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EXPERIMENTAL STUDIES ON METHYL-ETHYL-KET-OXIME TOXICITY

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SUMMARY

In order to protect membrane formation that might occur on the surface of paint or enamel, etc. during their preservation, MEK-oxime was developed. The usefulness of MEK-oxime for this purpose was discovered in the U.S.A. Recently MEK-oxime has been commercialized also in Japan and it is supposed that the number of workers exposed to it will increase hereafter.

Considering the fact that MEK-oxime is composed of an oxime and a ketone derivative, it is reasonable to assume that it may have some effects on the living body.

From the viewpoint of industrial hygiene, the author examined the toxicity of MEK-oxime using albino rats.

As the result of this study, it was found that MEK-oxime has many toxic effects on the living body, causing splenic tumor, hemorrhagic tendency of many organs, inhibition of cholinesterase activities of the specific and non-specific types, etc., though the toxicity of MEX-oxime is not so severe as far as the lethal dose is concerned. Therefore hygienic precautions are essential in the industrial handling of MEK-oxime.

INTRODUCTION

On the surface of paint, varnish, enamel, or lacquer kept in vessels for a long time, a hard membrane like gum is formed due to oxidation by air. Previously it was a problem to protect such membrane formation, and many chemicals such as phenolic, cresylic and aminetype derivatives have been used for this purpose. Later, it was found that organic antioxidants, especially aliphatic derivatives were superior for this purpose. MEK-oxime (Methyl-Ethyl-Ketoxime), one of the above derivatives with a boiling point below 160°C, was synthesized and the usefulness of MEK-oxime as a protective agent was recognized in the U.S.A. about 1940.

Prior to 1962 the consumption of MEK-oxime in Japan was low, and that utilized was imported. Since 1962 it has been synthesized and commercialized by some chemical companies. It is supposed that the consumption of MEK-oxime will increase hereafter.

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From the viewpoint of industrial hygiene, it is likely that during the production process, storage, transportation, and mixing with paints etc., the chances of exposure to MEK-oxime may increase for workers.

At present there have been no reports on the toxicity of MEK-oxime with the exception of a few reports concerned with its derivatives or metabolites. There are some reports[1)2)3)] on neural influences by oxime derivatives such as PAM, DAM, etc. and on narcotic effects[4)] by some ketone derivatives. It may be supposed that MEK-oxime will also have some influence on the living body from these facts. Therefore studies were pursued for the purpose of clarifing MEK-oxime toxicity.

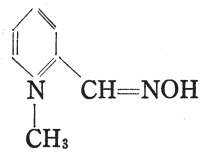
Properties of MEK-oxime

Chemical structure	$CH_3—C(=NOH)—CH_2—CH_3$
Molecular weight	87.12
Specific gravity[5)]	0.922 (25°C)
Boiling temperature[5)]	152°C (760 mmHg)
Flash point[6)]	24°C
Vapour pressure[5)]	1.06 mmHg (20°C)
	7.6 mmHg (50°C)
	60.5 mmHg (89°C)

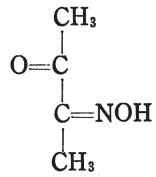
Fluid at ordinary temperatures.

For reference, the chemical structures of PAM and DAM are as follows:

PAM (Pyridine-2-Aldoxime Methiodide)



DAM (Diacetyl Monoxime)



ON THE ACUTE POISONING BY MEK-OXIME

Experimental Animals and Materials

The animals used were male albino rats about two months of age and were fed the standard diet of Oriental pellets.

Pure MEK-oxime, of over 99.8% purity, was used in these experiments. The experiments were performed between September and December.

Lethal Dose (LD_{50} to Rats)

Forty-two albino rats, weighing 125 g on an average, were used and were divided into seven groups of 6 rats each. Each group was injected with MEK-oxime subcutaneously in doses of 2.0 ml, 2.4 ml, 2 8 ml, 3.2 ml, 3.6 ml, 4.0 ml, and 4.4 ml respectively per kg of body weight.

The number of rats which died in 24 hours after injection are listed in Table 1, and on the basis of this table LD_{50} was calculated.

1) By Behrens-Kärber Method
   $LD_{50} = 2701.5$ mg/kg B.W. (2.93 ml/kg B.W.)
2) By Van der Wärden Method
   $LD_{50} = 2761.6$ to 2813.2 mg/kg B.W. (2.99 to 3.05 ml/kg B.W.)

TABLE 1. Acute Toxicity of MEK-oxime for Rats (subcutaneous injection)

Injected Dose of MEK-oxime (ml/kg)	No. of Rats Tested	No. of Rats died in 24 hrs after Inj.
2.0	6	0
2.4	6	3
2.8	6	3
3.2	6	4
3.6	6	4
4.0	6	5
4.4	6	6

Application on Skin of Rat

This test was made to study the influence of MEK-oxime by cutaneous contact.

To the necks of 3 rats of group A 0.4 ml of MEK-oxime was applied with white vaseline and to 2 rats of group B which served as controls only white vaseline was applied once a day for 5 weeks.

In 2 rats of group A, the following dermatological changes were observed; wettish erythema on the 4th experimental day, papule or local erosion on the 6th to 7th day, thin and small squamous like crusta on about the 10th day.

Thereafter, only slight hypertrophy of the corneous layer was observed after about 15 days. In another rat of group A and 2 rats of group B, abnormal changes of the skin were scarcely observed throughout the entire experiment.

Exposure to Saturated Vapour

This examination was carried out to study the influence of inhalation of a high concentrated vapour of MEK-oxime.

Two rats were reared in a chamber of about 0.008 sqm filled with saturated MEK-oxime vapour and their behavior was observed. (The temperature in the chamber was kept between 23°C and 24°C, and the ventilation was made by natural ventilation through 2 windows of 1 cm diameter.)

Exposure	Behavior of Rats
After 2 minutes;	Agitated and not at ease.
After 15 minutes;	Continued in the same state, sometimes wiped their heads.
After 35 minutes;	Crouched and drowsy.
On the 2nd day;	Slumbered away. (Eyes fatty, loss of appetite, and diarrhea were recognized.)
On the 4th day;	One died.
On the 5th day;	Another died.

Instillation into the Eye

This examination was carried out in order to clarify the action of MEK-oxime on the eye.

Soon after instilling a drop of MEK-oxime into one of the eyes of 3 rats, marked hyperemia appeared in the eye-lid and the bulb conjunctiva. The rats repeatedly wiped their eyes with their legs and shook their heads. This behavior may be due to strong eye-pain. After a few minutes, fatty eyes were recognized and hyperemia recognized in the other eye as well. These symptoms reduced gradually, and completely disappeared after 6 to 9 hours.

Discussion

In molecular structure, MEK-oxime consists of methyl-ethyl-ketone (butanone) and an oxime base (=NOH).

It has been reported by R. T. William[7] that oxime derivatives are metabolized to aldehydes or ketones and hydroxylamines or primary amines *in vivo*. Therefore, MEK-oxime also is metabolized to such substances *in vivo* and these metabolites may exert some influence on organisms.

In discussing the effects on the living body, one may compare MEK-oxime with these metabolites. As with the irritating effects of butanone[8] and aldehydes[4] on skin or mucous membranes, likewise contact dermatitis and mucous irritating symptoms by MEK-oxime were observed in this experiment though they were reversible, and also narcotic actions were observed in the MEK-oxime inspiratory test as previously demonstrated with ketone derivatives'[4].

