ATP and Glycolytic Enzymes of Stored Blood "Probable Cause of Loss of Phosphofructokinase during Storage"

Susumu ODA
Third Division, Department of Internal Medicine, Yamaguchi University School of Medicine, Ube, Japan
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INTRODUCTION

It has been well documented on the damages of erythrocytes during storage, for instance, shortness of survival time, reduction of adenosine triphosphate (ATP) and 2,3-diphosphoglycerate (2,3-DPG), reduction of some glycolytic enzyme activities, increased autohemolysis, increased osmotic fragility, and decrease of pH. It is also well known that ATP is a compound of high potential energy which is essential to maintain biconcave shape of erythrocyte and physiological ionic composition in the cell and that 2,3-DPG may serve as store of energy used only when glycolysis is inadequate.[1] Hexokinase, phosphofructokinase (PFK), and glyceraldehyde-3-phosphate dehydrogenase were said to decrease in their activity during storage. Glycolysis of stored blood remained suppressed on incubation with glucose, even if ATP level maintained high during storage by adding adenine to fresh blood.[2] Reduction of the enzymatic activity could result in reduction of glycolytic rate and ATP level. However, the blood stored for more than 7 weeks could regenerate ATP when they were incubated with adenine, inosine and glucose.[3] So far, the influence of pyruvate, adenine and inosine (PAI) to glycolytic enzymes remains unclear although PAI has been well investigated to preserve ATP and 2,3-DPG levels.

In this paper, (1) the difference of enzymatic activities between plain and PAI-treated ACD blood during storage, and (2) a probable cause of loss of PFK activity in stored blood were studied.

MATERIALS AND METHODS

Blood was collected in ACD solution from four clinically and hematologically healthy donors and each blood sample was separated into two groups. The blood of group I was stored 4°C for 28 days without any treatment. Blood of group II was centrifuged and the plasma was separated. Three mM adenine, 5 mM

inosine and 1 mM pyruvate were incorporated into the plasma. The plasma was returned to the packed red cells by sterile procedure after filtrating through membrane filter (pore size, 0.45 μ) (Sartorius, Membranfilter, 34 Göttingen, West Germany) and stored at 4°C for 28 days. Five ml. of blood from each blood sample was harvested after gentle shaking for the estimation of ATP, 2,3-DPG, glucose, lactate, glycolytic enzymes, osmotic fragility, autohemolysis, whole blood pH and hematocrit on 0 (fresh blood), 4th, 7th, 14th, 21th and 28th storage day, respectively.

Determination of erythrocytic glycolytic intermediates and adenine nucleotides were done enzymatically according to the method described by Minakami et al.,[4] except for 2,3-DPG which was assayed enzymatically by the method described by Rose and Liebowitz.[5]

Erythrocyte enzyme activities were determined according to the method as previously described,[6] which was similar to Beutler's method.[7]

Coil Planet Centrifuge, CPC Analyzer® (Sanki Engineering, Ltd. Kyoto, Japan) was used for erythrocyte osmotic fragility determination.

pH of whole blood was measured at room temperature using Precision pH-Meter (Model HM-8) (TOA Electronics Ltd. Tokyo, Japan).

Autohemolysis was observed by estimating plasma hemoglobin with the method described by Crosby and Furth's modification of that of Bing and Baker.[8]

For red cell incubation studies in order to estimate ATP and 2,3-DPG regeneration as well as lactate formation, the stored red cells of 21st day in both PAI-treated and plain ACD blood were washed three times with cold saline and then with incubation medium once. The red cells were then suspended in 3 volumes of the incubation medium and incubated at 37°C for 3 hours. The incubation medium contained the following: 100 mM NaCl; 100 mM glucose; 1 mM KCl; 10 mM sodium phosphate; 30 mM triethanolamine (TEA) and its pH was adjusted with HCl to 7.4.

In order to estimate the relationship between ATP regeneration and PFK activity, the blood stored in plain ACD blood was incubated either with the above-mentioned medium or with a medium which contained additional 3 mM adenine and 5 mM inosine.

