Sulfhydryl Groups of the Erythrocyte Membrane and their Relation to Glycolysis and Drug-Induced Hemolytic Anemia

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Research Article

Hemolytic anemia caused by oxidative drugs is thought to result from the oxidation of intracellular and membrane sulfhydryl groups of the erythrocyte. This process is more likely to occur in those erythrocytes in which the intracellular mechanism for reduction of disulfides is abnormal (e.g., glucose-6-phosphate dehydrogenase deficiency). If a membrane sulfhydryl group is critical in the pathogenesis of druginduced hemolytic anemia, it follows that this specific group must be dependent on intracellular reductive mechanisms for maintenance of the reduced state.

This report describes a sulfhydryl group(s), involved in membrane structure, which is (are) dependent on intracellular metabolism for maintenance of the reduced state. It is postulated that this metabolically dependent membrane sulfhydryl group may play a role in the pathogenesis of drug-induced hemolytic anemia.

Membrane sulfhydryl groups were studied by observing the effect of sulfhydryl blocking agents, e.g.,*N*-ethyl-maleimide (NEM), on the recovery of erythrocyte ghosts after osmotic lysis. It was shown that NEM interfered with ghost recovery by reacting with membrane sulfhydryl groups. The concentration of NEM (as determined by [^{14}C] NEM binding) necessary to cause this effect was lower than that necessary to produce changes in osmotic fragility or cation permeability, or to cause Heinz body formation.

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came disulfides, but could be returned to the reduced
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Phenylhydrazine hemolytic anemia was induced in pigs,
and membrane changes of the type described above oc-
curred early in the pathogenesis of the disease.

INTRODUCTION

Hemolytic anemia can result from the action of oxida-
tive drugs on erythrocytes (1). The effects of such drugs
in vitro are many, and include changes in hemoglobin
with the formation of Heinz bodies, alteration in mem-
brane function, and shortening of erythrocyte life span
(1, 2). That such changes result from oxidation of
sulfhydryl groups has been suggested in several studies
(3, 4). It has been postulated, furthermore, that the
critical changes affecting erythrocyte life span occur
in the sulfhydryl groups of the erythrocyte membrane
(4).

It has been noted that the susceptibility to this type of
hemolysis is greatest in those erythrocytes in which
metabolic defects render it less capable of reducing oxi-
dized sulfhydryl groups, as is the case in G6PD de-
ficiency (1). This suggests, therefore, that the pentose
phosphate pathway of glycolysis is necessary to main-
tain membrane sulfhydryl groups in their reduced state.

In the present study we have detected one or more
sulfhydryl groups in the erythrocyte membrane that are
closely related to its structure and can be oxidized by
treatment with phenylhydrazine both in vivo and in vitro.
Furthermore, it would appear that these membrane
groups are maintained in their reduced state by intra-
cellular glycolysis.

METHODS

Preparation of erythrocyte ghosts and
determination of ghost recovery

Erythrocyte ghosts were prepared according to a modi-
fication of Hoffman's technique (5). Each sample of
erythrocytes was hemolyzed in the following manner:
Whole heparinized blood was centrifuged, and the plasma
and buffy coat were removed. The erythrocytes were dis-
lodged from the bottom of the tube by gentle tapping; 2.4

ml of cold (4°C) hemolyzing solution (1–2 µCi ^{22}Na/25 ml ion-free water) was added to 0.4 ml of erythrocytes quickly, and the sample was mixed well immediately.[1]

The hemolyzates were allowed to stand for 20 min. Approximately 1 ml was removed from each sample and saved. The remaining hemolyzates were centrifuged at 20,000 g for 15 min at 4°C. The supernates were discarded, and the erythrocyte ghosts were washed three times with 10 ml of cold (4°C) MgCl$_2$ Tris solution (9 parts 17 mM MgCl$_2$, 1 part 17 mM Tris, pH 7.45 [25°C]), and centrifuged each time at 10,000 g for 10 min at 4°C. After removal of the last wash, the ghosts were resuspended in 1.2 ml of "reversal" solution (NaCl 135 mM, KCl 15 mM, Tris 10 mM, adenosine 5 mM, pH 7.45 [25°C]).