It has been reported that the dose of LD_{50} using albino rats was about 200 mg/kg[1)9)] for PAM, which belongs to the oxime derivatives, and 410 mg/kg[4] for hydroxylamine. Also guinea pigs lived for over 13 hours in 10,000 ppm

concentration of butanone[4]. Comparing these results, the lethal dose of MEK-oxime resembles comparatively the lethal dose of butanone.

In view of the results so far obtained, symptoms of acute poisoning by MEK-oxime seem to resemble those by methyl-ethyl-ketone (butanone) rather than those by other oximes and metabolites of MEK-oximes.

ON THE CHRONIC POISONING BY MEK-OXIME

In the preceding chapter, the author described the symptoms of acute MEK-oxime poisoning to resemble butanones.

However, workers who actually handle MEK-oxime may be exposed to low concentrations for a long period of time. In order to know the influence of chronic exposure to MEK-oxime, experimental studies of chronic poisoning were conducted using albino rats.

EXPERIMENT I

Experimental Subjects and Procedures

Male albino rats weighing about 130 g were used. They were divided into 4 groups of 6 rats each (except for the control group of 7 rats).

Every day for 4 weeks rats of each group were injected dorsally subcutaneously with MEK-oxime or olive oil in the following doses:

Group A:	MEK-oxime 1.0 ml/kg B.W.
Group B:	MEK-oxime 0.5 ml/kg B.W.
Group C:	MEK-oxime 0.1 ml/kg B.W.
Group D (Control):	Olive oil 1.0 ml/kg B.W.

Items of Measurement

The following together with the general status of rats were observed throughout the experimental period of 4 weeks: body weight, erythrocyte count, leucocyte count, hemoglobin content and hemogram.

After 4 weeks the rats were sacrificed by bleeding from *A. femoralis*, and the pathological changes in organs were observed macro- and micro-scopically. Also, serum protein protein concentration and its fractions were examined.

Methods of Measurement

Body weight: Weight balance.

Erythrocyte count and leucocyte count: Koizumi method[10], using a small test tube.

Hemoglobin content: CN-methemoglobin method.

Hemogram: Giemsa staining.

Pathological preparation: Fixed with Carnoy's solution and hematoxylin-

eosin stained.

Serum protein concentration: The hand protein reflectometer, manufactured by Hidachi Ltd.

Serum protein fractions: By a modified method[11] of Smithies' zone electrophoresis[12) 13) 14)] in which starch gel was used as the supporting medium. The experimental conditions and materials are as shown in Table 2. The general lay out of the apparatus is as shown in Fig. 1.

TABLE 2. Procedure for Starch Gel Electrophoresis

Hydrolyzed starch 15 g + Borate buffer 100 ml (pH 8.7)
↓
Heat with mixing
↓
Starch gel obtained
↓
Pour starch gel into tray
↓
Insert pieces of filter paper introduce sample of sera into slots
↓
Set up general layout of apparatus
↓
Apply 22 mA 220 V for 20 hrs
↓
Stain starch gel plate with Amido-Black 10 B for 10 min

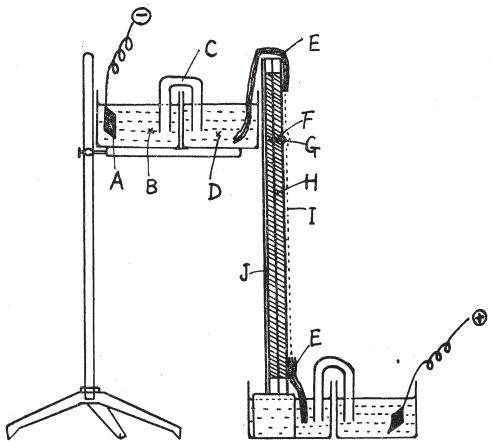


FIG. 1. General layout of apparatus.
A: Ag/AgCl electrode,
B: 5% of KCl solution,
C: agar-ager bridge,
D: 0.3 M H_3BO_3 0.6 M NaOH solution,
E: filter-paper bridge soaked in bridge solution,
F: position of sample insertion,
G: vaseline,
H: starch gel contained in plastic tray,
I: sealed paraffin,
J: tray.

Results of Experiment

i) General status of exposed rats: Rats in both groups A and B were irritated for about 15 minutes after injection. Thereafter a drowsy state ensued for about 3 hours in group A and for about 1 hour in group B. The drowsy state progressed gradually day after day, the state continuing for about 10 hours in group A and for about 8 hours in group B at the terminal stages of the experiment. On awakening, the movements of the rats grew slower by degrees and at the end of the experiment, all rats in group A and 3 rats in group B were sleeping almost the entire day.

Poor appetite was recognized in nearly all the rats in groups A, B and C,

TABLE 3. Body Weight (g) ( ): %

Group→ Date ↓	A	B	C	Control
Pre-Exper. Value	118.7±8.6 (100)	128.3±5.1 (100)	147.4±10.2 (100)	128.7±5.5 (100)
3rd day	115.8±8.0 (97.6)	127.0±4.5 (99.0)	152.3±10.1 (103.3)	139.6±5.1 (108.5)
1st week	118.5±9.6 (99.8)	127.2±5.6 (99.1)	149.7±9.0 (101.6)	138.3±4.9 (107.5)
2nd week	128.5±9.2 (108.9)	142.6±5.1 (111.1)	161.1±8.8 (109.3)	156.0±4.0 (121.2)
3rd week	139.2±10.6 (117.3)	149.9±3.9 (116.8)	169.1±8.9 (114.7)	161.6±3.6 (125.6)
4th week	146.9±9.9 (123.8)	153.7±3.9 (119.8)	173.6±9.5 (117.8)	175.6±3.8 (136.4)

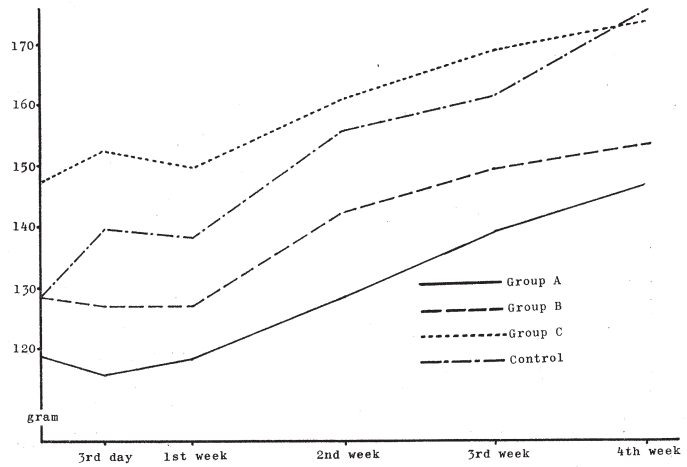


FIG. 2. Body weight.

and moreover, 1 rat of group A suffered from diarrhea on the 3rd day, 4 rats of group A and 2 rats of groups B and C on the 7th day.

ii) Body weight (Refer to Table 3 and Fig. 2): The average body weight of groups A and B decreased somewhat on the 3rd day and showed lower values in the 1st week than at the initial time, and thereafter gradually increased. The average body weight of group C increased smoothly, though it showed stagnation during the 1st week. Therefore, in general, the growth of the rats was inhibited in proportion to the injected dose of MEK-oxime.

iii) Erythrocyte count (Refer to Table 4 and Fig. 3): In each of groups A and B, erythrocyte counts followed a remarkable decrease tendency till the

TABLE 4. Erythrocyte Counts ($\times 10^4/mm^3$)

