

ERYTHROCYTE PRESERVATION. VI. THE STORAGE OF BLOOD WITH PURINE NUCLEOSIDES¹

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The addition of adenosine to stored blood results in a chemical restoration of deteriorated erythrocytes and sustains their viability during continued storage (1, 2). This phenomenon has been referred to as the *in vitro* reversibility of a biochemical lesion of storage. Apart from the implications of this reaction in red cell metabolism (3), it seemed of importance to evaluate the effect of adenosine in the preservation of blood. The present study indicates that adenosine and some related purine nucleosides retard the onset of the storage lesion and extend significantly the period of viability of stored blood. The effective concentration of these supplements, as well as their potential toxicity, is discussed.

METHODS

The techniques for the measurements of erythrocyte phosphate partition, blood glucose, sodium and potassium, osmotic fragility, and aerobic utilization of glucose have been described (1, 4). Oxygen content and oxygen capacity of erythrocytes were determined by the method of Van Slyke and Neill (5).

Isotonic saline solutions of adenosine and its analogs were employed in concentrations of 50 to 100 μ moles per ml. The nucleosides are soluble in warm saline. While relatively unstable with respect to re-precipitation at these concentrations, the nucleosides can be redissolved by subsequent heating. Sterilization of the solutions was accomplished by filtration through an ultrafine fritted pyrex glass disc. The method for the determination of adenosine concentration and absorption² has been reported (1).

In the experiments reported, the control sample refers to blood stored in ACD³ alone. On the basis of *in vitro* studies on the synthesis of organic phosphate esters

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² Although this phenomenon could be absorption into the cell and/or adsorption onto the membrane, we shall use the term, absorption, throughout.

³ National Institutes of Health, Formula B.

in stored blood, it was found unnecessary to maintain similar volumes in the samples within one experiment, and there were no additions made to the control aliquots, although nucleoside solutions were added to the experimental aliquots.

Post transfusion survival of stored red cells was measured with a double isotope technique in which Cr⁵¹ was employed as a tag for stored cells, and P³²-labeled fresh cells were used as a blood volume reference (6). When the measurements were performed in duplicate, the accuracy of the survival determination was considered to be ± 5 per cent. Theoretical survival was determined from the ratio of the two isotopes. Subsequent survival of the Cr⁵¹-tagged cells was determined over a period of seven days. An excess loss of Cr⁵¹ of about 10 per cent occurs during the first 24 hours when washed fresh cells are transfused. The reported survival data are thus somewhat lower than would be expected from comparable studies employing the technique of differential agglutination of erythrocytes (Ashby). Before transfusion, cells stored with nucleosides, as well as control cells, were washed three times with saline. Hemolysis incident to washing was not usually significant before the 60th day of storage.

Toxicity measurements were carried out in rabbits and dogs by the intravenous administration of sterile saline solutions of the purine nucleosides and related products. In addition, plasma, and hemolyzed and fresh red cells from blood stored in ACD plus adenosine, were tested. Rabbits were anesthetized with ether, and dogs were given sodium pentobarbital intravenously. The blood pressure, pulse pressure, and heart rate were recorded directly from the cannulated femoral artery by means of a Sanborn Electromanometer Model No. 120 and a Sanborn Poly-Viso Recorder. A simultaneous electrocardiogram was obtained.

RESULTS

I. Adenosine as a supplement to ACD in blood preservation

A. Rabbit blood. The effects of a single initial supplement of adenosine compared with intermittent addition of this nucleoside during storage are illustrated by the following typical experiment. Blood, collected in ACD, was divided into three parts. Aliquot 1 served as a control. To Aliquot

TABLE I
Storage of rabbit blood in ACD+adenosine at 4°C.*

Days storage	μmoles P/100 ml. RBC		Cumulative absorption of adenosine (%)	Survival of RBC (%)
	Inorganic	Organic		
0	142	2,442		
15				
C-1	1,130	1,036		74
A-2	184	2,353	58	95
A-3	174	2,443	62	86
22				
C-1	1,317	601		68
A-2	292	2,220	66	87
A-3	180	2,454	66	88
29				
C-1	1,400	519		53
A-2	459	1,997	77	86
A-3	188	2,541	71	86
36				
C-1	1,470	362		38
A-2	615	1,641	81	82
A-3	199	2,563	74	83
43				
C-1	1,497	348		20
A-2	775	1,523	86	66
A-3	151	2,278	77	76
50				
C-1	1,500	289		7
A-2	863	1,153	90	66
A-3	174	2,581	80	81

