safe concentration} = \frac{\text{48-hour } TL_m \times 0.3}{(\text{24-hour } TL_m/\text{48-hour } TL_m)^2} \quad (2)$$

where the exponent 3 (in Formula 1) and exponent 2 (in Formula 2), as well as the factor 0.3 are arbitrary "safety factors." The units in which the safe concentrations are expressed are the same as those in which the TL_m values are expressed—for example, if a waste has a 24-hour TL_m of 20% by volume and a 48-hour TL_m of 15% by volume, then from Equation 1 the safe concentration will be:

$$\text{Safe concentration} = \frac{20 \times 0.3}{(20/15)^3} = 2.54\% \text{ by volume}$$

The same value is obtained by applying Formula 2.

Numerous factors influence the results of the test and must be considered in the application of the test data to field conditions. The test conditions afford but a small volume of test solution per fish. Therefore, in some cases, it is possible for the fish to oxidize, absorb, or otherwise alter the composition of the test material. There are cases in which 24-hour and 48-hour TL_m results cannot be obtained as such because of the effect of the fish on the toxic material. Materials such as phenol, for instance, are unstable under the test conditions. In some cases, if the fish are not killed in the first hour or so they will survive indefinitely. In such a case, the test results may indicate a lower degree of toxicity—i.e., higher TL_m—than would occur in nature. In other cases, the absolute weight of toxic material required to kill a fish may necessitate a relatively high concentration in the small volume used for the test. If this is the case, the laboratory TL_m could be much higher than that for field conditions.

In some cases, the toxic material is volatile. Such materials would be lost from solution rapidly in turbulent streams and slowly in sluggish streams. The rate of loss for the test conditions reported herein simulates the anticipated losses from a sluggish stream. Because losses occur during the course of a test, the TL_m data reported for volatile materials are relatively high.

The composition of the dilution water in the test solution may affect the results appreciably. Experience has shown that hardness, alkalinity, pH, and organic matter content of the water may effect the toxicity of a given material. The precipitation of toxic ions, the inhibition of toxic effects due to the antagonism of one ion toward another, such as the antagonism of the calcium ion toward the sodium ion, and the formation of relatively nontoxic complexes all can affect toxicity. In some cases, combinations of ions or materials are more toxic than the individual ions or materials alone.

The test data obtained apply only to the species of fish being tested. More sensitive forms may be present in the stream. In general, "game" fish are more sensitive than "rough" fish—for instance, bluegill sunfish are much more sensitive to phenol than are goldfish. Therefore, it is advantageous to use fish in testing programs that have approximately the same sensitivity as the game fish indigenous to the stream under consideration.

The temperature of the receiving body of water will affect the toxicity of a given material. In general, higher temperatures are conducive to greater toxicity.

The test procedures outlined above measure short-time effects only. The long-term influence of the waste is not measured, nor is the effect of the waste on the food supply of the fish. Thus, the disposal of a waste may destroy fish life over a period of time by cumulative effect of toxic materials or by the destruction of fish food life without being responsible for a mass killing of fish.

The toxicity results indicate the toxicity for only that size of fish used in the test. There are times when fry are more sensitive to toxic materials than the adult fish. On the other hand, there have been times when the larger fish have been affected by toxic materials not affecting small fish or fry.

The test procedures are not well suited to control testing for continuous discharges, unless considerable storage time is available, because there is an appreciable delay in obtaining results. The procedures do provide an excellent research method for studying the relative effects of the many variables that may be encountered in practice.

The equipment required for stocking, acclimatizing, and testing the fish is relatively complex and expensive. Considerable space is required and temperature-control equipment is essential. The test procedures necessitate the killing of test animals. Fish are relatively expensive, and approximately 100 or more fish are required to obtain a measurement of median tolerance limit. In the Philadelphia area, goldfish cost 3 cents apiece and bluegill sunfish cost 15 cents apiece delivered to the laboratory. It can be expected that losses will be as much as 20%. Therefore, the cost of fish alone may amount to $18 per TL_m test in the case of bluegills. The cost of food and care may be expected to increase the unit cost per fish to 35 cents. In some cases, it is possible to recover and reacclimatize fish for the testing of other materials: but fish must be re-used with caution. Approximately 5 hours of a man's time is required to run a TL_m test, if no special chemical testing is required.

Values for the relative acute toxicity of a waste are useful for estimating safe disposal rates; they also provide information on the effectiveness of treatment processes. In proposing estimated safe disposal rates from relative acute toxicity data, an extensive survey of the receiving body of water may be required to confirm the validity of the estimates and establish the absence of harmful effects over a prolonged period of time.

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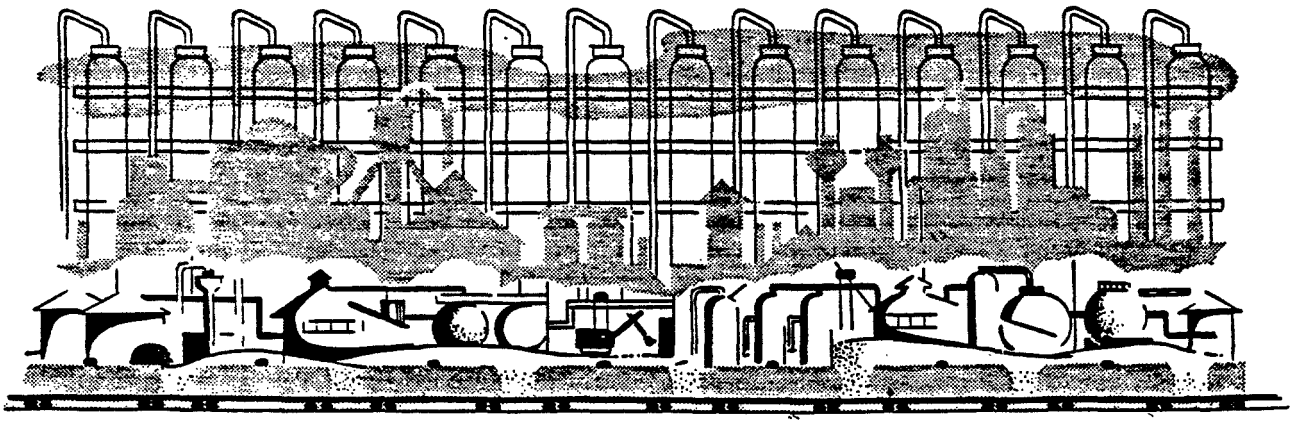
Received for review April 28, 1953. Accepted September 19, 1953