Group→ Date ↓	A	B	C	Control
Pre-Exper. Value	648.0±13.6 (100)	637.7±15.7 (100)	651.3±25.2 (100)	611.3±15.0 (100)
3rd day	613.2±22.7 (94.6)	627.2±13.7 (98.4)	643.5±26.7 (98.8)	594.6±9.2 (97.3)
1st week	578.5±29.0 (89.3)	602.8±25.6 (94.5)	671.5±18.6 (103.1)	679.3±9.1 (111.1)
2nd week	455.0±15.5 (70.2)	528.7±17.7 (82.9)	663.2±20.4 (101.8)	657.9±11.5 (107.6)
3rd week	475.8±26.3 (73.4)	565 0±8.2 (88.6)	709.8±18.4 (109.0)	724.6±16.7 (118.5)
4th week	549.2±12.7 (84.8)	603.5±23.5 (94.6)	698.3±20.5 (107.2)	717.4±21.2 (117.4)

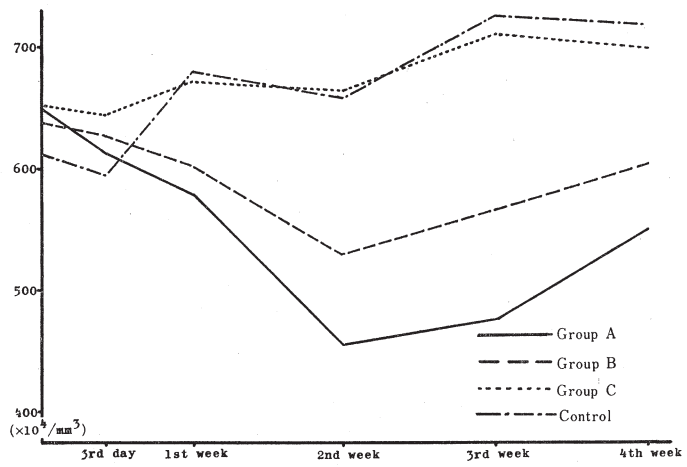


FIG. 3. Erythrocyte counts.

2nd week and thereafter recovered somewhat, but at the termination of the experiment, the final value of group A was about 85% of the initial value and the final value of group B about 95% of the initial value. In group C, it increased smoothly after a slight decrease on the 3rd day. The final value amounted to more than 7% increase above the initial value or one half of the increase rate of the control.

iv) Hemoglobin content (Refer to Table 5 and Fig. 4): The same tendency as was obtained in erythrocyte counts was observed in the hemoglobin contents for each group. Namely, in the 4th week of the experimental period, the contents of group A amounted to 91.3% of the initial value, and in group B to 99.2%. But in group C, a slightly increasing tendency was observed.

TABLE 5. Hemoglobin Content (g/dl)

Group→ Date↓	A	B	C	Control
Pre-Exper. Value	12.6±0.6 (100)	12.3±0.1 (100)	12.1±0.2 (100)	13.2±0.3 (100)
3rd day	11.6±0.6 (92.1)	11.7±0.3 (95.1)	12.4±0.2 (102.5)	12.6±0.3 (95.5)
1st week	10.8±0.2 (85.7)	11.8±0.6 (95.9)	12.8±0.2 (105.8)	13.3±0.3 (100.8)
2nd week	9.7±0.7 (77.0)	10.8±0.5 (87.8)	12.9±0.4 (106.6)	13.6±0.3 (103.3)
3rd week	10.6±0.7 (84.1)	11.9±0.5 (96.7)	13.0±0.3 (107.4)	13.8±0.3 (104.5)
4th week	11.5±0.6 (91.3)	12.2±0.4 (99.2)	13.0±0.5 (107.4)	13.8±0.3 (104.5)

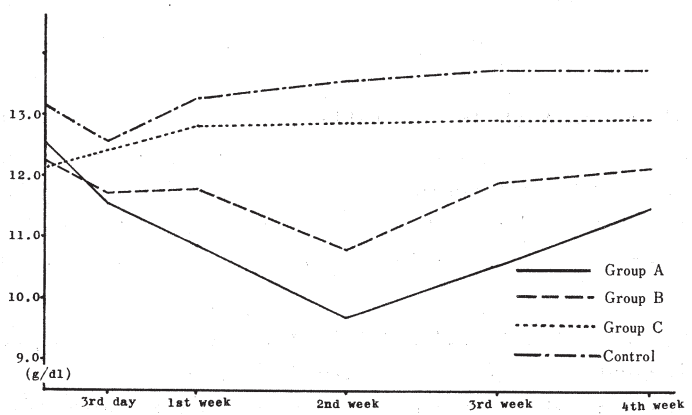


FIG. 4. Hemoglobin content.

TABLE 6. Leucocyte Counts ($1/mm^3$)

Group→ Date↓	A	B	C	Control
Pre-Exper. Value	16875±2966 (100)	14208±837 (100)	17850±1313 (100)	16614±810 (100)
3rd day	22100±1566 (131.0)	15500±455 (109.1)	17867±1393 (100.1)	16136±1023 (97.1)
1st week	18350±1245 (108.7)	17708±1289 (124.6)	16958±760 (95.0)	16971±996 (102.1)
2nd week	18525±1830 (109.8)	16675±1608 (117.4)	16142±1244 (90.4)	16764±877 (100.9)
3rd week	21740±3300 (128.8)	19083±2029 (134.3)	17458±1540 (97.8)	17200±1162 (103.5)
4th week	23850±3492 (141.3)	22117±2572 (155.7)	16017±883 (89.7)	15550±715 (93.6)

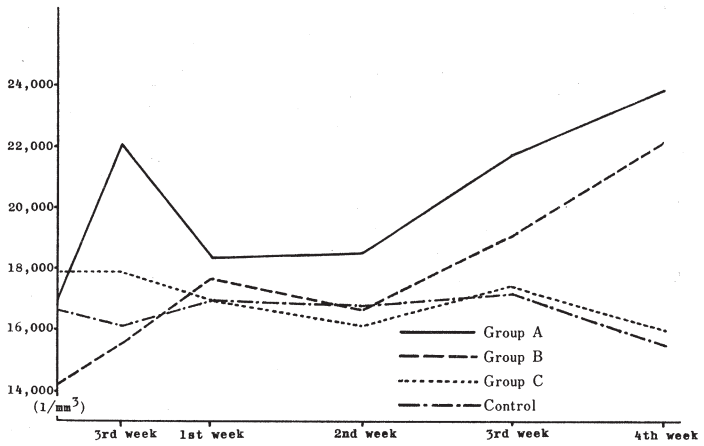


FIG. 5. Leucocyte counts.

v) Leucocyte count (Refer to Table 6 and Fig. 5): In group A, the leucocyte count recovered nearly to the initial value after an increase of 31% on the 3rd day, and after 3 weeks, it increased again. In group B, it showed a slightly decreasing tendency after an increase of 25% during the 1st week, but it again increased after 3 weeks. In group C, no noticeable change was recognized.

vi) Hemogram (Refer to Table 7): In group A, neutrophilia and a relative decrease in lymphocyte count were observed. An increase in neutrocyte was recognized on the 3rd day, thereafter it increased and its count attained finally 2.4 times that of the initial value.

