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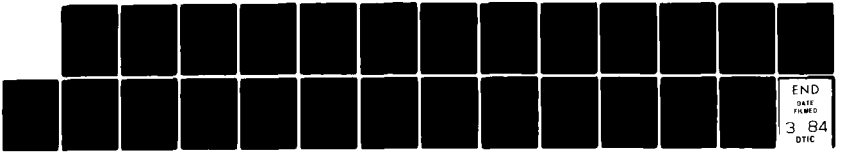

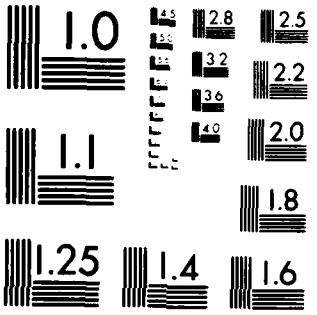

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Red cell Preservation Adenine

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report reviews progress made under contract DAMD17-79-C-9071. The effect of storage position on the shelf life of red cells was investigated; studies were also carried out on the correlation between red cell viability and complement binding, erythrocyte deformability, and peroxidation of membrane lipids.

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For the protection of human subjects the investigator has adhered to policies of applicable Federal Law 45CFR46.

BLOOD PRESERVATION STUDY

ANNUAL PROGRESS REPORT

E. BEUTLER

January 1982

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

FORT DETRICK, FREDERICK, MARYLAND 21701

Contract No. DAMD17-79-C-9071

Scripps Clinic and Research Foundation

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La Jolla, California 92037

TABLE OF CONTENTS

1. Study of blood stored in CPD and in modified CPD solutions containing adenine.

1.1. Background

CPD (citrate-phosphate-dextrose) has been routinely used as the standard medium for preservation of blood for many years. Red cells from blood which has been collected in CPD can only be stored for 21 d. After this, they are no longer sufficiently viable for transfusion. It has been clearly established that the addition of adenine to CPD preservative solution prolongs the length of time that red cells may be stored to 35 or 42 d (1). Moreover, higher concentrations of glucose than those present in CPD are required to sustain red cell concentrates for prolonged periods of time in adenine-containing media (2).

CPD-A1 contains 1.25 times the glucose content of CPD and sufficient adenine to provide a concentration of 0.25 mM in the blood-preservative mixture. Higher glucose concentrations were not incorporated into this preservative because of the existence of anecdotal reports that high glucose concentrations increased the rate of fall of the pH of platelet concentrates. CPD-A1 was studied by a cooperative group (3) with the demonstration that red cells from whole blood stored for 35 d had viability of 80.53 $\pm$ 6.44% ($x \pm 1$ S.D.) while red cell concentrates with a hematocrit averaging 75% had a mean viability of 71.38 $\pm$ 10.3% ($x \pm 1$ S.D.). After these studies were concluded, it was discovered that the manufacturer of the blood bags had inadvertently prepared the bags used from an obsolete plastic film, PL 130, rather than the new plastic formulation, PL 146, which had been intended. These studies

were then repeated in PL 146, with results quite similar to those obtained in PL 130.

In both of these studies, it was observed that most or all of the glucose was exhausted from cells in some units packed at hematocrits of 75 ± 5% and stored for 35 d. There is reason for concern that under field conditions, where the storage temperature may sometimes rise to higher than 4° and where packing of the red cells, a parameter which is difficult to control, may frequently exceed 80%, that CPD-A1 may prove to be suboptimal for the storage of red blood cells. In the meantime, in vitro studies failed to show any deleterious effect of higher glucose concentrations on platelet function (4). For this reason, two new preservatives, believed to represent improved formulations for storage of cell concentrates have been devised and manufactured by Fenwal Laboratories. These preservatives designated CPD-A2 and CPD-A3 contain respectively 1.75 and 2.00 times the amount of glucose present in CPD and sufficient adenine to provide a concentration of 0.5 mM in the blood-preservative mixture.

Data which have been accumulated over the past 15 years clearly indicate that ATP levels are a disappointing parameter with respect to predicting the viability of stored red cells (5-8). There is a great need to identify measurements which may prove to be more useful than ATP in predicting whether or not a given storage system provides red cells which will be viable on reinfusion.

