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# ULTRASTRUCTURE OF HEINZ BODIES IN HUMAN ERYTHROCYTES AS VISUALIZED BY THE FREEZE-CLEAVE TECHNIQUE

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13. ABSTRACT Heinz bodies were produced in red blood cells by exposing the cells to monomethyl- hydrazine (MMH) <u>in vitro</u> in the presence of oxygen. The freeze-cleave technique showed that these Heinz bodies were coarse aggregates and strands of polymerized, oxidatively denatured hemoglobin. After 2 hours incubation <u>in vitro</u> , Heinz bodies had not bound to the plasma membranes of the red cells. The freeze-cleave appear- ance of the Heinz body can be mimicked by hemoglobin precipitated with mercuric chloride. In addition, mercuric chloride caused binding of the denatured hemoglobin to the red cell membrane. Occasionally, the binding of hemoglobin with mercuric chloride caused a dramatic clustering of intramembranous particles. These results indicate that Heinz body binding to the plasma membrane is not an immediate conse- quence of exposure of red blood cells to hydrazines and that the freeze-cleave technique is useful for study of Heinz body ultrastructure.  Key Words: Hemoglobin Mercuric chloride Plasma membrane Freeze-fracture Monomethylhydrazine Freeze-etch technique			

## FOREWORD

This study was initiated by the Pathology Branch, Toxic Hazards Division of the Aerospace Medical Research Laboratory. The research was conducted by the Mixer Laboratory for Electron Microscopy, Massachusetts General Hospital, Boston, Massachusetts 02114, under Air Force Contract F33615-71-C-1578. Dr. N. Scott McNutt\* was the principal investigator for Massachusetts General Hospital. Mr. Shoukimas is presently associated with the Department of Anatomy, Boston University School of Medicine, Boston, Massachusetts 02111. Major Ronald S. Weinstein of the Pathology Branch, Aerospace Medical Research Laboratory, was the project engineer and monitor at the initiation of the research. Major Ernest E. McConnell, Chief of the Pathology Branch, was the technical monitor for the Aerospace Medical Research Laboratory. The research sponsored by this contract was started in May 1971 and was completed in November 1972.

Major Sheldon S. Diamond of the Pathology Branch, Aerospace Medical Research Laboratory, contributed invaluable technical comments to this effort.

This technical report has been reviewed and is approved.

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## INTRODUCTION

Heinz bodies are aggregates of oxidatively denatured hemoglobin that form in the red blood cell cytoplasm after exposure of red cells to a variety of compounds that have strong oxidation-reduction potential. The mechanism of Heinz body formation is thought to be mild oxidation of hemoglobin first to methemoglobin, then to sulfhemoglobin, and finally precipitation and polymerization of the oxidized derivatives (ref 1).

Standard thin section techniques for electron microscopy generally have been rather uninformative about the state of hemoglobin in the red blood cell. Normal hemoglobin stains darkly with the usual stains used in electron microscopy but the high concentration of hemoglobin in the normal red cell prevents the visualization of the individual hemoglobin molecules in standard thin sections. Heinz bodies stain slightly darker than normal red cell cytoplasm but thin sections do not reveal the arrangement of the altered hemoglobin molecules in the Heinz bodies (ref 26). In a thin section study of Heinz body formation, Rifkind and Danon (ref 26) found that Heinz bodies form in the central cytoplasm of the red cell and then rapidly bind to the plasma membrane. This binding causes severe deformation of the membrane, and may cause focal loss of the trilaminar appearance of the "unit" membrane (ref 26). Also Rifkind (ref 25) observed that the binding of Heinz bodies to the red cell membrane appeared to be important in the sequestration of the damaged erythrocytes in the spleen.

The freeze-cleave technique has produced a considerable amount of information on the structure of the mammalian erythrocyte (refs 23, 24, 29, 30, 31). In this technique, red blood cells are frozen and fractured or "cleaved." The topography revealed by cleaving is replicated in vacuo. The replica is cleaned and examined in an electron microscope (for a discussion of freeze-cleave technique see refs 5, 21, 30). Replicas of freeze-cleaved red cell cytoplasm demonstrate granules that are replicas of molecular hemoglobin (refs 10, 31). Hemoglobin molecules in the normal red cell appear closely

but randomly packed. When hemoglobin crystals are present in the red cell, the freeze-cleave technique demonstrates crystalline packing of the cytoplasmic granules, for example in sheep red cells (ref 33) and in human red cells from patients with homozygous hemoglobin C disease (refs 15, 17). Therefore the freeze cleave technique can be used to examine the distribution of hemoglobin molecules in the Heinz bodies within the red cell cytoplasm.

