ERYTHROCYTE PRESERVATION

IV. IN VITRO REVERSIBILITY OF THE STORAGE LESION*

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Previous investigations (1–3) have shown that the biochemical lesion occurring during the storage of blood in acid-citrate-dextrose at 4° is primarily an intrinsic metabolic failure of the erythrocyte. Alterations in extraerythrocytic factors such as plasma, leucocytes, reticulocytes, hemolysates, citrate, and gas phase were ineffective in alleviating or accelerating the degenerative process in the red cell during storage. The storage lesion was found also to be unrelated to senescence of the red cell *in vivo*.

The biochemical lesion of storage was rapidly reversed after the introduction of stored cells into the active circulation of a normal animal as measured by a regeneration of cellular organic phosphates, especially ATP.[1] A similar reversal was found to occur also in the transfused erythrocytes of man. These restorative changes were interpreted to indicate an increased metabolic potential of the cell and were paralleled by other physiological alterations of the cell. 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.

The present study is concerned with the reversibility of the biochemical lesion of storage *in vitro*, with special reference to the use of the nucleoside, adenosine.

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[1] ATP = adenosine triphosphate; ADP = adenosine diphosphate; TPN = triphosphopyridine nucleotide.

Materials and Methods

Acid-citrate-dextrose preservative[2] was used routinely for the storage of human and rabbit blood at 4°. The final concentration of the preservative components in the blood was approximately 0.264 per cent sodium citrate, 0.096 per cent citric acid, and 0.294 per cent glucose.

Phosphate partitions on suspensions of erythrocytes in 0.9 per cent NaCl were performed as described previously (1).

Adenosine concentration was calculated from the absorption at 258 mμ as measured with a Beckman model DU spectrophotometer; a millimolar extinction coefficient of 14.1 was used (4). For the calculation of adenosine concentration in plasma, the difference in absorption at 258 mμ between adenosine plus plasma and a plasma control was employed.

Glucose was determined colorimetrically (5) on a blood filtrate prepared according to the method of Somogyi (6).

The subcellular components of rabbit liver were prepared according to the procedure of Lee and Williams (7), except that the nuclear, mitochondrial, and microsomal + residual fractions were obtained by successive centrifugations of the homogenate at 600 × *g* for 10 minutes and at 29,000 × *g* for 30 minutes respectively.

EXPERIMENTAL

Effect of Subcellular Components of Liver on Storage Lesion—To test the action of liver particulates *in vitro*, stored blood was incubated at 37° for 1 to 2 hours with the liver fractions, and phosphate partitions were carried out on the red cells at the end of this time (Table I). Phosphate analyses of the particulate fractions alone indicated that, in the amounts used, there was negligible contribution of the particulates *per se* to the red cell phosphate values if any cross-contamination existed. Furthermore, in preparation of red cells for analysis, it was possible to remove most of the particulate fraction as well as white cells which sedimented above the erythrocytes. From the results of a typical experiment shown in Table I, it is apparent that the mitochondrial fraction provided some stimulus for the resynthesis of organic phosphates. The phosphorus was derived partially from the cellular inorganic phosphorus fraction, which had increased during storage, and from inorganic phosphorus withdrawn from the plasma medium. Apparently a diffusible substance from the mitochondria was the active agent. Although the regeneration of organic phosphates of the stored cells was significant, the reversibility was not comparable to that obtained *in vivo* as demonstrated previously. The cellular difficultly hydrolyzable phosphate fraction was not increased after treatment with liver particulates.

[2] National Institutes of Health, Formula B.

Substances such as succinate, α-ketoglutarate, fumarate, and malate, dinitrophenol, coenzyme A, various amino acid mixtures, and nicotinamide, when added to blood either alone or in combination with the liver fractions, neither enhanced nor inhibited the changes observed with incubated stored cells alone or those observed with only liver particulate additions.

