

[54] **BLOOD COAGULATION COMPLEX**

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[58] Field of Search **424/177**

[56] **References Cited**

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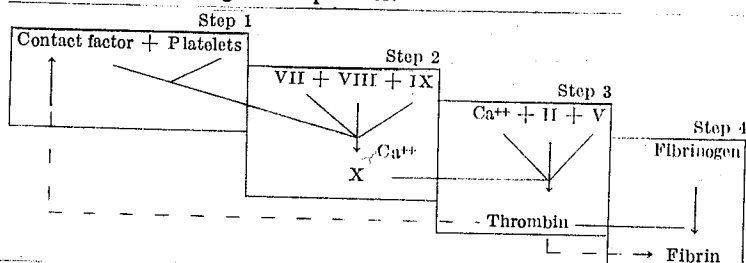
[57] **ABSTRACT**

A lyophilized mixture of coagulation components of human origin useful in the treatment of patients, who bleed due to a congenital or acquired deficiency of one or more of the coagulation Factors II, VII, IX, X, prepared from human plasma collected in a citrate anticoagulant, free of heparin, free of thrombin, free of the activated form of Factor X, free of depressor activity, free of anti-complement activity, and comprising the coagulation components Factors II, VII, IX, X in essentially the same proportions as normal human plasma and of a potency equivalent to a specific activity of more than 0.5 clinical unit of each component per milligram of protein; and the process of preparing such product by selective adsorption and selective fractional elution.

7 Claims, No Drawings

BLOOD COAGULATION COMPLEX

This invention relates to blood coagulation com-



played by Factor IX, as well as by Factors II, VII and X, is illustrated by the following simplified diagram of the coagulation process.

ponents and in general has for its object the provision of a purified composition of therapeutically useful blood coagulation components and to a method for producing such components from readily available materials.

There are estimated to be 100,000 cases of congenital hemophilia in the United States. Of these, approximately 20,000 are cases of hemophilia B, the blood of such patients being either totally devoid of plasma thromboplastin component or seriously deficient in plasma thromboplastin component. The disease therefore exists in varying degrees of severity, requiring therapy anywhere from every week up to once or twice a year. The completely deficient cases require replacement therapy once every week; the partially deficient cases require therapy only when bleeding episodes occur, which may be as seldom as once a year. The bleeding episodes in congenital, partially-deficient cases are generally caused by a temporarily acquired susceptibility rather than by injury alone. Intravenous injection of a sufficiently large amount of fresh plasma, or an equivalent amount of fresh blood, temporarily corrects the defect of a deficient subject. The beneficial effect often lasts for two or three weeks, although the coagulation defect as measured by in-vitro tests on the patient's blood appears improved for only two or three days. Such therapy with fresh plasma or fresh blood is effective but it has several serious drawbacks: (1) it requires ready availability of a large amount of fresh plasma; (2) requires hospitalization for the administration of the plasma; (3) a great many of the patients become sensitized to repeated blood or plasma infusions and ultimately encounter fatal transfusion reactions; (4) at best plasma can only partially alleviate the deficiency; and (5) prolonged treatment or surgery is not possible because the large amounts of blood or plasma which are required will cause acute and fatal edema.

Several investigators noted that the mixing of blood of certain hemophilic patients would result in the mutual correction of the clotting defect of each blood. Interpretation of these findings was eventually made by Aggeler and co-workers [Proc. Soc. Exptl. Biol. Med. 79:692-696 (1952)] and S. G. White et al [Blood 8:101-124 (1953)]. These workers, studying a male patient with a severe hemorrhagic diathesis associated with a prolonged clotting time which was clinically indistinguishable from classic hemophilia, postulated the existence of a new clotting factor. Aggeler et al, recognizing that the new factor was a precursor of thromboplastin, named it Plasma Thromboplastin Component (PTC). The work was confirmed by Biggs et al. [Brit. Med. J. 2:1378-1382 (1952)] in England, who gave it the name Christmas Factor, and by Soulier and Larrieu [New Eng. J. Med. 249:547-553 (1953)] in France, who called it Antihemophilic Factor B. This factor is now officially designated Factor IX. The role

In collaboration with Aggeler et al., the first fractionated PTC preparation was prepared [Revue d'Hematologie 9:447-453 (1954)]. The PTC was absorbed on barium sulfate from a solution of Cohn Fraction IV and eluted with 0.34 M sodium citrate. The yields are very small, and the post-infusion in-vivo activity was only about one-fourth that predicted from in-vitro assays by a prothrombin consumption test.

