ADENINE IN RED CELL PRESERVATION *

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Purine nucleosides have been reported to extend the preservation of red cells stored in acid citrate dextrose (ACD) (1–6). This effect was originally attributed to the production of phosphorylated ribose as an energy substrate within the erythrocyte (7–9) through the action of nucleoside phosphorylase (10). This enzyme was shown to act on inosine, guanosine (11, 12), and on adenosine after its conversion to inosine by the adenosine deaminase of the erythrocyte (3, 13–19). Since inosine was the preferred substrate over guanosine (11) and was much less toxic than adenosine (2, 3, 16, 18, 20), it was selected as the most suitable of the nucleosides for red cell preservation. Indeed, the initial studies indicated that inosine produced satisfactory preservation for 42 days and was even more effective than adenosine in regenerating depleted organic phosphate esters in erythrocytes stored for 25 days (3, 11).

While it is well established that purine nucleosides provide phosphorylated pentose as a source of energy for the red cell, subsequent observations suggested that the viability effects could not be explained by this mechanism. In the first place, the original viability results with inosine (3) could not be reproduced. Thus, in a subsequent investigation (5), only 4 of 23 units stored with inosine were satisfactorily preserved after 6 weeks, thereby raising the question of variability in the composition of the inosine prepara-

tions used. Secondly, unexpected differences occurred in studies comparing various nucleosides. Red cell viability was better maintained with adenosine than with inosine (6), and adenosine was more effective than inosine in maintaining and regenerating adenosine triphosphate (ATP) in the stored cells (6, 21, 22). Finally, the importance of the purine moiety of the nucleosides was emphasized by reports of *net* synthesis of adenine nucleotide occurring after incubation of nucleotide-depleted cells with adenosine or with inosine (22–26).

Recently, Nakao and his associates reported an effect of adenine on stored cells (27, 28). When blood which had been previously stored for 8 to 10 weeks was incubated with both adenine and inosine, the altered red cell appearance reverted to normal or near normal and regeneration of ATP took place. These changes did not occur when the incubation medium contained either inosine or adenine alone. Furthermore, when the ACD preservative was supplemented with both inosine and adenine at the beginning of storage, red cell shape, osmotic resistance, ATP and post-transfusion viability were better maintained than in samples supplemented with inosine alone or not supplemented at all (29, 30).

The present studies are concerned with the action of adenine alone on post-transfusion viability and on biochemical alterations of erythrocytes during storage. The results indicate that small amounts of this purine base are more effective than large supplements of inosine in the preservation of red cell viability.

MATERIALS AND METHODS

Purine compounds. Adenine (lot no. AN 6001) and inosine (lot no. I0 5802) were obtained from Schwarz BioResearch, Inc., and adenine sulfate (lot no. A89-70) from the Sigma Chemical Company. These compounds

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were examined by ascending paper chromatography using a) distilled water, b) n-butanol: 4 per cent boric acid (86:14), and c) n-butanol: H_2O (86:14) as solvent systems (31). Sufficient material was spotted to detect 0.5 to 1.0 per cent hypoxanthine in adenine, or 0.5 to 1.0 per cent adenine, adenosine, or hypoxanthine in inosine. Ultraviolet-absorbing impurities were not found. Chromatography of the adenine sulfate, in quantities sufficient to detect small contaminants, resulted in streaking and thus proved unsatisfactory; its spectral reference ratios were 0.76 at A 250/260, 0.35 at A 280/260, and 0.02 at A 290/260, which conformed to accepted values (32).

Solutions of adenine, adenine sulfate, and inosine were prepared in concentrations of 0.02, 0.02, and 0.15 M, respectively, in 0.15 M NaCl. When larger amounts of adenine were required, the purine was dissolved first in 1 N HCl and adjusted to pH 6.5 with 1 N NaOH, resulting in a final concentration of 0.045 M adenine in 0.36 M NaCl. All solutions were sterilized through Morton ultrafine fritted Pyrex filters. The concentrations of adenine and inosine were calculated by their absorption at 262 mμ (A_{mM} 13.1, pH 1) and 248 mμ (A_{mM} 12.2, pH 6), respectively (32).

