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TOLERANCE CRITERIA FOR CONTINUOUS INHALATION EXPOSURE TO TOXIC MATERIAL

II. Effects on Animals of 90-Day Exposure to H₂S, Methyl Mercaptan, Indole, and a Mixture of H₂S, Methyl Mercaptan, Indole, and Skatole

Dr. Curtis Sandage

Midwest Research Institute

December 1961

Contract No. AF 33(616)-7055

(Contract Monitor: Kenneth C. Back, Ph.D.)

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AEROSPACE MEDICAL LABORATORY
AERONAUTICAL SYSTEMS DIVISION
AIR FORCE SYSTEMS COMMAND
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Project No. 7165

Task No. 716501

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FOREWORD

This report was prepared by Midwest Research Institute under USAF Contract No. AF 33(616)-7055. The contract was initiated under Project No. 7165, "Health Hazards of Materials and Radiation," Task No. 716501, "Evaluation and Control of Toxic Chemical Materials." The work is administered under the direction of the Aerospace Medical Laboratory, Aeronautical Systems Division, with Dr. Kenneth C. Back, Toxic Hazards Section, Physiology Branch of the Biomedical Laboratory, acting as project initiator and contract monitor.

The period of research covered is from 15 January to 30 September 1961.

The experimental work on this project was directly supervised by Dr. Curtis Sandage, under whom Mr. W. E. Carter was responsible for the activities of Registered Medical Technologists A. M. Kilp, Ethel Nott, and B. A. Kenagy. This project was carried out in the Biochemistry Section of the Chemistry Division, Dr. B. W. Beadle, Director.

Gross and microscopic pathology on monkeys was performed by Dr. T. Y. Lou, Pathologist, Virus Research Laboratory, Kansas University Medical Center, Kansas City, Kansas. Microscopic examination of tissue sections of rat and mouse organs was done by Dr. Nelson Powell, Upshar Laboratories, Kansas City, Missouri.

Animal experimentation was performed in accordance with the "Rules for Animal Care" established by the American Medical Association.

ABSTRACT

Physiological changes in rats, mice, and monkeys were studied during continuous 90-day exposure to controlled atmospheres of toxic vapors and gases. Concentrations of test chemicals were those recognized as Industrial Threshold Limit Values and included: (a) hydrogen sulfide (20 ppm), (b) methyl mercaptan (50 ppm), (c) indole (10.5 ppm), and (d) a mixture of these three compounds plus skatole (3.5 ppm). Hematological and urine analyses and liver function tests were performed before exposure and at 30-day intervals thereafter. At the end of the 90-day exposure period, stress tests were done prior to autopsy for gross and microscopic pathology. Mortality rates were high only in the group exposed to a mixture of four compounds. There was evidence that animals can adapt to tolerate otherwise lethal concentrations of some of these compounds, and that there are great individual differences in the ability to so adapt.

PUBLICATION REVIEW

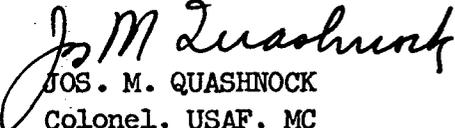

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I. INTRODUCTION

An earlier phase of this investigation of the effects on animals of long-term continuous exposure to toxic vapors was concerned with carbon tetrachloride, phenol, and a mixture of the four compounds included in the present study: methyl mercaptan, hydrogen sulfide, indole, and skatole (ref. 1). Phenol was included as representative of those compounds for which the Threshold Limit Values (T.L.V.) had remained at the same concentration for many years. Carbon tetrachloride represented a group of compounds for which the T.L.V. had been changed frequently. The mixture of four chemical agents was composed of compounds which might be expected to accumulate within the sealed capsule of a space vehicle, in concentrations which might constitute a hazard to health if inhaled over long periods of time. In the previous experiments, as in the present one, the atmospheric concentrations employed were those that represent the present T.L.V.

The results of the first phase of study were not entirely expected. Most of the animals survived 90-day exposure to toxic agents, with the exception of monkeys in the atmosphere containing a mixture of chemicals, in which case eight out of ten animals succumbed. It was doubtful, however, that death was due only to these chemical agents. Further unexpected results were obtained in the case of animals exposed to carbon tetrachloride. While all such animals displayed liver pathology, no deaths in this group could be attributed to carbon tetrachloride poisoning. The only additional inconsistency in pathology appeared in mice and rats of the group exposed to the mixture of vapors. A significant number of mice displayed evidence of lung damage on autopsy, and extent of liver damage was significant in both rats and mice.

Since the mixture of methyl mercaptan, H₂S, indole, and skatole caused the most extensive pathology of the experiment, including 80 per cent mortality to monkeys, it was of obvious interest to determine, if possible, which of these agents individually caused the most serious toxic injury to experimental animals. The present investigation was designed to answer this question.

II. METHODS AND PROCEDURES

A. Exposure Chambers

Four isolation and exposure chambers were utilized, each chamber being 10 feet long, 8 feet wide, and 7-1/2 feet high. Construction included effective insulation and moisture barrier. The chambers were entered by a door at one end. These chambers were located along one wall of a room

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with dimensions of approximately 30 x 40 x 14 feet. The control groups of animals were housed in this large room. Since this room was not as efficiently air-conditioned as were the chambers, the control animals were occasionally exposed to temperatures as high as 86°F, or 6° higher than allowed in the chambers. Minimum temperature differences did not exist, and caging facilities were identical with those in the chambers.

B. Experimental Animals

Rhesus monkeys were obtained from Asiatic Animal Imports, San Francisco, California. They were tuberculin tested and conditioned to monkey chow before shipment to Kansas City. "Sprague-Dawley" general purpose rats were obtained from the Holtzmann Company, Madison, Wisconsin. Mice were obtained from Porten-Woods, Inc., Chicago. Only male animals were ordered. Animal weights (average) at the beginning were as follows: monkeys, 1.7 kilograms; rats, 175 grams; and mice, 25 grams. Each experimental group consisted of ten monkeys, 50 rats, and 100 mice.

C. Environmental Control

Each isolation chamber was equipped with a one ton air-conditioner for maintaining constancy of temperature and of air input. Thermostatic controls were placed within each chamber, and there was no recirculation of air. The volume of air turnover was approximately one-tenth the room volume per minute, or 60 cubic feet. Temperatures were maintained at 75°F, $\pm 5^\circ$.

The chamber and group designations, and the concentrations of chemicals, were as follows:

A - MeSH (50 ppm), H₂S (20 ppm), indole (10.5 ppm), and skatole (3.5 ppm)

B - Hydrogen sulfide (20 ppm)

C - Methyl mercaptan (50 ppm)

D - Indole (10.5 ppm)

E - Controls. No chemical agents

Constancy of chemical input was maintained by the same control system as previously used, with respect to the two gases. The gas cylinders were equipped with noncorrosive, constant-flow, regulator valves. Beyond these tank valves, the gas lines led to solenoid safety valves energized by

a 117-volt AC line. In the event of power failure (which would cause the loss of the primary air supply), the solenoids would be de-energized, closing the valve and shutting off the gas flow. From the solenoid valves, the gas lines led to needle valves and rotometers and thence into the large duct of the primary air supply. Flow rates were controlled by the needle valves and reflected by the rotometers.

The method used for introduction of indole and skatole into chambers A and D was somewhat modified from the previous method. Two large metal boxes were constructed, 24 x 24 x 48 inches. A gasketed lid was clamped on with bolts. Each end of the box was entered by 6-inch pipe. Within the box, dividers were fastened as slots for retaining 24 x 24 filters of the kind used for furnaces. The boxes were attached to the outer walls of chambers A and D in the position shown in Figure 1. The 6-inch pipe extended into the rooms, the bottom ones being attached to small blowers within the chambers. In practice, solid indole was sprinkled over the entire surface of the bottom three filters, and, in the case of room A, skatole was placed on the next higher filters. With the lids clamped on, the blowers were turned on and room air was circulated constantly over these filters, carrying sublimed compound into the rooms through the upper pipe. In practice, the filters carrying the chemicals were weighed before and after new material was added (each day), enabling a fair estimate to be made of the amount of material by weight actually being used.