Measurement of ^{22}Na retention by ghosts. ^{22}Na activity and cell counts were determined in each hemolyzate and in each reversed ghost sample. Cell counts were estimated with a Model B Coulter Counter with aperture tube 100 (Coulter Electronics, Inc., Fine Particle Group, Hialeah, Fla.). The radioactivity in hemolyzates (0.5-ml aliquots) and in reversed ghost samples (1.0 ml) was measured in a Packard Auto-Gamma Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples were counted in triplicate for 20 min. Microhematocrits were determined on each hemolyzate with a 15-min centrifugation period. As it is difficult to assess the amount of trapping under the conditions of these experiments, the microhematocrit values are uncorrected. Though a possible error exists in the calculations, it was considered that this point could be disregarded, as all samples probably contain the same degree of discrepancy.

Calculations. Percent ^{22}Na retention ("ghost recovery percent") = ([cpm/10^9 reversed ghosts]/[cpm/10^9 hemolyzed ghosts]) × 100, cpm/10^9 ghosts in hemolyzate = (cpm/ml hemolyzate × hematocrit)/(cell count/ml hemolyzate × 10^{-9}), and cpm/10^9 reversed ghosts = (cpm/ml reversed ghosts)/(cell count/ml reversed ghosts × 10^{-9}). It is assumed that all the radioactivity in the reversed ghost sample would be inside the ghosts at the end of the last wash.

Reproducibility of the technique. As noted in the Results section, sodium retention (i.e., ghost recovery) of normal fresh blood averaged 38.7%. Duplicate blood samples of normal fresh blood were carried through an entire run on eight occasions. The mean difference of ghost recovery between duplicate samples was 3.8% with an SD of 2.84%.

Analytical methods and buffer solution

Glutathione and ATP concentrations of erythrocytes were determined by standard methods (7, 8), as were hemoglobin, hematocrit, reticulocyte, and Heinz body values (9). The "buffer" referred to in the text is a Tris buffer: NaCl 135 mM, KCl 15 mM, Tris 10 mM, pH 7.45, at 25°C.

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[1] Note: Hoffman (5) reported that this technique will produce virtually 100% lysis and complete mixing of the ^{22}Na throughout the hemolyzate. To test this, we compared the ^{22}Na activity of the whole hemolyzate with that of the hemolyzate supernate obtained after centrifuging the hemolyzate at 20,000 g for 15 min. If mixing were complete, the ratio of supernate ^{22}Na to total hemolyzate should have been one. In eight experiments, it varied from 0.987 to 1.01, and it was concluded, therefore, that mixing was virtually complete by our technique.

N-ethylmaleimide (NEM)[2] incubation

10 ml of blood was collected in 0.1 ml of heparin (1,000 IU/ml). The sample was centrifuged, and the plasma was saved. The erythrocytes were washed twice in cold (4°C) buffer. With this buffer as diluent, various concentrations of NEM were prepared from a 2 mM stock solution (fresh daily). Then 39.6 ml of buffer and of each NEM solution (0.02, 0.03, 0.035, 0.04, 0.10 mM) were placed in 50-ml centrifuge tubes and kept in a water bath at 37°C for 10 min. 0.4 ml of packed washed erythrocytes were then added to each tube, mixed by inversion, and the tubes were placed in a shaking waterbath at 37°C for 20 min. The samples were centrifuged for 10 min, and supernates were discarded. The erythrocytes were transferred to 15-ml centrifuge tubes (International Equipment Co., Needham Heights, Mass., Autoclear) and washed twice with cold (4°C) normal saline. The first wash was used to transfer erythrocytes to smaller tubes. After removal of the last wash, erythrocytes in each tube were resuspended in 0.5 ml of heparinized plasma. The samples were allowed to stand at 4°C for approximately 30 min. They were then centrifuged for 10 min, and the plasma was carefully removed and discarded. Other incubation procedures are described in Results section.

