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EXPOSURE OF DOG ERYTHROCYTES IN VIVO TO PHENYLHYDRAZINE AND MONOMETHYLHYDRAZINE: A FREEZE-ETCH STUDY OF ERYTHROCYTE DAMAGE

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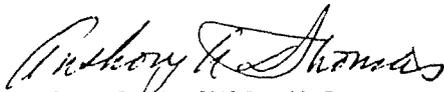
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FOR THE COMMANDER

ANTHONY A. THOMAS, M.D.
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Dog red blood cells were exposed in vivo to phenylhydrazine or monomethylhydrazine by injecting the dogs subcutaneously with these compounds. The cell damage was evidenced clinically by hemolytic anemia at all concentrations in the phenylhydrazine exposed animals. In contrast, there was little clinical evidence in monomethylhydrazine cells except at the highest concentration. The freeze-cleave technique showed that phenylhydrazine exposed erythrocytes contained Heinz bodies within two hours. The Heinz bodies formed in the central cytoplasm

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and migrated to the membrane. The membrane was evaginated and dimpled where Heinz bodies were near the membrane and this might represent the damage which initiates sequestration of erythrocytes by the spleen and liver. The freeze-cleave technique showed that monomethylhydrazine treated cells did not produce typical Heinz bodies, but did produce aggregates of granules arranged in ordered rows which may represent hemoglobin crystals.

These striking differences between the effects of phenylhydrazine and monomethylhydrazine exposure may be related to the doses used and route of administration or might indicate widely different toxicities of these compounds.

PREFACE

This study was initiated in the Pathology Branch, Toxic Hazards

Division of the Aerospace Medical Research Laboratory. The author

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pathology; and Ms. Marilyn George for assistance with the biochemistry.

INTRODUCTION

The Air Force has been interested in the toxic effects of mono-
methylhydrazine (MMH) because of its use as a propulsion fuel. Haun,[8]
MacEwen,[14] Leahy,[7] and others[12,13] have investigated the acute and chronic
effects of MMH exposure, via injection, and inhalation routes, using
mice, rats, dogs, monkeys, and in some cases, human volunteers. In all
of these studies, the hematologic system was the one most sensitive to
damage after exposure to low levels of MMH, with resultant anemia
and Heinz body formation. Heinz body formation using phenylhydrazine
has been investigated previously by Beaven[1] and by Jandl[3] who demonstrated
that the many drugs which induce Heinz body formation are highly reactive
redox compounds. These compounds, in the presence of oxygen, cause a
progressive oxidative destruction of hemoglobin which precipitates as
Heinz bodies.[1,3,15]

Two compounds were used in the present experiment, phenylhydrazine
and monomethylhydrazine. Both compounds are highly reactive redox
compounds which oxidize in the presence of oxygen to intermediates that
damage red cells. These two compounds basically can produce a similar
type of damage, but they do so to varying degrees and at different rates.
The freeze-cleave technique has been shown to be very useful for the
study of erythrocyte damage including Heinz body formation[16,17] and was
used in the present study.

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Previous studies of erythrocyte damage using dogs exposed to MMH have been done *in vivo* by inhalation, by intraperitoneal (ip) injection, or by *in vitro* studies. Erythrocyte damage induced by phenylhydrazine has been studied *in vitro* using various animal and human cells and *in vivo* using rabbits.[11] Rifkind used intramuscular (im) injections in rabbits and made significant observations using the standard thin section method.

The purpose of this paper is to present the findings of a study designed to investigate erythrocyte damage to dog red blood cells *in vivo* induced by subcutaneous (SC) injections of phenylhydrazine and MMH. Most other studies have used either light microscopy, the standard thin sections or, occasionally, freeze-etching. In this study, we used the freeze-etching technique to evaluate erythrocyte damage and attempted to verify Rifkind's results for phenylhydrazine using standard thin sections. We also compared the results of inhalation exposure of MMH to subcutaneous injection in an effort to determine the significance of the route of exposure versus the effect of exposure.

MATERIALS AND METHODS

Animals

Eight young adult (18 months old – average weight 9.6 kg) male

Beagle dogs (Hazelton Research Animals, Inc, Cumberland, Virginia)

were used. Three were exposed to phenylhydrazine, three to monomethyl-

hydrazine (MMH) and two were used as control animals. The agents were

administered by subcutaneous (SC) injection on two consecutive days

and the animals were sacrificed on the fifth day. The pure phenylhydrazine

(Matheson, Coleman, and Bell, Norwood, Ohio) was diluted to a 25% solution

by the addition of 0.9% NaCL. Enough hydrochloric acid (1N) was added

to reduce the pH to 6.6 in order to obtain a completely miscible solution.

Each of the three dogs received a different dose, i.e. 20mg/kg (0.74ml),

30mg/kg (1.0ml), and 40mg/kg (1.4ml). The MMH (Eastman Organic Chemical

Company, Rochester, New York) was diluted to a 2% solution with isotonic

phosphate–buffered saline, pH 7.4, (containing 0.011mm glucose) in a manner

similar to that described by Jacob[21]. The dogs exposed to MMH were injected

subcutaneously with either 0.5mg/kg (0.3ml), 1.0mg/kg (0.5ml), or 1.5mg/kg

(0.8ml). The control dogs were not injected. Daily blood samples (8ml)

were taken from each dog for four days prior to the first injection to serve

as baselines, and at two hours post-injection for each of the two exposure

days and subsequently at the same time sequence for two additional days.

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Hematology and Chemistry

Baseline values included hematocrit, red cell count, reticulocyte count, white blood cell count with differential, and blood glucose. The hematology was determined using routine clinical laboratory procedures, and the blood glucose was determined using the glucose oxidase method (Worthington Biochemical Corp.). Eight ml of blood was drawn from each dog two hours post injection. Six ml of the sample was heparinized, stored in ice, and centrifuged within twenty minutes for clinical laboratory determinations and freeze-cleaving. The remaining two ml of unheparinized blood was used to determine the methemoglobin and reduced glutathione (GSH) levels. Methemoglobin was determined by a modification of the Evelyn and Malloy method as described by Hainline.[19] The GSH level was measured by the Alloxan 305 method[20] and results reported as μg GSH/10^9 red cells.

