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EFFECTS OF MONOMETHYLHYDRAZINE ON
HUMAN RED BLOOD CELLS

Marilyn E. George

Aerospace Medical Research Laboratory
Wright-Patterson Air Force Base, Ohio

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The effects of monomethylhydrazine on red blood cells in vitro were studied. These effects are characteristic of oxidant damage and included methemoglobin formation, Heinz body production, and a decreased level of reduced glutathione. Cell morphology was examined by light microscopy and the cells showed a distorted appearance with a loss of the biconcave shape after 2 hours exposure. There was no change in cell osmotic fragility. In the presence of adequate (over)		

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substrate the effects were reversible with the exception of Heinz body precipitation. The number and size of Heinz bodies and the percentage of red cells affected was related to the concentration and length of exposure to MMH. After an extended period of exposure, multiple small Heinz bodies appeared to fuse into single large bodies.

MMH did not exhibit any effect on various protective enzyme systems involved in maintaining the redox equilibrium in the cell. The initial enzymes in the hexose monophosphate shunt, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid dehydrogenase, which provide NADPH, and the enzymes related to glutathione and methemoglobin reduction were not affected at the exposure levels and times employed.

It appears the hemolytic effect of MMH observed in vivo is caused either by a direct action of this oxidant on cell membrane which is not obvious on fixed morphological observation or by the effect of Heinz bodies on cellular integrity leading to a decreased cellular "deformability" and premature removal of the injured cells from the circulation by the spleen, or both.

FOREWORD

This study was performed in support of research Project 7163, "Research on Biomechanisms and Metabolism." The work was performed from January 1971 to June 1972 in the Toxicology Branch, Toxic Hazards Division of the Aerospace Medical Research Laboratory.

This Technical Report has been reviewed and is approved.

This technical report has been reviewed and is approved.

ANTHONY A. THOMAS, MD
Director
Toxic Hazards Division
Aerospace Medical Research Laboratory

INTRODUCTION

The use of monomethylhydrazine (MMH) in rocket propulsion systems has stimulated an intensive investigation into the toxic effects of this compound. There has been a substantial amount of data reported on the effects of both acute and chronic exposures to MMH by various routes of administration in several animal species including man (Clark et al., 1968).

Exposure to acute doses of MMH can cause a variety of symptoms and pathological changes involving the liver, kidneys, lungs and central nervous system depending on the concentration of MMH and the animal species. The range between a lethal or near lethal concentration of MMH producing major pathological changes with overt symptoms and a concentration causing minimal effects is very narrow. However, there are certain effects on the hematologic system which are consistently seen in animals exposed to MMH by all exposure routes and exposed to levels as low as inhalation of 0.2 ppm for 2 weeks. These changes are similar to those produced by other compounds which affect the redox state of the cell and are characterized by anemia with methemoglobinemia and Heinz body formation. Haun et al. (1969 and 1970) exposed dogs and rhesus monkeys continuously or intermittently to various concentrations of MMH in inhalation chambers and measured red blood count, hemoglobin, hematocrit, osmotic fragility, methemoglobin, Heinz body formation, and reticulocytes. Beagle dogs exhibited a significant anemia, reticulocytosis, increased osmotic fragility, and methemoglobinemia and Heinz body formation. Monkeys under the same experimental conditions had similar changes but to a much milder degree, indicating a distinct difference in susceptibility between dogs and monkeys. The dog red cell produces the highest level of methemoglobin followed by man, rat, and monkey red cells (Clark and De La Garza 1967).

Many anemias that are associated with methemoglobin formation and Heinz body production are also associated with alterations in glutathione metabolism. The sequence of events between the appearance of methemoglobin and Heinz bodies and the net loss of reduced glutathione (GSH) in these anemias is variable and a direct mechanistic relationship between these events is uncertain. Also, the mechanism by which red cells become increasingly susceptible to hemolysis in these anemias is unclear although it has been widely speculated that alterations in the red cell membrane may play an important role in the premature removal of injured cells from the circulation. (Rifkind, 1965.)