NEM uptake by erythrocytes

In order to standardize our NEM procedures, we determined the amount of NEM bound under various experimental conditions. The studies of Jacob and Jandl (4) used NEM concentrations calculated on the basis of moles of NEM per milliliter of erythrocytes. However, it is evident that at low NEM concentrations all the NEM will be bound, preferentially to the more avid sites (e.g., glutathione), and it is difficult to predict the amount of NEM on any specific site (e.g., membrane). Accordingly, our standard procedure employed an erythrocyte per NEM solution ratio of 1/99, which results in little change in NEM concentration during incubation.

To determine the amount of NEM bound to the erythrocytes and to the isolated membrane under the conditions of our experiment, and to compare this with the studies of Jacob and Jandl (4), we have studied the binding of [^{14}C]NEM. Erythrocytes separated from whole blood were washed twice in buffer. Then 0.4 ml packed erythrocytes was mixed with 39.6 ml NEM (containing trace amounts of [^{14}C]NEM) for 20 min at 37°C. The suspension was then centrifuged, and the supernate was saved. The erythrocytes were washed twice in saline and resuspended in plasma. Ghosts prepared by the method of Parker and Hoffman (6) were washed until hemoglobin-free and then were counted in a liquid scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). For counting, 0.2 ml of ghosts was mixed with 1.0 ml of solubilizing solution (Nuclear-Chicago Solubilizer); then 9.5 ml of scintillation fluid was added. The amount of NEM bound is shown in Table I. A cell count of the ghost suspension was performed on a Coulter counter. The NEM used was supplied by Schwarz Bio Research Inc., Orangeburg, N. Y., as [1-^{14}C]N-ethylmaleimide with a specific activity of 10.3 mCi/mmol.

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[2] *Abbreviations used in this paper:* NEM, N-ethylmaleimide; PCMBS, p-chloromercuribenzene sulfonic acid monosodium salt; PMB, p-hydroxymercuribenzoate.

Table I

[^{14}C]NEM Binding by Erythrocytes

Vol erythrocytes	Vol medium	Incubation time	Concn NEM	NEM bound	Erythrocytes
		min	*mM*	*µmol/10¹⁰ ghosts*	*µmol/ml*
1	99	20	0.02	0.016	1.77
1	99	20	0.035	0.053	2.9
1	99	20	0.1	0.13	4.2
				0.120	4.5
				0.143	4.8
				0.12	4.1
1	3	60	0.66*	0.025	1.73
1	3	60	3.33*	0.43	9.47
				0.47	9.46
1	3	60	6.66*	0.734	15.4

* These are equivalent to 2 µmol, 10 µmol and 20 µmol NEM/ml of erythrocytes, respectively, and represent the procedure employed by Jacob and Jandl (4).

RESULTS

^{22}Na retention by normal erythrocytes ghosts

Erythrocytes lysed in water containing ^{22}Na will, at the time of lysis, mix completely with the hemolyzing solution (5). As a result, at the moment of lysis, ^{22}Na enters the erythrocytes and exists in the same concentration in the ghosts as in the suspending medium (see Methods).

As these ghosts stand and are then washed, many of them will recover the original sodium-permeability characteristics of the intact erythrocytes (5). As a result, the repeated washing of the ghosts employed in this study will remove little of the ^{22}Na from these recovered or reversed ghosts. However, those ghosts that have not reversed will lose their ^{22}Na as a result of washing. Accordingly, the amount of ^{22}Na retained by the washed erythrocyte ghosts has been used as an index of ghost recovery. Complete retention (see Methods) would be expressed as 100% ghost recovery.

In 10 samples of fresh human blood, ghost recovery averaged 38.7%, with an SD of 10.9%.

The effect of NEM on ghost recovery

Erythrocytes were washed in buffer and incubated for 20 min at 37°C in buffer (control) or in buffer containing NEM, following the procedures described in Methods. They were then washed three times and resuspended in plasma; Ghost recovery was determined as described in Methods.

It was noted that ghost recovery was affected by preincubation of intact erythrocytes with NEM (Fig. 1). This effect was frequently seen at NEM concentrations as low as 3×10^{-5} M. The effect was consistent at concentrations of 1×10^{-4} M; at this concentration, ghost recovery in nine experiments was 1.4% (SD = 0.82).