Light Microscopy

Wet preparations of exposed and control cells were stained with 1% crystal violet to determine Heinz body formation. The crystal violet solution was prepared using a saturated solution of crystal violet 2-b stain[18] diluted 1 to 4 with 0.9% sodium chloride. Two drops of cells were mixed with four drops of stain and were incubated at 37°C for 15 minutes. A complete necropsy with routine histopathology was performed on all dogs.

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Electron Microscopy

Blood samples for freeze-etching were prepared by centrifuging the heparinized blood at 4°C for 10 minutes at 2000rpm. The buffy coat was removed and the red cells were washed three times in 0.9% sodium chloride. The cells were suspended in 10, 15, and 20% glycerol for 15 minutes each, and for thirty minutes in 30% glycerol. The glycerol solutions (pH 7.4) were made with isotonic phosphate buffered saline. The red cells were pelleted by centrifuging at 2000rpm for 10 minutes. Samples of the pellets were frozen in Freon 22 at -150°C and then stored in liquid nitrogen until ready for use. The specimens were fractured in vacuo (10^{-6} Torr) at -115°C by two to three strikes of the microtome blade in a Balzer's 360M Freeze-Etch Machine (Balzer's High Vacuum Co., Santa Ana, California). Replicas of the fractured faces were made by platinum-carbon shadowing according to a technique by Moore.[6] The replica was cleaned with Clorox®, washed with distilled water, and mounted on Formvar-coated 300 mesh copper grids. The replicas were examined and photographed in a JEM 100-B electron microscope. (JEOL, USA, Medford, Mass.)

RESULTS

Hematology and Chemistry

The hematology results of the animals exposed to phenylhydrazine showed a significant reduction in the hematocrit (Table I), hemoglobin (Table II), red cell counts (Table III), and high methemoglobin levels

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TABLE I

EFFECTS OF PHENYLHYDRAZINE
ON HEMATOCRIT (VOLUME %)

DOSE

	CONTROL	20mg/kg	30mg/kg	40mg/kg
6 JUNE 73	44	46	41	42
7 JUNE 73	42	45	41	44
8 JUNE 73	44	45	41	43
11 JUNE 73	41	47	42	44
12 JUNE 73*	40	45	40	41
13 JUNE 73*	42	33	41	X
14 JUNE 73	42	28	30	X
15 JUNE 73	40	27	24	X

X = dog died
* = Phenylhydrazine injected

TABLE II

EFFECTS OF PHENYLHYDRAZINE
ON HEMOGLOBIN (GRAMS %)

	CONTROL	DOSE 20mg/kg	30mg/kg	40mg/kg
6 JUNE 73	14.7	15.0	14.0	14.3
7 JUNE 73	14.3	16.3	14.3	15.0
8 JUNE 73	14.7	15.0	14.0	14.0
11 JUNE 73	14.3	16.3	14.3	15.7
12 JUNE 73*	14.0	16.3	14.0	15.0
13 JUNE 73*	14.3	11.1	14.3	X
14 JUNE 73	14.7	10.5	11.1	X
15 JUNE 73	14.0	8.7	8.5	X

X = dog died
* = Phenylhydrazine injected

TABLE III

EFFECTS OF PHENYLHYDRAZINE ON
RED BLOOD CELL COUNTS (MILLION CELLS)

| | DOSE | | | |
	CONTROL	20mg/kg	30mg/kg	40mg/kg
6 JUNE 73	6.09	6.59	6.23	6.07
7 JUNE 73	6.12	6.83	6.20	6.38
8 JUNE 73	5.84	6.32	5.91	6.12
11 JUNE 73	5.84	6.69	6.23	6.44
12 JUNE 73*	5.84	5.77	6.01	5.20
13 JUNE 73*	5.76	4.62	5.59	X
14 JUNE 73	5.72	4.02	4.20	X
15 JUNE 73	5.78	3.35	3.29	X

X = dog died
* = Phenylhydrazine injected

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(Table IV). The dog given 40mg/kg showed 46.9% methemoglobin level two

hours post injection on the first day and died 1 1/2 hours after the

second injection. The methemoglobin was as high as 85.5% two hours

after the last injection in one dog (20mg/kg) and 60.8% in the other

(30mg/kg). White blood cell counts, glucose and differentials were

within normal limits. Reticulocytes could not be counted because of the

presence of numerous Heinz bodies.

The MMH treated animals showed no significant change in hematocrit

(Table V) or reticulocyte count (Table VI), but at (1.5mg/kg) there

was a slight decrease in hemoglobin (Table VII). The biochemical data

did not indicate any change in GSH or production of methemoglobin (Table

VIII).

Light Microscopy

In each dog exposed to 20, 30, or 40mg/kg phenylhydrazine, 30-40%

of the red blood cells contained Heinz bodies at two hours post injection.

By 24 hours after the first exposure, Heinz bodies appeared in 95-100%

of the cells. The Heinz bodies first appeared as numerous small bodies

scattered throughout the cytoplasm; however, after 24 hours they were

fewer and larger.

By 24 hours after the second injection of 20, and 30mg/kg phenylhydrazine,

the dogs showed hematuria, and nearly 100% of their red cells contained

Heinz bodies. The dog given 40mg/kg could not be compared because of

death. In contrast, the dogs exposed to MMH at the levels used in this

experiment did not demonstrate formation of typical Heinz bodies.

