

Quantitative Relationship Between Heinz Body Formation and Red Blood Cell Deformability

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The ultimate cause of destruction of red blood cells (RBCs) after oxidative damage with Heinz body formation is not well understood. We correlated the changes in RBC morphology and membrane protein composition after oxidant treatment with the alterations in deformability of whole cells and cell membranes. The incubation of RBCs with phenylhydrazine concentrations of 0.3 to 100 mg/dL at 37 °C for one hour led to a dose-dependent formation of Heinz bodies, ranging from isolated Heinz bodies at 1 mg/dL to a confluent coating of the inner membrane surface at 100 mg/dL phenylhydrazine. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed the presence of a large quantity of hemoglobin bound to the ghost membrane of treated RBCs. Electrophoresis with and without dithiothreitol indicated that disulfide bridges are abundant between hemoglobin molecules and are also present among membrane proteins but are not the major

bond between hemoglobin and membrane. Changes of spectrin, ankyrin, band 3, and band 6 and the appearance of a 260,000-dalton complex were also observed. With phenylhydrazine concentrations below 30 mg/dL, even in the presence of multiple Heinz bodies, the RBC deformability measured by filtration through 2.6-, 4.5-, and 6.8- μ m pores and the membrane deformability determined by a filter aspiration technique were not altered. With 100 mg/dL phenylhydrazine, when the entire membrane was coated with Heinz bodies, RBC filterability and membrane deformability were drastically reduced. These results indicate that oxidative damage of RBCs with discrete Heinz body formation causes focal membrane rigidification but does not affect the global cellular deformability until the Heinz bodies nearly cover the entire cell endoface.
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AN INTRACELLULAR PRECIPITATION of hemoglobin with the formation of Heinz bodies occurs in congenital hemolytic anemias associated with unstable hemoglobins¹ and in certain congenital red blood cell (RBC) enzyme defects, eg, in glucose-6-phosphate dehydrogenase deficiency.² Heinz body formation can also be produced by oxidative agents, such as phenylhydrazine.³ RBCs containing Heinz bodies are sequestered in the spleen, where the Heinz bodies are "pitted" or the entire cell is phagocytized by a macrophage.⁴ Although this is well established, the ultimate cause of RBC destruction in oxidant-induced hemolytic disease is not well understood.

It is generally assumed that RBCs with Heinz bodies are less deformable.^{5,6} RBC deformability is determined by the cell geometry, the viscoelastic properties of the cell membrane, and the internal fluid viscosity.⁷ Oxidative damage with Heinz body formation can influence all these factors. These alterations in cell or membrane deformability could cause RBC sequestration in the spleen and facilitate erythrophagocytosis. It has been proposed that, due to the presence of Heinz bodies, the RBCs have a decreased filterability.^{5,6,8} Other oxidative damages with possible influence on RBC deformability include the inhibition of the calcium-ATPase with concomitant intracellular accumulation of calcium,⁹ the

loss of potassium and water,¹⁰ the peroxidation of membrane phospholipids with a subsequent polymerization of both membrane lipid and protein components,¹¹⁻¹⁴ and the change in the organization of the phospholipids within the membrane.¹⁵

In the present investigation we have correlated changes in cell morphology and membrane protein composition induced by the oxidant phenylhydrazine with the deformability of the RBCs and the cell membrane, with the aim of elucidating the role of deformability in the removal of oxidant-injured RBCs.

MATERIALS AND METHODS

Preparation of cells. Blood was drawn from healthy volunteers into heparinized Vacutainer tubes. The RBCs were washed three times in a Ringer solution containing 145 mmol/L NaCl, 4 mmol/L KCl, 2.2 mmol/L CaCl₂, 5.5 mmol/L dextrose, and 0.5 g/dL bovine albumin, adjusted to pH 7.4 with 0.1 N Tris buffer. The washed RBCs were resuspended in the Ringer solution to a hematocrit of 10.0%. Aliquots of this suspension were centrifuged at 1,500 g for two minutes to separate the RBCs from the suspending medium. A calculated volume of the suspending medium was then replaced by an equal volume of a stock solution of 500 mg/dL phenylhydrazine hydrochloride (molecular weight [mol wt] 144.6). The samples were resuspended and incubated aerobically in a waterbath at 37 °C, with gentle mixing from time to time.

Morphology of cells. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) were used. The specimens were fixed in 1% glutaraldehyde in cacodylate buffer (pH 7.4, 4 °C) and postfixed in 2% OsO₄. For SEM, the specimens were dehydrated in ascending ethanol series, air-dried, coated with gold-palladium, and examined in a scanning electron microscope (JSM-25, Jeol Corp, Peabody, MA). For TEM, the specimens were embedded in Epon, and thin sections were prepared, stained with 2% uranyl acetate and lead citrate, and examined in a transmission electron microscope (Model EM 9, Carl Zeiss, Oberkochen, FRG).