The effect of NEM on erythrocyte glutathione levels

Two experiments were performed in which washed erythrocytes were incubated with NEM in buffer for 20 min at 37°C and then resuspended in plasma at 4°C for 30 min, as described for NEM incubation in Methods. The samples were then prepared for the glutathione assay. The results indicated that an approximately 50% and 95% decrease in glutathione levels occurred after preincubation with 2×10^{-5} M and 3.5×10^{-5} M NEM, respectively.
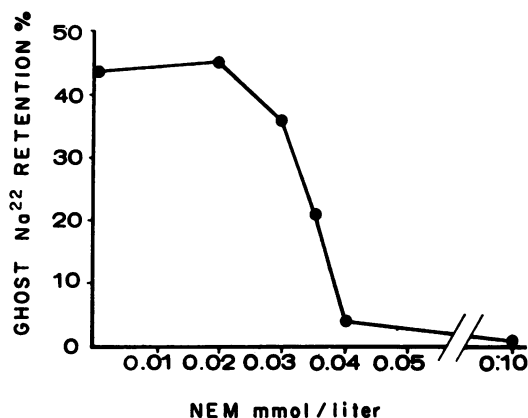

FIGURE 1 Effect of NEM on ^{22}Na retention by erythrocyte ghosts. Erythrocytes were washed in buffer and incubated for 20 min at 37°C in buffer with or without NEM. One part of erythrocytes was incubated in 99 vol of solution. The erythrocytes were washed three times and resuspended in plasma. Ghost recovery was then determined, as described in Methods.

The effect of phenylhydrazine on ghost recovery

In two experiments, erythrocytes were incubated with phenylhydrazine in buffer for 20 min as per the NEM experiments. Heinz bodies were seen only in samples incubated with 5×10^{-5} M phenylhydrazine and greater. Ghost recovery, however, was reduced to 30% and 8% of control values at 5×10^{-6} M and 1×10^{-5} M phenylhydrazine, respectively.

The effect of p-hydroxymercuribenzoate (PMB) and p-chloromercuribenzene sulfonic acid monosodium salt (PCMBS) on ghost recovery

Erythrocytes were preincubated for 20 min at 37°C with PMB and PCMBS. In two experiments at concentrations of 5×10^{-5} M, PMB pretreatment decreased ghost recovery to 50% and 51% of control values, and PCMBS reduced recovery to 70% and 77%. In five experiments, PMB and PCMBS at concentrations 2×10^{-4} M caused mean fall in recovery to 33% (range 27–40) and 44% (range 27–65), respectively.

It is evident that PMB is somewhat more effective than PCMBS. What is of interest, however, is the fact that PCMBS shows such a distinct effect, in view of its relatively poor ability to permeate the erythrocytes membrane (10). We have studied the uptake of PMB and PCMBS in the following experiments.

The uptake of PMB and PCMBS by erythrocytes

The uptake of PMB and PCMBS was studied by observing the change in OD in the supernate of a suspension of erythrocytes (10). For the experiment, 1.0 ml of erythrocytes was incubated with 20 ml of 10^{-4} M PMB or PCMBS. In the presence of 2×10^{-4} M cysteine the OD at 250 and 236 nm is an index of the concentration of PMB and PCMBS, respectively. A linear fall in OD was observed, which was calculated to represent an uptake of PMB of 28%, 39%, and 72% after 80 min in three separate experiments, respectively. PCMBS uptake performed at the same time with the same erythrocytes was 2%, 10%, and 12%, respectively.

The effect of prolonged incubation on erythrocyte ghost recovery and NEM reactivity

Incubation at 37°C. It was noted that with prolonged incubation at 37°C, erythrocytes became progressively more resistant to the effect of NEM on ghost recovery. Fresh erythrocytes were washed three times, resuspended to approximately 50% hematocrit, and incubated in sterile buffer for 15 h or more.

Under these conditions, ghost recovery of the erythrocytes was slightly, but significantly, less than that of fresh erythrocytes. However, a striking difference was observed when NEM reactivity was compared (Table II). Incubated erythrocytes were affected minimally by treatment with NEM. This phenomenon was examined further to determine whether erythrocyte metabolism could maintain NEM reactivity.