In the current study, we have used an in vitro system to examine the effects of MMH on the human red cell; GSH levels, methemoglobin and Heinz body production were measured. Biological reduction in the red cell is maintained by GSH and various reductases which require either NADH from the Embden-Meyerhof pathway or NADPH from the hexose monophosphate shunt (HMP) pathway. The activities of glutathione reductase (GSSG-R) and two enzymes in the HMP pathway, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconic acid dehydrogenase (6PGD), were determined since a decrease in these enzyme activities with a resultant loss of NADPH could partially explain the striking susceptibility of mammalian red blood cells to MMH exposure and a decrease in GSSG-R could explain the decrease in GSH. Osmotic fragilities of red cells were examined as a measure of red cell membrane integrity. The development of Heinz bodies was followed by light microscopy and an attempt made to correlate morphologic alterations in red cells exposed to MMH with biochemical changes.

METHODS

Whole blood was drawn by venipuncture from normal human subjects using heparin as the anticoagulant. The blood was centrifuged at 6C and the plasma and buffy coat removed. The red cells were washed three times with isotonic phosphate buffered saline, pH 7.4, containing 0.01M glucose, (Jacob, 1968) and resuspended in the buffered saline to a hematocrit of 40-50%. The white cell count was less than 100/cu mm. The cell suspension was incubated aerobically with MMH* at a concentration of 10 mM. This concentration provided a ratio of approximately 1 mole heme to 1 mole of MMH. In a few experiments additional samples were exposed to concentrations of 1mM and 0.1 mM MMH. Controls were run concurrently using buffered saline in place of MMH. After incubation the cell suspensions were washed three times with the buffered saline and resuspended to a hematocrit of 40-50%. An aliquot was taken for a red count, hemoglobin and hematocrit using standard hematologic techniques.

For enzyme activity measurements the cell suspensions were diluted to about 20% and lysed by adding 1 ml water, 0.3 ml digitonin and 0.7 ml triethanolamine buffer, 0.05 M, pH 7.5, per 1 ml suspension and placed in the refrigerator at 4C for 20 minutes. The lysate was centrifuged for 15 minutes at 1000 g to remove cell stroma. Glucose-6-phosphate dehydrogenase activity was measured on the lysate by the method of Lohr and Waller, (1963) following the formation of NADPH at 340 mu, 25C, spectrophotometrically. Cells and lysate were prepared in a similar manner for determining 6-phosphogluconic acid dehydrogenase activity except that a glycylglycine buffer, 0.25M, pH 7.5 was used. The activity was measured according to the method of Marks (1961). Glutathione reductase activity was determined using the method outlined by Salkie and Simpson (1970). The results were calculated and reported as International Enzyme Units/ 10^9 red blood cells. Cells were exposed to 10

*Eastman Organic Chemical Co., Rochester, N.Y.

mM MMH for times ranging from one hour to twenty-four hours and GSH levels measured by the Alloxan 305 method, (Patterson and Lazarow, 1955) and results reported as $\mu\text{g GSH}/10^9$ red cells. A limited number of experiments were run with various amounts of glucose, 0 to 400 mg/100 ml, added to the buffer in which the cells were suspended to determine the effect of glucose in the substrate on the depletion of GSH by MMH. Methemoglobin was determined by a modification of the Evelyn and Malloy method as described by Hainline (1965). Osmotic fragility of control cells and cells exposed for 2 or 4 hours to MMH was determined using the spectrophotometric method outlined by Wintrobe (1951).

Wet smears of control and MMH exposed red cells were prepared after incubation for 30 minutes, 1 hour, 2 hours and 4 hours and 24 hours. Heinz bodies were demonstrated by supravital staining of the cells with 1% crystal violet and examined by light microscopy.

RESULTS

The number of Heinz bodies per cell, size of Heinz bodies and the percentage of cells containing Heinz bodies was related to MMH concentration in the incubation medium and length of exposure. Incubation of cells with 0.1 mM MMH produced no apparent Heinz bodies at 2, 4, or 6 hours and small Heinz bodies in less than 20% of cells at 24 hours. Incubations of cells with 1.0 mM MMH produced small Heinz bodies in less than 20% of cells at 2 hours but 1 to 4 large Heinz bodies in over 90% of the cells at 24 hours. Incubation with 10 mM MMH produced 1 to 9 small Heinz bodies per cell in 95-100% of cells in 1 hour. These appeared to increase in size at 2 hours and many of the Heinz bodies were large (up to 2 microns) at 24 hours. Heinz bodies could not be demonstrated with certainty after 30 minutes of incubation with any of the concentrations of MMH used in this study.