END OF SYMPOSIUM



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HANDBOOK OF ENVIRONMENTAL DATA ON ORGANIC CHEMICALS

SECOND EDITION

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VAN NOSTRAND REINHOLD COMPANY
NEW YORK CINCINNATI TORONTO LONDON MELBOURNE

p,p'-methylenedianiline *see* 4,4-diaminodiphenylmethane

methylenedimethylether *see* dimethoxymethane

4,5-methylenephenanthrene

Manmade sources: in exhaust condensate of gasoline engine: 0.47–0.76 mg/l gasoline consumed. (1070)

A. PROPERTIES: solub. 1.1 mg/l at 24°C

2-(1-methylethoxy)phenolmethylcarbamate *see* baygon

methylethylcarbinol *see sec*-butanol

methylethylene *see* propylene

***sym*-methylethylethylene** *see* 2-pentene

methylethylketone (MEK; 2-butanone)

$CH_3COCH_2CH_3$

Manufacturing source: organic chemical industry. (347)

Users and formulation: solvent or swelling agent of resins; intermediate in mfg. of ketones and amines; flush-off paint stripper; extraction and production of wax from lube oil fractions of petroleum; solvent in nitrocellulose coatings and vinyl films; cements and adhesives; smokeless powder mfg.; cleaning fluids; printing catalyst and carrier. (347)

Man caused sources (*water and air*): general use as a solvent, evaporation from applied paints and coatings, cements, adhesives, cleaning fluids; lab use. (347)

A. PROPERTIES: colorless liquid; m.w. 72.1; m.p. −86.4°C; b.p. 79.6°C; v.p. 77.5 mm at 20°C; v.d. 2.41; sp.gr. 0.805 at 20/4°C; solub. 353 g/l; at 10°C; 190 g/l at 90°C; THC 583 kcal/mole; LHC 550 kcal/mole; log P_{oct} 0.26

B. AIR POLLUTION FACTORS: 1 mg/cu m = 0.340 ppm, 1 ppm = 2.94 mg/cu m

—Odor: characteristic: quality: sweet, sharp
hedonic tone: neutral to unpleasant

T.O.C.: recogn.: 32–80 mg/cu m (73)
25 ppm = 80 mg/cu m (278)(210)
30 mg/cu m (291)

$PIT_{50\%}$: 4.68 ppm

$PIT_{100\%}$: 10 ppm (2)

abs. perc. limit: 2.0 ppm

50% recogn.: 5.5 ppm

100% recogn.: 6.0 ppm

O.I. 100% recogn.: 15,350 (19)

T.O.C.: 25 mg/cu m = 8.5 ppm (279)
14 mg/cu m = 4.7 ppm (307)
distinct odor: 163 mg/cu m = 50 ppm (278)

—Manmade sources:

in cigarette smoke: 500 ppm (66)

in gasoline exhaust: <0.1–1.0 ppm (195)

in exhaust of a 1970 Ford Maverick gasoline engine operated on a chassis dynamometer following the 7-mode California cycle:
from API #7 gasoline: 2.6 ppm
from API #8 gasoline: 1.4 ppm (1053)

–Atmospheric reactions:
estimated lifetime under photochemical smog conditions in S.E. England: 1[illegible] hr (1699; 1700)

–Control methods: catalytic combustion: platinized ceramic honeycomb catalyst: ignition temp.: 175°C; inlet temp. for 90% conversion: 300–350°C (91)

–Sampling and analysis: photometry: min. full scale: 1,360 ppm (53)

C. WATER POLLUTION FACTORS:

–Oxidation parameters:

BOD_5:	1.92 NEN 3235–5.3	(277)
	1.515; 1.81	(30)(36)
	2.24 std. dil. sewage	(41)(27)
	46% ThOD	(220)
BOD_5^{20}:	1.81 at 10 mg/l, unadapted sew.: lag period: 1 day	
BOD_{20}^{20}:	1.92 at 10 mg/l, unadapted sew.	(554)
COD:	2.31 NEN 3235–5.3	(277)
	2.20	(36)
	100% ThOD	(220)
$KMnO_4$:	0.029	(30)
	acid: 7% ThOD	
	alkaline: 7% ThOD	(220)
TOC:	100% ThOD	(220)
ThOD:	2.44	(36)

–Odor threshold conc. (detection): 1.0 mg/l (998)

–Manmade sources:
PVC pipe cement used for joint connection caused 4.5 ppm, 6 months after PVC pipe installation and 2.2 ppm after 8 months (at equilibrium condition—reached in about 48 hr) (1670)

–Waste water treatment: A.C.: adsorbability: 0.094 g/g C; 46.8% reduction, infl. 1,000 mg/l, effl.: 532 mg/l; (32)
reverse osmosis: 72.9% rejection from a 0.01 M solution (221)
anaerobic lagoon: lb COD/day/1,000 cu ft infl. mg/l effl. mg/l
13 10 5 (37)
A.S.: after 6 hr: 0.4% of ThOD
12 hr: 0.8
24 hr: 3.1 (88)

D. BIOLOGICAL EFFECTS:

–Toxicity threshold (cell multiplication inhibition test):

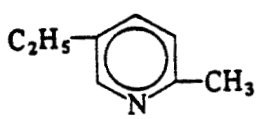
bacteria (*Pseudomonas putida*):	1150 mg/l	(1900)
algae (*Microcystis aeruginosa*):	110 mg/l	(329)
green algae (*Scenedesmus quadricauda*):	4300 mg/l	(1900)
protozoa (*Entosiphon sulcatum*):	190 mg/l	(1900)
protozoa (*Uronema parduczi Chatton-Lwoff*):	2830 mg/l	(1901)

–Bacteria:
Pseudomonas: LD_0: 2.5 g/l

—Algae:
Scenedesmus: LD_0: 12.5 g/l
Protozoa: *Colpoda*: LD_0: 5 g/l (30)
—Fish: mosquito fish: TLM (24–96 hr): 5,600 mg/l;
bluegill: TLM (24–96 hr): 5,640–1,690 mg/l in tapwater (41)(247)
goldfish: LD_{50} (24 hr): 5,000 mg/l, modified ASTM D 1345 (277)
—Mammalia:
rat: single oral lethal dose: 3.3 g/kg
guinea pig: inhalation: no serious disturbance: 10,000 ppm, 1 hr
rat: inhalation: no death: 2,000 ppm, 2 hr
4/6 deaths: 4,000 ppm, 2 hr (211)
—Man: no permanent ill effects: 700 ppm; complaints: >300 ppm (211)

methylethylketoxime *see* 2-butanoneoxime

2-methyl-5-ethylpyridine (aldehydine)



Manmade sources:
in coke oven emissions: 24.4–73.5 μg/g of sample
in coal tar (n.s.i.): 3.12 mg/g of sample (n.s.i.)
in wood preservative sludge: 0.37 g/l of raw sludge (993)

2-methylfluorene
Manmade sources:
in wood preservative sludge: 3.82 g/l of raw sludge
in coke oven emissions (n.s.i.): 8.9–98.7 μg/g of sample (993)