TABLE IV

EFFECTS OF PHENYLHYDRAZINE
ON GSH AND METHEMOGLOBIN

				DOSE			
				CONTROL	20mg/kg	30mg/kg	40mg/kg
8	JUNE	73	GSH	53.01	51.38	44.76	45.77
"	"	"	MET	0	0	0	0
11	JUNE	73	GSH	49.93	45.64	38.86	38.54
"	"	"	MET	0	0	0	0
12	JUNE	73	GSH*	54.60	37.47	39.92	37.69
"	"	"	MET*	0	42.9%	0	46.7%
13	JUNE	73	GSH*	50.61	32.08	33.49	X
"	"	"	MET*	0	85.5%	60.8%	X
14	JUNE	73	GSH	55.47	37.46	36.18	X
"	"	"	MET	0	43.4%	35.9%	X

GSH = Glutathione (μg.GSH/10^9cells)
MET = Methemoglobin (%)
X = dog died
* = Phenylhydrazine injected

TABLE V

EFFECTS OF MMH ON
HEMATOCRIT (VOLUME %)

DOSE

	CONTROL	0.5mg/kg	1.0mg/kg	1.5mg/kg
22 Aug. 73	47	47	49	44
23 " "	45	48	46	42
24 " "	48	42	48	47
27 " "	45	46	45	42
28 " "*	45	45	45	41
29 " "*	49	46	44	41
30 " "	45	43	44	41
31 " "	44	43	41	42

* = MMH injected

TABLE VI

EFFECTS OF MMH ON
RETICULOCYTES (%)

DOSE

	CONTROL	0.5mg/kg	1.0mg/kg	1.5mg/kg
22 Aug. 73	1.4	0.6	1.2	1.0
23 " "	0.9	0.8	0.7	1.4
24 " "	1.1	1.5	0.8	1.4
27 " "	0.4	0.4	0.6	0.6
28 " "*	0.5	0.6	0.6	0.5
29 " "*	0.4	0.5	0.8	1.2
30 " "	0.5	0.4	0.9	0.7
31 " "	0.4	0.4	0.4	0.8

* = MMH INJECTED

TABLE VII

EFFECTS OF MMH ON
HEMOGLOBIN (GRAMS %)

	DOSE			
	CONTROL	0.5mg/kg	1.0mg/kg	1.5mg/kg
22 Aug. 73	16.8	16.8	17.3	15.0
23 " "	15.0	16.3	15.7	14.4
24 " "	16.3	15.7	16.3	15.7
27 " "	15.7	16.3	16.3	14.3
28 " "*	15.7	15.7	16.3	14.0
29 " "*	16.3	16.3	14.3	13.7
30 " "	15.0	14.7	14.7	12.9
31 " "	16.3	14.8	14.7	13.2

* = MMH injected

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TABLE VIII

EFFECTS OF MMH ON
GSH AND METHEMOGLOBIN

DOSE

		CONTROL	0.5mg/kg	1.0mg/kg	1.5mg/kg
27 Aug. 73	GSH	57.28	53.04	63.45	45.12
" " "	MET	0	0	0	0
28 AUG. 73	GSH*	56.64	59.18	66.78	53.66
" " "	MET*	0	0	0	0
29 AUG. 73	GSH*	56.98	58.08	#	62.35
" " "	MET*	0	0	#	0
30 AUG. 73	GSH	49.84	51.08	64.27	57.51
" " "	MET	0	0	0	0

GSH = Glutathione (μg.GSH/10^9cells)
MET = Methemoglobin (%)
# = Specimen contaminated
* = MMH injected

At necropsy the dogs exposed to phenylhydrazine showed a very dark brown color in all visceral organs, and severe congestion in the spleen, liver, and kidney. The spleens were 3 to 5 times normal size. Microscopically, the spleen, liver, and kidney each contained large amounts of blood pigments that did not stain with the Prussian blue iron stain. The Kupffer cells in the liver and the epithelium lining the convoluted tubules of the kidney were hypertrophied and filled with blood pigment (Fig. 1) which was interpreted as hemoglobin based on their appearance and a negative iron stain. Also, there was a striking reduction in spermatogenesis in these exposed animals, and sperm was absent in the epididymis.

Necropsy of the dog injected with 0.5mg/kg MMH showed no macroscopic or microscopic lesions referable to the exposure. At 1.0mg/kg of MMH, bile stasis was suspected in the liver, but blood pigment was not prominent in the Kupffer cells. The kidney showed fine brown granules (probably hemoglobin) in the cells lining the convoluted tubules (Fig. 2). At 1.5mg/kg of MMH, there was increased blood pigment in Kupffer cells and mild fatty changes in the liver. The kidney showed more of the fine brown granular pigment in the cells lining the convoluting tubules, and the spleen showed an increased number of pigment laden macrophages.