Freeze-cleaving also reveals unique information on red cell membrane structure. Branton (refs 2, 3) and others (refs 8, 22, 28, 34) have shown that frozen plasma membranes are cleaved along an interior plane, thereby splitting each membrane into two lamellae. One membrane lamella (Lm 1) remains attached to the frozen cytoplasm and the other lamella (Lm 2) remains attached to the frozen extracellular fluid. When the lamellae are cleaved apart, the two new surfaces that are generated are called "faces." The new face of Lm 1 has been called face A and the new face of Lm 2 has been called face B (ref 18). Face A and face B were apposed to each other prior to freeze-cleaving. On the membrane faces there are small particles that have been interpreted as protein penetrating into the interior of the membrane (refs 3, 4, 24, 27, 29). With the freeze-cleave technique, the distribution of these particles in the plane of the membrane can be studied. In addition, the freeze-cleave technique allows examination of cells that have not been chemically fixed, so that chemical fixation artifacts can be eliminated.

A standard laboratory method for producing Heinz bodies in vitro is to expose red cells to hydrazine compounds in the presence of oxygen (refs 1, 14). In this study, monomethylhydrazine (MMH) was used to produce typical Heinz bodies. The chemical reactions of MMH with hemoglobin are similar to those reported for other hydrazines (ref 16). Also MMH is an important liquid fuel that presents an occupational exposure hazard. Further morphological information can be obtained from a comparison of the effects of MMH

with those of mercuric chloride, which will also precipitate hemoglobin primarily by binding to sulfhydryl groups (ref 1).

#### MATERIALS AND METHODS

Four-ml whole blood samples were obtained from thirty-one human blood bank donors in blood groups A or O. Sodium citrate was added to the blood as an anticoagulant. The red blood cells were washed using low speed centrifugation in 0.9% sodium chloride. Half-milliliter aliquots of packed red blood cells were divided into three groups.

Group I aliquots were control specimens. They were diluted to 10-ml with 0.9% NaCl and incubated in covered 15-ml centrifuge tubes at 37°C for 2 hours. The cells were then centrifuged and resuspended in graded glycerol solutions that were 0.9% in sodium chloride. The cells were processed for freeze-cleaving either in 20% or 40% glycerol - 0.9% NaCl solution.

Group II aliquots were exposed to MMH. Each aliquot was added dropwise to 9.5 ml of MMH solution. MMH concentrations used were  $10^{-1}$  M,  $10^{-2}$  M,  $10^{-3}$  M,  $10^{-4}$  M,  $10^{-5}$  M, and  $10^{-6}$  M. All MMH solutions contained 0.85% sodium chloride. The MMH solutions had been neutralized by the addition of hydrochloric acid. The red cells were incubated in these solutions in covered 15-ml centrifuge tubes at 37°C for 2 hours. During incubation, the red cells were periodically resuspended by gentle swirling. At the end of the incubation period, an aliquot was stained with 1% crystal violet and examined by light microscopy for the presence of Heinz bodies. The remainder of the incubated red cells were glycerinated and washed free of MMH in graded glycerol solutions that were 0.9% in sodium chloride. The cells were processed for freeze-cleaving either in 20% or 40% glycerol-0.9% NaCl solution.

Group III aliquots were exposed to mercuric chloride by adding 0.5 ml aliquots of packed red cells dropwise to 9.5 ml of mercuric chloride

solutions. The solutions were  $10^{-3}$ ,  $10^{-4}$ , or  $10^{-5}$  M in mercuric chloride and 0.85% in sodium chloride. Incubation and glycerination conditions of Group III specimens were the same as for Group II specimens.

All specimens were freeze-cleaved in a Bullivant Type II freeze-cleave device which was modified to accept multiple specimens (ref 20). The freeze-cleave technique was used to prepare platinum-carbon replicas of frozen-fractured red blood cells as previously described in other papers (refs 5, 6, 32). The platinum carbon replicas were examined in a Siemens Elmiskop I electron microscope.

#### RESULTS

Examination of Group I (control) specimens and Group II cells exposed to  $10^{-6}$  M MMH showed the typical morphology of human red blood cells as visualized in freeze-cleave replicas. The replica of the cleavage plane through the frozen specimen had four distinct types of surfaces (fig 1). The fractured surface of the extracellular space generally appeared smooth. The fractured surface of the red cell cytoplasm contained many closely-packed granules, 50 to 150 Å in diameter. The size variation probably reflects different heights that hemoglobin molecules project above the fracture plane. The distribution of the granules appeared random, giving the cytoplasmic region a uniformly granular appearance. When the fracture plane had an acute incident angle to the red cell membrane, the membrane was cleaved into two lamellae, each with a distinctive face appearance called face A and face B.