Effect of Adenosine Triphosphate Derivatives on Storage Lesion—Compounds such as adenine, ribose, inorganic orthophosphate, adenosine, and adenylic acid were incubated 1 hour with stored blood, and the phosphate partitions of the red cells were determined. In Table II are recorded the results of a typical experiment in which 20 μmoles of each substance were used. It is apparent that supplemental adenosine provided the greatest

TABLE I

Effect of Liver Particulates on Stored Erythrocytes

8 ml. aliquots of rabbit blood (27 days stored) were incubated for 1 hour at 37° with 0.5 ml. of liver particulate, adjusted so that the concentration of the particulate was equivalent to that present in a 10 per cent homogenate.

Sample	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Control stored blood	1207	131	155	704	2197
Blood + homogenate	1204	155	130	740	2229
" + nuclear fraction	1203	153	139	715	2210
" + mitochondrial fraction	1176	173	134	869	2352
" + microsomal + residual fraction	1205	153	118	758	2234

impetus to the resynthesis of red cell organic phosphates and that a noteworthy reconstitution was obtained in all fractions. Although both muscle and yeast adenylic acids caused some hemolysis of the red cells, they proved to be relatively stimulatory. The apparent uptake of the latter two compounds was a somewhat unexpected finding owing to the known limited permeability of phosphorylated compounds. Ribose-5-phosphate, ATP, and ADP were found to yield negative results in whole cell systems. There was no adenosine synthesis from the addition of adenine and ribose, as expected, since ribose-1-phosphate is required for this nucleoside synthetic mechanism.

The variation in the amounts of total cellular phosphorus in the experimental samples (Table II) can be accounted for by differences in retention of inorganic phosphorus after incubation. After exposure of stored blood to 37°, there is a diffusion of some of the accumulated inorganic phosphorus

to the extracellular environment (Table III). Treated stored blood retains varying amounts of this phosphorus for the resynthesis of organic phosphates and often withdraws phosphorus from the plasma, depending upon the effectiveness of the added substance. In other cases, in which there is no rejuvenation of treated cells, the membrane permeability may be changed by the agent, thus allowing more or less inorganic phosphorus diffusion.

In all of the experiments described, whole cell systems were used, and the uptake of the adenine-containing compounds tested except ATP and ADP was about the same; *i.e.*, 50 to 70 per cent in 1 hour at 37°.

TABLE II

Effect of ATP Derivatives on Stored Erythrocytes

9 ml. aliquots of human blood (stored 27 days) were incubated 1 hour at 37° with 20 μmoles of the specific compound indicated. The hematocrit of the cell systems was 30.

Sample	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Control	686	155	52	113	1006
Adenine	621	150	50	130	951
Ribose	653	134	39	163	989
Adenine + ribose	617	170	10	161	958
" + " + inorganic orthophosphate	780	182	32	78	1072
Adenosine	269	301	236	323	1129
" + inorganic orthophosphate	460	303	211	322	1296
Muscle adenylic acid	662	184	68	200	1114

Characterization of Adenosine Effect—A similar reversal was produced by adenosine in stored rabbit and human erythrocytes, and the following results are considered equally applicable to both species.

(*a*) *"Absorption" of Adenosine*—Inasmuch as the uptake of adenosine by the red cell could be absorption of the compound into the cell or adsorption onto the membrane, the phenomenon will be referred to hereafter as "absorption." The absorption of adenosine presented to the cells in optimal concentration to produce the red cell effect (2500 μmoles per 100 ml. of red blood cells) was tested at 37° with human blood (stored 30 days) over a period of 1.5 hours. The per cent absorption was as follows: 15 minutes 35 per cent, 30 minutes 44 per cent, 1 hour 61 per cent, and 1.5 hours 71 per cent. These values were reproducible in all experiments with stored blood. Accordingly, most of the reversibility studies have been

carried out over a period of 1 hour at 37° and about 60 per cent absorption of adenosine was obtained.

