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EFFECT OF MONOMETHYLHYDRAZINE ON RED BLOOD CELL METABOLISM

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AEROSPACE MEDICAL DIVISION
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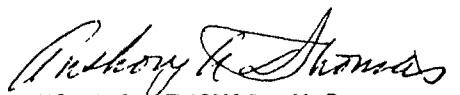

ANTHONY A. THOMAS, M.D.
Director, Toxic Hazards Division
6570th Aerospace Medical Research Laboratory

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Monomethylhydrazine is used in various Air Force propellant systems. Chronic exposure to low concentrations of this compound results in an anemia characterized by methemoglobinemia, decreased levels of reduced glutathione and Heinz body formation. The study reported here was designed to determine if Heinz body formation was the principal mechanism of the red cell destruction or if MMH also had effects on the glycolytic pathways and/or red cell membranes

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which would contribute to the hemolytic mechanism. Human red cells were exposed in vitro to three levels of MMH for two, four, or six hours. Glucose utilization, lactate production and ATP levels were measured to determine effects on glucose metabolism; and osmotic fragilities, red cell potassium concentration, and malonyldialdehyde levels were measured to assess membrane effects. The results support the thesis that the major mechanism of red cell destruction following exposure to MMH appears to be the physical presence of Heinz bodies in the red cell resulting in a decreased deformability of the cell and accelerated sequestration and destruction by the spleen.

INTRODUCTION

The use of monomethylhydrazine (MMH) in various Air Force propellent systems has prompted intensive investigations into the toxicity of this compound. The effects of acute exposures to high concentrations of MMH have been studied and reported. At lethal concentrations the principal effects were on the central nervous system. Death resulted from respiratory arrest; and the major findings at necropsy were hepatic and pulmonary congestion and moderate to severe kidney damage (Haun, 1968; MacEwen, 1969). It has also been shown that there is a significant difference in species susceptibility to MMH toxicity, the dog being the most sensitive species followed by man, rat, mouse, and rhesus monkey (Clark et al., 1967). Further studies performed to determine the toxic effects of chronic exposure of dogs and monkeys to low concentrations of MMH indicated that the only consistent pathological response involved the hematologic system (Haun, 1970; Kroe, 1971; MacEwen and Haun, 1971). MMH caused a dose related hemolytic anemia characterized by methemoglobinemia, Heinz body formation, and at some exposure levels an increased osmotic fragility. There was also a significant species difference in the severity of the anemia but in all cases the anemia was reversible after the exposure was terminated. At necropsy tissue examination showed hepatic, renal proximal tubular, and in mice, splenic hemosiderosis. These findings would indicate the principal target organ was the red cell with the resulting tissue changes being secondary effects typical of the species ability to clear products of hemolysis. The only irreversible damage observed was the denaturation and precipitation of hemoglobin as Heinz bodies.

Since this anemia was similar to other anemias associated with alterations in glutathione metabolism and in glutathione reductase activity, the in vitro effects of MMH on reduced glutathione (GSH) levels in red cells were measured. The amount of GSH in the cells was decreased although normal enzyme reductase activity was observed. This effect was reversible and the GSH levels eventually returned to normal (Weinstein and George, 1972).

The occurrence of Heinz bodies in red cells is a nonspecific type of response to many anemia causative factors, i.e., enzyme deficiencies, unstable hemoglobins and exposure to oxidant compounds. It is known that Heinz body anemias are characterized by accelerated splenic sequestration and destruction of the Heinz body containing red cell although the exact mechanism is not completely defined (Rifkind, 1965). It is also recognized, however, that Heinz body formation is not necessarily the only change leading to increased red cell destruction in the anemias caused by oxidizing compounds. Depending on the oxidant employed, these anemias may also show changes in intracellular metabolism and/or red cell membrane which may or may not be directly related to the Heinz bodies. The studies described here were undertaken to determine if Heinz body formation with the resultant accelerated destruction of the affected red cells was the principal mechanism of the anemia caused by exposure to MMH or if there were additional effects resulting in alterations in red cell metabolism or in cell membrane which would contribute to the hemolytic process.