Face A of the red cell membrane had many 60 to 120 Å particles distributed as a rather uniform network over its surface. Face B had fewer 60 to 120 Å particles which usually appeared as single particles scattered over the face. In addition, the background appearance of face B showed a fine texture that in high quality replicas could be resolved into many pits, small grooves, and ring-shaped depressions (ref 19). In occasional

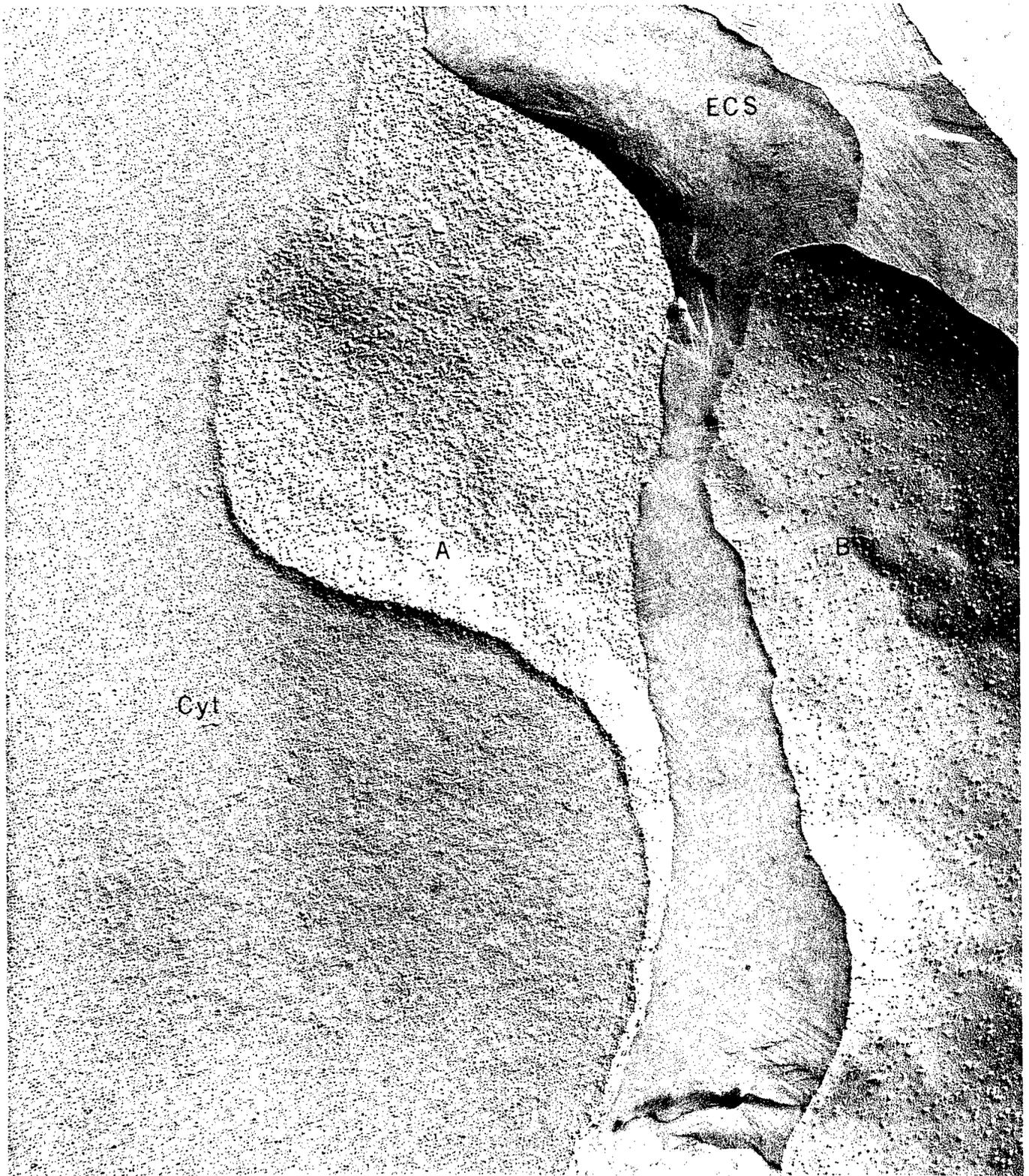


Figure 1. Replica of a Freeze-cleaved Red Cell Suspension from Group II.  
(See legend on p. 6.)

Figure 1. Replica of a freeze-cleaved red cell suspension from Group II, in which red cells are exposed to  $10^{-6}$  M MMH, without the formation of Heinz bodies. This replica reveals the four types of surfaces produced by the cleavage process. The fractured extracellular space (ECS) is smooth except for lines where the fracture sharply changes angle. The fractured red cell cytoplasm (CYT) displays many randomly but closely packed granules that probably represent replicas of molecular hemoglobin. The red cell membrane is split into two lamellae. One type of lamella remains attached to the cytoplasm and has a face A (at A) covered with many small particles. The other type of lamella remains attached to the extracellular matrix and has a face B (at B) with relatively few small particles. The distribution of these membrane particles reflects the distribution of certain types of membrane proteins in the plane of the membrane. (X59,500)

specimens, face B particles appeared as strands of fine filaments, 80 to 120 Å in diameter and of irregular length (ref 30).

