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Research Article

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EFFECTS OF SULFHYDRYL INHIBITION ON RED BLOOD CELLS. I. MECHANISM OF HEMOLYSIS *

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A variety of hemolytic processes is associated with diminished sulfhydryl activity in the red cell. This was shown by Beutler, Dern, Flanagan and Alving (1) in Heinz body anemias and has subsequently been observed in favism (2, 3) and in certain nonspherocytic congenital hemolytic anemias (4–6). Susceptibility of red cells to hemolysis in these conditions involves a failure to maintain the sulfhydryl compound, glutathione (GSH), in the reduced state; however, it is unclear whether the level of GSH, per se, is critical to red cell survival *in vivo,* or is merely itself a secondary and nonspecific aspect of the hemolytic process.

Recent investigations have associated the aging of hemoglobin and its precipitation into Heinz bodies (7) with thiol metabolism (8). It was shown *in vitro* that such aging and denaturation of heme proteins involves the oxidation of globin, including its sulfhydryl groups, and that such oxidation is inhibited by reduced GSH (8, 9). Although an indication of cell damage, the oxidation and denaturation of hemoglobin may not be directly involved in the actual mechanism of red cell destruction, which presumably results from alterations in the cell membrane itself. That oxidant compounds may inflict damage on the cell membrane was suggested by the observations of Emerson, Ham and Castle (10), who observed a gradual increase of osmotic fragility in red cells exposed to phenylhydrazine and related substances *in vitro*. Recently it was found by Weed, Eber and Rothstein (11) that relatively small amounts of such oxidant compounds cause membrane injury with leakage of potassium and entry of sodium.

Pertinent separate evidence relating hemolysis *in vitro* to the destruction of red cell sulfhydryl groups has been presented by several investigators. The hemolysis of equine erythrocytes by exposure to oxygen and to iodine was believed by Fegler (12) to follow from the marked decrease in reduced GSH of these cells. Benesch and Benesch (13) noted that hemolysis occurred on incubation of red cells with various mercurial compounds. Metabolic inhibition and hemolysis by such oxidants as oxygen or iodine (12), by organic mercurials (13–15), and other sulfhydryl-reactive heavy metals (16) were presumed to be secondary to specific sulfhydryl blockade by these agents; however, the mechanism of this hemolytic effect of sulfhydryl inhibition and its relevance to *in vivo* cell destruction remain unclear. Accordingly, studies were made of the behavior of sulfhydryl-inhibited red cells *in vitro* and *in vivo*.

As will be described in a forthcoming report (17), red cells previously exposed to sublytic doses of sulfhydryl inhibitors *in vitro* are subject to specific patterns of sequestration upon their injection into human subjects. Studies were made correlating the biochemical and morphologic changes in the inhibited cells with their behavior upon reinjection into the circulation. The present report describes observations made *in vitro;* a later one (17) will present data obtained *in vivo*. The findings have been reported in preliminary form elsewhere (18).

MATERIALS AND METHODS

Two sulfhydryl inhibitors were selected for study. *p*-Mercuribenzoate (PMB),[1] often called *p*-chloromer-

* This investigation was supported in part by Grant RG3507 (C8) from the National Institutes of Health, Bethesda, Md.

[1] PMB was obtained as the sodium salt of *p*-hydroxymercuribenzoate from Sigma Chemical Company, St. Louis, Mo. This product has been found by the manufacturer to be free of inorganic Hg^{++} by the HgS precipitation method.

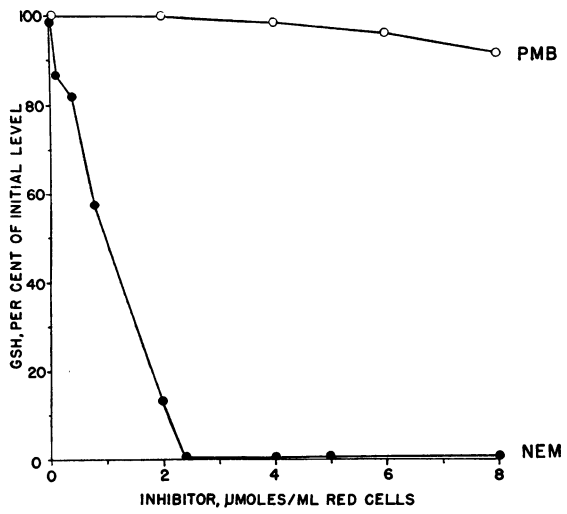

FIG. 1. EFFECTS OF VARIOUS QUANTITIES OF PMB AND NEM ON RED CELL GSH CONCENTRATIONS. The binding of red cell GSH by NEM (black circles) was found to be nearly stoichiometric. In contrast, PMB (open circles) produced negligible GSH inhibition in doses up to 4 μmoles per ml red blood cells, after which minimal depression occurred. The time of incubation was 1 hour.

curibenzoate (PCMB), reacts with sulfhydryl groups by forming reversible mercury mercaptides (19, 20). N-ethylmaleimide (NEM) [2] is an alkylating agent that forms stable addition products with sulfhydryl compounds (21–23). Both inhibitors react rather specifically with sulfhydryl groups in contradistinction to iodoacetate and the oxidizing agents (24). The inhibitors were freshly dissolved at appropriate concentrations in isotonic saline before their addition to the buffered cell suspensions.

Glutathione [2] was dissolved just prior to use in isotonic (0.12 M) phosphate buffer, pH 7.4, containing 0.01 per cent (wt/vol) EDTA; this preparation showed a 5 to 10 per cent increase in reduced GSH after reduction at the mercury cathode (25).

Red cells from heparinized or defibrinated human blood were washed thrice in isotonic saline, and made up to a 50 per cent cell suspension in a phosphate buffer, pH 7.4, that contained 7 mEq per L K^+ and 150 mEq Na^+. In experiments involving measurements of cation movements across the red cell membrane, phosphate buffer containing 155 mEq per L Na^+ and no K^+ was used as the suspending medium. The appearance of treated cells was examined microscopically in wet preparations after resuspension in autologous plasma. Supravital staining with aqueous solutions of crystal violet, made isotonic with NaCl, was employed in examinations for inclusion bodies. In osmotic fragility studies (26) correction was made for the "tonicity" of red cells by the method of Emerson and associates (27). Per cent hemolysis values were obtained directly from the ratio of the absorbance of the spun supernate of the sample in 1.0 per cent NaCl divided by that in H_2O; readings were made at 540 mμ using the Evelyn photoelectric colorimeter. Per cent swelling of the cells was determined by duplicate hematocrit determinations in Wintrobe tubes; no correction for trapped plasma was employed. Methemoglobin and so-called sulfhemoglobin were measured by the Evelyn-Malloy method (28). The Coombs test (29) and polyvinylpyrrolidone (PVP) test (30) were used to assess the agglutinability of red cells, and susceptibility to acid hemolysis was estimated (31).