Later, in collaboration with Aggeler et al., a process was developed by which PTC was adsorbed onto barium sulfate from EDTA anticoagulated plasma, eluted with a sodium chloride-sodium citrate buffer, and further purified by cold ethanol fractionation. This preparation was used clinically with excellent results [Aggeler, P. M. et al. Trans. 6th Congress, Internat. Soc. Hematol. Grune & Stratton, N. Y., pgs. 490-497 (1958)]. The process was later published in detail, and it was pointed out that the process was never commercialized because the Albumin and Plasma Protein Fraction obtained as by-products were contaminated with potentially hazardous levels of barium [Hink, J. H. and Johnson, F. F., The Hemophilias, CH. 18, page 156, edited by Brinkhous, Univ. Of North Carolina Press (1964)]. Biggs et al. in England [Brit. J. Haematol. 7:349-364 (1961)] and Janiak and Soulier [Thromb. et Diath. Haemorr. 8:406-424 (1962)] in France later prepared Factor IX by a similar process, substituting tricalcium phosphate for the barium sulfate. However, spontaneous formation of thrombin always occurred and it was always necessary to add heparin, both during and after processing to neutralize the potentially dangerous thrombin.

Tullis et al. [New Eng. J. Med. 273:667-674 (1965)] have prepared and studied a somewhat similar plasma fraction, which they refer to as "prothrombin complex". In the Tullis process, the blood was collected through a calcium-removing ion exchange resin, and the resulting resin-plasma adsorbed onto DEAE cellulose. Elution was accomplished with a sodium phosphate-sodium chloride buffer stabilized with EDTA. Clinical studies on the "prothrombin complex" are described in the reference, but other data characterizing the complex are not available.

The preponderance of blood generally taken and available for transfusion is protected from coagulation by treatment with a citrate anticoagulant which allows the blood to be utilized for only a limited period of time. After this limited time expires, this blood must be discarded or it can be made available for fractionation into certain useful components. As a matter of fact, the major source of whole blood converted to plasma for fractionation comes from outdated blood which has been protected by citrate anticoagulation. It is, therefore, important that processes for the fractionation of plasma, such as to obtain a Factor IX containing concentrate, be directed to citrate preserved blood. An ob-

ject of this invention is that the process for making the concentrate of this invention starts with plasma taken from citrate preserved blood.

It is also important that such concentrates do not contain thrombin since the injection of thrombin into a human would be considered highly dangerous. Although it is possible to neutralize thrombin activity with heparin, it would be preferable not to have the thrombin there in the first place. Heparin is undesirable in a Factor IX containing concentrate because it also is potentially hazardous to the patient and because it causes difficulties in assaying the coagulation factors in the concentrate. In the administration of a Factor IX containing concentrate, constant monitoring of the patient's coagulation status is required, and the presence of heparin will not only complicate this testing procedure, but will make the results thus obtained unreliable. An object of this invention is that the concentrate be free of both thrombin and of heparin.

It is a further object of this invention to provide a concentrate that comprises coagulation components II, VII, IX and X in proportions essentially equal to those found in plasma, yet free of the activated form of Factor X, thereby reducing the danger of precipitating intravascular coagulation.

It is a further object of this invention to provide a concentrate free of the anticomplement activity which is so often associated with plasma globulins and which would make the intravenous administration of the product highly dangerous.

It is a further object of this invention to provide a very pure product by selective adsorption of active components and further selectivity by fractional elution.

Other objects and advantages of the invention will appear from the following description of preferred embodiments thereof.

