Assay for Hexokinase Activity in Intact Red Cells and Its Alteration on Storage*

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To determine hexokinase activity in intact red cells, one may either measure the disappearance of glucose or the appearance of glucose 6-phosphate. The former is not absolutely specific for the hexokinase reaction. The latter can only be done after the cells are broken because phosphorylated compounds are reluctant to move across red cell membranes. Alternatively, studies of hexokinase activity can and have been made on hemolysates, but such studies violate intracellular environmental conditions including natural inhibitors, activators, and cofactors. For example, when red cells are hemolyzed, their adenosine triphosphate is rapidly destroyed (1), and adenosine triphosphate is necessary for the phosphorylation of glucose as the enzyme, hexokinase. Our objective was to assay continuously the rate of formation of glucose 6-phosphate in intact red cells.

The assay described in the present work depends on the continuing presence in the intact human red cell of adequate amounts of glucose-6-P dehydrogenase and triphosphopyridine nucleotide such that when methylene blue is added, oxygen uptake will become dependent on the rate of production of glucose-6-P. Oxygen uptake will then measure hexokinase activity. The use of methylene blue to enhance oxygen uptake via shunt pathways has been well established (2). Inasmuch as some standard assays for hexokinase activity in hemolysates are based on the spectrophotometric determination of reduced triphosphopyridine nucleotide in a suitably arranged system (3), it seemed reasonable to extend the principle to an assay based on following oxygen uptake of a suspension of intact red cells to which dye had been added. However, it remained to be established that adequate amounts of glucose-6-P dehydrogenase and triphosphopyridine nucleotide were already present inside the red cells, since adding them extracellularly would be of no use. Fortunately, inosine could be substituted for glucose as a glucose-6-P-generating source, and when this was done, the oxygen uptake with methylene blue proved the adequacy of supply of glucose-6-P dehydrogenase and triphosphopyridine nucleotide. In subsequent utilization of this assay system, an aliquot was always run with inosine to insure adequacy of glucose-6-P dehydrogenase and triphosphopyridine nucleotide stores.

Inasmuch as the hexokinase reaction is the first step in the conversion of glucose to lactate in the mammalian red cell, it is an attractive step to incriminate in the gradual failure of glycolysis that occurs in stored (banked) red cells. Both Denstedt and Rapoport have been advocates of this idea, the one on the basis that the pH decrease during blood storage forces hexokinase out of its optimal pH range (4), the other espousing the view that hexokinase activity falls during red cell ripening to a level barely sufficient to maintain normal glycolysis, and hence constitutes a vulnerable point in the curtailment of glycolytic ability (6). With the assay described here, it was possible to show that the main reason for failing glycolysis in banked human red cells was a lack of ATP; the enzyme, hexokinase, being essentially unharmed by its sojourn in cold acid-citrate solution.

EXPERIMENTAL PROCEDURE AND RESULTS

Details of Assay

The assay is performed much as described by Brin and Yonemoto (2), with 2.0 ml of prepared blood and 0.6 ml of buffer in the main chamber of the Warburg vessel, 0.2 ml of 15% KOH in the center well, and 0.2 ml of 0.05% methylene blue solution and 0.2 ml of either 3% glucose or 2.25% inosine solution in the side arm. Incubation is performed at 37°C. Manometers are read and reset about every half hour for about 3 to 4 hours. Values are expressed as microliters of O2 taken up per minute per 100 g of hemoglobin. The practice of expressing all values in terms of hemoglobin has become accepted in this laboratory because the hemoglobin of red cells remains quite constant during their life and it therefore is a conveniently determined measure of the number of red cells participating in a metabolic reaction.

Under the above conditions, fresh human red cells resuspended in Krebs-Ringer-phosphate buffer (6), at pH 7.4, and with the amounts of glucose mentioned earlier, had an oxygen uptake of about 600 μl per minute per 100 g of hemoglobin. With inosine in the aforementioned amounts, the O2 uptake was roughly the same. Increasing the amounts of glucose or inosine had no effect on the rate. It was conjectured, however, that the amounts of substrate indicated might not be adequate for longer periods of incubation since it was noticed that by 3 hours the O2 uptake sometimes began to fall off.