Red cell glutathione levels were measured in the presence of NEM by the nitroprusside method of Grunert and Phillips (32) as modified by Beutler (33). However, it was found that sodium cyanide, used in the alkalinization step of this procedure, freed GSH from its reversible mercaptide linkage to PMB. Hence the "alloxan 305" method of Patterson and Lazarow (34) was used in experiments involving this organic mercurial. Glucose concentration was determined by the Somogyi-Nelson procedure (35), and lactate levels were measured as described by Barker and Summerson (36). The Patwin flame photometer was utilized for sodium and potassium determinations. Extracellular cation levels were determined on the supernatant fractions from centrifuged incubation suspensions; these supernatant solutions had negligible (< 1 per cent) hemolysis. The Na^+ and K^+ levels of whole cell suspensions were measured in freeze-thawed preparations. In calculating intracellular cation concentrations the following formula was used [3]:

$$C^+_i = \frac{C^+_w - C^+_s (1 - Hct)}{Hct}$$

where C^+_i = intracellular concentration of cation (Na^+ or K^+) in milliequivalents per liter red blood cells, C^+_w = cation concentration in whole cell suspension in milliequivalents per liter, C^+_s = cation concentration in supernate in milliequivalents per liter, and Hct = hematocrit (not corrected for trapped plasma).

Procedures. Unless otherwise stated, the incubation mixture contained a washed 50 per cent red cell suspension in isotonic phosphate buffer, to which was added an equal volume of sulfhydryl inhibitor in saline. Glucose was added to provide a final concentration of 200 mg per 100 ml. The mixture was incubated in a Dubnoff shaker at 37° C and aliquots removed for study at various time intervals. The pH of the final incubated suspension never fell more than 0.2 pH unit from that of the initial pH (7.4).

[2] Obtained from Schwarz BioResearch, Inc., Mount Vernon, N. Y.

[3] It is realized that the calculation of intracellular cation concentrations derived by this formula must contain some inherent inaccuracy due to changes during the experiment in cell size and shape and thus in the volume of plasma trapped in the packed cell column of the hematocrit tube.

RESULTS

Sites of sulfhydryl blockade. The effects of NEM and PMB on red cell glutathione levels are shown in Figure 1. It is apparent that the uncharged alkylating agent, NEM, entered the cell freely and became bound to intracellular GSH in a nearly stoichiometric fashion, 2.5 μmoles of NEM completely blocking the 2 μmoles of GSH present per ml of red cells. This may be compared with the 2.3 μmoles of NEM found necessary to block 2 μmoles of GSH in aqueous solution under the same conditions. Reaction both in cells and in solution was rapid, being complete within 5 minutes. The charged molecule, PMB, on the other hand, caused little or no inhibition of intracellular GSH activity, although in free solution GSH was inhibited in a stoichiometric manner by this inhibitor. Only at doses of PMB that grossly affected cellular permeability (as evidenced by hemolysis) was there any appreciable lowering of GSH levels. The remote possibility that PMB might permeate the membrane but be preferentially bound by hemoglobin or other intracellular sulfhydryl groups, to the exclusion of GSH, is negated by the finding that GSH in destromatized hemolysates was readily blocked by this inhibitor. It is concluded that in the low doses used in these experiments PMB does not permeate the intact red cell membrane. As a corollary, effects of this inhibitor must be secondary to membrane, rather than to intracellular sulfhydryl blockade. These results are consistent with those recently reported by Tsen and Collier (37).

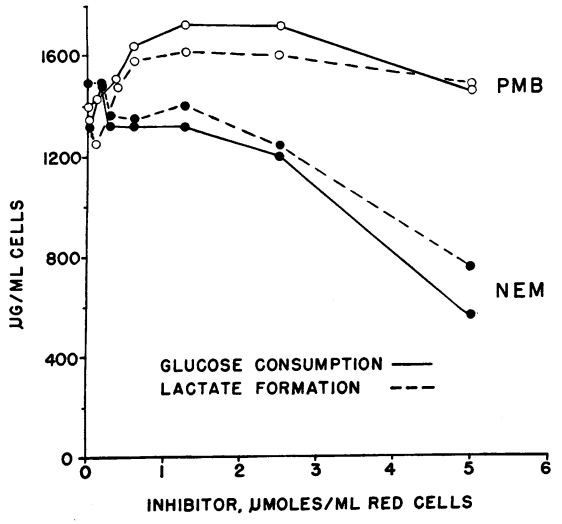

FIG. 2. EFFECTS OF VARIOUS QUANTITIES OF PMB AND NEM ON RED CELL GLYCOLYSIS. NEM (black circles) produced a depression of glucose consumption (solid lines) and lactate formation (broken lines) in concentrations in excess of 2.5 μmoles per ml red blood cells. PMB (open circles), in contrast, produced slight stimulation of glycolysis in low doses and no depression over the higher dose range studied. The duration of the experiment was 2 hours.

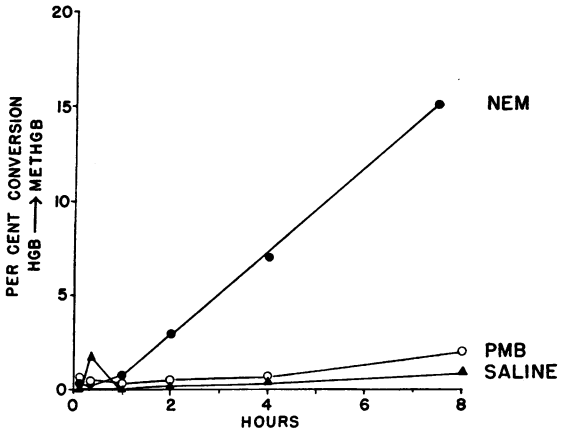

FIG. 3. EFFECTS OF NEM AND PMB ON THE FORMATION OF METHEMOGLOBIN IN RED CELLS. Prolonged incubation of red cells with NEM (black circles) caused marked methemoglobin accumulation, whereas incubation with an equally hemolytic concentration of PMB (open circles) or with saline (black triangles) resulted in negligible intracellular levels of methemoglobin.