Design of experiments. From healthy type O subjects who had not been previously bled for at least 6 months, 500 ml of blood was drawn directly into plastic bags[1] containing 72 ml of ACD.[2] Immediately thereafter, 50-ml portions were transferred to glass storage bottles[3] and supplemented with adenine, inosine, adenine plus inosine, or isotonic NaCl, as indicated. After mixing, the bottles were stored at 4° C and not disturbed again until 1 week prior to transfusion, when samples were removed for culture in fluid Sabouraud medium at 22° C and in thioglycollate broth at 32° C. On the day of transfusion the cultures were inspected, and smears of the stored blood were stained with Wright's stain and examined for bacterial contamination. No microorganisms were found.

Post-transfusion survival was measured in healthy volunteers using a double isotope technique (33) with slight modifications. Cr⁵¹ was employed for labeling stored cells; fresh cells, previously washed in citrate phosphate dextrose, were labeled with P³² (34) and served as a reference for the recipient's blood volume. Just before infusion approximately 1 ml of the P³²-labeled cell suspension was mixed with 10 ml of the Cr⁵¹-labeled cell suspension. From this mixture approximately 5 ml was injected into each of two recipients, and the remaining cells were used for the preparation of radioactivity standard solutions. Samples were obtained for radioactivity determinations 10 minutes, 1, 2, and 3 days after infusion of the labeled cells.

The theoretical 100 per cent survival, expressed as Cr⁵¹ counts, was determined from the ratio of Cr⁵¹ and P³²

radioactivity in the sample infused and from the P³² radioactivity of the 10-minute post-transfusion specimen. The actual Cr⁵¹ activity in each post-transfusion sample could then be expressed as per cent survival. The survival values for Days 1, 2, and 3 were plotted semilogarithmically and extrapolated to zero time. Hemolysis occurring during storage and preparation of the cells was measured and subtracted from the extrapolated survival figure. The value thus obtained was taken to represent the actual post-transfusion survival of the stored cells and is termed "viability." All viability figures listed are the average values obtained in two recipients from each blood specimen. A sample of fresh cells, suitably labeled with both isotopes, was included in each series of survival measurements as a check on the 100 per cent survival figure.

Transfusion studies were performed in 124 recipients with 62 specimens of blood from 10 donors. The standard deviation[4] of duplicate determinations was 2.7 per cent. The rate of disappearance of radioactivity from the recipient's circulation after the initial loss of nonviable cells averaged 2.5 per cent per day. All extrapolated survival figures were obtained from lines whose slope did not exceed 3 per cent per day.

Measurement of erythrocyte glycolysis in the presence of methylene blue. Blood was centrifuged for 5 minutes at 1,800 G in the International refrigerated centrifuge, model PR-2, at 4° C, and the cells were washed with 8 vol of cold 0.15 M NaCl, centrifuged as above, and resuspended, with continuous mixing, in 2 vol of cold 0.3 M glycine-KOH buffer, pH 9.0. After the suspension was kept at room temperature for 10 minutes to allow pH equilibration, centrifugation was repeated, and the supernatant fraction was removed. The final cell suspension was prepared by the addition of 8 ml of isotonic saline-phosphate buffer (0.075 M sodium chloride–0.04 M potassium phosphate), pH 8.0, containing 130 μmoles of glucose, to 4 ml of red cells.