Preliminary Experiments

In initial experiments to test the assay system, red cells were separated from ACD blood1 that had been under the usual storage

1 ACD Solution B. The bottle for the collection of 480 ml of blood contains 120 ml of a solution consisting of 0.44 g of citric acid, U.S.P. (anhydrous), 1.32 g of sodium citrate, U.S.P., and 1.47 g of dextrose, U.S.P., per 100 ml of solution. Blood is drawn aseptically by vacuum into the bottle and stored at 4°C. Such blood stored more than 21 days is considered outdated and is usually obtained for study in the first few days thereafter.
conditions for only a day or two. These cells were resuspended in buffer at pH 7.4. Various amounts of glucose or inosine were added as substrate until it was apparent that the amounts finally specified in the assay system were not limiting the rate of O2 uptake. Neither the amount of methylene blue nor the gassing mixture (air) was varied from the conditions arbitrarily selected at the outset on the basis of the work of Brin and Yonemoto (2). Fig. 1 shows O2 uptake curves of 1-day-old ACD blood with both inosine and glucose as substrates. It is apparent that the O2 uptake with glucose as substrate lags behind the O2 uptake with inosine, but that both finally run at about the same rate for the duration of the experiment. This behavior suggests that the oxidation of glucose-6-P is the limiting factor in both reactions since it is the only common portion of both systems.

The same blood was restudied on its 6th day of storage, at which time the second set of curves in Fig. 1 was obtained. The rate of oxygen uptake with glucose as substrate was now less than with inosine. Since the inosine flasks were able to maintain good oxygen uptake, we must conclude that the ability of the red cells to oxidize glucose-6-P in the presence of methylene blue has not been impaired by the period of storage. The oxygen uptake in the glucose flasks must therefore be lower because the hexokinase reaction has become rate-limiting. Hence, under these circumstances, it works out that hexokinase activity can be measured by oxygen uptake. This is caused by a fortuitous combination of reaction rates, availability of factors, and differential effects with storage. If the results with the two substrates had been reversed, the system obviously would not have been readily adaptable for hexokinase assay. It will be clear in the subsequent development that when hexokinase activity is mentioned the presence of both the enzyme and ATP, as well as other factors, is implied.

Experiments with Outdated Blood

It is common knowledge that outdated ACD blood has a low glycolytic rate even though its plasma has adequate supplies of glucose. Preliminary assays of such blood with the use of the methods just described showed that with glucose as substrate, outdated red cells resuspended in buffer at pH 7.4 had O2 uptake values of one-third to one-half of those seen in fresh blood (even these figures are above those likely to be seen if one incubates red cells at the pH encountered in outdated blood, which is usually pH 6.8 or below). However, when inosine was used as substrate at pH 7.4, the O2 uptake was similar to that seen in fresh blood. These observations suggested that (a) the enzymatic pathways from inosine to phosphogluconic acid were essentially unaffected by storage; and (b) the hexokinase reaction had become less competent during the storage period.

In reviewing the situation and keeping in mind that both the enzyme hexokinase and the cofactor ATP are required for the conversion of glucose to glucose-6-P, several possibilities were considered. If Denstedt's ideas about stored blood were correct that glycolysis failed because hexokinase would not operate efficiently at the lower pH values encountered later in storage, then resuspending the red cells in pH 7.4 buffer ought to restore their glycolytic ability. Since this had not occurred in our experiments and since in other experiments quite active glycolysis had been seen at pH values as low as 6.3, this explanation seemed inadequate. If Rapoport were correct that hexokinase was in limiting supply and if one made the further assumption that some hexokinase was destroyed or inactivated during storage, the end result would be consistent with our assay results. Proof of this explanation would depend on showing an actual decrease in enzyme activity during storage. Attempts to show this appear to have been made on hemolysates, not on intact red cells.

A third possibility had been alluded to by Gabrio, Hennessey, Thomasson, and Finch (7), who quoted E. C. Krebs as saying that the failure in hexokinase activity was related to the disappearance of ATP, not to incompetence of the enzyme. No data were cited in that communication. I (8) and others have published adequate data to show that ATP concentration does fall markedly in stored ACD blood. Furthermore, since ATP levels can be raised in red cells by incubating them with a combination such as inosine and adenine, it appeared possible to test the idea that ATP availability was indeed the limiting factor in the hexokinase reaction in stored blood. To pursue this idea, the following experimental design evolved. Two samples of recently outdated ACD blood were centrifuged, and the cells were washed once with cold Krebs-Ringer-phosphate buffer, pH 7.4. One sample (blood sample 1) was resuspended in the same buffer containing 40 mg of inosine and 10 mg of adenine per 10 ml of buffer, the hematocrit being about 50%, and incubated for 2 hours at 37°. At the end of this time, the cells were washed three times in the cold with fresh buffer, the other sample meanwhile having been washed a total of three times with cold buffer. Five pairs of duplicate Warburg flasks were then set up according to the previously described assay system. From the nonevolved blood (blood sample 2) one pair of flasks was set up with the standard amount of glucose as substrate. A second pair of flasks contained the standard amount of inosine as substrate. From the incubated blood (blood sample 1), three pairs of flasks were set up. One pair had no added substrate, one pair had glucose, and one pair had inosine. The necessity of setting up incubated blood without added glucose is obvious since if all the inosine were not washed out after the incubation, this inosine would act as substrate and simulate a glucose effect.