Details of Making Product

Very generally, the product is made by starting from Effluent I, prepared by the Cohn Method 6. The complex of Factors II, VII, IX, X is adsorbed onto DEAE Sephadex, which is an anion exchanger, diethylaminoethyl Sephadex, consisting of cross-linked dextran chains with diethylaminoethyl groups attached by ether linkages to the glucose units of the polysaccharide chains, supplied by Pharmacia Fine Chemicals, Inc., Piscataway, New Market, N. J. The spent Effluent I is returned to the plasma fractionation process so that none of the other plasma components are wasted. The DEAE Sephadex is fractionally eluted with increasing concentrations of ammonium bicarbonate solution. The complex of the invention is eluted in the fraction between 0.3 M and 0.75 M ammonium bicarbonate at pH 7.3-8.2.

More specifically, the preferred procedure is as follows:

A. Adsorb the Effluent I of the standard plasma fractionation process onto a column of freshly equilibrated DEAE Sephadex in an amount of 10 Gms. (wet weight) per liter of Effluent I, at 0° to -3°C. Return the spent Effluent I to the plasma fractionation process just prior to precipitation of Fraction II+III. B. Wash the DEAE Sephadex column with 0.2 M ammonium bicarbonate at pH 7.0-7.8 until no further protein is eluted and discard the wash.

C. In the same manner, wash the DEAE Sephadex

column with 0.3 M ammonium bicarbonate, at pH 7.0-7.8 until no further protein is eluted; the blue ceruloplasm is eluted with this wash; discard the wash.

D. In the same manner, elute the DEAE Sephadex column with 0.75 M ammonium bicarbonate, at pH 7.6-7.8 until the Factor IX is eluted.

E. Freeze and lyophilize the eluate of step D; the ammonium bicarbonate sublimates leaving a salt-free protein powder equivalent to about 350 mg. per liter of Effluent I; assay for Factor IX, and store in a moisture-proof container at 5°C or below.

A modification of the product of this invention is the product in an electrolyte buffer suitable for intravenous administration as follows:

F. Slowly dissolve the dry protein powder in a diluent of 0.05 M sodium citrate and 0.088 M sodium chloride to result in a final concentration of 25 units of Factor IX per ml.; as the powder dissolves, maintain the pH at 7.0-7.5 by additions of 1 N sodium hydroxide; adjust the final pH to 7.3 ± 0.1 .

G. Sterilize by filtration through a sterile 0.2 micron porosity membrane filter.

H. Aseptically fill convenient portions into sterile bottles, freeze, and lyophilize. Store at 5°C.

I. For human administration, the content of the vial in step H is aseptically redissolved in water for injection. Alternatively, the product of step D can be sterile filled and lyophilized to a salt-free product. In this instance it is reconstituted with a suitable isotonic diluent prior to human injection.

The above process recites the details which are preferred at this time. However, many variations can be made without departing from the general principles of the invention.

For example, the amount of DEAE Sephadex relative to the volume of Effluent I may be varied over a wide range. The use of larger amounts of DEAE Sephadex will result in a lower degree of purity and greater yield of the coagulation complex, and a greater loss of other plasma proteins normally recoverable in subsequent steps. The use of smaller amounts of DEAE Sephadex will result in even higher degrees of purity, but will also result in considerably lower yields. Although DEAE Sephadex is the preferred anionic exchanger, presumably other anionic adsorbents would work also.

The elution step with ammonium bicarbonate is extremely important to the invention of this process. The unique selectivity obtained by application of the ammonium bicarbonate salt gradient elution combined with the unique volatility factor of ammonium bicarbonate are very important aspects of this process for the following reasons:

1. Ammonium bicarbonate being a volatile, inorganic salt is completely removed from the desiccated product thus resulting in a completely salt-free protein preparation. This offers an advantage in that the final clinical preparation may be easily adjusted to isotonicity.

2. The recovery of a salt-free protein obviates the necessity of extensive salt removal procedures such as dialysis, gel filtration, ultra filtration, etc., thus a commercially feasible large scale operation becomes more practical.

3. Ammonium bicarbonate was found to offer selectivity in the elution of the bound proteins, allowing the ready separation of undesirable impurities such as

ceruloplasmin. As a result, the desired components are eluted in a higher state of purity than can be accomplished with conventional eluting solutions.

The above specified pH ranges for the elution steps are preferred, although higher pHs up to 8.2 could be employed, the higher pHs contributed to instability of the product.