Reaction mixtures were prepared by the addition of 2 ml of the cell suspension, as prepared above, into each of five 25-ml Erlenmeyer flasks containing 0.012 μmole of methylene blue (final concentration, 6×10^{-6} M). The flasks were fitted with rubber serum stoppers,[5] and all mixtures were equilibrated at 37° C for 60 minutes in a Dubnoff metabolic shaking incubator (60 oscillations per minute). The reactions in two flasks were stopped with 4.0 ml of cold 0.6 N perchloric acid. After these mixtures were shaken at 4° C for 80 minutes, they were centrifuged, the supernatant fractions neutralized with 20 per cent KOH, and stored frozen for subsequent glucose and lactate determinations. To each of two other reaction mixtures, 25 μl of a water solution of glucose-1-C¹⁴ (about 0.04 μc) was added, and the flasks were resealed with a serum stopper fitted with a glass center well. Incubation at 37° C was continued for 120 minutes.

The reactions were stopped and the $C^{14}O_2$ released from solution by injecting 4.0 ml of 0.6 N perchloric acid through the rubber stopper. Immediately thereafter, 0.5 ml of hydroxide of Hyamine (36) was injected through the stopper into the center well, and the flasks were shaken in the cold for 80 minutes to insure complete trapping of the CO_2 by the Hyamine. Finally, the perchloric acid extracts were prepared for glucose and lactate determinations as previously described. The remaining reaction mixture (flask no. 5) was used for hemoglobin, hematocrit, and pH measurements, performed at the beginning and end of the incubation period.

The center wells were flushed into counting vials with 10 ml of reagent grade toluene containing 0.4 per cent 2,5-diphenyloxazole (PPO) and 0.01 per cent 1, 4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) and their $C^{14}O_2$ content assayed with a Packard Tri-Carb liquid scintillation spectrometer. Recovery of C^{14} was compared with a standard vial containing 5 μl glucose-1-C^{14} solution, 0.5 ml hydroxide of Hyamine, and 10 ml of PPO-POPOP-toluene. Counting efficiency was about 60 per cent with a background of about 25 cpm. Periodically, a control reaction mixture containing glucose-1-C^{14} and buffer was incubated and analyzed; no significant radioactivity was released into the Hyamine (37).

Standard methods. Morphologic examinations of the stored cells were performed on smears stained with Wright's stain and on wet preparations suspended in a solution of 1 per cent formalin in 3 per cent trisodium citrate (38). Total hemoglobin was estimated colorimetrically as cyanmethemoglobin; plasma hemoglobin was measured as pyridine hemochromogen (39). Measurements of pH on whole blood and incubation mixtures were performed at room temperature with a Beckman model G pH meter and standard glass electrodes.

Glucose was estimated with glucose oxidase (40) and lactate with lactic dehydrogenase (41). ATP was measured in the bioluminescent reaction with luciferin-luciferase (42) by the G. K. Turner fluorometer. The above determinations were performed on duplicate neutral perchloric acid extracts of whole blood or incubation mixtures. ATP was measured on the same day the extracts were prepared.

Materials. Lactic dehydrogenase (type I), and desiccated firefly tails were obtained from Sigma Chemical Company; ATP (dipotassium) and diphosphopyridine nucleotide from Pabst Laboratories; glucose oxidase from Worthington Biochemical Company; glucose-1-C^{14} (specific activity 8.3 μc per mg) from the Volk Radiochemical Company; and hydroxide of Hyamine 10-X, PPO, and POPOP from the Packard Instrument Company.

RESULTS

In the experiments to be described adenine was added to the ACD-blood at the beginning of the storage period in order to evaluate its effect on:
1) the preservation of post-transfusion viability,
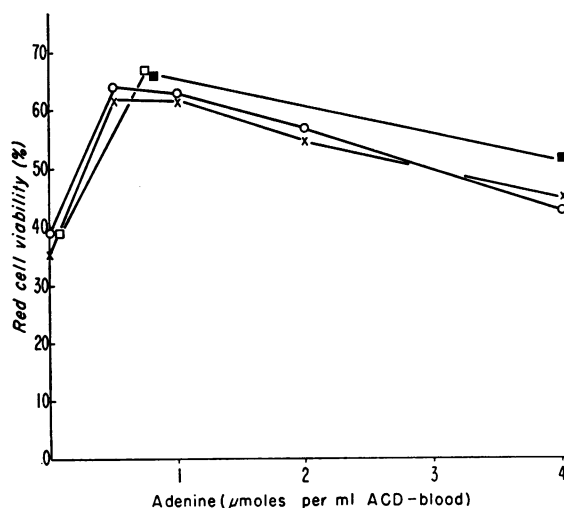2) chemical changes accompanying storage, and