The quantification of Heinz bodies was performed with standard morphometric techniques, as outlined by Weibel¹⁶ and applied to the study of Heinz bodies by Weinstein et al.⁵ The fractional volume of RBC cytoplasm occupied by Heinz bodies was obtained by point counting and expressed as volume percentage. The fraction of the

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cell membrane (expressed as percentage) in apposition with Heinz bodies was measured by intercept counting. The mean volume of a Heinz body was calculated using the method described by Weibel and Gomez¹⁷:

$$\bar{V}_{\text{HB}} = k \bar{S}^{3/2},$$

where k is a factor depending on the shape of the measured structure ($k = 1.382$ for spheres as assumed for Heinz bodies) and \bar{S} is the mean cross-sectional area of a Heinz body calculated from diameter measurements on random sections in any direction.

In order to estimate the relative hemoglobin concentration in single RBCs with Heinz bodies, normal RBCs and RBCs treated with phenylhydrazine were fixed separately, mixed together, and then processed for TEM as described. A video digitizing system (Eye Com II, Model 109 PT, Spatial Data System, Goleta, CA) was used to perform densitometric scanning of microphotographs along a line that crossed both treated and untreated RBCs.

Analysis of membrane proteins. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis¹⁸ was performed. RBCs washed in 0.01 mol/L phosphate buffer in 0.15 mol/L NaCl (pH 7.2) and resuspended in 0.02 mol/L phosphate buffer in 0.13 mol/L NaCl (with 5 mmol/L dextrose, 1 mmol/L adenine, and 10 mmol/L inosine, pH 7.4) were used. After treatment with phenylhydrazine (10 mg/dL for one hour or 100 mg/dL for two minutes), the RBCs were washed twice in ice-cold phosphate buffer, and RBC ghosts were prepared according to Bennett and Stenbuck.¹⁹ The ghosts were washed four times. Membrane protein concentrations were determined by the method of Lowry et al.²⁰ A standard solution of hemoglobin monomer and oligomers was prepared by incubating a hemoglobin solution with 0.02% glutaraldehyde for 30 minutes at room temperature. SDS-polyacrylamide gel electrophoresis of the RBC ghosts and hemoglobin solution was performed according to Fairbanks et al.¹⁸ in the presence or absence of dithiothreitol (DTT). The gels were stained with Coomassie blue. The bands were numbered according to Steck et al.²¹ Densitometric scanning was performed with the aid of the video digitizing system described.

Deformability of cells. For the determination of filterability, suspensions containing the same number of RBCs (hematocrit 10%) were incubated with phenylhydrazine at concentrations of 0.3, 1, 3,

10, 30, and 100 mg/dL, for one hour at 37 °C. The deformability was assessed by recording the filtration pressure with a pressure transducer (Model MP 45-14, Validyne Engineering Corp, Northridge, CA) during filtration of RBC suspensions under constant flow of 0.82 mL/min. The method has been described in detail elsewhere.²² Polycarbonate filters (Nuclepore Corp, Pleasanton, CA) with measured pore diameters of $2.6 \pm 0.2 \mu\text{m}$, $4.5 \pm 0.6 \mu\text{m}$, and $6.9 \pm 0.8 \mu\text{m}$, respectively, were used. The relative resistance in a pore containing a RBC to that in a pore with Ringer solution alone, β , was computed as²³:

$$\beta = 1 + [(P_i/P_o) - 1]V/h,$$

where P_i is the initial pressure reading, P_o is the pressure reading for the Ringer solution, V is the fraction of the pore volume occupied by RBC, and h is the fractional volume of RBCs in the suspension.

Membrane deformability. For the assessment of the deformability of RBC membrane, a modified version of a filter aspiration technique introduced by Brailsford et al.²⁴ was used. A Nuclepore filter with a pore diameter of $1.0 \mu\text{m}$ was mounted horizontally in a filter chamber. The chamber was connected to a reservoir bottle filled with saline. The RBC suspension (hematocrit 10%) was placed onto the wetted filter. By adjusting the relative height of the reservoir bottle to the fluid level above the filter, a total hydrostatic pressure of 5 mm H₂O was imposed on the RBCs across the filter, leading to the aspiration of a small part of the RBC into the filter pores. After 20 seconds, 1% glutaraldehyde was added and the RBCs fixed for ten minutes while maintaining the constant hydrostatic pressure. The filters were then carefully removed and fixed in 1% glutaraldehyde overnight. The specimens were dehydrated in increasing ethanol series and air-dried. A SEM specimen stub covered with a piece of double-sided Scotch tape was placed onto the Nuclepore filter (with the side containing the cells facing up). The filter was then gently stripped away from the Scotch tape, leaving the RBCs on the Scotch tape with the pressure-aspirated segments (tongues previously in the pores) protruding vertically.²⁵ The specimens were coated and viewed in the scanning electron microscope at an angle of 80 °. The length and the diameter of the protrusions were measured on the microscope screen at a magnification of 18,000 \times .