(*b*) *Concentration of Adenosine*—The effect of varying concentrations of adenosine on human red cells stored 25 days is shown in Table III. The optimal concentration of adenosine to produce maximal reconstitution of cellular organic phosphates was approximately 2500 μmoles of adenosine per 100 ml. of red cells. At this concentration of added substance, the total phosphorylated compounds were regenerated to a level which was comparable to that present in fresh blood. The absorption of adenosine by

TABLE III

Concentration of Adenosine and Rejuvenation of Stored Erythrocytes

11 ml. aliquots of human blood (stored 25 days) were incubated 1 hour at 37° with increasing amounts of adenosine.

Adenosine added per 100 ml. red blood cells	Absorption of adenosine	μmoles phosphorus per 100 ml. red blood cells				
		Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
μmoles	*per cent*					
Fresh		165	230	181	922	1498
Stored 25 days						
Non-incubated control		945	169	81	79	1274
Incubated control		680	185	71	67	1003
125	100	613	262	77	132	1084
375	74	440	325	151	256	1172
625	64	299	362	243	355	1259
1250	68	151	407	311	384	1253
2500	62	98	436	361	461	1356
7500	31	99	438	375	461	1373

the erythrocytes varied somewhat with the amount added to the blood. However, within a concentration range of 625 to 2500 μmoles per 100 ml of red blood cells, about two-thirds of the added nucleoside was absorbed.

An examination of the efficiency of adenosine indicates that over a wide range of adenosine concentration a resynthesis of organic phosphates occurs (Fig. 1). A linear relationship appears to obtain, and approximately 1 μmole of organic phosphate is resynthesized for 1 μmole of adenosine absorbed. At higher concentrations of the nucleoside the ratio of micromoles of organic phosphate resynthesized to micromoles of adenosine absorbed declines; this is probably a reflection of more absorption than utilization of the adenosine molecule.

A Michaelis constant, K_m, of 3.6×10^{-3} mole per liter was obtained from data in which 125 to 7500 μmoles of adenosine per 100 ml. of red blood cells

were incubated for 1 hour at 37°. In these computations, v = the organic phosphate resynthesized, expressed as μmoles of organic P per 100 ml. of red blood cells per hour, and s = the concentration of adenosine absorbed,

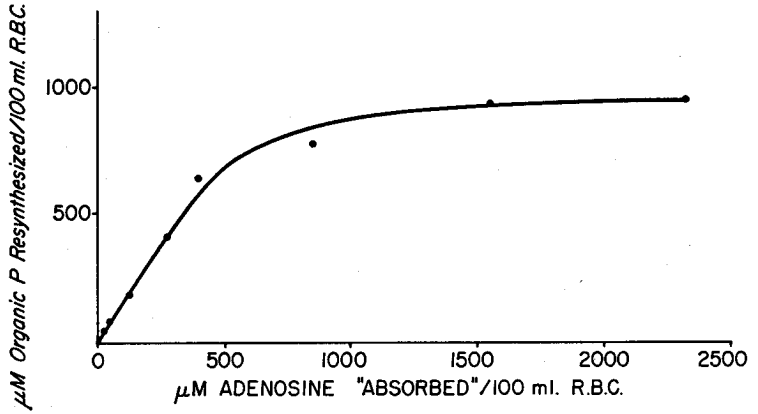

FIG. 1. The relationship between adenosine absorption and the resynthesis of erythrocyte organic phosphates. Stored blood was incubated for 1 hour at 37° with varying concentrations of adenosine.

TABLE IV

Effect of Temperature on Rejuvenation of Stored Red Cells with Adenosine

11 ml. aliquots of human blood (stored 28 days) were exposed to adenosine in the concentration of 2400 μmoles per 100 ml. of red blood cells at 2°, 23°, and 37° for 1 hour.

	Absorption of adenosine	μmoles phosphorus per 100 ml. red blood cells					
		Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total	Increase in organic P
	per cent						
2°. Control		808	163	87	155	1213	
2°. Adenosine	24	654	230	124	220	1228	169
23°. Control		754	199	80	127	1160	
23°. Adenosine	50	337	272	258	395	1262	519
37°. Control		559	206	47	177	989	
37°. Adenosine	69	88	376	376	473	1313	795

expressed as μmoles per 100 ml. of red blood cells per hour, and the conventional plot of $1/v$ and $1/s$ was employed.