Control red cells retained a normal biconcave configuration throughout the 24 hour incubation period. Alterations in the configuration of red cells were apparent in MMH exposed cells. They consisted of distortion of cells and loss of their central concavities. These changes were related to concentration of MMH, since they were observed earliest with 10 mM MMH.

The results of the measurement of the activities of G6PD, 6PGD, and GSSG-R as shown in table 1 indicate that exposure to 10 mM MMH for 2 hours at 37C had no significant effect on the activity of these enzymes.

The effects on red cell GSH levels after exposure to 10 mM MMH in vitro are shown in table 2. These data indicate there is about a 40% decrease in GSH levels in 2 hours if an adequate supply of glucose and oxygen is present.

The GSH levels after exposure for periods of time (1 hour, 2 hours, 3 hours and 4 hours) with and without glucose added to the incubation medium are shown in figure 1 and table 3. The control levels of samples with or without added glucose in the incubation medium are quite stable over the 4-hour period. The levels of GSH in control cells incubated with an adequate supply of glucose at 4 hours show a moderate rise over baseline levels which is probably due to the reduction of existing GSSG to GSH by the GSSG-R system. The cells exposed to MMH in an incubation medium containing 200 mg% glucose exhibit a decrease of GSH of approximately 40% at 2 hours with a subsequent return to baseline levels after 4 hours incubation. The cells exposed to MMH in an incubation medium with no added glucose show a progressive decrease in GSH over 4 hours with almost total depletion of GSH at four hours. Both control cells and exposed cells had extremely low levels of GSH at 24 hours when no glucose was added to the medium. As shown in figure 2, when glucose was added to the incubation medium after 5 hours exposure at a concentration of approximately 100 mg/100 ml, the GSH levels returned to near baseline levels.

TABLE 1
EFFECTS OF MMH ON RED CELL ENZYME ACTIVITY
m I.U./10⁹ red cells^a

	<u>G6PD</u>	<u>6PGD</u>	<u>GSSG-R</u>
Control ^b	140 ± 29	84.9 ± 8	84.0 ± 6.7
Exposed ^b	137 ± 20	79.5 ± 10	91.4 ± 8.8

a. Values are mean ± S.D. n = 10.

b. Control cells incubated with buffered saline 2 hours 37°C.

Exposed cells incubated with MMH, 10mM, 2 hours 37°C.

TABLE 2
EFFECTS OF MMH ON RED CELL GSH LEVEL

	GSH μ g/10 ⁹ red cells ^a
Baseline	63.7 ± 7.8
2 Hour Control ^b	60.5 ± 6.3
2 Hour Exposed ^b	38.3 ± 12.1 ^c

a. Values are mean ± S.D. n = 20

b. Control cells incubated with buffered saline at 37°C.

Exposed cells incubated with MMH, 10mM, 37°C.

c. Significantly different from baseline and 2 hour control at p < 0.01.

TABLE 3

EFFECTS OF MMH ON GSH AFTER VARIOUS EXPOSURE TIMES

	$\mu\text{g GSH}/10^9 \text{ Red Cells}^a$				
	<u>Baseline</u>	<u>1 hr.</u>	<u>2 hr.</u>	<u>3 hr.</u>	<u>4 hr.</u>
Control ^b	63.74	61.85	60.50	59.42	71.35
Exposed ^b	61.72	48.81	38.27	57.27	60.89
Control ^c	68.28	66.15	61.21	70.94	69.46
Exposed ^c	68.28	36.61	25.39	11.20	7.49