To investigate the reversibility of PFK activity, a special solution was used instead of water when stored blood was hemolized prior to assay. The solution contained 0.2 M $(NH_4)_2SO_4$; 0.05 M tris-phosphate (pH 8.0); 50 mM mercaptoethanol; 2 mM ATP and 1 mM disodium ethylenediaminetetraacetate (EDTA-Na_2).

To examine the significance of SH radical of PFK, effects of p-chloromercuribenzoic acid (p-CMBA) and mercaptoethanol to PFK activity were studied. When the effect of mercaptoethanol on PFK activity of hemolysate without stroma was examined, hemolysate was prepared with water and centrifuged at 2,500

r.p.m. for 5 min. and then the supernatant was filtrated through membrane filter (pore size, 0.45 μ).

RESULTS

The results of the serial estimations of glucose, 2,3-DPG, lactate and ATP of stored blood are shown in figure 1. In plain ACD blood, erythrocyte ATP decreased to one-half of original ATP level on 21st day. The decline in 2,3-DPG level was steep in the absence of PAI, reaching to 12% of original value on 14th day. Glucose level showed 72% of original value on 28th day. In PAI-treated ACD blood, ATP level was still kept over 95% of original value in 21st day, while 2,3-DPG level took 7 days to reach maximum (150% of original value) with rapid decline after that. Reduction of glucose concentration was negligible even after 28 days. Lactate accumulation was greater in PAI-treated ACD blood than in plain ACD blood.

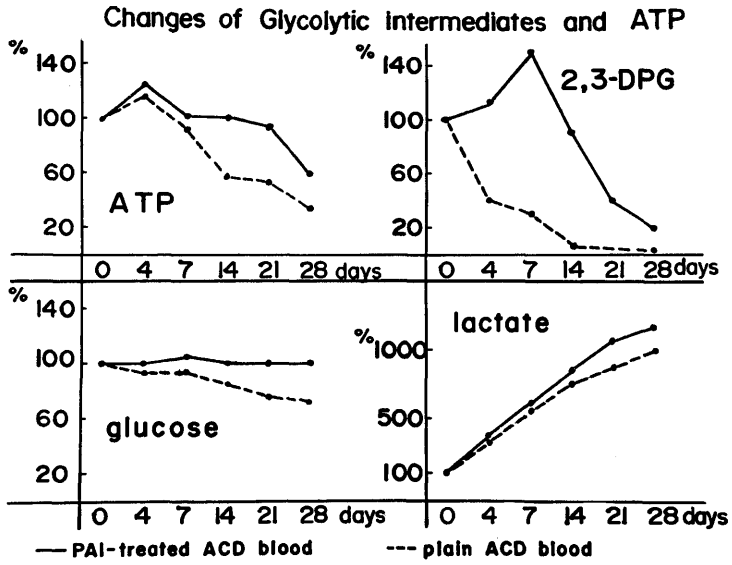

Fig. 1. Changes of glycolytic intermediates and ATP during storage. All contents at zero day is taken for 100%. PAI means pyruvate, adenine and inosine.

Changes of glycolytic enzyme activities including hexokinase (HK), aldolase (Ald), phosphofructokinase (PFK), triosephosphate isomerase (TPI), pyruvate kinase (PK) and lactate dehydrogenase (LDH) during storage are shown in figure 2. All enzymes which we could assay decreased gradually during storage although aldolase

did not show apparent reduction in its activity. All the enzymes of PAI-treated ACD blood kept lower in their activities for early days of storage but later surpass that of plain ACD blood with the exception of HK.

