

Reduction of in Vitro Autohemolysis in Hereditary Spherocytosis by Impermeant Molecules

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IN HEREDITARY SPHEROCYTOSIS (HS) the red cell membrane is abnormally permeable to the passive influx of Na.^{2,3} This leads to colloid osmotic lysis of the erythrocyte unless the accumulation of intracellular Na and water is prevented.³ The HS red cell compensates for this Na leak by increasing the active transport of Na out of the cell at a rate to keep pace with the increased passive influx. The hyperactivity of the Na pump in HS is manifested by an increase in Na turnover³ and in glucose^{3,4} and ATP⁴ utilization by the HS red cell compared to normal.

Under circumstances of glucose deprivation which occur in vivo with erythrocytosis in the spleen or after prolonged incubation in vitro, ATP generation ceases and energy for the active extrusion of Na from the cell is no longer available. This leads to Na and water accumulation and osmotic hemolysis. This principle is well illustrated by the autohemolysis test used in the diagnosis of HS.^{5,6} When erythrocytes are incubated for 48 hours without the addition of glucose, there is an increase in red cell volume secondary to osmotic swelling during the first 24 hours. During the second 24 hours there is a decrease in cation content and cellular volume with the formation of spherocytes.^{5,7} Reed and Swisher⁸ have demonstrated that the observed decrease in volume and surface area is caused by loss of membrane lipid and have shown that this loss occurs more rapidly with HS erythrocytes. Weed and Bowdler⁷ have emphasized the importance of this loss of red cell surface in decreasing the critical hemolytic volume of the HS red cell so that a smaller increment of osmotic swelling will lead to hemolysis compared to an erythrocyte with a normal surface area. The addition of glucose decreases the abnormal hemolysis which occurs after prolonged incubation of HS red cells by reducing the intracellular accumulation of Na^{5,7} and by lessening the loss of membrane lipid.⁸ Sucrose, a compound which does not enter the red cell, also lessens the hemolysis of HS erythrocytes in the autohemolysis test, indicating the importance of osmotic changes in the production of hemolysis after prolonged incubation.³

Although the autohemolysis test has been used extensively in HS, the effects of compounds other than glucose have been tested infrequently in this sys-

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tem. The results to be reported in this paper show that many compounds will protect against the abnormal autohemolysis observed in HS. The compounds having a protective effect either enter the red cell and provide energy in the form of ATP or are unable to enter the cell, have no effect on intracellular ATP, and appear to lessen hemolysis by creating an external osmotic force.

METHODS

Thirteen patients from 9 families served as donors of HS erythrocytes. At the time the studies were done 7 of the patient had had splenectomy. Routine blood studies⁹ were normal in the patients who had had splenectomy while there was a moderate anemia and reticulocytosis in those patients whose spleen had not been removed. However, the results of the autohemolysis studies were similar whether the spleen was present or not, and in the data to be presented the patients are treated as one group. The diagnosis of HS was made on the basis of spherocytosis on the peripheral blood smear, family history of HS, increase in osmotic fragility, increase in autohemolysis partially corrected by glucose, and response to splenectomy in those who had the operation.

Heparinized or defibrinated whole blood was incubated in 16 x 125 mm. screw-capped tubes at 37 C. for 24 or 48 hours in an incubator equipped with an attachment for continuous rotation of the tubes.⁹ Incubations were carried out under sterile conditions and, as an added precaution against bacterial contamination, penicillin (100 units per ml.) and streptomycin (100 μ g. per ml.) were added. The addition of these two antibiotics did not affect the amount of autohemolysis when compared to duplicate samples to which they were not added.

The compounds[†] used as additives in the autohemolysis experiments were dissolved in normal saline, neutralized with NaOH when necessary, and autoclaved before addition to the samples. Compounds were added to give a final concentration of either 10 mM or 20 mM. At the end of incubation, per cent hemolysis, red cell ATP content, and pH were determined. The pH did not fall below 7.0 in any of the samples except those to which glucose had been added.

Per cent hemolysis was determined as described by Jaffé.¹⁰ ATP was measured enzymatically by coupling the hexokinase and glucose-6-phosphate dehydrogenase reactions as described previously.⁴ The red cells were washed twice with saline to remove all added substrates prior to ATP measurement. The pH was measured in an open cup on a pH meter.[‡] All determinations were performed in duplicate.

RESULTS

The Effect of Added Compounds on 48-Hour Autohemolysis of HS Blood

Sulfhydryl-Containing Compounds. Because of previous work by others¹¹ and ourselves¹² which suggests that a derangement of membrane sulfhydryl metabolism may play a role in the altered membrane permeability of the HS red cell, we were interested in studying the effect of sulfhydryl-containing compounds on the autohemolysis of HS erythrocytes. Figure 1 shows that in addition to glucose the sulfhydryl-containing compounds reduced glutathione (GSH), and cysteine led to a significant reduction of hemolysis while the nonsulfhydryl-containing compounds—glutamine, glycine, and methionine—did not. However, the oxidized form of glutathione (GSSG) lessened hemol-

*Elconap incubator with a Wyble Engineering Corporation (Silver Springs, Md.) attachment for rotation of tubes.

