FOREWORD

The investigations described in this report were carried out during the period from June 1962 to August 1963. The research was conducted in the Toxic Hazards Branch, Physiology Division, Biomedical Laboratory of the 6570th Aerospace Medical Research Laboratories, under Project No. 6302, "Toxic Hazards of Propellants and Materials," and Task No. 630202, "Pharmacology and Biochemistry." Acknowledgment is made of the invaluable assistance lent the authors by Mildred K. Pinkerton, TSgt. G.W. Craig and A2C Charles Witchett, of the Toxic Hazards Branch.

The experiments reported herein were conducted according to the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

ABSTRACT

A simple procedure is described for measuring microgram amounts of hydrazine in the blood serum of rats. The procedure, with a minor modification, can be used for measuring microgram amounts of 1-methylhydrazine. The report presents calibration ranges of 0.5-5.0 μg/ml and 0.5-10.0 μg/ml of hydrazine and 1-methylhydrazine, respectively. Data are presented on the dose-blood-level relationship of hydrazine and 1-methylhydrazine in rats following intraperitoneal injection. Minimum detectable dose levels were 0.6 mg/kg and 3.0 mg/kg of hydrazine and 1-methylhydrazine, respectively.

PUBLICATION REVIEW

This technical documentary report is approved.

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DETERMINATION OF HYDRAZINE AND 1-METHYLHYDRAZINE IN BLOOD SERUM

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INTRODUCTION

The need to investigate the toxicological effects of hydrazine (H) and 1-methylhydrazine (MMH) in laboratory animals necessitated development of a suitable method for their identification and quantitation.

p-Dimethylaminobenzaldehyde (DMBA) has been a commonly used reagent for the in vitro determination of H (refs 1,2,4,5). Pesez and Petit (ref 4) observed that the reaction of H and DMBA in an acid medium resulted in the production of a yellow-colored solution. Watt and Chrisp further utilized this reaction and developed a spectrophotometric method for the quantitation of H (ref 5).

The method of Watt and Chrisp for the measurement of H has been modified and adapted for use with blood serum. An additional minor change has allowed this same procedure to be used in detecting and quantitating MMH. Using these modified procedures, information has been obtained on the dose-blood-level relationship of H and MMH in rats.

METHODS

Experimental Animals

Male Sprague-Dawley rats weighing 100-425 gm were used for the animal investigations. The rats were maintained in separate cages and were fed Purina Rat Chow and water ad libitum. Groups composed of 5 rats each received intraperitoneal

(i.p.) injections of aqueous H and MMH at doses ranging from 0.5 to 60 mg/kg and from 1.0 to 15 mg/kg, respectively. As a control, an additional rat was given an i.p. injection of 0.3 cc distilled water with each group of 5 test rats. Blood specimens were collected, using ether anesthesia and exsanguination, at 1, 2, 4 and 6 hours after injections.

Reagents

Trichloroacetic Acid (TCA) 10%

Dissolve 100 gm trichloroacetic acid in 1000 ml of distilled water.

Absolute ethyl alcohol

p-Dimethylaminobenzaldehyde (DMBA)

Melting point 73°-75° C. Dissolve 4 gm DMBA in 100 ml ethanol. If the DMBA solution is not colorless, purify the reagent as follows: Recrystallize from a hot 60:40 solution of ethanol and distilled water to which activated charcoal has been added. Filter the hot solution and allow to cool and crystallize. Using a Buchner funnel, filter and allow the crystals to dry at room temperature for at least 24 hours before using. The yield from this purification procedure is approximately 50%-60%. This color reagent should be stable for 3-4 weeks at room temperature.

Hydrazine

Purity assay 95% - 98%. Specific gravity 1.0034/25° C.

Hydrazine Working Standard (100 μg/ml)

Prepare by diluting 0.1 ml H to 1000 ml with distilled water in a volumetric flask. This solution should be freshly prepared.

Methylhydrazine

Boiling point 87° - 89° C. Specific gravity 0.8743/25° C.

Strong Methylhydrazine Standard (1000 μg/ml)

Prepare by diluting 0.11 ml methylhydrazine to 100 ml with distilled water in a volumetric flask. This solution should be freshly prepared.

Weak Methylhydrazine Standard (100 μg/ml)

Prepare by diluting 10 ml of the Strong MMH Standard to 100 ml with distilled water in a volumetric flask. This solution should be freshly prepared.