METHODS

Venous blood was collected from normal human subjects in heparinized tubes, centrifuged at 6°C, and the plasma and buffy coat removed. The red cells were washed three times with phosphate buffered isotonic saline, pH 7.4, containing 0.01M glucose and resuspended in the same buffer at a hematocrit of 40-50%. The white blood count was less than 200 per cu. mm. Monomethylhydrazine (Eastman Organic Chemical Co., Rochester, New York), diluted with the same buffered saline, was added to the red cell suspensions to provide final concentrations of 10 mM, 1 mM, and 0.1 mM. Buffered saline without MMH was added to cell suspensions at the same concentrations and these were run concurrently as controls. All red blood cell suspensions containing MMH or saline were incubated at 37° and samples taken for the various determinations at two, four, and six-hour intervals.

Total red cell counts, hematocrits and hemoglobin levels were measured by standard hematologic techniques. Osmotic fragility tests were performed using the method described by Dacie, 1954. The percent methemoglobin formed was determined by the method of Hainline, 1965 and reduced glutathione levels by the alloxan 305 method of Patterson et al., 1955. Methyl violet in 0.9% saline was used for supravital staining of Heinz bodies.

Glucose consumption, lactate production and ATP levels were run on perchloric acid protein free filtrates of the cell suspensions. Glucose was determined enzymatically by measuring the formation of NADPH in the reaction involving hexokinase and glucose-6-phosphate dehydrogenase (Slein, 1963). Lactate was measured by following the rate of formation of NADH in the presence of lactic dehydrogenase (Hohorst, 1963). ATP levels were run by the enzymatic

method of H. Adam, 1963, measuring the decrease in NADH in the reaction utilizing glycerate-3-phosphate, phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase.

Red cell potassium changes were studied by methods based on those described by Rasbridge and Scott, 1973. Aliquots taken from both the control and MMH exposed cell suspensions were centrifuged, the supernatant removed and the amount of hemolysis measured. The cells were washed twice with potassium free buffered saline and resuspended in the saline to a hematocrit of 50%. The cells were lysed with distilled water and the potassium content of the hemolysate measured with a flame photometer.

Malonyldialdehyde (MDA) levels were measured by the method described by Stocks and Dormandy, 1971. It has been shown that MDA is a secondary product of autoxidation of the polyunsaturated lipids found in the cell membrane. Therefore, the reaction of MDA with thiobarbituric acid was used as an indication of membrane lipid peroxidation.

RESULTS

Heinz body formation in red cells exposed to MMH was time and dose related. At the lowest concentration of MMH used, 0.1 mM, Heinz bodies were not observed during the six hour incubation; at the 1 mM concentration approximately 20% of the cells contained 1-3 small Heinz bodies after two hours with the size and number of cells affected increasing with longer times of exposure. Exposure to 10 mM MMH resulted in 2-5 Heinz bodies in 90-100% of the cells after two hours incubation. The control cells appeared normal throughout the six-hour test period.

The degree of oxidation of hemoglobin to methemoglobin was directly related to the concentration of MMH, Figure 1. The lowest concentration of MMH, 0.1 mM, did not cause any detectable methemoglobinemia at any of the time periods. Exposure to 1mM MMH produced about 10% in two hours and the 10 mM MMH concentration caused 22% methemoglobin in two hours. The maximum amount of methemoglobin was produced within the two-hour exposure period as the percent methemoglobin decreased at four hours and was less than 10% after six hours incubation with both 10 mM and 1 mM concentrations of MMH. These data are similar to the results reported earlier in which the peak methemoglobin production in response to 10 mM MMH was reached in 30 to 60 minutes and the peak response to 1 mM was reached in 90 to 120 minutes followed by a gradual decrease over five hours. This confirms the direct relationship of amount of methemoglobin formation to MMH concentration but, unlike Heinz body production which increases with extended exposure time, methemoglobin production is a reversible process and the concentration decreases on longer exposures.

