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BIMONTHLY PROGRESS REPORT NO. 2

RESEARCH ON PROCEDURES FOR THE

LOW-TEMPERATURE PRESERVATION OF BLOOD

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I. INTRODUCTION:

In Bimonthly Progress Report No. 1 was summarized the status of processes using polyvinylpyrrolidone (PVP) as the protective additive in the freezing of whole blood or red cell suspensions. The data presented there show that high recovery can be achieved of intact red cells, that these cells have high survival after transfusion, and that reproducible results can be achieved with pint quantities. Clinical trials are needed to establish the suitability of red cells preserved in this way in persons requiring them, and studies are needed of the physiological acceptability of PVP in the concentrations that would be administered if post-thaw separation were not used.

We are continuing to seek a red cell preparation that can be infused without post-thaw processing and in addition, have increased the effort on developing a rapid and simple post-thaw separation procedure.

This report summarizes the studies that were carried out in the period March 1 to May 1, 1963.

II. STUDIES OF HAEMACCEL AS A PROTECTIVE ADDITIVE:

A new plasma volume expander has become commercially available which can be metabolized and does not produce any substantial side effects. This plasma volume expander, Haemaccel, is a polymer produced by degrading gelatin and then crosslinking with urea bridges.[1]

Haemaccel as a plasma substitute is manufactured by Behringwerke A.G., Marburg-Lahn, Germany, as a 3.5% solution of polymer with a mean molecular weight of about 35,000 having a relative viscosity of 1.7 to 1.8 (water = 1). The gel point is below +4°C., the isoelectric point is pH 4.5 to 5.0 and the pH of the infusion solution is 7.1. The solution is mildly hyperoncotic in order to facilitate return of water from the tissues. Pharmacological investigations in the literature have shown that Haemaccel can replace up to 80% of circulating plasma in rats. Results obtained by circulation analysis, plasma exchange experiments and renal function tests, give evidence of the practicability as a plasma substitute.[2] In further

studies on duration of storage and pathology, these authors have found no signs of storage or secondary tissue reactions. Haemaccel is broken down by the endogenous proteolytic enzymes trypsin and cathepsin. Native gelatin has been observed to induce antibody formation but injections of Haemaccel have not been followed by antibody formation in rabbits or guinea pigs. In patients with high gelatin-antibody titers injection of Haemaccel lowered the serum-antibody level and did not produce a rise in titer; hence, Haemaccel has no antigenic character but behaves like a hapten.[3]

Preservation of red cells in gelatin has been reported previously (ONR VIII). One problem involved with the use of gelatin has been its high gel temperature whereas this problem has been eliminated with Haemaccel which gels at below 4°C. Since many of the problems involved with use of PVP as an additive such as lack of total excretion and ultimate storage can be circumvented with use of a polymer such as Haemaccel, this compound is currently being investigated as a protective additive for freezing whole blood.

A. Preparation:

The use of Haemaccel as a protective additive has been complicated by the fact that the material is not available as a powder. Haemaccel samples have been obtained through the courtesy of Professor Dr. H.E. Schultze of Behringwerke A.G., Marburg-Lahn, Germany, as a 10% solution in sterile 500-ml. volumes containing 100 mg-% sodium and 4 mg-% calcium with a pH value of 7.6. It was felt that the solution was not sufficiently concentrated for use as an additive without further processing.

B. Concentration by Dialysis:

The first technique used to obtain more concentrated material was dialysis against polyethylene glycol (Carbowax 20M) with an average molecular weight of 17,500. A total of 450 ml. of 10% solution (in two batches) was concentrated in slowly rotating bottles with dialysis bags containing solid Carbowax 20 M for a total of 60 hours during a 3-day period. Approximately 120 ml. of a solution containing 30% (w/v) Haemaccel were recovered. Analysis indicated that the Na^+ concentration had likewise been increased threefold.

Whole blood was mixed with varying amounts of the 30% concentrate to yield blood-additive mixtures with overall Haemaccel concentrations of 6 to 10% (supernatant Haemaccel concentrations were 9 to 13%). The mixtures were frozen and thawed, and RBC recovery and 1/100 saline EOP were determined. Data, presented in Table I, show that gross hemolysis occurred on slow controlled-rate cooling. Processing in BPU-1 allowed higher yields of intact cells, with best results obtained under conditions affording the lowest external heat transfer coefficient. Also, significantly better results, particularly EOP's were obtained at the higher additive concentrations.

Although dialysis against PEG had allowed concentration of the Haemaccel to practical levels for use as an additive, this technique was abandoned because of other problems. The time necessary to process even small quantities of concentrate is quite long. There is always danger of bacterial contamination of the material during these long periods at room temperature. The 10% solution forms a very firm gel on 4°C. storage, making processing at temperatures lower than ambient impossible.

C. Use of 10% Haemaccel as a Suspending Medium:

The possibility of using the 10% Haemaccel stock solution as a suspending medium to protect red cells from freeze-thaw damage was investigated. A quantity of 10% Haemaccel was deionized by passage through a mixed bed of Dowex 50W x 8 (H^+ form) cation exchange and Dowex 1 x 8 (OH^- form) anion exchange resins. NaCl was added to aliquots of the deionized material, to give concentrations of 0.05, 0.1, and 0.15 M. Packed erythrocytes from ACD-A blood were suspended in an equal volume of Haemaccel-saline mixtures and aliquots were processed in BFF-19110B containers. Duplicate samples were frozen in heavily insulated containers in an effort to lower heat transfer rates. Warming was by immersion in water with agitation. Duplicate aliquots of the one mixture (0.1 M saline) were thawed under conditions affording slower and faster warming. Data are shown in Table II.

Red cell recovery was uniformly poor, with only slight variation observed. In most cases cardboard jacketing during freezing resulted in lowered cell yields. Medium saline concentration showed little effect, nor did varying warming conditions. Difficulty in thawing the material was encountered, a problem which will be discussed more completely later in this section.

TABLE I

Initial Freeze-Thaw Processing

of Whole Blood-Haemaccel

Additive Concentration (% w/v)		Dilution	RBC Recovery Data (%)			
Overall	Supernatant	Vol. Blood/ Final Vol.	Controlled Rate	Uncoated	PVP-MeOH	Santocel-Glycerol
6	8.7	4/5	29	86.4 (59)	85.5 (48)	
7.5	10.6	3/4	29	90.1 (74)	85.8 (55)	64.2
10.0	13.3	2/3	35		88.7 (71)	71.3 (44)

NOTES:

Prefreeze Processing: Whole blood (ACD-B) was diluted as shown with 30% Haemaccel prepared by dialysis of 10% material against solid polyethylene-glycol (Carbowax 20 M).

Freezing:

Controlled Rate: 15 ml. aliquots placed in BFT 1420 containers, cooled from room temperature to the freezing point at 2.5°C./min., held for 3 min. at the freezing point, cooled to -21°C. in about 2 min., to -38°C. at about 3°C./min., then immersed in liquid nitrogen.

Other Samples: 50 ml. aliquot placed in BFF 19110 containers, coated as shown and frozen by immersion in liquid nitrogen with agitation (200 cpm) in BPU-1.

Thawing: Immersion in water at 45°C., controlled rate samples with gentle manual agitation for 2 min., other samples with agitation at 160 cpm for 42 sec. in BPU-1.

RBC Recovery: Normal recovery determined by the usual methods. The figures in parentheses are 1/100 saline EOP's.

TABLE II

Freeze-Thaw Processing of Red Cells

Suspended in 10% Haemaccel

Additive Saline Concentration (M)	Freezing Conditions	Thawing Conditions			Normal RBC Recovery (%)
		Water Temp. (°C.)	Time (sec.)	Agitation (cpm)	
0.05	uncoated	45	50 + 10*	160	58
0.05	insulated	45	55	160	57
0.1	uncoated	45	55	160	60
	insulated	45	55 + 10*	160	54
	uncoated	45	120	75	57
	insulated	45	180	75	50
	uncoated	50	45 + 10*	160	57
	insulated	50	50 + 10*	160	58
0.15	uncoated	45	55	160	61
	insulated	45	55	160	52

NOTES:

Prefreeze Processing: Aliquots of whole blood (ACD-A) were centrifuged and the plasma removed. The packed cells were suspended 1:1 in deionized 10% Haemaccel to which NaCl had been added to give the concentrations shown.

Freezing: 50 ml. aliquots of the above mixtures were processed in BFF 19110B containers. Freezing was by immersion in liquid nitrogen for 90 sec. with agitation at 200 cpm in BPU-1. As shown, some containers were covered with a 3/64-in. thick cardboard jacket during freezing to reduce external heat transfer.

*Thawing: Containers were immersed in water with agitation in BPU-1, under the conditions shown. The extra times shown are additional warming periods necessary to effect complete melting in some cases.

D. Deionization and Lyophilization:

Solid Haemaccel was prepared to provide a raw material for the preparation of solutions of the desired concentrations. A volume of the 10% stock material was deionized by passage through a mixed bed of Dowex 50W x 8 (H^+ form) cation exchange and Dowex 1 x 8 (OH^- form) anion exchange resins. Analyses showed that this technique lowered the Na^+ concentration to less than 5 p.p.m.

Aliquots of the deionized material (75 to 100 ml.) were shell-frozen in 500-ml. round bottom flasks by a slow rotation of the flasks in liquid nitrogen. The frozen flasks were then attached to a vacuum system containing a large liquid nitrogen cold trap, and lyophilized for about 24 hr. at pressures of approximately 1×10^{-3} torr, with a loss in weight averaging better than 90%, indicating that the material had been dried as completely as possible. The dried material was powdered in a high-speed blender, and stored at 4°C. for use as needed.

E. Freeze-Thaw Processing of Whole Blood--Haemaccel Mixtures:

Dissolving large enough amounts of the powdered Haemaccel in water to yield solutions with concentrations high enough for practical use as protective additives was found to be difficult. The material was very viscous, with a tendency to foam. In order to simulate the addition of concentrated Haemaccel solutions to whole blood the following procedure was used: the proper volume of blood was centrifuged and the plasma was removed. Solid Haemaccel was dissolved in the plasma, and NaCl and water were added to give the desired final volume, overall Haemaccel concentration, and "additive" saline concentration. This technique was found to be quite satisfactory, particularly with larger volumes (>100 ml.).

Data obtained with whole blood-Haemaccel systems prepared by the above procedure are shown in Tables III to VI. Results with blood containing 7% Haemaccel, approximating a mixture of four volumes of whole blood with one volume of 35% Haemaccel in various saline concentrations (Table III) show that under the conditions used (BFF-19110 container), additive containing 0.1 M NaCl appears to allow maximum recovery of intact cells. The supernatant Haemaccel concentration was 10.2%. Higher or lower saline content resulted in greater cell losses, and slower cooling (gelled shell formation followed by stagnant freezing in liquid nitrogen) resulted in gross hemolysis in the thawed material.