Further evidence of the membrane locus of PMB action was derived from experiments demonstrating the prevention of PMB-induced hemolysis by GSH. Addition of excess GSH to red cells within 15 to 30 minutes of their exposure to PMB largely prevented hemolysis, as also noted by Sheets, Hamilton and DeGowin (14). In our hands this protection was found to occur without increase in the concentration of intracellular sulfhydryl activity, a finding that substantiates a previous suspicion (38) that the red cell is impermeable to this sulfhydryl compound. GSH also was found to inhibit the sphering and lysis of red cells exposed to NEM, when added within 15 minutes of this agent. This protective effect was manifest despite the fact that no intracellular sulfhydryl activity could be detected, suggesting that NEM also owes its lytic effect, at least partially, to membrane interaction.

Effects of sulfhydryl inhibitors on red cell metabolism. As might be expected from the differences in permeability of these two inhibitors, their effects on red cell glycolysis also differ. At doses of NEM sufficient to block the cellular GSH (2.5 μmoles or more of NEM per ml red cells), the ability of red cells to consume glucose and to produce lactate was impaired (Figure 2). PMB, in contrast, caused no inhibition of glycolysis over the dose range portrayed. Indeed, a moderate increase in metabolic activity was consistently observed at PMB doses of about 1 to 3 μmoles per ml red cells (Figure 2). Similarly, the capacity of cells to maintain hemoglobin in the reduced ferrous state differed in cells treated with PMB as compared to NEM (Figure 3). Methemoglobin gradually accumulated in red cells treated with NEM (20 μmoles per ml red cells) but not in cells exposed to PMB (5 μmoles per ml red cells),[4] a result consistent with the respective effects of these agents upon cell glycolysis (Figure 2).

Effects of sulfhydryl inhibitors on red cell morphology. Cells treated with PMB for short periods appeared slightly larger than normal, and contained a minor fraction of small crenated spherocytes. As incubation continued, sphering became more generalized until all the cells appeared to be perfect spheres. At this point hemolysis and ghost formation became prominent and small coccoid bodies with the staining characteristics of Heinz bodies became evident in the periphery of the cells, as described previously (7).

NEM, on the other hand, did not produce an early, minor population of crenated spheres, but, like PMB, did cause gradually increasing spherocytosis of the whole cell population, which resulted eventually in hemolysis and in the appearance of small Heinz bodies at the cell periphery.

Osmotic fragility studies. The incubation of washed buffered red cells in various concentrations of NEM produced dose-related changes in osmotic fragility. Small, nonhemolyzing doses of

[4] PMB is approximately four times as hemolytic as NEM; these doses were selected because they induced similar rates of hemolysis.

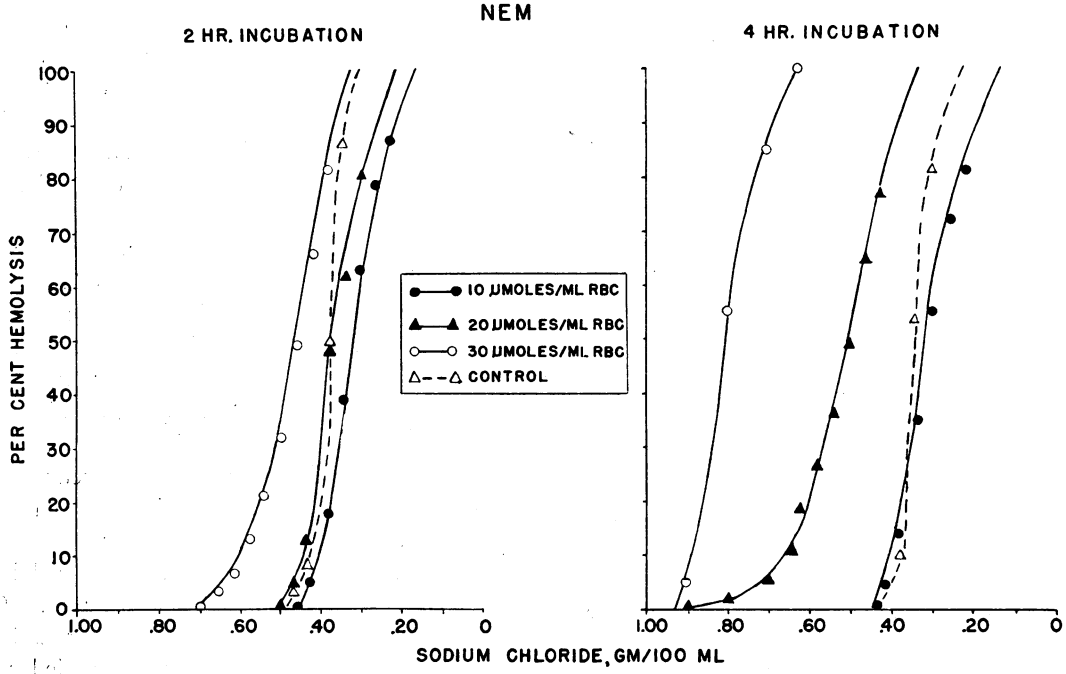

FIG. 4. EFFECTS OF VARIOUS QUANTITIES OF NEM ON THE OSMOTIC FRAGILITY OF RED CELLS. After 2 hours of incubation (left), red cells treated with small doses (black circles) and intermediate doses (black triangles) of NEM showed decreased osmotic fragility. Relatively large doses (open circles) caused a general increase in fragility. After more prolonged incubation (right) the intermediate dose of the inhibitor caused sphering and increased fragility of the red cells, while higher doses of inhibitor produced an extreme increase in fragility involving autohemolysis in isotonic saline.