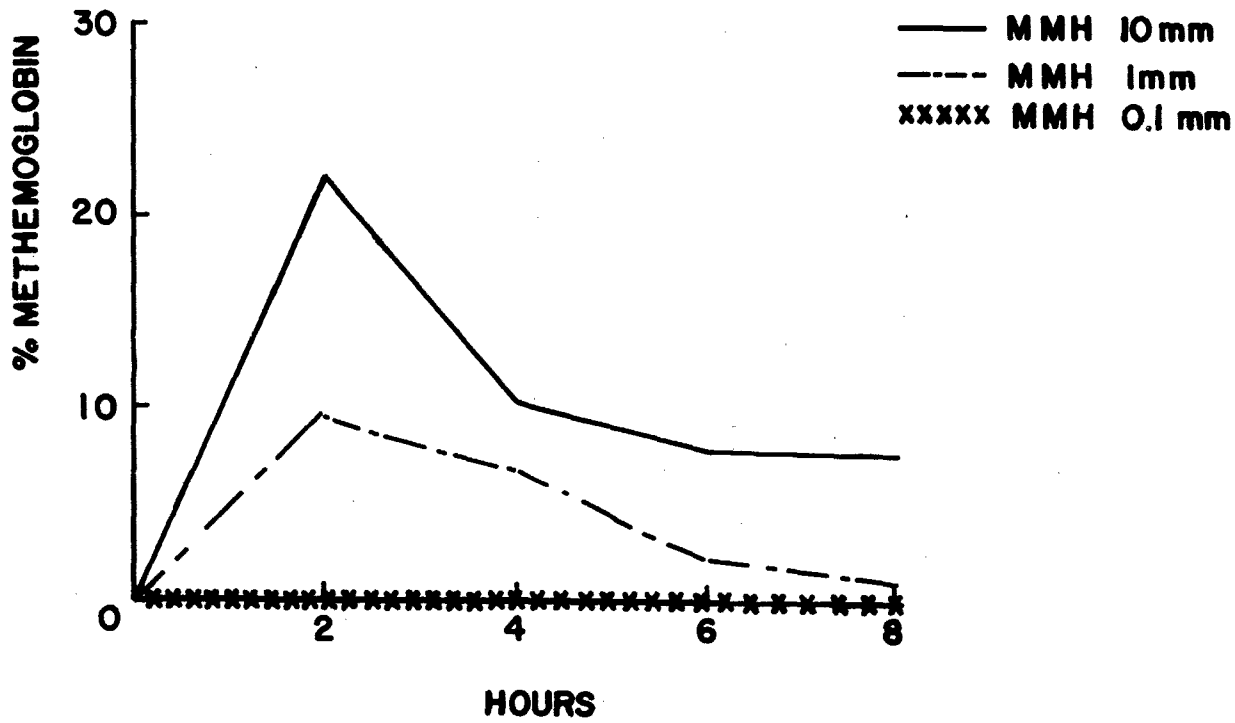

FIGURE 1

METHEMOGLOBIN LEVELS IN RED CELLS EXPOSED TO MMH

The levels of GSH measured show a similar pattern to methemoglobin formation. There is a significant decrease in GSH after exposure to 10 mM concentration of MMH which reaches the lowest level after four hours with a return to near normal levels at eight hours. Again these data confirm the reversibility of the decrease of GSH with extended exposure (Figure 2).

The glucose consumption and lactate production of red cells exposed to 10 mM MMH was significantly increased; there was no difference between control suspensions and exposed suspensions at the 1 mM or 0.1 mM levels (Table 1). The ATP levels of red cells showed no significant difference from control levels although there was a slight decrease in all samples at each concentration and at each time period (Figure 3). These results add weight to the earlier studies on the effects of MMH on enzymes in the glycolytic pathway, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. There was no decrease in activity of these enzymes and no apparent interference with the glycolytic process through the hexose monophosphate pathway.

The determinations performed to screen possible effects of MMH on the cell membrane were osmotic fragilities, MDA production and potassium loss from the cells. There were no significant changes in osmotic fragility or MDA levels between exposed cells and controls. Exposure to 10 mM MMH for six hours did cause a significant decrease in potassium content, Table 2, but not at the other exposure times or lower concentrations.

