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Adenosine Triphosphate Metabolism in Hereditary Spherocytosis *

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The red cell in hereditary spherocytosis (HS) is unusually susceptible to the damaging effects of erythrostasis. The spleen is the primary site of erythrostasis *in vivo*, and it is a feature of HS that splenectomy corrects the hemolytic anemia, although the intrinsic red cell defect persists. The characteristic laboratory findings in HS of an increase in osmotic fragility and in autohemolysis are enhanced by *in vitro* incubation that simulates *in vivo* erythrostasis. Since these *in vitro* abnormalities are partially corrected by the addition of glucose (2), efforts have been made to detect a defect in the carbohydrate metabolism of the HS erythrocyte. As yet no definite abnormality in glycolysis has been demonstrated (3–5).

In 1953 Harris and Prankerd (6) showed that there was an increased rate of extrusion of Na by the HS red cell, and in 1957 Bertles (7) demonstrated an increased rate of influx of Na into the HS erythrocyte. Jacob and Jandl (8) have confirmed this earlier work and have observed an increased turnover of Na by the HS red cell that is approximately twice normal. Based on these observations, they have postulated that the primary defect in HS is an abnormal permeability of the red cell to the passive influx of Na with secondary changes in glycolysis. To maintain a high concentration of K and a low concentration of Na within the red cell with respect to the concentration of these cations in the plasma, active transport against a gradient must take place. In the HS red cell there is an increased passive influx of Na (7, 8) that must be compensated by increased active efflux of Na if osmotic hemolysis is to be prevented. Jacob and Jandl (8) have demonstrated that maneuvers which interfere with this compensatory increase in active Na transport lead to an increased rate of destruction of HS red cells *in vivo*. Since the energy necessary for the active transport of Na is derived from glycolysis in the form of adenosine triphosphate (ATP) (9–12), increased utilization of ATP and glucose by the HS red cell would be anticipated. Furthermore, ouabain, which blocks the utilization of ATP by the Na-K pump (11–13), should nullify increased rates of ATP and glucose utilization by HS red cells if the increase is caused by hyperactivity of cation transport. The data presented in this paper indicate an increased rate of ATP and glucose utilization by the HS erythrocyte that is only partially abolished by ouabain. The results suggest that in addition to increased utilization of ATP for active cation transport, the HS erythrocyte has an increased requirement of ATP for other metabolic processes as well.

Methods

Subjects studied. HS red cells were obtained from eight patients. These patients were from three families, and all but one had undergone splenectomy. Routine blood studies (14) and reticulocyte counts were normal except for the patient whose spleen had not been removed. In this patient the hematocrit varied from 36 to 39%, and the reticulocyte count was usually about 5%. The diagnosis of HS was made on the basis of spherocytosis on the peripheral blood smear, positive family history of HS, increase in osmotic fragility, increase in autohemolysis partially corrected by glucose, and response to splenectomy in those who had had that operation. Normal red cells were obtained from normal subjects who had normal hematocrits and reticulocyte counts.

Preparation and incubation of blood. Anticoagulation was achieved by heparin (0.1 mg per ml of blood) or defibrination. The type of anticoagulation that was used

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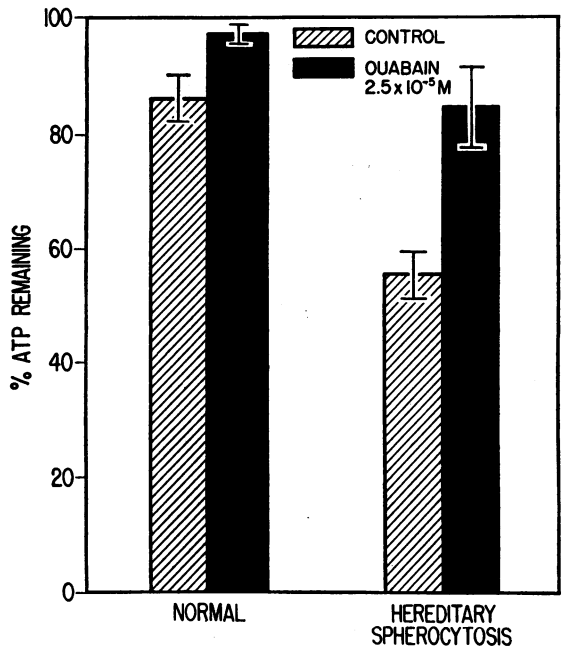

