Erythrocyte Preservation: A Topic in Molecular Biochemistry

By Beverly W. Gabrio, Clement A. Finch and Frank M. Huennekens

The transfusion of blood is required when sudden depletion of blood volume occurs either through blood loss or decrease in the number of red cells produced by disease. In addition to the customary demands of blood banks for hospital uses, there may be superimposed the needs of war areas, particularly in the event of atomic disaster. The latter requirements can be adequately met, not only by increased procurement of blood, but by the ability to "stock-pile" such blood through prolonged periods of in vitro storage. During storage it is the erythrocyte component of blood which is of major concern.

For a number of years the erythrocyte was considered to be simply a membrane-enclosed entity filled with hemoglobin. Although hemoglobin comprises more than 90 per cent of the total solids of the erythrocyte, more recent research has revealed that there is also an imposing collection of other substances contained therein. In addition to water, proteins, lipids, carbohydrates, certain vitamins and metal ions, there is a group of enzyme systems which function to preserve the integrity of the cell membrane and which, consequently, make the cell nearly an autonomous unit. While in the circulation, certain of the erythrocyte constituents are in a state of dynamic turnover with respect to their precursors whereas, in contrast, the hemoglobin remains static.

When whole blood is withdrawn from the donor and stored at 4 C. in standard ACD medium, the plasma component, as an osmotic fluid, is quite stable for extended periods of time. The erythrocytes, however, begin to deteriorate after the first few days of storage. Some of the physical and chemical changes which occur in stored cells include: the decreased ability to metabolize glucose, the loss of K+ from within the cells and the corresponding replacement by Na+, increased osmotic and mechanical fragility, and the decreased ability of the...
cells to survive when transfused into a recipient. Post-transfusion survival, as measured by the radioactive chromium or the differential agglutination techniques, is undoubtedly the most important criterion of physiologic viability of the cell. By general consent, 21 days has been considered to be the maximum permissible time of storage, since at this time approximately 70 per cent of the ACD-stored cells will survive after transfusion.3

There are two principal approaches to the problem of erythrocyte preservation: (1) to arrest metabolic activity by storing the cells at subzero temperatures in glycerol, and (2) to control the metabolism of the cells at 4 C. through the addition of metabolic substrates or inhibitors.

Studies in this laboratory have been directed toward the latter approach. It was established initially that the phenomenon of erythrocyte deterioration was not due to extraerythrocytic factors such as: (a) the composition of the preservative medium, ACD; (b) fresh, aged, or dialyzed plasma; (c) leukocytes, reticulocytes, platelets, or hemolysates (both with and without stroma); and (d) the gas phase (O2, N2, or air). From these results and the further observation that the ability to withstand storage was unrelated to the in vivo age of the cells, i.e., 0–120 days, it seemed evident that the deterioration must be the result of some internal metabolic failure.

Since deterioration and loss of membrane integrity imply a concomitant loss of energy potential, it appeared worthwhile to examine the qualitative and quantitative picture of the phosphate-containing compounds in the erythrocyte, with particular reference to ATP. As shown in table 1, a "phosphate partition" was determined on a fresh sample of erythrocytes and then after 28 days of storage. It is evident that upon storage there is a marked decline in the level of organic phosphate fractions: EH, which represents the two terminal phosphates of ATP, the terminal phosphate of ADP, and perhaps other phosphate esters of a similar high-energy nature; DH, which represents AMP and various sugar phosphates; and Non-H, which is essentially 2,3-diphosphoglycerate. Corresponding to the diminution of organic phosphate, the P level has increased

* AMP—adenosine monophosphate; ADP—adenosine diphosphate; ATP—adenosine triphosphate; P—total phosphorus; P—inorganic phosphorus; EH—easily hydrolyzable phosphorus; DH—difficultly hydrolyzable phosphorus; Non-H—non-hydrolyzable phosphorus; 2,3-DPG—2,3-diphosphoglycerate; DPN, TPN—di- and triphosphopyridine nucleotide; DPNH, TPNH—reduced DPN and TPN; MHb—methemoglobin.