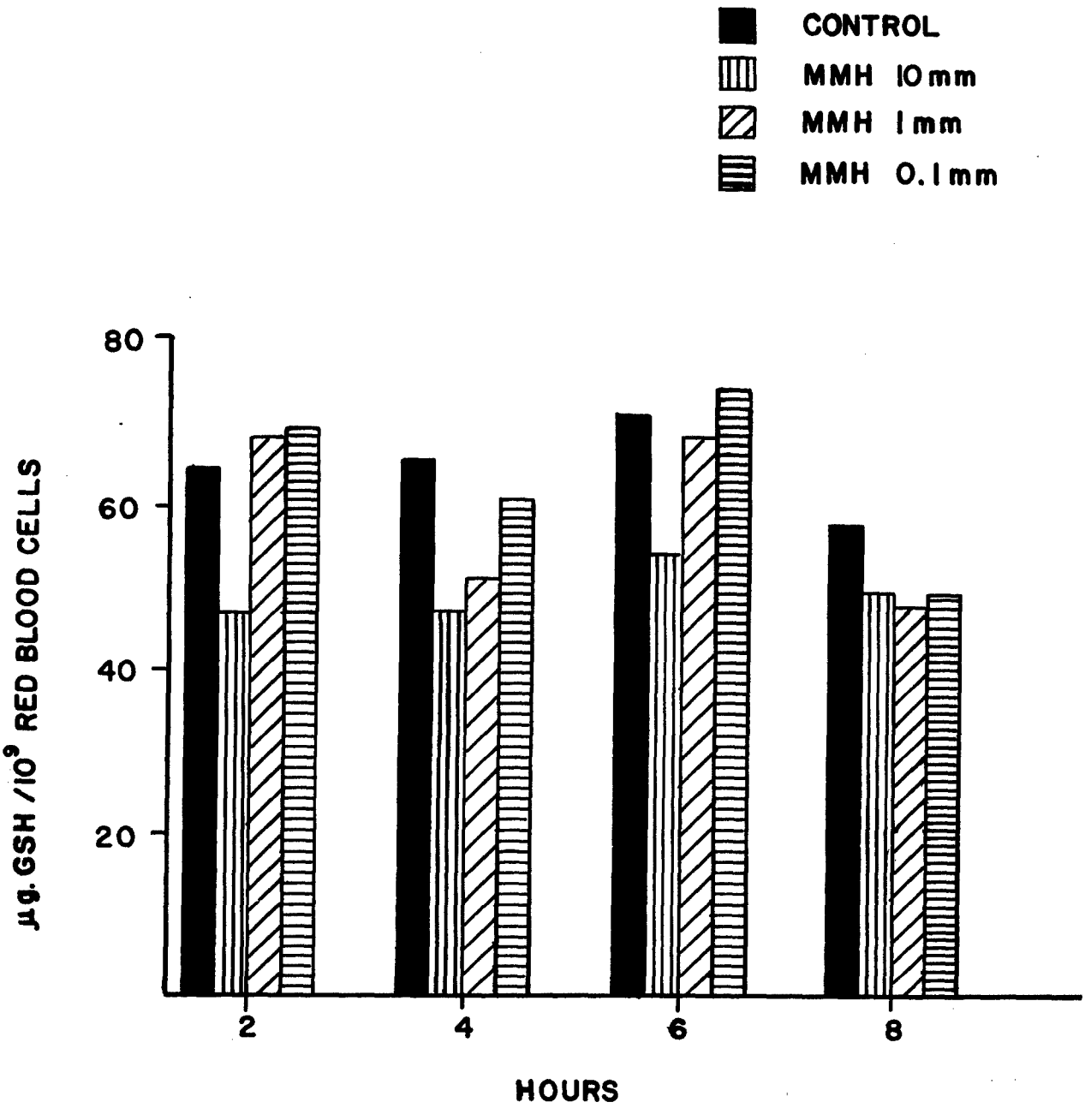

FIGURE 2

GSH LEVELS IN RED CELLS EXPOSED TO MMH

TABLE 1

GLUCOSE CONSUMPTION, LACTATE PRODUCTION
IN RED CELLS EXPOSED TO MMH

	Concentration MMH - mM			
	0-Cont.	10	1	0.1
Glucose Consumption mM glucose/l rbc/hr 37°	1.55±.44	2.70±.89[X]	1.64±.53	1.54±.45
Lactate Production mM lactate/l rbc/hr 37°	2.90±.60	4.26±.86[X]	3.21±.57	2.91±.61