FIG. 1. EFFECT OF OUABAIN ON ATP OF NORMAL AND HEREDITARY SPHEROCYTOSIS (HS) WHOLE BLOOD INCUBATED FOR 6 HOURS. ATP expressed as per cent remaining, considering ATP content of unincubated samples as 100%. Each column and brackets represent mean ± standard error. The ouabain values are based on eight experiments on samples from three normal subjects and four patients with HS. The normal control values are based on 14 experiments on samples from three normal subjects, and the HS control values on ten experiments on samples from four patients with HS.

made no difference in the results. Incubations were carried out in screw-capped tubes at 37° C in an incubator equipped with an attachment for continuous rotation of the incubation tubes.[1] Washed red cells were prepared by centrifugation at 2,000 × *g* and removal of the plasma and buffy coat. The cells were then washed twice with Krebs-Ringer phosphate solution buffered to pH 7.4 (15) and resuspended in Krebs-Ringer phosphate solution to approximately the original volume. The pH of the red cell suspension was measured in an open cup on a pH meter.[2]

Measurement of ATP, glucose, and per cent hemolysis. ATP was measured enzymatically by coupling the hexokinase and glucose-6-phosphate dehydrogenase (G-6-PD) reactions as described by Kornberg (16) but with several modifications. The 2-ml reaction mixture contained the following: 0.4 ml Tris(hydroxymethyl)aminomethane (0.21 M), 0.2 ml glucose (0.3 M), 0.2 ml $MgCl_2$ (0.1 M), 0.2 ml triphosphopyridine nucleotide (TPN)[3] (0.0026 M or 2 mg per ml), 5 µl crystalline hexokinase [3] suspended in 3 M $(NH_4)_2SO_4$ pH 6 (2,400 Kunitz-McDonald U per ml), 5 µl crystalline G-6-PD [4] suspended in 3 M $(NH_4)_2SO_4$ pH 6 (33 Kornberg U per ml), and 1 ml of ATP extract or ATP standard.[3] The reaction was started by the addition of G-6-PD, and the amount of ATP present was determined by the change in OD_{340} after 30 minutes at room temperature compared to the change for the ATP standards. All samples were run in duplicate. The ATP extracts were prepared by adding 1 ml of whole blood or red cell suspension to 3 ml of 0.96 M perchloric acid and neutralizing with K_2CO_3 after centrifugation. All procedures were performed at 5° C unless stated otherwise.

Glucose was measured by a glucose oxidase method,[5] and per cent hemolysis was determined as described by Jaffé (17).

Results

Rate of fall of ATP in normal and HS whole blood and the effect of ouabain on this fall. When

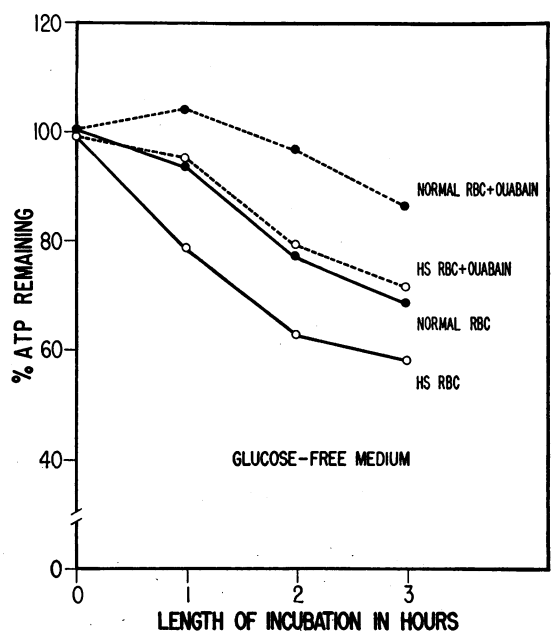

FIG. 2. EFFECT OF OUABAIN ON ATP OF WASHED NORMAL AND HS RED CELLS SUSPENDED IN A GLUCOSE-FREE MEDIUM. ATP expressed as per cent remaining if ATP content of the unincubated red cell suspension is considered as 100%. Each closed circle represents the mean value for ten experiments on samples from eight normal subjects, and each open circle represents the mean value for seven experiments on samples from seven patients with HS. Ouabain was added in 2.5×10^{-5} M concentration.