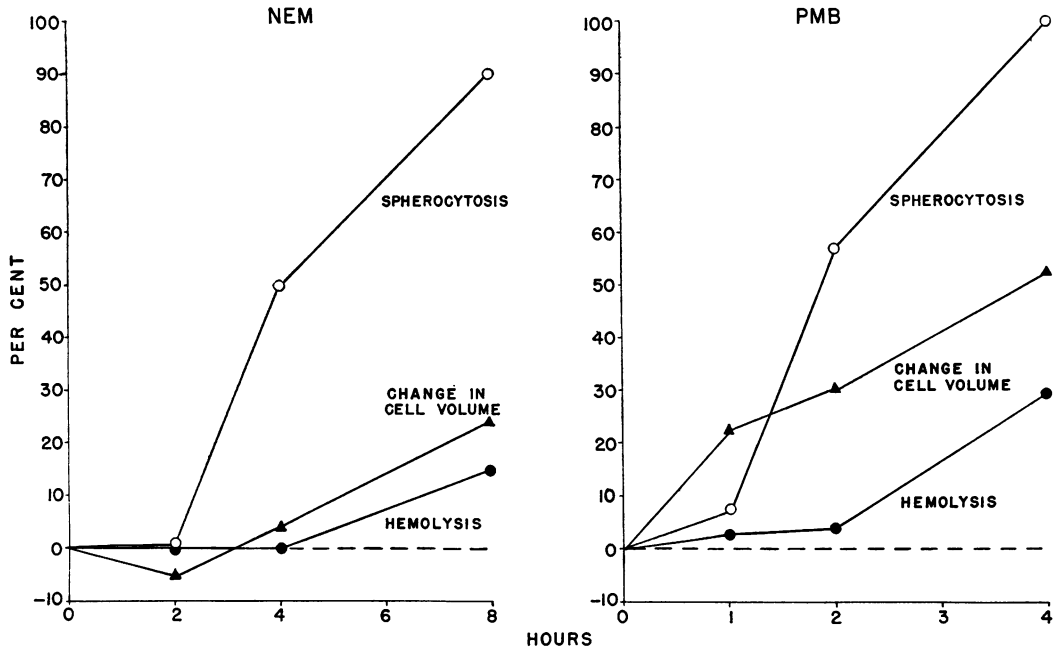

FIG. 5. SEQUENTIAL CHANGES IN RED CELL SIZE, SHAPE, AND INTEGRITY INDUCED BY NEM AND PMB. Red cells incubated with 20 μmoles NEM per ml red blood cells (left) underwent shrinkage followed in time by swelling (black triangles) and spherocytosis (open circles), measured by the per cent lysis in 0.50 per cent saline. Note that frank hemolysis (black circles) occurred only after these changes were well established. Similar relationships were obtained on incubation with PMB, 15 μmoles per ml red blood cells (right).

this inhibitor caused decreased red cell fragility associated with shrinkage of the cells (Figure 4). Larger doses produced increased osmotic fragility with swelling and spherocytosis of the treated cells. The change in osmotic fragility so produced was gradual in onset and increased with time until ultimately the cells hemolyzed in isotonic saline (right portion of Figure 4). An intermediate dose of NEM (20 μmoles per ml red cells) caused a time-related change in size and shape of the incubated cells, as also shown in the figure. Initially, this concentration of inhibitor caused cell shrinkage and decreased osmotic fragility. After 4 hours, a gradual sphering, swelling, and increase in osmotic fragility of the cells occurred (right portion of Figure 4); this process continued and led finally to moderate hemolysis in 8 hours. These relationships are summarized in the left portion of Figure 5 where changes in cell volume as measured by hematocrit determinations, hemolysis in 0.5 per cent NaCl (as an approximate measure of spherocytosis), and hemolysis in isotonic saline are portrayed as functions of incubation time. It is clear from this figure that swelling and spherocytosis precede frank hemolysis, leading to the hypothesis, further examined below, that hemolysis in these cells is caused by osmotic changes.

The effect of PMB on red cell osmotic fragility differed somewhat from that of NEM. As shown in Figure 6, small doses of PMB (5 μmoles per ml red cells) produced spherocytosis and hemolysis of a minor population of red cells (as demonstrated by the "tail" on the osmotic fragility curve), whereas the majority of cells maintained a normal osmotic character. Larger doses (15 μmoles per ml red cells), which produced gross hemolysis within 2 hours, caused a more general shift leftward of the fragility curve, indicating a more uniform population effect. As with NEM, these effects were progressive, and led ultimately to hemolysis of the cells in isotonic media. These gradual changes with time are illustrated in the right portion of Figure 5. Frank hemolysis with PMB (15 μmoles per ml red cells) occurred only after marked swelling and sphering of the red

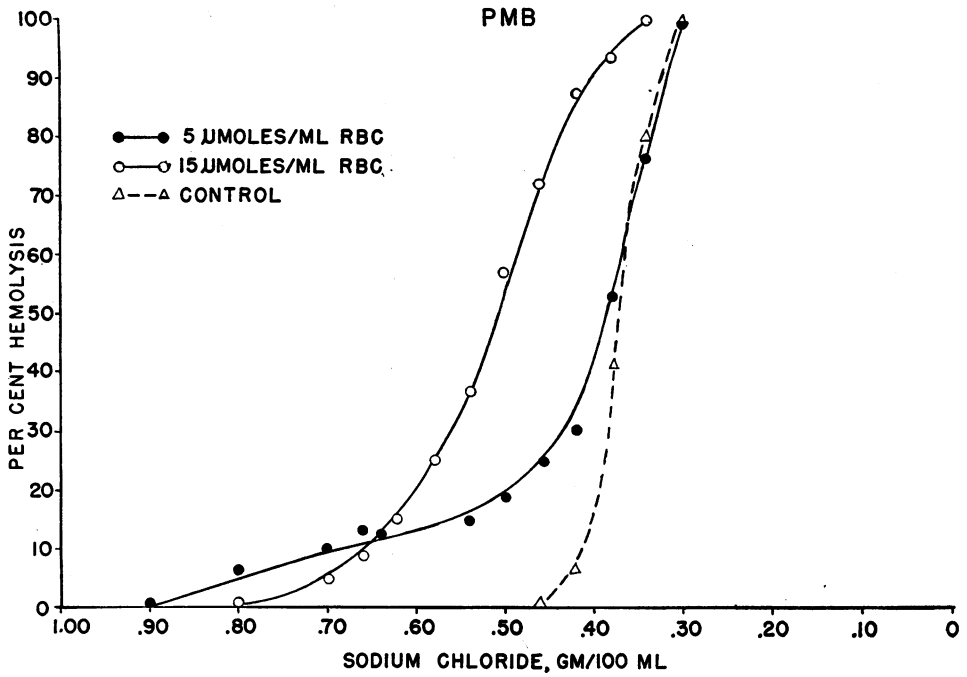

FIG. 6. EFFECTS OF VARIOUS QUANTITIES OF PMB ON THE OSMOTIC FRAGILITY OF RED CELLS. Small doses of PMB (black circles) produced spherocytosis of a hypersusceptible population of red cells represented by the "tail" on the osmotic fragility curve. Larger doses (open circles), sufficient to cause marked hemolysis in 2 hours of incubation, produced swelling and spherocytosis of the total population of red cells, demonstrated by the more symmetrical shift to the left of the entire fragility curve.

cells, a result comparable with and even more marked than that obtained with NEM (left portion of Figure 5). Again, such results suggest that hemolysis occurs by osmotic changes in these cells. Regardless of inhibitor dose or time of incubation, PMB did not cause cell shrinkage.