X: $P < .001$

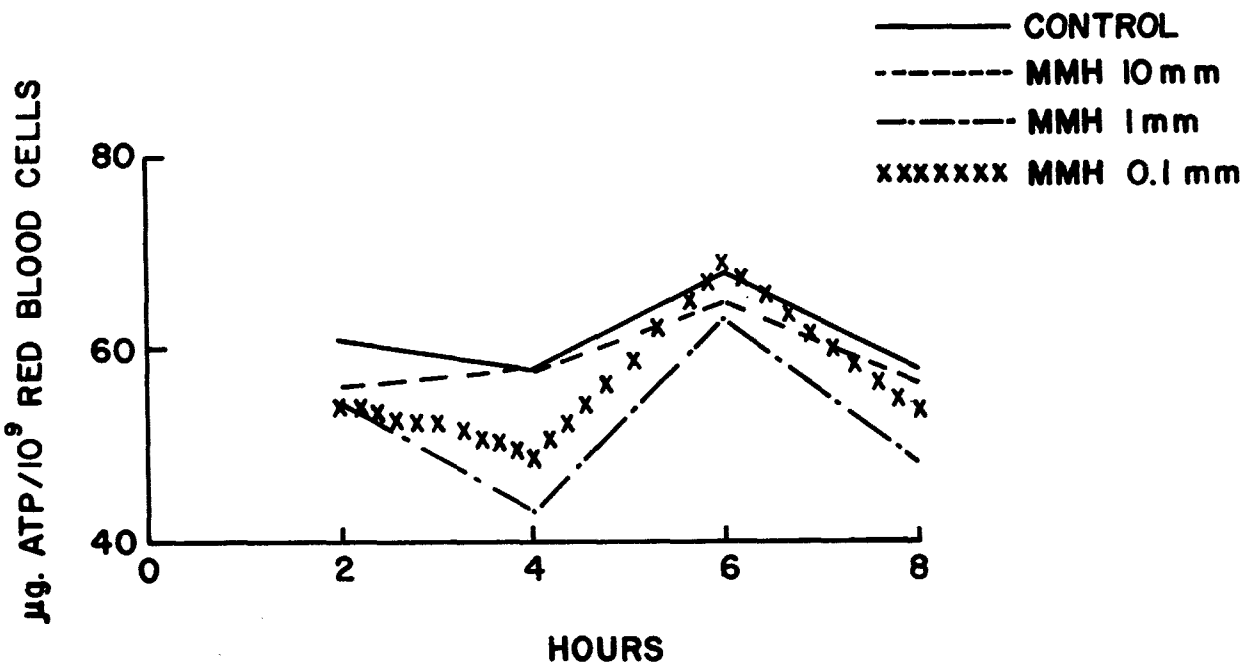

FIGURE 3

ATP LEVELS IN RED CELLS EXPOSED TO MMH

TABLE 2

K^+ CONTENT OF RED CELLS EXPOSED TO MMH

	Concentration MMH - mM			
	0-Cont.	10	1	.1
K^+ - mEq/l rbc 4 hr exposure 37°	98.07±3.94	93.20±4.10	96.31±2.78	98.32±2.57
K^+ mEq/l rbc 6 hr exposure 37°C	98.59±2.66	91.35±3.67[x]	93.88±2.19	97.82±2.86

x - $P < .01$

DISCUSSION

Hemolytic anemias characterized by Heinz body formation can be caused by a wide variety of oxidant compounds which differ in their effects and patterns of damage and in the time sequence and severity of toxic action, (Miller and Smith, 1970). Some of these compounds affect intracellular metabolism; oxidation of hemoglobin and/or GSH and decreased ATP levels have been reported by several authors (Mohler, 1961; Jacob and Jandl, 1966). Other oxidants may also damage the cell membrane causing increased osmotic fragility, changes in membrane permeability or lipid peroxidation of the cell membrane (Miller and Smith, 1970; Weed et al., 1961). It has been proposed that oxidizing compounds generate peroxide radicals which react with the polyunsaturated lipids in the cell membrane as well as with intracellular components, and this free radical production is the basic mechanism causing cellular damage (Cohen and Hochstein, 1964; Rasbridge et al., 1973). These effects may occur singly or in combination and there does not appear to be a direct correlation between membrane and intracellular effects.

The question arises whether the destruction of the red cell is a result of membrane damage due to the attachment of the Heinz body to the membrane, to membrane lipid peroxidation, or if other intracellular changes also contribute to the hemolytic process. Jacob (1970) has suggested that Heinz bodies attach to the membrane by disulfide bonding with the resultant inhibition of membrane sulfhydryl groups the major source of membrane changes. Winterbourne, (1973) however, has indicated that covalent bonding is not present and proposed that hydrophobic bonding is the mechanism involved in the membrane distortion and dysfunction. Studies in this laboratory with cells incubated with MMH indicate that Heinz bodies appear mainly in the central portion of the cell

and do not appear bound to the membranes at least during the peak exposure period. Therefore, the hemolysis from MMH exposure does not seem to be caused by Heinz body attachment to the membrane. The major factor in the destruction of Heinz body containing red cells may well be related to the physical presence of Heinz bodies in the cell with a resultant increase in cell rigidity and decrease in cell deformability. This would lead to an increased rate of splenic sequestration and destruction (Rifkind, 1965; Lubin and Desforges, 1972).