[1] Elconap incubator with a Wyble attachment for rotation of tubes, Wyble Engineering Corp., Silver Springs, Md.

[2] Radiometer-Copenhagen, London Co.

[3] Obtained from Sigma Chemical Co., St. Louis, Mo.

[4] Obtained from Sigma Chemical Co. and Calbiochem, Los Angeles, Calif.

[5] Glucostat, Worthington Biochemical Corp., Freehold, N. J.

whole blood was incubated for 6 hours, there was a greater fall in the ATP of HS samples than in normal samples ($p < 0.001$) (Figure 1). Part of this difference might be attributed to glucose depletion, which occurred in most of the HS samples by 6 hours, whereas some glucose remained in most of the normal samples. The blood glucose levels in normal and HS samples were comparable before incubation (mean value of 5.0 μmoles per ml for normal samples and 4.8 μmoles per ml for HS samples).

Incubations were also carried out in the presence of 2.5×10^{-5} M ouabain, a concentration designed to block active cation transport and prevent utilization of ATP by the Na-K pump. Under these circumstances the fall in ATP was less in both normal ($p < 0.02$) and HS samples ($p < 0.01$), but ouabain had a greater effect on the ATP levels of HS samples than on normal samples (Figure 1).

There was no significant difference between the ATP values of unincubated normal whole blood (mean 131 ± SE 2.9 μmoles per 100 ml red cells) and HS whole blood (mean 142 ± SE 6.7 μmoles per 100 ml red cells) [6] ($p > 0.2$).

Rate of fall of ATP in normal and HS washed red cells suspended in a glucose-free medium and the effect of ouabain on this fall. Experiments were performed with washed red cells suspended in Krebs-Ringer phosphate so that the amount of glucose in the medium could be controlled. The washed red cell suspension contained no glucose unless added, and the rate of fall in the ATP of such cells was an indication of ATP utilization, since no glucose was available for ATP generation. The amount of ATP remaining in the HS red cells after 1, 2, and 3 hours of incubation was less than in normal red cells, and the difference was highly significant at each time interval ($p < 0.001$). It should be noted, however, that the main difference in the rate of decline of ATP between normal and HS red cells occurred during the first hour. For the next 2 hours the rate of decline of ATP was essentially the same for both groups (Figure 2).

When ouabain was added to the red cell suspension in a 2.5×10^{-5} M concentration, the rate of fall in red cell ATP was significantly less ($p < 0.001$) for both normal and HS red cells. During

[6] The ATP values of the patient with HS who had not had splenectomy were not included.

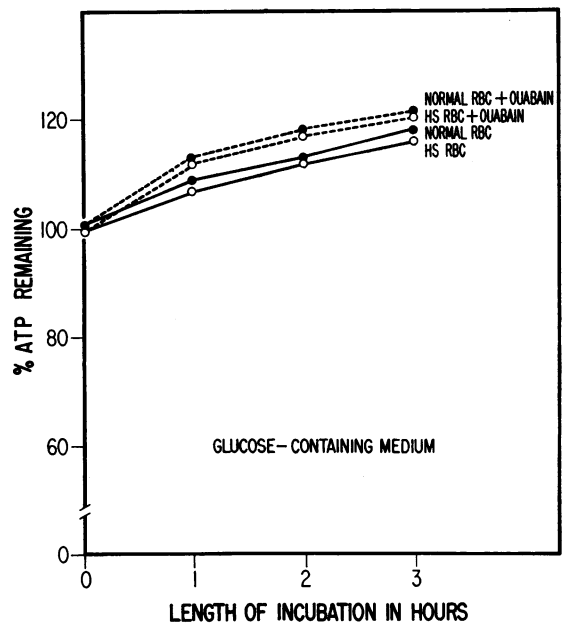

FIG. 3. EFFECT OF OUABAIN ON ATP OF WASHED NORMAL AND HS RED CELLS SUSPENDED IN A GLUCOSE-CONTAINING MEDIUM. Same as Figure 2 except glucose was added in a concentration of 5 μmoles per ml.

the first hour of incubation ouabain had a greater effect on HS red cells than on normal red cells ($p < 0.05$), but for the remainder of the incubation period there was no difference in the ouabain effect on normal and HS cells (Figure 2).