TABLE 7. The Leucocyte Differential Count

A-Group (1 cc/kg) ( ): %

Date	Baso-phils	Eosino-phils	Neutrophils			Lympho-cytes	Mono-cytes
			Band-Nt.	Segmented-Nt.	Total		
Pre-Exper. Value	0.3	1.5	8.8	5.2	14.0 (100)	83.2 (100)	1.2
3rd day	0.5	3.0	4.3	17.0	21.3 (152.4)	74.0 (89.0)	1.2
1st week	0.0	2.3	5.0	15.3	20.3 (145.2)	77.0 (92.6)	0.3
2nd week	0.3	1.8	3.8	23.7	27.5 (196.4)	69.8 (84.0)	0.5
3rd week	0.4	2.2	4.8	21.4	26.2 (187.1)	70.4 (84 6)	0.8
4th week	0.0	1.8	5.3	28.0	33.3 (237.5)	64.3 (77.3)	0.8
B-Group (0.5 cc/kg)							
Pre-Exper. Value	0.3	2.7	12.7	8.8	21.5 (100.0)	74.7 (100.0)	0.8
3rd day	0.2	2.7	4.5	14.5	19.0 (88.4)	77.3 (103.6)	0.8
1st week	0.5	2.3	4.7	16.8	21.5 (100.0)	75.2 (100.7)	0.5
2nd week	0.2	2.7	5.3	19.8	25.2 (117.1)	72.0 (96.4)	0.0
3rd week	0.0	2.2	2.8	16.2	19.0 (88.4)	78.0 (104.5)	0.8
4th week	0.3	2.3	3.3	20.5	23.8 (110.9)	72.8 (97.6)	0.7
C-Group (0.1 cc/mg)							
Pre-Exper. Value	1.0	2.5	10.0	12.8	22.8 (100)	73.3 (100)	0.3
3rd day	0.3	2.5	3.7	14.3	18.0 (78.8)	78.0 (106.4)	1.0
1st week	0.3	3.8	2.7	20.5	23.2 (101.5)	71.2 (97.0)	1.5
2nd week	0.3	3.0	2.3	15.7	18.0 (78.8)	78.3 (106.8)	0.3
3rd week	0.2	2.0	2.3	14.5	16.8 (73.7)	80.8 (110.2)	0.2
4th week	0.3	2.7	1.7	16.3	18.0 (78.8)	78.3 (106.8)	0.7
Cont.-Group (Olive oil 1.0 cc/mg)							
Pre-Exper. Value	2.9	1.1	2.9	14.0	16.9 (100)	81.3 (100)	0.4
3rd day	0.3	1.7	3.6	13.9	17.4 (103.4)	78.6 (96.7)	2.0
1st week	0.3	2.7	2.3	16.9	19.1 (113.6)	76.7 (94.4)	1.1
2nd week	0.1	2.0	2.6	13.9	17.9 (105.9)	81.0 (99.6)	0.4
3rd week	0.3	1.7	1.4	14.1	15.6 (92.4)	82.1 (101.1)	0.3
4th week	0.1	2.4	2.1	14.1	16.3 (96.6)	80.1 (98.6)	1.0

The tendency toward an increase in neutrocyte was recognized in group B, but reversely in group C, and the tendency toward a decrease in neutrocyte was slight.

vii) Pathological findings:

Macroscopic changes: Abnormal findings were remarkably recognized in the spleens and lungs of both groups A and B. Namely, the spleens became glaring dark brown and hypertrophy was noticeable. Moreover the ratio of spleen weight to body weight was about 3 times in group A and over 2 times in group B greater than in the control group (as shown in Table 8 and Fig. 6).

TABLE 8. The Ratio of Organ Weight to Body Weight

Organ→ Group↓	Liver	Spleen	Heart	Thymus	Kidney		Lung	
					right	left	right	left
A	517.6±35.9 (128.0)	109.6±11.3 (319.5)	44.1±2.1 (128.9)	13.3±1.7 (74.7)	43.6±4.4 (126.0)	42.8±4.2 (125.5)	51.6±6.3 (128.0)	26.6±3.8 (127.9)
B	445.0±34.7 (110.0)	73.6±14.0 (214.6)	38.8±1.7 (113.5)	16.5±1.5 (92.7)	38.1±0.5 (110.1)	39.6±0.5 (116.1)	42.3±0.9 (105.0)	22.2±0.7 (106.7)
C	411.0±15.5 (101.6)	58.2±28.1 (169.7)	36.2±1.4 (105.8)	16.1±1.8 (90.4)	34.8±2.9 (100.6)	37.6±2.2 (110.3)	45.3±4.0 (112.4)	24.1±1.6 (115.9)
Control	404.5±13.0 (100.0)	34.3±3.6 (100.0)	34.2±1.3 (100.0)	17.8±0.6 (100.0)	34.6±0.9 (100.0)	34.1±0.9 (100.0)	40.3±3.8 (100.0)	20.8±1.7 (100.0)

$\frac{\text{Organ Weight}}{\text{Body Weight}} \times 10{,}000$ Figures in parentheses show percentages to the values of control.

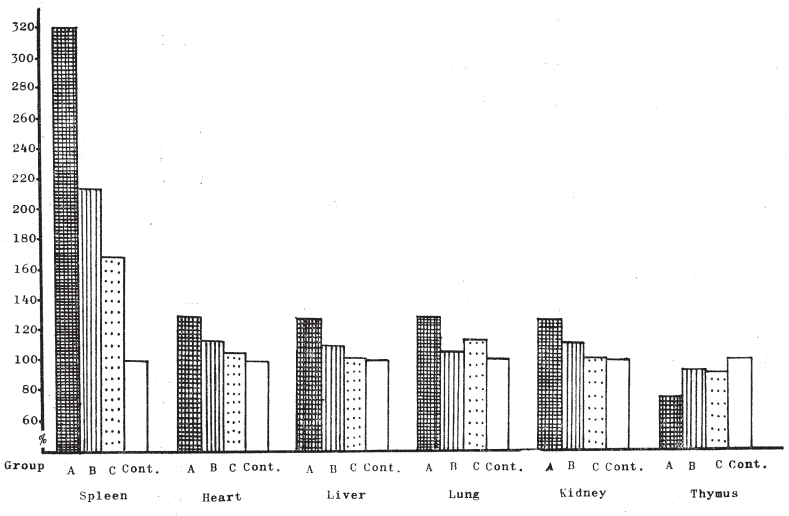


FIG. 6. The ratio of organ weight to body weight as percentage of control group.

In the lungs of groups A and B there was found abundant petechiae, partially flesh-tinted, tight atelectasis, and also some local emphysema.

In the main organs such as the lung, heart, liver, kidney, etc., the ratio of the organ weight to body weight was larger in proportion to the injected dosages of MEK-oxime, with the exception of the thymus where the ratio showed entirely an inverse tendency.

Microscopic changes: Examinations were made of the lung, heart, liver, stomach, spleen, kidney, sprarenal gland, testicle, thymus, abdominal lymphatic node, and skin.

In these organs, abnormal findings were recognized in groups A and B and are described below, but there were no remarkable changes in group C and the control group.

In the spleen (Photo. 1), a hemorrhagic tendency and erythrocyte wandering throughout the blood vessels were observed, while the major part of the tissue was occupied by red pulp. Therefore, noticeable shrinkage of the white pulp and lymphopenia were recognized.

In the lung (Photo. 2), acute inflammation was found. Wandering of such inflammatory cells as neutrophil, lymphocyte and plasma cell and stagnation in alveolar stroma were observed. On the other hand, the following chronic inflammatory findings were also recognized in some parts: wanderings of these cells into alveoli, slight fibrotic tendency, and fibrous hypertrophy and cell infiltration of bronchiole walls.