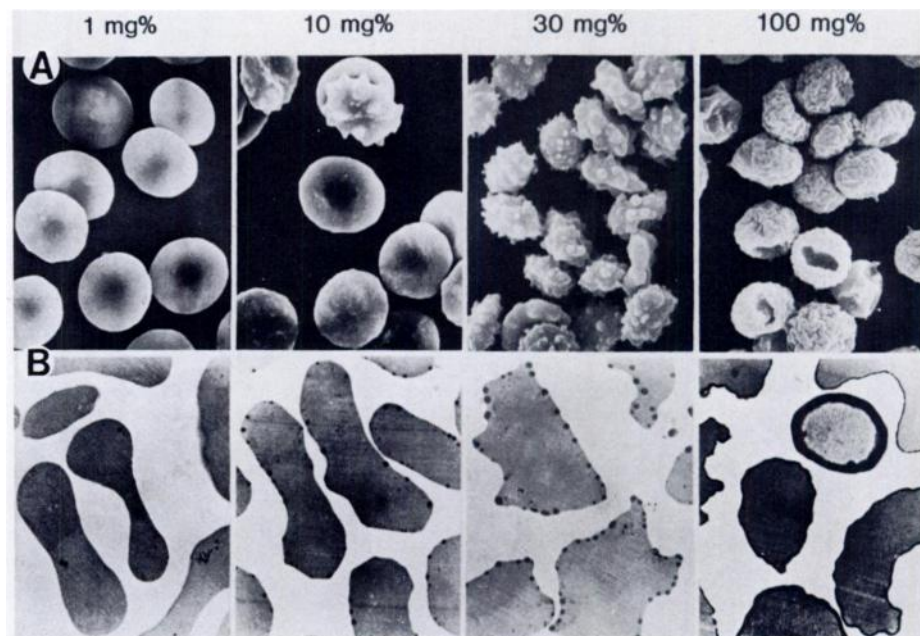


Fig 1. Scanning electron micrographs (A) and transmission electron micrographs (B) of red cells incubated for one hour at 37 °C with 1, 10, 30, or 100 mg/dL phenylhydrazine hydrochloride, as indicated.

Statistics. For statistical analysis, a one-way analysis of variance was used.

RESULTS

Morphology of cells. The incubation of RBCs with different concentrations of phenylhydrazine hydrochloride for one hour resulted in a dose-related formation of Heinz bodies in the cytoplasm (Fig 1). No Heinz bodies were detectable at a phenylhydrazine concentration of 0.3 mg/dL. 1 mg/dL induced Heinz bodies only occasionally, 10 and 30 mg/dL led to the formation of multiple Heinz bodies per cell, and 100 mg/dL generally resulted in a complete coating of the inner surface of the cell membrane with confluent Heinz bodies. The Heinz bodies were found to be in close apposition to the cell membrane. The portion of the cell surface containing the Heinz bodies showed an altered shape, ranging from a slight buckling to extended protrusions and severe membrane distortions, which gave the cell surface a bizarre appearance, like cerebral convolutions (Fig 1). At high magnifications (Fig 2) it can be seen that Heinz bodies were not confluent with the membrane, but that a small gap seemed to exist between the Heinz body and the membrane, which measured 10.7 ± 3.6 nm (mean \pm SD of 25 measurements). The onset of Heinz body formation was rapid; two minutes after the beginning of the incubation, small Heinz bodies were already visible along the endface of the membrane (not shown).

The morphometric data are given in Table 1. At phenylhydrazine concentrations >10 mg/dL, 100% of the cell sections showed Heinz bodies. The volume percentage of cytoplasm occupied by Heinz bodies gradually increased with increasing concentrations and reached 20% with 100 mg/dL phenylhydrazine. At this concentration, 97% of the cell membrane endface had Heinz bodies. The volume of the individual Heinz body was similar for 3, 10, and 30 mg/dL.

The cytoplasmic hemoglobin concentration of RBCs exposed to phenylhydrazine (100 mg/dL for one hour) was compared with that of untreated RBCs (Fig 3). Densitome-

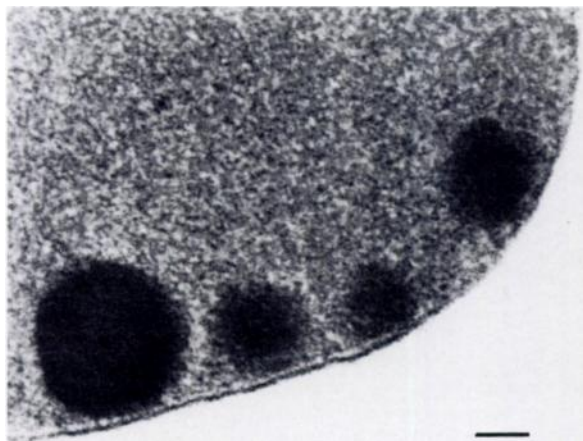


Fig 2. Transmission electron micrograph showing the attachment of Heinz bodies to the cell membrane. A small gap of about 10 nm between the Heinz bodies and the membrane can be seen. Bar = 100 nm.

try of TEM micrographs revealed that Heinz bodies had an increased density; the relative density of the remainder of the cytoplasm was 0.52 ± 0.05 and 0.34 ± 0.08 ($P < .001$) for untreated ($n = 43$) and treated ($n = 23$) RBCs, respectively. Lower concentrations of phenylhydrazine (30 mg/dL and lower) had no significant influence on cytoplasmic density.