Group II (MMH treated) cells contained typical Heinz bodies after exposure to  $10^{-1}$  M to  $10^{-5}$  M MMH. In whole mount preparations examined by light microscopy, the Heinz bodies stained dark purple with crystal violet and were generally from 0.2 to 1.0 micron in diameter. Variation in the MMH concentration caused variation in the size and number of Heinz bodies per cell. High MMH concentrations ( $10^{-1}$  to  $10^{-3}$  M) produced many small Heinz bodies in each cell, whereas low MMH concentrations ( $10^{-4}$  to  $10^{-5}$  M) produced a few large Heinz bodies. Heinz bodies were not observed after exposure of red cells to  $10^{-6}$  M MMH.

In freeze-cleave replicas, the Group II red cells had focal areas of increased cytoplasmic granularity that corresponded in size to the Heinz bodies visualized by light microscopy (fig 2). The freeze-cleaved Heinz bodies had a surface exhibiting distinctive clumps of granules and short strands, 100 to 250 Å in diameter (fig 3). Heinz bodies were located predominately in the central cytoplasm of the erythrocytes. Occasionally several small Heinz bodies appeared to be aggregated together as if in the process of fusion. There was no evidence of crystallinity in the Heinz body granules.

At concentrations of  $10^{-4}$  and  $10^{-3}$  M MMH, the red cells usually became spherical and the membranes of many cells were deformed by broad protrusions. On these protrusions, the membrane faces A and B usually appeared to have a similar particle distribution as the corresponding control membrane faces. The Heinz bodies usually were not directly applied to the cytoplasmic surface of the membrane, even at the broad protrusions. Only rare cells had Heinz bodies that appeared bound to the membrane, and then only after 2 hours incubation. These bound Heinz bodies did deform the membrane slightly.