Determination of H and MMH

Analytical Procedure for Serum

Prepare a protein-free solution of the unknown by adding 4.0 ml of 10% TCA to 1.0 ml of serum sample. Mix well and centrifuge for 6 minutes at 2000 rpm. A BLANK is prepared in the same manner using a commercial 'Control' serum or serum from an unexposed person. To 3 ml of the supernatant, add 5.0 ml DMBA color reagent. Mix well and let stand 10 minutes for color development. Set zero optical density with the BLANK using a wavelength of 470 mμ. Read the optical density of the unknown and convert to micrograms of H per ml serum using a previously constructed calibration curve.

The determination for MMH utilized the above procedure with two exceptions: (1) the color is allowed to develop for 30 minutes after mixing and (2) the color is read at a wavelength of 485 mμ.

Calibration for H

Add 1.0 ml of commercial 'Control' serum to each of eight tubes. Using a microburette, add 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 μg of Hydrazine Working Standard to the first seven tubes, respectively. Tube No. 8 is the BLANK. Add 4.0 ml of 10% TCA to each tube and mix well. Centrifuge at 2000 rpm for 6 minutes. Transfer 3.0 ml of each supernatant to cuvettes and add 5.0 ml DMBA color reagent to each tube. Mix well and let stand for 10 minutes. Set optical density at zero with the BLANK using a wavelength of 470 mμ. Plot optical density versus concentration of H on graph paper (figure 1).

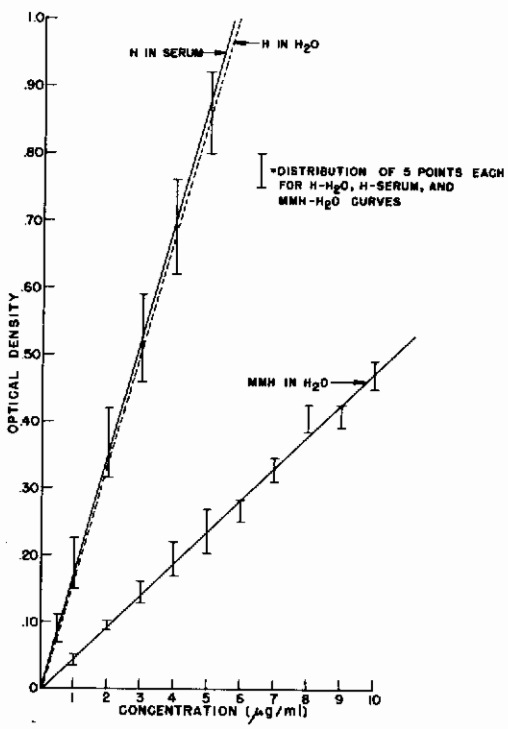

Figure 1

Hydrazine (H) and 1-Methylhydrazine (MMH) Calibration at 470 mμ (Coleman Jr. Spectrophotometer)

Calibration for MMH

Add 1.0 ml of commercial 'Control' serum to each of nine tubes. Using a microburette, add 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 μg of the Weak Methylhydrazine Standard, respectively, to the first six tubes and 8.0 and 10.0 μg of Strong Methylhydrazine Standard to tubes No. 7 and 8. Tube No. 9 is the BLANK. Add 4.0 ml of 10% TCA to each tube and mix well. Centrifuge at 2000 rpm for 6 minutes. Transfer 3.0 ml of each supernatant to cuvettes and add 5.0 ml of DMBA color reagent to each tube. Mix well and let stand for 30 minutes. Set optical density versus concentration of MMH on graph paper (figure 2).

