ERYTHROCYTE PRESERVATION. VII. ACID–CITRATE–DEXTROSE–INOSINE (ACDI) AS A PRESERVATIVE FOR BLOOD DURING STORAGE AT 4° C.[1]

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Previous investigations on erythrocyte preservation (1–4) have demonstrated a marked effect of purine nucleosides on the metabolism of the red cell throughout storage. Attention has been focused upon the effect of adenosine in the maintenance of glucose metabolism and of high energy phosphate compounds, such as adenosine triphosphate (ATP). As a result, not only are the physical and chemical characteristics of the erythrocyte preserved, but the storage period, during which satisfactory post-transfusion viability is maintained, is extended to approximately twice that observed in acid-citrate-dextrose (ACD) preservative alone. It was postulated that the effect of adenosine was the result of an initial enzymatic conversion to inosine by a deaminase and the subsequent splitting of inosine to yield hypoxanthine and ribose-l-phosphate, mediated by a nucleoside phosphorylase (4, 5). Thus, it appeared that inosine might be the actual substrate for the initial enzymic reaction, and hence the preferred substance for viability studies. In order to evaluate a new preservative, it is desirable to correlate 1) the chemical characteristics of the stored red cells, 2) the ability of the cells to survive after transfusion, and 3) possible toxic effects of the preservative. Studies employing acid-citrate-dextrose-inosine (ACDI) as a blood preservative are presented in this paper, as well as further investigations on the mechanism of action of purine nucleosides.

METHODS

Procedures for the determination of phosphate partition, glucose, and osmotic fragility of erythrocytes have been described elsewhere (3, 6).

Inosine, adenosine, deoxyadenosine, and adenine were obtained from Schwarz Laboratories; guanine, xanthine, hypoxanthine, guanosine, xanthosine, and ribose-5-phosphate from Nutritional Biochemicals Corporation. Adenosine mono- and tri-sulfate were gifts from Dr. A. Hock, and 2,6-diaminopurine riboside from Dr. G. B. Brown.

One-dimensional, paper chromatography on Whatman No. 1 filter paper was performed with several solvent systems; the R_f values for known standards are given in Table I. Identification of the purine compounds was accomplished by inspection of the papers under ultra violet light (Mineralite). After location of the spots, the materials could be eluted from the paper and measured spectrophotometrically.

The concentration of inosine in stock solutions was calculated from light absorption measurements at 247 mμ in the Beckman DU spectrophotometer, using a *millimolar* extinction coefficient of 13.2 at pH 7.0. This value was determined on a weighed sample of pure inosine. Similarly, hypoxanthine concentration was determined at 249 mμ using a *millimolar* extinction coefficient of 10.5 at pH 7.0. For a determination of total inosine concentration *in plasma* of blood treated with inosine, the light absorption of the sample was measured at 247 mμ using the untreated plasma as the control. In later experiments, it was found that hypoxanthine was present in the plasma of blood stored with inosine (or adenosine). Since the molar extinction coefficients of hypoxanthine and inosine are different, the relative amounts of each could not be estimated directly from light absorption measure-

TABLE I

Paper chromatography of nucleosides and bases

	R_f value in solvent system		
	1*	2	3
Inosine	.50	.08	.84
Adenosine	.82	.19	.53
Guanosine	.53	.10	.71
Xanthosine	.40	.04	.65
Hypoxanthine	.63	.26	.66
Xanthine	.51	.12	.65
Guanine	.66	.10	.45
Adenine	.89	.36	.40
Uric Acid	.32	.02	.71

* Solvent System 1—Isobutyric acid:concentrated ammonia:water (66:1:33), ascending. Solvent System 2—n-butanol:water (86:14), descending. Solvent System 3—Water adjusted to pH 10 with NH_4OH, ascending.

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ments alone. In this instance, the hypoxanthine was estimated enzymatically using xanthine oxidase (7) on a neutralized perchloric acid filtrate of the plasma, and this value used in conjunction with light absorption data to calculate the inosine concentration.