These conditions have been designed to make further fractionation of the spent Effluent I more practical or possible.

The dissolving of the dried protein, its concentration, the nature of the electrolyte buffer, and the filtration are all standard steps which can be varied by anyone familiar with the art.

CHARACTER OF PRODUCT

The product resulting from the above described process is a lyophilized product containing human Factors II, VII, IX and X in a suitable electrolyte buffer such as sodium chloride-sodium citrate buffer. The product is free of thrombin, thromboplastin activity, anticomplement activity, and depressor activity. It has a specific activity of at least about 0.5 Factor IX unit per mg. protein. One Factor IX unit is defined as that amount of Factor IX activity contained in one ml. of fresh average pooled plasma. Assay results are expressed in terms of a lyophilized reference standard which contains 0.7 unit per ml. upon reconstitution.

ASSAYS

In-Vitro Assay of Factor IX Activity

The assay was based on the partial thromboplastin time technic of Langdell, Wagner, and Brinkhous [J. Lab. Clin. Med. 41:637-647 (1953)] and the kaolin clotting time method of Proctor and Rapaport [Am. J. Clin. Path. 36:212-219 (1961)]. Platelet Factor 3 was supplied by a cephalin suspension. Maximum surface contact activation was achieved with a Celite powder. All other clotting factors (except Factor IX) were supplied by a substrate comprising plasma from a patient severely deficient in Factor IX mixed with barium sulfate adsorbed beef plasma. Quantitation of an unknown specimen was made by comparing its clotting time in the test with that achieved by dilutions of a normal standard.

REAGENTS

1. Calcium Chloride Solution. 0.05 M CaCl_2
2. Veronal Buffer
 - 2.94 gm Sodium Barbital
 - 3.67 gm. Sodium Chloride
 - 105 ml. 0.1 N Hydrochloric Acid
 - q.s. 500 ml. with distilled water
3. Celite. Analytical Filter Aid, Johns Manville.
4. Cephalin Suspension. Prepared from rabbit brain thromboplastin according to Bell and Alton [Nature 174:880-881 (1954)]. 470
5. Cephalin-Celite Reagent. Equal volumes of Cephalin Suspension diluted 1:50 in Veronal buffer at pH 7.3 $\pm$ 0.1, and Celite, 1.0 gm. suspended in 5 ml. 0.9 percent NaCl solution were mixed.
6. Diluting Fluid I (DF I)
 - 50 ml. 0.1 M Sodium Citrate
 - 300 ml. 0.15 M Sodium Chloride
7. Diluting Fluid II (DF II)
 - 20 ml. Veronal Buffer

60 ml. 0.15 M NaCl

8. Barium Sulfated Beef Plasma. Oxalated beef plasma was adsorbed with 100 mg./ml. barium sulfate and stored in small aliquots at -20°C .

9. Substrate Plasma

1 part citrated plasma from a patient severely deficient in Factor IX (stored in small aliquots at -20°C .) was mixed with 3 parts barium sulfated beef plasma.

10. Reference Standard

Lyophilized plasma reference standard No. 788-27. Assigned potency = 0.7 unit per ml.

The cephalin-celite reagent was thoroughly re-suspended before use. The substrate reagents were thawed with constant agitation in a water bath at 37°C , then mixed in a ratio of one part patient's plasma to three parts BaSO_4 beef plasma. It was then kept in a melting ice bath with all other reagents except the calcium solution, which was placed in a 37°C water bath. A standard reference plasma was diluted 1/10, 1/50, 1/100, etc., with diluting fluids I and II, according to the method Hjort, et al. [J. Lab. Clin. Med. 46:89-97 (1955)]. Unknowns were tested at suitable dilutions. One-tenth ml. of re-suspended cephalin-celite, 0.1 ml. of substrate plasma, and 0.1 ml. of diluted unknown or standard plasma were transferred to a glass tube in the 37°C water bath and a stopwatch started. The mixture was gently tilted during the three minute incubation period to keep the celite in suspension. At the end of three minutes, 0.1 ml. of the CaCl_2 solution was added and a second stopwatch started. The tube was tilted until clotting occurred. All tests were done in duplicate and the clotting times averaged.