* Blood was collected in ACD and divided into three aliquots: 1) Control (C-1); 2) Initial addition of 2,800 μmoles adenosine per 100 ml. RBC (A-2); 3) Addition of 1,400 μmoles adenosine per 100 ml. RBC weekly (A-3).

2 was added 2800 μmoles adenosine per 100 ml. erythrocytes (RBC) at the beginning of storage. To Aliquot 3, 1425 μmoles adenosine per 100 ml. RBC was added initially and the same amount each week throughout the storage period of 50 days. As shown in Table I, the per cent absorption² of adenosine was approximately the same in the treated samples, although the absolute amount taken up by the cells at 50 days in Aliquot 3 (7300 μmoles per 100 ml. RBC) was almost three-fold greater than the uptake in Aliquot 2 (2590 μmoles). Higher levels of cellular organic phosphates were maintained in Aliquot 3, suggesting an increased utilization for this purpose. Erythrocytes of both treated samples retained their organic phosphate components (adenosine triphosphate, hexose phosphate, and 2,3-diphosphoglycerate) considerably better than did the control.

The correlated post-transfusion survival meas-

urements (Table I) showed that at 15 days of storage, the erythrocytes stored in ACD alone were 74 per cent viable, while Aliquot 2 had 95 per cent survival, and Aliquot 3, 86 per cent. At this time the amount of adenosine available to the cells in the latter two samples was the same (2800 μmoles). This amount had been available to Aliquot 2 from the beginning of storage, while only 1400 μmoles was present in Aliquot 1 for the first week. The expected decline in survival of the control cells occurred subsequently. Both adenosine-supplemented samples showed about 85 per cent survival through 36 days. Thereafter, the viability of the erythrocytes of Aliquot 3 was better than that of Aliquot 2. Apparently the initial addition of adenosine, at the level employed, was adequate to maintain the red cells throughout the first five weeks of storage, and the weekly additions were not of additional benefit.

In Table II are shown the results of a long-term storage experiment in which rabbit blood was supplemented intermittently with 2300 to 2500 μmoles adenosine per 100 ml. RBC. Approximately 70 per cent of the adenosine was absorbed. A sediment, noted in the treated blood sample at 105 days of storage, was presumed to represent precipitated adenosine or its degradation product. This did not appear to affect the phosphate partition or the survival of these cells. The improved maintenance of the energy potential of

TABLE II
Storage of rabbit blood in ACD+adenosine at 4°C. for 105 days*

Days storage	μmoles Organic P/100 ml. RBC	Survival of RBC (%)
0	2,504	
25		
Control	847	44
Adenosine	2,000	65
54		
Control	408	5
Adenosine	1,417	56
77		
Adenosine	1,431	59
105		
Adenosine	1,271	44

* Blood was collected in ACD and divided into two aliquots: 1) Control; 2) Adenosine (2,300 to 2,500 μmoles per 100 ml. RBC) added on days 1, 25, 54, and 77.

TABLE III
*Post-transfusion survival of human blood stored in
 ACD+adenosine at 4°C.**

Days storage	Survival of RBC (%)
19	
Control	77
Adenosine	91
42	
Control	29
Adenosine	65
68	
Control	4
Adenosine	53

* Human blood was collected in ACD and divided into two aliquots: 1) Control; 2) 2,500 μ moles adenosine per 100 ml. RBC added at days 1, 19, and 42.

the cells, as indicated by organic phosphate levels, is evident in the adenosine-treated cells throughout storage. Prolongation of viability was demonstrated in the treated blood as compared with the control cells which were completely non-viable by the 50th day of storage.