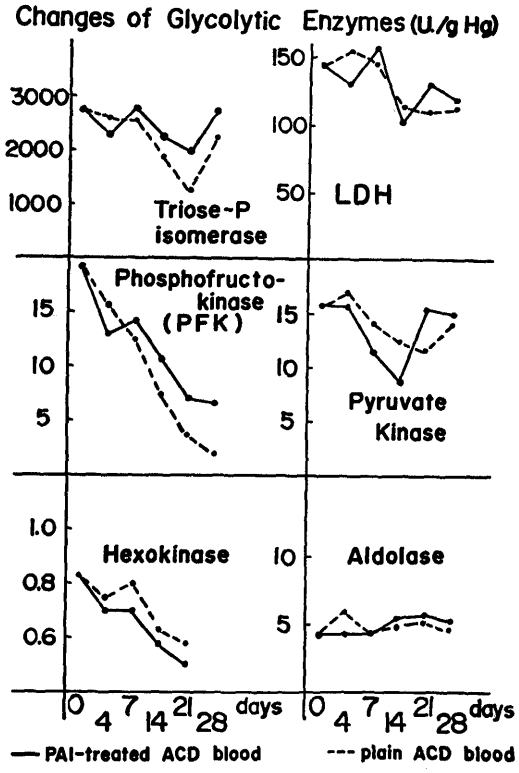

Fig. 2. Changes of glycolytic enzymes during storage.
All values are expressed as units/g. Hb.

The resutls of hematocrit (Ht), pH of whole blood and plasma hemoglobin (Hb) determinations during storage are shown in figure 3. Plasma Hb. showed that the difference between PAI-treated and plain ACD blood became apparent on 14th day of storage. The Ht values were variable, but it appeared that Ht of plain ACD blood became smaller than that of PAI-treated ACD blood on 14th day, when plasma hemoglobin became greater in plain ACD blood. On the contrary, during the first week Ht of PAI-treated ACD blood was smaller than that of plain ACD blood although the degree of autohemolysis of both PAI-treated and plain ACD blood was about the same. The pH of PAI-treated ACD blood was kept higher than that of plain ACD blood.

The results of osmotic fragility (O.F.) by means of CPC Analyzer revealed apparently increased O.F. on 21st day in both PAI-treated and plain ACD blood.

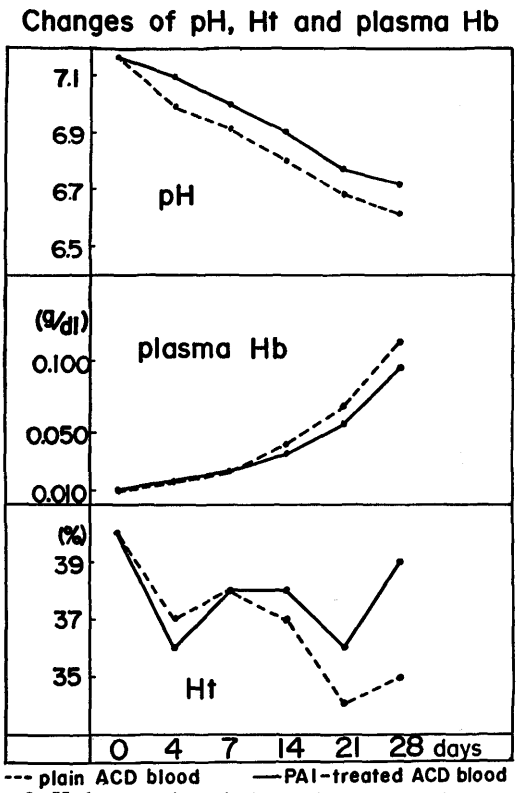

Fig. 3. Changes of pH, hematocrit and plasma hemoglobin during storage.

The result of red cell incubation study performed on 21st day is shown in table 1. Red cells of PAI-treated ACD blood were able to generate ATP up to fresh blood level after 3 hours, while red cells of plain ACD blood could regenerate only 60% of original ATP level. Lactate formation in PAI-treated ACD blood was greater than in plain ACD blood. 2,3-DPG level in both PAI-treated and plain ACD blood decreased gradually during incubation.

The relationship between ATP regeneration and PFK activity is shown in figure 4. After one hour of incubation, PFK activity became four times of that before incubation, while ATP level increased twice when stored blood was incubated with adenine, inosine and glucose. In the blood which was incubated with only glucose, PFK activity increased about three times of original value and ATP level rather decreased.

