ERYTHROCYTE PRESERVATION. III. THE REVERSIBILITY OF THE STORAGE LESION [1]

By BEVERLY WESCOTT GABRIO, ALEXANDER R. STEVENS, JR., AND CLEMENT A. FINCH

(From the Department of Medicine, University of Washington School of Medicine and the King County Central Blood Bank, Seattle, Wash.)

(Submitted for publication June 8, 1953; accepted September 30, 1953)

The progressive morphological and biochemical alterations which develop in the erythrocyte during *in vitro* storage are not the result of cell aging in a physiological sense (2), nor do they appear to be induced by harmful substances in the extra-erythrocyte environment (3). Rather, the "storage lesion" is considered to be a primary metabolic failure of the *in vitro* erythrocyte. The various measurable abnormalities are regarded only as indicators of this metabolic lesion, since no single abnormality in itself has been shown to be critical to cell viability.

Since the red cell derives its energy primarily from carbohydrate metabolism, the glycolytic scheme and associated high energy phosphate compounds would seem important in a consideration of an abnormality which progresses to ultimate loss of cell viability. Furthermore, it has been shown that characteristic changes appear in the glycolytic structure of the erythrocyte during storage (4) which can be correlated with the post-transfusion survival of blood. In this study of the reversibility of the storage lesion, the phosphate partition, particularly adenosine polyphosphates, was employed as an indicator of the metabolic potential of the cell. The significance of these measurements was evaluated by the post-transfusion survival of the stored erythrocytes.

MATERIALS AND METHODS

Rabbit blood was collected by intracardiac puncture using sterile precautions and stored at 4° C. in ACD (acid-citrate-dextrose) as previously described (2).

Exchange transfusion in the rabbit was performed by the simultaneous injection of stored blood into the ear vein and removal of blood from the cannulated femoral artery. The recipient cell mass had been tagged previously by the injection of fresh rabbit erythrocytes containing labeled hemoglobin (Fe^{59}). Thus the exact amount of exchange of the recipient's erythrocytes with stored blood was calculated from the decrease in circulating radioactivity immediately after the exchange transfusion. The subsequent destruction of stored blood over the following 12 hours was calculated from the increase in the specific activity of erythrocytes since only the non-radioactive transfused cells were destroyed at an increased rate. With this type of short-term experiment in animals, the reversal of the biochemical changes in the stored erythrocytes was investigated.

In man, studies were undertaken to determine the ability of an erythrocyte, after an initial period of storage and transfusion, to withstand a second period of *in vitro* storage. Patient 1 (H. S., Type A) with aregenerative anemia was transfused repeatedly over a period of three weeks with Type O blood which had been in storage for three weeks. Blood was then withdrawn from H. S. and stored for three weeks before transfusion into a Type A recipient. The survival of the Type O cells was determined in the second recipient by the differential agglutination technique. Patient 2 (K. K., Type O) with aregenerative anemia was given 20 units of three-week stored Type O cells over a period of 10 weeks. At the completion of these transfusions, when it was almost certain that all of his circulatory erythrocytes were composed of red cells which had undergone three weeks of storage, a unit of blood was collected from the patient, stored for three weeks and transfused to two Type A recipients. Thus, in each instance, normal blood was followed through an initial three week period of storage *in vitro*, a period in a recipient, a second three week interval of *in vitro* storage, and its subsequent survival in a second recipient.

Procedures for the measurement of phosphate partition, osmotic fragility, mechanical fragility, radioiron processing and counting, and post-transfusion survival of erythrocytes with the agglutination and radioiron techniques have been described elsewhere (2).

RESULTS

I. In Vitro Reversibility of Storage Lesion

In vitro experiments in which stored human and rabbit blood was incubated at 37° C. for one to two hours showed no significant metabolic re-

[1] This investigation was supported in part by research grants from the United States Atomic Energy Commission (Contract AT (45-1)-343) and United States Public Health Service (Contract H-1179(C1)). A preliminary report has appeared (1).

TABLE I

Erythrocyte phosphates after in vitro and in vivo incubation of stored rabbit blood

	mg. P/100 ml. erythrocytes				
	Inorganic	Organic	EH*	DH*	Non-H*
17 day stored blood	36.84	33.33	3.76	4.76	24.81
Recipient	6.24	76.55	9.20	7.27	60.08
Stored blood after 24 hrs. incubation:					
In vitro control	13.35	7.28	2.22	0.40	4.66
Peritoneal cavity	5.51	18.99	5.36	3.89	9.74
Pleural cavity	5.16	40.61	7.83	3.50	29.28