FIG. 1. RED CELL VIABILITY AFTER 6 WEEKS OF STORAGE AT 4° C IN ACD AND VARYING AMOUNTS OF ADENINE. Adenine was added at the beginning of the storage period. Experiment B, X——X; C, O——O; D, □——□ adenine and ■——■ adenine sulfate. Units B and C were stored for 42 days; unit D was stored for 46 days.

3) erythrocyte glycolysis measured at the end of the storage period.

Post-transfusion survival studies

Effect of adenine concentration. This was evaluated by comparing samples of blood, obtained from the same donor unit, to which different amounts of adenine had been added (Figure 1). After 42 to 46 days of storage at 4° C, 35 to 39 per cent of the control cells in ACD remained viable; by contrast 62 to 67 per cent of cells survived when supplemented with 0.5 to 1.0 μmole of adenine (all supplements of adenine and inosine are presented as μmoles per ml ACD-blood). Larger supplements were less effective in improving post-transfusion survival. This was observed whether adenine was added as a hypertonic solution (experiments B and C) [6] or as an isotonic solution of adenine sulfate (experiment D). Both adenine preparations were equally effective when added to provide a supplement of 0.75 μmole.

Duration of satisfactory storage with optimal adenine supplementation (0.75 μmole). This was evaluated in 3 donor units by performing survival

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[6] With 4 μmoles of adenine the tonicity in these specimens was about 12 per cent higher than that in the ACD controls.

TABLE I

Effect of duration of storage on the viability of red cells stored in ACD plus adenine

Experiment	Days stored	Post-transfusion viability	
		ACD	ACD +adenine*
		%	%
D	32	62	81
	39	48	74
	46	39	67
	46†		66
E	32	76	92
	39	63	84
	46	44	79
F	32	57	74
	39	41	76
	46	29	66

* At the outset of storage 0.75 μmole of adenine per ml ACD-blood (1.8 to 2.0 μmoles per ml red cells) was added in each experiment. Units D, E, and F were studied concomitantly. Survival results for the fresh controls were: Day 32, 99%; Day 39, 100%; Day 46, 98%.

† Adenine sulfate.

measurements after 32, 39, and 46 days of storage (Table I). After 39 days the viability varied from 74 to 84 per cent with adenine as compared with 41 to 63 per cent for the ACD controls. One week later the corresponding figures were 66 to 79 per cent with adenine and 29 to 44 per cent for the controls. In each of these units the presence of adenine extended the effectiveness of the ACD preservative by 2 weeks or more.

In all, 12 samples of blood from 6 donors were supplemented with an optimal level of 0.75 ± 0.25 μmole of adenine and stored 39 to 46 days; 9 specimens from the same units were stored in

TABLE II

Effect of adenine on the viability of red cells stored 42 days in ACD plus inosine*

Adenine supplement	Post-transfusion viability			
	Experiments			
	G	H	I	J
μmoles/ml blood	%	%	%	%
	47	44	63	30
0.05			62	43
0.10			74	
0.20			82	52
0.45			84	72
0.70	70	77		
0.90			91	77
7.0	75	63		

* Inosine supplement, 9 μmoles per ml ACD-blood.

ACD and served as controls. The mean post-transfusion viability with adenine was 70 per cent (range, 62 to 84 per cent) and 41 per cent (range, 29 to 63 per cent) for the controls.