1.2. Progress in current period (January 1981 through December 1981).

1.2.1. Biochemical studies and studies of viability of stored cells.

450 ml of blood has been collected from each of 10 volunteer donors. After an 8-hour holding period at room temperature the red cells were packed to a hematocrit of 80% and stored at 4°C, 2 units for 42 and 8 units for 49 d without agitation. Storage position of the blood was alternated between lying and standing. Viability studies were carried out on all 10 units. The results of biochemical studies and of the viability of the 8 units stored for 49 d is summarized in Table I. The other two units, stored for 42 d represented the last units of the study reported last year. In contrast to the studies (Table II) which we have reported earlier indicating greater glucose utilization in blood stored in the lying position than in blood stored in the standing position for 42 d, no significant difference has been observed in the studies, carried out at 49 d. The viability of these samples was 69.09% $\pm$ 5.2% ($\bar{x} \pm 1$ S.D.). There was no significant correlation between 24 hour viability, on the one hand, and post-storage ATP levels on the other ($r = 0.07 \pm 0.41$). An undesirable degree of hemolysis was also present, plasma hemoglobin levels ranging between 0.78 and 2.70 g/dl. Moreover the amount of hemolysis was significantly negatively correlated to viability ($r = 0.69 \pm 0.21$).

Subsequently two additional units of blood have been drawn to further investigate the effect of storage position but these have not yet been stored for 49 d.

1.2.2. Auxiliary studies.

In the course of these investigations various auxiliary studies were undertaken to aid in better definition of the "storage lesion."

1.2.2.1. Studies of osmotic fragility.

The osmotic fragility of stored red cells was investigated both before and after reinfusion into volunteer donors. The osmotic fragility of red cells stored in CPD-A2 was found to be greatly increased when measured directly by estimating lysis in graded phosphate-buffered salt solutions. Much of this increase in osmotic fragility was found to be due, however, to the accumulation of lactate in the stored cells. Lactate leaves red cells only slowly and therefore exerts a marked osmotic effect. When stored red cells were pre-equilibrated with isotonic sodium chloride solutions until the lactate had been removed from the cells the osmotic fragility was increased to a much smaller extent. However, a "tail" of osmotically fragile cells could still be detected. Studies to determine the fate of these cells were performed by devising a method of sequential hemolysis which permitted us to measure the osmotic fragility of the reinfused cells using the release of ^{51}Cr from the labeled cells as an indicator of hemolysis. These investigations demonstrated that the osmotic fragility of stored cells rapidly returns to normal after their reinfusion into the circulation. The normalization of the osmotic fragility of reinfused cells does not apparently depend principally upon removal of the most fragile cells, but rather on correction of the increased osmotic fragility of cells in the circulation. This correction is due to regeneration of 2,3-DPG after reinfusion.

We have also compared the size of the fragile tail with in vivo viability. These studies showed little correlation between these measurements (See Figure 1 to 6).

1.2.2.2. Studies of complement binding.

According to Szymanski (9), the binding of complement to red cells may prove to be a useful predictor or viability. In the course of some of the viability studies which were carried out last year red cells have been frozen in glycerol according to Dr. Szymanski's instructions and were shipped to her for study. Results of these investigations are given in Table III. In this small series, no correlation between C3 binding and viability could be demonstrated.

1.2.2.3. Studies of deformability

The measurement of erythrocyte deformability by use of the ektocytometer has been pioneered by Mohandas and Bessis (10). Recently, Mohandas has instituted certain marked improvements in this technology. Samples of blood which were studied for viability have been shipped to Dr. Mohandas in San Francisco for measurements of deformability using the ektocytometer. The results of these studies are summarized in Table IV. They do not show any correlation between deformability and viability of cells stored for 49 d.

2. Studies of lipid peroxidation.

2.1 Background

Little is known of the nature of membrane damage which occurs during red cell storage. It is well-established however, that peroxidation of membrane lipids occurs under many circumstances (11,12). A number of techniques have been developed to measure the products of red cell membrane lipid peroxidation. These include the thiobarbituric acid method for the measurement of malonaldehyde (13) and the measurement

of fluorescent products representing covalently cross-linked complexes of amino acid groups of protein or lipid with malonaldehyde (11). There are no data indicating whether such lipid peroxides accummulate during blood storage and whether the addition of vitamin E, a free radical scavenger, will prevent peroxidation of membrane lipids.