In experiments carried out on two normal subjects and two patients with HS, ouabain was increased to a 2.5×10^{-3} M concentration, and the rate of fall of ATP was not significantly different than when a 2.5×10^{-5} M concentration was used, indicating that active cation transport was maximally blocked with the lower concentration of ouabain (not shown). When active cation transport was maximally blocked by ouabain in both normal and HS red cell, there was still a greater rate of fall of ATP in HS red cells than in normal cells ($p < 0.001$) (Figure 2).

Effect of ouabain on the ATP content of normal and HS red cells suspended in a glucose-containing medium. When glucose was added to the medium in a concentration of 5 μmoles per ml, there was a net gain in ATP for both normal and HS red cells during the 3 hours of incubation and no difference between the two groups. When ouabain was added in a 2.5×10^{-5} M concentration, there was a greater rise in ATP in both normal and HS

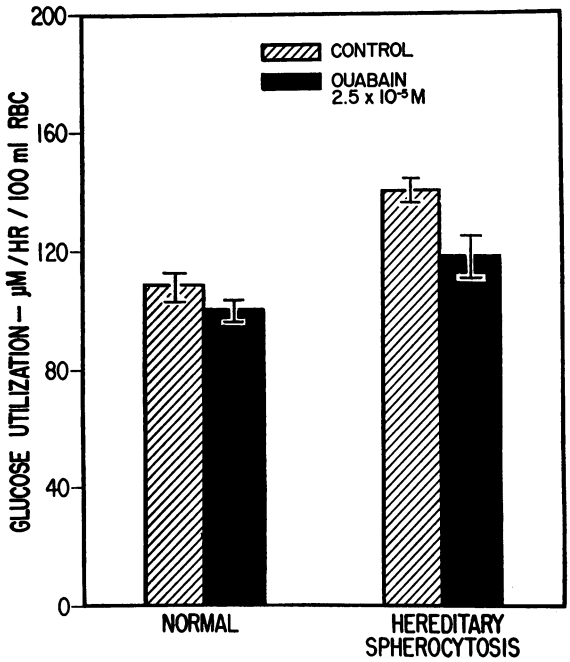

FIG. 4. EFFECT OF OUABAIN ON GLUCOSE UTILIZATION OF WASHED NORMAL AND HS RED CELLS INCUBATED FOR 3 HOURS. Each column and brackets represent mean ± standard error. The normal values represent ten experiments on samples from eight normal subjects and the HS values six experiments on samples from six patients with HS. The HS patient without splenectomy was not included. Glucose was added to the red cell suspension in a concentration of 5 μmoles per ml.

cells compared to samples to which only glucose was added ($p < 0.02$) but no difference in the rate of rise of ATP in normal and HS cells (Figure 3).

Effect of ouabain on the rate of glucose utilization in normal and HS washed red cells. When glucose was added to the red cell suspension in a concentration of 5 μmoles per ml, the rate of glucose utilization by HS red cells was significantly greater ($p < 0.001$) than in normal red cells. Ouabain in a 2.5×10^{-5} M concentration reduced glucose utilization by both normal and HS cells. However, the difference was not significant with normal red cells ($p > 0.2$), but was significant with HS cells ($p < 0.05$). When active cation transport was blocked by ouabain in both types of cells, there was still a greater rate of glucose utilization by HS red cells than by normal cells ($p < 0.05$) (Figure 4).

Effect of glucose and ouabain on the pH of normal and HS washed red cell suspensions. Table I shows that there was a fall in pH during 3 hours of incubation for all the normal and HS samples. When glucose alone was added to the suspension, there was a greater fall in pH in the HS samples than in the normal samples ($p < 0.01$). When ouabain was added in addition to glucose, the degree of fall in the pH of HS samples was significantly less than in the HS samples to which only glucose was added ($p < 0.05$). On the other hand, ouabain produced no significant change in the pH of the normal samples. When active cation transport was blocked in normal and HS cells by ouabain in the presence of glucose, there was still a greater fall in the pH of HS samples than in normal samples ($p < 0.05$).