Inosine plus adenine supplementation. Four donor units were stored with 9 μmoles of inosine and varying amounts of adenine (Table II). After 42 days, average viability for the ACD-inosine controls was 46 per cent (range, 30 to 63 per cent); with 0.45 to 0.9 μmole of adenine, 78 per cent of the cells survived (range, 70 to 91 per cent). A beneficial action was detectable with smaller adenine supplements as well.

The preceding studies indicated that adenine exerts a preservative action on red cells stored either in ACD or in ACD plus inosine. The following experiments were undertaken to ascertain whether the simultaneous presence of ino-

TABLE III

Effect of adenine and inosine on the viability of red cells stored 42 days in ACD

Supplement		Post-transfusion viability		
		Experiments		
Adenine	Inosine	A	B	C
μmoles/ml blood		%	%	%
		35	35	39
1.0		73	62	63
1.0	10	55	74	70

sine actually enhanced the adenine effect. One specimen from each of three donor units was supplemented with 1 μmole of adenine while another received 1 μmole of adenine plus 10 μmoles of inosine (Table III). The proportion of cells remaining viable after 42 to 46 days' storage with inosine plus adenine was 55, 74, and 70 per cent, respectively; with adenine alone it was 74, 62, and 63 per cent. These results suggest that any additional effect of inosine on the viability of cells stored in ACD plus adenine is slight when compared with the effect of adenine alone.

In vitro studies

General changes. Morphologic studies during storage showed some retardation in the progressive change from biconcave disk → crenated disk → crenated sphere → microsphere when adenine or adenine plus inosine was present, but quantita-

tion of these changes was not undertaken. In the various units studied, the pH was initially 7.1 to 7.2 and declined to values between 6.6 and 6.9 after 6 weeks of storage, without consistent differences related to the type of supplementation.

In vitro hemolysis amounted to about 1 per cent by 6 weeks of storage. It was slightly more with the larger supplements of adenine and somewhat less when inosine was present. Further hemolysis during processing of the cells was appreciable only in those specimens which contained 4 μmoles of adenine; the corrections for cell loss through hemolysis required in estimating viability ranged from 1 to 3 per cent.

Chemical changes accompanying storage. The total amount of glucose consumed and lactate formed during the storage period was calculated from the glucose and lactate content of the blood, measured at the beginning and end of the storage period. In each of the units tested (6 experi-