Inhibition of hemolysis by albumin and by sucrose. The possibility suggested above that the hemolytic action of PMB and NEM is the consequence of osmotic swelling was examined by washing and suspending inhibited cells (60 μmoles NEM per ml cells and 15 μmoles PMB per ml cells) in media containing nonpenetrating, osmotically active substances (sucrose or human serum albumin [5]) and comparing the amount of hemolysis with that of saline-suspended, inhibited cells. As seen in Table I, both 25 g per 100 ml albumin (0.0036 M) in physiologic saline and 0.31 M sucrose in water blocked the hemolytic effect of these inhibitors.

[5] Nutritional Biochemicals Corporation, Cleveland, Ohio.

The inhibition of such hemolysis by sucrose was quantified by washing and suspending red cells treated with a hemolytic dose of PMB (20 μmoles per ml cells) in media of varying sucrose concentrations. Hemolysis was almost completely prevented by concentrations of the sugar in excess of 50 mM whether the disaccharide was added to isotonic saline (300 milliosmolar), as shown in

TABLE I

Effect of various suspension media on the lysis and K^+ leakage of sulfhydryl-inhibited red cells

Inhibitor	Suspension media	Hemolysis in 3 hrs	Cellular K^+ loss
		%	%
PMB	Saline*	14	68
	Albumin†	0	81
	Sucrose‡	0	70
NEM	Saline	58	36
	Albumin	0	44
	Sucrose	0	72

* Na^+ = 143 mEq/L; K^+ = 0 mEq/L.
† Na^+ = 158 mEq/L; K^+ = 2.0 mEq/L.
‡ Na^+ = 37 mEq/L; K^+ = 0 mEq/L.

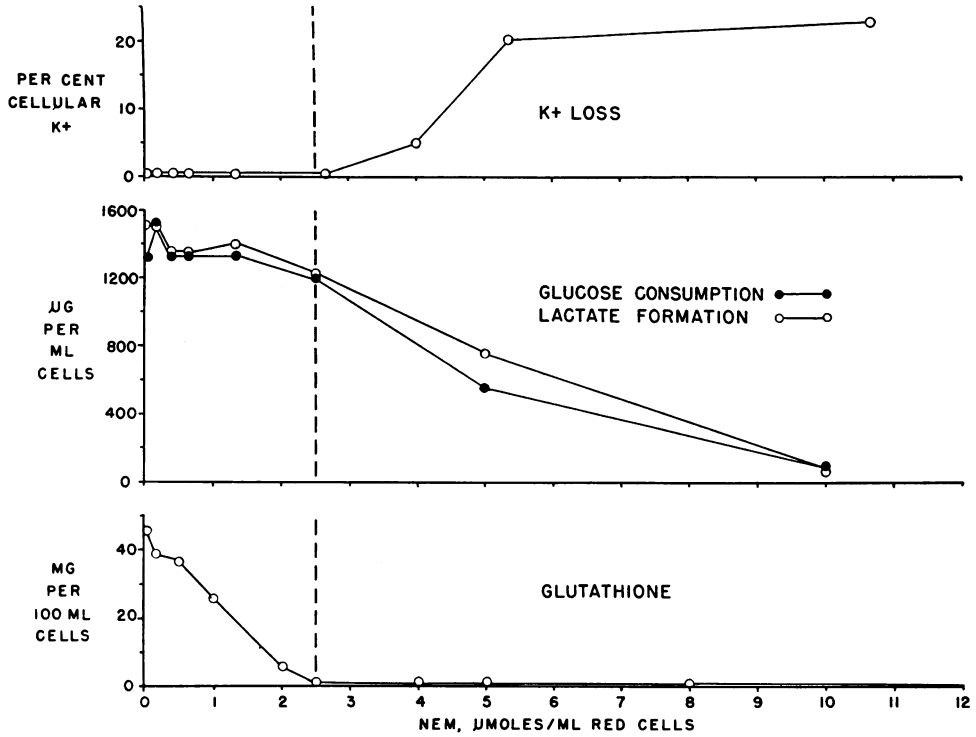

FIG. 7. EFFECTS OF VARIOUS QUANTITIES OF NEM ON RED CELL POTASSIUM LOSS (UPPER), GLYCOLYSIS (MIDDLE), AND GLUTATHIONE (LOWER) LEVELS. Potassium leakage from NEM-treated red cells occurred only after complete glutathione inhibition, when cellular glycolysis also became depressed. This coincidence is emphasized by the vertical interrupted line. The incubation time was 2 hours.

Table II, or added so as to displace an equal number of milliosmoles of NaCl, the final tonicity of the media remaining constant at 300 milliosmolar. Of special interest was the finding that hemolysis by PMB was reduced two-thirds by as little as 20 mM sucrose. In contrast, media containing large quantities of sucrose were without effect on the hemolysis of type A red cells treated with anti-A isohemolysin and complement, as also shown in Table II. In addition, immune hemolysis also differed from hemolysis by sulfhydryl inhibitors in being unattended by pre-lytic cellular swelling or by an increase in osmotic fragility. The assumption that both sucrose and albumin inhibit hemolysis simply through their osmotic properties was investigated by studying the effect of adding saline to PMB-treated cells that were fully protected from hemolysis by suspension in 67 mM sucrose or 20 g per 100 ml albumin. The addition of 6 vol of saline to such suspensions in sucrose caused rapid hemolysis. The addition of 6 vol of saline to the albumin-suspended cells resulted in very little hemolysis even after 2 hours.

TABLE II

Effect of varying concentrations of sucrose on PMB and complement hemolysis

Sucrose conc. of suspending media	Hemolysis of PMB-treated cells	Hemolysis of isohemolysin-treated cells
mmoles/L	*%/3 hrs*	*%/hr*
0	100	100
10	100	100
20	34	100
30	24	100
40	11	100
50	5	100
70	0	100
100	0	100

Various other tests for potential hemolytic injury to sulfhydryl-inhibited red cells, including tests of agglutinability, susceptibility to lysis at an acid pH, and susceptibility to hydrogen peroxide, were all negative.