9-methylfluorene
Manmade sources:
in wood preservative sludge: 1.44 g/l of raw sludge
in coke oven emissions: 6.7–102.8 μg/g of sample (993)

methylformate (methylmethanoate)
$HCOO—CH_3$
Use: organic synthesis; cellulose acetate solvent; military poison gases; fumigant; larvicides

A. PROPERTIES: colorless liquid; m.w. 60.05; m.p. -99.8°C; b.p. 32°C; v.p. 480 mm at 20°C; 700 mm at 30°C; v.d. 2.07; sp.gr. 0.975 at 20/4°C solub. 304 g/l at 20°C; satur. conc. 1,569 g/cu m at 20°C, 2,213 g/cu m at 30°C (57)

B. AIR POLLUTION FACTORS: 1 mg/cu m = 0.40 ppm, 1 ppm = 2.50 mg/cu m
—Odor: odor threshold values: recognition: 5,000 mg/cu m = 2,000 ppm
165–180 mg/cu m (73)(210)(610)
distinct odor: 6,900 mg/cu m = 2,750 ppm (278)

Mutation Research, 149 (1985) 339–351

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REF 60

~~OPTS-400026~~
E3-001 (4)

Acetone, methyl ethyl ketone, ethyl acetate, acetonitrile and other polar aprotic solvents are strong inducers of aneuploidy in *Saccharomyces cerevisiae*

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(Received 17 August 1984)
(Revision received 11 October 1984)
(Accepted 19 November 1984)

Summary

A diploid yeast strain D61.M was used to study induction of mitotic chromosomal malsegregation, mitotic recombination and point mutation. Several ketones (including acetone and methyl ethyl ketone) and some organic acid esters (including the methyl, ethyl and 2-methoxyethyl esters of acetic acid) and acetonitrile strongly induced aneuploidy but not recombination or point mutation. Only diethyl ketone induced low levels of recombination and point mutation in addition to aneuploidy. Related compounds were weak inducers of aneuploidy: methyl *n*-propyl ketone, the methyl esters of propionic and butyric acid, acetic acid esters of *n*- and iso-propanol and ethyl propionate. No mutagenicity was found with *n*-butyl and isoamyl acetate, ethyl formate, acetyl acetone (2,5-dipentanone) and dioxane. Methyl isopropyl ketone induced only some recombination and point mutation but no aneuploidy. Efficient induction was only observed with a treatment protocol in which growing cells were exposed to the chemicals during a growth period of 4 h at 28°C followed by incubation in ice for more than 90 min, usually overnight for 16–17 h. Aneuploid cells could be detected in such cultures during a subsequent incubation at growth temperature if the chemical was still present. Detailed analysis showed that there was a high incidence of multiple events of chromosomal malsegregation. It is proposed that the mutagenic agents act directly on tubulin during growth so that labile microtubules are formed which dissociate in the cold. When cells are brought back to temperatures above the level critical for reassembly of tubulin and allowed to grow, faulty microtubules are formed.

Abnormal chromosome segregation in human germ cells results in a number of severe genetic defects such as Klinefelter's, Turner's or Down's syndromes. Moreover, the most frequently observed genetic aberration found in spontaneous human abortions is chromosomal aneuploidy (Sankaranarayanan, 1979). Such untoward genetic defects represent a significant health problem to human populations (Newcombe, 1979). It has been recognized that some chemicals can cause chromosomal malsegregation in test organisms designed to detect aneuploidy and, by inference, may have the same effects in exposed human popula-

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0027-5107/85/$03.30 © 1985 Elsevier Science Publishers B.V. (Biomedical Division)

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tions. It is also apparent from recent studies that some chemicals can induce aneuploidy but no other types of genetic effects such as point mutation or mitotic recombination (Gualandi and Bellincampi, 1981; Parry et al., 1979, 1981; Zimmermann, 1983). To fully understand the genetic effects of an agent it is imperative that chromosomal malsegregation be investigated.

We developed a system with the yeast *Saccharomyces cerevisiae* for detecting the induction of mitotic aneuploidy (Parry and Zimmermann, 1976). The strain and system based on chromosome loss in mitotically growing cells are currently under investigation so as to enhance the responsiveness of the test. In the course of these studies a number of bipolar aprotic solvents including acetone, methyl ethyl ketone and ethyl acetate were found to induce aneuploidy but no other types of detectable genetic effects in yeast. We report here on a systematic investigation into the genetic activity of this class of chemicals and also propose a mutagenic pathway which is neither initiated by a primary reaction with DNA nor does it lead to an alteration of DNA. Rather, we propose that such agents are mutagenic because they interfere with tubulin assembly and therefore, formation and function of microtubules.

Materials and methods

The test system. The diploid strain used in this study is D61.M (Zimmermann and Scheel, 1984). The full genotype is:

cyh2	+	*leu1*		*ade6*	*ade2*	*ilv1-92*
+	*trp5*	+	centromere	+	*ade2*	*ilv1-92*
			MATa *MAL2*	*SUC*	*HIS*	
centromere			*MATα* *mal*	*suc*	*his*	

Cells of strain D61.M form red rather than the usual white colonies because of the homozygous condition of *ade2*. They require isoleucine because of the homozygosis of *ilv1-92*, and they can utilize maltose and sucrose/raffinose since *MAL* and *SUC* are dominant. They are sensitive to 1.5 ppm cycloheximide, and do not require leucine, tryptophan nor histidine because markers *cyh2*, *leu1*, *trp5* and *his* are recessive. The aneuploidy detection system is based on the uncovering of a group of coupled recessive markers on chromosome VII. The simultaneous expression of *cyh2* and *ade6* is indicative of mitotic chromosome loss of the homolog containing the dominant wild-type alleles. Confirming evidence is provided if the cells also express *leu1*. Expression of only one or two of the markers on the same side of the centromere is indicative of point mutation, chromosomal fragment loss or recombination. There are additional recessive markers on other chromosomes. The mating type alleles *MATa* and *MATα* confer the ability for diploid cells to undergo meiosis and to sporulate. In the event that one homolog III is lost, one or the other of the two alleles will be expressed and enable such a cell to act, in part, like a haploid cell i.e. to mate with another cell of the opposite mating type. Linked to *MATα* is a recessive inactive allele *mal2*. The dominant and functional allele *MAL2* enables maltose utilization. There is also a heterozygous condition for an unknown but dominant *SUC* gene which enables the utilization of sucrose and raffinose. Expression of its inactive and recessive counterpart can be used along with a heterozygous condition for an unidentified defect in histidine biosynthesis, *his*, to indicate losses of chromosomes other than number VII and to detect possible trisomic conditions using tetrad analysis.