a. Mean values $N = 5$

b. Cells incubated in buffered saline containing 200 mg% glucose

c. Cells incubated in buffered saline with no glucose added

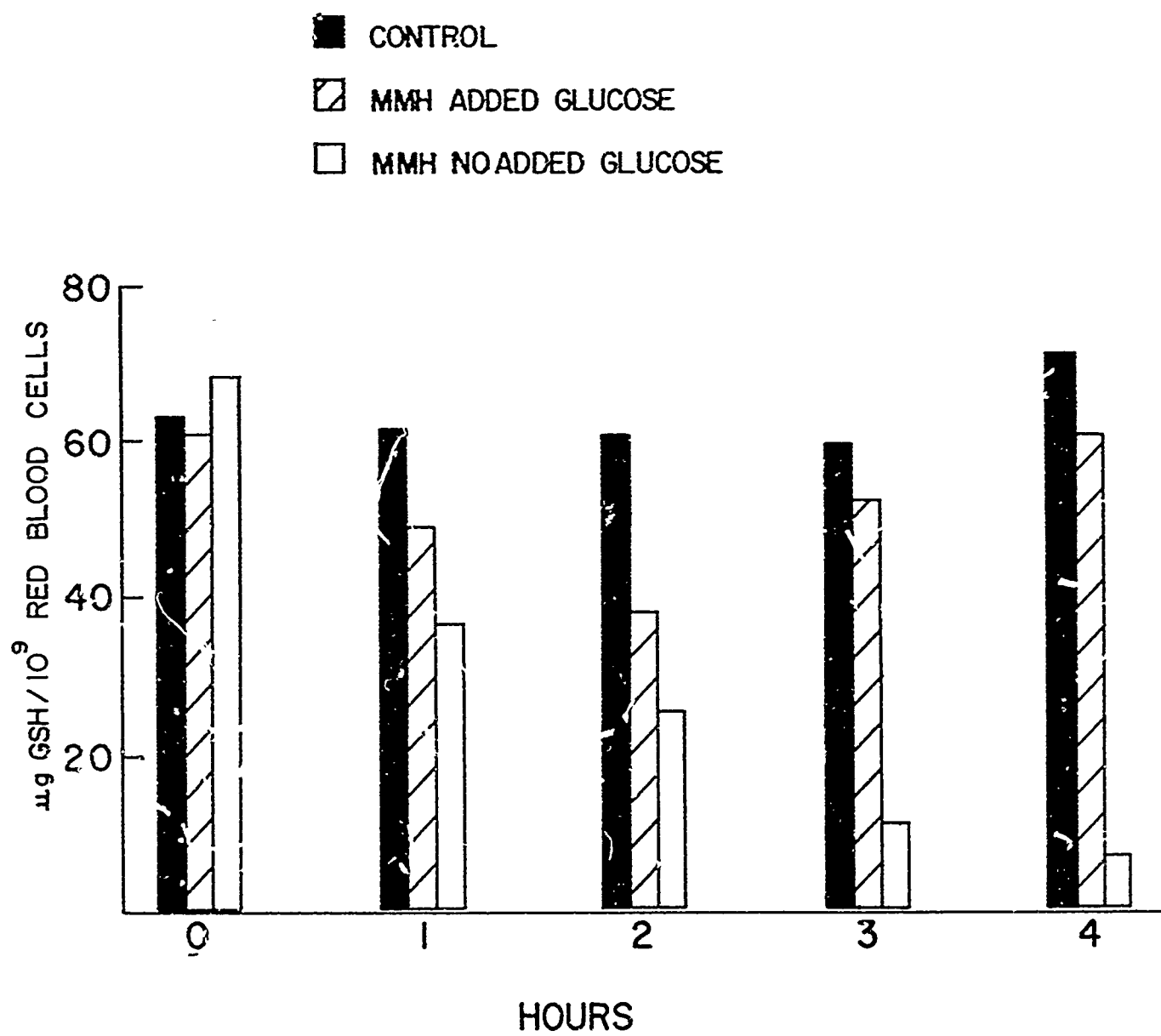


FIGURE 1 - Effects of MMH on Red Cell GSH Level With and Without Glucose.