Analysis of membrane proteins. The polyacrylamide gel electrophoresis on ghosts of RBCs treated with 10 mg/dL (one hour) and 100 mg/dL (two minutes) phenylhydrazine is shown in Fig 4; the densitometry of a gel from a similar experiment, in Fig 5. The incubation with 100 mg/dL phenylhydrazine was stopped after two minutes because it seemed to be more reasonable to study these early changes rather than the severe changes after one hour. Several alterations were found after phenylhydrazine treatment. (Hemo)globin was not found in controls, but was abundant in the ghosts of phenylhydrazine-treated cells, being visible by eye as a brown shade of the washed ghosts. The electrophoresis gel of the glutaraldehyde-treated hemoglobin solution (second well from right in Fig 4), which shows monomers, dimers (that band was split for an unknown reason), trimers, tetramers, and pentamers, is included as a reference. Electrophoresis of RBC ghosts with DTT showed a higher density of the hemoglobin monomer band ($\sim 14K$) than that without DTT. After phenylhydrazine treatment (10 and 100 mg/dL) the gels also exhibited peaks corresponding to Hb dimer (between bands 7 and 8), which was weaker in the presence of DTT. In the absence of DTT, the 100 mg/dL phenylhydrazine specimen showed a peak corresponding to Hb trimer (between bands 5 and 6) as well (Fig 5B). While the control gels showed the typical pattern of major membrane proteins, ghosts from RBCs treated with 100 mg/dL phenylhydrazine had some smearing between the protein bands on the gel. The smearing was more pronounced in the absence of DTT, which also led to reduced amounts of bands 1, 2, and 2.1. These findings indicate that S-S bonds may be involved, at least in part, in forming molecular complexes containing these bands. The higher density of band 6 after 10 mg/dL phenylhydrazine suggests that a newly formed complex comigrated with band 6. With 100 mg/dL phenylhydrazine, band 6 disappeared, suggesting that the comigrating complex and band 6 itself were bound to other complexes or that the high dose of phenylhydrazine dissociated the loosely bound band 6 from the membrane.²⁶ For both phenylhydrazine concentrations, a sharp band appeared at the lower boundary of band 3. Above band 1 a faint band of high mol wt (approximately 260,000 daltons) was detected (Fig 4).

Deformability of cells. The results of the RBC filtration experiments are shown in Fig 6. Up to a phenylhydrazine concentration of 10 mg/dL, the relative resistance of a RBC in a pore (β) was not different from control values for all three pore sizes. A concentration of 30 mg/dL resulted in an increase of β in 2.6- μ m pores (threefold), but not in 4.5- and 6.9- μ m pores. The concentration of 100 mg/dL had a dramatic effect on RBC filtration for all three pore sizes. The RBCs virtually could not pass 2.6- and 4.5- μ m pores, and β for 6.9- μ m pores was increased 20-fold.

Counting of the number of Heinz bodies per cell section on TEM pictures showed that it was unaffected by filtration

Table 1. Morphometric Data of Heinz Bodies (HB)*

	Phenylhydrazine Concentration (mg/dL)			
	3	10	30	100
Cell sections with HB (%)	22	97	100	100
Cytoplasm occupied by HB (%)	0.2 ± 0.2	1.8 ± 0.7	5.0 ± 1.4	19.8 ± 4.1
Membrane area adjacent to HB (%)	0.3 ± 0.3	7.8 ± 2.4	18.8 ± 3.2	97.3 ± 1.7
Mean cross-sectional diameter of HB (μm)	0.17 ± 0.02	0.18 ± 0.01	0.17 ± 0.01	Confluence
Mean volume of single HB (10 ⁻³ μm ³)	4.86	5.37	4.65	Confluence

*Values are mean ± SEM. The data for the first three lines were obtained from 12 to 18 cells, and the results for the last two lines were obtained from 50 Heinz bodies.

through 2.6-μm pore filters. Thus, for 10 mg/dL phenylhydrazine, the number was 4.2 ± 2.1 (mean ± SD) per cell section before and 4.2 ± 2.2 after filtration; for 30 mg/dL phenylhydrazine, 18.3 ± 5.9 before and 21.5 ± 7.8 after filtration.

Membrane deformability. The results of the membrane deformability study are shown in Fig 7. The length-radius ratio of the aspirated protrusions was used as a parameter for estimating membrane deformability. Phenylhydrazine concentrations up to 10 mg/dL did not affect the membrane deformability (Fig 7A through C); the length-radius ratio was 2.56 ± 0.38 , 2.65 ± 0.35 , and 2.74 ± 0.34 (mean ± SD, n = 40) for 0, 3, and 10 mg/dL phenylhydrazine, respec-

tively. With 30 mg/dL phenylhydrazine (Fig 7D), the length-radius ratio was significantly reduced (1.89 ± 0.30 , $P < .001$); with 100 mg/dL (Fig 7F), there was no detectable deformation (the small nodules were due to Heinz body attachment, as shown in Fig 1). The transmission electron micrograph for 30 mg/dL phenylhydrazine (Fig 7E) shows that the large number of Heinz bodies present may leave insufficient amount of intervening membrane area for deformational entry into the pore, and thus reduce the protrusion length.