In all, four experiments were run. Two samples of outdated blood were used. Duplicate flasks were set up on each of 2 days for either blood. The results are shown in Fig. 2.

From these data, it is apparent that in the unincubated red cells (i.e. unincubated with adenine and inosine), the rate of oxygen uptake in the assay system is considerably lower from glucose than from inosine. Although we have no uptake values

![Fig. 1. Oxygen uptake in the presence of methylene blue, with either inosine or glucose as substrate, in ACD blood either fresh (1 day) or stored for a few days.](image-url)
with glucose in these bloods before they were stored, later experiments will show that the values for glucose in freshly drawn blood are approximately the same as for inosine, and that the inosine values do not change much during 3 to 4 weeks of storage. In the aliquots that were incubated with adenine and inosine, the O\textsubscript{2} uptake with glucose as substrate was approximately doubled and brought into the range of the inosine values (i.e. restored to approximately prestorage levels). This effect could not have been caused by the residual inosine not washed out since the flasks of incubated blood without glucose showed essentially no oxygen uptake. The only apparent explanation is that the ATP levels in the incubated blood were now similar to those in fresh blood, and the hexokinase reaction now possessed both needed factors, namely enzyme and ATP.

The logical deduction from these experiments is that the enzyme hexokinase does not suffer any appreciable decrement during blood storage in ACD for 3 to 4 weeks, but that the cofactor in the hexokinase reaction, ATP, does decrease, and hence becomes the limiting factor in glucose phosphorylation. When the ATP level is restored, glycolysis (at least glucose phosphorylation) is restored. The chain of events in this system is unusual in that Embden-Meyerhof activity is required to produce ATP, and ATP is required to initiate the Embden-Meyerhof series of reactions. Thus, if ATP is consumed inordinately, the synthesis of ATP becomes jeopardized. Blanckaer, Brownstone, and Williams (9) concluded some years back that the vulnerable spots in glycolysis were at the hexokinase and phosphofructokinase reactions, especially the latter. Although they presumably had in mind the enzymes involved, these are precisely the reactions in glycolysis that require ATP and both would fail in its absence.

**Stored Bloods**

Six donors each contributed 2 half-pints of blood. One half-pint was drawn in the usual ACD solution (A samples), and the other half-pint was collected in a heparin-glucose-phosphate-adenine solution (B samples) which had been found to maintain ATP levels better during storage. The assay for hexokinase activity was performed on all samples after 0, 2, and 4 weeks of storage at 4°C both without and with incubation with adenine and inosine. These results are shown in Fig. 3. Among other determinations performed, the blood nucleotides were measured (10). The value for ATP (in micromoles per 100 g of hemoglobin) for each unincubated blood sample is shown over the appropriate bar in Fig. 3.

The findings in Fig. 3 confirm what had been predicated from the earlier experiments with incubated blood. Assay of fresh blood (either A or B samples) indicated good O\textsubscript{2} uptake with either inosine or glucose as substrate. After 2 and 4 weeks of storage of the A samples, their ATP concentrations declined as can be seen in Fig. 3, and simultaneously their hexokinase activity declined. Their ability to utilize inosine as substrate was little affected, however. Their ability to utilize glucose was partially or completely restored to initial levels after the red cells were incubated with adenine and inosine to restore the ATP levels. In the B series, on the other hand, ATP levels did not decline, and in general, hexokinase activity was undiminished. The O\textsubscript{2} uptake with inosine as substrate is not shown. It was little affected by storage.

![Fig. 2. Oxygen uptake in the presence of methylene blue in outdated ACD blood. Rejuvenated samples were those which had been incubated with inosine and adenine to restore the ATP levels in the red cell. After washing, glucose was added as substrate. Unrejuvenated samples were simply red cells resuspended in buffer at pH 7.4.](image)

![Fig. 3. Oxygen uptake in the presence of methylene blue in blood stored in ACD (A samples) or in heparin-glucose-phosphate-adenine solution (B samples), incubated either with inosine or glucose as substrate. Hatched bars (rejuvenated) are replicate blood samples which have been incubated with adenine and inosine and then washed, to raise the red cell ATP levels. The figures above the bars indicate ATP concentrations (micromoles of ATP per 100 g of hemoglobin).](image)

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2 For each 100 ml of blood, 20 ml of 0.11 M NaH\textsubscript{2}PO\textsubscript{4} plus 1 ml of heparin solution (1000 units) and 10 mg of adenine. The pH is adjusted to 5.6 with NaOH before autoclaving. In some cases, the solutions were made 0.09 M in phosphate and 0.02 M in citrate or EDTA. These samples were studied in collaboration with Wieslaw Kozek and will be reported in detail elsewhere.
DISCUSSION