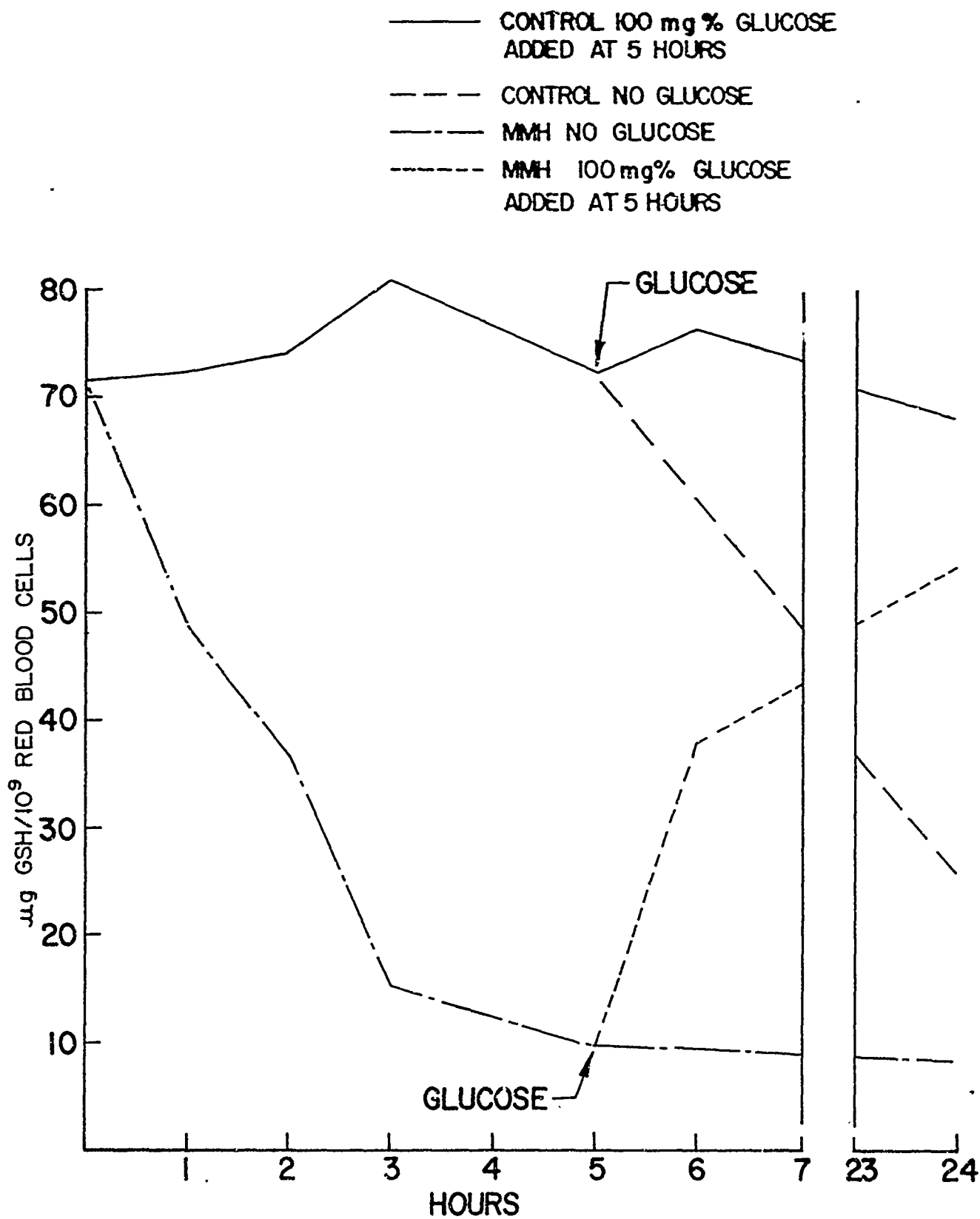


FIGURE 2 - Levels of GSH Before and After Added Glucose.

To assure an optimum amount of glucose for efficient reduction of GSSG to GSH by the GSSG-R enzyme system in vitro, various amounts of glucose were added to the incubation mixture and the cells exposed to MMH. The data indicate that a minimum glucose concentration of approximately 50 mg/100 ml was required for the glutathione reductase system to function efficiently.

The formation of methemoglobin was followed in cells exposed to mM MMH and 1mM MMH as is shown in figure 3. Maximum levels of methemoglobin were reached after 30 to 60 minutes of incubation followed by a gradual decrease over a 3-hour period in the cells exposed to 10 mM MMH. Cells exposed to 1 mM MMH reached a peak level between 90 and 120 minutes. Levels were still above baseline at 6 hours at both concentrations.

The determinations of osmotic fragility of cells exposed to MMH did not indicate there were any shifts in cell fragility after 2 or 4 hours exposure.