The standard reference plasma was tested with each set of unknowns. A graph was constructed on log-log paper with clotting time on the ordinate and plasma concentrations on the abscissa. The clotting times of the various dilutions of the standard reference plasma were plotted, and the best possible line drawn through the points. The Factor IX concentrations of unknown specimens were determined by reference to this graph. The values obtained for the various concentrations of the unknown were averaged.

In-Vitro Assay of Factor II (Prothrombin)

The Factor II assay was a typical Owren One-Stage assay. The results are expressed as units per ml., one unit of prothrombin being defined as that prothrombin activity contained in one ml. of a frozen plasma standard.

In-Vitro Assay of Thromboplastin and Thrombin

Thromboplastin and thrombin were estimated simultaneously, by a typical recalcification time test. The clotting time in seconds was compared for the sample and for a control, as follows:

Sample	0.1 ml. sample (at various dilutions)
	0.1 ml. citrated plasma
	0.1 ml. 0.05 M CaCl_2
Control	0.1 ml. 0.15 M NaCl
	0.1 ml. citrated plasma
	0.1 ml. 0.05 M CaCl_2

The "sample" was tested at various dilutions with distilled water because high concentrations of neutral electrolyte will inhibit clotting due to any thromboplastin or thrombin.

ASSAY FOR ANTI-COMPLEMENT ACTIVITY

The anti-complement assay was done as follows:

Reagents:

1. CF Saline
Stock, Ca-Mg solution

10 g. CaCl₂

q.s. 100 ml. with
distilled water.

4 g. MgCl₂

Add 1 ml. stock solution of 1 liter 0.85% NaCl

2. Alsever's Solution

20.5 g. Dextrose

4.2 g. NaCl

8.0 g. Na Citrate

0.55 g. Citric Acid

9.5.1 liter with
distilled water.

3. Sheep Red Blood Cells (S-RBC)

Approximately 20 ml. cells were drawn into 40 ml. Alsever's solution. For assay, a portion of cells were washed 3 times with normal saline, then a 2 percent suspension was prepared with CF saline as diluent.

4. Hemolysin

Stock solution 1:100 was prepared using phenol as preservative.

5. Complement

Reconstituted as per directions with supplied diluent.

6. Sensitized RBC

Warmed 15-30 minutes at 37°C before use.

2 units/0.2 ml. vol. Hemolysin
2% S-RBC

equal volume

Titration

1. Hemolysin

Doubling dilutions through 1:32 of stock 1:100 solution using CF saline as diluent.

0.2 ml. hemolysin dilutions

0.2 ml. Complement (1:30 with

CF saline)

0.2 ml. 2% RBC

0.5 ml. CF saline

Incubated 30
min. at 37°C

Highest dilution of hemolysin giving complete lysis = 1 unit. Amount used in assay = 2 units/0.2 ml. vol.

Controls (incubated 30 min. at 37°C)

- (1) 0.2 ml. hemolysin (undiluted)
0.2 ml. 2% RBC
0.7 ml. CF saline

All Controls should
be
negative (no lysis)

- (2) 0.2 ml. complement (1:30)
0.2 ml. 2% RBC
0.7 ml. CF saline
- (3) 0.2 ml. 2% RBC
0.9 ml. CF saline

2. Complement

Tube No.	1	2	3	4	5	6	7	8
Complement 1:30 ml.	.12	.11	.10	.09	.08	.07	.06	.05
CF saline (ml.)	.48	.49	.50	.51	.52	.53	.54	.55

Incubate dilutions 30 min. at 37°C

Add 0.4 ml. sensitized RBC to each.

Incubate 30 min. at 37°C

Least amount C' giving complete lysis = "exact" unit.

Next highest amount = "full" unit

- 5 Amount used in assay = 2 "full" units/0.2 ml. vol.

Control (incubated 30 min. at 37°C)

- 10 0.4 ml. sensitized RBC
0.6 ml. CF saline

Should be negative
(no lysis)

Test:

- 15 0.2 ml. Sample (doubling dilutions with CF saline as diluent)
0.2 ml. CF saline
0.2 ml. C' (2 units)
Dilutions were incubated 1 hour at 37°C.
0.4 ml. sensitized RBC was added to each.
Incubated 30 min. at 37°C
End point = highest dilution sample showing at least 50 percent lysis.