Tubes leading from the center of the chambers to a connection outside the rooms allowed for periodic sampling of the atmospheres for quantitative chemical analysis. Sample volumes were measured by wet test meters set after the double gas traps inserted in the line to collect the sample.

Methyl mercaptan was determined by absorbing the mercaptans from 10 liters of room air in 20 ml. 0.0081N silver nitrate and titrating the excess silver nitrate with 0.0083N potassium thiocyanate, using ferric alum as indicator. Hydrogen sulfide was removed in the mercaptan analysis by adsorption on a lead acetate column placed in the sampling train.

Hydrogen sulfide and methyl mercaptan were then determined together by absorbing 10 liters of room air in 20 ml. 0.0081N silver nitrate. The excess silver nitrate was titrated with 0.0083N potassium thiocyanate using ferric alum as indicator. The concentration of hydrogen sulfide in the sample was determined by difference (ref. 2).

Indole and skatole were determined together after absorption in chloroform by measuring the red color produced with p-dimethylaminobenzaldehyde reagent. In the presence of strong acid, the color is transferred to the aqueous phase and measured photometrically at 565 m μ (ref. 3).

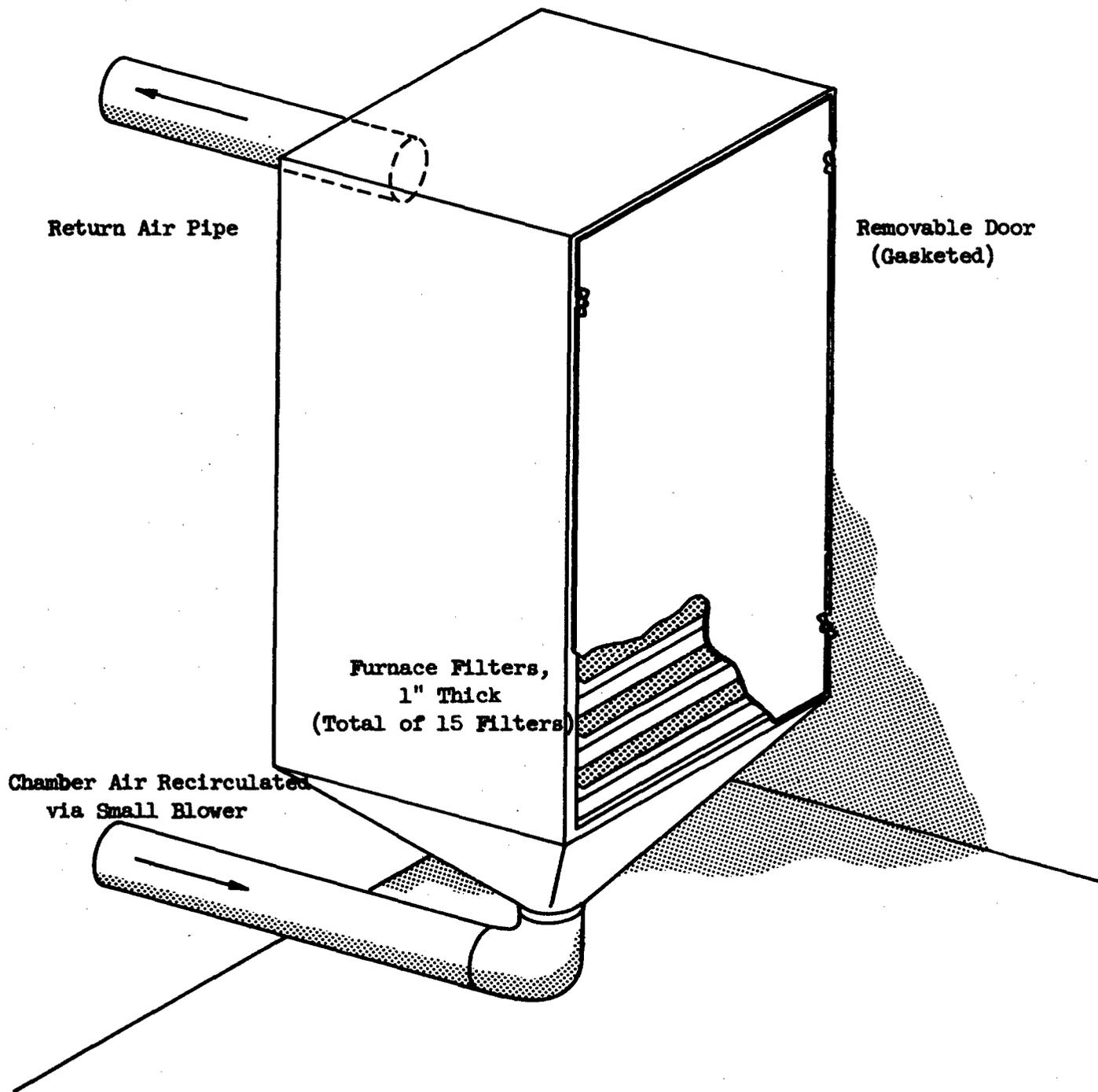


Figure 1. Device for Introduction of Indole into Test Chambers
A and D. Approximate Dimensions, 24" x 24" x 40"

Indole was determined alone by using a modified p-dimethylamino-benzaldehyde reagent. With this reagent, skatole is polymerized and the indole color read at 565 m μ . Values for indole were subtracted from the indole-skatole figures to determine the concentration of skatole.

The concentration of ammonia in room air was obtained by titration. The ammonia from 100 liters of room air was absorbed in 20 ml. 0.02N sulfuric acid and the excess acid was titrated with 0.02N sodium hydroxide solution, using methyl red as indicator (1 ml. 0.02N acid is equivalent to 0.00034 gram ammonia) (ref. 4).

The carbon dioxide from 10 liters of room air was absorbed in 20 ml. 0.10N barium hydroxide solution containing 0.01N barium chloride. The excess barium hydroxide was titrated with 0.0454N oxalic acid using thymolphthalein as an indicator (1 ml. of 0.0454N acid is equivalent to 1 mg. carbon dioxide) (ref. 5).

D. Clinical Laboratory Studies

In a toxicological study of this type, changes in three general categories of physiological observations are most pertinent. These may be roughly grouped as follows: departures from normal values with respect to urinalysis and hematology; functional abnormalities as reflected by liver and kidney function tests; and histological changes as disclosed by gross autopsy and microscopic examination of tissue sections.

The following laboratory tests, concerned with hematology, urinalysis, liver and kidney function tests were performed:

1. Hematology:

a. Erythrocyte and total leucocyte counts: by use of the Coulter Electronic Particle Counter, using a 100-micron aperture and correcting for coincident passage (ref. 6)

b. Differential leucocyte counts: routine procedure, Wright's stain

c. Reticulocyte counts: by the method of Brecher (refs. 7, 8)

d. Platelet counts: by standard methods (ref. 9)

e. Hemoglobin: as cyanmethemoglobin (ref. 10)

f. Sulfhemoglobin: by treatment of whole blood (0.05 ml.) with Drabkin's cyanide-ferricyanide reagent (5 ml.). After mixing, one

drop of concentrated ammonium hydroxide was added to a 3 ml. aliquot of the mixture and the optical density measured at 618 m μ .

g. Hematocrits: by routine methods (ref. 11)

h. Sodium and potassium: by flame photometry, using a Beckman DU flame photometer with photomultiplier attachment (ref. 12)

i. Glucose: by the Nelson-Somogyi micro procedure (ref. 13)

j. Mean values for corpuscular volume, corpuscular hemoglobin, and corpuscular hemoglobin concentration: by calculation from appropriate data

2. Urinalysis:

a. Total protein: by the sulfosalicylic acid method (ref. 14)

b. Urobilinogen: by reaction with Ehrlich's aldehyde reagent (ref. 15)

c. Specific gravity: by weighing a 5.00 ml. sample on the analytical balance to four-place accuracy. This weight was divided by the weight of the same volume of distilled water to obtain specific gravity

d. pH: by a Beckman Model G pH meter

3. Blood chemistry: (monkeys only)

a. Alkaline phosphatase: by the Bessey-Lowry method (ref. 16)

b. Cholinesterase: by measurement of released acetylcholine (ref. 17)

c. Amylase: by the method of Somogyi (ref. 18)

d. Lipase: by the method of Tietz (ref. 19)

e. Glutamic oxalacetic transaminase: by measurement of oxalacetate released by the enzyme and a catalyst (ref. 20)

4. Liver function tests: (monkeys only) These tests utilized the sulfobromophthalein method of Seligson et al. (ref. 21). The amount of dye injected was based on animal weight, and a 45-minute interval was allowed between injection and withdrawal of the second blood sample.