FIG. 3. SERIAL DETERMINATIONS OF ATP, GLUCOSE CONSUMPTION, AND LACTATE FORMATION IN BLOOD STORED AT 4° C WITH VARYING AMOUNTS OF ADENINE. Glucose consumption and lactate formation were calculated from the glucose and lactate concentrations in the blood at the beginning and at intervals throughout the storage period. Experiment K: ACD control, ●----● ; 0.75 μmole of adenine per ml ACD-blood, □——□ ; 3.0 μmoles of adenine per ml ACD-blood, ✕—·—✕. Similar results were obtained in an additional study (experiment L). Adenine sulfate was used.
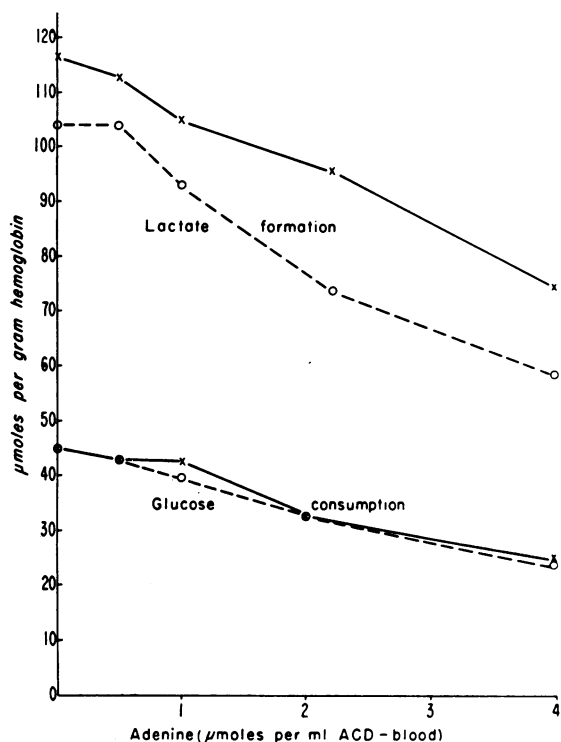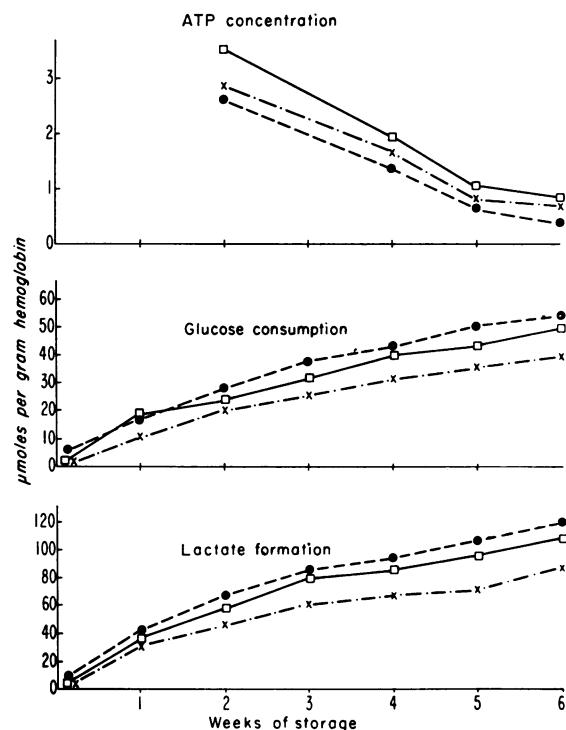
FIG. 2. GLUCOSE CONSUMPTION AND LACTATE FORMATION IN BLOOD STORED FOR 43 DAYS AT 4° C IN ACD AND VARYING AMOUNTS OF ADENINE. The values were calculated from the glucose and lactate concentrations in the blood at the beginning and end of the storage period. Experiment B, ✕——✕ ; C, ○----○.

ments) glucose consumption and lactate formation were reduced in those specimens supplemented with adenine when compared with the ACD controls. Whereas this effect was most pronounced with large amounts of adenine it was already discernible with supplements of 1 μmole or less of adenine (Figure 2). Serial glucose and lactate measurements showed that this reduction in glycolysis took place throughout storage despite the maintenance of higher ATP levels in the samples supplemented with adenine (Figure 3). The effect of adenine on ATP levels was small. The ATP concentrations after 4, 5, and 6 weeks of storage with 0.75 μmole of adenine were 1.93, 1.07, and 0.85 μmoles per g hemoglobin as compared with 1.43, 0.67, and 0.41 μmoles for the
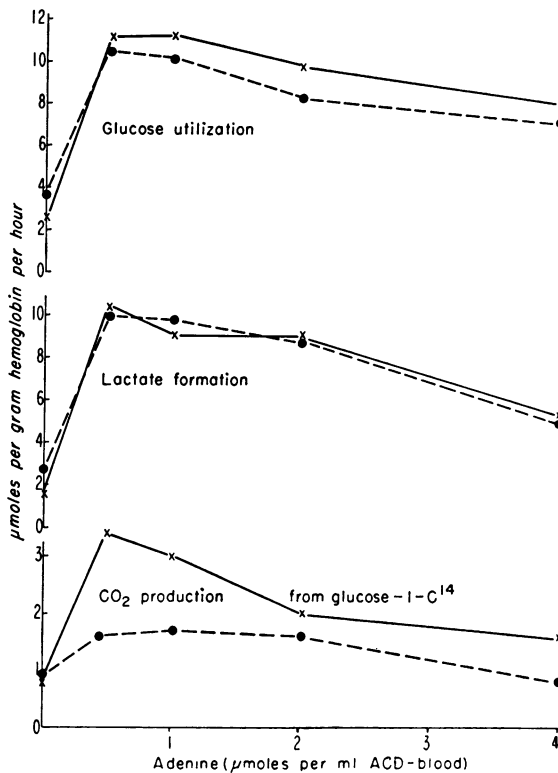
FIG. 4. ERYTHROCYTE GLYCOLYSIS AFTER 6 WEEKS OF STORAGE AT 4° C IN ACD AND VARYING AMOUNTS OF ADENINE. Glycolytic capacity was measured as glucose utilization, lactate formation, and CO_2 production from glucose-1-C^{14} at 37° C, pH 8.0 ± 0.1, and in the presence of methylene blue (see Methods). Experiments B, X——X; and C, ●————●. Values obtained with fresh blood (5 determinations) were: glucose utilization, 15.5 (range, 13.9 to 18.2); lactate formation, 22.2 (range, 20.6 to 23.2); $C^{14}O_2$ from glucose-1-C^{14}, 7.04 (range, 6.95 to 7.26). Figures 1, 2, and 4 depict data from the same storage units.