2.2. Studies performed (January 1981 through December 1981)

Various methods for the addition of water-insoluble vitamin E compounds into blood have been explored. D-α tocopherol acid succinate has been used as a source of vitamin E in these studies. The normal level of vitamin E in human plasma is approximately 1 mg/100 ml (14). We found it possible to prepare a solution of 100 mg α-tocopherol in 1 ml of acetone. Diluting the acetone in blood 1,000-fold provides a level of vitamin E which is approximately 10 times the physiologic level.

In preliminary studies we have stored two 60 ml portions of blood collected in CPD-A2 with α tocopherol dissolved in acetone. Acetone alone and blood without either acetone or vitamin E added served as controls. Since the thiobarbituric acid method gave negligible readings even with prolonged-stored blood in preliminary studies, the fluorescent method of Goldstein (11) was used, modified to use a filter fluorometer. The results of our preliminary investigation are given in Table V. Although there was some tendency for fluorescence to be less in the presence of vitamin E, the results are not statistically significant.

3. Publications

The following publications supported, in part, by this contract, appeared during 1981:

Beutler, E. The maintenance of ATP in maltose and galactose containing preservatives is due to endogenous blood sugar. Transfusion 21:232-233, 1981.

Beutler, E., Villacorte, D. Spectrin extractability in blood storage. Transfusion 21:96-99, 1981.

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13. Stocks, J., Dormandy, T.L.: The autooxidation of human red cell lipids induced by hydrogen peroxide. British Journal of Haematology 20:95-111, 1971.

14. Natta, C., Machlin, L.: Plasma levels of tocopherol in sickle cell anemia subjects. American Journal of Clinical Nutrition 32:1359-1362, 1979.

TABLE I - RESULTS OF BIOCHEMICAL AND RE-INFUSION STUDIES PERFORMED ON BLOOD COLLECTED FROM 8 DONORS AND STORED FOR 49 D IN CPD-A_2

Donor		KC	LH	KH	JW	LF	YM	KH	ML
Storage Position		L	L	L	L	S	S	S	S
L = Lying S = Standing									
	Day								
24 hr Viability (%)	49	75.1	66.0	65.1	68.9	72.2	66.2	62.2	77.0
Hematocrit (%)	0	33.9	37.5	32.0	39.0	39.7	33.8	32.0	36.4
	49	83.3	80.5	80.4	82.5	81.3	80.3	79.8	80.9
Glucose (mg %)	0	548.5	538.4	555.3	555.9	619.9	526.5	555.8	569.1
	49	60.0	94.6	163.5	47.6	40.1	99.4	146.9	112.8
ATP (uMoles per gm Hb)	0	3.54	3.85	3.33	3.89	4.28	4.45	4.47	4.78
	49	0.95	1.55	1.45	2.11	0.76	1.47	1.83	2.69
2,3 DPG (uMoles per gm Hb)	0	17.05	13.28	10.17	14.28	17.61	11.86	12.52	13.9
	49	0.10	0.10	0.08	0.12	0.10	0.05	0.09	0.07
Plasma Hb (mg %)	0	11.0	5.8	10.8	5.8	14.4	7.8	10.8	7.8
	49	1624	2700	2490	2700	1248	1192	2520	780
pH (4°C)	0	---	---	---	---	---	---	---	---
	49	6.755	---	6.89	6.785	6.655	6.695	6.755	6.790
Na^+ (mM)	0	164	166	166	167	164	169	165	162
	49	78	74	74	68	77	80	70	80
K^+ (mM)	0	2.8	3.3	3.3	3.1	2.8	3.1.	3.4	3.3
	49	115	111	111	128	117	112	113	113
Glucose consumption (mg consumed/ml RBC)	49	5.87	5.51	4.88	6.16	7.13	5.32	5.12	5.64

TABLE II - RESULTS OF BIOCHEMICAL AND RE-INFUSION STUDIES PERFORMED ON BLOOD COLLECTED FROM 2 DONORS AND STORED FOR 42 D IN CPD-A_2

Donor		AM	TB
Storage Position L=Lying S=Standing	Day	L	S
24 hr. Viability (%)	42	75.1	75.6
Hematocrit (%)	0	33.5	43
	42	81	80.8
Glucose (mg %)	0	598	564
	42	79	153
ATP (µMoles/gm Hb)	0	4.16	4.40
	42	2.07	2.30
2,3-DPG (µMoles/gm Hb)	0	10.0	14.0
	42	0.1	0.1
Plasma Hb (mg %)	0	7	21
	42	1875	1568
pH (4°C)	0	7.50	7.62
	42	6.82	6.83
Na^+ (mM)	0	170	174
	42	80	82
K^+ (mM)	0	3.3	3.3
	42	98	102
Glucose consumption (mg/ml RBC)	42	6.41	5.09