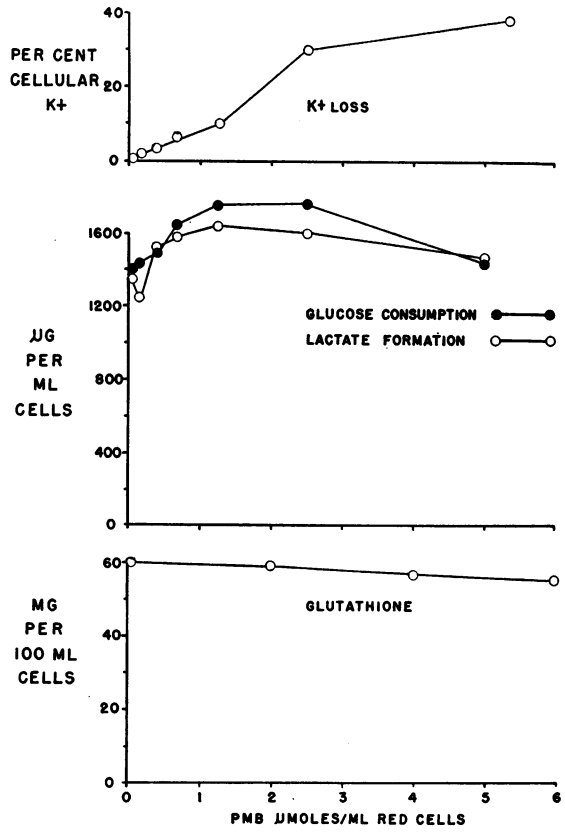

FIG. 8. EFFECTS OF VARIOUS QUANTITIES OF PMB ON RED CELL POTASSIUM LOSS (UPPER), GLYCOLYSIS (MIDDLE), AND GLUTATHIONE (LOWER) LEVELS. In contrast to the results portrayed in Figure 7, potassium leakage from PMB-treated red cells occurred at relatively low doses of inhibitor, and was not associated with decreases in glycolysis or intracellular glutathione concentrations. The incubation time was 2 hours.

Effect of sulfhydryl inhibitors on the cation content of red cells. The changes in red cell shape, size, and fragility described above involve disruption of sodium and potassium gradients across the red cell membrane. Potassium leakage from intact cells occurred with a dose of NEM in excess of 2.5 μmoles per ml red cells. With these doses, K^+ leakage was detectable within 5 minutes of incubation and increased progressively with dosage, as shown in the upper portion of Figure 7. The highest dose portrayed did not cause hemolysis within the experimental period (2 hours). It will be recognized that the smallest dose of NEM to cause K^+ leakage coincided with that just necessary to block completely intracellular GSH (lower portion Figure 7) and begin to diminish glycolysis (middle portion). These correspondences are emphasized in the figure by the vertical interrupted line.[6] PMB also produced K^+ leakage, and at lower doses than those needed with NEM (Figure 8); K^+ leakage occurred without inhibition of glycolysis or of intracellular GSH reactivity.

Na^+ gain by red cells paralleled K^+ loss during PMB inhibition. However, with NEM the cellular levels of these two cations responded differently. During the early period of NEM incubation, K^+ leakage exceeded Na^+ gain. A dose of NEM (20 μmoles per ml red cells) which caused shrinkage of the cell, followed later by swelling and spherocytosis, was used in the investigation of this phenomenon. The results of this study are portrayed in Figure 9, which demonstrates changes in Na^+ and K^+ with time and the relation of such changes to cell size. It is seen that cell shrinkage occurred during the early period of independent leakage of K^+; the swelling that occurred later was associated with Na^+ gain. The absence of cellular shrinking phenomena with low doses of PMB corresponds with the absence of this selective effect upon K^+ levels.

The studies so far described suggest that hemolysis induced by sulfhydryl inhibition involves two steps: *1*) sodium and potassium gradients across the red cell membrane are disrupted; and *2*) water (containing some Na^+) then diffuses into the cell, attracted by the unopposed oncotic activity of hemoglobin and other high molecular weight cellular components. If so, impermeable, extracellular, osmotically active agents such as albumin and sucrose should prevent hemolysis by inhibiting the entry of extracellular water, but should not affect the disruption of cation gradients. This was found to be the case and, as indicated in Table I, the loss of K^+ was augmented, rather than diminished, by these media.

Inhibition of hemolysis by Mg^{++}. If, in addition to the disruption of Na^+ and K^+ gradients, the inhibition of red cell sulfhydryl activity causes the loss of cellular divalent cation with its replacement by twice its number of extracellular uni-

[6] Data relating the effects of NEM and PMB on GSH levels and glycolysis have been presented in Figures 1 and 2. They are repeated in the middle and bottom portions of Figures 7 and 8 to clarify their relationship to cation changes in sulfhydryl-inhibited red cells.

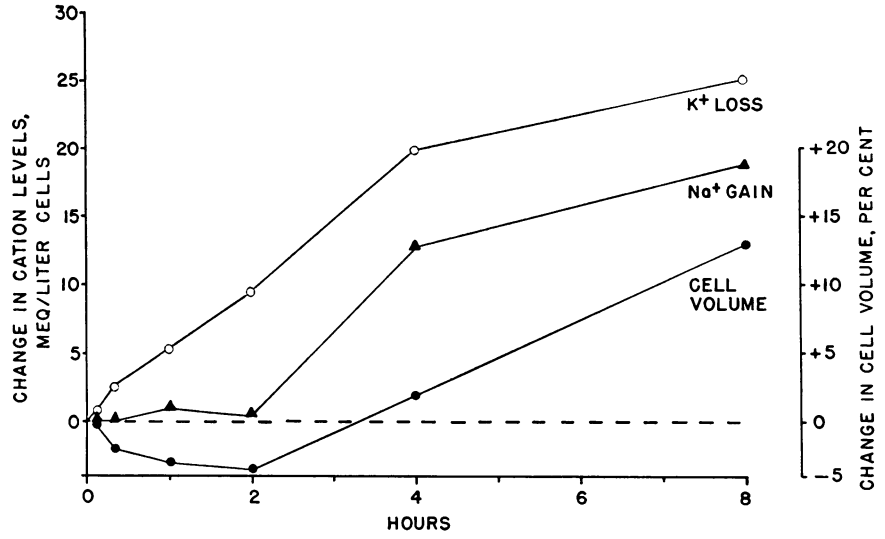

FIG. 9. RELATION BETWEEN THE ALTERATIONS OF SIZE OF NEM-TREATED RED CELLS AND THEIR CONCENTRATIONS OF Na^+ AND K^+. Incubation with 20 μmoles NEM per ml red blood cells caused rapid potassium loss (open circles) but negligible sodium gain (solid triangles) for the initial 2 hours of study. This was associated with cell shrinkage (solid circles). After this period sodium and presumably water gain by the cells became prominent and coincided with cellular swelling.

valent cations, a further increase in intracellular osmotic activity and hence in cell swelling and lysis would be expected. Mg^{++} is the only divalent cation present in red cells in appreciable amounts (about 2 to 3 mM). To prevent loss of this cation, increasing amounts of magnesium chloride were added to the physiologic saline in which red cells treated with PMB (15 μmoles per ml red cells) were suspended. As seen in Table III, hemolysis was inhibited by relatively low concentrations of Mg^{++}, 5 mM Mg^{++} being almost as inhibitory as the 20 mM dose.