Electron Microscopy

The freeze-etch technique has been shown to reveal four different surfaces in pure suspensions of red blood cells.[5,10] During the fracture step, the fracture plane passes within the interior of the membrane,
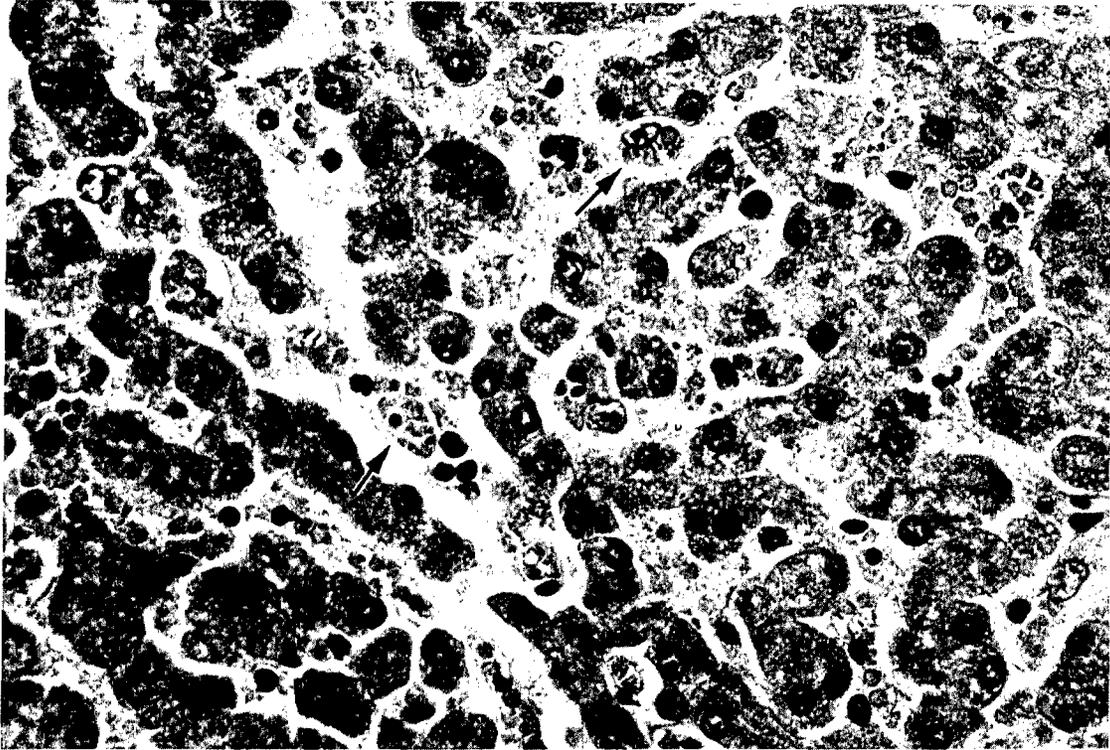
FIGURE 1. Photomicrograph of liver exposed to phenylhydra-
zine (30mg/kg) 48 hrs. post injection. Kupffer cells (arrow)
are enlarged and contain erythrocytes and blood pigment.
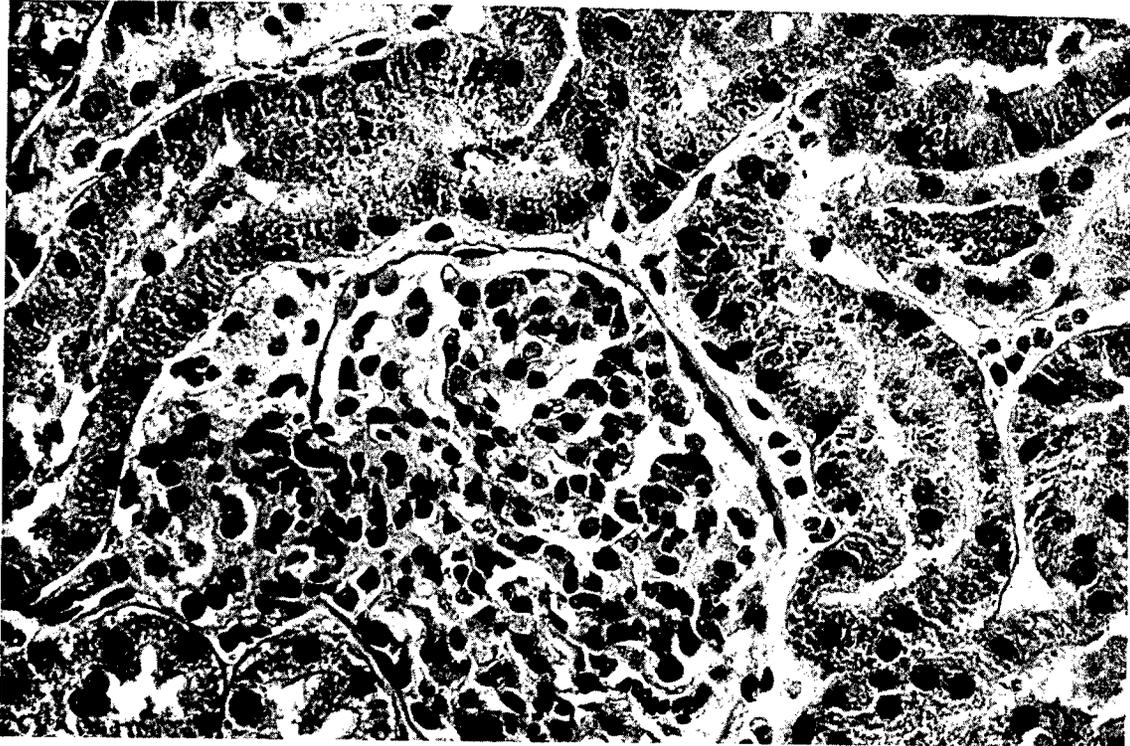Hematoxylin and eosin stain. x600.

FIGURE 2. Photomicrograph of kidney exposed to phenylhydra-
zine (30mg/kg) 48 hrs. post injection. Glomerulus surrounded
by convoluted tubules which contain fine brown granules
(probably hemoglobin) within epithelial lining cells. Hema-
toxylin and eosin stain. x600.

thus generating two distinct surfaces designated, by convention, as face A and B.[5] The other two surfaces are the fractured cytoplasm and the extracellular space. Face A is the exposed aspect of the inner half membrane and normally has many (60 to 120Å) particles randomly distributed over its surface. The cytoplasm has a granular appearance due to molecules of hemoglobin. Face B is the exposed aspect of the outer half membrane and has fewer particles distributed over its surface than face A. The extracellular space usually appears rather smooth in well washed preparations.

The majority of the cells exposed to phenylhydrazine had a normal distribution of particles on the A and B faces of the membranes. There were many small Heinz bodies in the cytoplasm and in some areas appeared to be clustering together to form larger aggregates. Crystalline arrays of granules were present in some of the cells which contained typical Heinz bodies (Fig. 3), and similar crystalline arrays were seen in some control cells. Some of the membranes were distorted and evaginated. Where a Heinz body was adjacent to the membrane surface, the membrane was pulled inward slightly or "dimpled" (Fig. 3 and 4).