Effect of glucose and ouabain on the autohemolysis of normal and HS whole blood. When normal blood was incubated for 48 hours, the amount of autohemolysis was slight and essentially unaffected by the addition of glucose or ouabain or both. On the other hand, the addition of glucose (30 μmoles per ml) lessened the amount of hemolysis occurring in HS samples ($p < 0.01$). When ouabain was added (2.5×10^{-5} M) to the HS samples, the corrective effect of glucose was abolished. Although the HS samples to which ouabain or glucose plus ouabain had been added

TABLE I

*Effect of glucose and ouabain on the pH of normal and hereditary spherocytosis (HS) washed red cell suspensions incubated for 3 hours**

	pH before incubation	pH after 3 hours of incubation			
	Control	Control	Glucose†	Ouabain‡	Glucose† and ouabain‡
Normal	7.57 ± 0.03	7.33 ± 0.01	7.32 ± 0.01	7.35 ± 0.01	7.34 ± 0.02
HS	7.60 ± 0.03	7.30 ± 0.01	7.26 ± 0.01	7.32 ± 0.01	7.30 ± 0.01

* Normal pH values represent mean ± standard error for eight experiments; HS pH values represent mean ± standard error for six experiments.

† Added in a concentration of 5 μmoles per ml.

‡ Added in a 2.5×10^{-5} M concentration.

showed slightly more hemolysis than untreated samples, these differences were not statistically significant (Figure 5).

Discussion

It is well established that the red cell membrane in HS is abnormally permeable to the passive influx of Na (7, 8). If the HS erythrocyte compensates for this increased influx of Na by increasing active efflux, then HS red cells should utilize greater amounts of ATP and glucose than normal red cells to provide the energy for increased active cation transport. The results of the studies presented in this paper indicate that there is increased utilization of ATP and glucose by the HS erythrocyte that may be partially accounted for by increased active cation transport. However, when active transport is completely blocked by ouabain, there remains an increased rate of ATP and glucose consumption by HS red cells compared to normal, which suggests that not all of the increased energy expenditure of the HS red cell can be attributed to hyperactivity of the Na-K pump.

ATP utlization. In both whole blood and washed red cells there was a greater rate of fall of ATP in HS samples than in normal samples (Figures 1 and 2). When whole blood was used, the difference between ATP values in normal blood and HS blood after 6 hours of incubation could be partially explained by increased glucose utilization by the HS samples. In most of the normal samples some glucose remained after 6 hours, whereas there was no glucose present in most of the HS samples after 6 hours. Hence, the lower ATP values in the HS samples could be attributed to both increased utilization of ATP and decreased generation of ATP in the absence of glucose. When washed red cells suspended in a glucose-free medium were used, the more rapid decline of ATP in the HS samples indicated increased utilization of ATP, since no glucose was available for ATP generation. Other workers have also demonstrated a more rapid fall in ATP of HS red cells compared to normal after incubation of washed red cells (18) and whole blood (19).

After it was demonstrated that the HS red cell consumed more ATP than the normal red cell, the question remained as to whether or not hyperactivity of cation transport was responsible for the increase in ATP expenditure. To investigate this possibility, the Na-K pump was blocked by ouabain. Ouabain inhibits that portion of the ATPase in the red cell membrane that is activated by Na and K and renders the energy of ATP unavailable for active cation transport (11, 13). Under these circumstances there was a decrease in the rate of fall of ATP in both normal and HS samples, but during the first hour of incubation ouabain had a greater effect on the ATP of HS cells than on normal cells (Figure 2). This suggests greater pump activity in HS cells than in normal cells during the first hour of incubation, which corresponds with the period in which there was the main difference in the rate of fall in ATP between HS and normal cells. After the first hour ouabain had an equal effect on HS and normal cells, and the rate of fall of ATP was the same in both groups. The change in the rate of fall of ATP and in the effect of ouabain that occurred after 1 hour of incubation in normal red cells (Figure 2) might have been due to some injury to the red cell. The stress of washing the red cells in a glucose-free medium (although at 5° C) followed by incubation in a glucose-free medium may have caused some damage to the red cell membrane so that after an hour of incubation there was increased active cation transport in the normal red cell as well as in the HS cell.