The data concerning the recovery of PFK activity of stored blood with a special hemolizing solution (A. solution) is shown in table 2. After two weeks of storage, hemolysate treated with A. solution showed over twice level of PFK activity as compared with the hemolysate treated with water, whereas in fresh blood such effect of the solution was not very marked. The A. solution prevented the loss

Incubation Study on 21st. Day of Storage

	original level		0 hr.	1 hr.	3 hrs.
ATP	1170	+	845	880	1210
		−	413	379	651
2,3 DPG	4430	+	1010	575	520
		−	574	442	372
Lactate		+	7060	15250	18200
		−	7600	11200	16100

+ PAI- treated ACD blood
− plain ACD blood

lactate : mμM/mL Whole Blood
2,3-DPG / ATP : mμM/mL Red Cell

Table. 1. Incubation study on 21st day of storage.
The original level is not shown in lactate. Lactate production could not compared with original level because lactate was lost considerably during the process of washing before incubation.

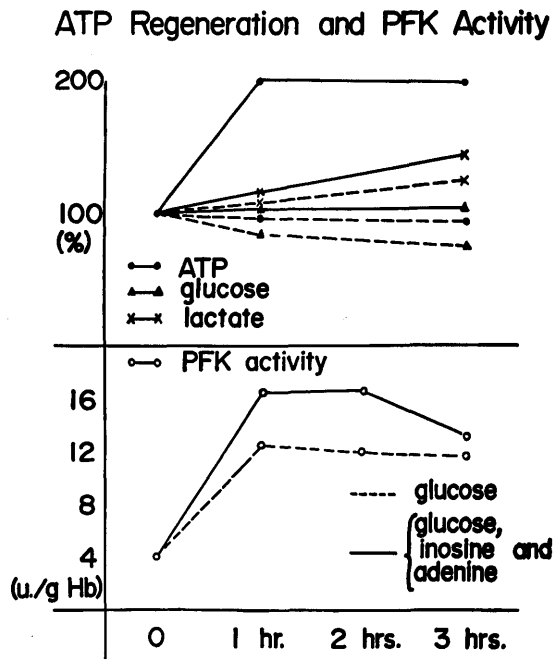

Fig. 4. Relationship between ATP regeneration and PFK activity.
After 3 weeks of storage, plain ACD blood incubated with glucose (·····) and with glucose, inosine and adenine (——).
PFK activity is expressed as units/g. Hb. ATP, glucose, and lactate levels at zero time is taken for 100%.

1) PFK activity of fresh blood

	0 hr.	12 hrs.	24 hrs.
A.Solution + Saponin	18.9	21.0	21.0
Saponin	18.3	13.8	11.1

2) PFK activity after 2 weeks storage

A.Solution + Saponin	26.7	27.5	21.8
Saponin	12.2	7.3	
Water	11.2	7.2	5.8

A. Solution: 0.2 M $(NH_4)_2SO_4$
0.05 M Tris-Phosphate
50 mM Mercaptoethanol
2 mM ATP
1 mM EDTA

Table 2. Riversibility of PFK activity of stored erythrocyte.
All values are expressed as units/g. Hb. Hemolysate was prepared with water, saponin solution and A. solution plus saponin before PFK assay.

of PFK activity at least for 24 hours. The effect of individual substances in A. solution on PFK activity was studies (table 3). It revealed that PFK activity was high in solutions which contained mercaptoethanol. Significance of SH radical in PFK was examined by using p-chloromercuribenzoic acid (p-CMBA) and mercaptoethanol (table 4). p-CMBA inhibited completely the PFK activity of erythrocytes and mercaptoethanol relieved PFK from its inhibition. The difference in PFK activity between hemolysate with stroma and without stroma is shown in table 5. In stored blood PFK activity of hemolysate with stroma showed more than twice than that without stroma, while fresh blood did not show such great difference. Activation of PFK of the hemolysate without stroma by mercaptoethanol is shown in table 6. Mercaptoethanol activated 9.4 times PFK activity in stored blood, while 1.5 times in fresh blood.

hemolizing solution	PFK activity(u./g Hb)
$(NH_4)_2SO_4$ Mercaptoethanol EDTA ATP Tris-phosphate	19.9
$(NH_4)_2SO_4$ Mercaptoethanol EDTA Tris-phosphate	17.5
$(NH_4)_2SO_4$ Mercaptoethanol Tris-phosphate	18.0
$(NH_4)_2SO_4$ Tris-phosphate	12.6
Tris-phosphate	12.6

Table 3. Effect of each substance in A. solution to PFK activity of stored blood. ACD blood stored 3 weeks was used for preparing hemolysate with each hemolyzing solution and the hemolysate was centrifuged before assay.