(*c*) *Effect of Temperature—In vitro* reversibility with adenosine occurred at 2° as well as 37° (Table IV). Although the increase in organic phosphates resynthesized in stored blood exposed to adenosine at 2° for

1 hour was only 169 μmoles per 100 ml. of red cells compared with 795 μmoles in blood incubated at 37°, the amount of adenosine penetrating the cell at the lower temperature was approximately one-third that found at 37°. The calculated activation energy for this process, which is a composite of the resynthesis of cellular organic phosphates and the diffusion of adenosine through the cell barrier, was 6540 calories per mole per degree over the temperature range of 2–37°. It was demonstrated in other experiments that full restoration did occur at 2° over a longer period of time.

(*d*) *Adenosine Effect As Related to Duration of Storage*—The effect of adenosine on blood stored for 4 weeks has been illustrated in Tables II to IV. In Table V the effect of adenosine on fresh blood is illustrated. It will be observed that even those cells containing a normal phosphate composition

TABLE V

Effect of Adenosine on Fresh and Stored Red Cells

10 ml. aliquots of human blood were incubated with adenosine (1500 μmoles per 100 ml. of red blood cells) for 45 minutes at 37°.

	μmoles phosphorus per 100 ml. red blood cells				
	Inorganic	Easily hydrolyzable	Difficultly hydrolyzable	Non-hydrolyzable	Total
Fresh. Control	245	304	161	776	1486
" Adenosine	39	375	230	1002	1646
Stored 12 days. Control	592	269	124	332	1317
" 12 " Adenosine	111	449	309	734	1603

increased in the organic phosphate fractions. In the same blood stored for 12 days and treated with adenosine there was a net resynthesis of organic phosphate which was almost twice that produced by stimulation of fresh cells. Apparently adenosine is more effective on the degenerate system. However, the absolute content of organic phosphate in the treated 12 day cells was less than that of the treated fresh cells, but more than was present in the control fresh cells. It is of interest that those erythrocytes not hemolyzed after 120 days of storage in acid-citrate-dextrose at 4° will also show some improvement in their phosphate partition after incubation with adenosine.

(*e*) *Glucose Concentration Related to Adenosine Effect*—In all of the reversibility experiments described previously, whole blood containing acid-citrate-dextrose was employed. Thus a factor to be considered is whether or not the reversal phenomenon will occur in red cell suspensions devoid of the substances (especially glucose) which are present in the plasma-acid-

citrate-dextrose medium. Optimal levels of adenosine were incubated for 1 hour at 37° with suspensions of stored erythrocytes in 0.9 per cent NaCl containing from 14 to 2600 μmoles of glucose per 100 ml. of red blood cells. The absorption of adenosine in each case was 65 to 69 per cent. The results indicated that the presence or absence of glucose did not affect the resynthesis of organic phosphate esters during adenosine treatment for 1 hour.

(*f*) *Aerobic Utilization of Glucose by Rejuvenated Cells*—The previous experiments have described the reversal effect of adenosine in terms of the

TABLE VI

Aerobic Utilization of Glucose by Adenosine-Treated Stored Erythrocytes

Aliquots of human blood (stored 28 days) were incubated 1 hour at 37° with 2100 μmoles of adenosine per 100 ml. of red blood cells. Cell suspensions of the incubated samples were prepared, after washing once, in Krebs-Ringer-phosphate solution. Hemolysates were prepared on twice washed cells by freezing and thawing three times. The stroma fraction was removed by centrifugation. O_2 uptake was measured for 2 hours in the conventional Warburg apparatus at 37°; O_2 gas phase.