B. *Human blood.* Comparable experiments were performed with human blood. In a typical study, 2500 μ moles adenosine per 100 ml. RBC was added every three weeks for approximately 10 weeks. The increased survival time of the adenosine-treated cells over a period of 68 days is shown in Table III. Other data which included phosphate partitions, osmotic fragilities, and determinations of the sodium-potassium gradient also indicated improved chemical characteristics of the erythrocyte when compared with the control blood stored in ACD alone.

C. *Miscellaneous studies.* Morphologic studies of red cells stored in ACD compared with those stored with the addition of adenosine showed some difference, but this could not be expressed quantitatively. The progressive spherocytosis, poikilocytosis, and crenation of stored blood was somewhat retarded by the addition of adenosine. Likewise, the flickering phenomenon, thought to bear some relation to normal function of the red cell (7), was maintained over a longer period of time. However, it was impossible to predict viability from these changes. The alterations in cell appearance, observed after 60 to 120 days of storage, were still compatible with appreciable cell survival (Tables II, III), which suggests that many

of these morphologic changes are reversible upon re-entrance of the erythrocyte into active circulation. Appreciable spontaneous hemolysis was not observed before the 50th day of storage in blood stored in ACD + adenosine. As the storage period approached the 100th day some hemolysis was clearly visible, although less marked than that in blood stored in ACD alone, and further lysis of cells occurred when the cells were washed before transfusion. Correction for hemolysis was included in the calculation of viability when cells were stored for prolonged periods.

Inasmuch as blood stored in ACD + adenosine is bright red after several weeks of storage, in contrast to the darker color of blood preserved in ACD alone, the oxygen content and capacity⁴ of such blood were measured. The results, recorded in Table IV, suggest a decreased oxygen utilization of red cells during storage in blood supplemented with adenosine. Concomitant analyses of aerobic utilization of glucose also showed the improved metabolic activity of erythrocytes stored several weeks in ACD + adenosine.

TABLE IV

*Oxygen content and capacity of human blood during storage**

Days storage	O ₂ Content (vol. %)	O ₂ Capacity (vol. %)	% Sat. with O ₂	O ₂ uptake† μ liters/hr./ml. RBC
1 day:				
Control	10.42	14.67	71.0	116
Adenosine	12.19	14.58	83.6	117
3 weeks:				
Control	6.50	13.02	49.9	72
Adenosine	8.89	13.40	63.3	106
7 weeks:				
Control	3.74	12.20	30.7	46
Adenosine	6.50	12.37	52.6	103

* Blood was collected in ACD and divided into two aliquots: 1) Control; 2) 2,500 μ moles adenosine per 100 ml. RBC added after 1-day sampling and after 3 weeks' storage. Both aliquots were equilibrated initially with nitrogen, and sampling was accomplished under anaerobic conditions.

† Aerobic utilization of glucose in cell suspension system: Main compartment: 0.01 ml. of 1 per cent methylene blue, 0.30 ml. of 1.8 per cent glucose, 1.00 ml. of red cell suspension, 1.70 ml. of M/10 phosphate buffer, pH 7.5; center well: 0.20 ml. of 5N NaOH. O₂ gas phase: conventional Warburg apparatus; 37°. (Huennekens, F. M., Liu, L., Myers, H., and Gabrio, B. W., Unpublished observations.)

⁴ We are indebted to Dr. Robert Bruce for these analyses.

TABLE V
Effect of glucose on the survival of blood
stored with adenosine *

Days storage	Glucose μ moles/100 ml. blood	Survival of RBC (%)
1		
Cit.	490†	
Cit. + G†	1,500	
Cit. + A†	490	
Cit. + G + A	1,600	
14		
Cit.	24	18
Cit. + G	800	70
Cit. + A	12	36
Cit. + G + A	1,160	82

* Human blood was collected in 4 per cent citrate (1 vol. + 9 vol. blood) and divided into four aliquots: 1) Control citrated blood; 2) Added 1,010 μ moles glucose per 100 ml. blood; 3) Added 1,100 μ moles adenosine per 100 ml. blood; 4) Added 1,010 μ moles glucose and 1,100 μ moles adenosine per 100 ml. blood. All samples were stored for 2 weeks at 4°C.

† G = glucose; A = adenosine.