†Obtained from Sigma Chemical Co.

‡Radiometer-Copenhagen, London Co.

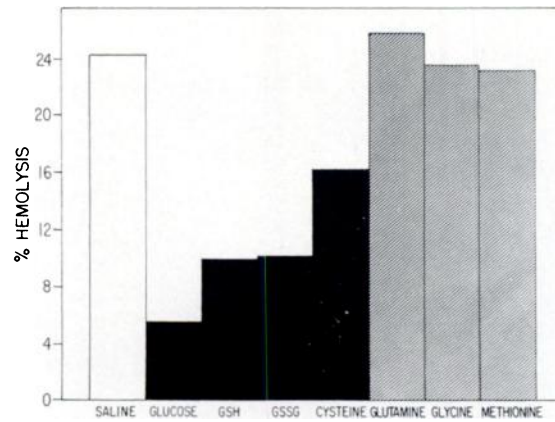


Fig. 1.—Effect of sulfhydryl-containing compounds on autohemolysis in hereditary spherocytosis. The height of the bar represents the mean value for the following number of experiments with each additive: saline, 38; glucose, 34; GSH, 21; GSSG, 20; cysteine, 18; glutamine, 4; glycine, 9; and methionine, 11. All compounds were added in a concentration of 10 mM, except for glucose which was 20 mM. The black bars represent a significant difference from control samples ($p < 0.01$).

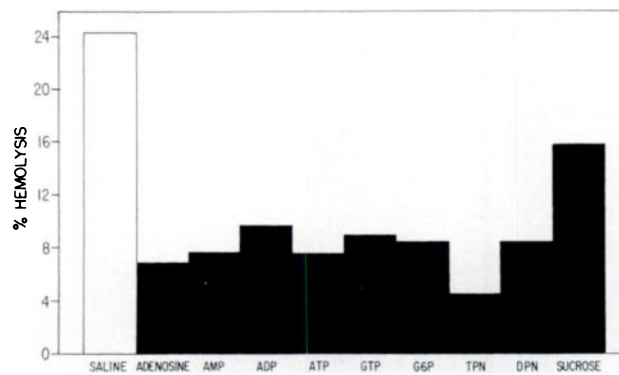


Fig. 2.—Effect of phosphorylated compounds on autohemolysis in hereditary spherocytosis. The height of the bar represents the mean value for the following number of experiments with each additive: saline, 38; adenosine, 7; AMP, 13; ADP, 9; ATP, 13; GTP, 12; G6P, 9; TPN, 19; DPN, 7; and sucrose, 27. All compounds were added in a concentration of 10 mM. All compounds significantly reduced hemolysis ($p < 0.001$).

ysis as much as the reduced form (GSH), and for this reason a wide variety of metabolic intermediates were tested.

Phosphorylated Compounds. Figure 2 illustrates that a large number of compounds partially protect against the abnormal autohemolysis seen in HS. All but two of the compounds depicted in Figure 3 are phosphorylated and do not enter the red cell,¹³ thus creating an external osmotic force. Adenosine readily enters the red cell but is metabolized to form ATP, thus creating a source of energy for cation transport.¹¹ Sucrose, like the phosphorylated compounds, does not enter the cell and provides an external osmotic force.³

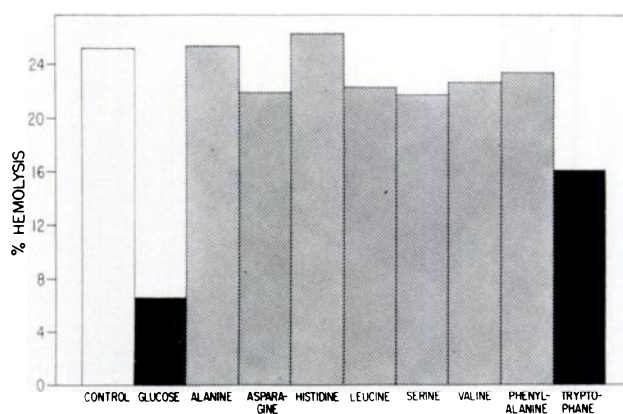


Fig. 3.—Effect of amino acids on autohemolysis in hereditary spherocytosis. The height of the bar represents the mean value for the following number of experiments with each additive: control, 38; glucose, 34; alanine, 2; asparagine, 3; histidine, 1; leucine, 3; serine, 5; valine, 3; phenylalanine, 4; and tryptophane, 6. All compounds were added in a concentration of 10 mM except for glucose which was 20 mM. The black bars represent a significant difference from control samples ($p < 0.05$).