		μl. O_2 per hr. per ml. red blood cells
Cell suspension.	Control	36
" "	Adenosine	121
Hemolysate.	Control	371
"	Adenosine	389

The *cell suspension system* contained, in the main compartment, 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of 1.8 per cent glucose, 1.00 ml. of cell suspension, 1.69 ml. of 0.1 M phosphate buffer, pH 7.5; in the center well, 0.20 ml. of 5 N NaOH. The *hemolysate system* contained, in the main compartment, 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of glucose-6-phosphate (100 μmoles per ml.), 0.30 ml. of TPN (1 mg. per ml.), 0.50 ml. of glucose-6-phosphate dehydrogenase (4 mg. per ml.), 0.30 ml. of nicotinamide (100 μmoles per ml.), 0.10 ml. of 1 M ethyl alcohol, 0.05 ml. of catalase, 1.00 ml. of hemolysate, 0.44 ml. of 0.1 M phosphate buffer, pH 7.5; in the center well, 0.20 ml. of 5 N NaOH. Huennekens, F. M., Liu, L., Myers, H., and Gabrio, B. W., unpublished observations.

resynthesis of organic phosphorylated compounds of red cells. It was of interest to determine whether adenosine-treated stored cells were able to utilize glucose at an increased rate. Two systems were employed to measure glucose utilization: red cell suspensions and hemolysates; the former employed the substrate glucose, the latter glucose-6-phosphate. The following electron transport mechanism obtains in both systems (8).

$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-phosphate} + \text{TPN} \rightarrow \text{6-phosphogluconate} + \text{TPNH} \quad (2)$$

$$\text{TPNH} + O_2 \rightarrow \text{TPN} + H_2O_2 \quad (3)$$

$$H_2O_2 + \text{EtOH} \rightarrow H_2O + CH_3CHO \quad (4)$$

Reactions 1 to 4 are catalyzed, respectively, by hexokinase, glucose-6-phosphate dehydrogenase, methemoglobin reductase with methylene blue as cofactor, and catalase.

The results presented in Table VI indicate that the utilization of glucose by the rejuvenated blood in the cell suspension experiment was about 4-fold greater than that of the control, while there was no difference in oxygen uptake in the hemolysate systems. It would appear that the limiting step in the stored blood is the one shown in Reaction 1. The specific limitation is apparently a greatly diminished ATP level, since it has been shown[3] that hexokinase activity is unchanged throughout storage. Adenosine stimulation results in the resynthesis of ATP, thus allowing the phosphorylation of glucose to proceed at a faster rate.

DISCUSSION

Prominent among the changes occurring during storage of blood are a decreased utilization of glucose and the depletion of organic phosphate content of the erythrocyte. The demonstration of a rapid return to normal of the phosphate partition of stored blood, once injected into the blood stream, led to a search for the mechanism of this reversal. In rabbits hepatectomized by the two-stage procedure (9), little or no reversal of the phosphate partition was observed over 1 hour after hepatectomy.[4] Therefore, liver fractions were prepared in the hope of isolating the substance or causative system responsible for the reversal *in vivo*. While definite increases in red cell organic phosphate were observed with the use of the mitochondrial fraction, the magnitude of these changes was not comparable to those obtained with reversal *in vivo*.

In a more direct attempt to reestablish glucose utilization and intracellular ATP, substances chemically related to ATP were added. Adenosine was found to produce repletion of ATP and other organic phosphates and to accelerate the aerobic utilization of glucose to even a greater extent than had been observed previously *in vivo*. Ribose, phosphorylated ribose, orthophosphate ion, and adenine were without effect. It appeared that the riboside was needed to produce both adequate penetration of the cell and to enter thereafter into the metabolic scheme of the cell.

Adenosine was similarly effective in the stored erythrocytes of both rabbit and man. This is consistent with the fact that the storage lesion is an intrinsic cellular defect and that the abnormalities occur at the same rate in both rabbit and human blood *in vitro*. Upon incubation at 37° for 1 hour with optimal concentrations of adenosine, about two-thirds of the nucleoside was absorbed by the erythrocytes, and the regeneration oc-

[3] Private communication, Dr. E. G. Krebs.