TABLE III

Freeze-Thaw Processing of Blood

Containing 7% Haemaccel

Additive Saline Concentration (M)	Supernatant Haemaccel Concentration (w/v%)	Normal RBC Recovery (%)	1/100 Saline EOP (%)
0	12.7	77.2	41.5
0.05	11.6	82.3	50
		42.7*	14*
0.1	10.2	90.1	66.7
0.15	10.0	88.7	63.6

NOTES:

Prefreeze Processing: Aliquots of whole blood (ACD-B) were centrifuged and the plasma removed. Haemaccel (lyophilized deionized powder) was dissolved in the aliquots of plasma, NaCl and H_2O were added, and the Haemaccel-plasma-saline mixtures were recombined with the red cells, simulating the addition of one-volume aliquots of 35% (w/v) solutions of Haemaccel in saline solutions of the concentrations shown to 4-volume aliquots of whole blood. The final overall Haemaccel concentration is 7 g./100 ml. of the blood-additive mixtures.

*Freezing: 53 ml. of WB-Haemaccel mixtures were placed in BFF 19110 containers and frozen by immersion in liquid nitrogen with agitation in BPU-1 (90 sec. at 200 cpm) except for the starred sample, in which a 5-mm. thick shell was formed on one side of a BFF 19110 by placing 28 ml. WB-Haemaccel in the container and allowing the mixture to gel at 4°C. for 1/2 hr., then repeating using another 28 ml. on the other side. The gelled specimen was then frozen by immersion in liquid nitrogen for 140 sec.

Thawing: Containers were thawed by immersion in water at 45°C. for 42-45 sec. with agitation at 160 cpm in BPU-1. Persistence of solid material (gel) was noted in the containers after thawing was thought to be complete. Further warming was done as needed to insure complete melting.

Increasing the overall Haemaccel concentration to 10%, which resulted in an increase of the supernatant Haemaccel concentration to about 15% (Table IV), gave no improvement in cell recovery under the same freezing and thawing conditions. A decrease in the "optimum" salt concentration is evident, however, less added saline being necessary for maximum recovery than at the lower Haemaccel concentration.

Use of a smaller container (BFT-1735) to increase the number of samples which can be processed from a given volume of blood-additive was attempted (Table V). Mixtures containing 10% Haemaccel (overall) at varying blood:additive ratios, thus varying the supernatant Haemaccel concentration, were frozen in liquid nitrogen and thawed at two different rates of agitation. Recovery was lower than in the larger volumes in all cases. It would seem that the mixture does not receive sufficient agitation in the small tubular containers. Saline EOP's in this experiment are extremely low and it is not certain whether this is significant, or is the result of a procedural error; however, it would seem unlikely, since all four containers thawed at one rate of agitation are similarly affected.

Other freeze-thaw studies with blood-Haemaccel are shown in Table VI. Tubular and flat containers (BFT-3365 and BFF-19110) were cooled with agitation in liquid nitrogen, while other tubular containers (BFT-3387) were frozen by "spin-freezing" (Shell formation by axial rotation of the container during cooling by immersion in liquid nitrogen). Several different thawing conditions were used. In all cases, agitation of the tubular containers yielded higher recoveries than did spin-freezing. Higher thaw bath temperature allowed slightly higher recoveries, while increased agitation during thawing increased cell losses. Slower thawing (37°C. bath with very gentle agitation) allowed almost as high yields as did the "standard" conditions. One sample, comprising a small amount of blood-Haemaccel in a flat container, frozen by floating on liquid nitrogen and thawed with agitation in a water bath at the higher temperatures used (50°C.), showed recovery and EOP comparable to the spin-frozen container thawed under the same conditions.

A problem arose in these experiments which has been a factor in all the blood-Haemaccel work, namely the formation of a rigid gel at temperatures above the freezing point. The effect of this gel on freezing has not been investigated. On thawing, however, it has been noted that there is often solid material in the containers after "thawing" is complete. It

TABLE IV

Freeze-Thaw Processing of Blood

Containing 10% Haemaccel

BFF-19110 Containers

Additive Saline Concentration (M)	Supernatant Haemaccel Concentration (w/v%)	Thawing Times (sec.)	Normal RBC Recovery (%)	1/100 Saline EOP (%)
0.05	15.8	45	89.5	63.8
0.075	15.9	56	88.5	56.5
0.10	15.7	60	86.3	59.5
0.125	16.0	70	86.0	55.6

NOTES:

Prefreeze Processing:

Aliquots of whole blood (ACD-B) were centrifuged and the plasma removed. Haemaccel (lyophilized deionized powder) was dissolved in the plasma aliquots, NaCl and H_2O were added, and the Haemaccel-plasma-saline mixtures were recombined with the cells, simulating the addition of one-volume aliquots of 50% (w/v) solutions of Haemaccel in salines of the concentrations shown to four-volume aliquots of whole blood. The final overall Haemaccel concentration is 10 g./100 ml. of the blood-additive mixtures.

Freezing:

Fifty-ml. aliquots of the blood-additive mixtures were placed in BFF-19110 containers and frozen by immersion in liquid nitrogen for 90 sec. with agitation in BPU-1 (200 cpm).

Thawing:

Containers were immersed in H_2O at 45°C. with agitation at 160 cpm (BPU-1) for the times shown. Increasing times were necessary because of incomplete melting of the gelled material.

TABLE V

Freeze-Thaw Processing of Blood

Containing 10% Haemaccel

BFT-1735 Containers With Varying Blood Dilutions

Blood Dilution		Supernatant	Thawing Conditions		Normal	1/100
Whole Blood Volume	Final Volume	Haemaccel Concentration (%, w/v)	Time (sec.)	Agitation (cpm)	RBC Recovery (%)	Saline EOP (%)
1	2	15.4	35	160	50.5	14.5
1	2	15.4	30	200	59.5	38
2	3	16.5	35	160	73.2	6
2	3	16.5	31	200	72.3	34
3	4	15.5	35	160	81.7	6.5
3	4	15.5	32	200	81.4	32.5
4	5	14.3	35	160	82.9	5.5
4	5	14.3	33	200	83.7	36

NOTES:

Prefreeze Processing:

Aliquots of whole blood (ACD-B) were centrifuged and the plasma withdrawn. Haemaccel (lyophilized deionized powder) was dissolved in the plasma; 0.45% saline was added, and the Haemaccel-plasma-saline mixtures were recombined with the cells, simulating the addition of one-volume aliquots of various Haemaccel-saline solutions to varying volumes of blood to yield a final concentration, in each case, of 10 g. Haemaccel/ 100 ml. of blood-additive mixture.

Freezing:

Twenty-ml. aliquots of the mixtures were placed in BFT-1735 containers and frozen by immersion in liquid nitrogen for 90 sec. with agitation at 200 cpm in BPU-1.

Thawing:

Containers were immersed in water at 45°C. for the times shown, with agitation as shown (BPU-1).

TABLE VI

Freeze-Thaw Processing of Blood Containing 10% Haemaccel

Some Freezing and Thawing Variables

Container	Volume Frozen (ml.)	Freezing Time (sec.)	Freezing Agitation (cpm)	Bath Temp. (°C.)	Thawing Time (sec.)	Thawing Agitation (cpm)	Normal RBC Recovery (%)	1/100 Saline EOP (%)
BFF-19110	50	90	200	45	45	160	80.9	36
BFF-19110	50	90	200	45	45	200	78.2	34
BFT-3365	30	90	200	45	45	160	80	30.6
BFT-3365	30	90	200	45	45	200	75.5	28
BFT-3365	30	90	200	50	35	160	85.3	35
BFT-3365	30	90	200	37	120	50	79.2	27
BFT-3387	30	90	1800*	45	45	160	75.4	25
BFT-3387	30	90	1800*	45	45	200	73	20.4
BFT-3387	30	90	1800*	50	35	160	78	17
BFT-3387	30	90	1800*	37	120	50	77.6	15
BFF-33200*	25	-	-	50	35	160	77.2	19

NOTES:

Prefreeze Processing: 300 ml. of whole blood (ACD-B) was centrifuged and the plasma removed. 37.5 g. Haemaccel (lyophilized deionized powder) was dissolved in the plasma. 70 ml. of 0.05 M saline was added, and the mixture was recombined with the cells, yielding approximately 375 ml. of a "4/5" dilution of whole blood and Haemaccel-saline with an overall concentration of 10% Haemaccel (w/v) or about 15% in the supernatant.

*Freezing: Aliquots of the above mixture (volumes shown) were placed in containers as shown and frozen by immersion in liquid nitrogen, agitated in BPU-1 as shown with the exception of the starred items. The 3387's were rotated axially (spin-freezing - rpm shown) and the 33200 was floated on one of its large sides on a liquid nitrogen bath.

Thawing: Immersion in water in BPU-1 under the conditions shown.

would seem that the effect of agitation during the thawing cycle on internal heat transfer due to the motion of the liquid is lost, necessitating lengthened thawing times. It is extremely difficult to determine when thawing is complete, i.e., all of the ice present in the sample has melted. Routinely in processing with other additives, completion of thawing is checked by feeling the container immediately after withdrawal from the water bath. The presence of ice can be detected by the movement of the solid as the container is gently shaken and also by the rapid cooling of the container. If blood-Haemaccel samples are subjected to this test, presence of a soft solid can be detected, but the container does not cool sufficiently to indicate the presence of ice.

It would seem desirable, then, that a study of the characteristics of Haemaccel solutions, such as temperature-viscosity relationships at various pH's and under various processing conditions (saline concentration, plasma concentration, etc.) be an important part of any further research of its applicability as a protective additive.

III. DEXTRAN AS A PROTECTIVE ADDITIVE:

Dextrans of molecular weights 10,000 and 40,000 were tested as protective additives in whole blood. Tables VII, VIII, and IX show they were inferior to PVP in affording protection, especially in giving low stability cells as demonstrated by marked lysis on resuspension in isotonic saline. Because dextran by itself is not a good protective additive, consideration is being given to the use of mixtures of dextran with other substances.

IV. SERUM ALBUMIN AS A PROTECTIVE ADDITIVE:

Red cells frozen and thawed in a medium containing PVP and serum albumin are the most stable on resuspension and on transfusion that we have obtained without an intracellular additive. We have now tested the effect of additional serum albumin on the protection of red cells in whole blood, without PVP, and found (Table X) that it is inferior to PVP or PVP-albumin.

Comparisons were made of the protective effect of Plasdone-C versus Light Company PVP and purified placental albumin versus human serum albumin. Results were not conclusive but are presented in Table XI. Indications are that we will find no difference in protective effect due to differences in albumin. Previous comparisons of Plasdone-C and Light Company PVP indicated no difference.

TABLE VII

Dextran-40 as a Protective Additive in Whole Blood:

Effects of Volume and Heat-Transfer Coatings

Volume Frozen (ml.)	Heat-Transfer Coating		
	None	PVP-MeOH	Glycerol-Santocel
35	88 (44)	81 (29)	5 (16)
55	87 (38)	74 (24)	5 (11)
75	87 (35)	76 (17)	17 (12)

WB-ACD (4 vol.) + Dextran, m.w. 40,000 in 30% aqueous solution (1 vol.). Frozen at 200 cpm BPU-1, LN_2, in BFF-19110's. Thawed 200 cpm, 45°C. Direct RBC recoveries, values in parentheses are saline EOP's (ONR P.R. VIII).