The schedule by which blood and urine samples were collected was simplified considerably, as compared with the previous operation. In the case of monkeys, all animals were treated individually throughout the study. Rats were also treated individually insofar as blood sampling was concerned, but groups of four rats served as a single source of urine. In the case of mice, five mice provided one blood sample, with care being taken that the same amount of blood was obtained from each animal. Ten mice were used for collection of one urine sample.

The sampling was divided into "phases." Phase I consisted of the samples taken in advance of exposure to toxic vapors. Phases II, III, and IV were at 30, 60, and 90 days, respectively. Although all the monkeys were treated individually, baseline (Phase I) data were obtained for rats and mice by pooling of data obtained on 20 per cent of the animals of each species. In the case of rats, for example, 50 of the animals were selected at random from the entire shipment. Urine and blood samples were collected from each of these animals, after which ten rats were randomly selected from the 50 for placing in each experimental group. The remaining 200 rats were then similarly distributed at random. After individual hematological determinations were made, the results were averaged, and this average value used as "baseline" value for all five rat groups. Mice were treated similarly, except that twice as many were used and each laboratory blood sample came from five mice instead of one. It will be noticed, then, as a result of this approach that the first vertical column of data for mice is the same for all five groups. The same is true for rat data.

Blood samples were obtained from monkeys by venepuncture, and from rats by cardiac puncture. Blood was obtained from mice by cutting off a short segment of tail. In the case of rat and monkey blood, a part of each sample was needed uncoagulated, and ethylenediamine tetraacetic acid was used as anticoagulant. This was not necessary in the case of mouse blood.

E. Stress Tests

In addition to clinical studies performed on animals, approximately 50 per cent of the animals surviving at the end of the 90-day test period were subjected to stress tests, performed in accordance with the method of Wilber (ref. 22). Conditions for the tests were determined for each of the three species of animals using control animals as a reference. Water temperatures were selected on the basis of practical limits of endurance. It was thought that 45-minute swimming time for the control animals would be sufficient to present a valid statistical difference in performance. In view of this, water temperatures were adjusted precisely at 22°C for monkeys and 17°C for rats and mice.

A smooth-walled tank 6 feet high and 6 feet in diameter, containing 4 feet of water was used to test monkeys and a 20-gallon reservoir was

employed for rats and mice. The animals were allowed to swim until completely exhausted; the end point taken as the time the animal could no longer keep its head above the surface of the water. In general, these animals were sacrificed shortly thereafter and autopsied. The remaining animals were held for a two-week observation period, after which they were also autopsied.

F. Pathology

Histological preparations were made of 5-6 organs from a large number of animals. These included all monkeys, and those surviving at the end of the test period were sacrificed after undergoing stress tests, as were about 25 per cent of the rats and mice. Tissues were fixed in 10 per cent buffered formalin. Sections were stained routinely with hematoxylin and eosin. Observations were recorded of both gross and microscopic autopsy examinations.

G. Statistical Analysis

The 95 per cent confidence limits of a difference were computed as in the following example, in which values of erythrocyte counts at Phase IV were compared for two groups of mice.

<u>Group</u>	<u>Number of Samples</u>	<u>Average RBC</u>	<u>Variance (s^2)</u>
B	5	7.27	0.2291
E	5	7.50	0.1531

$$\Delta = 7.27 - 7.50 = -0.23$$

$$s_{\Delta}^2 = \frac{0.1531}{5} + \frac{0.2291}{5} = 0.0764$$

$$s_{\Delta} = \sqrt{s_{\Delta}^2} = 0.277$$

$$\text{Degrees of freedom (harmonic mean)} = \frac{2}{\frac{1}{N_B} + \frac{1}{N_E}} = \frac{2}{\frac{1}{5} + \frac{1}{5}} = 5$$

$$\text{"t" value (95 per cent with 5 degrees of freedom)} = 2.57$$

$$\begin{aligned}
 \text{95 per cent confidence limits of } \Delta &= -0.23 \pm tS_{\Delta} \\
 &= -0.23 \pm 2.57 (0.277) \\
 &= 0.48 \text{ and } -0.94
 \end{aligned}$$

As the limits encompass zero, the difference is not significant at the 95 per cent confidence level.

Standard methods were used to calculate the variance (s^2) of each group (ref. 23, p. 91). The use of the harmonic mean in determining the degrees of freedom is a compromise between the assumption of homogeneity of variances and that of different variances. The number of samples is too small to resolve this question. The appropriate "t" value was selected from standard tables (ref. 23, p. 65).

III. RESULTS

A. Environmental Control

1. Maintenance of specified atmospheric conditions: Humidity and temperature in the test chambers were maintained within the specified ranges of 50 per cent \pm 10 per cent and 75° \pm 5°. Concentrations of test chemicals were maintained within the specified ranges as follows (in each case, plus or minus 10 per cent): indole - 10.5 ppm; skatole - 3.5 ppm; hydrogen sulfide - 20 ppm; and methyl mercaptan - 50 ppm. Carbon dioxide concentrations averaged 1,570 ppm in the four test chambers, and ammonia content of these rooms ranged from 3.1 to 17.3 ppm, depending upon time since animal cages were cleaned. Neither CO₂ nor ammonia determinations were made in the very large room housing the control animals.

2. Analysis of atmosphere for reaction products: Attempts were made to analyze the atmosphere of isolation chamber A (mixture) for compounds resulting from possible reaction between the four chemicals introduced into the chambers. Samples of room atmospheres were collected for these analyses by freezing out air samples. This was done before the animals were removed, in order to have ammonia included. Chemicals in the chamber air were also collected on activated charcoal columns of sufficient size to adsorb all material of interest. Frozen out samples and adsorbed samples were eluted with chloroform followed by water elution, and separated into chloroform and aqueous phases. Each phase was subjected to several analytical procedures.

a. Continuous flow paper electrophoresis: Chloroform and aqueous eluates were applied to the curtain of a Beckman CP cell, employing borate buffer, pH 10.0 at 40 ma. constant current. Results of this

procedure showed no separation of compounds other than a positive qualitative test for indole and skatole in several collected fractions. Upon chromatographing these fractions, no separation occurred. Examination of the curtain in ultraviolet light produced no evidence of additional compounds.

b. High voltage paper electrophoresis: Chloroform and aqueous phases were spotted on Whatman No. 1 paper and 1,000 volts constant voltage was applied, using 0.01M sodium borate as the electrolyte. There was no migration of compounds during several consecutive four-hour runs. Potassium chloride (0.1M) was also tried as an electrolyte, with essentially the same results.

c. One and two dimensional paper chromatography: Solvent systems specific for indole and skatole derivatives were employed as developers for the unknown chloroform and aqueous phases. One dimensional chromatographic solvents tried were: isopropanol - ammonia - water, 10:1:1; acetone - chloroform - ammonia - water, 30:5:4:0.2; butanol - acetic acid - water, 4:1:5; 0.01M borate buffer; and 20 per cent potassium chloride. Two dimensional solvents tried were: isopropanol - ammonia - water, 20:1:2 in the first direction and butanol - acetic acid - water, 12:3:5 in the second direction.

Standard mixtures of 5-hydroxy tryptamine, tryptamine, and urea were clearly separated with all solvents. Known mixtures of indole and skatole were separated with isopropanol - ammonia - water, 10:1:1 and appeared as red and violet spots at r_f 95 and 98, respectively. The color reagent used was 0.5 per cent paradimethylaminobenzaldehyde in 1N HCl.