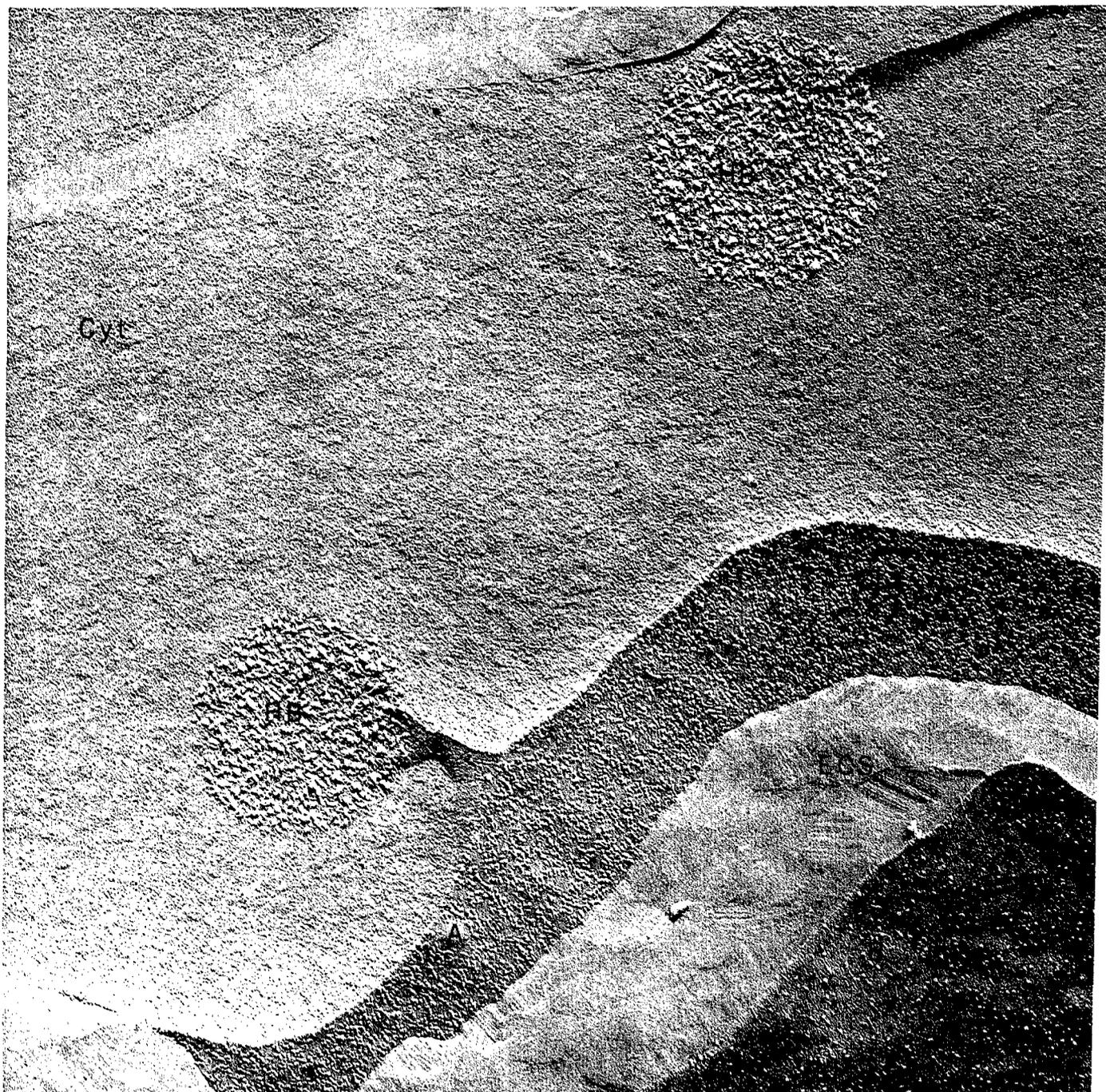


Figure 2. Replica of Freeze-cleaved Group II Red Cells Exposed to  $10^{-3}$  M MMH, with the Formation of Heinz Bodies. The four regions exposed by the fracture face are: extracellular space (ECS); cytoplasm (CYT); intramembranous face A (A); and intramembranous face B (B). The fracture through Heinz bodies (HB) reveal aggregates of large granules and strands of denatured, polymerized hemoglobin. (X63,400)

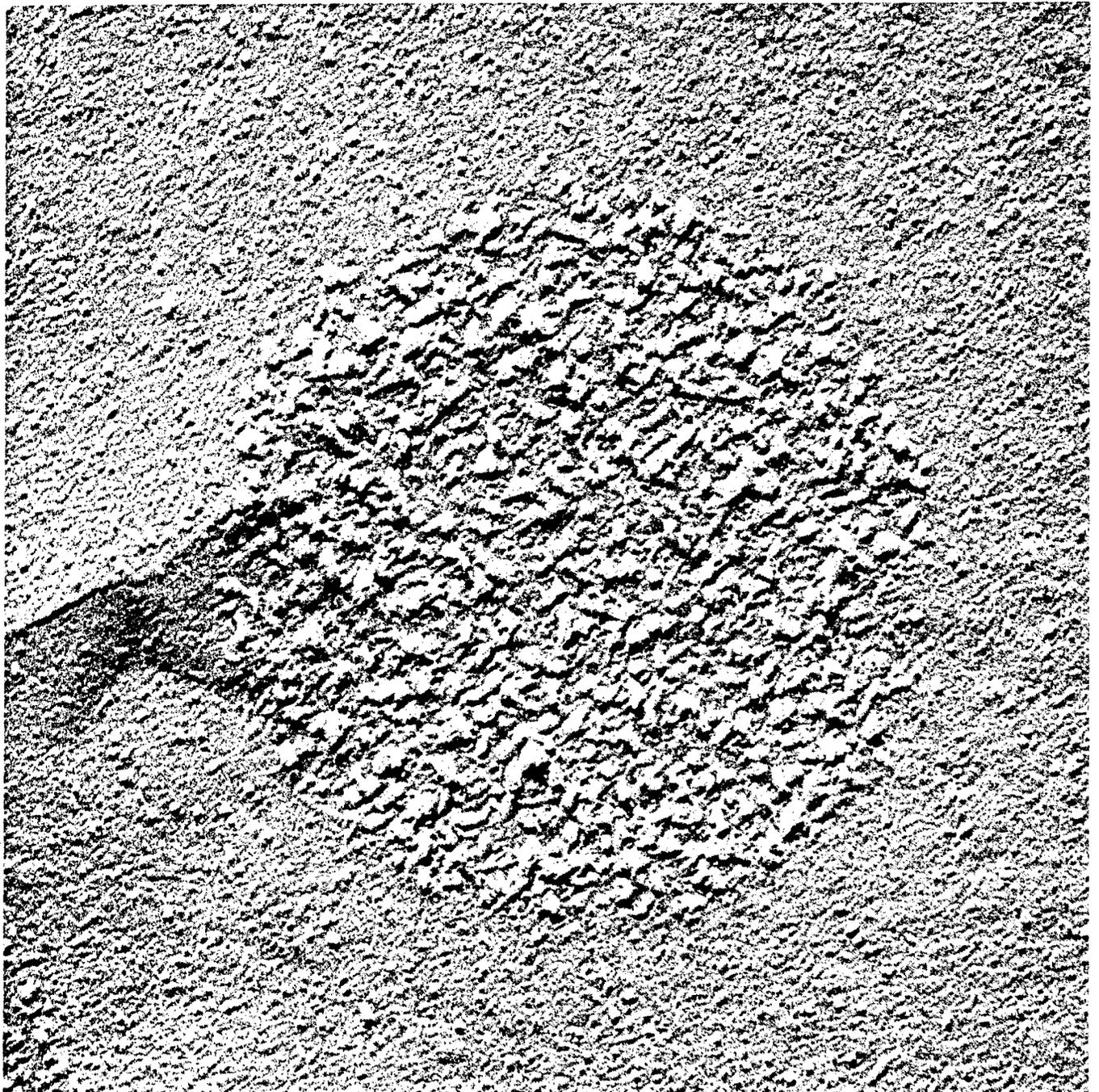


Figure 3. High Magnification of a Heinz Body from Figure 2. The Heinz body surface contains elongate granules, 150 to 250 Å in diameter, and short strands, 150 to 250 Å in diameter and of variable length. (X214,000)

