Effect of Additions to ACD Blood on Erythrocyte Preservation.* (20429)

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Human blood, when collected routinely in ACD solution and stored at 4°C is considered suitable for transfusion purposes only during the first 3 weeks after collection. There occurs during storage a gradual change in the red cells so that they lose the ability to survive after entering the circulatory system of the recipient. This process of erythrocyte deterioration is manifested *in vitro* by a progressive increase in osmotic fragility on exposure of red cells to hypotonic sodium chloride solutions. The decisive chemical events responsible for the limited *in vitro* "survival time" of red cells are not known. Enzymatic processes might be at work changing the red cell envelope, either directly through attacks on the lipids and proteins forming the membrane, or, indirectly, through the formation of products injurious to the membrane.

This report describes the effect of a variety of enzyme inhibitors, enzyme activators, and related compounds on the rate of the progressive development of osmotic fragility of human erythrocytes during storage.

Methods. ACD blood collected by the Red Cross was used in this work. In each bottle, as supplied, was a mixture of 480 ml blood and 120 ml ACD solution (NIH solution B, each 100 ml containing 1.47 g dextrose, U.S.P., 1.32 g sodium citrate, U.S.P., and 0.48 g citric acid, U.S.P.). The compounds to be tested for their effect on the development of osmotic fragility were prepared in aqueous solutions, adjusted to pH 6-7, made isotonic by addition of the calculated amount of NaCl, and sterilized by passage through a Seitz filter. If this sterilization method was accompanied by undesirable reactions (for example oxidation of the phenothiazine derivative mentioned below to a blue product) filtration through a Morton Filter Apparatus with ultrafine fritted glass disc (Corning No. 33990) was used instead. ACD blood in 50 ml portions was distributed into rubber stoppered sterile bottles, which contained small volumes (up to 5 ml) of the solutions to be investigated. The mixtures, together with a few control bottles containing samples of the ACD blood from the same donor without further additions (unmodified ACD blood) were stored at 4°C. After various periods of storage time, test samples as well as the corresponding controls were examined for chemical changes (N.P.N., glucose, inorganic phosphate, plasma potassium), for changes in red cell volume (hematocrit), and for osmotic fragility. The tests for *osmotic fragility* were carried out as follows. Three 0.1 ml samples of the blood to be tested were transferred to three test tubes containing 20 ml of, respectively, distilled water (tube 1), 0.9% NaCl solution (tube 2), and 0.6% NaCl solution (tube 3). After gentle mixing, the cell debris as well as the cells remaining intact in the NaCl solutions (tubes 2 and 3) were centrifuged down and the supernatant solutions were transferred to colorimeter tubes. Absorbancy measurements were carried out using a Lumetron Photoelectric Colorimeter, Model 402-E, Filter M-550, after addition of a drop (about 0.05 ml) of 2 *N* NH_4OH to the contents (about 10 ml) of each tube. Identical results were obtained, whether the NaCl-blood mixtures were centrifuged immediately when prepared or whether they were left standing for 30 min before centrifugation. The absorbancy of the completely hemolyzed sample (tube 1) was a measure of the total hemoglobin content. The percentage of this total hemoglobin released into isotonic (0.9%) NaCl solution, calculated from the absorbancy of the content of tube 2, represented the percentage of hemolysis which took place during storage. The percentage of the total hemoglobin found additionally in tube 3, containing 0.6% NaCl solution, gave an indication of osmotic fragility

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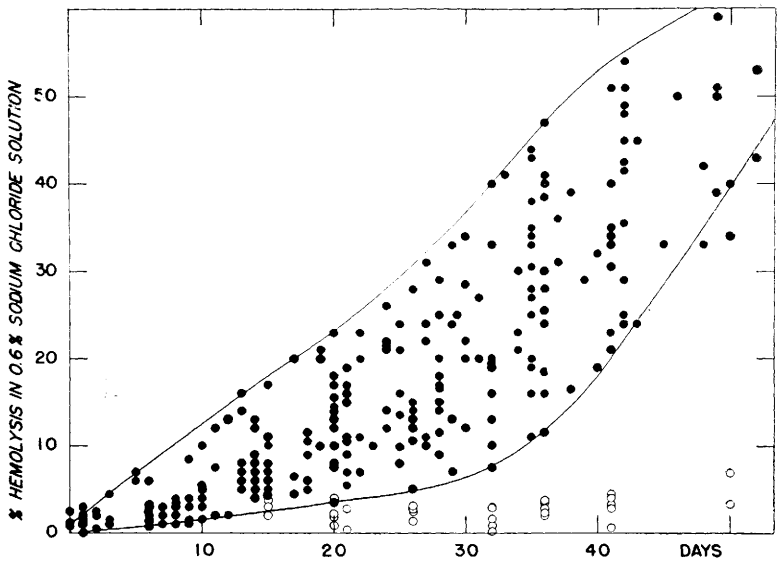