The unknown chloroform phase consistently produced a diffuse blue-green spot at the solvent front sometimes characteristic of 6-hydroxy indole compounds. Outside of this, no other compounds were separated.

d. Ultraviolet spectral analysis: Both chloroform and aqueous phases were subjected to spectral analysis on a Beckman DK-1 recording spectrophotometer. The unknown samples showed gradually increasing spectral absorption through the UV range from 400 $m\mu$ to 200 $m\mu$, but no definitive absorption peaks were demonstrated.

e. Infrared spectral analysis: Complete absorption curves were run on the chloroform and aqueous phases throughout the infrared range of a Perkin-Elmer IR recording spectrophotometer. Spectral curves were obtained for a known indole-skatole mixture as well as for chloroform alone. The transmittance curves for the unknown samples were essentially those of chloroform with only very minor differences. IR curves could not be plotted for the aqueous phase because of the solubility of the photometric cell material.

B. Clinical Laboratory Studies and Stress Tests

1. Routine physiological analyses: The data which are statistically significant, when evaluated by the methods described above (II, g), have been abstracted, and are summarized in Tables I, II, and III.

Inspection of the tables reveals that in no single instance did all three species of the same group show the same physiological response to the toxic agent. In the case of glucose levels, there were changes in all species of Group A (mixture) but the change was upward in the monkeys while rats and mice showed a decrease. Hemolytic processes were in evidence in Group A animals (data in Table III, Column A, were obtained from only 30 per cent of the required number of mice at Phase II because most of the animals died during the first month). In the case of monkeys, no other group showed signs of red cell destruction, Group B (H_2S) actually showing a significant increase in erythrocyte count during the 90-day period. Table II is somewhat misleading in that it appears that rats are more extensively affected by the mixture of gases in Room A than are the other two species, when, in fact, the death rates of mice and monkeys in this group were so great that statistical treatment of results has little meaning.

As reflected by red-blood cell data, indole alone (Room D) and methyl mercaptan (Room C) have an adverse effect on the blood picture of both rats and mice, while this was not true of H_2S (Room B). This is in contrast to the effect on monkeys, in which case there was little evidence of hemolytic influence in any group except A. Both hydrogen sulfide and methyl mercaptan appeared to be less toxic alone than was indole alone, as indicated by hematological data, particularly hematocrit and hemoglobin determinations in rats and mice (see 2 below). The leucocyte change in the case of monkeys exposed to indole may be more significant than some of the other changes, and is consistent with the results in rats and mice. For the first time, significant changes in serum sodium, amylase, and cholinesterase content were noted in monkeys exposed to indole alone. These were the only monkeys showing significant increases in urobilinogen. On the other hand, the death rate of monkeys in Room D cannot be considered significant, and fewer rats and mice died in this room than in any other test chamber -- despite the fact that weight loss of rats and mice in this case was greater than in any group except A. Swimming time of mice in this group was the lowest of any tested group, although there was no similar difference in the case of rats or monkeys.

Hydrogen sulfide alone was more toxic than methyl mercaptan alone for rats, while the reverse was true for mice. In the methyl mercaptan group, four of ten monkeys died, a significant number, but evidence of toxic effects is not apparent in the data on blood and urine. A similar phenomenon occurs in the case of rats in Group B -- the second highest death rate, and the least evidence of damage as reflected by hematology and urinalysis data.

TABLE I

SUMMARY OF STATISTICALLY SIGNIFICANT DATA:
MONKEYS*

	A**		B		C		D	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
Erythrocytes	S-	S-	S+					
Leucocytes							S-	S-
Reticulocytes	S+	S+						
Hematocrit	S-							
Hemoglobin	S-	S-						
MCHb	S-	S-	S-					
MCHbc	S-	S-	S-	S-				
Sodium					S+		S+	S+
Glucose	S+	S+						
Amylase	S+-		S+				S+	S+-
Cholinesterase						S+	S+	S+
Alk Phosphatase		?		S+		S+		
Urobilinogen							S+	S+
Weight			S-		S-			
Swim Test					S+			
Mortality	80%		0		40%		20%	

* Column 1: Comparison group Phase I with its own Phase IV.

Column 2: Comparison group Phase IV with Phase IV of control Group E.

** Both columns: Phase II values substituted for Group A, Phase IV, because of 30-day mortality.

TABLE II

SUMMARY OF STATISTICALLY SIGNIFICANT DATA:RATS*

	A		B		C		D	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
Erythrocytes	S-	S-			S-	S-	S-	S-
Leucocytes	S-	S-		S+		S+	S-	S-
Platelets	S+	S-	S+	S-		S-	S+	S-
Reticulocytes	S+							
Hematocrit	S+	S-	S+		S+		S+	
Hemoglobin		S-	S+		S+	S-	S+	S-
MCV	S+	S+	S+		S+		S+	S+
MCHb	S+	S+	S+		S+		S+	S+
MCHbC	S-	S-					S-	S-
Sodium	S-	S-						
Glucose	S-	S-						
Urobilinogen	S+	S+						
Weight	S-		S-				S-	
Swim Test								
Mortality	S		S					

* Column 1: Phase IV of group versus baseline data.

Column 2: Phase IV of group versus Phase IV of control group E.

TABLE III

SUMMARY OF STATISTICALLY SIGNIFICANT DATA:
MICE*

	A**		B		C		D	
	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>	<u>1</u>	<u>2</u>
Erythrocytes			S-		S-	S-	S-	S-
Leucocytes			S+	S+	S+		S+	S+
Platelets		S-		S-	S+	S+	S+	S+
Reticulocytes				S+		S+		
Hematocrit	S+	S+	S+	S+			S-	S-
Hemoglobin		S+	S+	S+			S-	S-
MCV			S+	S+	S+	S+	S+	S+
MCHb			S+	S+				S-
MCHbC		S-				S-		
Glucose	S-	S-						
Urobilinogen						S+	S+	S+
Weight	S-		S-				S-	
Swim Test	0		S-		S-		S-	
Mortality	S		S?		S			

* Column 1: Phase IV of group versus baseline data.

Column 2: Phase IV of group versus Phase IV of control group E.

** Both columns: Phase II values substituted for Group A, Phase IV, because of 30-day mortality.

2. Special blood pathology: A hematological anomaly was observed in animals exposed to indole, the chief characteristic of which was the appearance of numerous small granules, 1-2 microns in diameter which interfered with enumeration of cells by the Coulter Counter. Their occurrence within red cells often rendered the cell indistinguishable from reticulocytes while extracellularly the particles were easily confused with platelets.

On the basis of our observations, and review of the literature, these granules are probably Heinz bodies. Photomicrographs representative of the granules observed in this study are shown in Figures 2-5. While most of our observations were made in animals exposed to indole alone (Group D), the presence of indole in the mixture of vapors to which Group A animals were exposed caused the same effects. Mice in this group, however, died too early in the test period for the phenomenon to be clearly seen. Heinz body occurrence was most prominent in mice and appeared after a short exposure period (3-4 days), while it was slightly less prominent in rats and required 7-10 days of exposure to indole. No Heinz bodies were observed in monkeys until after 70 days of exposure..

The granules appeared as irregularly shaped, red-brown particles, dense, and slightly refractile. They occurred within red cells and extracellularly. There is little doubt concerning their genesis in erythrocytes, and they are liberated by cell lysis. Both intra- and extracellular granules may be seen in Figure 2, and in Figure 4 they may be seen adhering to red cell ghosts following hemolysis. The appearance of Heinz bodies was accompanied by anisocytosis (Figure 2-A), and polychromatophilia with many erythrocytes exhibiting diffuse basophilia (Figure 2-B). There is a general leucocytosis, and marked reticulocytosis with about 80 per cent of the reticulated cells appearing morphologically atypical. Instead of the fine thread-like reticulum of normal reticulocytes (Figure 5-A), there is a profuse, densely staining nuclear material that may fill the cell and which appears to consist of coarse granules (Figure 5-B).

C. Pathology - Tissue and Organ

Table IV summarizes the results of gross and microscopic pathology, and Table V shows mortality. In the case of monkeys, there did not appear to be any significant pathology in any group -- with the possible exception of lung pathology in animals of Group A. On the other hand, rats in this group had significantly extensive pathology of lung and liver tissue, and, perhaps, of kidney also. Mice showed only lung pathology referable to exposure to the gas mixture.

LEGEND - FIGURES 2-5

Figure 2 - Photomicrograph of mouse blood (Room D - Indole). Dry blood smear showing (A) anisocytosis, (B) diffuse basophilia of erythrocytes, and (C) faintly staining intracellular and extracellular Heinz granules.