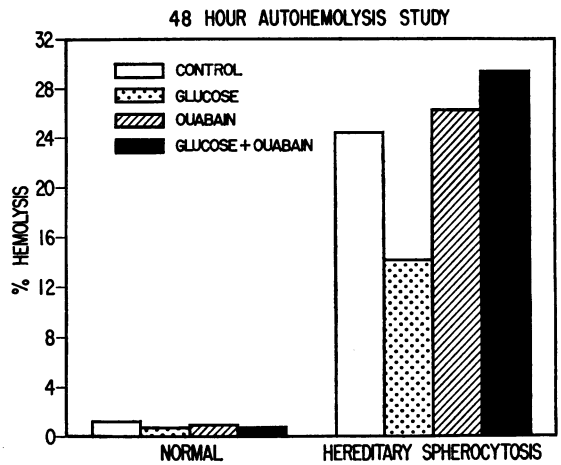

FIG. 5. EFFECT OF GLUCOSE AND OUABAIN ON THE AUTOHEMOLYSIS OF NORMAL AND HS WHOLE BLOOD INCUBATED FOR 48 HOURS. Each column represents the mean for each group. The normal values represent six experiments on blood from three subjects, and HS values represent ten experiments on blood from eight patients with HS. Ouabain was added in a 2.5×10^{-5} M concentration and glucose in a concentration of 30 μmoles per ml.

If the decline of ATP depicted in Figure 2 was the result of utilization of ATP for active cation transport, then there should have been little if any fall in ATP when the Na-K pump was blocked by ouabain, and under such circumstances the fall of ATP in normal and HS cells should have been similar. This was not the case. There was only a slight fall of ATP in normal cells treated with ouabain after 3 hours of incubation with 86% of the ATP remaining. On the other hand, there was a greater rate of decline of ATP in ouabain-treated HS cells with only 73% of the ATP remaining after 3 hours. The increased rate of fall of ATP in HS red cells in spite of ouabain inhibition might have been due to incomplete blockage of Na-K transport as a result of a suboptimal concentration of ouabain. However, when ouabain was increased 100-fold to a 2.5×10^{-3} M concentration, there was no significant difference in the rate of fall of ATP when compared to the 2.5×10^{-5} M level, indicating that the Na-K pump was maximally inhibited at the lower concentration. These findings imply that the HS red cell utilizes ATP at a greater rate than the normal red cell for some energy requirement in addition to that of active cation transport.

In the studies by Post, Merritt, Kinsolving, and Albright (11) and by Dunham and Glynn (13), approximately half of the ATPase activity of the red cell membrane was inhibited by cardiac glycosides, the portion activated by Na and K and responsible for active cation transport. It is unclear how the energy released by the breakdown of ATP by the ouabain-insensitive ATPase is used by the red cell. A recent report by Weed, Bowdler, and Reed (20) suggests that there is an energy-dependent mechanism for the maintenance of the lipid content of the red cell membrane that is insensitive to ouabain. Since they have also shown that HS red cells lose membrane lipid at a greater rate than normal red cells, it is possible that the increased utilization of ATP by HS cells in addition to that used for active cation transport may be for maintaining the lipid integrity of the membrane.

When a glucose-containing membrane was used as shown in Figure 3, the ATP values of normal and HS samples were similar, and both showed an increase in ATP content during incubation.[7] This indicates that ATP generation exceeded ATP utilization and that the HS red cell can maintain its ATP content as long as sufficient glucose is available. This is also suggested by the observation that the ATP content of HS red cells was not significantly different from normal red cells before incubation.

When active cation transport was inhibited by ouabain in the presence of adequate glucose, the rise in ATP content in both normal and HS red cells was greater than when glucose alone was added (Figure 3). A greater rise in the ATP values of HS cells would have been expected when active cation transport was blocked than in normal cells similarly treated if there were greater activity of the Na-K pump in HS red cells. However, there was no observed difference in the effect of ouabain on the ATP of normal and HS red cells in the presence of glucose. This observation might be explained by the fact that ouabain caused a reduction in glucose consumption by HS red cells, whereas it had no effect on glucose utilization by normal red cells (Figure 4). Thus in the presence of ouabain and glucose there may have been a greater depression of ATP utilization by the HS cell compared to the normal red cell, but this would have been balanced by a greater reduction of ATP production in the HS red cell compared to the normal, yielding the same net changes in ATP content.

Glucose utilization. When the rate of glucose utilization was determined with washed red cells in a suspension containing a physiological amount of glucose (5 μmoles per ml or 90 mg per 100 ml), HS red cells consumed 28% more glucose than did normal red cells during 3 hours of incubation (Figure 4). Jacob and Jandl (8) observed a similar magnitude of increase in glycolysis by HS red cells during a 4-hour incubation period (an increment of 34%), but other workers (21, 22) have found no significant difference in glucose consumption between normal and HS erythrocytes.