DISCUSSION

MMH is a strong reducing agent that produces biologic effects on red cells considered characteristic of oxidative damage. These changes include methemoglobin production, hemoglobin denaturation in the form of Heinz bodies, and oxidation of GSH to GSSG. Other similar compounds, such as phenylhydrazine and acetylphenylhydrazine, are also highly reactive redox compounds which produce some or all of these same changes in red cells. All of these compounds oxidize in the presence of oxygen into intermediates that may be responsible for damaging red blood cells.

Recent studies by Kosower et al. (1969a, 1969b, 1969c) suggest that a free radical mechanism may explain the kinds of changes that we have observed in MMH exposed red cells. They produced intracellular oxidation of GSH to GSSG

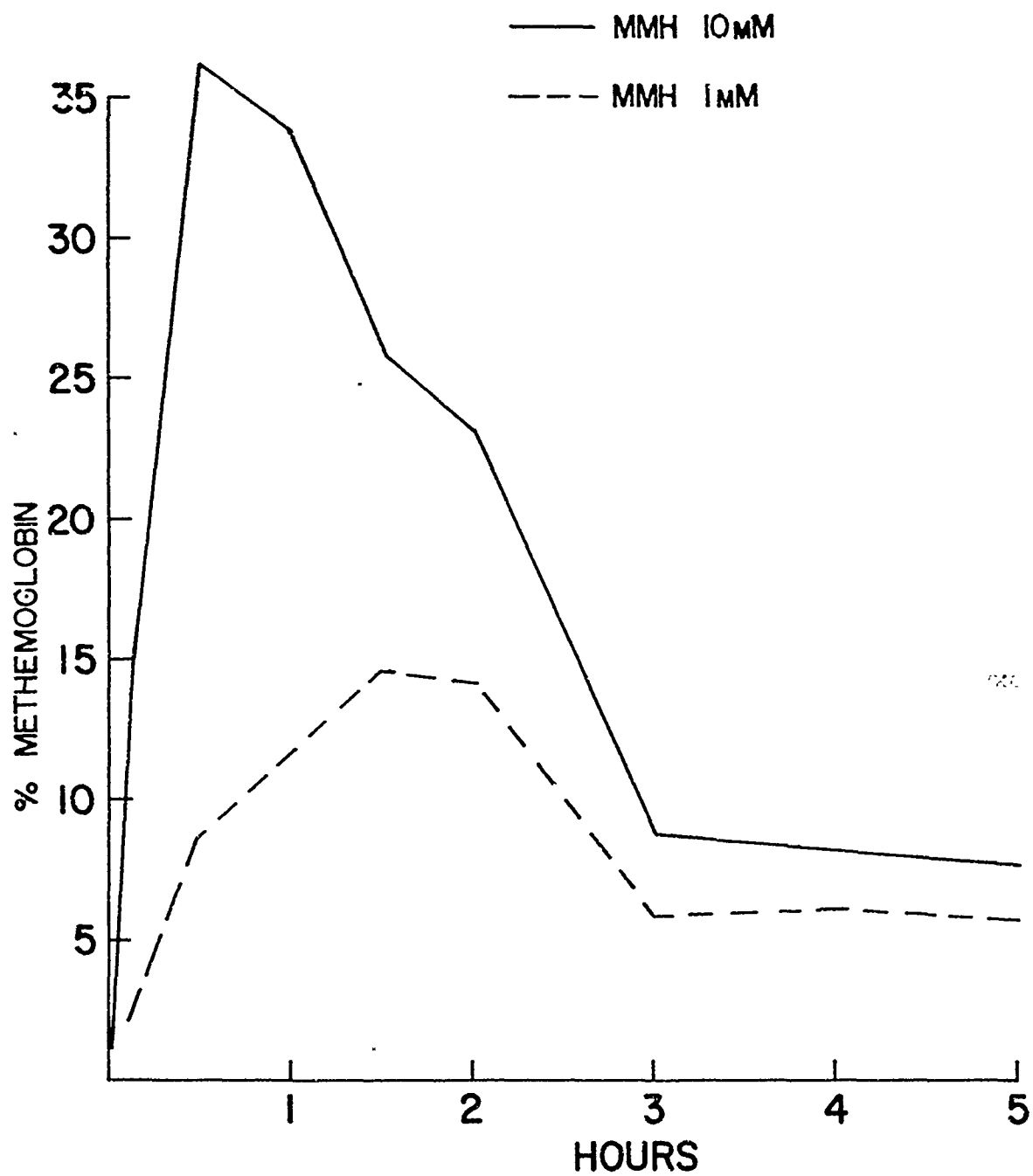


FIGURE 3 - Methemoglobin Levels After Exposure to 10mM MMH and 1mM MMH.