In the liver (Photo. 3), though there were no noticeable changes, it was found that there were basophil granules in some protoplasm and slight disturbances of some trabeculae.

Concerning the lymph nodes, swelling of some abdominal lymph nodes were observed, and moreover there were inflammatory pictures in some of them such as stasis of lymphocyte and plasma cells and fibrosis.

After local skin injection, some necrosis and cell infiltrations were observed in the subcutaneous layers. Among 4 rats of group A, abscesses of about 5 to 10 mm diameter were formed in the subcutaneous tissues injected.

In the interstitial tissues of the cardiac muscle fibers (Photo. 4), the medulla of the kidneys (Photo. 5), and the cortex of the suprarenal glands, many erythrocyte wanderings were observed and a bleeding tendency was noticeably recognized. The above described findings and tendencies were recognized markedly in proportion to the injected dosage.

viii) Serum protein concentration: The examinations were performed using blood drawn from *A. femoralis* of each rat immediately after the medicaion period of 4 weeks. The values obtained were as shown in Table 9. The average serum protein concentrations of the groups were 6.96 mg/dl in group A, 7.23 mg/dl in group B, 7.67 mg/dl in group C and 7.88 mg/dl in the control group.

TABLE 9. Serum Protein Concentration

Group A		Group B	
No. 1	6.94 mg/dl	No. 1	7.46 mg/dl
No. 2	7.22	No. 2	6.95
No. 3	7.30	No. 3	7.52
No. 4	6.48	No. 4	6.98
No. 5	6.85	No. 5	7.35
No. 6	6.96	No. 6	7.28
Mean	6.96	Mean	7.23
Group C		**Control Group**	
No. 1	7.68 mg/dl	No. 1	7.42 mg/dl
No. 2	7.95	No. 2	8.26
No. 3	7.72	No. 3	7.70
No. 4	7.54	No. 4	7.92
No. 5	7.74	No. 5	8.24
No. 6	7.30	No. 6	7.60
		No. 7	8.04
Mean	7.67	Mean	7.88

It was found that these concentrations showed statistically significant differences ($p<0.01$) between groups A and B, and between group B and the control group.

ix) Serum protein fractions: Serum protein electrophoresis was carried out using the same sera as that for measuring the serum protein concentrations. Serum protein fractions obtained by starch gel electrophoresis were classified as shown in Fig. 7, by the analysis method of Beaton[15]. It was found that the serum proteins of the experimental rats were composed of albumin, post-albumin IV, post-albumin II, fast-α_1-globulin, 2 bands of fast-α_2-globulin, transferrin II, transferrin I, slow-α_1-globulin and γ-globulin components.

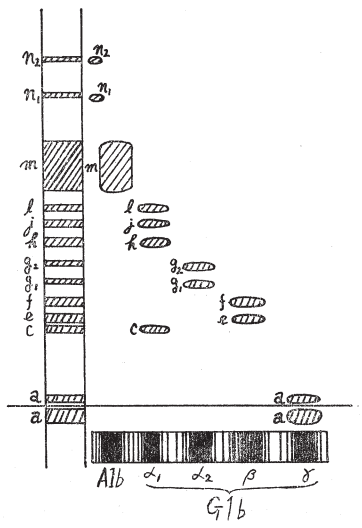


FIG. 7. Scheme of serum protein fractions of rat by Beaton.
n : pre-albumin,
m: albumin,
l : post-albumin I,
j : post-albumin III,
h : fast-α_1-globulin,
g : fast-α_2-globulin,
f : transferrin I,
e : transferrin II,
c : slow-α_1-globulin,
a : γ-globulin.

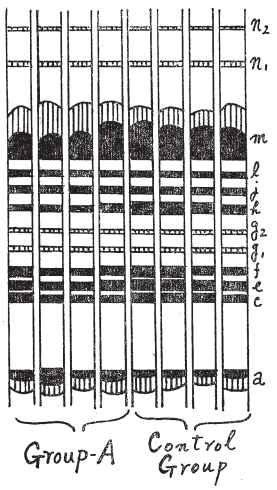


FIG. 8. Results of starch gel electrophoresis.

The results of serum protein electrophoresis of group A and the control group are shown in Photograph 6 and Fig. 8.

Generally, noticeable changes were recognized in groups A and B, compared with the control group. First, in both groups A and B the band of fast-α_1-globulin was fairly thin when compared with the control. Therefore, this phenomenon indicates a decrease in fast-α_1-globulin. Secondly, similar tendencies to decrease were also seen in transferrin I of both groups. Thirdly, γ-globulin was comparatively abundant in rats No. 1, No. 4 and No. 6 of group A indicating a slight increase of γ-globulin. No noticeable change was observed in group C.

Discussion

Generally speaking, poisoning symptoms were clearly observed in rats of groups A and B, which were injected with more than 0.5 mg/kg of MEK-oxime, while they were barely recognizable in rats of group C injected with 0.1 ml/kg of MEK-oxime.

Drowsiness of the rats continued much longer in proportion to prolongation of the exposure period, and therefore it is supposed that this results from accumulation of MEK-oxime into the body.

It appears that the temporary decrease in body weights of rats in groups A and B, notwithstanding the growing stage, was caused by loss of appetite and diarrhea (dyspepsia) due to the irritating action of MEK-oxime on the mucous membrane of the digestive organs.

In groups A and B, erythrocyte counts and hemoglobin contents recovered gradually after the 3rd week, after an initial decrease, but anemia tendency was recognized in general. These findings agreed with the hemorrhagic tendencies of a series of organs which were observed in the pathological examinations.

Noticeable initial leucocytosis in only group A was temporarily observed on the 3rd day. In groups A and B, leucocyte counts increased gradually after the 3rd week, but in group C, noticeable changes were barely recognized. From the fact that the initial leukocytosis was not observed in groups other than group A, it seemed that the findings were caused not by the pharmacological action of MEK-oxime itself but by the stimulation[16)17)] of a foreign body. And moreover, it is supposed that the leukocytosis in groups A and B after the 3rd

week resulted from inflammation since there was the existence of subcutaneous abscess, pathological pictures of organs, and leukocytosis, especially neutrocytosis.

Dissection examinations revealed stagnation and hypertrophy in substantial organs and especially, the ratio of spleen weight to body weight was about 3 times higher in group A as compared with the control group.

From pathological examinations, erythrocyte wanderings in the spleen were found to be remarkable. All the white pulp was shrunken, and for the most part was occupied by red pulp.

In the lymphatic tissues shrinkage tendency was strongly observed, and a decrease in the ratio of thymus weight to body weight, disappearance of white pulp in the spleen, and destruction or necrosis of lymphatic nodes were also observed.

In other organs, except the lung which showed inflammatory findings, erythrocyte wanderings or hemorrhagic tendencies were generally observed. As these hemorrhagic findings have been frequently observed in cases of general industrial poisoning, and also as there is a report[18] concerning brain edema or congestion of many organs in PAM poisoning, these phenomena must be pursued in a supplemental experiment.

Average serum protein concentrations of groups A and B decreased distinctly and there was a statistically significant difference ($p<0.01$) when compared with the control, but in group C there was seen no statistically significant difference. These decreasing tendencies of serum proteins may be related to loss of appetite, decrease of body weight, anemic tendency, and liver damage.

It is well known that in general serum protein fractions of man or animal might become varied by chronic diseases, poisonings, etc.