Ammonia was measured with Nessler's reagent and adapted for use in the Beckman DU spectrophotometer (8).

Post-transfusion survival of blood stored in ACDI was determined by the differential agglutination technique (Ashby) (9) with slight modifications, and by a double isotope method employing Cr^{51} and P^{32} (10). The blood was collected from Type O-RH negative donors directly into plastic bags (kindly supplied by Cutter Laboratories) containing 75 ml. of ACD.[2] Saline solutions of inosine were sterilized by filtration through an ultrafine fritted pyrex disc. The inosine was added immediately, and the blood was refrigerated at 4° C.

On the day of transfusion, the total blood volume of the recipient was determined with the use of P^{32} tagged fresh cells. Smears of the stored blood were stained and examined as a precaution against bacterial contamination. The red cell count of the stored blood was enumerated, and the blood was weighed in order to determine the volume of blood to be infused. The stored blood was then infused over a 30-minute period, and an initial sample for counting of unagglutinable cells was obtained 10 minutes later. A previously prepared sample of Cr^{51} tagged stored cells was then injected, and samples for radioactive counting were obtained after 10, 20, and 30 minutes and at intervals throughout 10 days subsequently. Post-transfusion survival was calculated from the above data obtained with P^{32} and Cr^{51} measurements.

Samples for counting by the differential agglutination technique were obtained at intervals for 60 days following transfusion. The theoretical post transfusion unagglutinable count was calculated as follows:

$$\text{Theoretical count} = \frac{\text{RBC/ml.} \times \frac{\text{gm. blood injected}}{1.08}}{\text{total blood volume (ml.)}}.$$

Baseline unagglutinable counts were less than 2.5×10^4 cells per mm.[3] using powdered Anti-A serum.[3]

The per cent survival, by both isotope and Ashby techniques at any given time, was determined from the ratio of the actual count to the theoretical count. These data were expressed graphically, and a straight line extrapolation was made to zero time. The value so obtained was designated "post-transfusion survival" and represents the percentage of cells which was viable after storage.

The hypotensive effect of solutions of inosine and hypoxanthine, when administered intravenously, was measured by recording directly the arterial pressure in dogs as described previously (3). In addition, 250 ml. of plasma from blood stored in ACDI was infused intravenously into a volunteer recipient. The blood pressure, pulse, and respiratory rate were recorded at one-minute intervals during the infusion, and for one hour subsequently. Similar recordings were made for each recipient of blood stored in ACDI.

RESULTS

A. *Reversal of the storage lesion with inosine*

The relative order of activity of purine nucleosides in the regeneration of phosphorylated esters of the stored erythrocyte was studied with human blood which had been stored 25 days. The blood was divided into 5 aliquots, and 1,300 μmoles of nucleoside were added per 100 ml. of red cells. The compounds tested were adenosine, inosine, xanthosine, and guanosine; one aliquot without nucleosides served as a control. All samples were incubated at 37° for 45 min., and erythrocyte phosphate partitions were determined subsequently. From a series of experiments, a representative of which is shown in Table II, it was found that inosine was the most effective agent in promoting resynthesis of phosphate esters. In hemolysate systems the same relative order of activity of the nucleosides has been demonstrated (5). Deoxyadenosine and 2,6-diaminopurine riboside were somewhat less effective than xanthosine in the intact cell system, while adenosine mono- and trisulfate were inactive. The purine bases, adenine, hypoxanthine, guanine, and xanthine, and ribose, alone, or in combination, were all likewise inactive, although ribose-5-phosphate was shown to have comparable activity to inosine in the hemolysate system. The inability of ribose-5-phosphate (and other phosphate esters) to penetrate adequately the intact cell made it impossible to assess its effectiveness in this system.