	PFK activity (u./g Hb)
reaction mixture p-CMBA (0.02 mM)	0
reaction mixture p-CMBA (0.02 mM) Mercaptoethanol (4 mM)	15.3
reaction mixture Mercaptoethanol (4 mM)	14.7
reaction mixture	14.0

Table 4. Effect of mercaptoethanol to the inhibitor (p-chloromercuribenzoic acid) of PFK. Reaction mixture contained 1.7 mM F-6-P; 0.83 mM ATP; 0.15 mM NADH; 0.4 mg. aldolase; 0.04 mg α-GPDTPI; 0.05 M TEA; 6 mM EDTA; 8 mM $MgCl_2$.

	PFK activity (u./g Hb)	
	stroma(+)	stroma(−)
Fresh Blood	26.8	25.4
Stored Blood	8.5	3.0

Table 5. Comparison of PFK activity between hemolysate with stroma and that without stroma. Hemolysate was prepared with water after freezing and thawing three times.

	PFK activity (u./g Hb)		
	ME(+)	ME(−)	ME(+)/ME(−)
Fresh Blood	27.4	18.1	1.5
Stored Blood	13.1	1.4	9.4

Table 6. Difference of effectiveness of mercaptoethanol to hemolysate without stroma betweeen stored blood and fresh blood.
ME (+): 4 mM mercaptoethanol was added in reaction mixture.
PFK assay was done after ten minutes incubation at room temperature.

DISCUSSION

In recent years, adenine and inosine (AI) have been well investigated as the compounds to preserve ATP and 2,3-DPG levels of stored blood and to improve erythrocyte viability.[9)10)11)12)13)14)] Oski et al.[15)] reported that pyruvate appeared necessary to provide sufficient NAD for maximum 2,3-DPG regeneration. Beutler et al.[11)] observed that ATP preservation was favored by slightly higher preservative pH when adenine was used and pyruvate prevented the rapid loss of ATP in higher pH medium. According to Paniker et al.[12)] the reaction of the lactate dehydrogenase (LDH):

$$\text{lactate} + NAD^+ = \text{pyruvate} + NADH + H^+$$

is shifted to the left and the hydrogen ions are removed uppon addition of pyruvate. Produced NAD by LDH reaction promote the reaction of glyceraldehyde-3-phosphate dehydrogenase and its higher pH is also advantageous for maintaining the 2,3-DPG level. As shown in figure 3, pH of PAI-treated ACD blood was higher than that of plain ACD blood throughout the storage period. But preservation of 2,3-DPG was not so good after 2 weeks of storage. Preservation of 2,3-DPG may be partly improved by changing the way of addition and quantity of AI, especially inosine. Because Dawson[13)] and Strumia[14)] have shown that multiple additions of inosine were more effective to preserve ATP and 2,3-DPG. But in our experiment, AI was added only once in fresh blood in order to minimize the chance of bacterial contamination and mechanical impairment to stored blood. Up to date, PAI addition still seems to be one of the best way to preserve ATP and 2,3-DPG levels effectively according to the previous reports. By adding PAI, ATP level remained 95 % of original value on 21st day of storage and 2,3-DPG level 80 % on 14th day, whereas in plain ACD blood ATP level decreased to 54 % on 21 st day and 2,3-DPG level 4 % of the original level on 14th day. PAI addition was also effective in order to decrease the degree of autohemolysis as shown in figure 3. Osmotic fragility (O.F.) was found to be slightly improved by adding PAI with our method during 28 days. Recently, Coil Planet Centrifuge (CPC)[16)] has been developed in order to uncover subtle change in osmotic fragility. Rapoport[17)] reported that osmotic fragility examination by noting only the points of begining and of completed hemolysis not infrequently caused a highly misleading picture of the real change owing to the fact that a small proportion of the cells showed greatly increased fragility after a few days of storage. This mistake could be avoided when CPC was utilized for O.F., because it showed two apparently different hemolysed hemoglobin bands, one being composed of a small proportion of fragile cells and the other the bulk of the cells which were less fragile. So we regarded as O.F. was increased when the latter band of stored blood was seen in more hypertonic side than that of fresh blood. With this criteria, PAI did not have apparent effect on O.F. during 28 days of storage. The results of glycolytic

intermediates, autohemolysis, O.F., pH and Ht of whole blood reconfirmed the effectivness of PAI addition to the stored blood, which were reported by previous authors.