When red cells are hemolyzed, their ATP is destroyed (11). Under these circumstances, the assay of hexokinase activity can only be for the enzyme since ATP must be added. The present experiments, in which the rate of phosphorylation of glucose can be measured in the intact cell under reasonably normal conditions, reveal the importance of ATP in the hexokinase reaction. In retrospect, this is not surprising since it is well known that absence of a cofactor can slow a reaction to the stopping point. The stored human red cell happened to be a particularly good system with which to show such an effect with respect to glucose phosphorylation. The particularly happy circumstance here is that glycolysis does slow following storage and that this slowing can be related to reduction in hexokinase activity. Furthermore, when such ATP-deficient red cells are incubated with adenine and inosine to restore the ATP content of the red cells, the hexokinase activity tends to revert to prestorage levels. In addition to this finding, red cells stored in systems in which the ATP level does not fall retain essentially all of their hexokinase activity.

Previous assays for hexokinase activity have generally coupled glucose-6-P oxidation with TPN reduction and have followed the rate of synthesis of TPNH at 340 m,u, spectrophotometrically. The present assay, while following the same basic approach, utilizes the TPN as a carrier, coupled through the methylene blue to oxygen uptake. As a result of this combination, the assay not only is applicable to the intact cell, but has the advantage that the presence of oxygen enhances both the rate of the reaction and the analytical result. In most assays involving TPNH generation, the amount of TPNH measured is determined not only by the activity of the reducing reaction but also by the amount of TPN added, and the amount of TPN lost through air oxidation in the cuvette. These two latter complications are removed in the present assay approach.

Other interesting points are brought out if one calculates how fast glucose is phosphorylated under assay conditions as compared to that under normal conditions. Under assay conditions, the rate of oxygen uptake of fresh blood is of the order of 600 μl of O₂ per minute per 100 g of hemoglobin. This is equivalent to about 27 μmoles of O₂ or 54 μmoles of TPNH oxidized, which is equivalent to the number of micromoles of glucose-6-P oxidized or glucose phosphorylated. When blood is incubated at 37° in spinner flasks, I have found that about 20 μmoles of glucose are utilized per liter of whole blood per 24 hours. Since this amount of whole blood would contain about 140 g of hemoglobin, the number of micromoles of glucose phosphorylated per minute per 100 g of hemoglobin would be

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20,000 \times \frac{100}{140} \times \frac{1}{1,440} = 9.9
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The latter calculation may be somewhat rough but it serves to show that in our assay system, glucose is phosphorylated several times faster than it would be phosphorylated under normal glycolytic circumstances. This increase serves two useful purposes: (a) to make the assay more sensitive; and (b) to stress the system, thus revealing inherent incapabilities which might not show up otherwise.

Yet other interesting points emerge from the previous rough calculations. Among these is the fact that the hexokinase reaction in the red cell is capable of operating faster than it is normally required to operate. If in stored blood, the reaction has such a capability, then glycolytic failure in stored blood can hardly be blamed on failure of the enzyme, hexokinase. Of course, as I have shown, inadequate levels of ATP can cause failure of the hexokinase reaction.

There has always been a question as to what sets the rate of glycolysis in mammalian red cells and why only about 10% of the glucose phosphorylated passes through the shunt reactions. The rate of glycolysis is likely determined by the amount of ADP present, but with the overriding consideration that if ATP concentration falls too low, glucose phosphorylation will stop and all glycolysis will cease. In the light of the present studies, the insignificant shunt activity normally present in red cells would seem to be entirely explicable on the basis that no carrier is normally present in the red cell that will efficiently couple TPNH with oxygen. This would seem to be quite fortunate since otherwise glucose would simply be oxidized with no energy trapping. This simple explanation suggests that the red cell has quite adequate supplies of glucose-6-P dehydrogenase and TPN for much more shunt activity than it normally exhibits, but that this activity is limited or determined by the demand for TPNH (i.e. rate of regeneration of TPN).

SUMMARY

Hexokinase activity has been assayed in intact human red cells by following O₂ uptake in the presence of added methylene blue in Warburg apparatus. The substitution of inosine for glucose as substrate shows that adequate amounts of glucose 6-phosphate dehydrogenase and triphosphopyridine nucleotide are already present in the cells. Stored red cells are a good system with which to show that glycolysis slows not because of failure of the enzyme, hexokinase, but because the cofactor for the hexokinase reaction, adenosine triphosphate, is in limiting amounts. When blood is stored in systems that maintain good adenosine triphosphate levels or if red cells are incubated with adenine and inosine to restore adenosine triphosphate levels, glucose phosphorylation is restored to essentially normal levels. It is suggested that the hexokinase reaction in the human red cell is normally not running at full capacity and also that the portion of glucose 6-phosphate metabolized via the shunt is determined by the availability of oxidized triphosphopyridine nucleotide.

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