FIG. 1. Increase in osmotic fragility of human erythrocytes during storage of ACD blood. Data obtained with ACD blood from 55 different donors at various intervals are represented by solid black dots. Seven of these specimens were also stored with addition of Phenergan (0.4 mM/l). Results obtained with these modified samples are shown as circles near the abscissa.

and was used as an estimate of *"in vitro survival"* of red cells. This *in vitro* procedure follows a suggestion of and is based on findings by Gibson and coworkers(1) who had reported that the results obtained with this method showed a fair correlation with the post-transfusion survival measured directly by the Ashby technic.

Results of chemical analyses. Neither unmodified ACD blood nor blood samples containing those of the various additions to be discussed later, which retarded the increase in osmotic fragility, showed any significant changes in non protein nitrogen during storage periods of 42 days. This indicated that proteolytic processes leading to *small* breakdown products (soluble in trichloracetic acid) were not taking place. The glucose content of ACD blood and of the modified samples, with the exception of those containing NaF, decreased during storage. A specimen of ACD blood containing, for example, 366 mg glucose per 100 ml just after collection, showed a glucose content of 303 mg after 21 days, and of 269 mg per 100 ml after 42 days of storage. The *plasma potassium* content of ACD blood, of the samples with less pronounced osmotic fragility, and of those containing NaF, increased at about the same rate during storage, for example from 5 meq potassium per liter plasma at the time of collection to 17 meq after 21 days, and 30 meq/liter after 42 days. The concentration of inorganic phosphate in whole ACD blood and plasma increased, for example from 3.2 mg per 100 ml blood on the first day to 13 mg after 21 days, and 15.8 mg per 100 ml of blood after 42 days. The various additions to ACD blood, with the exception of NaF, did not alter significantly the rate of increase of inorganic phosphate.

Changes in hematocrit and osmotic fragility of ACD blood. The hematocrit of ACD blood increased regularly during storage, for example from 44 to 48 in 42 days. There was little spontaneous hemolysis of ACD blood during the early part of the storage period, as measured by the amount of hemoglobin released into 0.9% NaCl solution. Hemolysis after 42 days ranged from 1-3%, but reached 10-13% after 92 days of storage. The osmotic fragility of ACD blood varied with different specimens. Blood from 55 different donors showed on exposure to 0.6% NaCl solution after 21 days storage 5 to 23% (median

TABLE I. Additions to ACD Blood Accelerating the Progressive Increase in Osmotic Fragility during Storage.

Addition	Concentration, mM/l blood	Storage time, days	Hemolysis factor*
L-Arginine	13.6	32	1.28
L-Histidine	.40	24	4.1
Ascorbic acid	1.12	21	1.51
	3.31	21	2.07
Nicotinic acid	.16	29	1.96
Nicotinamide	9.0	57	1.09
Calcium pantothenate	.006	41	1.14
Acetylcholine chloride	1.4	43	1.19
	4.1	43	1.40
p-Amino salicylic acid	.41	39	1.25
	3.27	36	1.34
Benzoic acid	8.33	26	1.62
m-Hydroxy benzoic acid	8.50	34	1.46
p-Hydroxy benzoic acid	8.50	35	2.06
Pyruvic acid	3.0	28	1.18
	11.4	28	1.67
N-Ethyl maleiimide	.73	23	1.83
Methylene blue	.05	41	1.41
	.24	41	1.50
3.7-Diamino-5-methyl-acridinium chloride	.20	30	1.50
α-Hydroxy phenazine	.46	23	1.84
Copper sulfate	.16	10	6.20
	.73	10	10.50
Trypsin (1:300, NBCo.)	10.0†	42	1.32

* Hemolysis factor calculated by dividing percentage hemolysis (in 0.6% NaCl) of test sample by that of control blood.
† mg/liter blood.

13%), and after 42 days 20 to 58% hemolysis. A graphic presentation of such measurements is shown in Fig. 1.