The effect of glucose during incubation. Erythrocytes were washed in buffer and then incubated under sterile conditions at 37°C for 16 h and 40 h either with or without added glucose (55 mmol/liter). The results showed that glucose is able to maintain ghost recovery and NEM reactivity for up to 40 h of incubation at 37°C.

The effect of glucose and adenosine on previously incubated erythrocytes. The results of one typical experiment are shown in Table III. It is evident that adenosine improves NEM reactivity compared with the almost complete ineffectiveness of glucose. This is consistent with studies of metabolic recovery in incubated ghosts.

Glutathione and ATP levels were measured before and after 1 h recovery incubation, at 37°C, of previously incubated erythrocytes. The results of a typical experiment are also shown in Table III. It is clear, that under the conditions of this experiment, ATP and glutathione are regenerated upon reincubation with adenosine, but almost no ATP regeneration occurs upon reincubation with glucose.

In all experiments performed, the pattern seen in Table III was observed. In six experiments on preincubated erythrocytes glutathione levels after 1 h incubation in glucose or in adenosine were 48% (SD = ±15) and 77% (SD = ±15) of the 0-h values, respectively. ATP levels in five experiments were 17.6% (SD = ±11.5) and 60% (SD = ±16), respectively. Sodium retention in the presence of NEM was 15.5% (SD = ±7) and 3.5% (SD = ±5.7) after incubation in glucose or in adenosine, respectively, in six experiments. All these differences among samples incubated in glucose and in adenosine were significant ($P < 0.01$).

TABLE II

Ghost Recovery of Erythrocytes Preincubated at 37°C*

| | Number of subjects | Ghost recovery | |
		0 NEM	1×10^{-4} M NEM
		(%)	
Fresh cells	10	37.7 (±10.9)	1.4 (±0.82)
Incubated cells	9	24.3 (±9.2)	19.0 (±9.0)

* Washed erythrocytes were incubated at 37°C for 15–22 h in buffer (without glucose). Ghost recovery was determined after the incubation and after an additional 20 min incubation at 37°C in buffer or in buffer containing 1×10^{-4} M NEM.

TABLE III

TABLE III

The Effect of Glucose or Adenosine on Erythrocytes Previously Incubated for 22 h at 37°C*

		22 h plus 1 h at 37° in		
	0 h	Buffer	Buffer plus glucose	Buffer plus adenosine
Ghost recovery‡ after pretreatment with 0.1 mM NEM		20	20	1
Reduced Glutathione, mg/100 ml erythrocytes	81	42	50	63
ATP, μmol/ml erythrocytes	1.0	0.16	0.04	0.41

* Washed erythrocytes, suspended in sterile buffer (without glucose) were incubated for 22 h at 37°C. The erythrocytes were separated and washed in buffer. They were then incubated for 1 h at 37°C in one of the three solutions.
‡ After the 1-h incubation, the erythrocytes were washed and further incubated for 20 min at 37°C in 0.1 mM NEM. Ghost recovery was then determined.

The effect of incubation with dithiothreitol on NEM reactivity. Dithiothreitol (Cleland's reagent) is an agent with a very low redox potential, capable of maintaining sulfhydryl groups in the reduced state (11). It was reasoned that the loss of NEM reactivity after prolonged incubation might be due to the oxidation of sulfhydryl groups in the erythrocyte membrane and the formation thereby of disulfides. If so, incubation with Cleland's reagent should prevent the formation of such disulfides; furthermore those erythrocytes which had lost reactivity (due to prolonged incubation) should regain it upon exposure to Cleland's reagent. These hypotheses were tested in the following experiments.