Wright's stain. X 900.

Figure 3 - Photomicrograph of rat blood (Room D - Indole). Dry blood smear showing various forms of reticulated cells and Heinz bodies. (A) normal reticulocytes, (B) atypical reticulocytes with dense nuclear material, (C) reticulum without cytoplasm and cellular membrane, (D) intracellular Heinz body, (E) extracellular Heinz bodies may be seen as pale grey spots interspered between the cells.

Supravivally stained with Brecher's new methylene blue and counterstained with Wright's stain. X 900.

Figure 4 - Photomicrograph of mouse blood (Room D - Indole). Wet preparation of cells lysed in water showing (A) Heinz bodies contained in the erythrocyte "ghost," (B) erythrocyte "ghost."

Lightly stained with 1 per cent crystal violet. X 900.

Figure 5 - Photomicrograph of mouse blood (Room D - Indole). Dry blood smear showing (A) normal reticulocytes, and (B) atypical reticulocytes with dense nuclear material.

Supravivally stained with Brecher's new methylene blue. X 900.

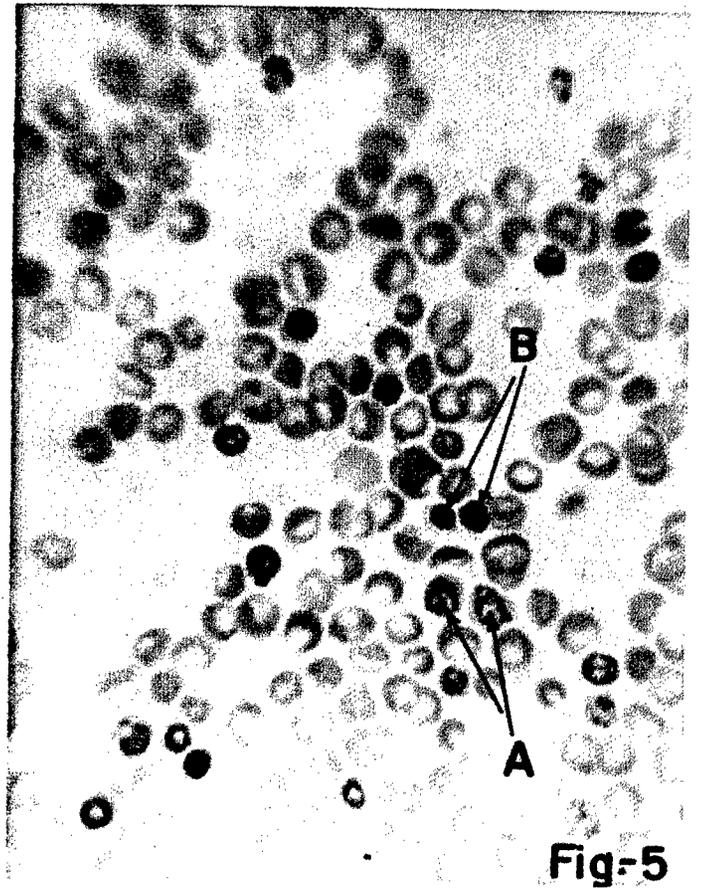
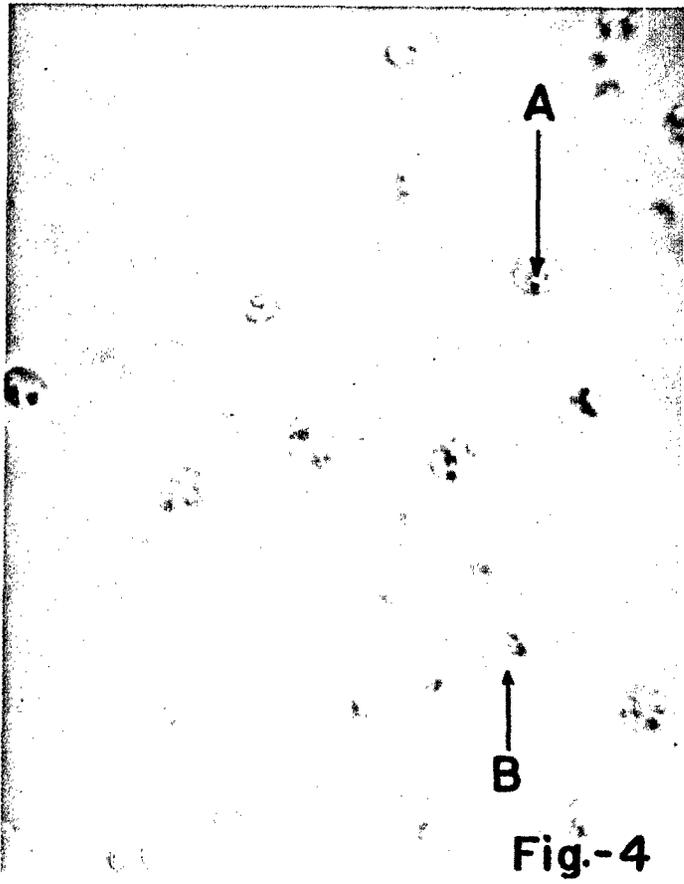
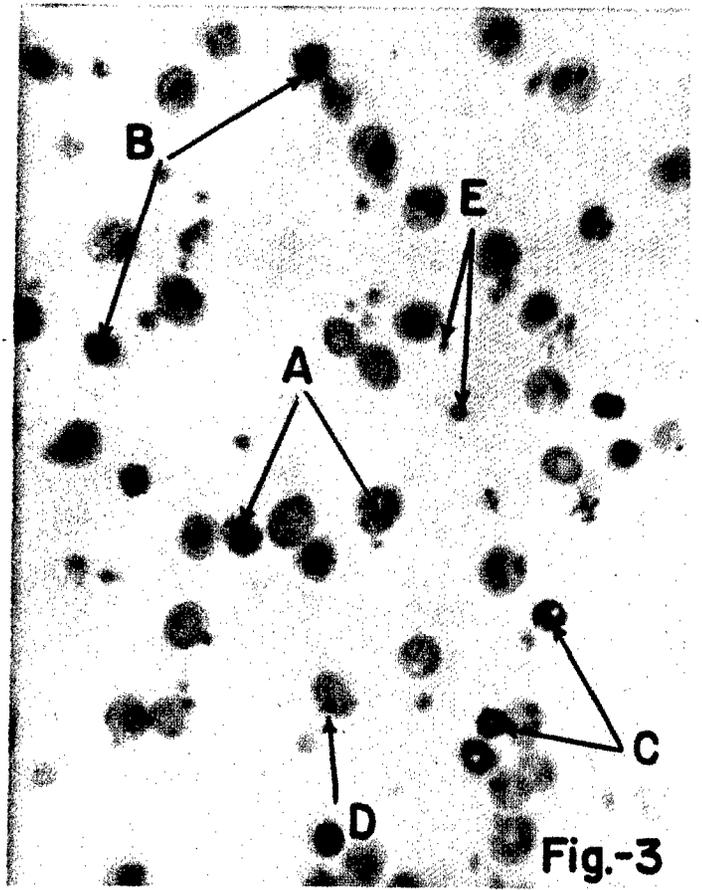
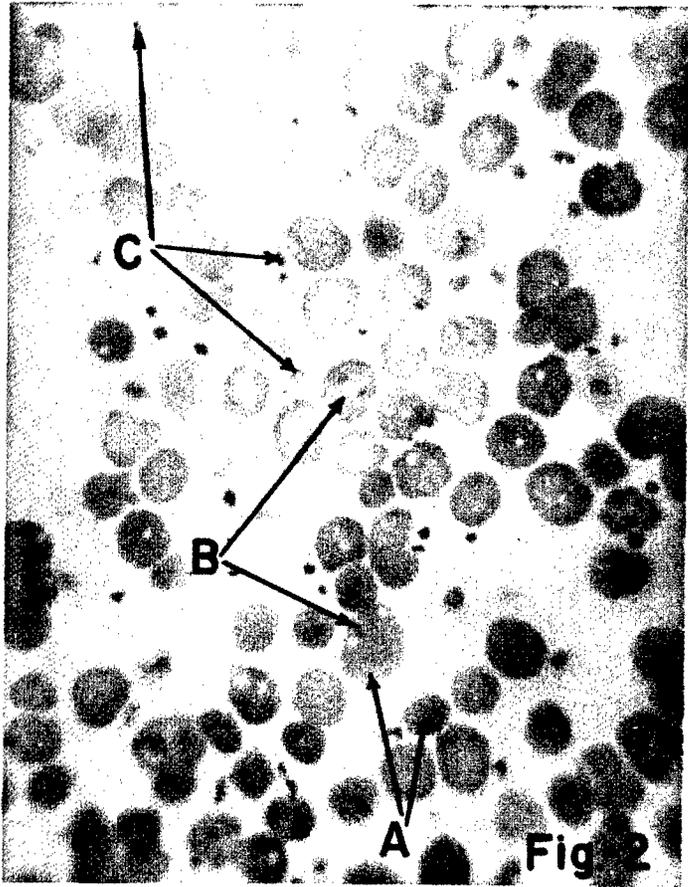