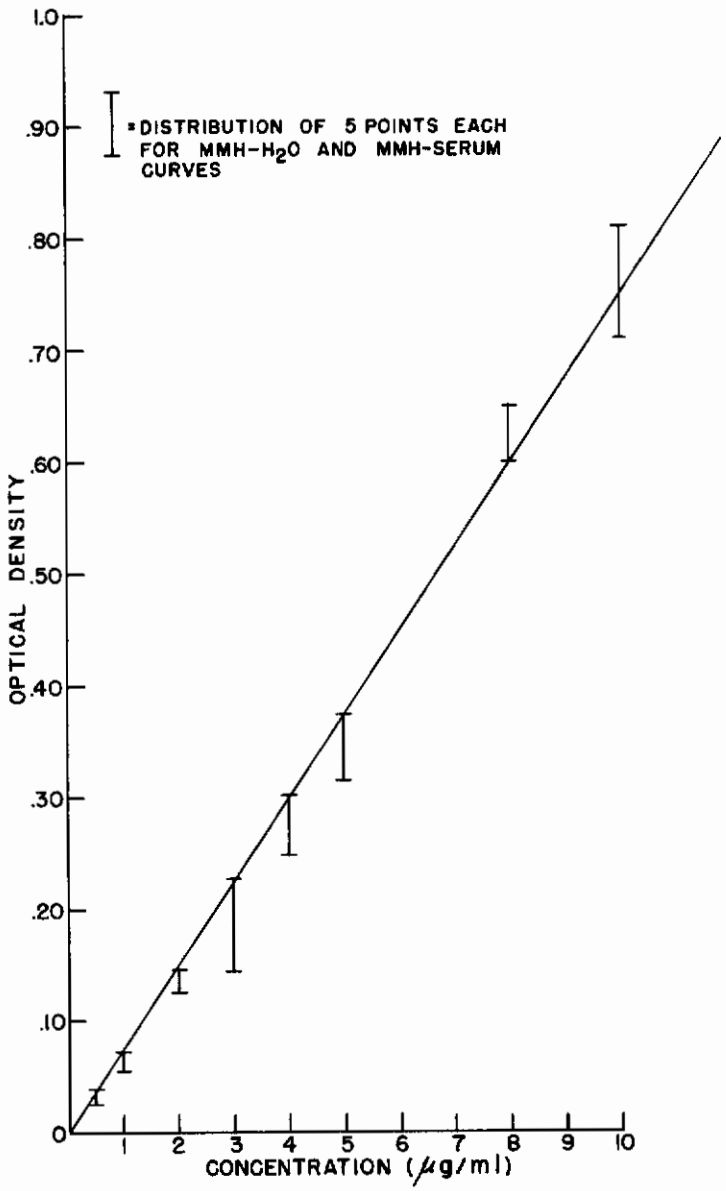

Figure 2. 1-Methylhydrazine (MMH) Calibration in H_2O and Serum at 485 mμ (Coleman Jr. Spectrophotometer)

Change the last sentence in the paragraph to read:

> Set optical density at zero with the BLANK using a wavelength of 485 mμ. Plot optical density versus concentration of MMH on graph paper (figure 2).

Figures 1 and 2 show calibration curves for H and MMH both in water in and serum. Adherence to Beer's Law is demonstrated through 5 μg/ml H and 10 μg/ml MMH. Extrapolation of the line through zero is arbitrary since actual measurements were not made for values less than 0.25 μg/ml and 0.5 μg/ml of H and MMH, respectively. Increased sensitivity of the serum determination may be accomplished by use of a water-reagent BLANK and a negative control serum, the optical density being subtracted from that of the unknown serum. Dilution of the original serum sample with distilled water is necessary for all concentrations greater than 5.0 μg/ml of H and greater than 10.0 μg/ml of MMH. The identical water and serum curves demonstrate 100% recovery from serum (figures 1 and 2).