O. Smithies (1955)[12] reported a method of zone electrophoresis in which starch gel was used as the supporting medium, and it was found that in many cases the method was superior in resolving power to the Tiselius or other methods'.

Moreover, Latner and Zaki (1957)[13] studied serum protein fractions from many species of animals including the albino rat using the starch gel electrophoresis method, and they recognized species difference. On the other hand C. Rimington[19] examined the serum protein fractions of sporidesmin poisoning in the albino rat by the electrophoretic method, and characteristic changes were observed in the fractions.

In accord with the above data, the serum protein fractions of the albino rats used in this experiment were examined by a modified method of Smithies.

Initially serum protein fractions of normal albino rats (control group) were classified by the method of Beaton[15] but our patterns of serum protein fractions of normal albino rats differed somewhat from Beatons' reports.

In this experiment, post-albumin I and III were obtained instead of post-albumin II and IV as observed by Beaton, and also the site of trasnsferrin was nearer the site of slow-α_1-globulin than that of Beatons' description.

Though the decrease in serum protein concentrations by the MEK-oxime injections were generally observed in groups A and B as mentioned before, there was merely a slight decrease of fast-α_1-globulin and transferrin I and a slight decrease of γ-globulin in some rats of group A. Therefore, characteristic changes were observed.

Though the changes in serum protein fractions are minor thus far, these findings are most likely correlated with the decrease in body weight, anemia, and liver damage.

In a preceding experiment, it was found that acute butanone poisoning resembled the symptoms of acute MEK-oxime poisoning.

In comparing chronic MEK-oxime poisoning with chronic butanone poisoning, several facts may be derived.

Though there are only a few reports[20) 21) 22)] on chronic butanone poisoning, it has been described by Browning that congestion or emphysema of the lung, and stagnations in parenchyma of organs such as liver, kidney, brain and etc. are observed in chronic butanone poisoning. Chronic MEK-oxime poisoning resembles chronic butanone poisoning as far as these pathological findings are concerned.

EXPERIMENT II

Some oxime derivatives such as PAM, DAM etc. have recently come to attract much attention as new therapeutic drugs for organophosphate poisoning, because of the reactivating action of organophosphate inhibited by acetylcholinesterase. However, Inoki[23)] and Loomis[1)] have reported that when these drugs are taken singly by the living body, they themselves show restrictive action on the cholinesterase activity.

The following report involves the study of cholinesterase activity and some supplemental examinations concerned with the bleeding tendency observed in the preceding experiment.

Experimental Subjects and Procedures

A group injected with MEK-oxime and a control group consisted of 5 male albino rats, weighing 130 to 190 g.

Rats in the experimental group were subcutaneously injected with 1.5 ml/kg body weight of MEK-oxime every other day for 4 weeks and similarly rats in the control group were injected with 1.5 ml/kg body weight of olive oil.

Measurements

After the MEK-oxime administration for 4 weeks, the following items were

examined: namely, cholinesterase activity of erythrocyte and plasma, capillary fragility test, and resistance of erythrocyte.

Methods

i) Cholinesterase activity: There have been a few reports[24)25)] concerned with the standard cholinesterase activity value of albino rats. After qualitatively measuring the standard plasma cholinesterase activity values of normal albino rats by the instant test paper Acholest[26)], as a preliminary test, the cholinesterase activity values of the experimental rats were measured quantitatively by the modified method of Michel[27)] as shown in Table 10.

ii) Capillary fragility test: Experimental rats' abdomens were depilated with the depilatoria "Ebacream"* two days before the test day, in order to avoid influences from the depilation.

The measurement instrument used was the petechio-meter by the Sato-method[28)]. This instrument was manufactured by Iwashiya Co.

The pressure in the sucking-cup set on the experimental rat's abdomen was accurately kept within -100 or -200 mm Hg for 1 minute, and after

TABLE 10. Measurement of Cholinesterase Activity

Cholinesterase Activity of Erythrocyte	Cholinesterase Activity of Plasma
1.0 ml of 0.01% saponin + 0.02 ml of erythrocyte; 1.0 ml of buffer for erythrocyte*	1.0 ml of buffer for plasma** + 1.0 ml of distilled water; 0.02 ml of plasma
↓ Incubate in a constant temperature bath for 15 min. at 37°C	↓ Incubate in a constant temperature bath for 15 min. at 37°C
↓ Measurement of pH_1; 3.6 mg of acetyl choline	↓ Measurement of pH_1; 5.4 mg of acetyl choline
↓ Incubate in a constant temperature bath for 1 hour at 37°C	↓ Incubate in a constant temperature bath for 1 hour at 37°C
↓ Measurement of pH_2	↓ Measurement of pH_2

The cholinesterase activity value (ΔpH) is calculated by the following formula.

$$\Delta pH/1 \text{ hour} = (pH_1 - pH_2 - b) \times f$$

(The b and f value being the corrective indexes have been shown in the original work by Michel[27)])

* One liter of the buffer contains 4.1236 g of soluble barbiturate, 0.5446 g of potassium phosphate monobasic, 44.73 g of sodium chloride and 28.0 ml of N/10 hydrochloric acid.

** One liter of the buffer contains 1.237 g of soluble barbiturate, 0.1361 g of potassium phosphate monobasic, 17.535 g of sodium chloride and 11.6 ml of N/10 hydrochloric acid.

* Medical preparation of the Tokyo Tanabe Pha. Co. Ltd. of Japan.

removing the sucking-cup, it was left intact for 5 minutes more. Then, the number of petechiae appearing on a 10 mm diameter portion of the sucked-skin were counted.

iii) Resistance of erythrocyte: A drop of erythrocyte was added to a mechanical mixture in each test tube, consisting of sodium chloride solutions from 0.5% to 0.28% with a difference of 0.02% each, and each degree of hemolysis was examined after leaving it at room temperature for 1 hour. (The erythrocytes examined were purified by centrifuging 3 times and by washing twice with physiological sodium chloride solution.)

Results

i) Erythrocyte and plasma cholinesterase activity: As a preliminary examination, the plasma cholinesterase activity of normal albino rats was measured by the instant test paper "Acholest", and it was found that plasma cholinesterase values of albino rats were very low when compared with those for man, by

TABLE 11. Plasma Cholinesterase Activity

	pH_1	pH_2	pH/hour
MEK-oxime Injected Group			
No. 1	7.44	7.33	0.08787
No. 2	7.39	7.30	0.07070
No. 3	7.40	7.31	0.06969
No. 4	7.43	7.33	0.07777
No. 5	7.41	7.32	0.06868
Mean			0.07494
Control Group			
No. 1	7.44	7.33	0.08787
No. 2	7.39	7.29	0.08072
No. 3	7.43	7.32	0.08888
No. 4	7.41	7.31	0.07979
No. 5	7.44	7.32	0.09898
Mean			0.08725
Erythrocyte Cholinesterase Activity			
MEK-oxime Injected Group			
No. 1	7.43	7.29	0.14000
No. 2	7.41	7.29	0.12000
No. 3	7.38	7.25	0.13000
No. 4	7.40	7.26	0.14000
No. 5	7.42	7.31	0.10989
Mean			0.12798
Control Group			
No. 1	7.46	7.31	0.14980
No. 2	7.42	7.28	0.14000
No. 3	7.41	7.26	0.15000
No. 4	7.43	7.29	0.14000
No. 5	7.39	7.23	0.16000
Mean			0.14790

the fact of having elapsed over 20 hours on reaction of these test papers.