TABLE IV

SUMMARY OF AUTOPSY AND PATHOLOGY DATA
IN THREE SPECIES

(Figures are per cent of all animals autopsied)

<u>Groups</u>	<u>Heart</u>	<u>Lung</u>	<u>Liver</u>	<u>Kidney</u>	<u>Brain</u>	<u>Miscellaneous</u>	<u>None</u>
<u>Monkeys</u>							
A	0	80	10	0	0	0	20
B	0	50	0	0	0	0	50
C	0	60	0	0	20	0	30
D	0	60	0	0	0	0	40
E	0	40	10	0	10	0	40
<u>Rats</u>							
A	9	59	45	13	4	0	18
B	0	33	0	6	0	6	60
C	0	16	0	0	0	0	84
D	0	0	0	0	0	0	100
E	0	0	9	0	0	0	91
<u>Mice</u>							
A	1	84	33	5	1	0	10
B	0	33	50	7	0	0	40
C	1	26	75	22	0	0	41
D	5	90	64	-	-	0	20
E	7	17	50	20	2	0	45

TABLE V

SUMMARY OF MORTALITY RATES IN THREE SPECIES OF ANIMALS

(As per cent of original group)

<u>Group</u>	<u>Monkeys</u>		<u>Rats</u>		<u>Mice</u>	
	<u>% Mortality</u>	<u>No. Deaths No. Used</u>	<u>% Mortality</u>	<u>No. Deaths No. Used</u>	<u>% Mortality</u>	<u>No. Deaths No. Used</u>
A	80	8/10	64	32/50	100	99/100
B	0	0/10	24	12/50	26	26/100
C	40	4/10	10	5/50	43	43/100
D	20	2/10	10	5/50	22	22/100
E	0	0/9	4	2/50	16	16/100

Only rats appeared to have pathology referable to H₂S exposure, and this in the lung. Methyl mercaptan alone appeared to increase liver injury in mice and perhaps lung damage in rats. The observations of pathology in animals exposed to indole alone would seem to compound confusion. In an atmosphere in which both rats and mice lost significant weight and showed extensive signs clinically of toxic effects, the rats are reported as being 100 per cent free of pathology. Monkeys are essentially free also except for a degree of lung pathology not significantly different from the other groups. Mice, however, were found to have very extensive lung damage, comparable to that observed in Group A mice.

The general comments of the pathologist, concerning microscopic pathology of monkeys, follow.

Most of the pathological changes of monkeys were in the lungs. Of 14 monkeys which expired before the end of the test, 12 showed a mild to moderate edema, often associated with congestion and/or accumulation of inflammatory cells, predominantly polymorphonuclear leukocytic. With no evidence of other etiologic factors, such as circulatory disturbances or infectious processes, the acute inflammatory reaction in the lung tissue was presumably due to the noxious effects of chemicals these animals were exposed to. One of these monkeys (Room C, Monkey 6) had a recent, small softening in the cortex of the left frontal lobe, the nature of which is not clear. It may be due to anoxia.

The remaining 35 monkeys were sacrificed at the end of the test. There were 15 of these which also showed a recent mild inflammation of the lungs, probably as a result of the swimming test. Two animals exhibited lesions in the central nervous system -- Room C, Monkey 10 had a recent small softening of the cortex of the right parietal lobe, and Room E, Monkey 4 had a marked atrophy of the brain of uncertain duration.

Other changes included: moderate fatty change of the liver in one monkey (Number 10, Group A); lipid pneumonia following aspiration in another (Number 6, Group B); and nonspecific chronic pleuritis in two others (Number 10, Group C; Number 7, Group D).

Observations on rat and mouse pathology were summarized as follows:

Room A (mixture): The mice in this room all expired, as did most of the rats autopsied. Mice consistently exhibited four plus lung hemorrhages with or without pneumonia. There was very scant evidence of liver or kidney pathology. Most of the rats, whether expired or sacrificed, showed bronchopneumonia, but there was no lung hemorrhage. A large number showed central degeneration of the liver.

Room B (H₂S): The expired mice exhibited abscesses of the brain, liver, and lung. In the sacrificed animals there were a number of instances of bronchopneumonia and hepatitis. The respiratory involvement was characterized by alveolar cell proliferation in most instances. Most of the rats were normal. There were five instances of lung pathology showing congestion and edema, probably resulting from swim tests.

Room C (methyl mercaptan): Most of the mice and rats were normal, except for a persistent hepatitis in mice. There were a few cases of bronchopneumonia and of pyogenic abscesses of the liver and lungs in mice.

Room D (indole): In the expired mice, focal pyogenic abscesses of the lungs and kidneys were extremely common. In the sacrificed mice, there was a specific pattern of central degeneration of liver plus pigmentation of reticuloendothelial cells in all cases. Ninety-five per cent of the animals with this abnormality showed pigment in the renal tubule cells. No comparable pathology was observed in rats from this group. A few cases of terminal congestion of the lung were observed.

Room E (control): Pyogenic abscesses of liver, lung, and kidney were common, many of them being multiple. There was some hepatitis and almost no pneumonia. The expired animals appeared to have died of systemic bacterial infections. In rats, there was one case of focal necrosis of the liver and one cystic liver.

IV. DISCUSSION

A. Group A - H₂S, MeSH, Indole, and Skatole

It should be noted that statistical consideration of mouse and monkey data in this group is difficult, due to rapid death of many animals. In the statistical data, Group A monkeys and mice were evaluated by use of Phase II instead of Phase IV data, i.e., data collected at 30 days rather than 90 days. Death, however, can hardly be considered insignificant even though it obscures hematological and other changes.

As in the previous experiment, the effects of exposure to this combination of gases were reflected most uniformly by hematological data. There appeared to be low-grade hemolytic activity without evidence of interference with erythropoietic functioning. All three species evidenced lung pathology and rats showed additional pathology of liver and kidney. Except for the fact that in the first experiment only 30 per cent of the monkeys were reported as having lung pathology, there is good agreement between the two experiments with respect to Group A pathology. Exactly the same number of monkeys

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died, and in approximately the same time. It is difficult to account for the difference in rat and mouse deaths, however. In the first experiment they were reported as "negligible" whereas in this case mortality was 60-65 per cent for rats and 84-99 per cent for mice.

Significant changes in this experiment which were not observed previously involved glucose (increased in monkeys, decreased in rats and mice); serum sodium, reduced in rats; urobilinogen increased in rats (probably obscured in other animals by early death); and probably a significant increase in alkaline phosphatase in monkeys.

Cause of death in this group is again in doubt. In view of the high rate of lung pathology in mice exposed to indole alone, and the fact that all three species show lung pathology in Group A, a combination of anoxia and secondary respiratory infection would seem to be a reasonable conjecture as cause of death. Further consideration of the significance of these results is found under "General," below.