TABLE III

EVALUATION OF COMPLEMENT ON TRANSFUSED RBC

Sample	Storage Length	% Agglutination with anti-C3c+ After Deglycerolization	After Deglyc./Incub.*	% Viability
R.P.	42 days	71.6	55.1	85.4
P.N.	42 days	0.87	2.3	79.2
P.G.	Fresh	0	3.9	---
P.G.	42 days	73.9	44.1	72.9
T.B.	Fresh	5.25	8.3	
T.B.	42 days	77.8	62.3	75.6

* After incubation with heated plasma
+ Data of Dr. I. Szymanski

TABLE IV

	Deformability Index % of Normal[+]	Viability (%)
P.N.	85.4	79.2
K.L.	80.4	81.6
R.P.	76.5	85.4
P.G.	87.3	78.9
A.M.	81.6	75.1
T.B.	77.3	75.6

[+] Data of N. Mohandas

TABLE V

FLUORESCENCE OF EXTRACTED LIPIDS FROM STORED RED BLOOD CELLS
(Fluorescence units per gm Hb)

Donor		Day					
		0	7	14	21	35	49
1	Control	506.8	518.0	869.8	794.5	689.6	634.8
1	Acetone control	679.4	513.1	863.6	916.7	885.2	452.0
1	Vit. E + acetone	590.2	383.7	877.4	689.2	685.6	526.3
2	Control	965.7	384.1	853.9	679.8	573.1	516.8
2	Acetone control	581.9	449.1	838.9	637.6	638.9	496.6
2	Vit. E + acetone	554.3	430.8	799.6	815.4	656.5	476.2