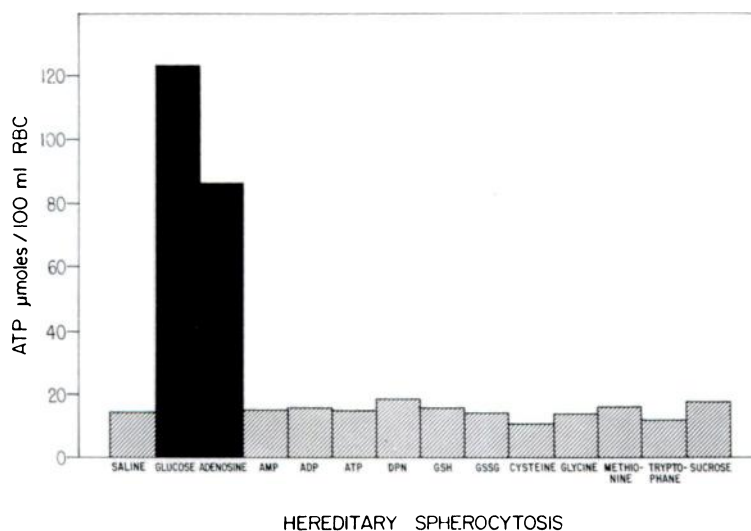


Fig. 4.—Effect of compounds on red cell ATP content after 24-hour incubation. The height of the bar represents the mean value for five experiments with each additive. All compounds were added in a concentration of 20 mM. The black bars are significantly higher than the control samples ($p < 0.001$).

Amino Acids. To test further the hypothesis that compounds improve autohemolysis in HS by either providing energy in the form of ATP or an external osmotic force, a number of amino acids which should enter the red cell¹⁴ and not provide energy were tested.

None of the amino acids tested afforded significant protection against in-

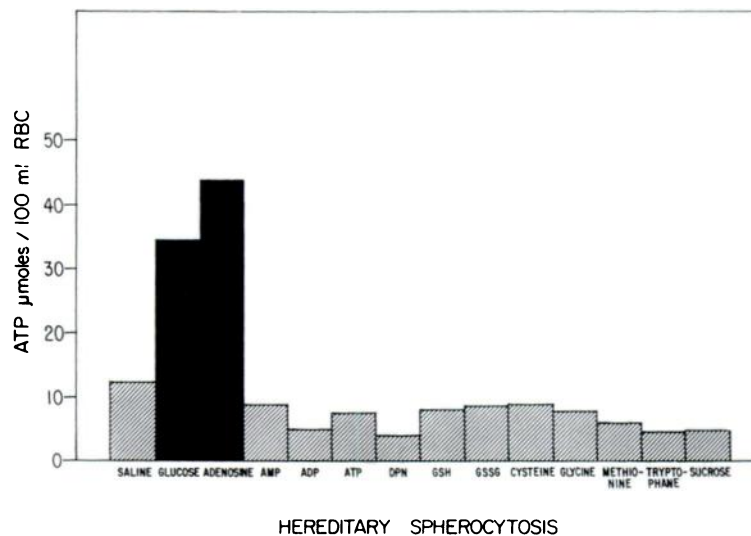


Fig. 5.—Effect of compounds on red cell ATP content after 48-hour incubation. The height of the bar represents the mean value for five experiments with each additive. All compounds were added in a concentration of 20 mM. The black bars are significantly higher than the control samples ($p < 0.05$).

creased hemolysis except tryptophane, shown in Figure 3, and cysteine, shown in Figure 1.

Effect of Added Compounds on Red Cell ATP Content After Prolonged Incubation

When red cell ATP levels were measured after 24 and 48 hours of incubation, there was significant preservation of ATP content with adenosine and glucose, while the addition of the adenine nucleotides, DPN, glutathione, amino acids, and sucrose had no effect on red cell ATP levels compared to control samples to which only saline was added (Figs. 4 and 5).

DISCUSSION

The increase in hemolysis which occurs after prolonged incubation of HS erythrocytes is secondary to abnormally permeable red cell membrane with subsequent colloid osmotic hemolysis.³ The addition of compounds which do not enter the red cell and thereby create an external osmotic force, and compounds which supply energy in the form of ATP for the active extrusion of Na from the cell, would be expected to protect against this type of osmotic injury to the red cell. On the other hand, compounds which are not a source of energy and enter the red cell so that no external osmotic gradient is produced would not be expected to lessen the hemolysis of incubated HS erythrocytes.