The utilization of adenosine can be attributed to its initial conversion to inosine, according to equation (*1*),

$$\text{adenosine} + H_2O \rightarrow \text{inosine} + NH_3 \quad (1)$$

since ammonia was found to be released in equimolar amounts when adenosine was incubated with stored erythrocytes (*cf.* Table III).[4] This enzymatic conversion is rapid, since, after one hour incubation of adenosine with erythrocytes, inosine and hypoxanthine are the only substances present,

[2] National Institutes of Health, Formula B.

[3] We are indebted to Lederle Laboratories for the antiserum used in these studies.

[4] It is of interest in this respect that 2 moles of ammonia are produced for each mole of 2,6-diaminopurine riboside utilized in the restoration of stored cells.

TABLE II

The effect of purine nucleosides on phosphate esters of stored erythrocytes

Human blood stored 25 days in ACD was incubated with 1,300 μmoles nucleoside per 100 ml. red cells for 45 minutes at 37°. Phosphate partitions* were performed after incubation.

	μmoles P/100 ml. RBC					
	P_i	EH	DH	Non H	Total	Total organic
Fresh blood†	130	215	124	903	1,372	1,242
Incubated control	667	146	54	200	1,067	400
Inosine	90	394	326	585	1,395	1,305
Adenosine	172	323	305	507	1,307	1,137
Guanosine	260	295	312	373	1,240	980
Xanthosine	315	251	268	375	1,209	894

* P_i = inorganic phosphorus; EH, DH, and Non H = easily, difficultly, and non-hydrolyzable phosphorus, respectively.

† These values represent an average obtained from determinations made on a large number of samples of fresh, human blood collected in ACD.

as revealed by paper chromatography of the mixture. Furthermore, only the latter two compounds can be found in the plasma of blood stored for as short a period as 4 days with adenosine.[5] The presence of adenosine deaminase in erythrocytes has been demonstrated previously by Drury, Lutwack-Mann, and Solandt (11) and by Schaedel and Schlenk (12). Although it has been reported (12) that the activity of adenosine deaminase declines rapidly upon storage of blood, our present results would indicate that the enzyme is, in fact, stable to storage.

The finding of inosine, hypoxanthine and ammonia in adenosine-treated cells, the presence of an adenosine deaminase, and the previous observation that the nucleoside phosphorylase splits inosine but not adenosine, all suggest strongly that the sequence of events is:

$$\text{adenosine} \xrightarrow{-NH_3} \text{inosine} \rightarrow \text{hypoxanthine} + \text{ribose-1-phosphate} \quad (2)$$

rather than:

$$\text{adenosine} \rightarrow \text{adenine} + \text{ribose-1-phosphate}$$
$$\downarrow -NH_3 \quad (3)$$
$$\text{hypoxanthine}$$

This latter scheme has been invoked (13) as one of several possible pathways of purine metabolism in other tissues. Prankerd (14) has suggested that adenosine is cleaved directly to adenine by intact erythrocytes, but his identification of the purine was based upon paper chromatography in a single solvent system, *viz.* n-butanol: formic acid: water (77: 10: 13), and it was not stated whether hypoxanthine and adenine were separable under these conditions,[6] or even whether hypoxanthine was considered as a possible product.

Paper chromatographic evidence indicated that the purine reaction product of the utilization of inosine or adenosine during storage is hypoxanthine, and that of guanosine is guanine. The pathway of the utilization of xanthosine is being investigated, and an initial conversion to guanosine seems likely.

TABLE III

Release of ammonia from adenosine

Twelve-ml. aliquots of human blood stored 32 days were incubated 1 hour at 37° with adenosine, inosine, or 2,6-diaminopurine riboside. The control sample contained saline. The total volume in each case was 14 ml., but the results were calculated for 100 ml. of whole blood.

	Nucleoside "absorbed" (μmoles)	Ammonia produced (μmoles)	Ammonia produced / Nucleoside "absorbed"
Control		100	
Adenosine	510	620	1.02
Inosine	595	130	0.05
Diaminopurine riboside	150	405	2.03

[5] This recent observation suggests that the figures which have been presented heretofore on the "per cent absorption" of adenosine into red cells in various experiments must be modified, since the spectral measurements have not taken into account the presence of both hypoxanthine and inosine which have different extinction coefficients. The previously reported values, thus, have been too low.