with methyl phenyldiazene carboxylate, $C_6H_5N=NCOOCH_3$, "azoester." Azoester in excess of that required to oxidize GSH caused oxidation of ferrohemoglobin to ferrihemoglobin and Heinz body formation. These biological effects were explained on the basis of free radical formation. Hydrolysis of the azoester produces phenyldiazene which reacts with oxygen to generate free radicals. Free radicals could provide a mechanism for the denaturation of hemoglobin and the formation of Heinz bodies.

Homolytic reactions may generate free radicals from MMH in our in vitro system although reactive intermediates were not measured in this study. The degradation of MMH to CH_4 , N_2 , NH_3 , and methanol is very rapid in the presence of oxygen at room temperature (Vernot et al., 1967). It has been suggested that the initial reaction of MMH and oxygen involves the formation of methyldiazine. Methyldiazine can react with oxygen to produce several free radicals. The methyldiazine may also undergo a bimolecular coupling reaction to form symmetrical dimethylhydrazine which in the presence of excess oxygen is oxidized to azomethane, a potent free radical source. Hydrogen peroxide generated by the system could in turn form free radicals, although hydrogen peroxide is rapidly destroyed catalytically or by catalase, an enzyme found in great abundance in red cells.

Adequate levels of GSH have an essential role in maintaining the integrity of the red cells. GSH is required for certain enzyme activities and for maintaining the redox state of the cell. It also serves as an agent for the maintenance of enzyme SH groups in the reduced form, helps maintain structural integrity of proteins through disulfide interchange reactions, and is a hydrogen donor to scavenge potentially harmful free radicals. Decreased GSH levels are observed in several different clinical settings. Chronic low levels of GSH are associated with a spectrum of nonspherocytic hemolytic anemias that are caused by deficiencies of hexose monophosphate shunt enzymes such as G6PD, 6PGD, etc.

Inadequate HMP activity results in a deficiency of NADPH, an essential cofactor for GSSG-R and methemoglobin reductase. GSH levels are also lowered in certain congenital Heinz body hemolytic anemias. In those anemias unstable mutant hemoglobins spontaneously precipitate as Heinz bodies. It has been postulated that the cysteines in the beta chains of the abnormal hemoglobins excessively bind GSH in stable mixed disulfide linkages resulting in diminished intracellular GSH (Jacob et al., 1968) which in turn apparently stimulates HMP metabolism. Reportedly, GSH levels must be markedly lowered before cells are adversely affected. For example, Jacobs and Jandl (1962) lowered red cell glutathione content to 10% of normal with *n*-ethylmaleimide without affecting red cell glucose metabolism in vitro or red cell survival in vivo. Also Kosower (1971) found that oxidation of 70-80% of intracellular GSH with azoester was necessary to produce observable effects. In our experiments MMH lowered the intracellular GSH only about 40% after 2 hours exposure. In the presence of adequate glucose, GSH levels were restored to normal at 4 hours of incubation. It has been suggested that GSH may be lowered by binding free GSH to denatured protein during the formation of Heinz bodies. If this were the case, however, the decrease would not be reversible in 4 hours. More likely, GSH is oxidized to GSSG and this stimulates the HMP and glutathione reductase activities.