MMH, although a strong reducing agent produces effects in the red cells characteristic of oxidative damage. It has been postulated that MMH in the presence of oxygen forms methyldiazine which further reacts to provide a potent source of free radicals. The oxidation of intracellular constitutents indicated by the formation of methemoglobin and Heinz bodies and the oxidation of GSH supports this view. However, at the peak methemoglobin level when the cells contained several large Heinz bodies, and the GSH level had decreased to its lowest point there was no lipid peroxidation of the cell membrane detectable by the MDA thiobarbituric acid reaction. The cell fragility was not altered and the only noticeable effect was a small, though significant, loss of potassium at this most severe MMH exposure. This loss of potassium is minimal and the implications of this are somewhat unclear. An in vivo exposure to this level of MMH would be fatal but the question remains whether prolonged exposure to lower concentrations of MMH might produce the same result. This is unlikely in view of the reversibility of the hemolytic process seen in in vivo studies over a long exposure time.

At the same peak exposure times there was an increase in glucose consumption and lactate production indicating an increased glycolytic rate.

This would be expected as Jacob and Jandl (1966) have shown that a decrease in the level of GSH stimulates the rate of glycolysis through the hexose monophosphate shunt. This acts as a compensatory mechanism to reestablish the normal redox state of the cell since the activity of glutathione reductase is dependent on an adequate supply of NADPH produced in the HMP pathway. Previous studies (Weinstein and George, 1972) have shown that enzymes which have protective actions against oxidative agents, glutathione reductase, methemoglobin reductase and glucose-6-phosphate dehydrogenase activities, are not affected by MMH. This stimulation of glycolysis with normal reductase enzyme activity would account for the reversibility of the oxidative process indicated by the decrease in methemoglobin and GSH and the return to normal levels with time.

These data show no severe irreversible effects on intracellular metabolism from MMH exposure except for Heinz body formation. Further there was no lipid peroxidation of the membrane noted and the concentration of MMH required to cause a change in membrane permeability to potassium is extremely high. These results suggest that the major damage to red cells from MMH exposure is Heinz body formation with an increase in cell rigidity which probably causes an increased rate of cell sequestration and destruction in the spleen.

REFERENCES

1. Adam, Hans, "Adenosine 5 Triphosphate Determination with Phosphoglycerate Kinase"; In Methods of Enzymatic Analysis, Hans-Ulrich Bergmeyer, editor, Academic Press, New York, 1963.

2. Bunyan, J., J. Green, E.E. Edwin and A.T. Diplock, "Lipid Peroxidation in Dialuric Acid Induced Haemolysis of Vitamin E Deficient Erythrocytes"; Biochem. J., Vol 77, p 47, 1960.

3. Clark, D.A. and M. De La Garza, "Species Differences in Methemoglobin Levels Produced by Administration of Monomethylhydrazine"; Proc. Soc. Exp. Biol. Med., Vol 125, p 912, 1967.

4. Cohen, G. and P. Hochstein, "Generation of Hydrogen Peroxide in Erythrocytes by Hemolytic Agents"; Biochemistry, Vol 3, p 895, 1964.

5. Dacie, J.V., The Haemolytic Anemias, Congenital and Acquired, Grune and Stratton, New York, 1954.

6. Hainline, A. Jr., Standard Methods of Clinical Chemistry, Vol 5, S. Meites, editor, Academic Press, New York, 1965.

7. Haun, C.C., "The Acute Inhalation Toxicity of Monomethylhydrazine"; Proceedings of the 4th Annual Conference on Atmospheric Contamination in Confined Spaces, AMRL-TR-68-175, Wright-Patterson Air Force Base, Ohio, 1968.

8. Haun, C.C., "Chronic Exposure to Low Concentrations of Monomethylhydrazine"; Proceedings of the 1st Annual Conference on Environmental Toxicology, AMRL-TR-70-102, Wright-Patterson Air Force Base, Ohio, 1970.