In the presence of ouabain there was a significant decrease in glucose utilization by HS red cells but not by normal red cells (Figure 4). This

[7] The rise in ATP during incubation may have been the result of some injury to the red cell during washing, as there was some fall in ATP during the washing procedure.

observation was further supported by the changes in the pH of the red cell suspensions that occurred during incubation. Samples with increased glucose utilization would be expected to have a greater fall in pH during incubation because of the formation of increased amounts of lactic acid as shown by Jacob and Jandl (8). This was the case when normal and HS samples to which glucose had been added were compared; the HS samples utilized more glucose and had a significantly greater fall in pH than did the normal samples. The addition of ouabain resulted in less glucose utilization by the HS samples associated with a significant lessening in the fall of pH, but it had no significant effect on either glucose utilization or change in pH when added to normal samples (Table I).

The amount of decrease in glucose utilization induced by ouabain in the HS red cell of 16% was similar to that observed by Jacob and Jandl (8), who found that ouabain decreased glucose consumption in HS red cells by 18% and also found no significant effect on normal red cells during a 4-hour period. Murphy (23) found that ouabain did decrease glucose consumption in normal red cells but after prolonged incubation of 20 hours.

If the increased glycolytic rate of the HS erythrocyte is secondary to increased breakdown of ATP by a Na-K activated membrane ATPase to provide energy for increased active cation transport, then inhibition of the Na-K pump by ouabain should abolish the increase in glucose consumption. However, this was not the case. When active cation transport in normal and HS cells was completely blocked by ouabain, there was still a greater rate of glucose utilization and a greater fall in pH in HS red cells than in normal red cells (Figure 4 and Table I). This suggests that the increase in glycolysis observed in the HS red cell is stimulated not only by an increased rate of ATP breakdown for active cation transport but also by increased degradation of ATP for some other pathway.

Effect of glucose and ouabain on autohemolysis. Although ouabain *in vitro* decreases the rates of glucose and ATP utilization in the HS red cell towards normal, it does this by blocking active Na transport and thus inhibits the protective mechanism by which the HS red cell prevents osmotic hemolysis from occurring. This is illustrated by the data shown in Figure 5 concerning autohemolysis of normal and HS red cells. Normal blood incubated for 48 hours showed less than 2% hemolysis and was essentially unaffected by the various additives. On the other hand, the mean value for hemolysis of HS samples to which no additions were made was 24.1% and decreased to 14% when glucose was added. The corrective effect of glucose was most likely the result of ATP generation supplying energy for active Na transport. When ouabain was added, the corrective effect of glucose was abolished. The ATP generated through glycolysis was not then available for active cation transport because of ATPase inhibition by ouabain. When ouabain alone was added, there was no significant difference in the amount of hemolysis compared to the untreated HS samples. This was to be expected since both types of samples were depleted of ATP after prolonged incubation in the absence of glucose, and the blocking effect of ouabain on ATP utilization was of no consequence.

The results of our experiments indicate that there is an increased utilization of ATP by the HS red cell for metabolic processes in addition to active cation transport. Nevertheless, the observation that ouabain abolishes the corrective effect of glucose in the autohemolysis test suggests that increased utilization of ATP for active cation transport is of paramount importance in protecting the HS erythrocyte against hemolysis.

Summary

1. The adenosine triphosphate (ATP) content of incubated whole blood or washed red cells declines more rapidly in samples obtained from patients with hereditary spherocytosis (HS) than in normal samples, indicating a greater rate of ATP utilization by HS red cells.

2. The increased utilization of ATP by HS red cells is partly caused by increased activity of the Na-K pump because ouabain, which blocks active cation transport, has a greater effect on glucose and ATP utilization in HS red cells than in normal cells.

3. When active cation transport in normal and HS cells is completely blocked by ouabain, there remains an increased rate of ATP and glucose consumption by HS cells compared to normal, suggesting that the HS erythrocyte has an increased energy requirement in addition to active cation transport.

4. The corrective effect of glucose in diminishing autohemolysis of HS blood is abolished by ouabain, indicating that the utilization of ATP for active cation transport is of paramount importance in protecting the HS erythrocyte against hemolysis.

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