[4] Gabrio, B. W., Maguire, R., Donohue, D., and Finch, C. A., unpublished observations.

curred independently of glucose content. However, this should not be interpreted to mean that a portion of the adenosine molecule may replace glucose completely, although this possibility does exist, at least for short term experiments. While the reversal is nearly complete in 1 hour at 37°, several hours at 2° are required to produce the same effect. It is significant that the reversal will occur at the standard storage temperature used in the preservation of blood. However, it was found that glucose was essential for adenosine to function in its maximal capacity in maintaining the erythrocyte during long term storage at 4°. This aspect will be dealt with further in a subsequent communication.

It is of interest that adenosine can increase the organic phosphate content of fresh cells and can produce also some effect on non-viable cells stored for as long as 120 days in acid-citrate-dextrose. The latter observation would indicate that the metabolic pathway through which adenosine exerts its effect is a stable one and not easily destroyed.

The ultimate test of treated stored cells is the ability of these cells to survive after transfusion. It has been found that posttransfusion viability of adenosine-treated erythrocytes is considerably prolonged (10).

The mode of action of adenosine at the molecular level in the repair of the biochemical lesion of stored erythrocytes is unknown at the present time. However, inasmuch as there is an almost 1:1 stoichiometry involving adenosine and organic phosphate resynthesized, the reaction appears to be a chemical rather than a physical effect. Assuming then a chemical reaction, and with the knowledge that ribose and adenine alone are ineffective, adenosine apparently carries ribose into the cell where the nucleoside is subsequently cleaved to its purine base and ribose-1-phosphate. The existence of a nucleoside phosphorylase in this reaction is implicit in the work of Dische (11) and has been demonstrated in this laboratory with inosine as a substrate (12). Ribose-1-phosphate would then participate in the resynthesis of the organic phosphate esters, possibly through the aerobic shunt pathway. The K_m of 3.6×10^{-3} mole per liter and activation energy of 6540 calories per mole per degree both suggest that enzymatic reactions are involved in the stimulation by adenosine. Studies are currently in progress to investigate this problem.

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SUMMARY

The *in vitro* reversibility of the biochemical lesion of the stored erythrocyte has been demonstrated to occur to a slight extent with subcellular

components of the liver, to a moderate extent with some ATP derivatives, and completely with adenosine. It would appear that the repair of the storage lesion with adenosine involves enzymatic reactions in which the ratio of micromoles of organic phosphorus resynthesized to micromoles of adenosine absorbed is unity. A K_m of 3.6×10^{-3} mole per liter and activation energy of 6540 calories per mole per degree were calculated for this system.

BIBLIOGRAPHY

1. Gabrio, B. W., and Finch, C. A., *J. Clin. Invest.*, **33**, 242 (1954).
2. Gabrio, B. W., Stevens, A. R., Jr., and Finch, C. A., *J. Clin. Invest.*, **33**, 247 (1954).
3. Gabrio, B. W., Stevens, A. R., Jr., and Finch, C. A., *J. Clin. Invest.*, **33**, 252 (1954).
4. Schmitz, H., Hurlbert, R. B., and Potter, V. R., *J. Biol. Chem.*, **209**, 41 (1954).
5. Nelson, N., *J. Biol. Chem.*, **153**, 375 (1944).
6. Somogyi, M., *J. Biol. Chem.*, **160**, 69 (1945).
7. Lee, N. D., and Williams, R. H., *Endocrinology*, **54**, 5 (1954).
8. Huennekens, F. M., and Gabrio, B. W., *Federation Proc.*, **13**, 232 (1954).
9. Drury, D. R., *J. Exp. Med.*, **49**, 759 (1929).
10. Gabrio, B. W., Donohue, D. M., and Finch, C. A., *J. Clin. Invest.*, in press.
11. Dische, Z., in McElroy, W. D., and Glass, B., Phosphorus metabolism, Baltimore, **1**, 171 (1951).
12. Gabrio, B. W., and Huennekens, F. M., *Federation Proc.*, **14**, 217 (1955).