TABLE VIII

Dextran-40 as a Protective Additive in Whole Blood:

Effects of Concentrations of Additive and Salt

Dextran Concentration (%)	Dilution of Blood	Concentration of Salt in Additive (%)			
		0.9	0.6	0.3	0.0
6	4:5	88 (3)	89 (29)	92 (37)	91 (29)
9	3:10	92 (41)	94 (39)	94 (27)	90 (26)
12	2:5	92 (40)	94 (36)	95 (48)	85 (41)
15	1:2	95 (66)	92 (65)	75 (76)	67 (53)

WB diluted as shown with Dextran-40 30% w/v in salt solutions indicated. All freezing in BFF 19110's in LN_2 200 cpm, BPU-1 (uncoated containers). Thawing at 200 cpm, 45°C. Values are direct RBC recoveries; values in parentheses are saline EOP's (ONR P.R. VIII).

TABLE IX

Dextran-10 as a Protective Additive in Whole Blood:

Effects of Volume and Heat-Transfer Coatings

Volume Frozen (ml.)	Heat-Transfer Coating		
	None	PVP-MeOH	Glycerol-Santocel
35	81 (27)	76 (18)	13 (10)
55	80 (23)	77 (16)	14 (0)
75	80 (23)	74 (11)	24 (5)

See footnotes, Table VII.

Dextran m.w. 10,000 used in above studies.

TABLE X

Albumin as a Protective Additive in Whole Blood

Albumin Concentration (%)	Heat-Transfer Coating	
	None	PVP-MeOH
12.5	88 (66)	73 (47)
10	91 (68)	80 (52)
7.5	87 (64)	84 (50)
5	80 (62)	68 (46)
Control PVP-7%	94 (90)	94 (87)

50 ml. volumes of whole blood diluted with 25% human serum albumin (salt-poor) to give required overall albumin concentrations shown. Frozen in BFF 19110's 200 cpm; thawing 200 cpm, 45°C. Results are direct RBC recovery and, in parentheses, saline EOP's.

TABLE XI

PVP and Albumin Studies

(1/2 Pint)

Sample	PVP Used	Albumin Used	Recovery (%) Normal	Recovery (%) In 40 Vol. Saline	Comments
A_1	Plasdone-C	Placental	95.7	85.9	Incomplete thaw
A_2	Plasdone-C	Serum	96.5	88.4	
B_1	Light Co.	Placental	96.3	86.9	Incomplete thaw
B_2	Plasdone-C	Placental	96.9	89.2	

Blood sample A is ANRC No. 39770 and B is ANRC No. 39774.

One volume of packed cells was resuspended in an equal volume of additive containing 14% PVP, 3% albumin and 0.62% NaCl. Mixtures were frozen in PVP-coated FC-1/2 containers using 200 cpm agitation of BPU then thawed in a 45°C. bath at 160 cpm.

BPU with new agitator was not operating properly.

In vivo survival studies of erythrocytes protected with Plasdone-C and serum albumin are continuing at the VA Hospital, Buffalo. Cells from one pint of blood are frozen and thawed in the presence of PVP and albumin, then resuspended in a small quantity of autologous plasma prior to transfusion. Results as summarized in Table XII indicate that fasting prior to donation of blood improves cell survivals. Of interest also are the identical results attained with the nonfasted blood collected into ACD-A in both 1-pt. and 1/2-pt. quantities: 88, 82, and 78% survival for 24, 48, and 72 hr., respectively. The effect of fasting on cell stability was not observed with unfrozen controls.

The PVP-albumin protective system is the most effective of the PVP additive preparations. The quantity of PVP used to protect a unit of cells is less than or equal to that required in the whole blood system. Also, as shown in Table XIII, over 80% of the PVP in the additive can be removed from the blood mixture after thawing with one centrifugation.

V. PVP AS A PROTECTIVE ADDITIVE:

For a blood process requiring washing before transfusion, the quantity of PVP used to protect erythrocytes from freeze-thaw damage becomes less important clinically. It was thus suggested that an excess quantity of PVP be used to replace the 3% albumin of the PVP-albumin protective additive. Results of attempts to optimize the PVP and NaCl concentrations in the additive are presented in Table XIV. Recoveries can be increased over that obtained with 7% PVP; however, the cells are not as stable as those frozen using a small amount of albumin. Stability is measured by 1:40 dilution of the thawed blood in physiological saline. Thus there is a possibility that a gradual decrease in the oncocity and tonicity of the resuspending media will give rise to more intact cells in the final isotonic medium.

Light Company PVP (M.W. 24,500) and glucose solutions were studied as additives. Plasma was used with PVP as an additive supplement while retaining sufficient plasma for resuspending of thawed cells if necessary. The data in Table XV indicate that glucose has little effect on the stability of thawed cells.

TABLE XII

PVP-Albumin Blood for Clinical Study

Anti-coagulant	Free Hemoglobin (%) Thawed Blood	Free Hemoglobin (%) Resuspended Blood	No. Processed	Survival (%) 24 hr.	Survival (%) 48 hr.	Survival (%) 72 hr.	
ACD-A	2.8	1.1	8	93	90	86	fasting
ACD-A	2.8	1.2	7	88	82	77	nonfasting
ACD-B	2.8	1.4	3*	95	92	79	fasting
ACD-B	3.0	1.8	4	84	85	80	nonfasting

Collection: Approximately 450 cc. of blood were collected into 72 cc. of ACD-A or 480 cc. into 120 cc. of ACD-B.

Additive Composition: Plasdone-C (14%) and albumin (3%) in 0.6% saline solution.

Blood Mixture: For bottle collection use equal volume packed cells and additive. For blood packs 300 cc. of additive were used.

Processing: Entire volume frozen and thawed in an FC-1 container using a Linde Blood Processing Unit. Freeze in liquid nitrogen at 200 cpm and thaw in 45°C. water bath at 160 cpm agitation.

Transfusion Product: Centrifuge, remove supernatant and resuspend packed cells in ~ 100 cc. of autologous plasma.

*Survival data from one patient.

TABLE XIII

PVP Analysis of PVP-Albumin Protected Blood

Patient	Volume Additive (cc.)	Supernatant Removed (cc.)	PVP in Supernatant (%)	PVP Left in Packed Cells (g.)
Donner	300	265	13.6	6
Dorsey	300	285	11.9	8
Stavisky	300	255	11.3	13

Blood collected into ACD-A and processed in packs.

Additive solution consisted of 14% PVP, 3% albumin and 0.62% NaCl in H_2O.

After freezing, thawing and supernatant removal, cells were diluted with 100 cc. of autologous plasma and transfused.

TABLE XIV

Process I Optimization Studies

Sample No.	Additive Concentrations		Recovery (%)	
	Plasdone-C (%)	NaCl (%)	As Thawed	In 40 Vols. Saline
1	20	0.3	96.1	84.0
2	20	0.45	96.8	83.9
3	25	0.45	97.3	85.2
4	25	0.6	97.6	83.3
5	30	0.3	97.3	81.4
6	30	0.45	97.3	81.0
7	35	0.3	98.1	76.7
8	35	0.6	98.2	73.1
11	25	0	96.4	83.3 (2% glucose**)
12*	25	0	92.6	71.4 (2% glucose**)
13	25	0	91.6	70.7
14	25	0.15	96.2	83.7
15*	25	0.15	93.5	78.3
16	25	0.3	96.8	84.9
17	25	0.45	97.2	84.7
18*	14	0.62	96.9	90.3 (3% albumin**)

* Container coated with 500 cp PVP-methanol for freezing.

** Supplementary component of additive.

Samples 1-8 from ANRC blood No. 39814, drawn 4/4/63.

Samples 11-18 from ANRC blood No. 34624, drawn 4/10/63.

Blood mixture: packed cells plus equal volume of additive.
Freeze 50 cc. in BFF-19110 at 200 cpm BPU agitation in liquid nitrogen.
Thaw for 45 sec. in 45°C. bath at 160 cpm.

TABLE XV

Light Company PVP

Sample	Glucose In Additive (%)	Thawed Blood Recovery	
		Normal (%)	Saline EOP (%)
A_1	0	96.7	85.2
A_2	3	96.2	86.9
B_1	3	96.7	86.6
B_2	6	96.9	84.0

Sample	Resuspended Blood Recovery			
	Immediate		3 Days' Storage	
	Normal	Saline EOP	Normal	Saline EOP
A_1	98.0	88.2	96.3	85.2
A_2	98.1	90.2	96.8	87.7
B_1	98.3	89.7	96.8	86.4
B_2	98.2	89.2	96.3	85.2

Blood samples A and B were divided into 270-cc. aliquots. From each was removed 68 cc. of plasma and the plasma replaced with 68 cc. of 28% K-25 PVP in 0.15% NaCl solution containing glucose as shown in the table.

The samples in FC-1/2 containers were frozen in liquid nitrogen using 200 cpm agitation of BPU, then thawed in a 45°C. bath at 160 cpm.

Supernatant fluid was removed after thawing and the available autologous plasma used to dilute packed cells.

VI. ION EXCHANGE COLLECTION OF BLOOD:

Blood can be withdrawn from a donor through a cation exchange resin bed. Coagulation is prevented by removal of Ca^{+2} from the blood, replacing it with Na^{+}, the blood remaining undiluted by any "foreign" material. Com-

TABLE XVI

Freeze-Thaw Processing of

Ion Exchange-Collected Blood

Additive		Resultant PVP Concentration (g./100 ml.)		Cooling Conditions (Container Surface)	Normal Red Cell Recovery (%)	Saline EOP (%)
Plasdone C (% w/v) in	NaCl (M)	Overall	Supernatant			
19.6	0.05	3.9	6.3	PVP-MeOH	95.1	79.5
22.4	0.05	4.5	7.2	PVP-MeOH	96.3	85.9
28	0	5.6	9.8	PVP-MeOH	(97.0)*	(90.6)
28	0.05	5.6	9.0	PVP-MeOH	97.4 (97.4)	91.1 (90.5)
35	0	7	11.9	PVP-MeOH	97.2	91.8
35	0.05	7	11.7	Uncoated	97.1	88.4
				PVP-MeOH	97.2	91.1
				Santocel-Glycerol-MeOH	85.4	63.6
35	0.15	7	11.0	PVP-MeOH	97.5	90.9
14 (+ 3% albumin)	0.1	7.3	14	PVP-MeOH	(97.4)	(91.6)

NOTES:

*Prefreeze Treatment: Aliquots of human blood from two units (data from the second unit are in parentheses) collected into Fenwal JB-2 ion exchange blood packs were processed by addition of 1/4 vol. of one of the additive solutions shown (except PVP-albumin; equal volume of additive added to saline-washed red cells).

Freezing: Containers were immersed in water at 45°C. for 40-43 sec. with agitation at 160 cpm (BPU-1).

Normal Recovery: Calculated a % of the total hemoglobin remaining in the red cells of the thawed materials.

Saline EOP: Calculated as % of the total hemoglobin remaining in the red cells after resuspension of the thawed material 1/40 in isotonic saline followed by equilibration for 1/2 hr. at room temperature.

efficiency. It appears that less PVP is necessary to process the cells and that the system is fairly insensitive to variations in additive salt concentration.

Aliquots of blood and blood-additive mixtures from the second unit of ion exchange-collected blood, both before and after freeze-thaw processing were assayed for total and supernatant Na^+ and K^+. Cellular cation concentrations were then calculated from these values by difference. Data are shown in Table XVII.