Following exposure to high MMH concentrations, the red cell membranes were easily vesiculated during the washing and glycerination procedure. The cleaved faces of the vesicle membranes had an abnormal particle distribution in that the particles were frequently clumped and fewer in number than control membrane face A. Occasional preparations in Group II had fibrils attached to face B (fig 2), a feature also present in some of the control specimens.

Group III ( $\text{HgCl}_2$  treated) red cells were easily vesiculated by the exposure and subsequent preparation procedures. By light-microscopy, the red cell cytoplasm was frequently clumped into large aggregates of hemoglobin that did not stain as intensely with crystal violet as the Heinz bodies in Group II cells. In freeze-cleave replicas, the fracture plane revealed aggregates of hemoglobin that were indistinguishable morphologically from the Heinz bodies produced by MMH (figs 4, 5). The aggregates of hemoglobin had cleaved surfaces containing large granules and short strands, 100 to 250 Å in diameter (fig 5). The Group III cell membranes were usually vesiculated and most of the hemoglobin aggregates were extracellular. At high mercuric chloride concentrations (e.g.,  $10^{-3}$  M) The hemoglobin aggregates were adherent to the cytoplasmic surface of the membrane. In occasional cells, the binding of hemoglobin aggregates to the membrane appeared to be at regions of clustering of particles on membrane face A (fig 4).

#### DISCUSSION

This study demonstrates, for the first time, that the freeze-cleave technique can be used to visualize Heinz bodies in the cytoplasm of red blood cells so that the arrangement of the altered hemoglobin in the Heinz body can be studied. The substructure of the Heinz body indicates that the altered hemoglobin is coarsely aggregated. The presence of large granules and short strands of altered hemoglobin suggests that the Heinz body is a globular mass of randomly aggregated strands of polymerized, altered hemoglobin. The granules may simply represent cross-fractured strands. This substructure