Media. Two basic media were used: rich and synthetic. The rich medium consisted of 2% peptone, 2% glucose and 1% yeast extract (YEPD). The media used to diagnose the presence of dominant genes *MAL* and *SUC* media contained in addition to yeast extract and peptone either 2% maltose or 3% raffinose and were supplemented with 2 ppm antimycin A in order to block respiration so that fermentation of the two diagnostic sugars was the only means for growth. Expression of *cyh2* was detected on a YEPD medium with 1.5 ppm cycloheximide. A synthetic medium, consisting of 1.7 g/l Difco yeast nitrogen base without amino acid and ammonium sulfate, was supplemented with 5 g/l $(NH_4)_2SO_4$ and 2% glucose. The complete synthetic medium was supplemented with 10 mg/l adenine, 10 mg/l histidine, 60 mg/l isoleucine, 30 mg/l leucine and 10 mg/l tryptophan (free L-forms). In order to diagnose

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expression of recessive *ade2* which causes the formation of red colonies, the adenine content was reduced to 4 mg/l. All the other nutritional markers were diagnosed on the synthetic medium with one of the supplements omitted (drop out media). Sporulation was induced on a solid 1% potassium acetate medium. Media were solidified with 10 g/l Oxoid agar.

Preparation of cultures. Approximately 200 cells were inoculated into 5-ml volumes of YEPD in metal capped test tubes and incubated on a reciprocal shaker at 28°C for about 36 h to a titer of about 5–8 × 10^7 cells per ml. Samples of 0.1 ml were spread on YEPD plates with 1.5 ppm cycloheximide; the tubes were stored in the refrigerator until the resistant colonies could be scored (about 5 days later). Only cultures with a normal background of red and white resistant cells were used for experiments and were kept for up to 3 weeks. Before an experiment, such refrigerated cultures were diluted 1:50 into fresh YEPD medium and shaken at 28°C for about 4 h to bring cells back into growth phase.

Purity of chemicals. Ethyl formate was only 95% pure. The other chemicals were at least 97% pure or better and most were of analytical grade quality.

Treatment protocol. The chemicals studied were all liquid and were pipetted directly into a uniform volume of 2 ml of a growing culture in YEPD at cell titers between 3 and 8 × 10^6 cells/ml in metal capped 10-ml centrifuge tubes. They were incubated for 4 h, then placed into an ice bath for 16 or more hours. The cultures were incubated again on a shaker at 28°C for another 4 h before plating. Cultures were adjusted if necessary to cell titers of between 20 and 30 × 10^6 cells/ml according to hemocytometer counts. Samples of 0.1 ml were plated without washing on the selective cycloheximide medium (usually 3 plates per concentration). Platings on a non-selective synthetic medium were done from 1:10000 or 1:1000 dilution in order to determine the viable titers.

The chemicals tested are quite volatile and a loss by evaporation from the metal capped tubes led to an impaired reproducibility. The alternative would be hermetic sealing of the tubes. However, this would have created another problem because such a regimen leads to anaerobic conditions and induction of respiratory-deficient mitochondrial mutants which can be white on the selective medium and create unnecessary testing of white colonies for expression of *leu1*. Therefore, in spite of occasional variations in reproducibility between experiments, we routinely used capped tubes.

Evaluation of experiments. The selective cycloheximide containing media plates could be scored after about 6–7 days for the colony color and colony numbers. The red colonies expressed as frequency per 10^5 colony-forming units were usually scored. They reflect the cumulative effects of other types of genetic events like point mutation, mitotic recombination or deletion of chromosomal fragments. Control values ranged between 10 and 30 red colonies per 10^5 colony-forming units. The presumptive monosomics are found among the white resistant colonies. In the absence of high levels of recombination, chromosomal loss events are confirmed by the concomitant leucine requirement. The white cycloheximide-resistant colonies were examined further both for leucine requirement and to confirm that they were really white. Mere counts of white colonies are only permissible when there is a strong induction of white colonies on the selective medium and previous testing has proved that most of these are in fact requiring leucine. The spontaneous frequencies of such colonies are usually very low (generally less than 1×10^{-6}).

The normal incidence of white and leucine-requiring resistant colonies is usually too low for a reliable determination of the frequencies of aneuploids in control cultures. Therefore, the use of a positive control agent was indicated, such as ethyl acetate, methyl ethyl ketone or acetonitrile in order to demonstrate the proper functioning of the test.

Presentation of data. All treatments were done with growing cells. Final titers of all control and experimental samples are given in the first column of the tables. The next entry is the total number and frequency of resistant (both red and white) colonies. Finally, the number and frequency per 10^6 colony-forming units of white, leucine-requiring resistant colonies is given.

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Results

The agent acetone which initially was used as a solvent for various chemicals was found to induce aneuploidy albeit in an inconsistent fashion. We found, however, that overnight storage on ice of cells in growth medium containing acetone resulted in a very strong induction as shown in Table 1. A considerable fraction of the cycloheximide-resistant colonies were white and almost all of those were leucine-requiring. The relative frequencies were also very high, 100 times above the value in the concurrent control. In contrast to this, red resistant colonies did not increase and only 4.1% of the red resistant colonies showed a leucine requirement demonstrating that acetone does not induce point mutation or recombination under these conditions. Based on these results, the white and leucine-requiring cycloheximide-resistant colonies are concluded to arise from chromosome malsegregation.

The dramatic effect of storage in ice was in-

TABLE 1

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH ACETONE: ANALYSIS OF WHITE RESISTANT COLONIES

Sample	Titer $\times 10^6$	Resistant colonies		White resistants		Monosomics $\times 10^6$
		Total	White	Tested	leu	
Control	84.5	755	6	6	2	0.26
Acetone						
6.98%	20.2	937	187	57	54	19.95
7.41%	15.5	782	210	37	36	26.85
7.83%	7.7	570	161	51	50	34.26

The numbers in columns with headings 'Resistant Colonies', 'Total' and 'White' refer to counts on 3 plates with cycloheximide medium. 171 red resistant colonies were also picked from plates seeded with cells after exposure to 7.83% acetone; only 7 (4.1% of the red colonies tested) were found to require leucine. Acetone was added to a growing culture at a titer of 7.1×10^6 cells/ml and incubation continued for 5 h before a 17-h storage in ice. This was followed by a 7-h period at growth temperature. Cell titers were adjusted by dilution before plating. The titers are based on colony counts on a non-selective medium. Monosomic control frequencies are based on the number of actually leucine-requiring white resistant colonies. In case of the samples exposed to acetone, only representative samples of white colonies were tested. The fraction of leucine-requiring white resistant colonies was then used to compute the presumptive numbers of leucine-requiring colonies among the overall numbers of white resistant colonies. This was then used to compute the frequencies of monosomics.