‡ 490 μ moles glucose per 100 ml. blood represents the amount of glucose endogenous in the whole blood in each sample.

II. Adenosine as a substitute for glucose in blood preservation

It has been shown (1) that adenosine can reverse the biochemical lesion of stored red cells in the absence of glucose. These results were obtained upon incubation of the aged cells with the riboside at 37°C. for one hour. In addition, Pranker and Altman (8) have reported that erythrocytes can utilize adenosine in place of glucose to promote phosphate exchange when incubated over a six-hour period. It was of interest, therefore, to ascertain whether adenosine could replace glucose in maintaining viability of the red cell over a long period of storage at 4°C. The data shown in Table V indicate that, with only endogenous levels of glucose present in blood, there was almost complete glucose utilization in the presence of 1100 μ moles of adenosine per 100 ml. whole blood, and the preservation of citrated blood was improved only slightly. At higher levels of glucose supplementation, a sparing action of adenosine on the utilization of glucose and improved viability of red cells could be demonstrated.

Inasmuch as the level of adenosine employed in the previous experiment was about equimolar to glucose, it was thought that a replacement mechanism might require greater amounts of the ri-

boside. Consequently, citrated rabbit blood was stored with excess adenosine for four weeks (total of 15,000 μ moles per 100 ml. RBC added at intervals). There was an appreciable sparing of glucose utilization in the presence of a large amount of adenosine. The viability of cells stored in citrate + excess adenosine was 59 per cent and that of citrate + glucose was 50 per cent at four weeks of storage. However, the combination of both glucose and adenosine resulted in even better survival (85 per cent).

III. Effects of related nucleosides

Nucleosides related to adenosine, such as inosine, deoxyadenosine, guanosine, and xanthosine, similarly stimulated a resynthesis of organic phosphates in the stored erythrocyte after one hour incubation at 37°C. (3). The implications of this and the mechanism of action of nucleosides will be discussed in a subsequent communication.

Inasmuch as significant reversal of the chemical changes of the storage lesion can be accomplished with purine nucleosides other than adenosine, and since adenosine has been found to have potentially toxic properties, the viability of red cells stored with other nucleosides was determined.

A detailed study of deoxyadenosine, the least effective of the nucleosides on the basis of *in vitro* studies, is shown in Tables VI and VII. When this substance was added at the beginning of storage only, the optimal level for viability appeared to be 2600 μ moles per 100 ml. RBC, regardless of the fact that the level of organic phosphate esters was maintained better in the presence of an excess of the compound. The effect

TABLE VI
Concentration of deoxyadenosine in the storage
of human blood *

Deoxyadenosine added (μ moles/100 ml. RBC)	48 days' storage	
	Organic P (μ moles/100 ml. RBC)	Survival of RBC (%)
0	154	5
230	223	8
600	240	15
1,200	418	32
2,600	660	51
5,200	925	52

* Human blood was collected in ACD, divided into aliquots, supplemented with increasing concentrations of deoxyadenosine, and stored at 4°C. for 48 days.

TABLE VII
Storage of human blood in ACD + deoxyadenosine

	μ moles Nucleoside/ 100 ml. RBC		Nucleo- absorp- tion (%)	Survival of RBC (%)
	Added Initially	Other additions		
Exp. I. 42 da. storage				
Control				12
D*1	3,500		75	69
D2	3,500	7,000	69	74
Exp. II. 48 da. storage				
Control				12
A*	2,000		74	45
D1	2,000		73	41
D2	2,000	5,000	62	64

* D = deoxyadenosine; A = adenosine.

of the deoxyriboside was comparable to that of adenosine in prolonging viability of stored red cells. Similarly, it was most efficacious when added at intervals throughout storage in order to maintain a higher concentration of the nucleoside and a greater absolute absorption.

Blood stored with xanthosine resulted in a sediment formation and was not tested for survival.

IV. Toxicity of nucleosides

Preliminary studies were undertaken in order to evaluate the relative toxicity for animals of adenosine and its analogs. Of the effects reported, hypotension appeared to be the most important and sensitive for assay purposes (9). A study was conducted on the effects of the intravenous administration of commercially available nucleosides⁵ as well as plasma and washed red cells from blood stored in ACD + adenosine.