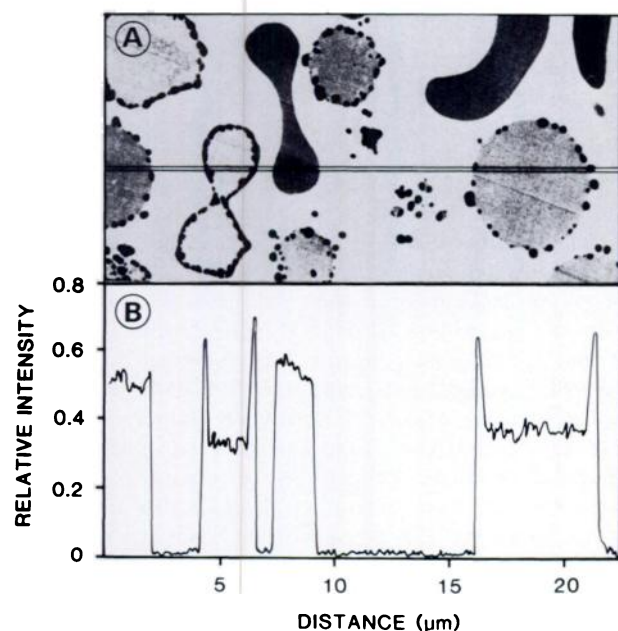


Fig 3. Estimation of the relative hemoglobin concentration in RBCs containing Heinz bodies. (A) Untreated and treated (100 mg/dL phenylhydrazine for one hour) cells were fixed separately and then mixed and embedded together. Densitometric scanning was performed on the transmission electron micrographs along a line that crossed both treated and untreated cells. (B) Tracing of the densitometry performed along a line indicated in the top graph. The relative density of the cytoplasm was measured: Heinz bodies have a higher density and the cytoplasm of these cells a lower density than do controls.

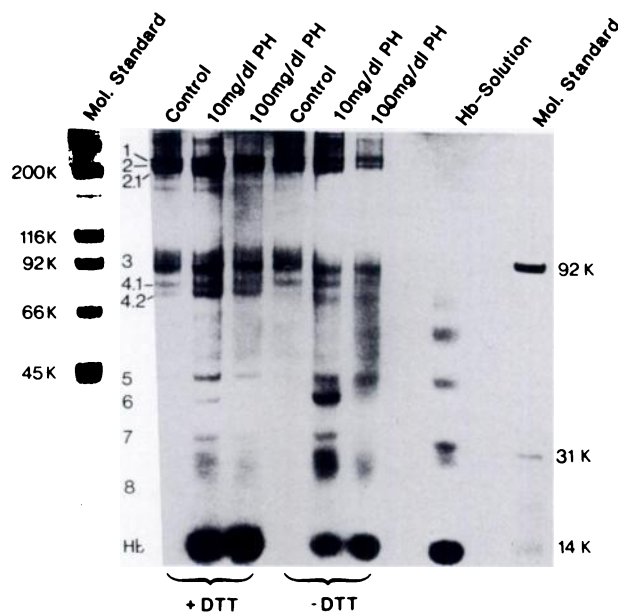


Fig 4. Polyacrylamide gel electrophoresis of ghost membrane proteins in the presence (+ DTT) or absence (- DTT) of dithiothreitol, an agent that reduces S-S bonds. The RBCs were treated with 10 mg/dL phenylhydrazine for one hour or 100 mg/dL phenylhydrazine for two minutes at 37 °C. A known volume (35 μL) of ghost membrane was dissolved in the solubilization solution (65 μL), and 40 μL of each solution was applied to each lane. Each lane contained 50 μg of ghost membrane protein. Protein bands are numbered according to Steck.²¹ High- (left margin) and low- (right margin) mol-wt standards are shown. A hemoglobin solution treated with 0.02% glutaraldehyde for 30 minutes was electrophoresed with DTT simultaneously, showing (from top to bottom) penta-, tetra-, tri-, di-, and mono-mers of (hemo)globin. The gel was stained with Coomassie blue.