The cation concentration gradients maintained by the cells are evident, particularly with respect to the K^+ ions. Freeze-thaw processing caused shifts in cation concentrations, possibly affecting the cells' ability to maintain these gradients through membrane damage. Regardless of the cause, it is apparent that Na^+ ions are moving into the cells, and K^+ ions are moving out into the supernatant. The amount of K^+ appearing in the supernatant after freezing does not seem to differ greatly from that appearing after freezing of ACD blood (Table XXV).

Blood was collected through an ion exchange column for study with the PVP-albumin system in one-pint quantities. Table XVIII contains results which are lower than those previously attained with blood collected into ACD-A or ACD-B. It may be necessary to vary the freezing and thawing conditions to get the same results with ion-exchanged blood.

TABLE XVII

Cation Shifts Following Freeze-Thaw Processing of PVP-Protected Ion Exchange-Collected Blood

	Na^+ (mEq/L)				K^+ (mEq/L)			
	Red Cells		Supernatant		Red Cells		Supernatant	
	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw	Pre-freeze	Post-thaw
Whole blood	39	-	139	-	82	-	5	-
WB + 28% Plasdone C in H_2O	26	35	109	96	74	56	3	20
WB + 28% Plasdone C in 0.05 M NaCl	43	74	117	104	89	59	3	18
Washed cells + 14% Plasdone C, 3% albumin in 0.6% NaCl	35	70	126	104	87	61	negligible	24

NOTES:

See Table I for processing details (post-thaw recovery data shown in parentheses).

Total and supernatant Na^+ and K^+ assayed by flame photometry (Analytical Group) and cell Na^+ and K^+ calculated by difference using hematocrits (uncorrected for trapped plasma).

TABLE XVIII

Ion Exchange Blood

Sample	Thawed Blood Recoveries		Resuspended Recoveries	
	Normal	Saline EOP	Normal	Saline
Getzman	97.0	86.8	98.1	87.5
Kratz	96.7	80.9	97.1	83.0

Blood: Collected using Fenwal Ion Exchange Pack.

Blood Mixture:

To packed cells was added a solution of 14% Plasdone-C, 3% serum albumin and 0.6% NaCl.

Freezing Conditions:

Blood frozen in FC-1 containers coated with 500 cp PVP-methanol using 200 cpm agitation of the BPU.

Thawing Condition:

160 cpm BPU agitation in 45°C. water bath.

Resuspension in Plasma:

Supernatant removed from 300 cc. of thawed blood and 50 cc. of plasma added to packed cells.

VII. ION EXCHANGE OF HEMOGLOBIN:

Numerous reports have appeared in the literature on the use of ion exchange resins in extracorporeal circulation systems for treatment of hyperkalemia, ammonia intoxication, and in cardiac surgery for removal of citrate from banked blood.[4] Stored ACD bank blood which has undergone undesirable biochemical alterations such as elevated potassium, ammonium and citrate concentration and a pH below 6.8 can be restored to near normal by use of ion exchange resins.[5] These investigators also report a reduction in plasma hemoglobin by passing the blood over resins. Ion exchange chromatography has been used for isolation of preparative amounts of various hemoglobins.[6]

After thawing of blood that has been rapidly frozen in PVP there is an increase in plasma potassium and hemoglobin. With appropriate ion exchange resins the extracellular potassium level can be reduced. The following set of experiments were designed to investigate the possibility of removing or reducing extracellular hemoglobin from the frozen cell preparations prior to transfusion. If such a system could be developed it would be possible to directly transfuse the thawed blood through an ion exchange column in a manner similar to that which is now used for collection of blood.

An experimental procedure was set up so as to measure the hemoglobin retained by different cationic and anionic resins at various pH's.

Hemoglobin solutions were prepared by repeatedly freezing and thawing of whole blood in liquid nitrogen. A solution containing 0.7% w/v hemoglobin was subsequently prepared in the buffer system under study. This corresponds to a 1:20 dilution of lysed whole blood, and represents 5% lysis.

The weakly acidic resin, Amberlite IRC-50, and the strongly acidic resin, Dowex 50 WX8, were converted from the hydrogen ion form to the sodium form by 10% solutions of sodium chloride or sodium hydroxide. The weakly basic resin, Dowex 3, was in the free base form and was converted to the chloride form with hydrochloric acid and sodium chloride.

Columns containing 10 ml. of resin were washed with isotonic sodium chloride and then with either the phosphate or citrate buffer at the pH desired. Two milliliters of the 5% hemoglobin solution (containing 1.4 g-% hemoglobin) was layered on the resin and followed by a buffer wash. The effluent was collected in 1 ml. fractions and each was

then analyzed for hemoglobin by the carbonate method and read at 540 mμ. The observed optical densities were converted to grams of hemoglobin per 100 ml. after appropriate corrections or dilutions. Table XIX summarizes the hemoglobin retention resulting at different pH's and buffers with each of the ion exchange resins used in Figures 1 to 6. In all of the figures it should be noted that the greatest hemoglobin concentration was collected in the fifth to sixth fraction.

The weakly acidic resin IRC-50$^{(Na^+)}$ did not appear to retain to any great extent (~ 14%) the hemoglobin solution when buffered with 0.1M phosphate buffer (Figure 1). There was some holdup of the hemoglobin solution when 0.2M citrate buffer was used (Figure 2) and the holdup increased as the pH decreased (Table XIX). However, when the peaks of both curves are compared at pH 6 they are not too dissimilar.

The strong cationic resin Dowex 50 did not retain any hemoglobin when buffered with phosphate at pH 6.0 but retained 16% when buffered with citrate buffer at pH 6.2. The graph of the fractional distribution of the eluate hemoglobin is shown in Figure 3.

The strong base anionic resin Dowex 21K when buffered with phosphate buffer at various pH's did not hold up more than 20% of the added hemoglobin (Figure 4). When the citrate buffer was used with this resin (Figure 5) there was 63% recovery of the added hemoglobin at pH 4.6 and 77% overall recovery at pH 6.2.

The weakly basic anionic exchange resin, Dowex 3 showed the greatest retention of added hemoglobin in comparison to the other resin types (Figure 6). A 51% recovery of hemoglobin in the effluent was observed with pH 6.0 phosphate buffer and a 57% recovery was noted with the pH 6.2 citrate buffer.

These studies with strong and weak cationic and anionic exchange resins show that some retention of hemoglobin can be effected depending upon the conditions. In most instances the pH for best hemoglobin retention, i.e., the more acid pH's would not be practical for a whole blood system. The most promising resin appeared to be Dowex 3 the weakly anionic resin at pH 6. Further work is necessary before a resin formulation could be made for at least partial removal of supernatant hemoglobin resulting from freeze-thaw hemolysis of blood.

TABLE XIX

Summary Table for Hemoglobin Retention

by Ion Exchange Resins

Resin	Type	Buffer	Total Hb Recovered* pH	g./100 ml.	%	Hemoglobin Retained %
Amberlite ARC 50 (Na^+)	weak acid	phosphate	5.7	1.20	86	14
			6.0	1.23	88	12
			7.0	1.20	86	14
			8.0	1.14	81	19
		citrate	3.0	0.65	46	54
			4.6	0.76	54	46
			6.2	1.12	80	20
Dowex 50 Wx8 (Na^+)	strong acid	phosphate	6.0	1.18	84	16
		citrate	6.2	1.30	93	7
Dowex 21K (Cl^-)	strong base	phosphate	5.7	1.17	84	16
			6.0	1.13	81	19
			7.0	1.25	89	11
		citrate	4.6	0.88	63	37
			6.2	1.08	77	23
Dowex 3 (Cl^-)	weak base	phosphate	6.0	0.709	51	49
		citrate	6.2	0.798	57	43

* Two milliliters of 5% solution of hemoglobin (1.4 g./100) added to resin

FIGURE 1

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH AMBERLITE IRC-50(Na^+)

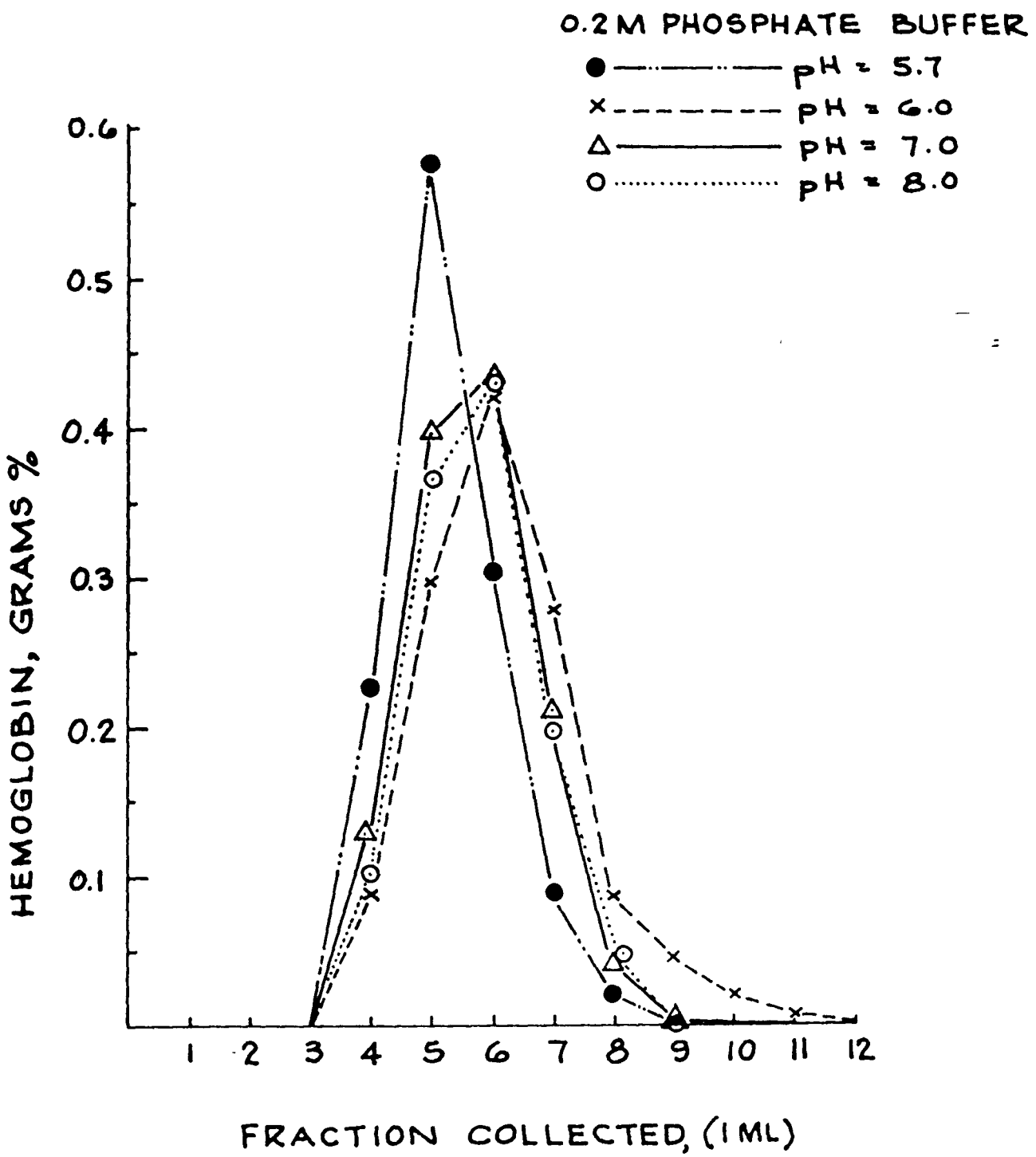


FIGURE 2

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH AMBERLITE IRC-50(Na^+)