The freeze cleaved cells exposed to MMH had A and B faces of the membranes that were relatively undistorted but had evaginations and dimples. The particles were randomly distributed over the A and B faces in a normal manner. Typical Heinz bodies were not seen. Instead, there was a crystalline organization to the hemoglobin into regular parallel rows of granules which in some instances were near evaginations of the membrane. These crystalline structures were numerous and varied in size, but were generally larger than Heinz bodies.
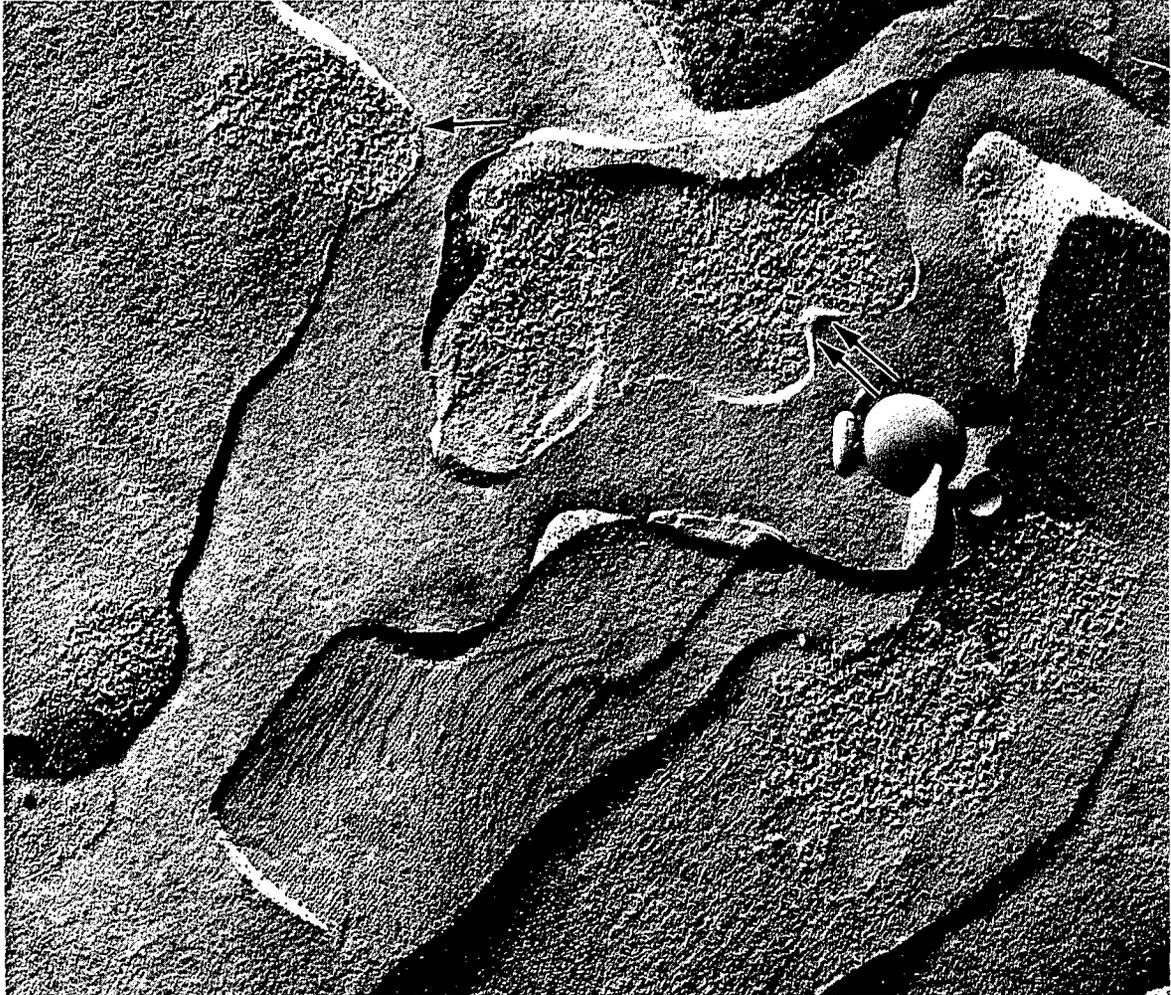
FIGURE 3. Freeze-etched replica of phenylhydrazine treated red cells (30mg/kg) 48 hrs. post injection, showing crystalline arrays of granules. The single arrow shows a distorted membrane evaginated by a Heinz body pushing outward. The double arrow shows a Heinz body adjacent to the membrane and pulling it inward. The direction of shadowing is from bottom to top. x72,000.
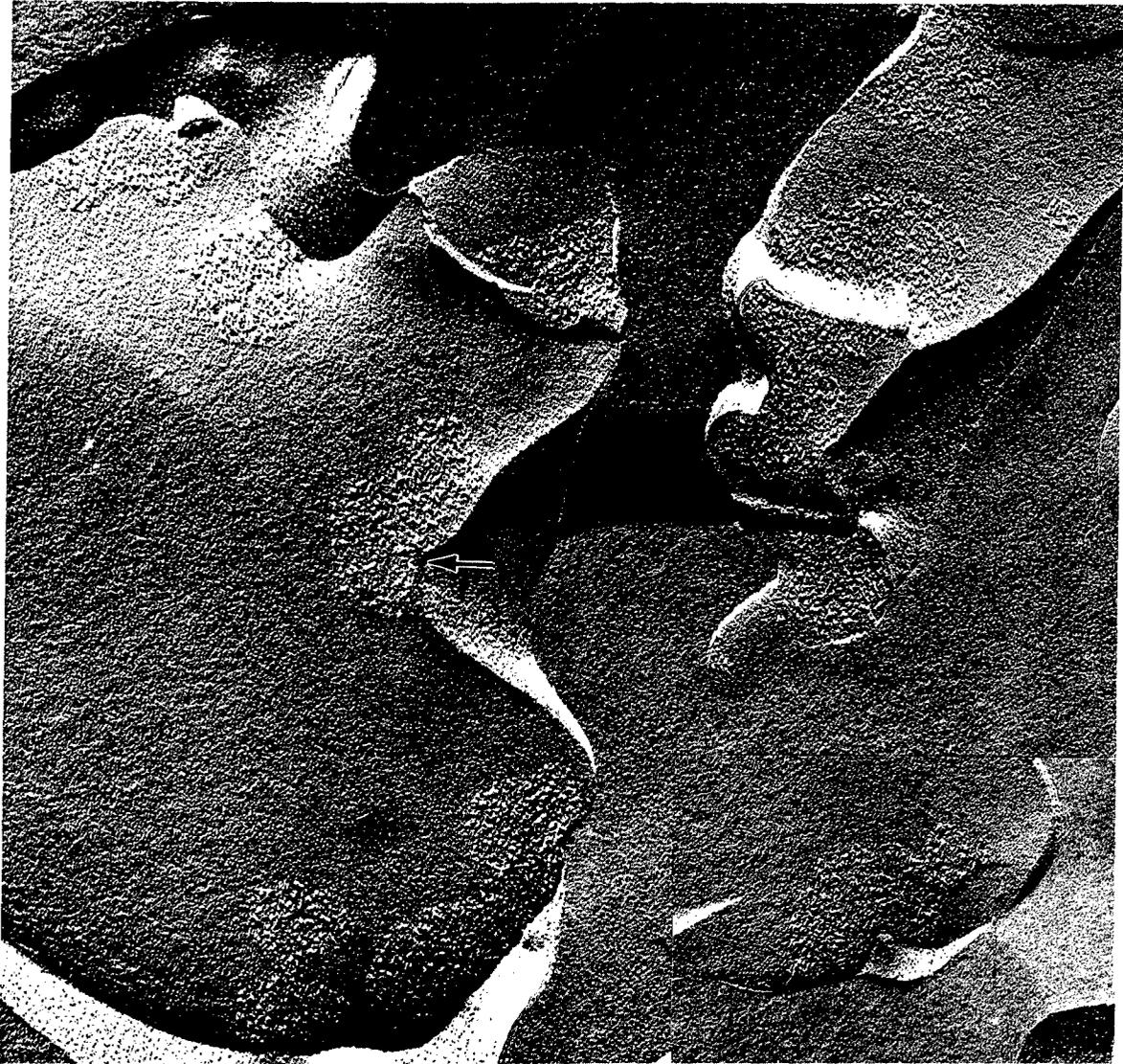
FIGURE 4. Freeze-etched replica of phenylhydrazine treated
red cells (20mg/kg) 48 hrs. post-injection. The arrow shows
a Heinz body invaginating the inner half membrane (Face A)
where they come in contact. x62,000. INSERT: The outer half
membrane is pulled in or "dimpled" by a Heinz body. The
direction of shadowing is from bottom to top. x60,000.