TABLE 2

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH 7.83% ACETONE: THE INFLUENCE OF STORAGE TIME IN ICE

Sample	Titer $\times 10^6$	Total resistants		White resistants	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control					
17 h 30 min	70.9	954	15.69	3	0.49
7.83% Acetone					
0 min	48.6	994	23.48	1	0.24
15 min	48.1	1004	24.39	5	1.21
30 min	13.3	579	28.94	0	–
1 h 30 min	20.0	775	25.33	3	0.98
17 h 30 min	8.5	789	27.00	98	38.28

Acetone was added to a growing culture at a titer of 9.9×10^6 cells per ml. Incubation was continued for 4 h before cultures were transferred into ice for the times indicated. The 0 min sample was plated 17 h 30 min later, the other samples after an accordingly shorter time. The 17 h 30 min control and acetone samples were plated after an additional incubation period of 4 h.

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TABLE 3

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH 6.82% ACETONE — TIME COURSE OF INDUCTION

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control					
before ice	43.6	879	20.13	3	0.23
after ice	50.3	–	–	3	0.20
2 h at 28°	61.3	–	–	5	0.27
4 h at 28°	53.8	–	–	4	0.25
6.82% Acetone					
before ice	5.5	287	17.29	2	1.20
after ice	5.4	271	16.73	2	1.85
2 h at 28°	2.7	330	40.74	38	46.92
4 h at 28°	4.9	482	32.57	67	45.27

Acetone was added to a growing culture at a titer of 7.5×10^6 cells per ml. A parallel culture without acetone was used as a control. The first platings were done 4 h after addition of acetone (designation 'before ice'). The next samples were plated after an additional storage period of 17 h in ice (designation 'after ice'). Both cultures were then further incubated at 28°C. Samples were again plated after 2 h and 4 h of incubation at growth temperature.

TABLE 4

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH 2.44% ETHYL ACETATE: AGENT ADDED AFTER DIFFERENT GROWTH PERIODS

Sample time of plating	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
(a) Agent added 4 h before transfer into ice					
before ice	10.00	354	11.70	3	1.00
end of ice period	5.5	207	12.62	5	3.05
1 h after ice	4.5	252	18.67	24	17.78
2 h after ice	5.5	437	26.65	210	128.05
3 h after ice	12.2	393	31.95	242	196.75
4 h after ice	16.0	578	36.12	368	230.00
6 h after ice	36.3	1260	34.71	673	185.40
(b) Agent added at time of transfer into ice					
before ice	34.1	856	16.72	0	–
end of ice period	27.9	491	11.75	0	–
2 h after ice	29.2	677	15.46	2	0.46
4 h after ice	36.4	711	19.53	9	2.47
6 h after ice	43.6	912	20.92	11	2.52
(c) Agent added after overnight storage in ice					
end of ice period	30.6	783	17.06	1	0.22
2 h after ice	36.9	783	14.16	4	0.72
4 h after ice	53.4	677	12.68	0	–
6 h after ice	52.2	891	16.97	8	1.51

A culture of strain D61.M was brought into the growth phase and divided into two volumes. To one was added ethyl acetate; the other part was incubated for another 4 h and divided again, with one part receiving ethyl acetate, the other left without addition. All 3 cultures were then placed into ice for 17 h, after which ethyl acetate was added to the final culture. Plating before ice in variant (b) and plating end of ice in variant (c) can be taken as controls. The monosomic frequencies below 100×10^{-6} are based on colonies which were white and found to be leucine-requiring. All higher frequencies were computed on counts of white colonies without further tests for a leucine requirement.

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vestigated further. As shown in Table 2, growing cells were exposed to acetone for 4 h and then placed in ice for various periods of time. No effect was seen for up to 90 min exposure. In a different experiment, a culture exposed to 7.41% acetone was stored in ice for 5 h 15 min; this raised the incidence of monosomics from 0.06×10^{-6} (only one colony counted) in the control to 27.14×10^{-6}, a value based on a count of 133 colonies. It is important to notice that there was hardly any increase in red resistant colonies indicative of other types of genetic effects. Monosomic colonies started to appear only after the cultures had gone through a growth period, followed by storage in ice, and through a second growth period as shown in Table 3 for acetone. This protocol had no effect on the control culture.

After the strong induction of aneuploidy by acetone had been established, we tested more solvents for induction of aneuploidy. The strongest inducer of mitotic aneuploidy was ethyl acetate. To determine the critical parts of the complex treatment protocol, a growing culture was divided into subcultures. One received ethyl acetate immediately (Table 4a); a second subculture was removed 4 h later and received ethyl acetate immediately before storage in ice (Table 4b). After overnight storage in ice, a third subculture received ethyl acetate immediately before transfer to growth temperature (Table 4c). It was obvious that only the full regimen of two growth periods interrupted by storage in ice led to strong induction of mitotic aneuploidy. Moreover, it was equally obvious that there was no increase in the frequencies of

TABLE 5

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH 3.54% METHYL ETHYL KETONE: REMOVAL OF AGENT AFTER DIFFERENT GROWTH PERIODS

Sample time	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control culture					
before ice	32.2	1024	10.60	1	0.10
end of ice	29.9	933	10.74	3	0.35
2 h after ice	33.1	1326	13.37	11	1.11
(a) Methyl ethyl ketone present throughout					
before ice	3.9	168	14.48	2	1.72
end of ice	3.9	197	16.69	0	–
1 h after ice	3.3	332	33.88	118	120.40
2 h after ice	2.7	373	45.49	138	168.29
3 h after ice	3.4	423	41.88	166	164.36
5 h after ice	4.1	533	42.98	176	141.94
(b) Methyl ethyl ketone removed after 4 h growth at 28°C					
end of ice	3.8	168	14.74	1	0.88
1 h after ice	5.4	123 *	11.32	3	2.76
2 h after ice	6.2	255	13.78	3	1.62
3 h after ice	10.6	521	16.38	1	0.31
5 h after ice	27.5	623	15.12	6	1.46
(c) Methyl ethyl ketone removed after overnight storage in ice					
1 h after ice	3.7	210	18.75	2	1.82
2 h after ice	6.2	334	17.96	4	2.15
3 h after ice	12.5	540	14.40	5	1.33
5 h after ice	31.6	759	16.01	6	1.27

Methyl ethyl ketone was added to a growing culture at a cell titer of 6.95×10^6 cells per ml. Part of this culture was removed after 4 h at 28°C, centrifuged and resuspended in fresh medium. Both cultures were stored overnight in ice. After 17 h in ice, part of the culture containing methyl ethyl ketone was removed, centrifuged and resuspended in fresh medium. All 3 cultures and the control culture were incubated again for the times indicated in column 1 and plated. * Only 2 instead of the standard 3 plates scored.

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red resistant colonies which would indicate point mutation or mitotic recombination. Their frequencies remained at the control level if the frequencies of white colonies were subtracted from the total resistant frequencies.