9. Hohorst, Hans-Jurgen, "Lactate Determination with Lactic Dehydrogenase and DPN"; In Methods of Enzymatic Analyses, Hans-Ulrich Bergmeyer, editor, Academic Press, New York, 1963.

10. Jacob, H.S., "Mechanisms of Heinz Body Formation and Attachment to Red Cell Membrane"; Seminars in Hematology, Vol 7, p 341, 1970.

11. Jacob, H.S. and J.H. Jandl, "Effects of Sulfhydryl Inhibition on Red Blood Cells. II. Glutathione in the Regulation of the Hexose Monophosphate Pathway"; Journal Biological Chemistry, Vol 241, p 4243, 1966.

12. Kroe, D.J., "Animal Pathology Resulting from Long Term Exposure to Low Levels of Monomethylhydrazine"; Proceedings of the 2nd Annual Conference on Environmental Toxicology, AMRL-TR-71-120, Wright-Patterson Air Force Base, Ohio, 1971.

13. Lubin, A. and J.F. Desforges, "Effect of Heinz Bodies on Red Cell Deformability"; Blood, Vol 39, p 658, 1972.

14. MacEwen, J.D., "Determination of Proposed Emergency Exposure Limits for Monomethylhydrazine"; Proceedings of the 5th Annual Conference on Atmospheric Contamination in Confined Spaces, AMRL-TR-69-130, Wright-Patterson Air Force Base, Ohio, 1969.

15. MacEwen, J.D. and C.C. Haun, "Chronic Exposure Studies with Monomethylhydrazine"; Proceedings of the 2nd Annual Conference on Environmental Toxicology, AMRL-TR-71-120, Wright-Patterson Air Force Base, Ohio, 1971.

16. Miller, A. and H.C. Smith, "The Intracellular and Membrane Effects of Oxidant Agents on Normal Red Cells"; British Journal Haematology, Vol 19, p 417, 1970.

17. Mohler, D.N. and W.J. Williams, "The Effect of Phenylhydrazine on the Adenosine Triphosphate Content of Normal and Glucose-6-Phosphate Dehydrogenase Deficient Human Blood"; Journal Clinical Investigation, Vol 40, p 1735, 1961.

18. Patterson, J.W. and A. Lazarow, Methods of Biochemical Analyses, Vol 2, D. Glick, editor, Interscience Publishers, New York, 1955.

19. Rasbridge, M.R. and G.T. Scott, "The Haemolytic Action of Dapsone: Changes in the Red Cell Membrane"; British Journal Haematology, Vol 24, p 183, 1973.

20. Rifkind, R., "Heinz Body Anemia: Ultrastructural Study. II. Red Cell Sequestration and Destruction"; Blood, Vol 26, p. 433, 1965.

21. Slein, M.W., "Glucose Determination with Hexokinase and Glucose-6-Phosphate Dehydrogenase"; In Methods of Enzymatic Analyses, Hans-Ulrich Bergmeyer, editor, Academic Press, New York, 1963.

22. Stocks, J. and T.L. Dormandy, "The Autoxidation of Human Red Cell Lipids Induced by Hydrogen Peroxide"; British Journal Haematology, Vol 20, p 25, 1971.

23. Weed, R.I., J. Eher, A. Rothstein, "Effects of Primaquine and Other Related Compounds on Red Blood Cell Membrane. I. Na^+ and K^+ Permeability in Normal Human Cells"; Journal Clinical Investigation, Vol 40, p 130, 1961.

24. Weed, R.I. and C.F. Reed, "Membrane Alterations Leading to Red Cell Destruction"; American Journal of Medicine, Vol 41, p 681, 1966.

25. Weinstein, R.S. and M.E. George, "Interrelationships of Methemoglobin, Reduced Glutathione and Heinz Bodies in Monomethylhydrazine Induced Anemias. In Vitro Studies of Human Red Cells"; Proceedings of the 3rd Annual Conference on Environmental Toxicology, AMRL-TR-72-130, Wright-Patterson Air Force Base, Ohio, 1972.

26. Winterbourne, C.C. and R.W. Carrell, "The Attachment of Heinz Bodies to the Red Cell Membrane"; British Journal Haematology, Vol 25, p 585, 1973.

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