TABLE IV

The Effect of Cleland's Reagents during 16 h Incubation at 37°C on Erythrocyte Ghost Recovery*

Experiment	Incubation solution	Ghost recovery 0.1 mM NEM
		%
1	Buffer	9.85
	Buffer plus Cleland's	1.45
	Buffer plus glucose	1.58
2	Buffer	34.6
	Buffer plus Cleland's	1.46
	Buffer plus glucose	2.12

* Whole blood was centrifuged, and the erythrocytes were washed three times with buffer and then resuspended to a 10% suspension. The samples were further diluted by the addition of an equal volume of either buffer, buffer with 5.0 mM Cleland's reagent, or buffer with 55 mM glucose. All procedures were carried out with sterile techniques. Ghost recovery was determined on erythrocytes after a 20-min exposure to 0.1 mM NEM (see Methods).

Erythrocytes were incubated 16 h at 37°C., with buffer only, buffer plus Cleland's reagent (2.5 mM), or buffer plus glucose (55 mM). The results of two such experiments are shown in Table IV. As anticipated, glucose maintained NEM reactivity. Incubation in the presence of dithiothreitol also maintained these groups. Furthermore, as shown in Table V, Cleland's reagent was effective in restoring NEM reactivity to previously incubated cells.

The effect of prolonged preincubation on binding of [14C]NEM by erythrocytes. We interpreted the data, to this point, to mean that during in vitro incubation, certain erythrocyte membrane sulfhydryl groups were depleted, rendering them nonreactive with NEM. We wished to determine, therefore, whether the total number of NEM reactive sites were diminished under these circumstances.

TABLE V

The Effect of Cleland's Reagent on Erythrocytes Previously Incubated for 16 H at 37°C*

Experiment	1 h incubation in	Ghost recovery 0.1 mM NEM
		%
1	Buffer	18.8
	Buffer plus Cleland's	0.35
2	Buffer	8.4
	Buffer plus Cleland's	0.11

* Erythrocytes were washed in buffer and incubated under sterile conditions for 16 h at 37°C. They were removed, washed, and resuspended for 1 h incubation at 37°C in one of the two solutions (buffer, buffer plus 2.5 mM Cleland's). Ghost recovery was determined on the erythrocytes after 20 min exposure to 0.1 mM NEM (see Methods).

TABLE VI

[^{14}C]NEM Binding to Incubated Erythrocytes*

Experiment	μmol NEM/10^{10}	μmol/ml erythrocytes
0 glucose	0.114	2.85
With glucose	0.115	3.60
0 glucose	0.122	2.80
With glucose	0.131	3.76

* Washed erythrocytes were incubated for 16 h at 37° in buffer with or without glucose (55 mmol/liter). [^{14}C]NEM binding to erythrocytes and to ghosts was then determined.

Washed erythrocytes were incubated for 16 h at 37°C with or without glucose (55 mmol/liter). The erythrocytes were incubated with [^{14}C]NEM as described in Methods. The results of ^{14}C binding are shown in Table VI. These data suggest that 16 h incubation in the absence of glucose reduces the total amount of NEM bound to the erythrocytes, but has no detectable effect on the amount of NEM bound to the membrane.
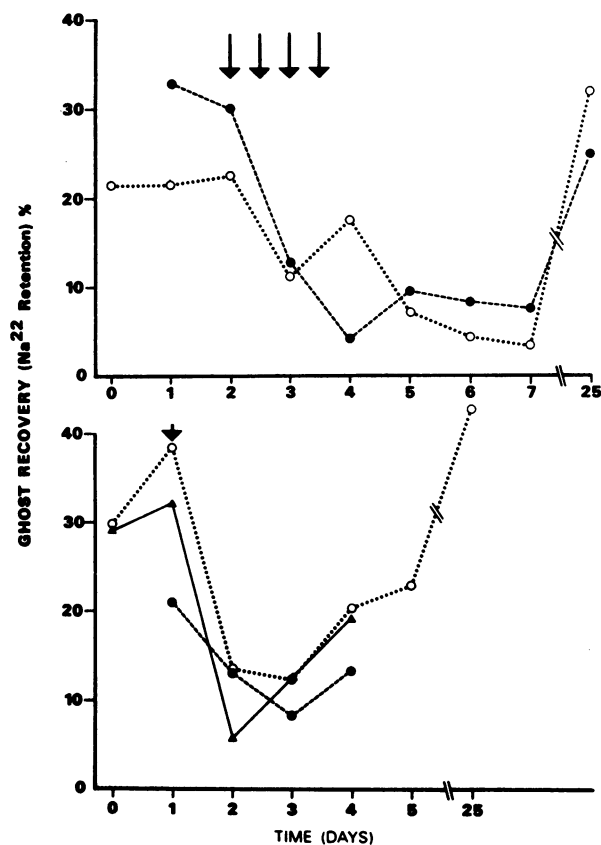

FIGURE 2 Erythrocyte ghost recovery after phenylhydrazine injections. Above: two pigs receiving four injections (7.5 mg/kg per injection); below: three pigs receiving one injection of 7.5 mg/kg.