Another type of experiment was performed with

TABLE 6

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH DIFFERENT ACETIC ACID ESTERS

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Methyl acetate					
Control	64.5	1532	23.75	23	3.57
2.91%	33.0	994	20.08	39	7.88
3.38%	16.0	666	27.63	69	28.63
3.85%	5.0	384	15.66	40	26.67
2-Methoxyethyl acetate					
Control	80.3	1031	6.85	1	0.12
2.91%	52.6	741	10.57	27	3.85
3.85%	34.0	590	13.02	38	8.39
4.76%	17.6	889	12.63	44	6.25
5.66%	4.7	339	18.72	73	39.04
n-Propyl acetate (1 out of 4 Expts.)					
Control	83.9	2577	10.24	5	0.20
Positive control: 2.44% ethyl acetate					
	3.9	571	47.98	367	308.40
0.50%	63.9	2625	13.69	6	0.31
0.74%	38.6	1631	14.07	2	0.17
0.99%	30.2	1189	13.12	1	0.11
1.23%	6.7	253	12.52	13	6.44
Isopropyl acetate (1 out of 3 Expts.)					
Control	160.3	5036	26.17	9	0.47
Positive control: 1.96% ethyl acetate					
	1.6	375	78.12	133	277.08
0.74%	44.2	725 *	24.58	3 *	1.02
0.99%	12.6	1209	32.11	12	3.19
1.23%	14.6	1322	30.29	11	2.52
n-Butyl acetate and isoamyl acetate					
Control	70.6	1160	16.42	1	0.28
Positive control: 2.44% ethyl acetate					
	2.3	209	29.65	51	72.34
n-Butyl acetate					
0.25%	31.9	829	25.95	3	0.94
0.32%	33.4	663	19.82	2	0.60
0.40%	6.5	373	19.13	1	0.51
Isoamyl acetate					
0.15%	52.5	878	16.72	0	–
0.20%	18.0	778	14.36	7	1.29
0.24% no survivors					

All treatments were started by pipetting the chemicals to be tested into a growing cell culture at a titer between 3 and 10 million cells per ml. 4 h of incubation at 28°C were followed by a storage of about 17 h in ice. Cells were plated after 4–5 h of additional incubation at 28°C. * Only 2 instead of the standard 3 plates scored.

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methyl ethyl ketone to determine whether the chemical needed to be present continuously. A growing culture received the agent and subcultures were prepared after the initial 4 h at 28°C and after the overnight storage in ice. Parts of the cultures were removed, the cells spun down and the supernatant replaced by fresh medium without methyl ethyl ketone. As shown in Table 5, induction was only strong in that part of the culture which was continuously exposed to the agent. Again there was no significant increase in the frequencies of red resistant colonies.

The required length of the growth period before storage in ice was investigated with methyl ethyl ketone. The agent was added to a growing culture and samples were removed and placed into ice at various times. All samples were then shaken at growth temperature and incubated for another 4 h before plating. Although in this experiment induction was low, a significant level (8.62×10^{-6} based on account of 28 colonies) was reached only after 225 min.

While ice treatment is important with these agents to elicit a response, induction of aneuploidy is possible without the ice treatment; however it is much weaker. The frequency of monosomic cells in a growing culture exposed for 8 h of incubation at 28°C to 2.44% ethyl acetate was 6.83×10^{-6} monosomics (based on a count of 45 leucine-requiring white resistants) as opposed to 0.14×10^{-6} in the control. The intermittant cold storage only enhances the potential to induce aneuploidy.

A series of acetate esters was tested as shown in Table 6. Methyl and 2-methoxyethyl acetate were found to be positive but at concentrations higher than ethyl acetate. The *n*-propyl and iso-propyl acetates were marginally active, however, the general toxicity of the two propyl esters occurred at lower concentrations. This toxicity was still more pronounced with the *n*-butyl and the isoamyl acetate.

Experiments with additional esters are shown in Table 7. Methyl propionate and methyl butyrate were positive but not as efficient as ethyl acetate. Ethyl formate and ethyl propionate each showed a weak induction in only one experiment but were negative in three others.

Acetone (dimethyl ketone) and methyl ethyl ketone were strong inducers of mitotic aneuploidy;

TABLE 7

INDUCTION OF MITOTIC ANEUPLOIDY WITH DIFFERENT FATTY ACID ESTERS OF METHANOL AND ETHANOL IN STRAIN D61.M

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Methyl propionate (1 out of 2 Expts.)					
Control	94.2	2165	22.98	7	0.74
1.23%	58.4	1998	22.81	12	1.37
1.36%	21.8	1784	27.32	12	1.84
1.48%	6.2	463	24.76	29	15.51
Methyl butyrate (1 out of 2 Expts.)					
Control	53.8	627	19.41	0	–
Positive control: 2.44% ethyl acetate					
	4.5	868	64.78	388	388.06
0.50%	22.5	847	14.05	2	0.33
0.99%	3.8	215	19.03	20	17.70
Ethyl formate (1 out of 4 Expts.)					
Control	93.6	1820	25.92	3	0.43
Positive control: 1.36% methyl *n*-propyl ketone					
	12.8	1488	38.75	37	9.64
1.36%	31.2	1652	17.63	19	2.03
2.32%	23.9	1499	20.88	9	1.25
2.44%	13.9	781	18.73	5	1.20
2.56%	1.3	68	17.89	0	–
Ethyl propionate (1 out of 4 Expts.)					
Control	75.9	1965	25.89	0	–
0.84%	44.3	3049	22.96	5	0.35
0.94%	31.5	2163	22.89	3	0.32
1.04%	7.6	614	26.70	18	7.83

The treatment protocol followed was the same as the one described in the legend of Table 6.

methyl *n*-propyl ketone also induced aneuploidy but less strongly (see also Table 7 where it was used as a positive control agent with ethyl formate). Diethyl ketone was found to induce aneuploidy also and, in addition, a slight increase was seen in the frequencies of red resistant colonies as shown in Table 8. The methyl isopropyl ketone also induced an increase in red resistant colonies indicative of the induction of point mutation and/or mitotic recombination, but only weakly induced mitotic aneuploidy at levels of high toxicity. A diketone, 2,5-dipentanone, was also tested but it was negative.