Fraction:	*Predominant constituent:*
* EH—easily hydrolyzable	(adenosine polyphosphates)
DH—difficultly hydrolyzable	(hexose phosphates, adenylic acid)
Non-H—non-hydrolyzable	(2,3-diphosphoglycerate)

storation. These manipulations included incubation of 16 to 21 days stored blood with old plasma, fresh plasma, and a synthetic medium composed of a Krebs-Ringer bicarbonate solution containing 150 mg. per cent glucose and 5 per cent albumin. Mechanical and osmotic fragilities remained abnormal. The organic phosphates, including ATP, were not appreciably restored by incubation. It is noteworthy that after incubation in all mixtures, the high inorganic phosphate retained within the erythrocyte during storage against a concentration gradient favoring the passage of the ion, was partially released into the surrounding media. This was probably related to membrane permeability changes at higher temperatures (5).

II. In Vivo Reversibility of Storage Lesion

A. Extravascular environment

In order to simulate more closely the normal red cell environment, erythrocytes, either enclosed in a differentially permeable membrane or as free cells, were placed in the body cavities of the rabbit. A typical experiment is shown in Table I. Defibrinated rabbit blood stored in ACD for 17 days was divided into three 10 ml. aliquots. Aliquot No. 1, contained in a sterile cellophane sac (8/32 inch diameter), was suspended in a Krebs-Ringer bicarbonate solution containing 200 mg. per cent glucose and incubated at 37° C. Aliquot No. 2 was placed in a cellophane sac which was introduced into the peritoneal cavity of a rabbit. Aliquot No. 3 was injected into the pleural cavity of the same animal. Phosphate partitions of these fractions were determined after 24 hours. Aliquot No. 1 showed a further reduction of cellular organic phosphates, but the accumulated inorganic phosphate had declined by diffusion due to increased permeability of the cell membrane at 37° C. Erythrocytes within the cellophane sac in the peritoneal cavity showed a normal inorganic phosphate content, some regeneration of adenosine polyphosphates, but further depletion of other organic phosphate, notably 2,3-diphosphoglycerate. In the blood which was free in the pleural cavity, the cellular inorganic phosphate declined to normal values and significant improvement was noted in the easily hydrolyzable and non-hydrolyzable phosphate fractions. This could be ascribed either to cellular repair or removal of defective erythrocytes since the amount of free blood in the chest cavity had decreased slightly over the 24 hour period.

It was evident, however, that there was a partial reconstitution of the phosphate compounds after exposure of the erythrocytes to *in vivo* environments as compared with the *in vitro* environment, although the magnitude of the restorative changes was limited.

TABLE II

Phosphate partition of stored rabbit erythrocytes after exchange transfusion

	mg. P/100 ml. erythrocytes			
	Inorganic	Organic	EH*	DH* and Non-H*
I. Stored blood—0 day	6.5	68.7	8.9	59.8
Stored blood—16 days	32.1	35.0	4.7	30.3
Recipient rabbit	4.3	85.7	13.3	72.4
Stored blood—after exchange transfusion†:				
30 min.	9.2	42.5	9.7	32.8
3 hrs.	2.2	55.7	12.0	43.7
6 hrs.	1.0	65.2	13.0	52.2
11 hrs.	9.1	76.3	13.6	62.7
II. Stored blood—0 day	7.5	70.9	9.2	61.7
Stored blood—16 days	36.5	35.8	6.7	29.1
Recipient rabbit	3.1	87.2	14.2	73.0
Stored blood—after exchange transfusion†:				
0	35.9	51.1	6.9	44.2
15 min.	17.0	52.8	8.1	44.7
30 min.	9.4	58.4	8.9	49.5
45 min.	4.6	59.8	9.1	50.7
60 min.	3.4	64.7	10.3	54.4

* See legend, Table I.

† By appropriate correction, the values represent the stored transfused cells only with the exception of the 11 hrs. values which include a slight reticulocyte contribution.