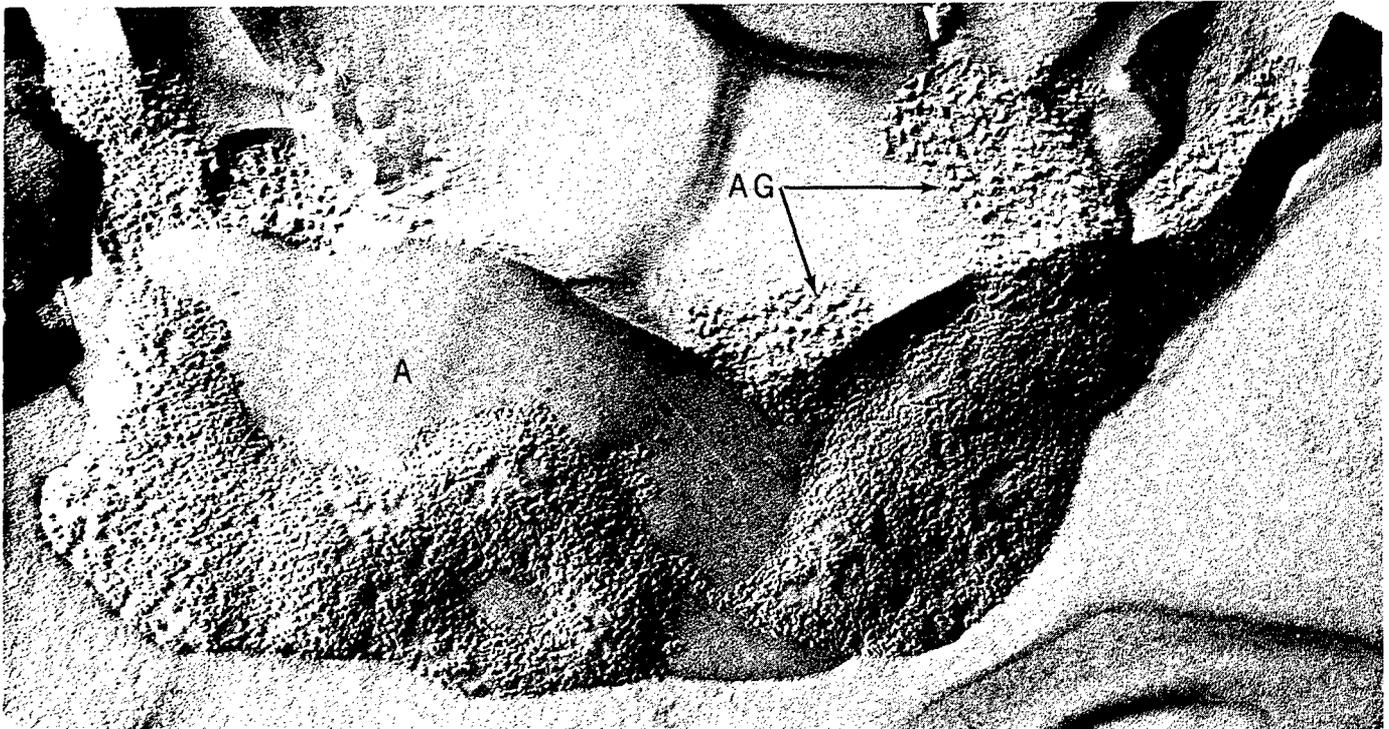


Figure 4. Replica of a Freeze-cleaved Group II Red Cell Vesicle Exposed to  $10^{-3}$  M Mercuric Chloride. The cytoplasmic region of the red cell has lost most of its granularity except at aggregates (AG) of hemoglobin. The hemoglobin aggregates are bound to the cell membrane. In this unusually dramatic example, the intramembranous particles on face A (at A) show an abnormal clustering of particles that correspond to areas adjacent to the aggregated hemoglobin. The extracellular space is smooth. (X71,700)