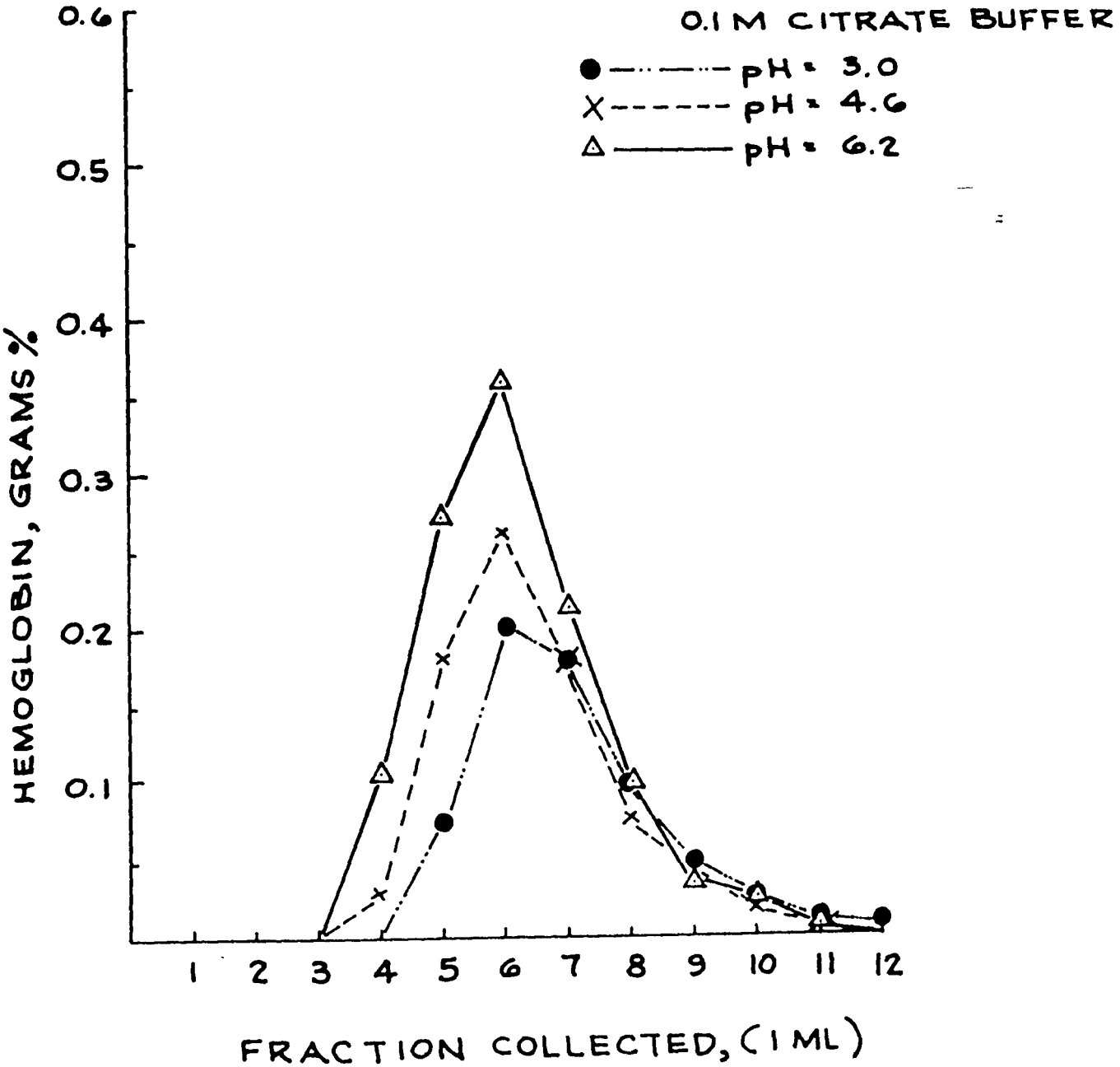


FIGURE 3

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH DOWEX 50W x8 (Na^+)

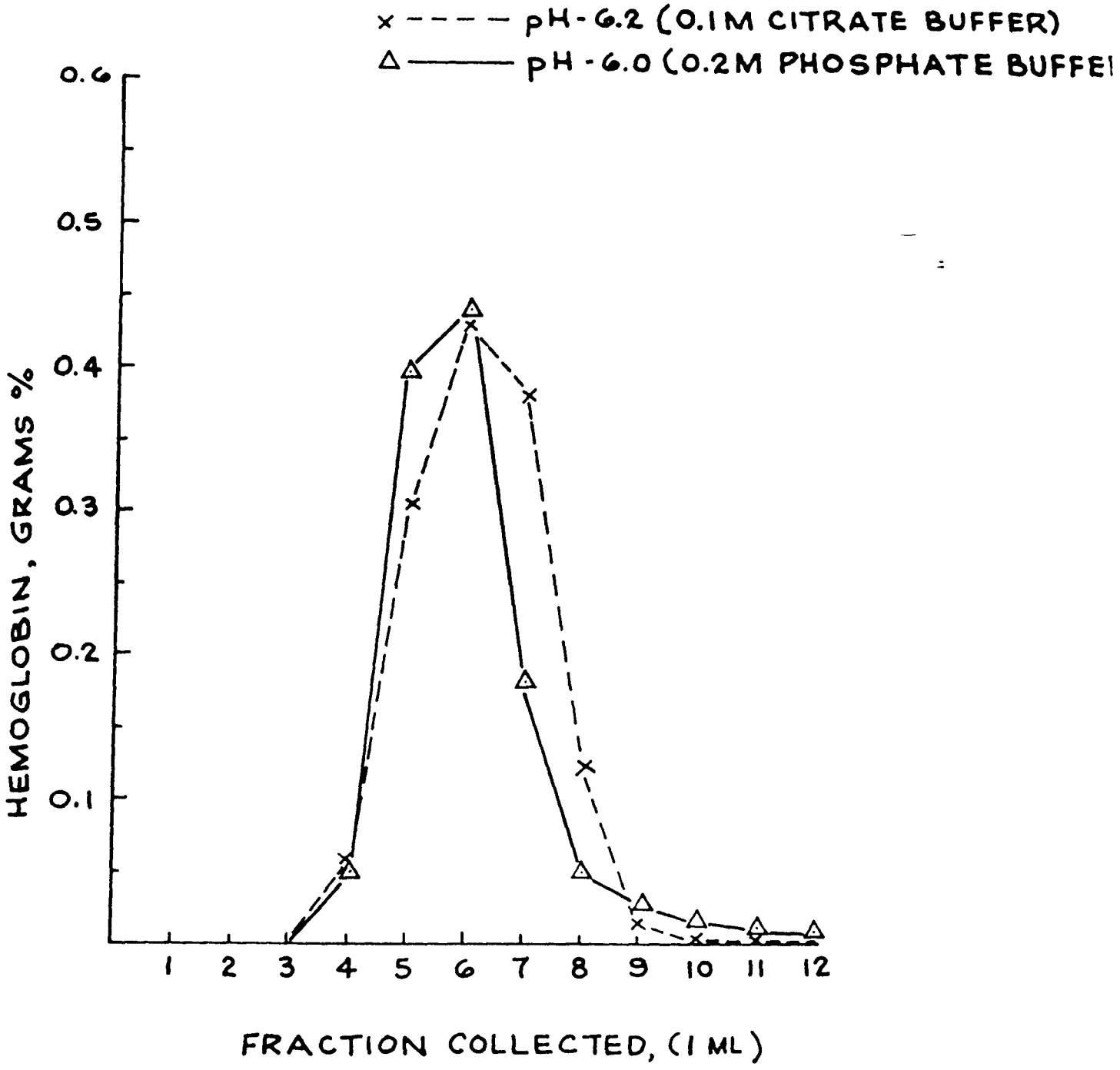


FIGURE 4

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH DOWEX 21K (Cl^-)

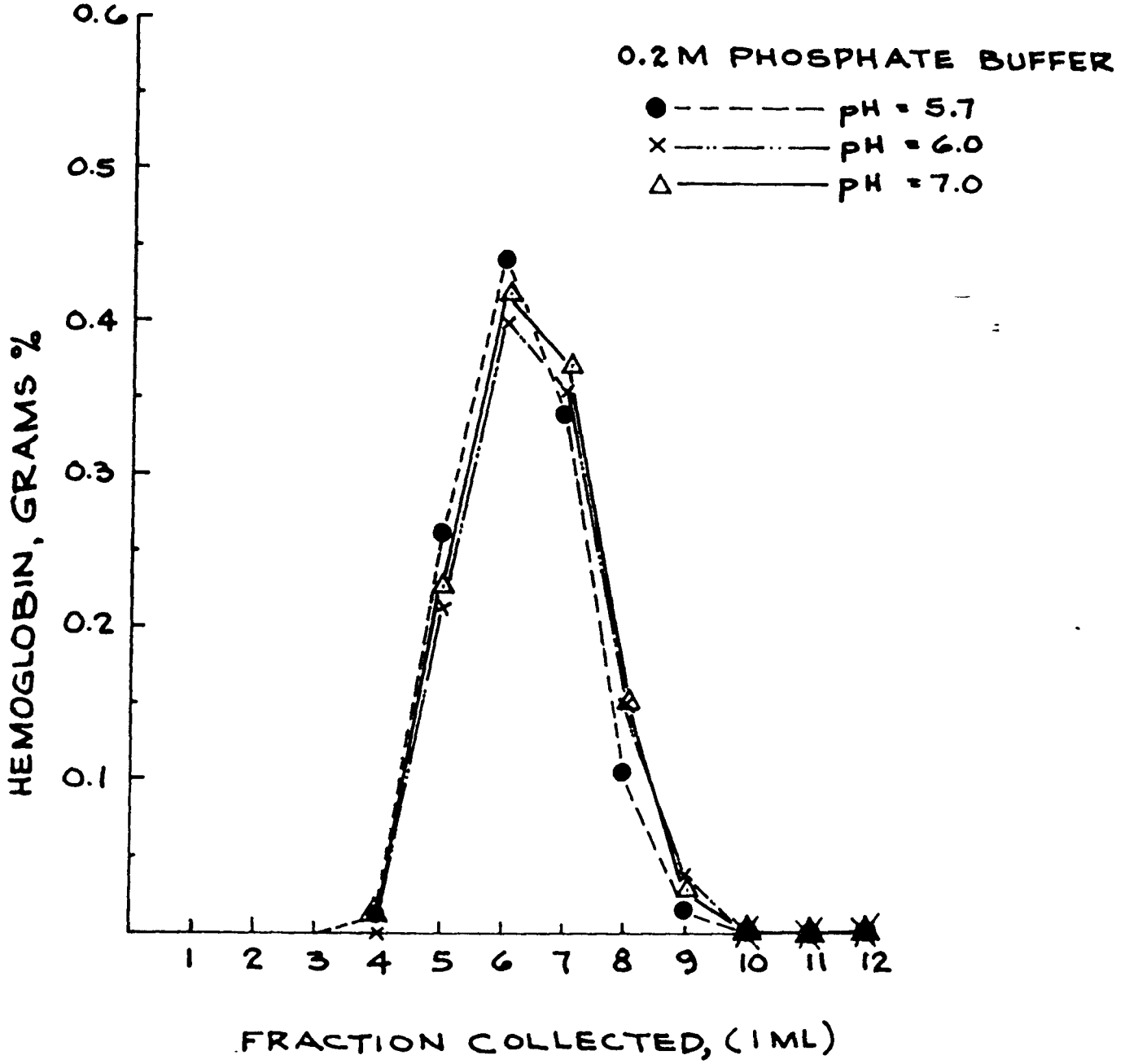


FIGURE 5

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH DOWEX 21 K (Cl⁻)