Next, cholinesterase activity values of erythrocyte and plasma were measured by the modified method of Michel as shown in Table 10 and the results are shown in Table 11. Acetylcholine, commercial name Ovisot, which was added to the sample was decomposed into choline and acetic acid by cholinesterase. The solution after reaction becomes acidic by the released acetic acid. Therefore the pH (pH_2 in the Table 11) of the sample solution after reaction becomes more acidic than the pH (pH_1 in Table 11) of the solution before reaction in proportion to the strength of the cholinesterase activity in the sample solution.

From the results, as shown in Table 11, it was found that both cholinesterase activities of erythrocyte and plasma were inhibited by nearly 15% in the MEK-oxime injected group in comparison with the control, and these values, including both activities of erythrocyte and plasma were statistically significant between the injected and the control group ($p<0.05$).

ii) Capillary fragility test: The number of petechiae produced on the abdominal skin by the application of pressures of -100 or -200 mmHg (by the petechio-meter) for 1 minute are shown in Table 12.

With a pressure of -100 mmHg, petechiae were not recognized in more than one half of the total rats in the 2 groups. And moreover, the number of petechiae appearing per rat was merely 1 or 2.

Similarly with -200 mmHg, petechiae appeared on the majority in both groups, but the number of petechiae was within 10 in the 2 groups.

These differences were not observed to be statistically significant between the injected and the control group.

iii) Resistance of erythrocyte (Table 13): In comparing the 2 groups, the beginning of hemolysis (the maximum resistance of erythrocyte) was on the average 0.464% of sodium chloride solution in the MEK-oxime injected group and 0.448% of sodium chloride solution in the control group. Completion of

TABLE 12. Resistance of Capillary

	No. of Petechiae produced on Skin	
	In -100 mmHg	In -200 mmHg
MEK-oxime Injected Group		
No. 1	0	1
No. 2	1	4
No. 3	0	3
No. 4	0	0
No. 5	2	3
Control Group		
No. 1	0	0
No. 2	0	2
No. 3	0	0
No. 4	2	3
No. 5	0	0

TABLE 13. Erythrocyte Resistance

	Beginning of Hemolysis	Completion of Hemolysis
MEK-Oxime Injected Group		
No. 1	0.44% of Sodium Chloride Solut.	0.32% of Sodium Chloride Solut.
No. 2	0.48%	0.34%
No. 3	0.46%	0.32%
No. 4	0.46%	0.32%
No. 5	0.48%	0.32%
Mean	0.464%	0.324%
Control Group		
No. 1	0.44% of Sodium Chloride Solut.	0.30% of Sodium Chloride Solut.
No. 2	0.46%	0.32%
No. 3	0.44%	0.30%
No. 4	0.46%	0.32%
No. 5	0.44%	0.30%
Mean	0.448%	0.308%

hemolysis (the minimum resistance of erythrocyte) was on the average 0.324% of sodium chloride solution in the MEK-oxime injected group and 0.308% of sodium chloride solution in the control group.

The differences in the maximum resistance were not statistically significant between the 2 groups, but the differences in the minimum resistance were statistically significant ($p<0.05$) between the 2 groups.

Discussion

There are two kinds of cholinesterase, namely the specific cholinesterase, which can decompose only cholinester, and the non-specific cholinesterase, which can decompose other esters.

In the living body, the specific cholinesterase is conteined in the erythrocytes, brain, and other nervous tissues, and the non-specific cholinesterase in plasma, liver, pancreas, and other organs.

The cholinesterase in plasma decreases in proportion to the diminution of serum albumin caused by liver diseases and inanition, etc. and regenerates according to the regeneration of serum proteins.

Therefore, it is supposed that plasma cholinesterase may serve as an indicator of liver function and protein metabolic function, though these function have no direct relation to acetylcholine metabolism.

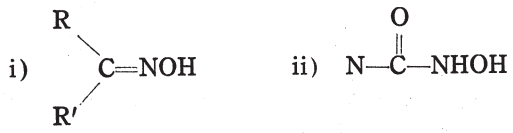
Cholinesterase in erythrocytes is the specific cholinesterase, having strong activity, but it does not have such direct concern with nervous functions as the specific cholinesterase in brain and other nervous systems.

On the other hand, it has been reported[23] that when an animal was independently medicated with a high concentration such as 10^{-3} mol. of PAM,

possessing reactivating action of the cholinesterase inhibited by organophsphate, the specific cholinesterase in brain and erythrocyte and moreover the non-specific cholinesterase in plasma were inhibited.

Cholinesterase is composed of an esteratic site and an anionic site in chemical structure. Parathion, an organophosphatic preparation, has affinity for the esteratic site, and PAM, one of oxime derivatives, has affinity for the anionic site. Therefore, there is non-competitive antagonism between PAM and parathion for cholinesterase, as has been related by Inoki.

From the results[29] that chemicals with the following general formulae (example, such oxime derivatives as PAM, MEK-oxime etc.) are superior as reactivation drugs of cholinesterase inhibited by organophosphate, it is supposed that the anti-cholinesterase effects observed by the single medication of PAM may appear likewise by a single medication of MEK-oxime.



Moreover, if it can be assumed from the report of Augstinsson[30] that rat thymus is very rich in specific cholinesterase, the decrease in the ratio of thymus weight to body weight in spite of hypertrophy of other organs may be connected with the anti-cholinesterase effect of MEK-oxime.

It may be concluded that the decrease of erythrocyte cholinesterase activity was caused by the direct anticholinesterase action of MEK-oxime and by the decrease of thymus function. And, on the other hand, the decrease of plasma cholinesterase activity was caused by nutritional disturbances of the experimental rat together with the direct action of MEK-oxime.

As frequently discussed, the fragility and permeability of the blood vessel wall become important as one of the causative factors in the hemorrhagic tendency.

Capillary resistances were measured as an index of hemorrhagic tendency, namely, by the application of 200 mmHg negative pressure for 1 minute, and the number of petechiae increased in the MEK-oxime injected group in contrast to the control. However, the number of petechiae was less than 10 and therefore showed no statistically significant difference between the both groups.

In shortening the time of the erythrocyte life as for example in hemorragic diseases, chronic liver diseases etc., the resistances of erythrocyte may diminish, as has been said, but in this experiment, both resistances of the minimum and maximum showed a 5% decrease in the MEK-oxime injected group in comparison with that of the control.

Moreover, a statistically significant difference ($p<0.05$) was recognized in

the minimum resistance but not in the maximum resistance.

From these facts it is conjectured that the hemorrhagic tendency in the MEK-oxime injected group was caused rather by the blood clotting mechanism than by the decrease of capillary fragility.

Diminution in the erythrocyte resistance, decrease of the peripheral erythrocyte count, change of the serum protein fractions, and pathological changes in the liver were observed in these experiments. Delay of the blood clotting time of rabbits treated with some oxime derivatives, having a reactivating action for acetylcholinesterase was reported recently by Albanus *et al.*[31] Also, an antagonistic relation between vitamin K and acetylcholine *in vivo* has been reported by Okinaka *et al.*[32] From the above results, it may be concluded that these hemorrhagic tendencies of rats treated with MEK-oxime are caused by some defects in the blood clotting factors or the blood clotting mechanism that is closely connected with liver function.

CONCLUSION

The results obtained by experimental exposure to MEK-oxime using albino rats are as follows.

i) LD_{50} by subcutaneous injection of MEK-oxime into the back of rat.

By Behrens-Kärber method.

$LD_{50} = 2701.5$ mg/kg B.W.

(2.93 ml/kg B.W.)

By Van der Wärden method.