Compounds without effect on osmotic fragility. In blank experiments it was ascertained that the addition of 0.1 volume of isotonic (0.9%) NaCl solution to ACD blood before storage had no effect on the rate of increase of osmotic fragility observed with the corresponding unmodified ACD blood. The following substances were also found to have no influence on the progressive increase in osmotic fragility of red cells during storage (in parentheses are concentrations in millimole per liter blood): ethylenediamine tetraacetic acid (2.0; 4.0; 9.0), iminotriacetic acid (9.0), orthodiaminocyclohexane tetraacetic acid (9.0), nicotinamide (3.0; 6.0), riboflavin (0.001; 0.005), p-amino benzoic acid (0.63; 3.05), creatine (1.2; 3.4), leucine amide (1.0; 4.1), D,L-malic acid (0.4; 9.2), tartaric acid (0.04; 1.96), urea (1.2; 5.8), thiourea (0.12; 0.59), guanidine (0.60), semicarbazide (1.58), sodium thiosulfate (0.40), sodium diethyl dithiocarbamate (0.60; 2.95; 13.7), uric acid (0.02; 0.54), thioglycolic acid (0.78; 3.84), cobaltous acetate (0.28; 1.10), nickel chloride (0.41; 0.81), zinc sulfate (0.20; 0.77), and N,N-dimethyl-N'-(2-pyrimidyl)-ethylenediamine hydrochloride (0.39). Likewise without effect were pepsin (1:10,000; Pfanstiehl, 10 mg/l blood) and cryst. trypsin inhibitor from soybeans (Armour Laboratories; 20 mg/l blood).

Compounds which accelerated the increase in osmotic fragility. Addition to ACD blood of a number of compounds decreased the "*in vitro* survival time" of erythrocytes to a varying degree, as shown in Table I. Results obtained with NaF are given separately in Table II. The presence of NaF during storage did not change the rate of diffusion of potassium into the plasma from that observed with control blood (corrections were made for the potassium released by spontaneous hemolysis during storage).

Compounds which retarded the increase in osmotic fragility. a) *Sodium ferrocyanide* (5.45 mM/l blood). After 21 days storage, the control showed 4.6%, the test sample 2.9% hemolysis in 0.6% NaCl solution. After 50 days the corresponding figures were 28.4% (control) and 14.0% (test sample). b) *Nickel chloride* (1.91 mM/l blood). The effect was very slight. After 29 days, the control showed 29.0%, the test sample 22.7% hemolysis in 0.6% NaCl solution. After 35 days, the corresponding figures were 46.6 and 38.8%, respectively. Lower concentrations of nickel chloride had no effect, as mentioned before. c) *Salicylic acid* (13.63 mM/l blood) showed good protective action for the first 20 days, but this effect all but disappeared when the storage period was extended (Table III). d) *10-(2-Dimethylaminopropyl)phenothiazine.* The addition of optimal amounts of this substance (0.4 mM/l) retarded the development of osmotic fragility to a considerable degree. After 21 days there was observed 0.4 to 4% (controls 5 to 23%), after 42 days

TABLE II. Effect of Sodium Fluoride on ACD Blood during Storage.

Blood No.	NaF, mM/l	Storage, days	% hemolysis in 0.9% NaCl Control	% hemolysis in 0.9% NaCl Test	% hemolysis in 0.6% NaCl Control	% hemolysis in 0.6% NaCl Test	Inorganic phosphate, mg/100 ml blood Control	Inorganic phosphate, mg/100 ml blood Test
2341	13.9	2	0	.8	.4	18.7	3.77	3.72
		15	.2	2.4	4.8	62.6	13.54	6.05
		29	.5	8.8	13.4	71.8	16.72	7.57
		35	2.1	12.7	19.3	78.7	18.05	8.66
2342	13.9	2	0	.9	1.9	14.8	3.26	2.86
		15	.6	4.0	3.0	65.5	11.28	4.32
		29	1.0	8.1	7.1	70.5	13.19	5.21
		41	2.1	14.1	21.2	87.5	15.27	5.92
2355	1.5	2	0	.5	0	16.5	3.24	3.03
		16	1.3	5.5	6.1	74.9	12.55	5.06
		30	1.0	11.0	21.7	89.4	15.27	7.79
		49	5.5	18.5	49.7	94.4	17.59	11.44

The following changes in blood glucose were observed during storage periods: Blood 2341, control, glucose decreased from 344 to 250, test sample no change with 354 and 350 mg/100 ml, respectively; Blood 2342, control, glucose decreased from 360 to 286, test sample constant at 368 mg/100 ml; Blood 2355, control, glucose decreased from 332 to 216, test sample no change with 337 and 336 mg/100 ml, respectively. Mixtures of NaF (1.5 mM/l) with cobaltous acetate (2 mM/l), or manganese sulfate (2 mM/l) gave essentially the same results as NaF alone.