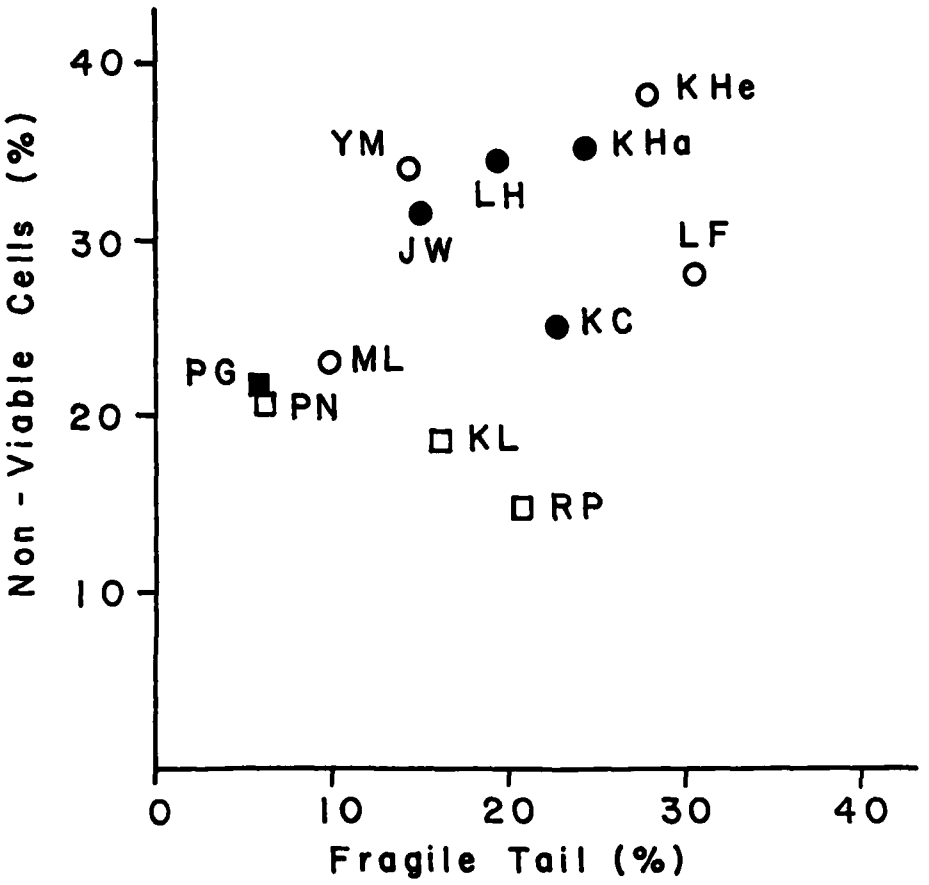

Figure 1. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position; open symbols represent which were stored in the standing position. Units stored for 42 days are designated by squares; units stored for 49 days are designated by circles. The size of the fragile tail in samples stored for 42 days was measured from the osmotic fragility as determined by chromium release from washed labelled erythrocytes. In the case of samples stored for 49 days the size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution at room temperature for 1 hr and then performing an osmotic fragility curve. The fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of 0.55% or greater.

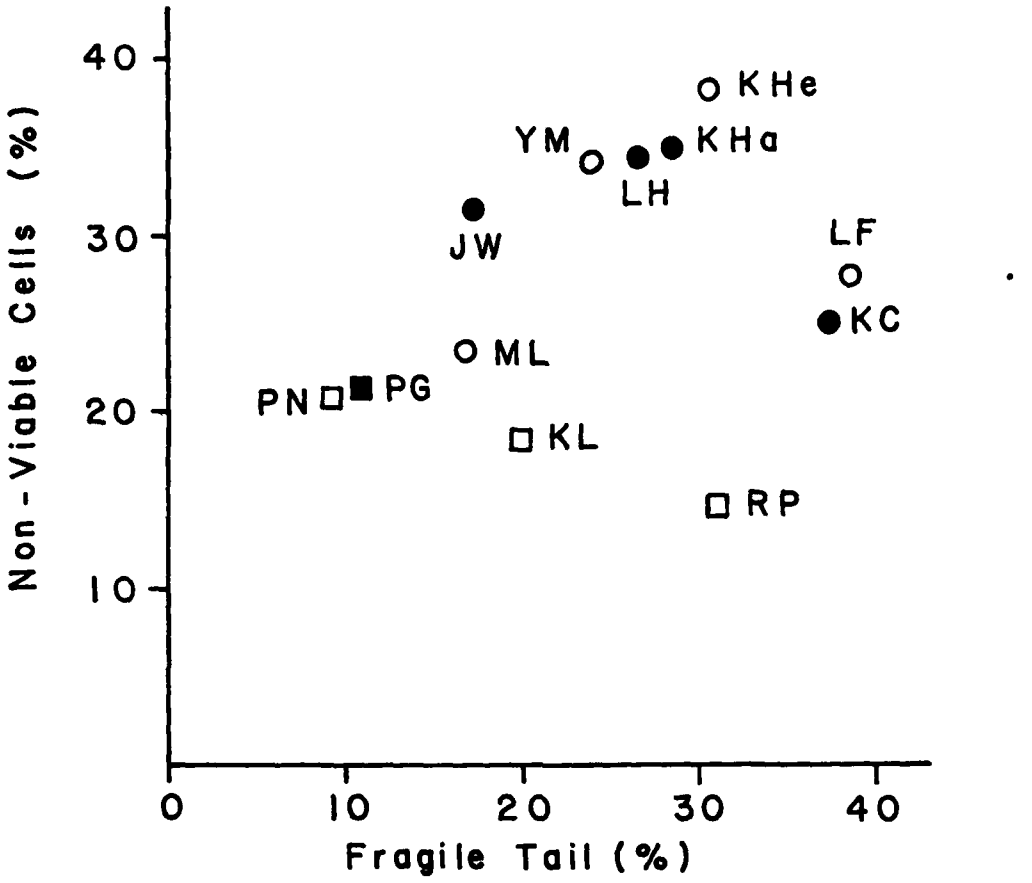

Figure 2. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position; open symbols represent which were stored in the standing position. Units stored for 42 days are designated by squares; units stored for 49 days are designated by circles. The size of the fragile tail in samples stored for 42 days was measured from the osmotic fragility, as determined by chromium release washed labelled erythrocytes. In the case of samples stored for 49 days the size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution at room temperature for 1 hr and then performing an osmotic fragility curve. The fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of 0.5% or greater.