† Erythrocytes are unique in containing a very high level of 2,3-diphosphoglycerate. Although it has been shown to participate as a catalyst in the conversion of 2- to 3-phosphoglycerate in other tissues, the abnormally large level of this substance suggests an additional metabolic role in the red cell.
markedly, although the concomitant small drop in $P_i$ indicates that most of the $P_i$ is retained within the cells against a concentration gradient. Since the "phosphate partition" method deals with classes of compounds rather than specific compounds, a more complete separation of the individual constituents of the erythrocyte was clearly desirable. At this time, Dr. Van R. Potter called to
our attention the elegant separations of nucleotides which could be effected by the method of gradient elution from ion exchange columns. Figure 1 illustrates a typical elution profile of nucleotides in fresh and stored erythrocytes. It should be emphasized that this figure shows only those substances which absorb light at 260 m\(\mu\), but other anions such as sugar phosphates and 2,3-diphosphoglycerate likewise are eluted from the column at various points and can be detected by appropriate analytical methods. Confirming the data of table 1, the ATP level declines over the storage period, but the problem is somewhat more complex than originally envisioned, inasmuch as the ATP region shows evidence of multiple components, perhaps similar in nature to ATP. There are also several unknown substances in the elution profile which remain to be identified. It is evident from the above results that a general parallelism exists between a decline in certain high-energy phosphate compounds and a decline in the physiological viability of the cells.

The next question which arose was whether or not this “storage lesion” could be either arrested or reversed. The method of tagging individual cell populations with separate iron isotopes (Fe\(^{55}\) and Fe\(^{59}\)) was used to show that deteriorated cells were restored to normal when transfused back into active circulation. This in vivo reversibility of the changes occurring in the stored cell appeared to be an all-or-none phenomenon. If the storage damage had not reached a critical point whereby the cells were lost from circulation within 24 hours after transfusion, the “phosphate partition” and other attendant systems of the remaining cells returned to normal within a few hours. In hepatectomized animals this effect was not observed. Further amplification of this point was obtained by fractionating rabbit liver homogenates by standard techniques into mitochondrial, microsomal, nuclear, and soluble fractions, and incubating stored erythrocytes with each of these fractions. Only with mitochondria was any restorative effect observed. In order for an interaction of this sort to occur between two formed elements, i.e., erythrocytes and mitochondria, either some soluble component must diffuse between the two, or else they must come into contact with the responsible enzymes and substrates on adjacent outside surfaces. The localization of many of the enzymes in the erythrocyte is still in doubt, but from experiments to be described subsequently, it is certain that at least a major portion of the enzymatic system responsible for the restoration phenomenon is found in the soluble, and presumably the interior, portion of the erythrocyte.

As a consequence of the above experiments, it seemed reasonable to search for a relevant chemical substance which could duplicate the restorative action of the more complex systems. Obviously, one cannot add ATP or other phosphate esters, since it is well known that such phosphorylated substances do not readily penetrate the erythrocyte membrane. However, when non-phosphorylated substances related to ATP were added to stored cells, it was found that the nucleoside, adenosine, served as an excellent rejuvenant. When cells stored for 28 days were incubated at 37 C. for one hour with adenosine (cf. table 1), there was a rapid and marked return of the phosphate pattern to its normal state. The rejuvenation is shown also in terms of the nucleotide profile

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* Prankerd and Altman have shown also that the entry of labeled P\(_i\) from the medium into erythrocytes is stimulated upon the addition of adenosine or glucose.
The adenosine effect is two-fold: (a) when cells have already deteriorated upon storage, adenosine exerts a rejuvenating effect; and (b) if adenosine is added to fresh cells, there is a prolongation of the effective period of storage. The latter point is demonstrated in figure 2, where per cent survival as a function of storage time is shown for cells which have been stored in ACD and in ACD supplemented with adenosine. It is apparent that cells stored with adenosine have a considerably better survival than those stored in ACD alone. Adenosine-supplemented cells can be stored for 40–50 days and still be suitable for transfusion, in contrast to the previous limit of 21 days.