TABLE III. Effect of Salicylic Acid (13.63 mM/l Blood) on ACD Blood during Storage.

Blood No.	Storage, days	% hemolysis in 0.6% NaCl Control	% hemolysis in 0.6% NaCl Test
2379	10	3.2	1.2
	20	13.6	4.9
	42	50.8	40.3
2382	10	3.7	2.9
	20	22.8	11.3
	42	47.6	45.5
2385	10	5.9	3.1
	20	21.3	11.0
	42	57.5	46.7

0.6 to 4.5% (controls 20 to 36%), and after 92 days 26 to 39% (controls 83 to 85%) hemolysis on exposure to 0.6% NaCl solution. A few examples of the experimental findings are listed in Table IV (see also Fig. 1). The presence of this phenothiazine derivative, which is manufactured under the trade name of Phenergan, did not modify glucose utilization, plasma potassium increase, or inorganic phosphate increase, but it inhibited by 20% plasma lipase activity, determined by the method of Goldstein *et al.*(2), as modified by Alper(3). The increases in hematocrit were less, usually one-half of those observed with ACD alone.

It is important, for reasons to be discussed later, that the addition of Phenergan to ACD blood which had been stored for various periods of time did not modify or reverse the osmotic fragility existing at the time of addition, but showed a marked protective action against further deterioration. This is illustrated by the data shown in Table V.

The methosulfate of Phenergan was also tested but proved to be considerably less effective in retarding the increase in osmotic fragility during storage than the parent compound. The control bottles from 3 different ACD bloods showed, for example, after 25 days 13 to 21%, and after 60 days 47 to 69% hemolysis in 0.6% NaCl solution. Phenergan methosulfate (0.4 mM/l blood) reduced the hemolysis figures at 25 days insignificantly to 11 to 20% and only slightly after 60 days to 41 to 53%. The corresponding values for Phenergan by contrast were 4.6 to 6.8% (25 days) and 12 to 19% (60 days).

Discussion. Metal ions, compounds reacting with sulfhydryl and disulfide groups and certain complex forming substances did not protect red cells from the gradual increase in osmotic fragility. The results with NaF add further weight to the belief that a functioning glycolytic system is essential for *in vitro* preservation of red cells.

A few substances were found to delay the progressive increase in osmotic fragility of

TABLE IV. Protective Effect of 10-(2-Dimethyl-amino-propyl) Phenothiazine on ACD Blood during Storage.

Blood No.	Storage, days	% hemolysis in 0.6% NaCl Control	Test
606	6	2.4	.8
	20	7.4	1.8
	41	33.1	2.9
2092	11	2.1	2.1
	17	4.5	2.5
	26	9.9	3.2
	32	12.8	3.5
	45	33.0	6.5
	52	43.2	12.8
	59	55.2	19.5
899	21	22.8	2.8
	50	62.9	7.1
	92	84.7	25.6

TABLE V. Effect of Phenergan on Aged ACD Blood.

Age of blood, days	Days stored with Phenergan	% hemolysis in 0.6% NaCl Control	Test
Blood No. 1014, stored 14 days before addition of Phenergan			
15	1	13.2	12.6
24	10	25.8	13.6
34	20	41.0	20.2
Blood No. 2487, stored 37 days before addition of Phenergan			
38	1	54.4	52.8
59	22	80.8	57.3

erythrocytes during storage. The most effective agent among the compounds studied so far was Phenergan hydrochloride. The mechanism of its "protective" action is open to speculation. This substance is known to be an antihistamine, but another antihistamine drug (dimethyl-pyrimidyl-ethylenediamine hydrochloride or Neohetramine) had no stabilizing influence on red cells.