[6] R_f values of 0.33 and 0.30 have been reported for adenine and hypoxanthine in this solvent (G. R. Wyatt *in* Nucleic Acids, Vol. I. ed. by E. Chargaff and J. N. Davidson, New York, Academic Press, Inc., 1955, p. 252).

B. *Studies on erythrocytes stored in ACDI*

Inasmuch as inosine appeared to be the nucleoside of choice for reversal of the storage lesion, chemical and post-transfusion survival studies were carried out on blood stored in ACDI. Three units of Type O-RH negative blood were drawn into plastic bags containing ACD. Approximately 3400 μmoles inosine per 100 ml. red cells were added to each. The blood was stored at 4° for 42 days (Unit 1), 43 days (Unit 2), and 46 days (Unit 3). At these times, chemical analyses and post-transfusion survival measurements were performed.

1. *Chemical studies*

Illustrative data are shown in Table IV, which indicate that certain physical and chemical characteristics of these red cells were maintained well throughout 6 to 7 weeks of storage. The glucose level of the whole blood had not declined appreciably from its initial level of about 315 mg. per cent, and the osmotic fragility of the cells, while somewhat increased, was not as great as that of cells stored in ACD alone for 6 weeks (15). The acid soluble organic phosphate fraction of cells stored in ACDI had declined slightly (normal, fresh blood = about 1,300 ± 300 μmoles P/100 ml. RBC) but still represented the preservation of most of the organic phosphate esters.

TABLE IV

Characteristics of blood stored in ACDI

Days' storage	Inosine added initially (μmoles)	Blood glucose (mg. %)	Erythrocyte organic phosphate (μmoles P/100 ml. RBC)	% NaCl at 50% hemolysis
Unit 2–43	6,900	287	1,076	0.44
Unit 3–46	6,600	288	736	0.50

There is a considerable quantity of hypoxanthine (measured enzymatically with xanthine oxidase) in the plasma of blood after storage in ACDI. This is assumed to be the result of the uptake of inosine and its metabolism by the cell [7] with subsequent outward diffusion of hypoxanthine into the plasma. Whether this reaction occurs at the membrane or in the cell interior [8] is unknown. Studies illustrating this conversion were carried out on Blood Unit No. 2. To the blood were added initially 6,900 μmoles of inosine. After 43 days of storage, there were approximately 1,500 μmoles of inosine and 3,000 μmoles of hypoxanthine in the plasma. The remaining 2,400 μmoles (obtained by subtraction of 1,500 plus 3,000, or 4,500, from 6,900 μmoles) would represent the actual "intracellular" amount and could be in the form of either inosine or hypoxanthine. From the finding that 1,500 of the initial 6,900 μmoles of inosine remained in the plasma, the percentage "absorption" of inosine was therefore (6,900–1,500)/6,900, or 78 per cent. These amounts are only order-of-magnitude approximations, and further analytical details will be reported in a subsequent communication. It is important to recognize, however, that the amount of purine base, hypoxanthine, in the plasma increases during storage as a reflection of the metabolic breakdown of inosine. In another experiment it was shown that even after a short period of storage in ACDI, *i.e.*, 15 days, there were 300 μmoles hypoxanthine in the plasma derived from the 1,300 μmoles of inosine added initially to the blood sample.

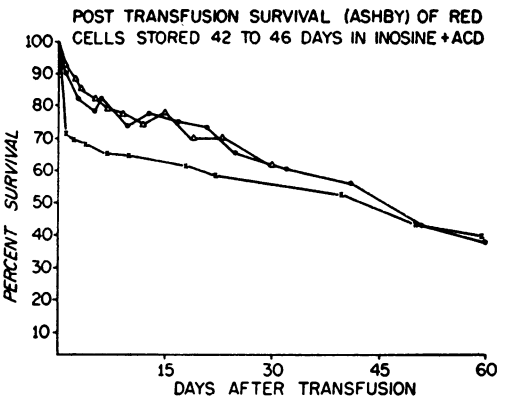

FIG. 1. EACH CURVE REPRESENTS THE TRANSFUSION OF ONE UNIT OF BLOOD STORED IN ACDI

△ = Unit 1, stored 42 days;
● = Unit 2, stored 43 days;
× = Unit 3, stored 46 days.