Both esters and ketones are polar aprotic solvents. This prompted us to test acetonitrile, another commonly used aprotic solvent. It was

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TABLE 8

INDUCTION OF MITOTIC ANEUPLOIDY WITH DIFFERENT KETONES IN STRAIN D61.M

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Diethyl ketone					
Control	38.3	2384	41.53	3	0.52
0.99%	15.9	1581	33.14	0	–
1.23%	11.2	1057	31.36	0	–
1.48%	3.8	1312	114.09	146	126.96
Control	64.5	1532	23.75	23	3.57
0.99%	40.8	1570	25.65	5	0.82
1.23%	29.9	1185	26.39	19	4.23
1.48%	11.7	1590	45.43	85 *	72.58
Methyl *n*-propyl ketone (1 out of 4 Expts.)					
Control	49.9	2833	37.82	0	–
1.48%	15.5	2390	51.40	21	4.52
1.72%	6.5	848	43.71	103	53.09
Methyl isopropyl ketone (2 out of 4 Expts.)					
Control	77.2	1405	27.03	1	0.13
Positive control: 3.38% methyl ethyl ketone					
	1.72	222	42.86	38	73.36
1.23%	2.2	933	143.35	3	4.62
1.36%	1.6	674	143.40	7	14.89
Control	93.6	1820	25.92	3	0.43
0.84%	28.6	1632 **	57.13	3	0.35
0.94%	23.7	1114	70.51	1	0.63
1.23%	2.29	693	100.73	2	0.29
2,5-Dipentanone (acetyl acetone)					
Control	80.3	1031	6.85	1	0.12
Positive control: 3.38% methyl ethyl ketone					
	16.0	862	13.39	245	38.04
0.74%	42.0	980	14.58	1	0.15
0.99%	33.1	1298	19.47	3	0.43
1.48%	23.3	1874	20.37	2	0.22
1.96%	7.8	696	22.38	2	0.64

The treatment protocol followed was the same as the one described in the legend of Table 6. * Leucine-requiring whites determined on only 1 instead of 3 plates. ** Total resistants scored on only 1 instead of 3 plates.

TABLE 9

INDUCTION OF MITOTIC ANEUPLOIDY WITH ACETONITRILE IN STRAIN D61.M

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	74.8	1119	24.95	3	0.67
2.91%	78.6	1793	22.81	11	1.40
4.76%	8.8	823	31.29	91	34.60

The treatment protocol followed was the same as the one described in the legend of Table 6.

TABLE 10

TREATMENT OF STRAIN D61.M WITH DIOXANE

Sample	Titer $\times 10^6$	Total resistants		Monosomics	
		Colonies	$\times 10^{-5}$	Colonies	$\times 10^{-6}$
Control	132.2	1143	17.29	5	0.76
Positive control: 3.38% methyl ethyl ketone					
	11.2	1014	30.27	175	52.24
	9.8	860	29.15	185	62.71
1.48%	99.4	1538	15.47	4	0.40
1.96%	83.8	1424	16.99	2	0.24
2.44%	58.9	1671	18.92	6	0.68
2.91%	30.9	1137	24.56	2	0.43
3.38%	20.9	1331	21.23	4	0.64
4.31%	8.4	573	22.30	0	–
4.75%	2.9	179	20.34	0	–
Repeated plating after an additional incubation period of 5 h					
3.85%	18.4	1491	27.01	2	0.36
4.31%	15.1	1110	22.98	9	1.86

Treatment conditions as described in the legend of Table 6 except for the repeated platings.

found to be positive (Table 9), and showed no effect on the induction of red resistant colonies.

Dioxane was also tested and found to be negative even with extended incubation (Table 10). It did have severe effects on cell morphology; at concentrations above 3%, aggregates were formed which looked like hyphal clusters rather than aggregates of yeast cells.

As shown by Wood (1982), mitotic aneuploidy can be induced in yeast with bavistan, an agent known to prevent self-assembly of yeast tubulin (Kilmartin, 1981). Because Wood found that selecting for monosomy of one chromosome often yielded clones with multiple aneuploidies, we examined further white cycloheximide-resistant colonies which arose after exposure to ethyl acetate. Cells from these colonies were spotted on cycloheximide medium. After overnight growth, the plates were replicated onto different diagnostic media to determine the expression of recessive markers including mating type. The results are shown in Table 11. All of the 100 isolates expressing α mating type also expressed the coupled marker *mal*. Five of these expressed the recessive *his* on another chromosome. Most isolates expressing *a* mating type were able to use maltose; only 4 expressed a maltose negative phenotype. Additional recessive markers were expressed in

TABLE 11

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M WITH ETHYL ACETATE: ANALYSIS OF WHITE, LEUCINE-REQUIRING RESISTANT COLONIES FOR THE SEGREGATION OF UNLINKED RECESSIVE MARKERS

Total numbers of colonies analysed		1248
(1) Expressing α mating type	100	
with additional expression of *mal*	95	
with additional expression of *mal* and *his*	5	
(2) Expressing *a* mating type	83	
with additional expression of *mal*	4	
with additional expression of *his*	4	
(3) Expresssing only *his*	16	
(4) Expressing only *mal*	7	
(5) Expressing *his* and *trp5*	1	
Total number of white and leucine-requiring resistant colonies expressing additional unlinked recessive markers		207 = 16.6%

Cells from white resistant colonies were picked onto the selective cycloheximide medium and the plates incubated for 36 h. Plates were then replica plated on diagnostic media for the determination of *his*, *mal* and *trp5*. Replication was also done on rich-media plates seeded with either cells of a strain of α or *a* mating type both carrying the recessive marker *ade1*. After an additional incubation for 24 h, these plates were again replicated onto a synthetic minimal medium where only those isolates could grow which could form a prototrophic triploid with one of the two mating-type tester strains. 200 red resistant colonies were tested in the same way. 31 expressed *leu1*, 5 *trp*

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16.6% of all white and leucine-requiring resistant isolates. 200 red resistant colonies were tested in the same way. The only additional recessive markers expressed were the linked alleles *leu1* and *trp5*, probably reflecting the occurrence of recombinational events at those loci which are located proximally to *cyh2*. These data indicate a high coincidence of mitotic malsegregation of other chromosomes with aneuploidy of chromosome VII and no coincidence with recombination.

To further characterize the genetic changes in the aneuploid variants, white and leucine-requiring resistant clones from controls and mutagen-treated cultures were sporulated and tetrad analysis was performed. True monosomic diploids should segregate two live and two dead spores in each meiosis. This occurred in only 7 of the 52 clones analysed (Table 12), indicating that most of the leucine-requiring and white resistant colonies had returned to a normal diploid condition by the time of sporulation. This conclusion is in agreement with the observations of Strömnaes (1967), Parry and Zimmermann (1976) and Wood (1982).

The experimental design used detects mutants which are monosomic or have gone through a transient stage of monosomy. It was most interesting to find that 5 of the 52 clones showed an additional trisomy for chromosome III which carries the mating-type locus. Cells that are trisomic for this chromosome can segregate haploids which are disomic for chromosome III carrying both mating type alleles *MATa* and *MATα*. Such disomic haploids cannot mate with a haploid mating-type tester strain, but they can sporulate. This was actually observed in most of the segregating non-maters. We conclude from this that when chemicals induce monosomy of chromsome VII, the treatment frequently induces malsegregation of additional chromosomes as well because of the high co-incidence of monosomies (Table 11) and trisomies (Table 12) seen in chromosome III.