DISCUSSION

In the present phenylhydrazine study, hemolytic anemia was observed within the first 24 hours and Heinz bodies were present in 95-100% of the red cells. The dogs exposed to 20 and 40 mg/kg of phenylhydrazine showed moderate concentrations (42-46%) of methemoglobin the first day, and the 20mg/kg dog showed a methemoglobin level of 85% the second day. The 40mg/kg dog died 1 1/2 hours following the second injection. Twenty-four hours after the last injection, the methemoglobin level was reduced to 43% in the 20mg/kg dog (a 50% reduction), and to 35% in the 30mg/kg dog (a 45% reduction). The 30mg/kg dog showed no methemoglobin the first day but 65% the second day. An explanation for this is not known but may be related to an error in determination the first day.

It has been well documented that various reactive redox compounds produce similar hematological changes in red blood cells. Rifkind and Danon[9] injected rabbits with 15mg/kg of phenylhydrazine and observed a prompt hemolytic anemia and reticulocytosis, while Ham and coworkers[4] injected 10-15mg/kg of acetylphenylhydrazine in dogs and observed hemolytic anemia, reticulocytosis, and reduced GSH within 24 hours. MacEwen[14] found that inhalation exposure of dogs and monkeys to MMH at low concentrations also caused a hemolytic response.

Heinz body formation started in the central cytoplasm and after 48 hours was seen near the cell surface, with the cell membrane being evaginated and dimpled. At the highest concentration of phenylhydrazine (40mg/kg), the Heinz bodies were smaller and were for the most part restricted to

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the central cytoplasm. This dog expired at 25 1/2 hours post-injection (1 1/2 hours post-second-injection); therefore, Heinz bodies may not have had sufficient time to migrate to the cell membrane.

There was severe congestion in the spleen, liver, and kidney at each exposure but it was greater in severity at the highest concentration. Histopathological examination of all visceral organs showed evidence of hemolysis and biochemical determinations showed methemoglobin formation in the blood. This indicated that the hemolytic process and oxidative denaturation of hemoglobin had taken place very rapidly. Hemoglobin is oxidized to methemoglobin and the cell was unable to reverse the process at the exposure levels used. A methemoglobin intermediate, an insoluble brown "sulfhemoglobin-like" denatured pigment, appears which precipitates to form Heinz bodies.[3] Heinz body formation mechanism is reversible up to the point of precipitation of pigment from the hemoglobin. Because these erythrocytes have been altered by Heinz body formation they are rapidly removed by the spleen and liver. The abnormal cells are unable to pass through the splenic chords and liver sinusoids thereby causing congestion. As expected, the highest dose level induced the greatest response and caused the most congestion.

It is difficult to understand why the dogs injected with MMH showed no clinical signs of anemia and no indication of a notable change in GSH production or methemoglobin since other investigators[7,8,13,14] using this compound have shown hemolytic changes and Heinz body formation. For

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example, Haun[8] exposed dogs to MMH by continuous inhalation exposure and produced Heinz bodies and hemolytic anemia, and Weinstein and George[16] exposed human red cells to MMH $\underline{in}$ $\underline{vitro}$ and obtained Heinz bodies and decreased glutathione levels within two hours. Ham and coworkers[4] found similar results in an $\underline{in}$ $\underline{vivo}$ study with dogs using acetylphenylhydrazine.