[7] Since erythrocytes or plasma are not thought to contain xanthine oxidase, it is not unexpected that hypoxanthine should accumulate under these conditions.

[8] Nucleoside phosphorylase is found as a soluble enzyme in hemolysates (5) and hence must be assumed to be localized initially in the cell interior, or, if on the stroma, or cell membrane, to be easily detached during hemolysis.

2. *Survival studies*

The survival data of erythrocytes stored in ACDI are plotted in Figure 1. Each curve represents the transfusion of one of the three units of stored blood described previously in this paper. The average post-transfusion survival of these cells, stored about 6 to 7 weeks, was 82 per cent. Comparable survival values were obtained with the use of Cr^{51} tagging, as shown in Table V. Following the initial phase of rapid destruction of non-viable cells, the destruction rate over the following two months approximated 1 per cent per day and was comparable to that obtained when fresh cells were transfused.

C. *Toxicity studies with inosine*

In order to determine the systemic effect of the intravenous administration of the inosine component of the ACDI preservative, studies were carried out in the dog, human, and rabbit. It was demonstrated previously that the intravenous administration of 1 μmole of adenosine per Kg. in the dog and rabbit produced a fall in arterial pressure of approximately 80 mm. Hg with a return to normal in 4 to 10 seconds (3). In contrast, 70 to 80 μmoles of inosine per Kg. were required to produce the same hypotensive effect observed with only 1 μmole of adenosine. The purity of the inosine sample used in these experiments was estimated by paper chromatographic analysis, considered sensitive enough to detect contamination by adenosine of 1 to 2 per cent, and by assay with adenosine deaminase, sensitive to 0.5 per cent. Both assays gave negative results. Despite these results it cannot be said with certainty that the hypotensive effect was specifically related to inosine rather than to a trace of adenosine (< 0.5 per cent). Hypoxanthine, in amounts up to 100 μmoles per Kg. produced no hypotensive effect.

TABLE V

Post-transfusion survival of blood stored in ACDI

Sample	Days' storage	Post-transfusion survival (%)	
		Ashby Method	Cr^{51} Method*
Unit 1	42	89	87
Unit 2	43	85	78
Unit 3	46	72	68

* Results with the Cr^{51} method average 5 to 10 per cent lower than those obtained with the Ashby technique due to the excessive loss of Cr^{51} during the first 24 hours (10).

Plasma from rabbit blood stored 42 days in ACDI (2500 μmoles inosine per 100 ml. of red cells) was injected into a recipient animal without effect on the arterial pressure. Similarly, 250 ml. of compatible plasma from human blood stored 42 days in ACDI (3,000 μmoles inosine per 100 ml. of red cells) was injected into a human recipient at rates up to 15 to 20 ml. per minute without effect on the respiration, pulse, or blood pressure. There were, likewise, no reactions in the recipients during the infusion of the 3 units of whole blood stored in ACDI.

DISCUSSION

There is a characteristic pattern of chemical and morphological deterioration of stored erythrocytes which is associated with a decrease in post-transfusion viability. It has been shown previously that these changes are not the result of an acceleration of normal *in vivo* senescence of the red cell (6), and that they are unaffected by variations in the extra-erythrocytic environment during storage (16). With the use of an exchange transfusion technique, it was shown that these effects of storage are reversible *in vivo* and that cells surviving the immediate post-transfusion period are capable of withstanding another interval of storage (17). These observations demonstrated that the limiting factor is an intrinsic metabolic failure of the cells which leads to loss of cell viability.