The average minimal intravenous dose of adenosine producing a depressor response was 1 μ mole in the rabbit and 10 μ moles in the dog. The average blood pressure fall was 80 mm. Hg in four to ten seconds after injection. The duration of the effect was variable, increasing with greater doses to as much as 200 seconds when 500 μ moles was given intravenously to a dog. There were no significant electrocardiographic changes in rabbits given 10 times the minimal hypotensive dose. The administration of amounts up to 100 times the minimal dose did not produce fatalities. The results were comparable in the two species.

⁵ Obtained from Schwarz Laboratories, Mt. Vernon, New York.

Solutions of guanosine, xanthosine, and inosine did not cause an observable effect in amounts up to ten times the minimal hypotensive dosage of adenosine. A significant fall in blood pressure was observed in the dog after 400 μ moles of deoxyadenosine was given. Thus the relative hypotensive effect of deoxyadenosine was estimated to be less than 5 per cent that of adenosine. Adenosine-5-phosphate was equal to adenosine in toxicity but ribose and adenine were without effect. It was demonstrated further that injected plasma from blood stored 14 days in ACD plus adenosine produced transient and mild hypotension, while injection of washed intact erythrocytes was without effect.

DISCUSSION

The incubation of stored blood with adenosine and other nucleosides results in a repletion of cellular organic phosphates and an increased ability of the cells to utilize glucose aerobically (1). This restoration of the energy potential has been associated, as well, with improvement in osmotic fragility, an increase in intracellular potassium and improvement in post-transfusion viability (2). From the data in the present communication, it is evident that the addition of adenosine (2500 μ moles per 100 ml. RBC) as a supplement to ACD during the storage of blood at 4°C. prolongs the period of useful storage to about 40 to 50 days. Supplementation at weekly intervals was shown to improve viability over an additional period of time.

The use of citrate alone as a preservative is associated with a rapid loss of cell viability, while the addition of glucose prolongs the useful storage period from 5 to 15 days, and an acid-citrate-dextrose medium extends this period to 21 days. Although adenosine added to citrated blood also improves viability, supplementation with both adenosine and glucose provides the best results. This implies that adenosine and glucose are complementary and neither completely substitutes for the other.

The hypotension produced in rabbits and dogs with small amounts of adenosine (1 to 10 μ moles) indicates the potentially hazardous effects of this substance. Only a small amount of adenosine-

treated blood has been transfused and only after repeated washing of the cells. Related purine nucleosides were found to be less "toxic," but only deoxyadenosine was studied in some detail.

It has been suggested (1, 3) that nucleosides provide the necessary substrate through phosphorylytic cleavage, and that the resultant ribose-1-phosphate participates through the aerobic shunt pathway in the energy-yielding reactions to maintain the cell. The existence of a nucleoside phosphorylase in this reaction is implicit in the work of Dische (10) and has been isolated in purified form from human erythrocytes (3). Inasmuch as inosine and guanosine are split readily by this phosphorylase and adenosine only at a very slow rate, it would appear that the action of adenosine could be explained by an initial conversion to inosine and subsequent cleavage to ribose-1-phosphate and hypoxanthine (3). This conversion can occur by means of an adenosine deaminase which has been found in the erythrocyte (11, 12). Thus this reaction sequence would implicate inosine as the actual effective agent in erythrocyte preservation, and further studies with this nucleoside will be reported subsequently.

SUMMARY

The addition of adenosine to rabbit or human blood stored at 4°C. in acid-citrate-dextrose improves the preservation of erythrocytes. The chemical characteristics of the red cell are improved, and the effective period of storage is approximately twice as long as with acid-citrate-dextrose alone. In high concentrations adenosine without glucose extends the period of viability of citrated blood and reduces the glucose consumption. However, both adenosine and glucose are necessary to provide optimal cell preservation. A similar effect is produced by related purine nucleosides, such as deoxyadenosine. The relative toxicity of these compounds when administered intravenously, and a possible mechanism of action of adenosine in blood preservation are discussed.

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