ACD controls (experiment K). The corresponding values for experiment L were 2.19, 1.39, and 0.76 μmoles with adenine, and 1.48, 0.65, and 0.28 μmoles for the controls.

The effect of inosine supplementation on glucose consumption and lactate formation throughout storage differed considerably from the adenine effect. Glucose consumption was almost completely inhibited while lactate formation was increased when compared with the ACD controls. In none of the stored samples was glucose consumed completely; at least 9 μmoles per ml blood remained in each of the specimens after 6 weeks of storage.

Erythrocyte glycolysis after storage with ade-

nine. The capacity to utilize glucose via glycolysis as well as via the hexose monophosphate shunt pathway and the pentose cycle was measured at the end of the storage period at 37° C in the presence of methylene blue, after the stored cells were washed and their pH was adjusted to a value of 8.0 ± 0.1. In the ACD controls (5 experiments), rates of glucose utilization deteriorated to 21 per cent (range, 16 to 27 per cent), lactate formation to 9 (range, 5 to 13) per cent, and $C^{14}O_2$ production from glucose-1-C^{14} to 10 (range, 7 to 13) per cent of values obtained with fresh blood. Specimens which had contained 0.5 to 1.0 μmole adenine (7 experiments) gave corresponding values of 61 (range, 43 to 72), 46 (range, 40 to 52), and 33 (range, 23 to 48) per cent. The most effective dose range for the preservation of glycolytic capacity, as estimated with these measurements, was the same as in the *in vivo* survival studies. Likewise, larger adenine supplements resulted in suboptimal preservation not only of viability but of glycolytic capacity as well (Figure 4).

DISCUSSION

The studies reported herein indicate that adenine will improve the preservation of red cells stored at 4° C in ACD. With optimal adenine supplementation the post-transfusion survival of blood stored 39 days (3 donor samples in 6 recipients) was better than 70 per cent. After 42 to 46 days of storage, 62 to 79 per cent of the adenine-supplemented cells survived (samples from 6 donors in 18 recipients). These preliminary studies indicate that the duration of satisfactory storage with adenine is at least 5 weeks.