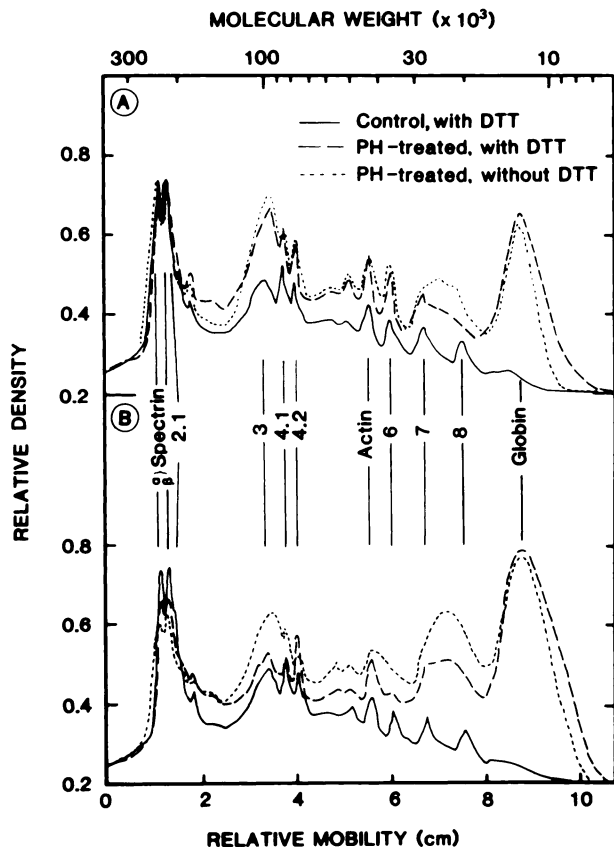


Fig 5. Densitometry of a polyacrylamide gel after membrane protein electrophoresis of untreated and phenylhydrazine-treated RBCs in the presence or absence of DTT. (A) Treated with 100 mg/dL phenylhydrazine for one hour at 37 °C. (B) Treated with 100 mg/dL phenylhydrazine for two minutes at 37 °C.

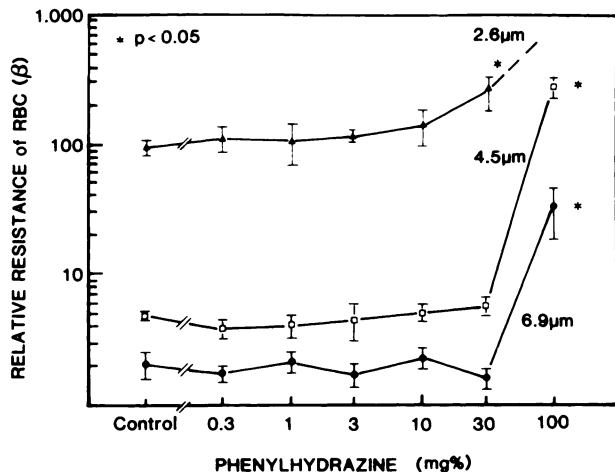


Fig 6. Log-log plot of the relative resistance for a red cell in a filter pore (β) against the concentration of phenylhydrazine for three pore sizes (2.6-, 4.5-, and 6.9- μ m). The data are means \pm SD of four experiments. Asterisks indicate $P < .05$.

DISCUSSION

The formation of Heinz bodies in intact RBCs induced by phenylhydrazine is dose- and time-dependent. Concentrations below 1 mg/dL did not induce any Heinz bodies, indicating that fresh RBCs with normal glutathione levels are capable of compensating for the oxidative effect of such phenylhydrazine concentrations for at least one hour. The other extreme was the complete coating of the cellular membrane with a continuous layer of Heinz bodies observed at a concentration of 100 mg/dL after one hour. The oxidative injury may vary with the redox agent used, and it would be interesting to obtain such dose-response curves for other oxidants.

With few exceptions, all Heinz bodies were found in proximity to the cell membrane. It has been claimed that Heinz bodies form in the cytoplasm and migrate to the membrane with time.²⁷ Our findings suggest that the formation takes place in close relation with the membrane. This may be the result of the vulnerability of the outermost layer of hemoglobin when the oxidative agent diffuses into the red cell or an early involvement of the membrane constituents, eg, phospholipids and membrane skeleton proteins.

We found a thin space of 10 nm between the Heinz bodies and the membrane on TEM at high magnification (Fig 2). Although we cannot exclude a fixation artifact, it could indicate that the denatured hemoglobin binds to components of the membrane skeleton that underlie the lipid bilayer, eg, spectrin. This hypothesis is supported by the observation of Ziparo et al,²⁸ who have shown that ferritin-conjugated antispectrin antibodies line up against the membrane, with the iron core of the ferritin approximately 10 nm apart from the apparent edge of the cell membrane.

How Heinz bodies are bound to membrane constituents has been a subject of debate for some time. Earlier investigations suggested that disulfide bonding may account for the attachment of unstable hemoglobin to the membrane²⁹; others have found that the membrane attachment of Heinz bodies was through hydrophobic bonding.^{30,31} Our results from polyacrylamide gel electrophoresis (Figs 4 and 5) showed that without DTT, ie, with intact S-S bridges, hemoglobin oligomers appeared in the phenylhydrazine-treated specimens at the expense of hemoglobin monomer, which supports the contention that S-S bridges are formed between (hemo)globin chains during oxidative hemoglobin denaturation (Heinz body formation) and are responsible for most oligomer formation.^{32,33} If Heinz bodies were associated with spectrin by S-S bonds, one would expect to see a high-mol-wt complex above spectrin on the gel in the absence of DTT. Electrophoresis of ghost membrane treated with 100 mg/dL phenylhydrazine without DTT, however, revealed little, if any, high-mol-wt complex larger than spectrin. This suggests that S-S bridges are probably not the major type of bonding between the denatured hemoglobin and spectrin. The smearing distribution of proteins between bands 3 and 5 may be the result of an association between the denatured hemoglobin with membrane proteins other than spectrin or an association among various membrane proteins. The prevention of this smearing with the addition of DTT indicates the involvement of S-S bonds in these associations.