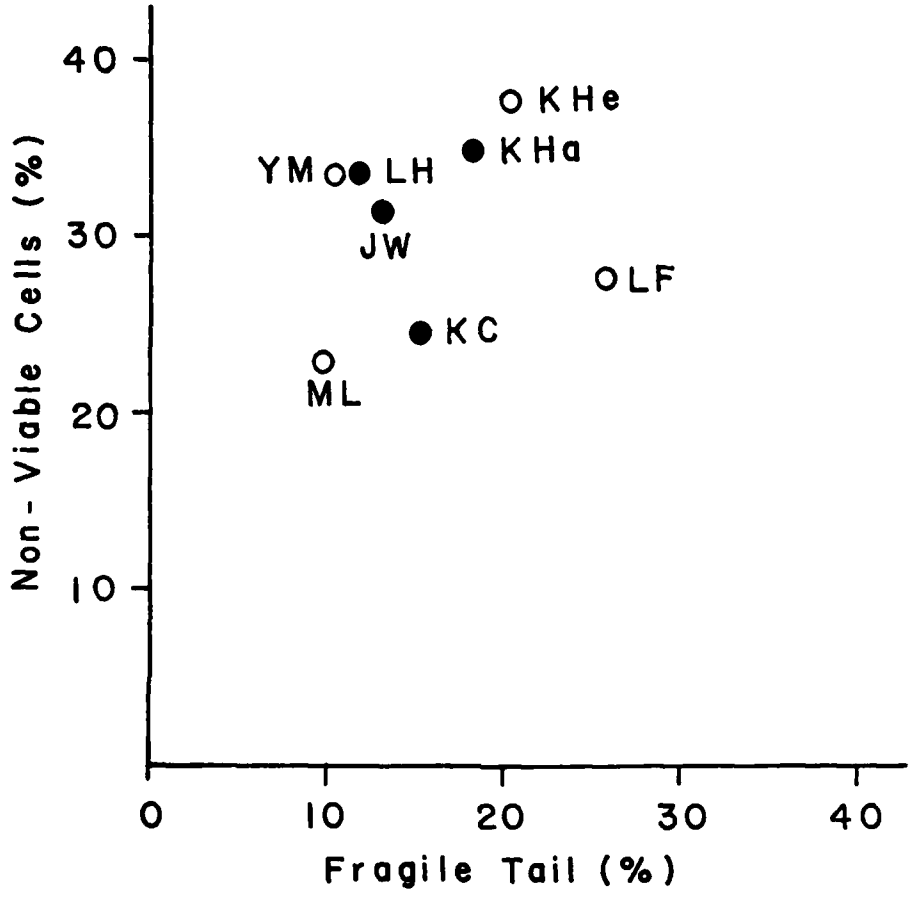

Figure 3. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position for 49 days; open symbols represent which were stored in the standing position for 49 days. The size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution containing 10mM pyruvate and 10mM inosine at room temperature for 1 hour, which regenerated the 2,3DPG to near normal levels. After performing an osmotic fragility curve, the fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of 0.55% or greater.