B. Group B - Hydrogen Sulfide

Hydrogen sulfide would not appear to be a major contributor to the hemolytic anemia observed in Group A, for the monkeys in Group B actually enjoyed an increase in red cell numbers and showed no reticulocytosis nor decline in hematocrit or hemoglobin. In general, similar results were observed in rats and mice. All animals showed a significant weight loss. Only rats, however, seemed to suffer significant mortality, the rate being 20 per cent even when corrected. The pathology in this group, when compared with mortality differences and pathology in Group A makes it apparent that the 80 per cent lung pathology of Group A monkeys is without significance. Half the Group B monkeys had similar lung pathology, but there were no deaths, and this is compatible with the fact that 40 per cent of the control monkeys also had lung pathology. On the other hand, rat controls were apparently free of lung pathology, and the 33 per cent of Group B rats showing such pathology is undoubtedly related to the mortality rate. Group B mice showed 33 per cent lung pathology, too, but if the 17 per cent figure for control mice is subtracted, we find that the significant figure for lung pathology in Group B mice is probably about 16 per cent. The ratio, lung pathology/mortality when rats and mice are compared -- rats 33/20, and mice 16/10 -- supports the suggestion that the cause of death in Group B rats was related to the lung pathology, and that, to the extent that Group B mice exceeded controls in lung pathology, their mortality was also due to this.

C. Group C - Methyl Mercaptan

No pathology reported for monkeys accounts for the death rate of 40 per cent in this group. Nor does the reasoning in the paragraph above apply for rats in this group, for with half as many rats showing lung pathology as in Group B, only one fourth as many died.

Mouse mortality is significantly higher than in any group except Group A, and would seem to be referable to observed liver pathology. On the other hand, mice in Group D had about as much liver pathology reported, but an insignificant mortality rate. The 40 per cent mortality of monkeys in this group was unaccompanied by liver pathology. The general hematological picture offers little in the way of clues to the cause of death of either mice or monkeys. In the latter species, the data differ little from that of Group B, in which no monkeys died, and the Group D mouse data are more suggestive of severe hematological response than are those of Group C, in the face of a fourfold higher death rate in Group C.

D. Group D - Indole

In mice and rats exposed to indole alone, the hemolytic effects are much clearer than was the case in Group A, perhaps because the animals survive long enough to manifest these changes. No similar changes are noted in monkeys, although there is significant elevation of serum sodium, amylase, and cholinesterase, which may provide a clue to the neurological symptoms manifested by monkeys in Group A prior to death. The fact that Table I indicates no comparable significance in Group A animals (for sodium and the two enzymes) is probably a statistical artifact, for four Group A monkeys which survived long enough for Phase II testing showed an increase in cholinesterase during the first month. This is true also of urobilinogen in Group A monkeys, the two survivors having very high excretion levels at the 30- and 60-day periods.

Certain observations in Group D are difficult to reconcile with each other, such as weight loss, stress tests, and mortality. Data on all three categories are without significance in the case of monkeys; mice and rats have the lowest mortality rate of any group, despite the appearance of more severe hematological pathology than in any group except Group A, and with respect to mice, in addition to severe changes in the blood picture, there is the greatest weight loss and the most serious shortening of swimming time. Nevertheless, rat and mouse death rates are not statistically significant, while in Group A, they are overwhelmingly so. Finally, the lung pathology of mice in this group, while it may suggest some clue to the 100 per cent mortality in Group A mice, is difficult to reconcile with the very insignificant death rate of mice in Group D, or with the reported total absence of lung pathology in rats.

The phenomenon of Heinz body formation in the erythrocytes of animals exposed to indole has not been definitely studied. Such investigations would necessarily constitute a separate research program, and interpretation of these observations is, therefore, tentative. The term "Heinz body" should probably be considered a "generic" term, i.e., it is likely that this term has been given to more than one kind of abnormal particle observed within erythrocytes. While it seems generally assumed that they owe their origin in some manner to hemoglobin, and that their occurrence is causally related to the presence of certain types of chemical agents, it is possible that nucleoprotein is concerned with their genesis.

With respect to the observations reported here, our tentative conclusion is that these "Heinz bodies" are "degradation products" of nucleoprotein material in new erythrocytes. We make the assumption that the material in new erythrocytes which takes up stains in such manner as to justify our calling them "reticulocytes" is residual nuclear substance. Normally, such cells "mature" in a matter of hours, i.e., cease to stain in this characteristic manner. It will be noted that, in most cases, our observations of Heinz bodies usually coincided with high reticulocyte counts. Indeed, they were first noted partly because their intracellular presence interfered with reticulocyte counts. Furthermore, the time of their appearance coincides with the occurrence of increased numbers of reticulocytes. If Heinz bodies were formed as the result of hemoglobin degradation or polymerization, it is difficult to explain why they do not appear independently of increases in number of reticulocytes, particularly when the latter are sometimes delayed for many weeks, although the toxic chemical concentration remains constant.

If our interpretation is correct, it implies a "double-barreled" hematological insult, and, therefore, crisis. For the new red cells "called" into circulation as the result of red cell destruction may be more susceptible to damage by certain toxic agents than are the mature cells. Such situation has precedence in observations with saponins which are more lytic for reticulocytes than for mature erythrocytes.

E. General

In a number of ways, the results of this experiment are consistent with those of the previous one: mortality rate of Group A monkeys, evidence of low-grade hemolytic anemia, and essentially similar pathology in all three species in this group. The discrepancy in mortality rates of both rats and mice, however, requires explanation.

There are a number of reasons for suspecting that individuality differences in response to simple chemical agents such as those used here

are more significant than we might assume. In the first place, it was observed that the monkeys which did survive in Group A past the middle of the run appeared to have adapted to the environment. The single mouse in this group that survived (which is still living in the laboratory) did so, apparently none the worse for the experience, despite the fact that only one other mouse in that group had survived beyond the 35th day of the run, and it died on the 58th day. As a matter of fact, 70 mice died in the first five days (see Figure 6). Now, if we ignore the statistical evaluation of weight data, and closely inspect instead the individual weight changes of the rats and monkeys surviving in Group A, we find a rather surprising picture. The monkeys were heavier than the average weight of those in any other group. Those rats that survived beyond 30 days had almost a normal rate of gain during the second 30 days, and during the last 30 days had a rate of gain higher than that of any other group of rats, including the control animals.

Twice during this experiment, we introduced small groups of mice into the experimental groups. Of 12 mice introduced into Room A on the 30th day, two died in 24 hours, five died in the next 24 hours, and they were all dead in 72 hours. On the 75th day of the run, this was repeated, with a different shipment of mice. Again, no mouse survived more than 72 hours. Nevertheless, of the 100 mice originally placed in this room, 24 survived longer than a week, 13 longer than two weeks, five lived four weeks or more, and one survived the entire 90 days.

These observations would seem to be entirely compatible with an assumption that there is a genuine adaptive process operating -- or operable -- and that there are quite serious and significant differences in individual tolerance for these toxic compounds. These differences would seem to justify the use of inbred animals in future work of this kind. Another change which might contribute much to the significance of such work would be the inclusion of a second set of control animals for the sole purpose of autopsy at the time the exposure period begins, perhaps five monkeys, 15 rats and 30 mice. We might thus avoid the confusion imposed by discovering at the end of the run that "normal" pathology was quite different than that observed previously (note lung pathology in rats and liver pathology of mice).

Finally, while it is possible to determine the chronic toxicity for animals of a mixture of compounds such as that used in Room A, it may be that the apparent toxicity is something other than the sum of the toxicities of the individual components of the mixtures. No monkeys died in Group B, and the mortality rate of mice was quite low. Only one monkey died in Group D, and the mouse mortality was even lower. Nevertheless, we are not able to predict the consequences of combining indole and hydrogen sulfide insofar as effects on animals are concerned. What would be the effect on monkeys of exposure to H_2S and MeSH -- without indole and/or skatole? Would we