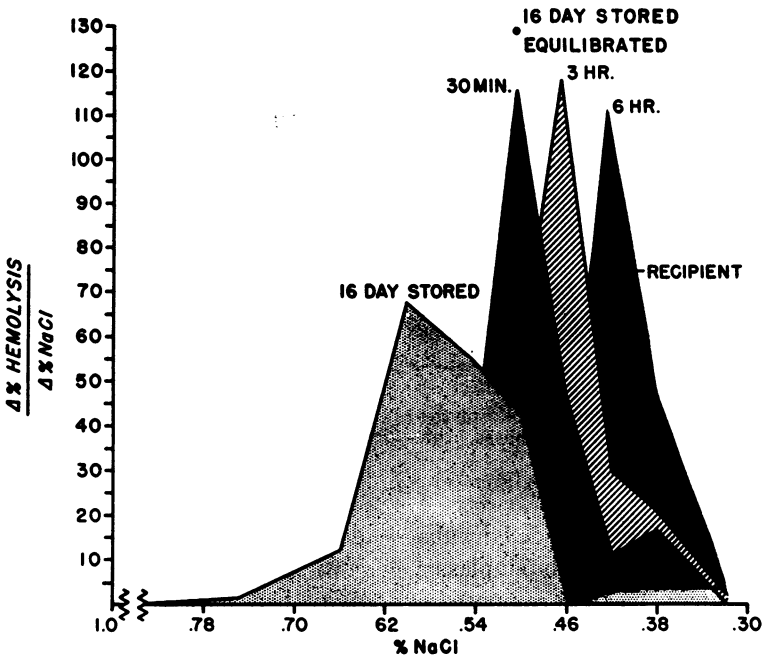

FIG. 1. OSMOTIC FRAGILITY MEASUREMENTS OF STORED RABBIT BLOOD 30 MINUTES, 3 HOURS, AND 6 HOURS AFTER EXCHANGE TRANSFUSION COMPARED WITH STORED BLOOD EQUILIBRATED IN VITRO WITH FRESH PLASMA

B. Intravascular environment

1. Exchange transfusion in animals: In order to follow the metabolic alterations of the stored erythrocyte subsequent to its return to active circulation, it was necessary to replace a large fraction of an animal's erythrocytes by stored blood. In the first exchange transfusion 67 per cent of the red cell mass of a normal rabbit was replaced by blood stored in ACD for 16 days. During the ensuing 11 hours, 20 per cent of the transfused cells were destroyed, and the values reported are corrected so that they represent only the transfused cells (Table II). Regeneration of the organic phosphate compounds of stored blood had begun at 30 minutes and was essentially completed at 6 hours, the values approaching those of the recipient animal. The accumulated cellular inorganic phosphate, which changed even more rapidly, exhibited a considerable decline explicable by a simple diffusion out of the cell as well as reutilization to a lesser extent. At 11 hours there was an increase of reticulocytes to 17 per cent, presumably responsible for the rise in cellular inorganic phosphate. The change in organic phosphates, particularly adenosine polyphosphates and 2,3-diphosphoglycerate, was of such a magnitude that, even assuming that there was none in the stored erythrocytes which disappeared from circulation, the remaining cells must also have been depleted and undergone a regeneration of organic phosphate. Osmotic fragility studies indicated a return to normal in contrast to the partial shift towards normal achieved by re-equilibration of these erythrocytes with fresh plasma (Figure 1). A second exchange transfusion (Table II) illustrates the reconstitution of the cellular phosphate partition occurring within the first 60 minutes.

2. Reconstitution of stored blood in man: A sub-

TABLE III

Regeneration of phosphate partition of stored erythrocytes after transfusion in man

	mg. P/100 ml. erythrocytes			
	Inorganic	Organic	EH*	DH* and Non-H*
Recipient before transfusion	2.4	52.4	9.6	42.8
Transfused cells (stored 3 wks.)	24.0	12.4	4.1	8.3
Recipient 3 hrs. after transfusion	2.3	48.4	10.7	37.7

* See legend, Table I.

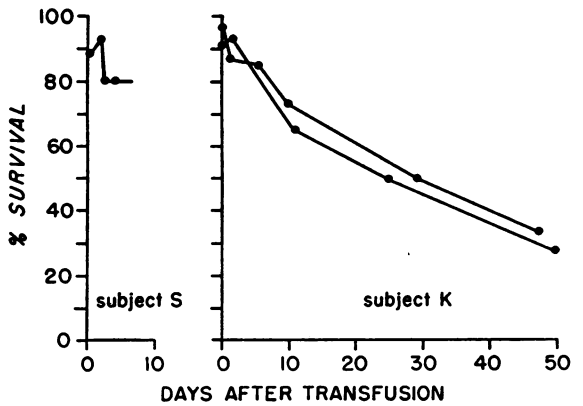

FIG. 2. SURVIVAL OF ERYTHROCYTES AFTER TWO THREE-WEEK PERIODS OF IN VITRO STORAGE

Normal erythrocytes stored three weeks and transfused to subjects with aregenerative anemia were again withdrawn and stored for a second three-week period. The subsequent survival of 2 units of such blood from subject K. K. and one from subject H. S. in normal recipients as illustrated is equivalent to blood undergoing only a single period of storage for three weeks.

ject with aregenerative anemia was transfused for ten weeks with three-week stored erythrocytes in an amount each week equivalent to two-thirds of the subject's red cell mass. The reversion to normal of the phosphate partition of the stored cells which occurred after each transfusion is illustrated in Table III.