The results of the experiments reported in this paper support this concept. Adenosine and glucose caused significant protection of ATP levels and reduced hemolysis, while the phosphorylated intermediates (AMP, ADP, ATP, DPN), the glutathiones (GSH, GSSG), and sucrose provided no intracellular

increment in ATP, but by virtue of their inability to traverse the red cell membrane they provided an external osmotic force and also significantly lessened hemolysis. On the other hand, the addition of a number of amino acids (cysteine, glutamine, glycine, methionine, alanine, asparagine, histidine, leucine, serine, valine, phenylalanine, tryptophane), which did not serve as a source of energy and presumably freely entered the red cell, did not significantly reduce the autohemolysis of HS erythrocytes with the exception of cysteine and tryptophane. Cysteine may have become partially oxidized during incubation to the impereant cystine and thus would have produced an osmotic effect. Why tryptophane caused some reduction in hemolysis is unclear. Although we have stressed the point that compounds which protected against hemolysis did so through their oncotic effect or by supplying energy, other mechanisms of protection should be considered. It is possible that compounds reduced hemolysis by lessening membrane permeability or lipid loss. We are currently investigating the effect of these compounds on membrane permeability to Na and K and the rate of lipid loss.

It can be noted in Figure 2 that sucrose caused less protection against hemolysis than equimolar amounts of the phosphorylated compounds. This can be explained by the fact that sucrose did not require neutralization while the phosphorylated compounds were neutralized with NaOH, and because of their greater ionizability would be expected to have a higher osmolality. It was important to neutralize all additives, as Young and his associates have shown that lowering the pH will lessen the autohemolysis of HS erythrocytes.⁶ In our experiments the pH did not fall below 7.0 after 48 hours of incubation with any of the added compounds except glucose.

In pyruvic kinase-deficiency hemolytic anemia, there is an increase in autohemolysis after 48 hours of incubation, which is usually not improved by glucose but is partially corrected by ATP.¹⁵ It has been postulated by deGruchy and his associates¹⁶ and by Tanaka and his colleagues¹⁵ that ATP added to the plasma may lead to an increase in intracellular ATP by the following mechanisms: ATP does not penetrate the red cell membrane¹³ but is hydrolyzed to ADP and AMP by plasma phosphatases,¹⁷ and the ADP so formed may be converted to ATP and AMP in the red cell membrane by adenylate kinase.¹⁸ Some of the ATP formed in this way may enter the red cell to provide a source of energy and reduce hemolysis. However, no data have been presented which show that ATP added to plasma leads to an increase in ATP within the red cell. Also, this mechanism would not explain the observation by Tanaka and associates¹⁵ that in addition to the adenine nucleotides such compounds as DPN, TPN, GSH, and coenzyme A also lessen the autohemolysis of pyruvic kinase, deficient red cells.

In our studies we found no significant preservation of red cell APT content after 24 and 48 hours of incubation of HS erythrocytes with any added compound other than glucose and adenosine (Figs. 4 and 5). The added compounds included the adenine nucleotides, DPN, glutathione, amino acids, and sucrose. The ATP measurements after 24 hours of incubation also indicate that there was not a significant hydrolysis of the adenine nucleotides to adenosine by plasma phosphatases, as the samples to which they were added had no

higher red cell ATP than did samples to which only saline was added. It is possible that the addition of adenine nucleotides to whole blood may have led to a transient rise in intracellular ATP during the early hours of incubation or to a small but undetectable rise in membrane ATP content, but it seems unlikely that this would lead to a significant reduction of autohemolysis over a 48-hour incubation period.

Although it is possible that the phosphorylated compounds which lessen hemolysis in the autohemolysis test may do so through some mechanism other than their osmotic effect, it seems quite clear from the data we have presented that they do not afford protection against hemolysis by the generation of intracellular ATP.

SUMMARY

It has been shown that a variety of compounds will reduce the abnormal autohemolysis which occurs after 48 hours of incubation of HS erythrocytes. These compounds either provide energy in the form of ATP, as is the case with glucose and adenosine, or provide an external osmotic force by virtue of their inability to cross the red cell membrane, as is the case with phosphorylated compounds, glutathione and sucrose. None of these latter compounds caused a significant preservation of erythrocyte ATP content. In assessing the effect of added compounds in the autohemolysis test, their osmotic behavior should be taken into account.

SUMMARIO IN INTERLINGUA

Ha essite monstrate que un varietate de compositos reduce le anormal autohemolyse que occorre post 48 horas de incubation de erythrocytos SH. Iste compositos provide energia in le forma de ATP (como il es le caso con glucosa e adenosina) o provide un externe fortia osmotic gratias a lor incapacitate de transversar le membrana erythrocytic (como il es le caso con compositos phosphorylate, con glutathiona, e con sucrosa). Nulle de iste ultime compositos causava un significative preservation del contento erythrocytic de ATP. In evaluar le effecto de addite compositos in le test de autohemolyse, lor comportamento osmotic deberea esser prendite in consideration.

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