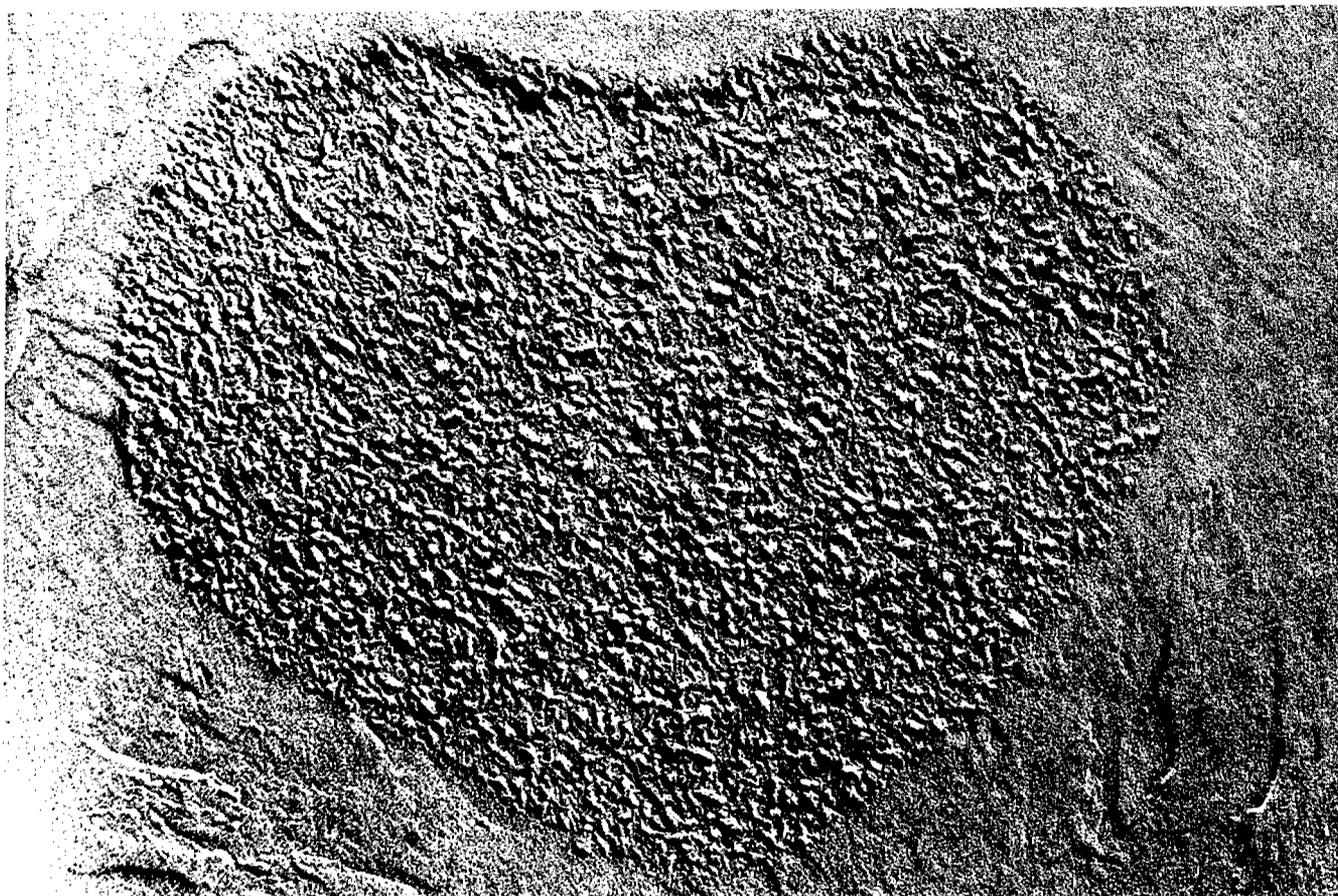


Figure 5. High Magnification of a Replica of an Aggregate of Hemoglobin precipitated with mercuric chloride. The aggregate contains many granules and strands of polymerized hemoglobin and bears a striking resemblance to the Heinz bodies formed in red cells exposed to MMH. (X110,000)

of the Heinz body is not specific for monomethylhydrazine exposure since a similar appearance is found in hemoglobin precipitated with mercuric chloride. Mercuric chloride precipitates and polymerizes hemoglobin predominately by strong binding of bivalent mercuric ions to sulfhydryl groups on globin proteins (ref 1). The measurement of the replicated granules and strands of polymerized hemoglobin cannot be interpreted as directly representing the actual size of the denatured hemoglobin molecules. Replicas enlarge the dimensions of projecting structures in proportion to replica thickness (ref 11). Also the freeze-cleave technique can plastically deform proteins during the cleaving process (ref 7) so that additional correction factors may be required to calculate molecular dimensions.

In Heinz body formation, the globin proteins are known to have an increased sulfhydryl reactivity (refs 1,12). The abnormal globin molecules are thought to attach the Heinz body to the red cell membrane by disulfide linkages (refs 13, 22). This altered sulfhydryl reactivity would suggest that the main linkages between Heinz body proteins may be disulfide bridges. However, Allen and Jandl (ref 1) found that phenylhydrazine-induced precipitation of hemoglobin from solution can be reversed by 8 M urea but not by mercaptoethanol. This observation led them to conclude that, in hemoglobin precipitation induced by hydrazines, strong hydrogen bonding is more important than disulfide bridges.

Our morphological study suggests that in Heinz bodies the proteins are bound to each other by linkages that are strong enough to resist the freeze-cleave process as indicated by the increased granularity and strands of proteins visible at the fracture face of the cytoplasm. The freeze-cleave appearance of the Heinz body can be mimicked by mercuric chloride precipitation of hemoglobin. The binding of mercuric ions to sulfur atoms has a bond strength that resembles covalent bonding. This morphological comparison suggests that the altered hemoglobin molecules in Heinz bodies are covalently bound to each other, such as by disulfide linkages, rather than by simple hydrogen bonding.

In this study, the Heinz bodies appear predominately in the central cytoplasm of the red cell and are not usually bound to the red cell membrane. Rifkind and Danon (ref 26) observed that Heinz bodies bind to the red cell membrane within 2 hours after exposure to phenylhydrazine. In their study, the Heinz bodies caused severe distortion of the cell membrane. Our freeze-cleave study did not confirm this observation. One explanation might be that phenylhydrazine and MMH react differently with the red cell and cause different degrees of Heinz body binding to the membrane.

Further studies will be necessary to examine the sources of differences between the freeze-cleave results in this study and the thin section results of Rifkind and Danon. It is not likely that the freeze-cleave protocol artifactually separates Heinz bodies from the plasma membrane, since strong binding of hemoglobin to the membrane can be demonstrated in the mercuric chloride experiments. An important conclusion from the freeze-cleave study is that rapid Heinz body binding to the plasma membrane is not an invariable result of exposure of red cells to hydrazines, specifically MMH.

In red cells exposed to low concentrations of MMH, the distribution and appearance of the small particles on cleaved membrane faces are essentially the same as in control red cells. Vesiculation and abnormal particle distributions are found only after treatment of red cells with high concentrations of MMH. In contrast, mercuric chloride treatment caused extensive vesiculation and alteration of membrane particle distribution. The aggregation of intramembranous particles in the presence of mercuric chloride can occasionally be extreme and suggests an intramembranous precipitation of the membrane proteins. Similar aggregation of intramembranous particles has been observed after proteolytic treatment of red cell ghost membranes (refs 4, 27, 29). The abnormal particle distribution observed at high concentrations of MMH is found primarily in vesicles rather than in the whole red cell membranes.

These results suggest that Heinz body formation per se is the predominant abnormality in the red cells exposed to low concentrations of MMH. Since the size of passages for red cells in the spleen has recently been estimated to be as small as 0.2 to 0.5 micron (ref 9) the presence of Heinz bodies in the cytoplasm of red cells may be sufficient to account for their sequestration in the spleen (c.f. ref 25).

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