In vitro measurements also demonstrated the beneficial effect of adenine. The ability of the stored cells to catabolize glucose, when measured at 37° C at the end of the storage period, was much better maintained in red cells stored with adenine than in control cells kept in ACD alone. ATP levels were also higher in the adenine-supplemented samples. While it has been shown that human red cells can incorporate adenine into adenine nucleotides (28, 43–45) it is not known whether this mechanism was responsible for the effects observed here. In our studies, the effect of adenine on viability was more marked than the

effect on ATP maintenance, which suggests that the level of ATP per se may not completely relate to the post-transfusion viability. Mollison and Robinson in their experiments with nucleosides (6) reached a similar conclusion. Whether the action of adenine is more closely reflected in the maintenance of *total* adenine nucleotide levels remains to be determined. It is significant that small amounts of adenine were consistently *more* effective than larger quantities in maintaining the viability, glycolytic capacity, and ATP levels of the stored cells. Furthermore, as the adenine supplement increased, glucose consumption and lactate formation by the cells during storage at 4° C was progressively reduced. In this connection it is of interest that, while adenine is incorporated by mammalian cells in tissue culture, it will inhibit their growth at concentrations near 10^{-3} M (46–49), the same order of magnitude which produced deleterious effects on red cell viability and glycolytic metabolism in the present study.

In comparison with adenine, inosine (9 to 10 μmoles per ml added at the outset of storage) exerted a very limited effect on red cell viability, whether added singly or simultaneously with adenine. However, an optimal inosine supplement has not been established. From the most extensive investigation (5), it appears that inosine (13 μmoles per ml) may extend preservation for approximately 7 days beyond that of ACD alone. Among the inosine studies there are, nevertheless, some results which stand out as being far better than this, and indeed are comparable with those obtained with adenine (3, 5). These discrepancies could be explained if some of the lots of inosine had been contaminated with small amounts of adenine or some other impurity beneficial to red cell preservation which escaped chromatographic detection. The present data suggest that the amount of adenine required to achieve optimal improvement in viability is 6 per cent or less on a molar basis (3 per cent on a weight basis) of the inosine added in previous studies, and even quantities in the neighborhood of 1 to 2 per cent of the prior inosine supplement may extend viability (Table II).

There are stronger resemblances between the present results with adenine and the previous

work with adenosine. Not only are the survival data more comparable, but with adenosine, as with optimal amounts of adenine, glucose consumption during storage is well maintained while it is depressed in favor of ribose consumption with inosine (3, 18, 21, 50). The consistent results obtained with adenosine make contamination a less likely explanation for its effects, but this possibility cannot be completely dismissed.[7] It remains to be determined whether adenosine and adenine share a common underlying mechanism of action. To date, cleavage of adenosine to adenine has not been demonstrated with human red cells (3, 17–19). While significant direct phosphorylation of adenosine to form adenine nucleotides (51) does not appear to occur in rabbit erythrocytes (52, 53), such data for human red cells have not been reported.

SUMMARY

Supplementing the ACD-preservative with small amounts of adenine (0.75 ± 0.25 μmole per ml ACD-blood) at the beginning of the storage period, preserves satisfactory viability (post-transfusion survival greater than 70 per cent) of stored human erythrocytes for at least 5 weeks. Larger amounts of adenine are less effective. The improved viability is associated with higher intracellular ATP levels throughout storage and better preservation of erythrocyte glycolytic capacity, measured at 37° C at the end of the storage period. The simultaneous addition of relatively large amounts of inosine (10 μmoles per ml ACD-blood) has little further effect in extending viability.

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[7] In the studies in which incubation with adenosine resulted in net adenine nucleotide formation (23–26) the magnitude of the increase was such that incorporation into nucleotide of a purine contaminant, which comprised 2 to 6 per cent of the adenosine, could account for the data. In one report (54) the adenosine contained a 10 per cent impurity, having the mobility of adenine or hypoxanthine on paper chromatography. Dr. Mollison has kindly furnished us a sample of the adenosine used in their study (6). No adenine was found after sufficient adenosine was chromatographed to detect a 0.5 to 1.0 per cent adenine contaminant. However, an ultraviolet-absorbing impurity having the mobility of guanosine was detected [n-butanol: water (86:14), distilled water] which comprised about 4 to 5 per cent of the material present.

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