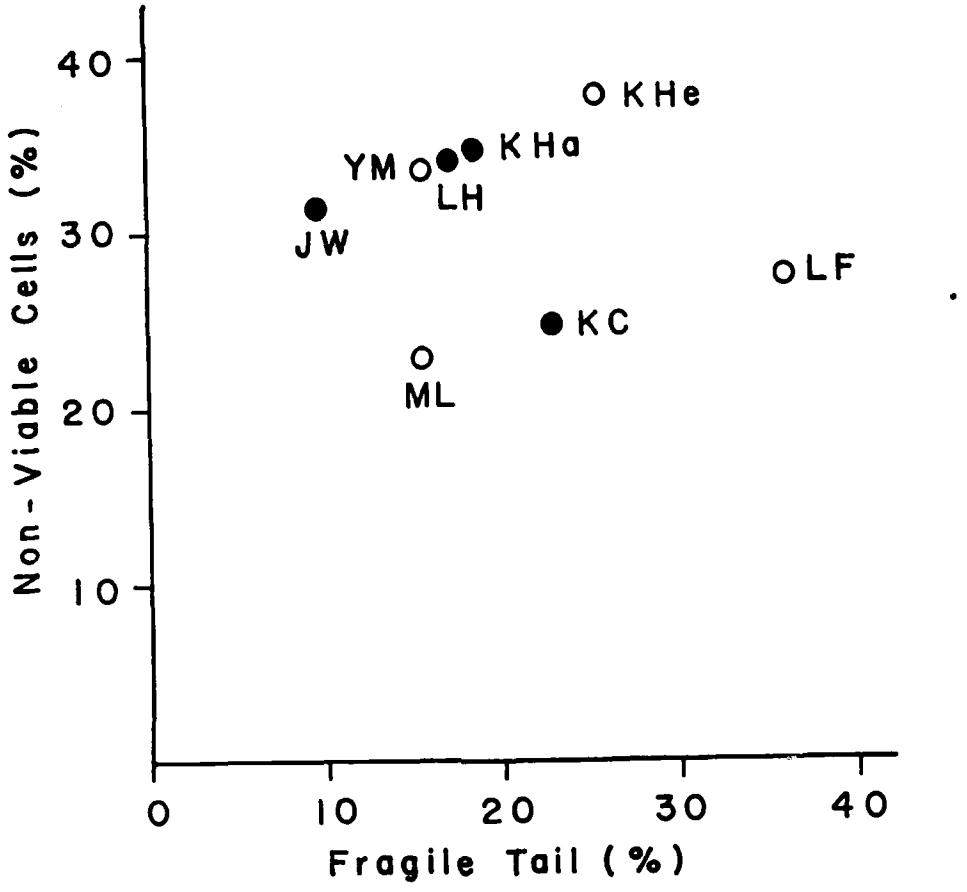

Figure 4. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position for 49 days; open symbols represent which were stored in the standing position for the same length of time. The size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution containing 10mM pyruvate and 10mM inosine at room temperature for 1 hour, which regenerated the 2,3DPG to near normal levels. After performing an osmotic fragility curve, the fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of 0.5% or greater.

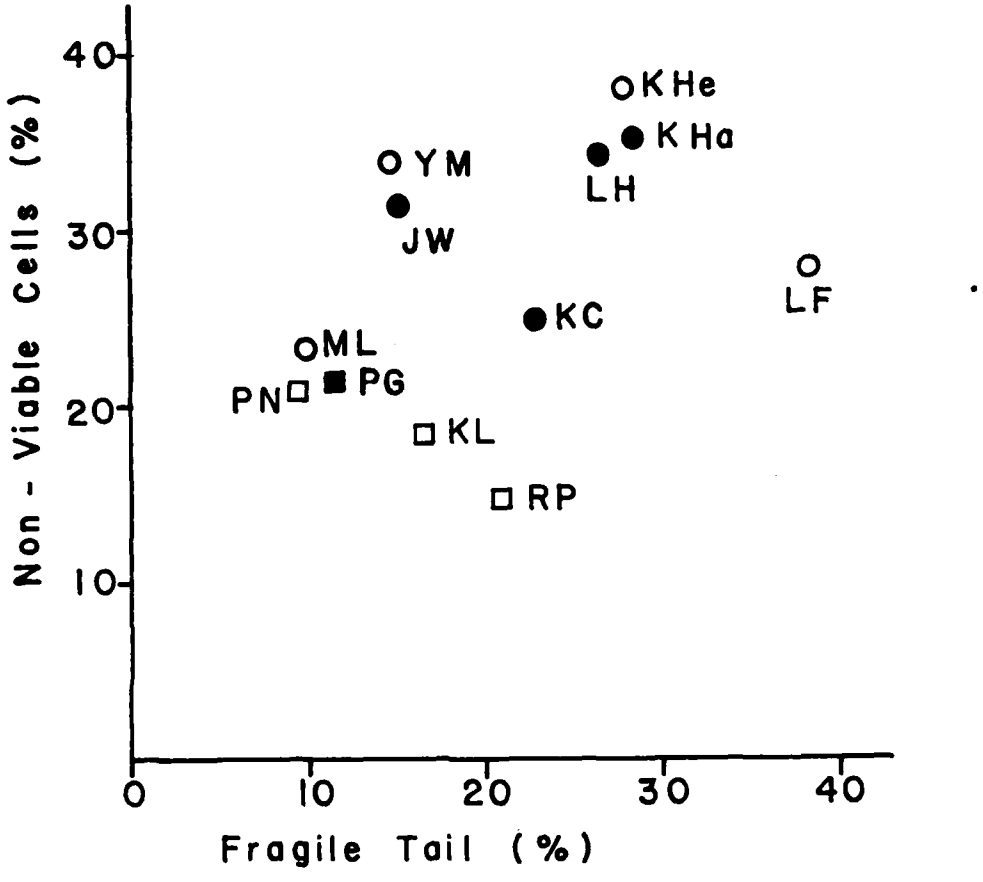

Figure 5. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position; open symbols represent which were stored in the standing position. Units stored for 42 days are designated by squares; units stored for 49 days are designated by circles. The size of the fragile tail in samples stored for 42 days was determined from the osmotic fragility, as determined by chromium release from washed labelled erythrocytes. In the case of samples stored for 49 days the size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution at room temperature for 1 hr and then performing an osmotic fragility curve. The fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of either 0.55% or 0.5%, whichever produced first hemolysis in fresh cells.