It has been reported that Phenergan preserved tissues and inhibited trypsin and papain(4) and the antiproteolytic activity of this material was our reason for testing its influence on the development of osmotic fragility, especially since Halpern *et al.*(5) found that it retarded morphological changes in stored blood. However, other compounds, as for example ethylenediamine tetraacetic acid (EDTA), which have been shown in a recent report from this laboratory(6) to inhibit certain proteolytic processes, did not duplicate the effect of Phenergan on stored erythrocytes. This is particularly surprising since EDTA, which was used by Dyckerhoff *et al.*(7) as an anticoagulant by virtue of its reaction with calcium, was reported, as had been Phenergan, to keep blood morphologically intact longer than sodium citrate or the oxalates(8).

A third property of Phenergan, found during the course of this study, was its inhibitory effect on plasma lipase activity. While only 20% inhibition was observed when this substance was present in a concentration of 0.4 mM/l blood and when tributyrin was used as substrate, one has to keep in mind that plasma lipase activity is apparently not due to a single enzyme but to a mixture of enzymes. It is conceivable, therefore, that a 20% inhibition of tributyrin hydrolysis might signify a complete inhibition of that part of plasma lipase activity which may be directed against lipids in the red cell membrane. Quinine (2.7 mM/l blood) inhibited plasma lipase activity 66% but caused no significantly increased "*in vitro* survival" of red cells, though these experiments, not reported here in detail, gave somewhat uncertain hemolysis data as a result of blood pigment changes. Tests with p-amino salicylic acid were carried out as this substance was claimed to inhibit plasma lipase quantitatively when present in a concentration of 170 mg/l(9). Experiments in this laboratory showed no inhibition of tributyrin hydrolysis even with p-amino salicylic acid levels of 918 mg/l plasma (6.0 mM/l) and the compound did not interfere with the normal rate of increase of osmotic fragility of red cells during storage. Inability to reproduce the Phenergan effect by using other antihistamines, other antiproteolytic agents, or other lipase inhibitors, makes it impossible at this time to explain the mechanism through which this phenothiazine derivative exerts its protective action.

The data shown in Table V are of particular significance and require additional comment for the following reason. Chaplin *et al.*(10) investigated the effect of a related phenothiazine derivative (R.P. 3300) on red cell preservation, and found that their compound

interfered with the hemolysis of fresh red cells by hypotonic NaCl solution. They observed strikingly less hemolysis when small amounts of R.P. 3300 were added to the hypotonic salt solutions. This property of R.P. 3300 made it appear (when blood was stored in its presence for longer periods of time) as if this compound had a protective action, whereas, according to Chaplin *et al.*, it only interfered with the determination of "*in vitro* survival". Transfusion experiments showed that R.P. 3300 did not increase posttransfusion survival.

The behavior of Phenergan in hemolysis experiments was quite different, as can be seen in Table V. In contrast to R.P. 3300, this compound, which was present in the hemolysis tests in a concentration of 1:1.6 million, did not (even after 24 hours contact) decrease the degree of hemolysis from that measured just prior to its addition. Once Phenergan was present, however, it retarded the *further* increase in osmotic fragility observed on continued storage of the control blood.

Experiments are in progress to determine whether Phenergan prolongs the useful shelf life of transfusion blood. It is also planned to study the effect of Phenergan on blood preserved with ethylenediamine tetraacetic acid.

Summary. 1. Based on the working hypothesis that the deterioration of erythrocytes during storage may be due to enzymatic processes affecting the cell membrane, experiments were carried out in which a variety of compounds known to modify enzymatic reactions were added to ACD blood. The effect of these additions on the rate of development of osmotic fragility (hemolysis in 0.6% NaCl solution) was determined. 2. The "*in vitro* survival time" (resistance toward 0.6% NaCl solution) of red cells was considerably shortened by a number of compounds, for example by sodium fluoride, which also prevented the utilization of glucose, delayed the rate of increase of inorganic phosphate during storage, but had no effect on the gradual increase of plasma potassium concentrations. 3. The most promising results in retarding the development of osmotic fragility were obtained with 10-(2-dimethylamino-propyl)phenothiazine. ACD blood from 55 donors showed on exposure to 0.6% NaCl solution after 21 days storage 5 to 23%, and after 42 days 20 to 58% hemolysis. The addition of the phenothiazine derivative (0.4 mM/l blood) retarded the development of osmotic fragility considerably, so that after 21 days there was observed 0.4 to 4%, after 42 days 0.6 to 4.5%, and after 90 days 26 to 39% hemolysis when the blood samples were added to 200 volumes of 0.6% NaCl solution.

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