Since it was established that adenosine improved the storage of erythrocytes, it was of interest to elucidate the mechanism of action of this substance. Undoubtedly, adenosine is not merely penetrating the cells and then being itself directly phosphorylated to give ATP, since no counterpart of this reaction exists in other cells. More likely, adenosine is being metabolized in such a way that one of its breakdown fragments is utilized ultimately in a reaction which results in the net synthesis of ATP. Support for this belief was given by the observation that adenine and ribose, alone or in combination, did not give any restoration. Some time ago, Dische suggested that adenosine is rapidly metabolized by erythrocytes via a nucleoside phosphorylase reaction to yield adenine and ribose-1-phosphate as shown in equation 1, whereupon ribose-1-phosphate is further metabolized to yield various other phosphate esters.

* It seems reasonable from analogy with other tissues to assume that adenosine brings about first the synthesis of ATP, and that this substance, in turn, resynthesizes many of the other phosphate esters through various kinase reactions.
Adenosine $+ P_i \rightarrow$ Adenine $+ \text{Ribose-1-phosphate}$

In addition, it was determined that various nucleosides such as inosine, adenosine, guanosine, xanthosine, and deoxyadenosine, in that order, were effective in the restoration of stored cells. All of these substances can give rise to ribose-1-phosphate and the corresponding purine base except deoxyadenosine, which would give deoxyribose-1-phosphate. Only purine nucleosides were effective; the pyrimidine nucleosides (cytosine, uridine, and thymidine) were lacking in ability to effect the rejuvenation, although they did not interfere with the utilization of purine nucleosides. In order to achieve the maximum effect, it was necessary to add about 2500 $\mu$M of adenosine (of which about 60 per cent is absorbed) per 100 ml. of red cells. This corresponds approximately to a one-to-one ratio of adenosine utilized to organic phosphate resynthesized. On the other hand, cells which have been rejuvenated at these high levels of adenosine must be washed by centrifugation to remove the unabsorbed nucleoside, which produces transient hypotension upon intravenous administration. Because of this potential toxicity, we decided to explore the problem further in the hope of encountering an alternate, nontoxic substance which would achieve the same restorative effect. Fortunately, biochemical aspects of this problem can be studied in stroma-free hemolysates in which adenosine still retains its capacity to resynthesize phosphate esters. Of course, the various cellular criteria, including physiological viability, cannot be studied under these conditions, but since resynthesis of phosphate esters parallels the restoration of cellular viability, it seemed permissible to continue our investigations using lysed cell preparations.

The resynthesis of ATP from adenosine by way of a nucleoside phosphorylase reaction focuses attention upon alternate pathways of carbohydrate breakdown existing within the erythrocyte. As shown in the abbreviated scheme (fig. 3), glucose may be metabolized via glycolysis to yield lactic acid, a reaction sequence which is considered to be the major energy source for the erythrocyte. However, the existence of an alternate pathway, whereby glucose is metabolized oxidatively (viz., glucose-6-phosphate to 6-phosphogluconate) is known to occur also in the erythrocyte. In glycolysis, ATP is required to prime the reaction although there is a net synthesis of two moles of ATP per mole of glucose metabolized in the over-all reaction. However, when adenosine is metabolized via the shunt mechanism, no ATP is required initially, and there is a net synthesis of ATP. This is consistent with our earlier observation that at least one mole of adenosine is required for each mole of organic phosphate resynthesized.