TABLE 12

INDUCTION OF MITOTIC ANEUPLOIDY IN STRAIN D61.M: GENETIC ANALYSIS OF PRESUMPTIVE MONOSOMICS

Origin of clones	Number analysed	2 dead: 2 viable	Additional trisomy
Control	6	1	0
Bavistan	12	3	1
Ethyl acetate	20	1	2
Methyl ethyl ketone	14	2	2
Total	52	7	5

The category 2 dead:2 viable is expected in the case of persisting monosomy. Additional trisomy can be recognized if it affects chromosome III which carries the mating-type locus. A diploid trisomic for this chromosome will segregate non-mating spores which are *a*/*α* and consequently should sporulate. This additional criterion for trisomy could be verified in 4 out of 5 such cases.

Discussion

The ability to induce chromosome malsegregation need not be associated with the induction of other genetic events. We have identified 6 chemicals that are very effective in inducing chromosomal malsegregation but no other type of genetic effects in our system: acetone, methyl ethyl ketone, acetonitrile and the acetic acid esters of methanol, ethanol and 2-methoxyethanol.

The most effective treatment protocol which included a holding period in ice might be expected in the light of the nature of the chemicals and their genetic effects. Most of the positive chemicals tested in this investigation induced only chromosomal malsegregation but none of the other types of genetic events, mitotic recombination or point mutation, easily studied in yeast (Zimmermann, 1984). This suggested that the primary targets were not DNA nor the DNA-metabolizing systems. The most likely target producing malsegregation would be the spindle apparatus. Tubulin is an essential component of microtubules including those of yeast (Kilmartin and Adams, 1984). It has an unusual property: microtubules dissociate in vitro below a certain critical temperature and aggregate again above this temperature when GTP is present as an energy source (Timasheff and Grisham, 1980). This reversible dissociation and reassembly can be used in general to purify tubulin and associated proteins (Williams and Lee, 1982) including yeast tubulin (Kilmartin, 1981).

The behavior of tubulin and microtubules provides an explanation for the unusual protocol required for efficient induction of aneuploidy with

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the aprotic bipolar solvents. It is possible that microtubules formed during growth in the presence of such agents are somehow weakened and function poorly during mitosis resulting in the observed chromosomal malsegregation. The enhancement of aneuploidy by cold treatment could be due to a greater dissociation when the agents are present. Upon return to growth temperature, microtubule formation in the presence of the agents could be perturbed resulting in poorly functional microtubules. The observation that considerable toxicity can be detected over a small concentration range may relate to the need for a critical amount of functional microtubules.

It has to be emphasized that there is some induction of aneuploidy by the solvents without the ice treatment. Induction of aneuploidy by acetone was indeed observed using the normal treatment protocol with continuously growing cell cultures. Cold storage merely amplifies the effects.

The 6 chemicals that were strong inducers of aneuploidy are widely used as solvents. The strongest aneuploidy inducing mutagen, ethyl acetate, is used as a nail polish remover and is consequently applied to humans at high concentrations. Only one of the strong aneuploidy-inducing agents, diethyl ketone, induced other genetic effects but at a low level. Methyl isopropyl ketone was the only agent which weakly induced mitotic recombination/point mutation but not aneuploidy in yeast.

It must be emphasized that not all the aprotic bipolar chemicals tested were positive. Butyl and isoamyl acetate, used as flavoring agents in sweets, were negative. Moreover, we have obtained negative results (not reported here) for a large number of alcohols and fatty acids.

Comparison of the 19 chemicals investigated leads to some speculation on chemical structure/biological effect relationships. The smallest ketone, dimethyl ketone or acetone, was the least effective. The next larger molecule, methyl ethyl ketone, was effective at lower concentrations (between 3 and 4%) and diethyl and the propyl ketones below 2%. A size relationship also applies to the esters. Methyl acetate was the least effective whereas butyl acetate was so toxic that no more than 0.5% was tolerated. The increasing toxicity of the larger homologs may mask the genetic effects, since only the smaller compounds were found to be active and these are the ones with lower toxicity. Only tests in vitro can reveal the actual capacity to interact with tubulin or microtubules.

It must be noted that two of the ketones, diethyl and methyl iso-propyl, but not the *n*-propyl methyl ketone also induced classical types of genetic effects, mitotic recombination and possibly point mutation. It is conceivable that the ketones form Schiff bases with amino groups of nucleobases and thus produce genetic effects. However, it is noteworthy that a distinct difference was found between the *n*-propyl and the iso-propyl ketones.

The 2,5-dipentanone is structurally a diketone but it lacks the properties of the other ketones as it is more reactive chemically but was negative in our tests. Dioxane is actually a double ether and was included in the present investigation only because its dramatic effects on cell morphology suggest that it interferes at some point with cellular activities.

Acetonitrile which is used in industry in large quantities and in paints was tested because of its pronounced nature as an aprotic polar solvent. It was indeed a good inducer of mitotic aneuploidy but at relatively high concentrations (close to 5%).

All of the strong aneuploidy-inducing agents were active in yeast at relatively high concentrations; this could be interpreted to mean that they pose no potential genotoxic hazard to man. However, the opposite may be true. Human tubulin could be more sensitive than yeast tubulin. Even though tubulin is a highly conserved protein (Little et al., 1981) there are species differences in its susceptibility to drugs. Kilmartin (1981) showed that yeast tubulin is hardly affected by colchicine, whereas bovine brain tubulin is highly sensitive. Nocodazole (oncodazole) was active on both types whereas another benzimidazole derivative, bavistan, was only active on yeast tubulin.

We shall demonstrate in a subsequent publication that the aneuploidy-inducing activity of aprotic polar compounds in yeast is associated with their ability to block assembly in vitro of porcine brain tubulin. In the case of acetonitrile, this block could be achieved at concentrations much lower than those necessary to induce aneuploidy in yeast. We believe that the yeast test for aneuploidy may have qualitative predictive value in respect to mammalian conditions.

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Acknowledgements

This investigation was supported by contract No. NO1-ES-1-5005, Development of a Yeast Aneuploidy Test System, from the National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709. It is a great pleasure to thank Dr. G. Schröder of Röhm GmbH, Darmstadt, for his generous gifts of chemicals and for explaining to us the function of the solvation coat of macromolecules which dissociate in the cold and reassociate at higher temperatures; Doz. Dr. Gais, Department of Organic Chemistry of our University, pointed out to us that all the chemicals we found mutagenic belonged to the group of polar aprotic solvents, suggested more for testing, especially acetonitrile, and also provided us with samples. V.W.M. was supported by a National Science Foundation Award under Agreement No. INT-8304930. Any opinion, findings and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the National Science Foundation.

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