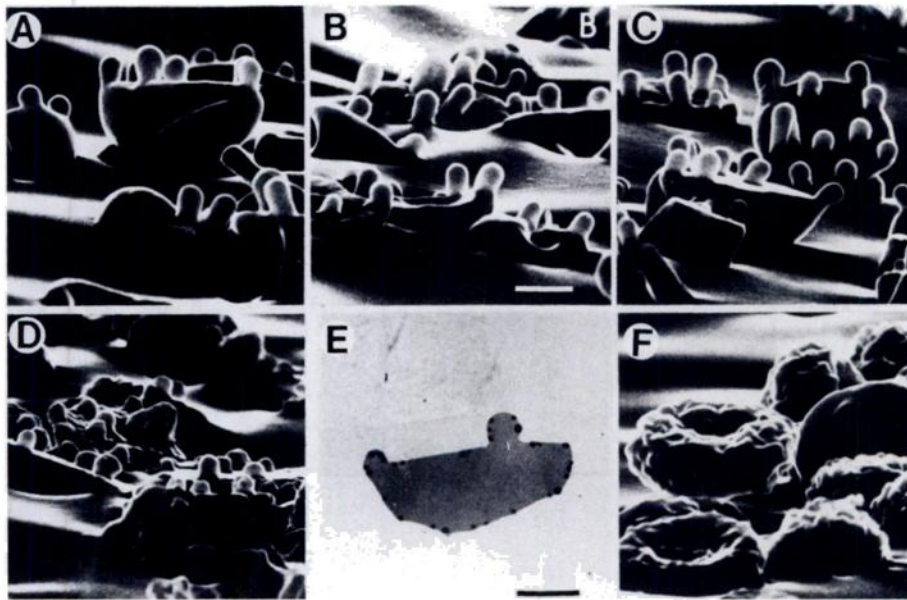


Fig 7. Assessment of the membrane deformability of RBCs with a filter aspiration technique.^{24,25} The scanning electron micrographs show the protrusions produced by a hydrostatic pressure of 5 mm H₂O for RBCs treated with (A) 0 mg/dL, (B) 3 mg/dL, (C) 10 mg/dL, (D) 30 mg/dL, and (F) 100 mg/dL phenylhydrazine. The length-radius ratio of the protrusions was, for (A), 2.56 ± 0.38 (mean \pm SD, $n = 40$); for (B), 2.85 ± 0.35 ; for (C) 2.74 ± 0.34 ; for (D) 1.89 ± 0.30 ($P < .001$); and for (F) zero. The transmission electron micrograph shown for 30 mg/dL phenylhydrazine (E) demonstrates that at this concentration (and above), the decrease in membrane area unoccupied by the large number of Heinz bodies can cause a reduced penetration length into the pore. The bars represent 2 μ m.

Of special interest are the minor complex with a mol wt of about 260,000 daltons, the sharp band within band 3, and the disappearance of band 6 with high phenylhydrazine concentrations. Palek et al³² found a 260,000-dalton complex in RBCs deficient in glucose-6-phosphate dehydrogenase that were treated with an oxidant (acetylphenylhydrazine). A 255,000-dalton complex formed *in vivo* in senescent RBCs has recently been described by Snyder et al,³⁴ who assumed a spectrin-hemoglobin crosslinking due to an oxidative damage occurring *in vivo*. It has been shown that denatured hemoglobin binds with high affinity to the cytoplasmic domain of band 3, where it competes for the binding with band 6,³⁵ and that band 3 becomes clustered in phenylhydrazine-treated RBCs.³⁶ Changes in the transmembraneous protein band 3 could play an important role in the recognition of damaged areas of RBCs by macrophages.

The present filtration study demonstrates that the presence of a limited number of small Heinz bodies in the RBC does not affect cell deformability. Apparently, the cytoplasmic viscosity and membrane viscoelasticity, two of the determinants of cell deformability,⁷ are not influenced by small quantities of denatured hemoglobin underlying the membrane endoface. The cytoplasmic viscosity is determined by the remainder of the hemoglobin in the cell interior; the cytoplasmic hemoglobin concentration is near the control level for 30 mg/dL phenylhydrazine, and actually lower for an incubation of one hour with 100 mg/dL, where no loss of cell water has yet occurred.¹⁰ When Heinz bodies are attached to the inner surface of the cell membrane, this area of the membrane becomes rigid, as can be seen by the small bumps of the membrane. Beside these rigid areas that

overlay the Heinz bodies, the membrane is presumably not altered in its flexibility, analogous to the canvas parts of a tent. Thus, at a phenylhydrazine concentration of 30 mg/dL, 20% of the membrane becomes rigidified, while the other 80% is essentially normal (Table 1). Such a cell is still capable of passing through narrow channels, eg, the 4.5- and 6.9- μ m pores; its passage through 2.6- μ m pores, however, is already impaired. A higher concentration of phenylhydrazine (100 mg/dL) results in the involvement of the entire cell membrane, which is now plastered with denatured hemoglobin (Fig 1). These cells are unable to traverse 2.6- and 4.5- μ m pores and barely pass through 6.9- μ m pores.