$LD_{50} = 2761.6$ to 2813.2 mg/kg B.W.

(2.99 to 3.05 ml/kg B.W.)

ii) By application of MEK-oxime on the skin of rat, reversible simple dermatitis was recognized, though individual differences existed.

iii) Instillation of MEK-oxime into one eye resulted in heavy hyperemia of both eye-lids, bulb conjunctivae, and eye pain.

iv) In cases of breathing a saturated vapour of MEK-oxime or the injection of large dose of MEK-oxime, experimental rats fell into a drowsy state after an agitated stage, and moreover rats died in the worst case.

v) In case of breathing saturated vapour of MEK-oxime, appetite loss and indigestion were recognized in the majority of rats.

vi) Symptoms of acute poisoning of MEK-oxime resembled those of butanone.

vii) As a results of the study on chronic poisoning of MEK-oxime, abnormal findings were generally recognized in groups injected with over 0.5 ml/kg B.W. and were as follows: inhibition of animal's growth, slight decrease of erythrocyte count and hemoglobin content, secondary leukocytosis, caused by inflammation after the initial leukocytosis, relative lymphopenia in spite of the absolute

neutrophilia on the hemogram, atrophy of lymphatic tissue, namely decrease of the ratio of thymus weight to body weight, disappearance of white pulp in the spleen and destruction of lymphatic nodes, stagnation hypertrophy of organs (lung, heart, liver, kidney, spleen, suprarenal body) exept lymphatic tissues, especially noticeable hypertrophy of spleen, hemorrhagic tendency and erythrocyte wandering throughout blood vessels in many organs, enlargement of red pulp and sedimentation of erythrocytes in spleen, and partial appearance of pulmonary atelectasis, pulmonary emphysema and bronchial pneumonia in lung tissues.

viii) Serum protein concentration decreased distinctly in proportion to the injected dosage of MEK-oxime. A statistically significant difference was recognized between groups A or B and the control. ($p<0.01$)

ix) As a result of the analysis of serum protein by the starch gel electrophoresis method modified from Smithies', fast-α_1-globulin and transferrin I of groups A and B showed a decreased tendency compared with the controls'. A slight increase of γ-globulin was observed in some rats of group A.

x) Both activities of the specific cholinesterase in the erythrocyte and non-specific cholinesterase in the plasma were inhibited by nearly 15% in chronic MEK-oxime poisoning, and also there was a statistically significant difference between the MEK-oxime injected group and the control. ($p<0.05$)

xi) Concerning capillary fragility, there was no noticeable difference between the MEK-oxime injected group and the control by the application of 100 or 200 mmHg of negative pressure for 1 minute.

xii) Concerning the resistance of erythrocyte, the average resistance of the MEK-oxime injected group was 0.464% of sodium chloride solution at the maximum and 0.324% at the minimum, and also the average resistance of the control was 0.448% at the maximum and 0.308% at the minimum. However, a statistically significant difference between both groups was observed only for the minimum resistance. ($p<0.05$)

xiii) From these experimental results and supporting literature, it is hypothesized that the hemorrhagic tendency of many organs which was observed by chronic exposure was caused by some defects of the blood clotting mechanism.

The toxicity of MEK-oxime is not so severe as the lethal dose is concerned, but from the fact of its having a bad influence on the living body, such as the development of splenic tumor, hemorrhagic tendency of many organs, and inhibition of both cholinesterase activities of the specific and the non-specific types, etc., industrial handling of MEK-oxime warrants careful hygienic attention.

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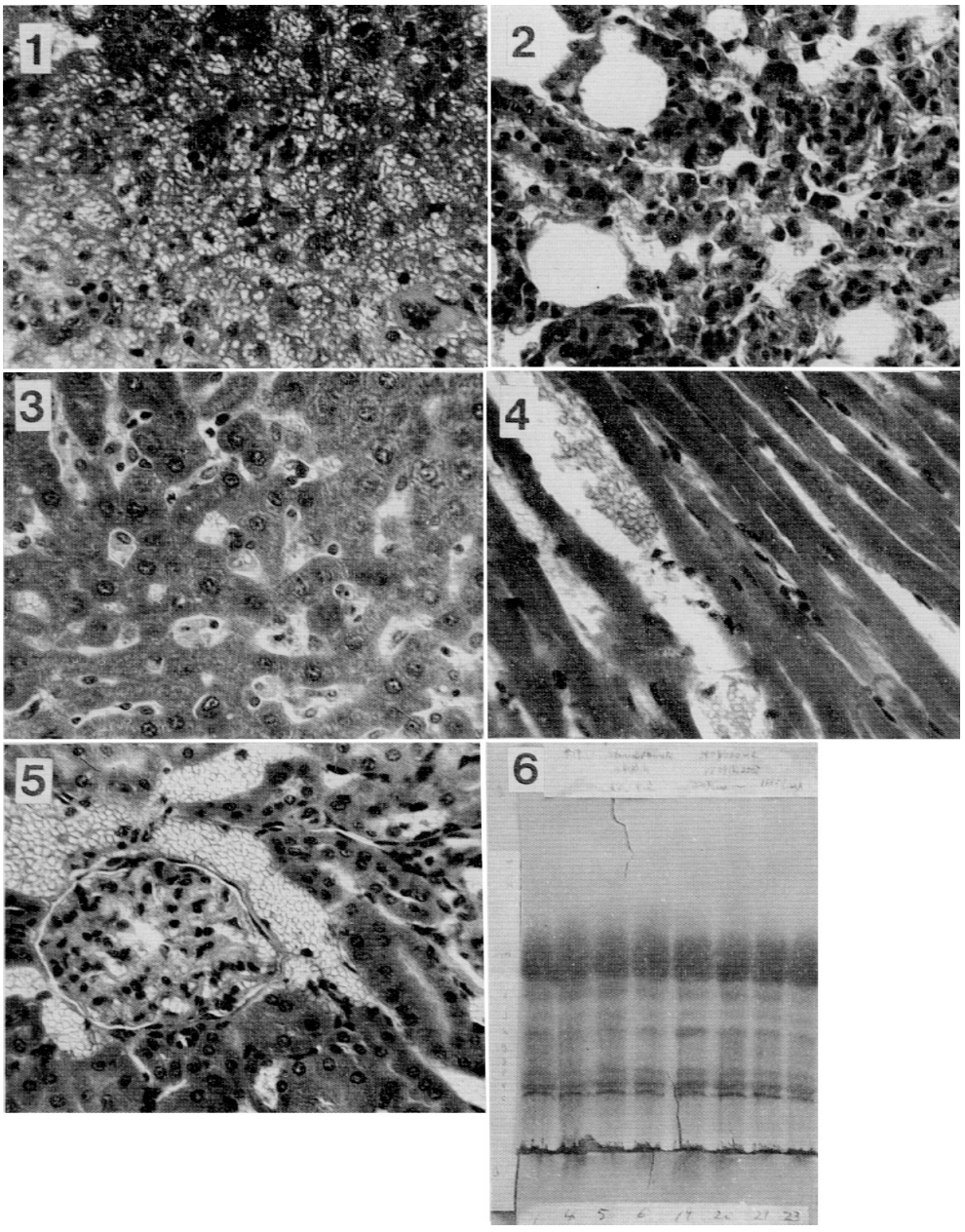

PHOTO

1: Spleen (×400) 2: Lung (×400)
3: Liver (×400) 4: Heart (×400)
5: Kidney (×400)
6: Result of Starch Gel Electrophoresis