DISCUSSION

These studies indicate that, regardless of their ability to permeate the red cells and to affect their level of GSH and their over-all rate of glycolysis, sulfhydryl inhibitors have the following effects on red cells: *1*) cation gradients are disrupted, *2*) spherocytosis and cellular swelling ensue, and *3*) finally the cells undergo osmotic hemolysis. These observations permit certain deductions as to the functioning and malfunctioning of red cells.

Site of sulfhydryl inhibition. The permeability of the two inhibitors employed clearly differs. As judged by its failure to lower intracellular GSH levels and diminish glycolysis, at least until hemolysis commences, the charged particles of PMB appear to be impermeable to the intact red cell membrane. Positively charged ions, such as PMB, are known to be transported very slowly across this membrane as compared to negatively charged ions (39). The net positive charge on PMB, therefore, may determine the site and thereby its mode of action on the cell, as is the case with many metallic inhibitors (40). For example, low concentrations of Hg^{++} have been shown to produce marked potassium leakage from muscle cells well before glycolysis is affected (41). Both in the present experiments with red cells and in those on muscle cells, metabolic or

TABLE III

Inhibition of PMB-induced hemolysis by Mg^{++}

Mg^{++} conc. of suspending medium	Hemolysis
mmoles/L	%
0	100
1.25	85.0
2.5	79.2
5	70.8
10	68.2
20	63.2

structural injury by mercurial sulfhydryl inhibitors must occur primarily on the cell membrane. Accordingly, PMB impaired cation gradients, caused cellular swelling, and produced spherocytosis, without impairing glycolysis or methemoglobin-reducing capacity within the cell.

In contrast to PMB, NEM rapidly permeated the red cell and combined with intracellular GSH. This ease of permeability probably explains the greater doses of NEM required to produce comparable effects on red cell fragility (Figures 4 and 6). Presumably this is an effect of the "dilution" of cell-permeable NEM in a large sulfhydryl pool within the cell, a pool not available to the impermeable PMB.[7] The rapid permeability of NEM confirms the report of Morell, Ayers and Greenwalt (43) that complete cellular sulfhydryl interaction with this inhibitor occurs within 60 seconds. The ready diffusion of NEM throughout the red cell is also attested to by its ability to stifle glycolysis, once all GSH is blocked (Figure 8). Nevertheless, as with PMB, the hemolytic and prehemolytic changes in the cell must result ultimately from the blockade of membrane sulfhydryl groups, since these effects of NEM were reversed by relatively impermeable glutathione, despite the persistent absence of intracellular sulfhydryl activity.

Metabolic derangements. The inhibition of glucose consumption and lactate production by NEM was found to occur only after complete inhibition of GSH. Presumably, at this point NEM is free to react with sulfhydryl-containing glycolytic enzymes such as aldolase and possibly with glucose-6-phosphate dehydrogenase, or with enzymes such as 3-phosphoglyceraldehyde dehydrogenase that may require GSH as a coenzyme (44). It is tempting to postulate that the disruption of cation gradients and spherocytosis produced by NEM is related to such metabolic blockade, since glycolysis produces energy in the form of ATP that is necessary for maintenance of normal cation gradients across the cell membrane (45, 46). Thus, it would seem reasonable to suspect that ATP deficiency secondary to the glycolytic inhibition of NEM is responsible for the cation leak found in the altered cells. However, there are reasons to doubt this interpretation. The rate of K^+ loss in red cells incubated with NEM (Figure 7) is 4 to 5 times greater than the rate of tracer K^+ exchange in the normal cell (47). This would indicate that NEM directly increases the membrane permeability for K^+ rather than simply inhibiting the active transport of this ion. Similarly, iodoacetate and fluoride have been shown to have lesser, yet direct, effects on membrane K^+ permeability independent of their alterations of glucose metabolism (48). Furthermore, inhibition of anaerobic glycolysis by fluoride causes marked K^+ leak with comparatively little Na^+ gain, leading to red cell shrinkage and decreased osmotic fragility (49, 50). Marked spherocytosis, however, is not seen for some 6 hours after the complete inhibition of glycolysis by fluoride (51, 52), at which time 30 per cent of original cell ATP is present (51). In contrast, with comparable doses of NEM, spherocytosis with concomitant hemolysis occurs within 1 hour. It would seem, therefore, that ATP levels decline relatively slowly after cessation of glycolysis, and that it is thus unlikely that a fall in ATP is responsible for the changes in size and shape of these NEM-treated cells. The impermeability of PMB would seem also to exclude intracellular metabolic alterations as primary in the cation disruptions found with this inhibitor. However, metabolic inhibition confined to the membrane, which presumably is the critical region as regards the control of cation levels, may underlie our findings. It is of interest in this regard that many enzymes appear to be present in the red cell membrane, including adenosine triphosphatase (53) and the sulfhydryl-dependent enzyme, pyrophosphatase (54, 55).

The mechanism by which inhibition of membrane sulfhydryl groups impairs cation gradients remains unclear, but certain analogies to other chemically induced red cell alterations seem of interest. Schatzmann (56) has demonstrated that the breakdown of normal cation relationships of

[7] The concentration of hemoglobin in red cells is about 5 mM. Human globin contains six sulfhydryl groups per molecule, of which two are readily reactive with metallic sulfhydryl inhibitors (42). Since GSH and hemoglobin contribute the only appreciable sulfhydryl activity to the red cell and since the former is present in 2 to 3 mM concentration in normal cells, it follows that the rapidly reactive sulfhydryl pool of these cells is 12 to 13 mM or 12 to 13 μmoles per ml packed cells. Similar considerations place the total red cell sulfhydryl concentration somewhat over 30 μmoles per ml packed cells.

cardiac glycoside-treated cells occurs in the absence of any measurable alteration in glucose metabolism, oxygen consumption, or lactic acid formation. In this regard, it is of especial interest that cardiac glycosides may act as sulfhydryl inhibitors by virtue of their unsaturated lactone structure (57, 58), although this is contested (59).