The reasons why these enzymes as shown in figure 2 were selected for assay in this study were as follows: HK, PFK and PK are regarded to be rate limiting enzymes in Embden-Meyerhof pathway. Activities of HK, Ald, PFK and LDH have been reported to be decreased during storage period.[18)19)20)] PFK, Ald, TPI and LDH all retarded in loss of their activity in PAI-treated ACD blood, but only HK activity in PAI-treated ACD blood of which glucose consumption was negligible for 4 weeks with our method, had been lower for first three weeks, in spite of higher ATP than that of plain ACD blood. The behaviour of HK activity does not contradict with the result of the glucose consumption but reason why HK activity of PAI-treated ACD blood kept lower than that of plain ACD blood is unknown. Simon[21)] showed that glucose consumption was completely inhibited in the presence of inosine. Strumia[10)] reported that adenine and inosine reduced the rate of glycolysis and inosine was consumed as a substrate and that ATP synthesis from inosine was as good as from glucose. Nakao et al.[9)] reported that red cells could regenerate ATP after long storage when they were incubated with inosine and adenine. So it is conceivable that inosine and adenine are more readily metabolized to lactate than glucose. Decrease of PFK activity in both PAI-treated and plain ACD blood was more rapid than the other enzymes. It may indicate that PFK is also a rate limiting enzyme in stored blood. Yoshikawa and Minakami[22)] reported that PFK was the primary cause of reduced glycolysis in stored blood because they found increased glucose-6-phosphate, fructose-6-phosphate, and decreased fructose-1,6-diphosphate of stored blood.

Red cell incubation study performed on 21st day of storage showed that erythrocytes with higher enzymatic activities and ATP level could produce faster and more ATP and lactate. As Hurn[2)] suggested, loss of ATP was not the only factor leading to the failure of red cell metabolism during storage. The loss of enzymatic activities may also be a factor to reduce glycolytic rate. Higher enzymatic activities seem to be advantageous for transfused erythrocytes to restore ATP level as soon as possible. PAI is also probably effective to keep the glycolytic enzymatic activities high as well as ATP and 2,3-DPG levels.

As shown in figure 2, PFK activity decreased to about 35 % and 20 % of original level on 28th day of storage in PAI-treated and plain ACD blood, respectively. However, the loss of PFK activity due to storage recovered when the stored blood returned to more physiological condition. In addition, recovery of PFK activity was marked under the condition in which ATP production was high. The blood incubated with only glucose regenerated ATP by consuming glucose and resultant lactate production. Glycolysis per se may consume more ATP than metabolism of adenine and inosin does. PFK activity could be restored in con-