III. Viability Studies in Man

Since the final criterion must be the effect on post-transfusion survival, further studies were conducted in man to determine whether the metabolic reversal of the storage lesion also represented an ability of the cell to withstand further storage. Cells stored for three weeks were transfused to two patients with aregenerative anemia. Blood was then withdrawn from these patients, stored a second three-week interval and transfused. In one patient the total cell mass had been replaced by repeated transfusions of three week old cells. In the second it was possible by differential agglutination to distinguish between the erythrocytes of the patient and previously transfused cells. The post-transfusion survival of these cells after the second period of storage (Figure 2) was as good as would be expected following a single period of storage. In addition, the changes in erythrocyte phosphate and potassium during the second storage period were somewhat less than those typical of only three-week storage.

DISCUSSION

The two major criteria of the storage lesion studied herein have been the alterations in erythrocyte phosphate compounds, notably adenosine polyphosphates, and the ability of the cells to survive in the circulation after transfusion. The phosphate changes are considered to be an indicator of disturbed metabolism of the cell as they reflect changes in overall carbohydrate metabolism and are metabolically linked to alterations in electrolytes. The post-transfusion viability is the ultimate criterion in the problem of blood preservation and must be measured directly.

Although the reported results do not delineate the origin of the storage lesion, they do indicate that when the stored erythrocytes are replaced in active circulation, the changes due to storage can be rapidly reversed. Depending upon the duration of storage, certain red cells become non-viable and rapidly leave the circulation. With ACD preservative this phase of destruction is complete within the first 24 to 48 hours, and thereafter cell destruction proceeds at a normal or near-normal rate. Thus storage damage in terms of post-transfusion survival appears to be an all-or-none phenomenon. If damage has not reached the critical point, the erythrocyte fragility and phosphate partition return to normal within a few hours. Maizels and Paterson (6) have observed sodium and potassium reconstitution under similar conditions.

It has been further demonstrated that the cell, after the rapid reversal of storage changes to normal, is able to withstand further storage as well as would fresh blood. Since blood stored continuously for 40 days in ACD shows less than 20 per cent survival, the beneficial effect of introducing the cells into circulation midway in this storage interval is apparent.

Initial attempts to produce a reversal of the storage lesion *in vitro* were unsuccessful. The lack of regeneration of organic phosphates under these conditions is reminiscent of the work of Ponder (7) in which the red cell was found to lack the ability to restore potassium *in vitro* after 10 days storage. Studies in progress indicate that

fractions of liver tissue have some effect on the phosphates of stored erythrocytes, but with these particulates complete reconstitution has not been obtained.

Pertinent to the problem of preserving red cells is the mechanism whereby the erythrocyte regains normality in the blood stream. That this is not due wholly to the freely diffusible nutrients which appear in the serous fluids is evident from the lack of complete regeneration of stored red cells placed in serous cavities. However, the fact that partial reconstitution was accomplished might indicate that the concentration or availability of such metabolites was one of the limiting factors in these environments.

SUMMARY

The biochemical storage lesion of the erythrocyte has been shown to be rapidly reversible, once the erythrocyte is introduced into active circulation. After its reconstitution, the erythrocyte also regains its ability to withstand further storage. *In vitro* manipulations thus far have not produced the reversal demonstrable *in vivo,* and extra-circulatory *in vivo* experiments have shown only limited regeneration. Implications of this reversibility are discussed.

ACKNOWLEDGMENT

The authors wish to acknowledge the valuable technical assistance of Mrs. Alice Rupen and Mrs. Olga Matson.

REFERENCES

1. Gabrio, B. W., and Finch, C. A., A reversible biochemical lesion in stored erythrocytes. Federation Proc., 1952, **11**, 52.
2. Gabrio, B. W., and Finch, C. A., Erythrocyte preservation. I. The relation of the storage lesion to *in vivo* erythrocyte senescence. J. Clin. Invest., 1954, **33**, 242.
3. Gabrio, B. W., Stevens, A. R., Jr., and Finch, C. A., Erythrocyte preservation. II. A study of extra-erythrocyte factors in the storage of blood in acid-citrate-dextrose. J. Clin. Invest., 1954, **33**, 247.
4. Rapoport, S., Dimensional, osmotic, and chemical changes of erythrocytes in stored blood. I. Blood preserved in sodium citrate, neutral, and acid-citrate-glucose (ACD) mixtures. J. Clin. Invest., 1947, **26**, 591.
5. Harris, J. E., The influence of the metabolism of human erythrocytes on their potassium content. J. Biol. Chem., 1941, **141**, 579.
6. Maizels, M., and Paterson, J. H., Survival of stored blood after transfusion. Lancet, 1940, **239**, 417.
7. Ponder, E., Accumulation of potassium by human red cells. J. Gen. Physiol., 1950, **33**, 745.