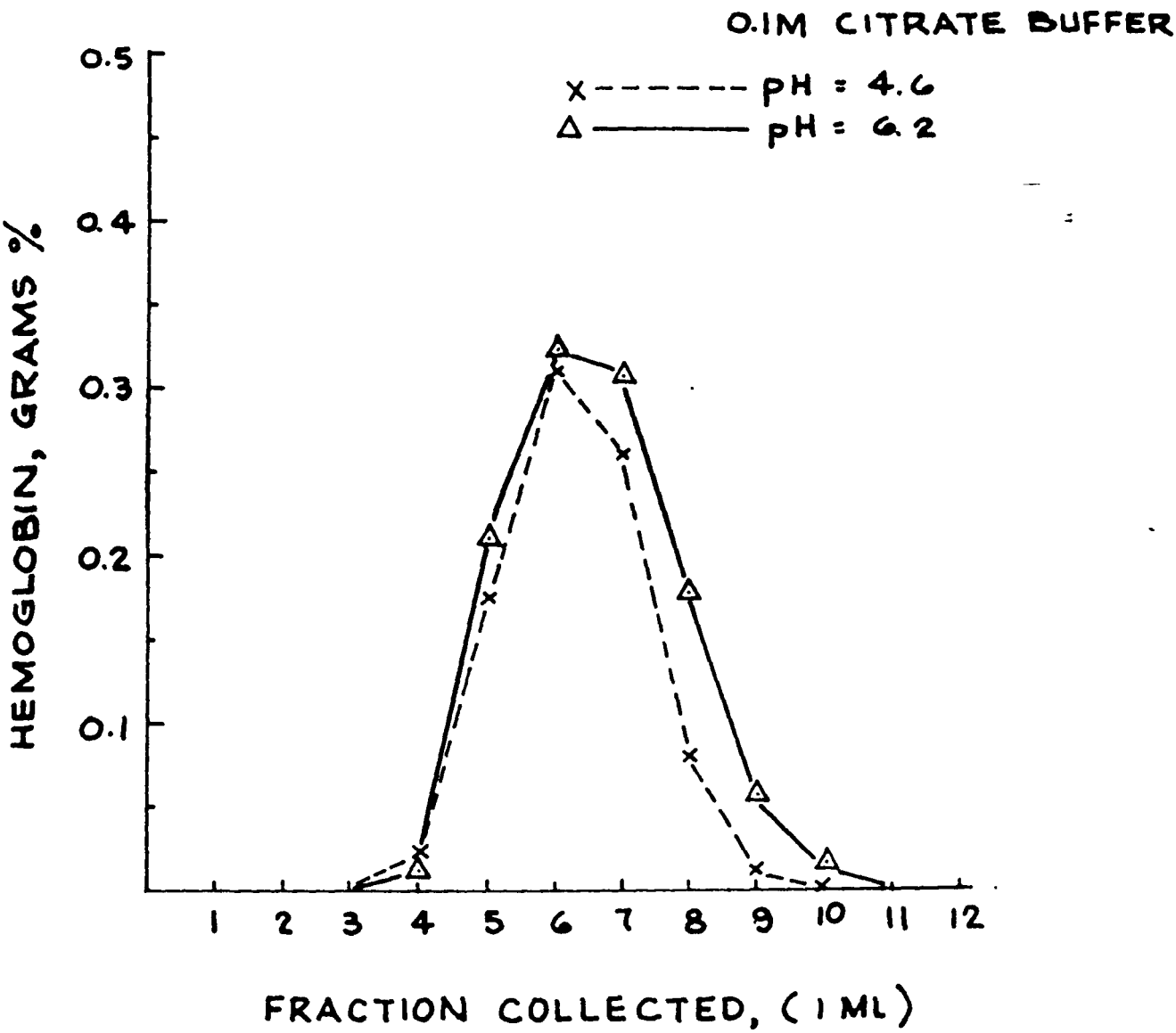
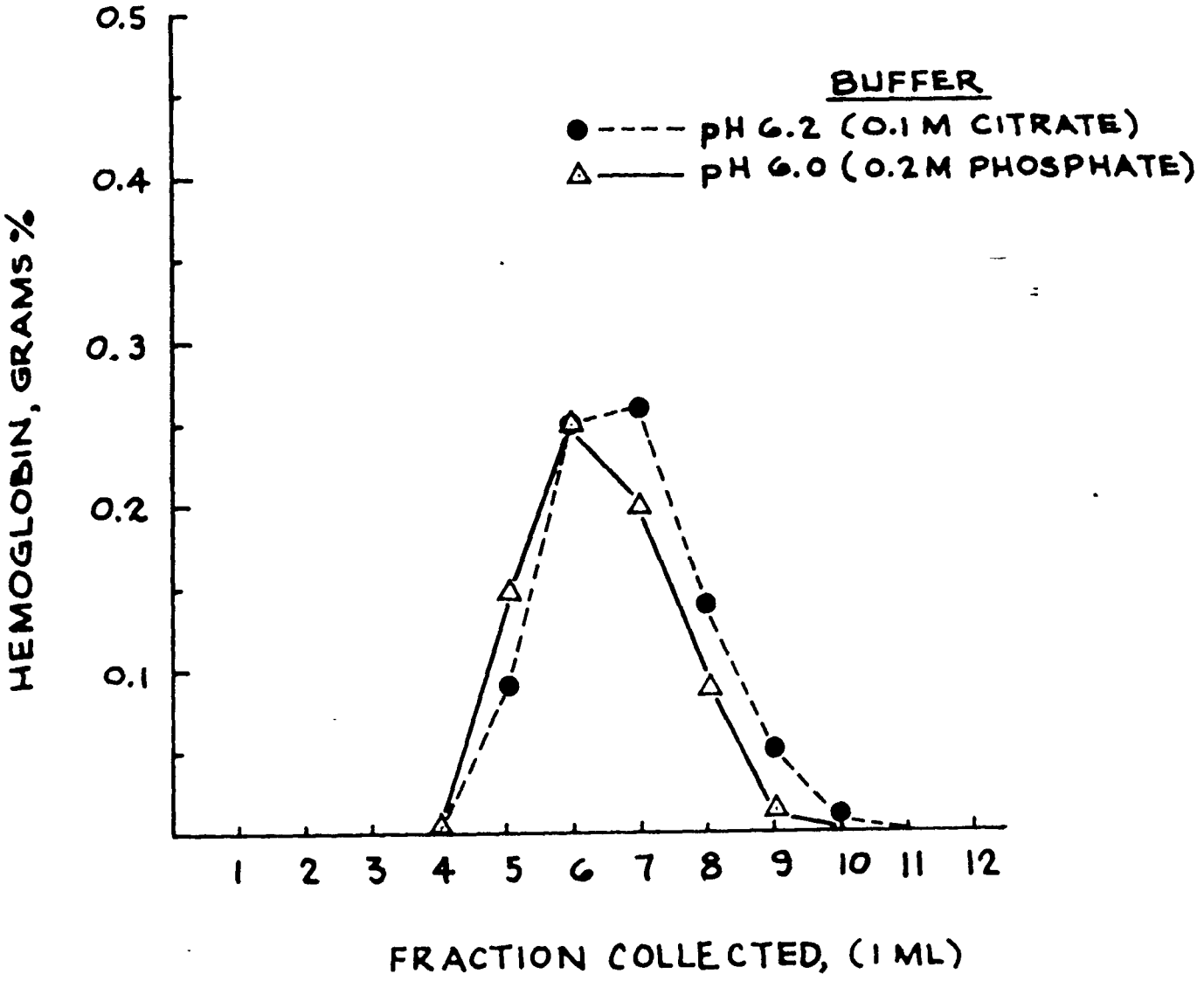


FIGURE 6

ION EXCHANGE OF A 5% HEMOGLOBIN SOLUTION WITH DOWEX 3 (Cl^-)



VIII. POST-THAW PROCESSING:

The primary objective of our blood program has been a process yielding a transfusible product with no post-thaw processing. Other investigators have developed processes utilizing additives which must be removed from the blood following thawing and while such techniques are more cumbersome than the whole blood-PVP process, the recovered erythrocytes are presumably quite stable. The question has arisen as to whether PVP-processed blood is adaptable to post-thaw processing, i.e., can such blood be washed to remove the PVP and free hemoglobin, what percentage of the erythrocytes are lost in the process, and are the intact washed cells as stable as unfrozen cells.

Saline and mannitol-saline dilution of thawed whole blood-PVP (7%) frozen by Process III were investigated so as to determine if additional hemolysis occurred. The data in Table XX show that a 1:2 dilution of the thawed Process III blood with saline resulted in additional hemolysis based on the initial amount of hemoglobin in the supernatant. Dilution of the blood 1:10 with saline resulted in even greater hemolysis. In contrast, resuspension of the thawed blood in 10% mannitol-saline did not depend upon dilution (1:5 or 1:10) and was less than with saline alone.

Additional studies were carried out using successive saline and mannitol washes to determine how many washes are necessary to reduce the supernatant hemoglobin concentration to below 100 mg-%. Two units of blood were frozen and thawed, one by Process III and the other by the PVP-albumin process. After thawing two aliquots of each were centrifuged and the supernatants were removed. One aliquot of cells from each unit was washed with isotonic saline, and the other with 10% mannitol-H_2O to observe the effect of a hypertonic, nonelectrolyte wash solution. In each case, sufficient wash solution was added to bring the total volume to that of the original blood aliquot. Washing was repeated until the supernatant fluid in each aliquot contained less than 100 mg-% free hemoglobin.

The intact washed cells were then resuspended, the Process III cells in 5% albumin-saline and the PVP-albumin cells in autologous plasma. As in the washings, sufficient medium was added to bring the total volume to that of the original blood aliquot. Red cell recovery was assayed immediately following resuspension and after storage at 4°C. Table XXI and Table XXII present the results obtained with Process III blood. The data show that 3.6% of the cells hemolyzed during freezing and thawing

TABLE XX

Supernatant Hemoglobin Levels After Resuspension

of Thawed Whole Blood-PVP 7%

Resuspension Solution	Dilution of Whole Blood-PVP	Supernatant Hemoglobin (mg. %)	Relative Hemoglobin Levels
none	-	350-380	-
saline (0.15 M NaCl)	1:2	310-325	620-650
saline	1:10	85-93	850-930
10% Mannitol-saline	1:5	97-100	485-500
10% Mannitol-saline	1:10	37-55	370-550

NOTE:

Aliquots from 1/2 pt. (FC-1/2) whole blood-PVP 7% system frozen by Process III subjected to above dilutions in saline or Mannitol-saline.