ditions in which Embden-Meyerhof pathway became active. Moreover, PFK activity of the hemolysate of stored blood was also restored (table 2). Tarui et al.[23] reported that purified PFK had tendency to aggregate readily and large polymers were formed by storage, but they could be redissolved in a freshly prepared solution containing 0.2 M $(NH_4)_2SO_4$; 0.05 M tris-phosphate (pH 8.0) ; 50 mM ditiothreitol; 2 mM ATP and 1 mM EDTA and in the solution the enzyme was fully active. Hemolysate was prepared by a special solution (A. solution) which was similar to the solution described by Tarui, when PFK activity of stored blood was assayed. The A. solution was effective to restore the decreased PFK activity caused by storage, although it was not very effective to fresh blood, if any. It has been uncertain as to which substance in the A. solution contributed in restoring the loss of PFK activity of stored blood. To clarify this point, the effect of individual substance on PFK activity was examined (table 3). It revealed that mercaptoethanol and ATP were important effectors which restored the loss of PFK activity. Mercaptoethanol protects sulfhydryl groups. In addition, Chapman et al.[24] reported that PFK of rabbit muscle was inhibited by p-chloromercuribenzoate and reactivated by addition of excess thiol and he confirmed sulfhydryl groups were closely associated with the catalytic site of PFK. On the other hand, it has also been reported that PFK is bound to the membrane of erythrocyte. PFK in stored red cells may be more tightly bound to membrane than that of fresh blood (table 5). PFK might have been removed together with stroma when hemolysate was centrifuged before assay. In conventional method, stroma was removed before the assay, and this may cause decreased PFK activity of stored blood. Thus, the presence of a large amount of PFK remain bound to stroma probably exaggerated the loss of PFK activity when the activity per unit hemoglobin basis was used for the expression of enzymatic activity in stored blood. But following two studies contradict that strict adhesion of PFK to membrane is the only cause for the marked loss of PFK activity. Firstly, aldolase did not show apparent decrease of its activity for 28 days of storage although aldolase is also said to be one of membrane-bound enzymes. Secondly, PFK activity of stored blood activated with mercaptoethanol even when it did not contain stroma (table 6). Moreover, the activation by mercaptoethanol of PFK activity was greater in stored blood than in fresh blood. Reduced glutathione (GSH) has been said to activate the PFK activity. Normal red cell contains a large amount of GSH which protect sulfhydryl groups. Mature red cells can produce GSH in the presence of ATP. In stored blood, the production of GSH may be suppressed partly due to lack of ATP. Bunn et al.[25] showed that GSH gradually decreased its concentration in ACD blood. The concentration of GSH was well maintained when adenine and inosine were added to ACD blood. ATP seems to be important to maintain GSH level in stored blood. High concentration of ATP may reduce the loss of enzymatic activity of stored blood partly through maintaining GSH. It seems reasonable to

conclude that PFK molecules change into inactive forms by inhibition of sulhydryl group of PFK by oxidation during storage, but it is reversible into active form by regaining sulfhydryl group. Exogenous agents to protect sulfhydryl groups appear to have advantage in blood storage. Previous authors[26] tried to increase the concentration of glutathione of stored blood by adding methylene blue (MB). But its availability of MB to stored blood remains unclear.

SUMMARY

1. To clarify the influence of pyruvate, adenine and inosine (PAI) to glycolytic enzymes of stored blood, the difference of erythrocyte enzyme activities between PAI-treated and plain ACD blood during storage was studied. As far as the glycolytic enzymes are concerned, phosphofructokinase (PFK), aldolase, triosephosphate isomerase, pyruvate kinase and lactate dehydrogenase all retarded loss of their activities in PAI-treated ACD blood than in plain ACD blood. Only hexokinase kept lower in PAI-treated ACD blood than in plain ACD blood during 21 days of storage. PAI-treated ACD blood could preserve better ATP and 2,3-DPG levels throughout storage period and produce faster and more ATP and lactate when incubation studies were performed on 21st day of storage than plain ACD blood could. These data probably indicate that PAI are effective to keep the glycolytic enzyme activities high, which may be advantageous for transfused erythrocytes to restore ATP level rapidly.

2. PFK activity decreased to about 20 % and 35 % of original level on 28th day of storage in plain and PAI-treated ACD blood, respectively. However, the loss of PFK activity recovered up to original level when stored blood was incubated with adenine and inosine in response to ATP regeneration. In addition, PFK activity of stored blood remained original level when they were hemolized with a special solution (A. solution) which contained ATP, ammonium sulfate, mercaptoethanol, EDTA and tris-phosphate (pH 8.0). Among these reagents, mercaptoethanol was the most effective to restore the loss of PFK activity during storage. On the other hand, PFK activity was completely inhibited with p-chloromercuribenzoic acid and its inhibition did not occur when mercaptoethanol was present. In addition, PFK activation by mercaptoethanol was greater in stored blood than in fresh blood. These data suggest that the loss of PFK activity of stored blood is produced by affecting SH group at the catalytic site of PFK, probably by oxidation, and the PFK activity is reversible when stored blood return to more physiological condition accompanied with restored red cell metabolism probably through regaining SH groups of PFK.

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