The lack of response in the present study could be related to the low dose of MMH used. A level of 1.5mg/kg was chosen as maximum dose level to insure adequate time for Heinz body formation. This was because an unpublished experiment showed that dogs given 2.5mg/kg MMH daily for 2 days died before Heinz bodies could form (Back and George, verbal communication, August 1973). In addition, the lack of observed response to MMH may be related to reversibility of the response. Decreased GSH level and production of methemoglobin are reversible reactions because of strong protective mechanisms in the cell.[16] It is probable that methemoglobin was produced and GSH levels decreased, but were not detected at the time sequence used for sampling.

The aggregates of granules found arranged in rows within the cytoplasm gave the appearance of an ordered structure, possibly hemoglobin crystals (Fig. 5). Although these crystalline structures were more numerous and larger than typical Heinz bodies, they may be related to an early phase of Heinz body formation similar to that observed by Rifkind and Danon.[9] Their ultrastructural study of Heinz body formation used standard thin sections. They separated Heinz body formation into three stages, an initial, an intermediate, and an end stage. The initial stage was an ordered and possibly crystalline structure which they presumed to be an early phase of hemoglobin oxidation.
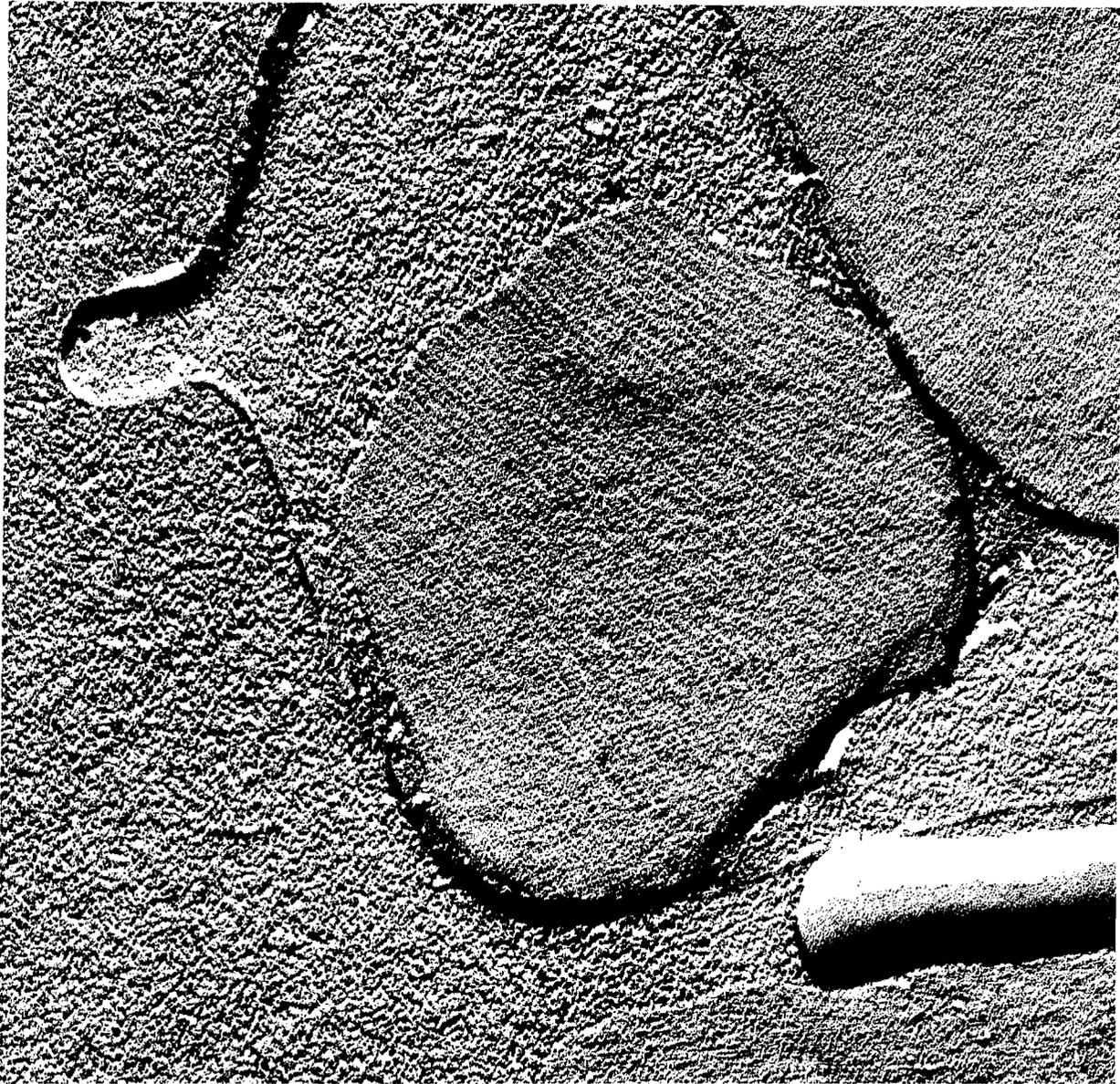
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FIGURE 5. Freeze-etched replica of monomethylhydrazine (MMH) treated red cells (1.5mg/kg) 48 hrs. post-injection showing the arrangement of granules that give the appearance of an ordered structure, possibly hemoglobin crystals. The direction of shadowing is from bottom to top. x120,000.

Histopathological sections of MMH exposed dogs showed no evidence of Heinz body formation and red cell destruction except at the highest dose. At 0.5mg/kg MMH there was only suspected bile stasis in the liver, and at 1.0mg/kg MMH there were a few fine granules in the cells lining the convoluted tubules. At 1.5mg/kg of MMH there were fatty changes in the liver and hemosiderosis in the spleen, liver, and kidney. These same changes were seen in MMH exposed dogs by Sopher and Robinson.[12] Since the two lower concentrations did not show significant changes, it was felt that the dose levels were at the low end of the response curve.[13,14] The response curve for MMH in dogs is very steep, and to obtain a non-convulsive dose level with a hematologic response would be difficult and would require further study.