TABLE XXI

Post-Thaw Processing of Blood Frozen by Process III

Isotonic Saline Wash

	Supernatant Fluid		Hemoglobin	
	Hg Conc. (g./100 ml.)	Vol. Removed (ml.)	Amt. Removed (g.)	Fraction of Total (%)
Thawed blood	0.495	102	0.505	3.2
Wash No. 1	0.89	110	0.98	6.3
Wash No. 2	0.29	118	0.342	2.2
Wash No. 3	0.115	116	0.133	0.8
Wash No. 4	0.092	119	0.110	0.7
Wash No. 5	0.078	120	0.094	0.6
Total hemoglobin removed (%)				13.8

Recovery of Washed Cells Following Resuspension in 5% Albumin-Saline

	Immediate	After Storage at 4°C. 24 Hr.	96 Hr.
Norman recovery (%)	99.8	97.5	93.9
1/40 saline EOP (%)	98.6	95.5	90.8
Hematocrit (%)	23		
Hemoglobin (g./100 ml.)	7.9		

NOTES:

Freeze-Thaw Processing: 600 ml. (approx.) of a mixture of 4 vol. whole blood (ACD-B) + 1 vol. of 35% Plasdone C in 0.05 M NaCl were processed in FC-1 container. Container was coated with PVP-MeOH (500 cp), frozen by immersion in liquid nitrogen with agitation in BPU-1 (120 sec. at 200 cpm) and thawed by immersion in water at 45°C. with agitation in BPU-1 (75 sec. at 160 cpm).

Post-Thaw RBC Recovery Data:

Normal recovery (%)	96.4
1/40 saline EOP (%)	88.7
Hematocrit (%)	31
Hemoglobin (g./100 ml.)	9.5

Post-Thaw Washing: A volume of thawed blood (164 ml.) was centrifuged and the supernatant removed (volume shown). The cells were diluted to the

TABLE XXI Cont'd

original volume with isotonic saline, mixed, and the centrifugation and washing were repeated as shown, to reduce supernatant hemoglobin to less than 100 mg-%. Following the final wash the intact cells were re-suspended to the original volume in 5% albumin-saline. Hemoglobin was assayed as cyanmethemoglobin.

TABLE XXII

Post-Thaw Processing of Blood Frozen by Process III

10% Mannitol Wash

	Supernatant Fluid		Hemoglobin	
	Hg Conc. (g./100 ml.)	Vol. Removed (ml.)	Amt. Removed (g.)	Fraction of Total (%)
Thawed blood	0.495	100	0.495	3.5
Wash No. 1	0.125	110	0.138	0.97
Wash No. 2	0.22	113	0.248	1.74
Wash No. 3	0.06	118	0.071	0.5
Total hemoglobin removed (%)				6.7

Recovery of Washed Cells Following Resuspension in 5% Albumin-Saline

	Immediate	After Storage at 4°C., 24 Hr.
Normal recovery (%)	93.2	90.0
1/40 Saline EOP (%)	90.6	86.5
Hematocrit (%)	23	
Hemoglobin (g./100 ml.)	8.9	

NOTES:

Freeze-Thaw Processing: See Table XXI.

Post-Thaw RBC Recovery Data: See Table XXI.

Post-Thaw Washing: Same as Table XXI except 150 ml. was processed.

and an additional 7.7% were sufficiently damaged to hemolyze on saline resuspension. Post-thaw saline washing (Table XXI) resulted in the loss of less than 14% of the cells or only slightly more than were damaged during freezing and thawing as indicated by the saline EOP. Four washings were required to lower the hemoglobin level to less than 100 mg-%, although an extra washing was done since the supernatant hemoglobin concentrations were not accurately measured until the entire procedure had been completed. Negligible hemolysis occurred on suspension of the saline-washed cells in albumin-saline. About 4-5% of the cells hemolyzed upon saline resuspension after 24-hr. storage at 4°C. It is quite possible that all or part of this hemolysis was due to the lack of nutrient in the medium and to the nature of the suspending medium.

Use of the mannitol wash solution (Table XXII) required only three washings to lower the free hemoglobin to less than 100 mg-%, with less than 7% of the cells hemolyzing during processing (including freeze-thaw losses). However, overall hemolysis was not decreased, since another 7% of the washed cells lysed on resuspension in the albumin-saline. Actually, overall cell damage as indicated by the EOP of the resuspended washed cells was slightly greater, and the cells were markedly less stable when stored at 4°C. Gross agglutination of the cells by the mannitol solution was observed after the first washing. This is probably the same phenomenon recently described by Huggins.[7] Apparently the mannitol does serve to decrease hemolysis by maintaining tonicity during washing, but with the result that the damaged cells then lyse on resuspension.

In Tables XXIII and XXIV are shown the results of post-thaw processing of PVP-albumin protected red cells. Here, the thawed material contained over 9% intact cells, with 92.5% remaining intact after 1/40 saline resuspension (EOP). Again, saline washing of the thawed cells (Table XXIII) resulted in a total loss after five washes of only about 1% more cells than were damaged during freezing and thawing (saline EOP). Suspension of the washed cells in autologous plasma yielded an extremely stable product, with 96% of the cells, representing over 87% of the original unfrozen cell population, remaining intact on saline resuspension after 22 days' storage at 4°C.

Mannitol washing of PVP-albumin cells (Table XXIV) gave about the same results as had been seen with the Process III blood. Few cells were hemolyzed during washing, with free hemoglobin reduced to 58 mg-% after two washes. Again gross agglutination was seen during the second wash.

TABLE XXIII

Post-Thaw Processing of Blood Frozen

by the PVP-Albumin Process

Isotonic Saline Wash

	Supernatant Fluid		Hemoglobin	
	Hemoglobin Concentration (g./100 ml.)	Volume Removed (ml.)	Amount Removed (g.)	Fraction of Total (%)
Thawed Blood	0.495	80	0.40	2.3
Wash No. 1	0.600	85	0.510	3.0
Wash No. 2	0.365	92	0.336	1.95
Wash No. 3	0.124	92	0.114	0.66
Wash No. 4	0.100	95	0.095	0.55
Wash No. 5	0.068	94	0.064	0.37
Total Hemoglobin Removed (%)				8.8

Recovery of Washed Cells Following

Resuspension in Autologous Plasma

		After Storage at 4°C.		
	Immediate	24 Hr.	96 Hr.	22 Days
Normal Recovery (%)	99.6	99.5	99.0	96.4
1/40 Saline EOP (%)	99.3	99.3	98.4	95.9
Hematocrit (%)	33			
Hemoglobin (g./100 ml.)	9.6			

NOTES:

1. Freeze-Thaw Processing:

530 ml. of whole blood (ACD-B) were centrifuged. 270 ml. plasma were extracted and saved for resuspension. To the cells were added 300 ml. of 14% Plasdone C-3% albumin in 0.6% NaCl. This mixture was by immersion in liquid nitrogen with agitation in BPU-1 (120 sec. at 200 cpm), and thawing was by immersion in water at 45°C. with agitation in BPU-1 (75 sec. at 160 cpm).

2. Post-Thaw RBC Recovery Data:

Normal Recovery (%)	97.3
1/40 Saline EOP (%)	92.5
Hematocrit (%)	37
Hemoglobin (g./100 ml.)	11.5

3. Post-Thaw Washing:

150 ml. of thawed blood were processed as described in the notes to Table XX.

TABLE XXIV

Post-Thaw Processing of Blood Frozen

by the PVP-Albumin Process

100% Mannitol Wash

	Supernatant Fluid		Hemoglobin	
	Hemoglobin Concentration (g./100 ml.)	Volume Removed (ml.)	Amount Removed (g.)	Fraction of Total (%)
Thawed Blood	0.495	85	0.42	2.3
Wash No. 1	0.095	98	0.093	0.54
Wash No. 2	0.58	105	0.061	0.35
Total Hemoglobin Removed (%)				3.2

Recovery of Washed Cells Following

Resuspension in Autologous Plasma

	Immediate	After Storage at 4°C. 24 Hr.
Normal Recovery (%)	94.7	94.4
1/40 Saline EOP (%)	92.5	92.1
Hematocrit (%)	33	
Hemoglobin (g./100 ml.)	10.9	

NOTES:

1. Freeze-Thaw Processing: See Table XXIII

2. Post-Thaw Recovery Data: See Table XXIII

3. Post-Thaw Washing: See Table XXIII

Resuspension of the washed cells in autologous plasma resulted in the immediate hemolysis of over 5% of the cells, but those remaining intact were fairly stable, with hemolysis increasing only slightly in 24 hours' storage at 4°C.

It is obvious that in both units of blood the mannitol wash merely prevented hemolysis of the damaged cells, which then hemolyzed upon resuspension. Thus one purpose of post-thaw processing, that of removal of free hemoglobin, is defeated. In fact supernatant hemoglobin content of the resuspended mannitol-washed cells in both units was almost twice that in the blood immediately following thawing. Saline washing, while a longer process with greater cell loss during washing, yields cells which can be resuspended with negligible hemolysis, and particularly in the case of PVP-albumin cells, are quite stable.

IX. BIOCHEMICAL DAMAGE TO RED CELLS DURING FREEZING AND THAWING:

A. Adenosine Triphosphatase:

Adenosine triphosphate (ATP) and the enzyme, ATPase, are involved in ion transport through the red cell membrane. We have studied the distribution of ATPase as affected by freezing and thawing.

Soluble ATPase activity is markedly increased in blood or red cell suspensions following partial or complete lysis by freezing and thawing. In the case of cells protected by PVP, the recovered red cells, after lysis by freezing, yield ATPase activity per unit of hemoglobin equal to that of control cells that are lysed by freezing. Soluble ATPase activity appears also after immunologic lysis.

When red cells are separated from buffy coat mechanically or by washing, the ATPase activity released from them by freezing lysis is less than that from red cells not so treated. Thus, leukocytes, platelets, and plasma appear to contribute to overall ATPase activity of blood. In fact, ACD-plasma can reverse the effect of washing in decreasing activity. Calcium inhibits ATPase activity; magnesium is required and some competition exists between Ca and Mg ions which is partially altered by the presence of citrate.

B. Hemoglobin:

Totally lysed blood, centrifuged to remove stromata and freed of potassium by passage over Dowex-50 (Na^+ form), failed to harm rabbits when infused in 20 to 50-ml. volumes of blood. Hemoglobin levels are, therefore, not the significant factor in the toxic effect of frozen and thawed blood reported on page 11, Bimonthly Progress Report No. 1.

Small volumes of hemoglobin (1 and 2 ml. of lysed blood) elevate the serum hemoglobin but a rapid and progressive drop occurs within minutes after infusion (Figure 7). It is doubtful that single serum hemoglobin values are a reliable measure of intravascular hemolysis in the rabbit.

C. Potassium:

Considerable redistribution of potassium and sodium ions occurs in blood subjected to freezing and thawing even under conditions resulting in high recoveries of viable red cells (Table XXV and Figure 8).

X. PROCESS PARAMETERS:

A. Role of Agitation During Freezing and Thawing:

The agitator system in the BPU has been replaced by one with a range of agitation rates from 0-250 cpm instead of 160-300 cpm. More precise studies of the effect of agitation rates are now possible.

In vitro and in vivo studies indicate that rapid shaking is advantageous in both the freezing and thawing of whole blood-PVP mixtures (Tables XXVI and XXVII).