Mechanism of hemolysis. The temporal relationship between swelling and hemolysis in sulfhydryl-inhibited cells indicates that such hemolysis is due mainly to osmotic swelling of the cell. The results can best be accounted for in terms of the colloid-osmotic theory of hemolysis (60, p. 242). The initial step appears to be the blockade of red cell membrane sulfhydryl groups. This leads to an increase in the permeability of the membrane to cations with disruption of the usual cation gradients across this membrane. Such disruption upsets the poorly understood mechanism by which the red cell normally compensates for the oncotic activity of cellular hemoglobin, equal to some 5 per cent of total red cell osmolality (61), and the even greater osmotic activity contributed by other impermeable or poorly permeable intracellular compounds, particularly the organic phosphate esters. As a result, water (as hypotonic saline) enters the cell, leading to swelling and membrane distention with enlargement of its "pores." This in turn leads to a loss of molecules too small to pass the normal membrane and eventually to the leakage of hemoglobin; i.e., hemolysis. The ability to prevent this process by balancing intracellular oncotic activity by extracellular oncotically active material (albumin), or nonpenetrating osmotically active material (sucrose), validates this interpretation. In addition to the osmotic effect of relatively large and impermeable intracellular molecules, it is conceivable that replacement of intracellular divalent cation (Mg^{++}) by extracellular monovalent cations results in a further increment in intracellular osmotic activity. Support for this hypothesis was provided by experimental evidence (Table III) that relatively low concentrations of extracellular Mg^{++} did indeed partially inhibit hemolysis by PMB. The replacement in this manner of intracellular divalent cation by twice its osmotic equivalent of monovalent cation may contribute to osmotic hemolysis caused by a variety of different agents.

Hemolysis induced by saponin (62) has been reported to be inhibited by albumin media and hence may involve mechanisms similar to that of sulfhydryl-inhibition hemolysis. In contrast, however, hemolysis by complement, although inhibited by albumin, as reported by Green, Barrow and Goldberg (63), differs from the lysis induced by sulfhydryl inhibitors in that in our studies with complement, cellular swelling did not occur and sucrose was ineffective in preventing complement hemolysis. Further studies of this discrepancy revealed that, with respect to PMB-treated red cells, sucrose behaved predictably as a simple impermeable particle; thus its antihemolytic effect was exerted only so long as it was present in sufficient concentration in the medium, and when this concentration was lowered by the addition of saline, hemolysis commenced. With albumin this was not the case. The addition of large volumes of saline to PMB-treated red cells protected by albumin was not followed by appreciable hemolysis. Thus albumin, although an oncotic agent, appears also to confer a protective effect apart from its osmotic properties in this system. Although our inability to demonstrate cellular swelling or spherocytosis during lysis by complement is incompatible with the conclusions of Green and his associates (63), it is possible that complement opens relatively large holes in the cell membrane, as indeed these authors have demonstrated in the case of ascites tumor cells (63), and as is suggested by the inefficacy of sucrose. If so, only a very slight osmotic swelling of the cell might then be sufficient to cause the leakage of hemoglobin. Therefore, it is suggested that immune hemolysis involves a more direct and extensive alteration of membrane structure, by creating even larger pores than do sulfhydryl inhibitors, through which hemoglobin leakage occurs with little prior osmotic swelling of the cell. This view is consistent with the fact that under optimal conditions complement-mediated hemolysis occurs more rapidly than does hemolysis by sulfhydryl inhibitors.

It is of some interest that hemolysis occurs in these sulfhydryl-inhibited cells at cell volumes of only 120 to 150 per cent that of normal cells. If hemolysis were solely due to osmotic swelling, a greater critical cell volume should be expected prior to cell lysis (60, p. 90). Two possible implications of this disparity are suggested: *1*) that

some loss of cell surface area is produced by the inhibitors, and/or *2*) that a hypersusceptible population of cells swells and lyses to a greater extent than do the remaining cells. Some evidence for the latter interpretation was obtained with the PMB-treated red cells (Figure 6), but not with NEM-treated cells in which less prehemolytic swelling took place (Figure 4).

In conclusion, these studies have shown that the shape and integrity of red cells exposed to sulfhydryl inhibitors *in vitro* depend ultimately upon membrane and not upon intracellular sulfhydryl activity. Presumably, injury to membrane sulfhydryl groups may arise through either of two mechanisms: *1*) a failure to maintain intracellular (and secondarily membrane) thiols because of the entry of oxidant compounds, as in the Heinz body anemias, or a failure of glycolytic enzymes, or both, in which case a fall in cellular glutathione is found; or *2*) a direct, localized membrane inhibition (i.e., by such agents as the heavy metals), without a decrease in cell glutathione concentration. In either case, the ability of cells to preserve their shape and hemoglobin content *in vitro* is dependent on their maintenance of membrane cation gradients, which in these studies was unrelated, per se, to over-all cellular glycolysis or glutathione levels.

SUMMARY

The behavior *in vitro* of human red cells exposed to two different sulfhydryl inhibitors was studied.

The addition to red cells of the freely permeable sulfhydryl inhibitor, N-ethylmaleimide, caused the following sequence of events: *1*) intracellular glutathione was blocked; *2*) glycolysis was inhibited; *3*) cation gradients across the cell membrane were disrupted, with a loss of cellular K^+ and a gain of Na^+ and water; and *4*) the influx of water caused by the unopposed oncotic activity of hemoglobin and other macromolecules led to spherocytosis and swelling of the cells, which resulted eventually in the leakage of hemoglobin (i.e., hemolysis). Comparative studies of immune hemolysis revealed that the lysis of red cells by complement does not occur by a comparable osmotic mechanism.

The addition to red cells of the nonpermeable sulfhydryl inhibitor, *p*-mercuribenzoate, also disrupted cation gradients and thereby caused spherocytosis and osmotic hemolysis; however, this inhibitor produced such alterations without blocking intracellular glutathione or inhibiting over-all glycolysis.

It is concluded that the shape and viability of red cells depend primarily upon membrane, and not upon intracellular, sulfhydryl activity.

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