The relationship between oxidation of ferrohemoglobin to ferrihemoglobin, and the precipitation and denaturation of the hemoglobin in the form of Heinz bodies is uncertain. Jandl et al. (1960) suggested that methemoglobin invariably occurs in the early stages of Heinz body anemias and that those instances in which methemoglobin is not observed can be explained by the transient nature of the reaction. Allen and Jandl (1961) proposed that oxidant drugs and chemicals produce the following sequence of events: oxidation of GSH to GSSG; oxidation of ferrohemoglobin to methemoglobin; oxidation of

sulfhydryl groups on the hemoglobin molecule and binding of GSSG to hemoglobin sulfhydryl groups resulting in an alteration of the configuration of the hemoglobin molecule; oxidation of other hemoglobin sulfhydryl groups and finally precipitation of the altered hemoglobin as Heinz bodies. In this scheme, oxidation of both ferrohemoglobin and GSH play an essential role in the mechanism of Heinz body formation. Rentsch (1968) has questioned the validity of the Jandl scheme. He believes that methemoglobin formation may occur concurrently with or before Heinz body production but that it is not a prerequisite in the sequence of events leading to hemoglobin precipitation. He based his argument on the observation that certain chemicals, such as sodium nitrite, cause methemoglobinemia without GSH oxidation or Heinz body formation and that any correlation between the time of appearance of Heinz bodies and the time of methemoglobin production and disappearance varies with exposure to different compounds. Most arguments against a cause-effect relationship of GSH oxidation, methemoglobinemia and Heinz body formation are based on the fact that these events do not always occur in concert. In some congenital anemias associated with decreased GSH, one consistent abnormality is the formation of Heinz bodies but without methemoglobinemia whereas other anemias lead to methemoglobinemia without Heinz body precipitation.

This study and other studies on MMH induced Heinz body anemias provide further examples of a lack of direct relationship between methemoglobin production, Heinz body formation and decreased GSH levels. In our in vitro system, ferrohemoglobin is oxidized to ferrihemoglobin and GSH levels are diminished during the time interval when Heinz body formation is initiated. However, Heinz body growth continues to be prominent after 2 to 4 hours of incubation with high levels of MMH although methemoglobin and GSH levels are returning to normal. The time span between maximum methemoglobinemia

and Heinz body formation is difficult to explain if there is a direct relationship. Notably, there are large species differences in the response to MMH. Monkey and rat red cells produce little methemoglobin but do show decreases in GSH levels and do form Heinz bodies. Inhalation of MMH by humans produces no measurable increase in methemoglobin although Heinz bodies were present in red cells up to 2 weeks after exposure (MacEwen et al., 1970).

The results of our morphologic studies on Heinz bodies in MMH exposed red cells are significantly different from the changes reported by Kosower et al. (1970) for azoester treated red cells and from the usual findings in non-spherocytic hereditary anemias in which Heinz body production is associated with oxidation stress in the face of low GSH levels. Kosower et al. found few Heinz bodies produced in their in vitro system when GSH oxidation by azoester was incomplete. However, when we exposed cells to MMH, Heinz bodies became prominent when intracellular GSH levels were 60% of normal levels. In studies in this laboratory of cells exposed to lower concentrations of MMH there was no measurable decrease in GSH but Heinz bodies were formed. This finding would appear to argue against a free radical mechanism as an explanation for Heinz body formation. The intracellular GSH levels in red cells in our system should be adequate to scavenge harmful free radicals and protect hemoglobin from denaturation by radical coupling. One possible explanation is that GSH is nonuniformly distributed in the cytoplasmic compartment of red cells. This might explain the distribution of Heinz bodies and the focal vulnerability of hemoglobin to denaturation.

The hemolytic effects of MMH must be caused by membrane damage either by direct action of the oxidant on membranes or by the effect of Heinz bodies

on membrane integrity. If there is a change in membrane composition resulting in a shift in the action gradient across the membrane it was not reflected by spherocytosis or swelling or by a change in osmotic fragility after 4 hours exposure. MMH causes oxidation of hemoglobin and possibly of the globin sulfhydryl groups which could cause conformational changes in the molecule with eventual denaturation and precipitation as Heinz bodies. Some of these Heinz bodies may attach to the membrane probably through disulfide linkage with membrane thiol groups, thus effectively blocking them. Cells with membrane thiol groups artificially blockaded with para-mercuribenzoate are rapidly trapped and destroyed in the spleen and this probably occurs with cells in which membrane thiols are blocked by Heinz bodies. Cells containing Heinz bodies attached to the membrane also have a decreased deformability ultimately leading to an increase in splenic entrapment and destruction of the cells with resultant anemia.

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