B. Hematocrit:

There appears (Table XXVIII) to be no effect of hematocrit of blood, as collected, on recovery or stability of cells frozen and thawed in presence of PVP.

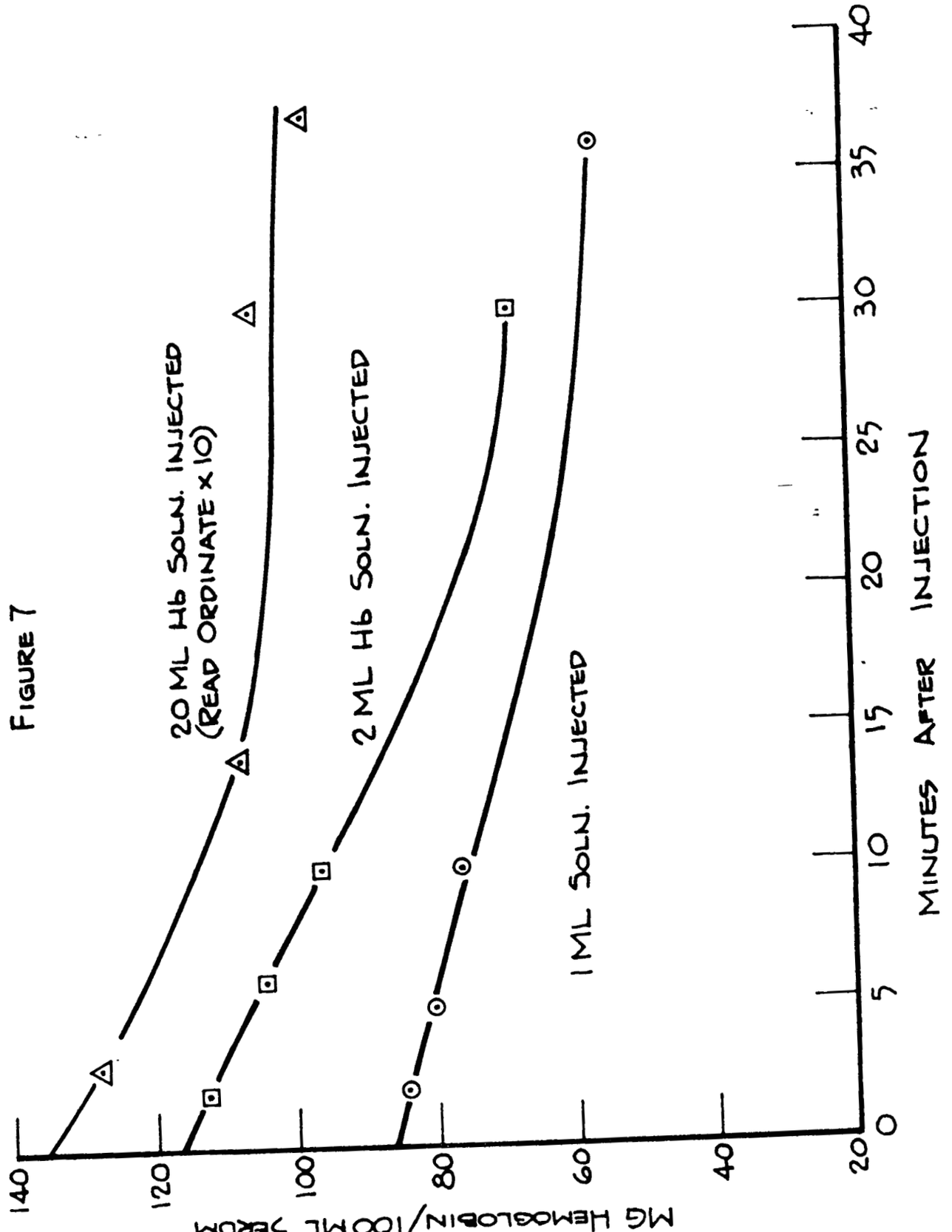

20 ML Hb Soln. Injected
(Read Ordinate x 10)
2 ML Hb Soln. Injected
1 ML Soln. Injected
MG Hemoglobin/100 ML Serum
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
40
Minutes After Injection

Figure 7

FIGURE 8

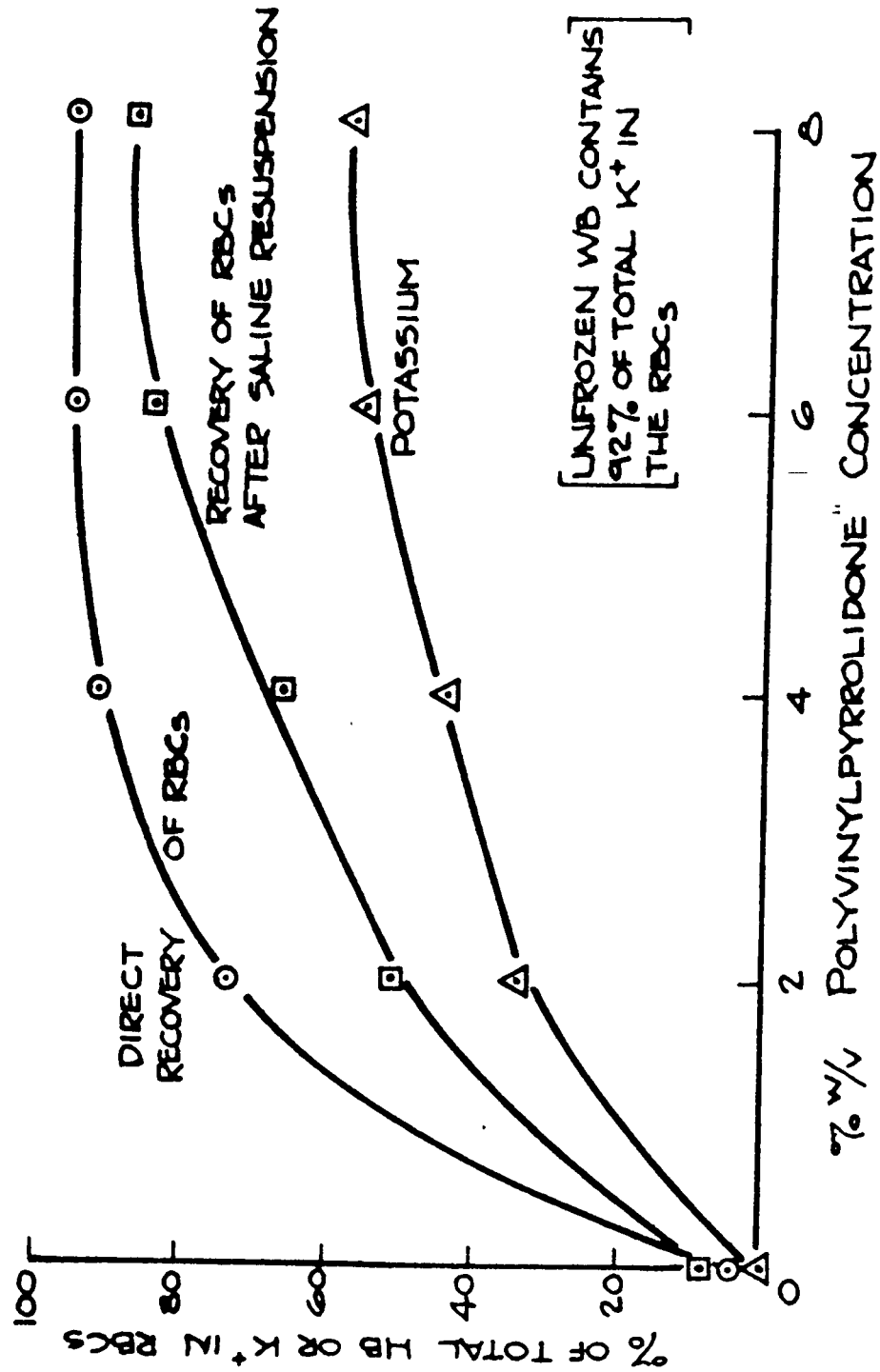

% OF TOTAL Hb OR K+ IN RBCs
100
80
60
40
20
0
2
4
6
8
% W/V POLYVINYLPYRROLIDONE CONCENTRATION
DIRECT RECOVERY OF RBCs
RECOVERY OF RBCs AFTER SALINE RESUSPENSION
POTASSIUM
[UNFROZEN WB CONTAINS 92% OF TOTAL K+ IN THE RBCs]

TABLE XXV

Potassium Distribution in Frozen-Thawed Human Blood

Process	No. Samples	Meq. K^+/Liter	
		Supernatant	Whole Blood
WB-7% PVP[1]	2	18.1	25.5
WB-7% PVP[2]	2	15.0	28.0
WB-7% PVP[3]	2	18.5	26.8
RBC-Alb-PVP[4]	7	22.0-34.0	26.8-41.2
RBC-Alb-PVP[5]	7	12.3-16.2	41.2-42.0

[1] Shaken 75 rpm thaw.

[2] Shaken 300 rpm thaw.

[3] Shaken 200-100 rpm thaw.

[4] Directly after thawing.

[5] After resuspension in plasma.

Usual supernatant (K^+), prefreeze 4-6 meq./l.

TABLE XXVI

Relationship of Volume and Agitation During Freezing

Volume Frozen (ml.)	Agitation Frequency (Freezing) cpm		
	100	200	300
25	94 (78)	95 (83)	97 (89)
50	94 (78)	95 (82)	94 (85)
100	91 (72)	93 (78)	96 (83)

WB-ACD (4 vol.) + PVP K30 35%-0.05 M NaCl (1 vol.)
Frozen in uncoated BFF 19110's at frequencies shown; thawed 200 cpm, 45°C.

TABLE XXVII

Survival of Human Red Cells in Whole Blood-PVP 7%

Subjected to Various Thawing Conditions of Agitation

Thawing Conditions*	No. Tests	Direct RBC Recovery (%)	% RBC Survival 1/2 hr.	% RBC Survival 24 hr.
BPU-1, 75 cpm	2	96 ± 0	88 ± 1	81 ± 11
BPU-1, 150 cpm	6	96 ± 1	94 ± 4	79 ± 4
BPU-1, 200-100 cpm**	1	96	98	82
Manual - slow	3	96 ± 1	88 ± 3	70 ± 3
Manual - rapid	3	97 ± 0	94 ± 3	88 ± 9

* Cell specimens agitated in 45°C. water.

** Agitated for 20 sec. at 200 cpm followed by 16 sec. at 100 cpm.

TABLE XXVIII

Effects of Hematocrit

Volumes, ml.			Hematocrits				
RBC	Plasma	PVP*	Before Additive	After Additive	After Thaw	Direct % Recovery	Saline EOP %
40	0	10	92	76	60	96	79
35	5	10	82	66	55	96	80
30	10	10	70	53	41	95	80
25	15	10	56	43	39	95	78
20	20	10	46	35	33	95	80
15	25	10	33	25	23	95	80
10	30	10	21	15	15	96	83
5	35	10	11	9	7	94	84
1	39	10	2	2	2	84**	77

* PVP K30 35%-0.05 M NaCl.
50 ml. frozen in uncoated BFF-19110's, BPU-1, 200 cpm; thawed 150 cpm, 45°C.

** 2% hematocrit specimens of doubtful accuracy because of low Hb concentrations during analyses.

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