In order to understand the adenosine effect on a molecular level, our efforts were necessarily directed toward the entire oxidative shunt pathway. As shown in Fig. 3, the hexokinase-catalyzed reaction, wherein glucose is phosphorylated by ATP to yield glucose-6-phosphate, is the same initial reaction for both glycolysis and the shunt pathway. A rather high level of ATP, as reflected by the Michaelis constant, $K_m \approx 10^{-2}$, is required in the hexokinase reaction, and this fact suggests an explanation for the storage lesion. Deterioration of the cells begins when the destruction of ATP exceeds its synthesis. Immediately, then, the declining level of ATP decreases the further utilization of glucose owing to the unfavorable Michaelis constant of the hexokinase reaction.
In turn, this decreases the synthesis of additional ATP, so that the cycle perpetuates itself until the cell exhausts its primary energy source, ATP. An investigation of the erythrocyte ATP-ase has shown that this enzyme is localized largely in the soluble fraction of the cell, and that it is activated by Mg\(^{2+}\) and inhibited by Ca\(^{2+}\). As in other tissues, it appears that this enzyme exists in the viable cells in a latent state whereby it does not attack ATP readily. However, as deterioration of the cell occurs, possibly with a corresponding loss of structural integrity, the enzyme becomes more active and thus hastens the deterioration process by destroying the very substance (ATP) which could obviate the original structural breakdown.

The next enzymatic step investigated was the oxidation of glucose-6-phosphate to 6-phosphogluconate. The first coenzyme for this reaction is TPN, and the ultimate electron acceptor is molecular oxygen.\(^7\) The problem was to establish the nature of the electron transport system which mediates the transfer of electrons between TPNH and oxygen. It has been known for a number of years that methylene blue stimulates this particular reaction in erythrocytes,\(^6\),\(^7\) although its precise mode of action was not clear. In this laboratory it has been possible to isolate from the erythrocyte in highly purified form an enzyme, methemoglobin reductase,\(^8\) which catalyzes the reaction:

\[
\text{TPNH} \quad \text{or} \quad \text{DPNH} \quad \text{\(e^-\)} \quad \text{Bound Hematin} \quad \text{Soluble Cofactor} \quad \text{O}_2 \quad \text{or} \quad \text{methylene blue} \quad \text{MHb}
\]

Electrons are transferred from the reduced pyridine nucleotides to a hematin component (absorption maximum at 410 m\(\mu\)) firmly bound to the enzyme and then to a soluble electron carrier (absorption maximum at 400 m\(\mu\)) or methylene blue and finally to oxygen. The latter is reduced to hydrogen peroxide. Either
As yet we have not investigated the conversion of 6-phosphogluconate to a 5-carbon compound, but from results obtained by others, it is probable that this reaction involves a decarboxylation to ribulose-5-phosphate followed by isomerization to ribose-5-phosphate. Adenosine is fed into the shunt system at this junction by being split to ribose-1-phosphate which, in turn, is converted through an isomerase to ribose-5-phosphate. The initial enzyme of the rejuvenation process, nucleoside phosphorylase, has been isolated recently in this laboratory. In order to accomplish the isolation, it was necessary to employ an assay system suitable for use in hemolysates containing large quantities of hemoglobin. The method of choice was a manometric system, used previously with rat liver phosphorylase, whereby inosine serves as the substrate and is broken down to hypoxanthine and ribose-1-phosphate. In the presence of added xanthine oxidase, hypoxanthine is oxidized to uric acid with a corresponding uptake of oxygen, as shown in the following diagram:

\[
\begin{align*}
P_i & \rightarrow \text{Ribose-1-phosphate} \\
\text{Inosine} & \xrightarrow{\text{Phosphorylase}} \text{Hypoxanthine} \quad \xrightarrow{x.o. \ [O]} \quad \text{Xanthine} \quad \xrightarrow{x.o. \ [O]} \quad \text{Uric acid} \\
\text{AsO}_4 & \rightarrow \text{Ribose-1-arsenate} \\
& \quad \text{Ribose}
\end{align*}
\]