Three other filtration studies on RBCs containing Heinz bodies have been reported. In these studies RBCs were incubated at a single high concentration of monomethylhydrazine,⁵ acetylphenylhydrazine,⁶ and phenylhydrazine⁸ over a period of several hours, and RBC filterability was tested through 5- μ m pores^{5,6} and 8- μ m pores⁸ under a constant, high negative pressure. RBC filterability decreased progressively between two and five hours of incubation, but there was no significant change after a one-hour incubation. This latter finding is in agreement with our results with 4.5- and 6.9- μ m pores for phenylhydrazine concentrations ≤ 30 mg/dL. Weinstein et al⁵ performed a morphometric quantification of the Heinz bodies induced by 0.02 mol/L monomethylhydrazine. After six-hour incubation they found 5.2% of the cell membrane in contact with Heinz bodies and 5.2% of the cytoplasm occupied by Heinz bodies. These values are comparable to our results on RBCs incubated in 10 to 30 mg/dL phenylhydrazine for one hour. In contrast to our findings on RBC filtration, this small amount of Heinz bodies decreased

the RBC filterability in the study of Weinstein et al⁵ by a factor of >50. One possible explanation for the discrepancy between the two studies is that the six-hour incubation with a different oxidative compound damaged the remainder of the cell (membrane or cytoplasm) more profoundly than the number of Heinz bodies per se, eg, by the loss of potassium and cell water,¹⁰ or a slow disulfide exchange between the denatured hemoglobin and spectrin.⁸ A four-hour incubation of RBCs with acetylphenylhydrazine also resulted in decreased deformability as measured by ektacytometry.³⁷ Another possible explanation is the difference in filtration methodology. We used a constant flow and measured the initial rise of filtration pressure, whereas the previous authors used a constant pressure and measured the filtration time for a volume of 4 to 20 mL. Plugging of filter pores by cells is progressive with time³⁸; therefore, their results obtained from the filtration of such a volume probably reflect more the plugging effect of cells containing Heinz bodies. In our experiments, however, Heinz bodies up to 5% of the cytoplasm did not result in a higher rate of pore plugging than control, as calculated from the rate of pressure rise with time³⁸ over the period of measurement (not shown).

Cell filtration is commonly used to assess RBC deformability in a variety of disease states and its alterations induced by drug actions. Our results show that focal changes of the membrane or cytoplasm do not affect the global RBC filterability. This finding has significant implications in the interpretation of filterability tests in clinical investigations; thus, a normal filterability, especially through larger pores, does not rule out such focal abnormalities in RBCs.

Moderate degrees of oxidative damage and Heinz body formation with phenylhydrazine up to 10 mg/dL did not affect the membrane deformability as assessed by the filter aspiration method using 1- μ m pores. This indicates that the RBC membrane between Heinz bodies has normal elastic properties, although the portion of the membrane associated with Heinz bodies has become rigid. An increase in phenylhydrazine concentration to 30 mg/dL caused a significant decrease in membrane deformability. This may result from a reduced deformability of the RBC membrane between Heinz

bodies or the insufficient areas of such residual membrane between large numbers of Heinz bodies to effect a full deformational entry into the pore. The complete coating of the membrane endoface with precipitated hemoglobin seen after 100 mg/dL phenylhydrazine rigidified the entire membrane such that no membrane deformation occurred in response to the applied aspiration pressure of 5 mm H₂O. The results with a moderate Heinz body formation (<30 mg/dL phenylhydrazine), which are probably more relevant to pathophysiological conditions in vivo, indicate that the deformability of the RBC membrane between Heinz bodies remains unaffected.

Our results suggest that a limited number of small Heinz bodies per se do not affect cell deformability. It is therefore unlikely that in such diseases as glucose-6-phosphate dehydrogenase deficiency^{2,39} or unstable hemoglobins,^{1,33} the Heinz bodies cause the change in RBC deformability and the in vivo hemolysis. Red cells containing giant Heinz bodies, which are larger than the opening of the slit in the splenic sinus wall to be passed, will naturally be trapped and undergo erythrophagocytosis.⁴ This, however, is probably an uncommon situation in vivo. Red cells with smaller Heinz bodies are most likely not sequestered by their "filterability" characteristics in the spleen. It seems more likely that macrophages recognize the membrane areas with attached Heinz bodies by their focal rigidity or biochemical changes of the membrane, eg, changes in the integral membrane proteins band 3 and glycoporphins. Depending on the size of the involvement, this would result in either a focal pitting or an extended engulfment with subsequent destruction of the whole RBC. Thus, our studies suggest that an oxidative damage with a limited number of small Heinz bodies represents primarily a biochemical hazard for the RBC (eg, a loss of water and potassium¹⁰) and that a significant coverage of membrane endoface by Heinz bodies is needed for the RBC to encounter biophysical disturbances in the microcirculation.

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