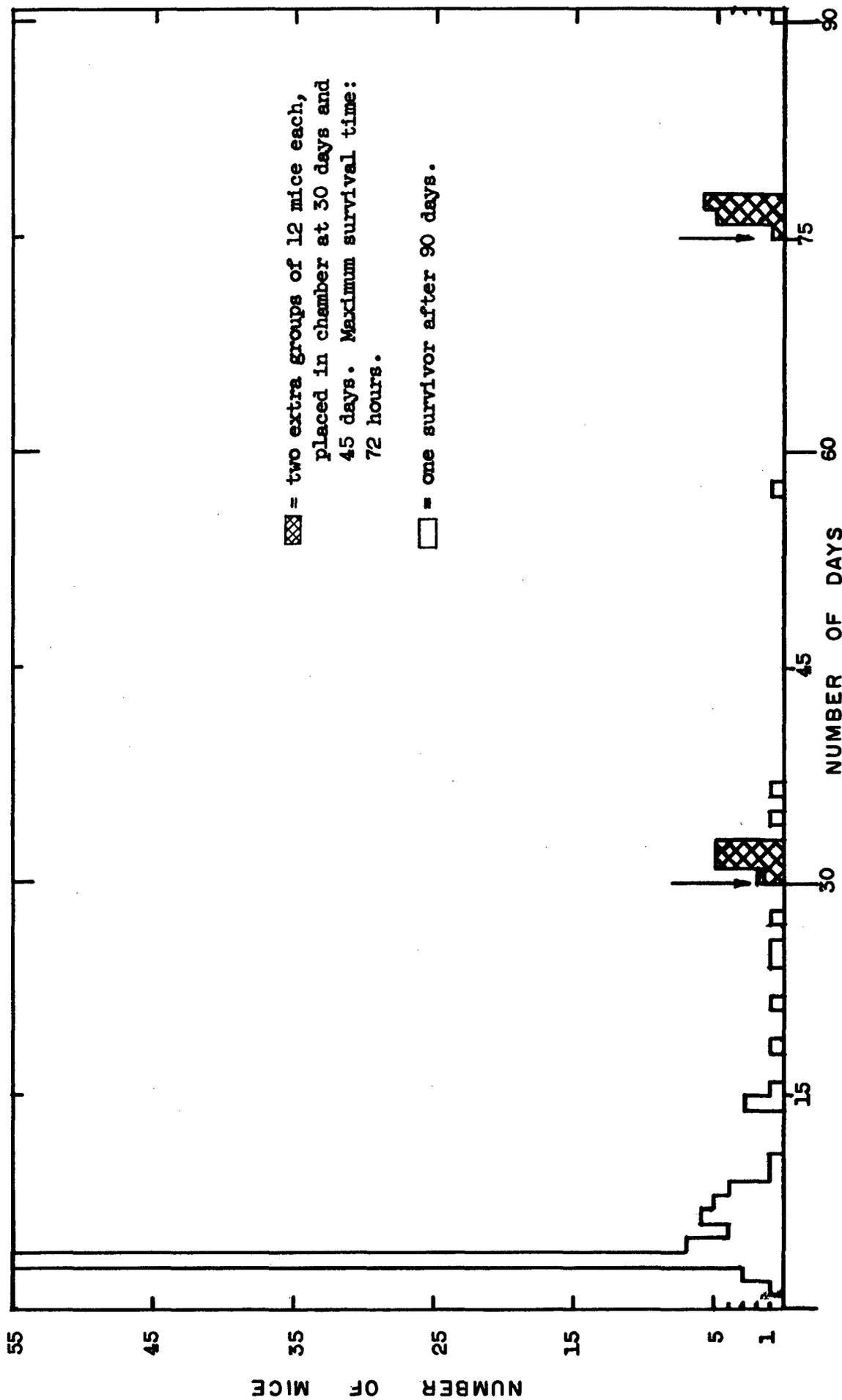


Figure 6. Distribution of Deaths (Mice) in Group A (Mixture of Vapors)

predict the 40 per cent mortality of Group C, since Group B had no deaths? Would a combination of indole and H_2S cause a 20 per cent mortality rate? Similarly, would methyl mercaptan and indole cause merely a 20 per cent (or 10 per cent more than controls) mortality in rats? Obviously, we are not able to answer these questions, and this may be pertinent also in the planning of future experiments of this kind. If we are to understand the mechanisms of these toxicological effects, perhaps all possible combinations should be used if mixtures of vapors are studied at all.

V. SUMMARY

1. Mortality rates for monkeys, rats, and mice, in the group exposed to a mixture of gases, were 80 per cent, 65 per cent, and 100 per cent, respectively. Monkey mortality confirmed previous results, but rat and mouse mortality was very much higher.

2. A low-grade hemolytic response was evident in Group A animals, although impairment of erythropoietic function did not appear to be involved.

3. There was fair agreement between the results of this experiment and a previous one with respect to pathology. All three species evidenced lung pathology, but this was of significance primarily in the case of mice.

4. The real cause of death in monkeys is once again obscure. In the case of mice and rats, however, the cause of death in Group A was probably anoxia and secondary respiratory infection, both of which are compatible with the lung pathology observed.

5. Hydrogen sulfide alone had less hematological effect than either indole or methyl mercaptan. While no monkey or mouse deaths could be attributed to H_2S exposure, 20 per cent of the rats succumbed. These deaths could not be clearly correlated with specific pathology, although there is some suggestion that lung pathology was associated.

6. The deaths of four monkeys in the group exposed to methyl mercaptan could not be explained on the basis of observed pathology. A significant number of mouse deaths occurred in this group, but rats were not affected. Liver pathology may be related to the high mouse mortality rate.

7. In animals exposed to indole alone, the hematological effects were more severe than in any other group except A. Furthermore, significant blood enzyme changes were observed for the first time in monkeys of this group. Nevertheless, no animal deaths could be attributed to this exposure.

This is all the more surprising in view of very extensive lung pathology in mice, and significant weight loss in both rats and mice.

8. Chronic indole exposure appears to be causally related to the appearance of abnormal sub-cellular (red cells) particles which correspond to those described in the literature as Heinz bodies. There is circumstantial evidence that they originate in new, as opposed to mature, erythrocytes.

9. Rats and mice exposed to the mixture of compounds in Group A displayed a higher mortality rate in this experiment than in the previous one. There are a number of reasons for believing that this difference reflects the existence of significant individuality differences with respect to sensitivity to toxic compounds. There is also evidence that an adaptation to the toxic atmospheres occurs if the animals are able to survive the first severe effects.

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<p>Aeronautical Systems Division, Aerospace Medical Research Laboratories, Wright-Patterson AFB, Ohio Rpt. No. ASD TR 61-519 (II). TOLERANCE CRITERIA FOR CONTINUOUS INHALATION EXPOSURE TO TOXIC MATERIAL. II. Effects on Animals of 90-Day Exposure to H₂S, Methyl Mercaptan, Indole, and a Mixture of H₂S, Methyl Mercaptan, Indole, and Skatole. Final report, Dec 61, 36p. incl. illus., tables, 23 refs. Unclassified report</p> <p>Physiological changes in rats, mice, and mon- keys were studies during continuous 90-day exposure to controlled atmospheres of toxic vapors and gases. Concentrations of test chemicals were those recognized as Industrial Threshold Limit (over)</p>	<p>UNCLASSIFIED</p> <p>1. Toxicity (Toxicology) 2. Indoles 3. Skatoles 4. Methyl Mercaptan 5. Hydrogen Sulfides 6. Pathology I. AFSC Project 7165, Task 716501 II. Biomedical Labora- tory</p> <p>III. Contract AF 33(616)- 7055 IV. Midwest Research Institute, Kansas City, Mo. UNCLASSIFIED</p>	<p>Aeronautical Systems Division, Aerospace Medical Research Laboratories, Wright-Patterson AFB, Ohio Rpt. No. ASD TR 61-519 (II). TOLERANCE CRITERIA FOR CONTINUOUS INHALATION EXPOSURE TO TOXIC MATERIAL. II. Effects on Animals of 90-Day Exposure to H₂S, Methyl Mercaptan, Indole, and a Mixture of H₂S, Methyl Mercaptan, Indole, and Skatole. Final report, Dec 61, 36p. incl. illus., tables, 23 refs. Unclassified report</p> <p>Physiological changes in rats, mice, and mon- keys were studies during continuous 90-day exposure to controlled atmospheres of toxic vapors and gases. Concentrations of test chemicals were those recognized as Industrial Threshold Limit (over)</p>	<p>UNCLASSIFIED</p> <p>1. Toxicity (Toxicology) 2. Indoles 3. Skatoles 4. Methyl Mercaptan 5. Hydrogen Sulfides 6. Pathology I. AFSC Project 7165, Task 716501 II. Biomedical Labora- tory</p> <p>III. Contract AF 33(616)- 7055 IV. Midwest Research Institute, Kansas City, Mo. UNCLASSIFIED</p>
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