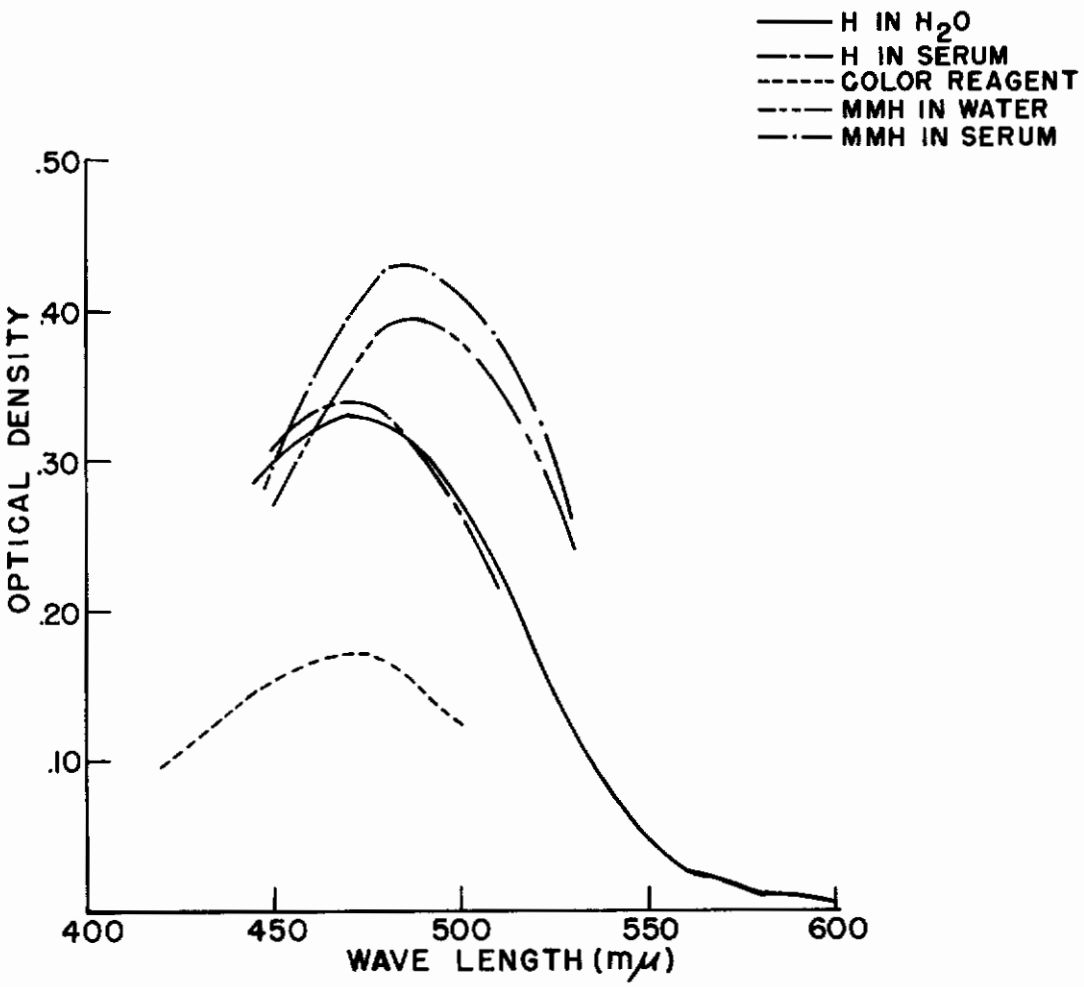

Figure 3. Absorption Characteristics

General Remarks

The reaction between low concentration of H and MMH and DMBA produces a yellow colored solution that, for H, exhibits maximum adsorption between 460-480 mμ and, for MMH, shows a maximum peak between 480-490 mμ (figure 3). Color development is optimum with both H and MMH at pH 1-2. Under test conditions, the intense yellow color developed with H is rapid and reaches a maximum in 5-10 minutes. The color remains stable for several hours at room temperature. The MMH reaction requires 30 minutes for maximum color development and significant fading does not occur until after 60 minutes.

When the serum is removed from the clot after collection of the sample, there is no apparent loss of H after 24 hours either at room temperature or under refrigeration. Hemolysis has little or no effect on the analysis.

Since urea and uric acid are normal constituents of blood and known to react with DMBA under other test conditions, it was necessary to investigated their possible interference in our procedure. These compounds when tested at concentrations of 15 and 30 mg% demonstrated no interference problems. Of the hydrazines tested (H, MMH, UDMH) only H and MMH reacted to produce a yellow color. The intensity of the color obtained with MMH as shown in figure 1 represents 30% of that which formed with an equal amount of H under identical test conditions. Comparison was limited to 5 μg/ml concentration since this represented full scale (100%) deflection of the galvanometer in the case of the H-DMBA color complex. A slightly yellow color is produced with deproteinized serum. This effect is nullified, however, by use of a serum BLANK which is also slightly yellow. Fifteen blood bank serum samples and 28 rat serum samples produced no false positives.

RESULTS AND DISCUSSION

Dose-blood-level relationship following both H and MMH injection was similar. The average serum concentrations at 1 hour tend to increase with increasing dosage. The same trend is evident for both 2- and 4-hour samples. The highest concentrations noted were in the 1-hour samples. (See tables 1 and 2.)

The individual serum concentrations obtained at various times were extrapolated to an estimated total quantity present in the circulating blood using 31.3 ml/kg as the average blood plasma volume for rats (ref 3). Based upon total dose administered the percent recovery was calculated and mathematical averages are shown in tables 3 and 4. At no time, for any dose tested, did the total percent recovered exceed approximately 2.5% in any animals. A fairly constant 1% recovery was obtained at 1 hour for all exposed animals.

TABLE 1

μg/ml of HYDRAZINE RECOVERED IN BLOOD SERUM*
(Average/Range)

Time (Hrs)	I.P. Dose of Hydrazine (mg/kg)												
	0.5	0.6	0.7	0.8	0.9	1.0	3.0	5.0	10.0	15.0	30.0	60.0	Controls
1	0.20 0.00-0.10	0.25 0.15-0.30	0.25 0.10-0.40	0.24 0.20-0.30	0.26 0.15-0.40	0.31 0.25-0.45	1.39 0.55-2.25	1.63 0.80-2.25	3.13 1.90-4.40	3.82 2.50-4.90	8.10 3.60-11.00	18.75 17.20-21.60	0.00∞ 0.00-0.00∞
2	0.00 0.00-0.00	—	—	—	—	0.11 0.05-0.25	0.26 0.20-0.30	0.47 0.35-0.60	0.72 0.35-1.25	1.37+ 1.15-1.80	2.82+ 1.10-4.00	10.26 7.80-16.50	0.00‖ 0.00-0.00
4	—	—	—	—	—	0.00 0.00-0.00	0.12 0.05-0.20	0.18 0.15-0.20	0.27 0.15-0.35	0.51 0.40-0.75	1.67 1.45-1.95	4.20 3.00-5.20	0.00‖ 0.00-0.00
6	—	—	—	—	—	—	—	—	—	—	—	2.18‡ 1.90-2.50	0.00** 0.00-0.00