Further studies indicated that the red cell can utilize purine nucleosides, especially adenosine, in two ways (1–3) : 1) To reverse the storage lesion *in vitro,* and 2) To retard the onset of the storage lesion with a resultant prolongation of the effective period of storage.

In the present study the relative order of effectiveness of these nucleosides in the reversal of the storage lesion, as indicated by a resynthesis of organic phosphate esters, was found to be: inosine $>$ adenosine $>$ guanosine $>$ xanthosine. This could be demonstrated in both hemolysate and intact cell systems. The above evidence, and the fact that red cell rejuvenation involves the reincorporation of inorganic phosphate into the organic phosphate fraction, suggested that a purine

nucleoside phosphorylase mediated the primary reaction. In a separate study this enzyme has been isolated from human erythrocytes in highly purified form (5). In hemolysate systems, the nucleosides were shown to exhibit the same relative order of activity for enzymatic phosphorolysis as in the rejuvenation phenomenon. Studies with the purified enzyme, however, indicated that only inosine and guanosine were the actual substrates for the enzymatic reaction, and thus implied that adenosine and xanthosine must be converted to either inosine or guanosine prior to phosphorolysis (5). It is, of course, possible that adenosine or xanthosine phosphorylases may exist in the cell or hemolysate and may be lost subsequently upon isolation. However, it should be noted that phosphorylases for these substrates have not been found elsewhere, and that other purine nucleoside phosphorylases split only inosine and guanosine.

It has been shown previously (5) and in the present communication that adenosine undergoes a rapid conversion to inosine with the concomitant production of ammonia and that there is a subsequent cleavage of inosine which yields hypoxanthine as one of the reaction products. Consequently, inosine appears to be the actual effective agent in blood preservation, and the use of adenosine not only introduces an unnecessary side product, ammonia, but may be unsafe for transfusion of whole units of blood due to its marked hypotensive properties when present even in trace amounts. Hypoxanthine had no depressor effect, and that of the inosine preparations used was in the order of magnitude of about 1/100 that of adenosine. Previously, it has been reported (18) that inosine is 1/20 as hypotensive as adenosine. This higher value may be in error owing to the possibility that the inosine, available at that time, may have been contaminated with adenosine.

Blood stored in ACDI contains increasing amounts of hypoxanthine in the plasma as the time of storage progresses, and further studies of the fate of hypoxanthine and its metabolic products in the body, *i.e.*, xanthine and uric acid, are desirable before multiple transfusions of blood stored in ACDI can be advocated.

A study of the viability of erythrocytes stored in ACDI indicated, with both Ashby and Cr^{51} techniques (when allowance is made for excessive Cr^{51} loss during the first 24 hours after transfusion), an average post-transfusion survival of about 82 per cent at 6 weeks of storage. After the immediate destruction of non-viable cells, subsequent loss of transfused cells occurred at a normal rate (approximately 1 per cent per day). Comparable studies on blood stored in ACD alone revealed approximately 10 per cent survival 1 to 2 days after transfusion (10). In the present investigation, a concentration of about 3,400 μmoles inosine per 100 ml. of red cells was employed at the beginning of storage. The optimal level of this nucleoside for preservation has not been determined as yet, and it is possible that the storage period of satisfactory viability may be extended even further than the 6 weeks indicated by the present data.

SUMMARY

The relative order of activity of purine nucleosides in the regeneration of phosphorylated esters of the stored erythrocyte has been established as follows: inosine > adenosine > guanosine > xanthosine. The utilization of adenosine by the red cell involves an initial conversion to inosine, and the subsequent purine metabolite is hypoxanthine. The plasma of blood stored in ACDI contains increasing amounts of hypoxanthine during storage.

The intravenous administration of inosine or hypoxanthine has only a questionable hypotensive effect in comparison with the pronounced hypotensive properties of adenosine.

The post-transfusion survival of cells stored in ACDI for 6 to 7 weeks is about 82 per cent as indicated by the differential agglutination and Cr^{51} techniques.

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