where x.o. = xanthine oxidase. A component study of this system with the purified enzyme indicates that either phosphate or arsenate is obligatory and that both the phosphorylase and the xanthine oxidase are required for oxygen uptake. After considerable purification of the enzyme, it is possible to employ a spectrophotometric assay wherein the same assay system is used but the appearance of uric acid is measured by an increased light absorption at 290 nm. About a 100-fold purification of the enzyme has been achieved using adsorption and elution from calcium phosphate gel, ammonium sulfate fractionation and selective heat denaturation. The enzyme appears to have no cofactor or metal ion requirement. An interesting observation is that the enzyme is inhibited competitively by pyrophosphate \((K_i = 4 \times 10^{-4} \text{ M})\), a finding which might be anticipated since phosphate is an obligatory reactant \((K_m = 1 \times 10^{-2} \text{ M})\). With the purified enzyme, various nucleosides can be assayed in the presence of arsenate, whereupon the ribose liberated is estimated colorimetrically. In this manner it was possible to show that inosine and guanosine are the only two nucleosides which are split readily by the purified phosphorylase. This is consistent with the observation of Kalckar, who found that rat liver phosphorylase was specific for the same two nucleosides. Adenosine, and to a lesser extent, xanthosine appear to be broken down but at a very slow rate. It would seem that the effectiveness of adenosine during storage can be explained either by the assumption that there
exists another phosphorylase with different specificity or that adenosine is converted first to inosine and split subsequently by the phosphorylase. The latter possibility is more likely, since an adenosine deaminase has been demonstrated previously in the erythrocyte, and in restoration experiments with adenosine, we have observed an accumulation of both inosine and hypoxanthine, but not adenine. The mechanism of breakdown and utilization of deoxyadenosine and xanthosine is currently under investigation.

From a consideration of the chemical and physiological evidence, we believe that inosine, rather than adenosine, is the nucleoside of choice for the preservation of blood. Erythrocytes can be stored equally well with inosine or adenosine, but the former does not exhibit the toxic properties shown by adenosine upon intravenous administration. Since inosine is the actual substrate for the phosphorylase reaction, the use of adenosine introduces an additional substance, ammonia, into the system.

It is planned to investigate further the metabolic fate of the 5-carbon sugars in the erythrocyte. At present it is not known whether pentose phosphate undergoes a simple aldolase splitting to give 2- and 3-carbon fragments, or whether the more complicated "pentose cycle" obtains. It is likely that a triose such as 3-phosphoglyceraldehyde, is utilized ultimately to synthesize ATP. It is hoped that studies in the area of the trioses will disclose additional information on the role of 2,3-diphosphoglycerate in erythrocyte metabolism. In this latter connection it is of interest that in a type of hereditary, nonspherocytic, hemolytic anemia, the level of 2,3-diphosphoglycerate is higher than in the normal cell, indicating a specific metabolic lesion which, in turn, reflects a specific physiological lesion.

Obviously, much remains to be done in the field of erythrocyte metabolism. However, at this point we are beginning to understand, at least to a certain extent, some of the physiological functions of the erythrocyte and how these events are controlled by the underlying enzyme systems. It is the belief in the utility of the biochemical approach to medical problems and the examples presented in this paper which have prompted the title, "Erythrocyte Preservation: A Topic in Molecular Biochemistry."

**Summary in Interlingua**

Le problema del preservatiomi de erythrocytos es de importantia cardinal in le complexo del problemas del immagazinage de sanguine. Le rapide deterioration de erythrocytos, que se initia intra alicun dies post le comenciamiento del immagazinage, es le effecto de activitates metabolic involvente constituentes erythrocytic altere que hemoglobina.

Le solution del problema pote esser cercate in duo manieras: (1) Per arrestar le activitate metabolic del cellulas per immagazinar los a temperaturas sub zero in glycerol e (2) per regular le activitate metabolic del cellulas al temperatura de 4 C per le addition de substratos e inhibitores metabolic. Le presente reporto es concernite con studios in le secunde del duo mentionate directiones.

Post demonstrar que le phenomeno del deterioration erythrocytic non resulta...
de factores extraerythrocytic sed presenta un interno disfallimento metabolic, le autores se proponeva examinar le qualitative e quantitative aspectos del destino del phosphatic compositos intraerythrocytic, con attention special a triphosphato de adenosina.

Il esseva constata te que le nivello del triphosphato de adenosina se reduce in le curso del immagazinage. Il appareva que adenosina meliora le immagazinage de sanguine.

triphosphato de adenosimia.

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KREBS, E. G.: Personal communication.

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NORBERG, B.: Personal communication.


RUNDLES, W.: Personal communication.

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