The effect of phenylhydrazine administered in vivo on erythrocyte ghost recovery

Preliminary studies on rabbits and dogs indicated that erythrocyte ghost recovery in these animals was quite poor by the techniques we employed. In 12 pigs studied, ghost recovery was substantial (27%±5.8), and we therefore used pigs for in vivo studies. Five young pigs were studied. They varied in weight from 10 to 25 kg. Two regimes of phenylhydrazine therapy were used. Two animals received four daily injections of 7.5 mg/kg. Three pigs received single injections of phenylhydrazine at a dose of 7.5 mg/kg. A hemolytic process appeared by the 2nd or 3rd day, as evidenced by a fall in hematocrit, and a rise in reticulocyte count. Heinz bodies appeared by the 2nd day. In Fig. 2, ghost recovery changes are shown for all five animals and demonstrate a consistent fall in ghost recovery after phenylhydrazine therapy. Normal levels appear approximately 1 wk after the end of phenylhydrazine treatment.

DISCUSSION

The results of the present studies suggest that there are specific sulfhydryl groups that are intimately related to membrane structure and are relatively sensitive to in vivo and in vitro oxidation, or to in vitro blockade by agents that react with sulfhydryl groups. The maintenance of these groups in the reduced state is dependent on metabolic processes within the erythrocyte. The evidence for these conclusions follows.

We have used the technique of ghost recovery after osmotic lysis to study some of the sulfhydryl groups of the erythrocyte membrane. Using this technique, our initial experiments showed that ghost recovery could be affected by pretreating the erythrocytes with NEM or PMB, reagents that react with sulfhydryl groups. We concluded from these studies that there was one or more sulfhydryl groups in the erythrocyte membrane that reacted with these reagents and thereby interfered with ghost recovery. It was also noted that phenylhydrazine, which can oxidize sulfhydryl groups, had a similar effect.

To quantitate more accurately the effect of NEM, we have determined the binding of [^{14}C]NEM at various concentrations (Table I) and related these findings to our data and those published by Jacob and Jandl (4, 12). We have calculated the amount of NEM bound by intact erythrocytes and by membranes in their experiments by simulating their technique in some of our ^{14}C-binding experiments (Table I).

Both studies showed that glutathione reacted with low concentrations of NEM. Thus, we observed a fall in glutathione when erythrocytes were incubated in 0.02 mM NEM where there were 1.77 μmol of NEM bound/ml erythrocytes. Since there are approximately 2.0 μmol

of GSH/ml erythrocytes, then one would expect that at a concentration of 0.035 mM (at which 2.9 μmol are attached/ml erythrocytes [Table I]), glutathione would be nearly totally blocked, which our data confirm. The findings of Jacob and Jandl (4) were similar (Table I).

It is apparent, however, that all the other effects of NEM noted by Jacob and Jandl (4, 12) occur at considerably higher concentrations than those creating the membrane changes noted in the present study. A significant effect on potassium levels occurs when there is 0.43 μmol NEM bound/10^{10} ghosts, a level that is significantly higher than the minimum concentration necessary to diminish ghost recovery. A measurable decrease in erythrocyte life span in vivo occurs only when there is 0.43 μmol NEM bound/10^{10} ghosts.

Structural changes (osmotic fragility, and erythrocyte size and shape) and microscopic Heinz body formation occur only at membrane concentrations (> 0.43 μmol/10^{10} ghosts), much greater than those necessary to affect ghost recovery. Furthermore, all the studies of Jacob and Jandl (4, 12) employed incubation periods of 1 h or longer, compared with the standard 20 min incubation used in the present studies. It is likely, therefore, that NEM membrane and erythrocyte concentrations were even higher than those in Table I.