* Average of 5 animals
+ Average of 4 animals
‡ Average of 3 animals
∞ Average of 12 animals
‖ Average of 7 animals
** 1 animal

TABLE 2

μg/ml 1-METHYLHYDRAZINE RECOVERED IN BLOOD SERUM*

(Average/Range)

Time (Hrs)	I.P. Dose of 1-Methylhydrazine (mg/kg)				
	1.0	3.0	5.0	10.0	15.0
1	0.00 0.00-0.00	0.46 0.00-0.80	1.52 1.00-2.30	4.06 3.30-5.00	5.36 4.50-5.90
2	0.00 0.00-0.00	0.62 0.00-0.80	1.20 1.10-1.30	2.70 2.20-3.00	3.52 2.90-4.00
4	0.00 0.00-0.00	0.00 0.00-0.00	0.30 0.00-0.80	1.40 1.10-1.80	1.94 1.40-2.70
6	—	—	—	0.00 0.00-0.00	0.70 0.00-1.00

* Average of 5 animals at each level and time

TABLE 3

PERCENTAGE OF INJECTED DOSE OF HYDRAZINE RECOVERED IN BLOOD SERUM*

Time (Hrs)	I.P. Dose of Hydrazine (mg/kg)												
	0.5	0.6	0.7	0.8	0.9	1.0	3.0	5.0	10.0	15.0	30.0	60.0	Control
1	0.12 0.00-0.62	1.25 0.78-1.82	1.12 0.45-1.79	0.98 0.78-1.17	0.91 0.52-1.39	0.97 0.78-1.41	1.45 0.57-2.36	1.02 0.51-1.40	0.98 0.60-1.38	0.80 0.52-1.02	0.95 0.69-1.08	0.97 0.87-1.12	0.00∞
2	0.00 0.00-0.00					0.34 0.16-0.78	0.28 0.21-0.37	0.27† 0.22-0.38	0.23 0.11-0.39	0.26 0.24-0.38	0.32 0.14-0.42	0.58 0.41-0.86	0.00‖
4						0.00 0.00-0.00	0.13 0.05-0.21	0.10 0.09-0.12	0.08 0.05-0.11	0.11 0.09-0.16	0.17 0.15-0.20	0.21 0.16-0.27	0.00‖
6												0.11‡ 0.10-0.13	0.00**

* Average of 5 animals
† Average of 4 animals
‡ Average of 3 animals
∞ Average of 12 animals
‖ Average of 7 animals
** 1 animal

TABLE 4

PERCENTAGE OF INJECTED DOSE OF 1-METHYLHYDRAZINE RECOVERED IN BLOOD SERUM*
(Average/Range)

Time (Hrs)	I.P. Dose of 1-Methylhydrazine (mg/kg)				
	1.0	3.0	5.0	10.0	15.0
1	0.00 0.00-0.00	0.48 0.00-0.83	0.95 0.63-1.44	1.26 0.99-1.56	1.12 0.94-1.23
2	0.00 0.00-0.00	0.64 0.00-0.83	0.75 0.69-0.81	0.84 0.69-0.94	0.73 0.60-0.83
4	0.00 0.00-0.00	0.00 0.00-0.00	0.19 0.00-0.50	0.43 0.34-0.56	0.40 0.29-0.56
6	—	—	—	0.00 0.00-0.00	0.19 0.17-0.21

* Average of 5 animals at each level and time

SUMMARY

A method has been developed for the identification and quantitation of hydrazine and 1-methylhydrazine in the blood serum of rats and was utilized in a study of dose-blood-level relationship of blood levels following injection.

Based upon the total dose of the hydrazines administered, the percent recovery in blood was found to be a fairly constant 1% at 1 hour after intraperitoneal injection for all exposed animals. The absolute blood concentrations tend to increase with increasing dosage and decrease with time after exposure. Blood levels were detectable when injected doses were 0.6 mg/kg and 3.0 mg/kg for hydrazine and 1-methylhydrazine, respectively.

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