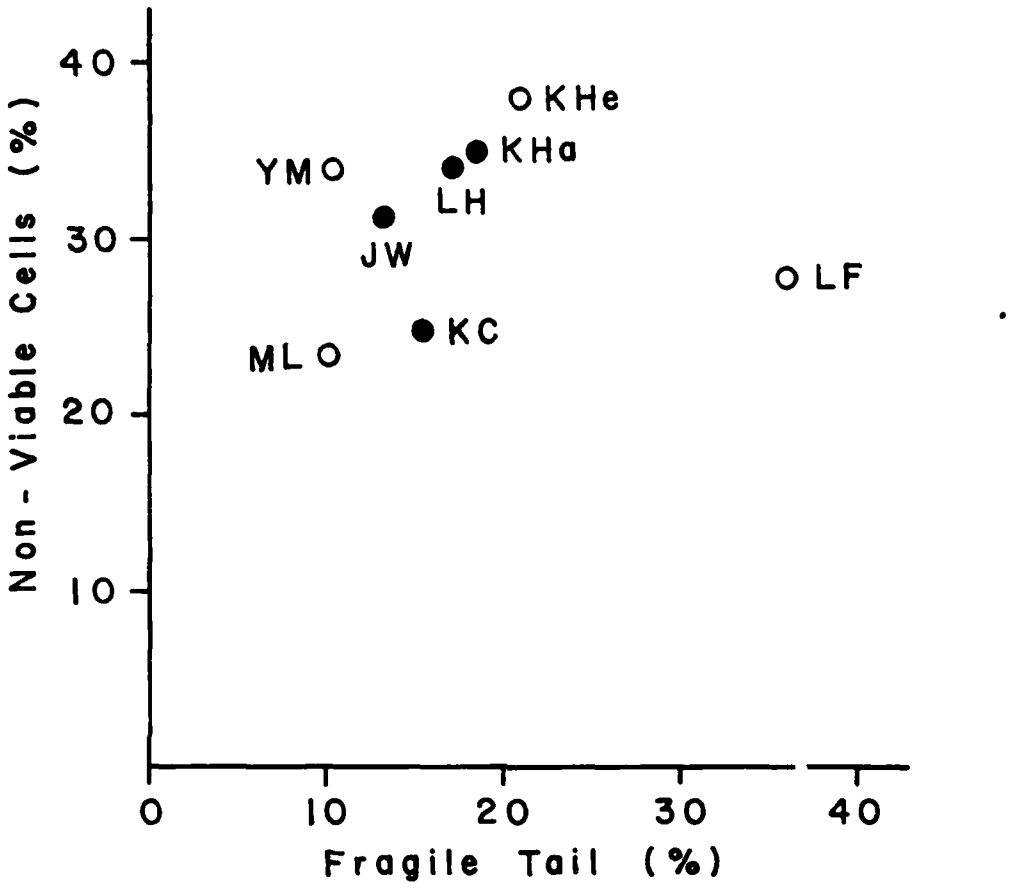

Figure 6. The relationship between the percentage of the cells lysed in the osmotically fragile tail and the percentage of the cells which were non-viable, as measured by ^{51}Cr loss after re-infusion. Solid symbols represent units which were stored in the lying position for 49 days; open symbols represent which were stored in the standing position for the same length of time. The size of the fragile tail was determined by equilibrating erythrocytes with an isotonic buffered saline solution containing 10mM pyruvate and 10mM inosine at room temperature for 1 hour, which regenerated the 2,3DPG to near normal levels. After performing an osmotic fragility curve, the fragile tail was defined as that portion of the erythrocytes hemolysing at a sodium chloride equivalent of either 0.55% or 0.5% whichever produced the first hemolysis in fresh cells.

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