It has been suggested that the critical changes affecting erythrocyte survival in drug-induced hemolysis is an alteration in membrane permeability (12) or the formation of Heinz bodies (13). It would appear, from the above observations, that ghost recovery is affected at significantly lower NEM concentrations than are necessary to induce Heinz body formation (as determined by light microscopy) or to alter sodium permeability of intact erythrocytes. At this point, we cannot state whether the sulfhydryl groups we study (i.e., by ghost recovery technique) are significant in the survival of the erythrocyte. Their involvement in the earliest demonstrable membrane defect in phenylhydrazine or in NEM-treated erythrocytes suggests that this point should be studied further.

The nature of this membrane sulfhydryl group and of its relation to cellular metabolism was then studied. It was found that incubated erythrocytes (in the absence of glucose) were no longer reactive with NEM (Table II), (i.e., NEM did not interfere with ghost recovery). This change could be prevented by providing glucose to the erythrocyte during incubation and could be reversed by incubating the nonreactive erythrocytes with adenosine (Table III). A 1-h incubation with adenosine reestablished glycolysis (i.e., ATP and GSH concentrations increased) and restored NEM reactivity. Incubation in glucose alone failed to reestablish glycolysis (no significant increase in GSH or ATP) and did not restore NEM reactivity. We postulated, therefore, that

the sulfhydryl groups were maintained in, or restored to, a reduced state by the pentose phosphate pathway of glycolysis; without glycolysis the sulfhydryl groups were oxidized and no longer reacted with NEM. The experiments with Cleland's reagent support this hypothesis (Tables IV and V), since that reagent can reduce disulfides and was able to restore NEM reactivity.

The mechanism by which glycolysis reduces membrane sulfhydryl groups (or possibly, mixed disulfides between hemoglobin and membrane sulfhydryl groups [14]), is not established by these experiments. It may be that this is achieved by reduced glutathione which can maintain hemoglobin and other proteins in the reduced state and depends itself upon glycolysis for reduction from the oxidized state. This would mean, however, that the glutathione must reach these sulfhydryl groups in the membrane. Reduced glutathione cannot cross the membrane (15). This would suggest that the critical sulfhydryl group in question exists near the inner surface of the membrane. The location of these membrane sulfhydryl groups is not clear from our studies. The similar effects of PMB and PCMBS suggest that these groups can be reached by water soluble agents (i.e., PCMBS).

These findings all suggest that in the membrane of the human erythrocyte there are sulfhydryl groups that are intimately related to the structure of the membrane. Since oxidant drug-induced hemolytic anemias are thought to result from oxidation of membrane sulfhydryl groups (1, 4, 12), it seemed likely that the particular group studied in this paper would be involved as well. That this does occur in vivo during drug-induced hemolytic anemia is shown in Fig. 2, which depicts phenylhydrazine-induced hemolytic anemia in pigs.

Although it is clear that these sulfhydryl groups are affected during oxidant drug-induced hemolytic anemia, their role in the pathogenesis of this disease is not clear. What is evident, however, is that these groups are affected and that they are normally dependent upon metabolic processes for reduction. This latter property is not shared by all membrane sulfhydryl groups as shown in Table VI. Under conditions that cause a fall in NEM reactivity (16 h incubation at 37°C), in the absence of glucose, the [^{14}C]NEM content of the membrane is unchanged. Total [^{14}C]NEM content of the erythrocyte is lower in the absence of glucose. It would appear, therefore, that the critical sulfhydryl groups represent only a small fraction of the total NEM-reactive groups of the membrane. The PMBS data are consistent with this because only a small proportion of membrane NEM-reactive sulfhydryl groups are reached by this reagent (10). This may also explain the observation that total membrane sulfhydryl groups are normal in patients with glucose-6-phosphate dehydrogenase deficiency (16),

since the membrane sulfhydryl groups that are dependent on glycolysis represent only a small fraction of the total.

ACKNOWLEDGMENTS

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