In both phenylhydrazine and MMH exposed red blood cells, the distribution of intramembranous particles was not obviously altered in the majority of cells when compared to control preparations. However, the evaginations and dimpling of the membrane when Heinz bodies were adjacent to the membrane may represent a type of red cell damage that causes the red cell to be removed by splenic and hepatic phagocytes.[11] Red cells containing Heinz bodies are removed by sequestration in fixed macrophages. These are easily recognized by their engorgement with Heinz bodies and pigments derived from the breakdown of hemoglobin.

CONCLUSIONS

This study has shown that red blood cells in dogs exposed _in vivo_ to phenylhydrazine develop hemolytic anemia, decreased GSH, increased methemoglobin levels and develop Heinz bodies. The phenylhydrazine

induced red cell damage in rabbits by intramuscular injection previously shown by Rifkind with standard thin sections was verified by this study using dogs _via_ subcutaneous injections and the freeze-etch technique. It was apparent that the route of administration did not alter the effect of the phenylhydrazine.

The negative results with MMH exposure will require further investigation of the dose levels and routes of administration in order to obtain an effective non-lethal dose but still a hemolytic response. Also needed is an investigation into the kinetics of red cell turnover _in vivo_ in order to be adequately related to the previous _in vivo_[8,13,14] and _in vitro_[16] studies of MMH.

REFERENCES

1. Beavin, G. H., and J. C. White, "Oxidation of Phenylhydrazines in the Presence of Oxyhaemoglobin and the Origin of Heinz Bodies in Erythrocytes," Nature, 173:389, 1954.

2. Beutler, E., R. J. Dern, and A. S. Alving, "The Hemolytic Effect of Primaquine. VI. An in vitro Test for Sensitivity of Erythrocytes in Primaquine," J. Lab. Clin. Med., 45:40, 1955.

3. Jandl, J. H., L. K. Engle, and D. W. Allen, "Oxidative Hemolysis and Precipitation of Hemoglobin. I. Heinz Body Anemia as an Acceleration of Red Cell Aging," J. Clin. Invest., 39:1818, 1960.

4. Ham, T. H., J. A. Gravel, R. F. Dunn, J. R. Murphy, J. G. White, and R. W. Kellermeyer, "Physical Properties of Red Cells as Related to Effects in vivo. IV. Oxidant Drugs Producing Abnormal Intracellular Concentration of Hemoglobin (Eccentrocytes) with a Rigid-Red-Cell Hemolytic Syndrome," J. Lab. Clin. Med., 82:898, Dec. 1973.

5. McNutt, N. S., and R. S. Weinstein, "The Ultrastructure of the Nexus. A Correlated Thin-Section and Freeze-Cleave Study," J. Cell Biol., 47:666, 1970.

6. Moor, H., "Use of Freeze-Etching in the Study of Biological Ultrastructure," Int. Rev. Exp. Path., 5:179, 1966.

7. Leahy, H. F., "Monomethylhydrazine Effect on Blood, In Vitro," In: Proceedings of the 1st Annual Conference on Environmental Toxicology, 365, 1970, AMRL-TR-70-102, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio, December 1970.

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. Rifkind, R. A., and D. Danon, "Heinz Body Anemia: An Ultrastructural Study. I. Heinz Body Formation," Blood, 25:885, 1965.

10. Weinstein, R. S., A. W. Clowes, and N. S. McNutt, "Unique Cleavage Planes in Frozen Red Cell Membranes," Proc. Soc. Exp. Biol. Med., 134:1195, 1970.

11. Rifkind, R. A., "Heinz Body Anemia: An Ultrastructural Study. II. Red Cell Sequestration and Destruction," Blood, 26:433, 1965.

12. Sopher, R. L., and F. R. Robinson, "The Effects of Methylhydrazine by Inhalation or Injection in Dog's Kidneys," Proceedings of the 4th Annual Conference on Atmospheric Contamination in Confined Spaces, AMRL-TR-68-175, Wright-Patterson Air Force Base, Ohio, 1968.

13. Haun, Charles C., "The Acute Inhalation Toxicity of Monomethyl-hydrazine Vapor," Proceedings of the 4th Annual Conference on Atmospheric Contamination in Confined Spaces, AMRL-TR-68-175, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio, 1968.

14. MacEwen, J. D., "Chronic Exposure Studies with Monomethylhydrazine," Proceedings of the 2nd Annual Conference on Environmental Toxicology, AMRL-TR-71-255, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio, 1971.

15. Mills, G. C., and H. P. Randall, "Hemoglobin from Oxidative Breakdown in the Intact Erythrocyte," J. Biol. Chem., 232:589, 1958.

16. Weinstein, R. S., and M. George, "Interrelationship of Methemoglobin, Reduced Glutathione, and Heinz Bodies in Monomethylhydrazine-Induced Anemia. In Vitro Studies on Human Red Cells," In: Proceedings of the 3rd Annual Conference on Environmental Toxicology; page 281, AMRL-TR-72-130, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio, 1972.

17. McNutt, N. S., and G. A. Shoukimas, "Ultrastructure of Heinz Bodies in Human Erythrocytes as Visualized by the Freeze-Cleave Technique," AMRL-TR-72-129, Aerospace Medical Research Laboratory, Wright-Patterson AFB, Ohio 1972.

18. Todd-Sanford, Clinical Diagnosis by Laboratory Methods, Thirteenth Edition, W. B. Saunders Co., Philadelphia, 1962, London.

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

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

21. Jacob, H. S., M. C. Brain, and J. V. Dacie, "Altered Sulfhydryl Reactivity of Hemoglobins and Red Blood Cell Membranes in Congenital Heinz Body Hemolytic Anemias," J. Clin. Invest., 47:2664, 1968.

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