Controls (incubated 30 min. at 37°C)

- 25 (1) 0.2 ml. sample
(lowest dilution used)
0.4 ml. sensitized RBC (no lysis)
0.4 ml. CF saline
Should be negative
(no lysis)
- (2) 0.2 ml. C' (2 units)
0.4 ml. sensitized RBC
0.4 ml. CF saline
Should be negative
(no lysis)
- (3) 0.4 ml. sensitized RBC
0.6 ml CF saline
Should be negative
(no lysis)

Depressor Activity

The depressor activity was measured by the Depressor Substances Test described in the USP XVII, page 843.

The results obtained by these various techniques are set forth in Table I.

TABLE I

TEST	Lot No.:	* RESULTS			
		A	B	C	D
Factor IX		480 ^a	540 ^a	500 ^a	550 ^a
Factor II		700 ^a	640 ^a	510 ^a	370 ^a
Factor VII		380 ^a	500 ^a	480 ^a	570 ^a
Factor X		1100 ^a	500 ^a	670 ^a	570 ^a
Protein		962 ^b	606 ^b	484 ^b	340 ^b
Specific Activity		0.5	0.89	1.03	1.6
pH		6.9	6.8	6.8	7.2
Na ⁺		242 ^c	237 ^c	240 ^c	237 ^c
Cl ⁻		90 ^c	94 ^c	88 ^c	87.1 ^c
Citrate (C ₆ H ₅ O ₇)=		51 ^c	48 ^c	51 ^c	50.2 ^c
Thrombin- Thromboplastin		none	none	none	none
Depressor Substances		pass	pass	pass	pass
*Expressed as values per vial, or as per unit volume when reconstituted with 20 ml.					
55 ^a —units					
^b —mg.					
^c —Eq./L.					

A batch of coagulation complex of this invention was prepared according to the process described herein. Tests showed the lot to be free of depressor activity, thrombin and thromboplastin, and anticomplement activity. The batch was stored lyophilized at ambient temperature and re-tested 3 months and 6 months later. No depressor activity, thrombin, or thromboplastin were found.

To demonstrate the stability of the complex of this invention, a large number of bottles from each of three

lots were placed at three different temperature stations and assayed at appropriate intervals. Stability data on lot PR 2240 (1.8 percent moisture), after reconstituting each bottle with 20 ml. water, is presented as an example in Table II.

TABLE II

Assay Date	Elapsed Time	Units Factor IX per ml.	5°C.	Room Temp.	40°C.
2/9/68	initial	33.6	33.6	33.6	33.6
3/19/68	5 weeks			34.6	34.6
4/19/68	9 weeks		35.4	29.0	
5/9/68	3 months	28.0			
5/20/68	3½ months			28.7	
6/19/68	4 months	34.2	32.6	32.6	
7/22/68	5 months			21.8	
8/8/68	6 months	35.0			

The potency of the complex of this invention was demonstrated by reconstituting a portion of a batch prepared according to the process described herein and assaying it for clotting factors. The results, expressed as percent of normal plasma are shown in Table III.

TABLE III

II	2550%
VII	2400%
IX	2500%
X	2350%

(Specific activity of Factor IX: 1.03 units/mg. protein)

TABLE V

Sample tested	Clotting time (secs.)
Saline (control)	135
Invention sample undiluted	180
Invention sample diluted 1:3	135
Invention sample diluted 1:10	135

The failure of invention sample to enhance the clotting time of normal human plasma is evidence of the absence of thrombin, thromboplastin substances, and the activated form of Factor X.

CLINICAL RESULTS

Human assays have been conducted with the coagulation complex of this invention with patients deficient in Factor IX and with patients deficient in Factor VII. The coagulation data from one of these human assays in a Factor IX deficient patient are presented in Tables VI and VII. During 48 hours subsequent to the administration of the product of this invention containing 2,030 units of Factor IX, there were no abnormalities in blood pressure, pulse, temperature, and respiration. Furthermore, two administrations of the coagulation complex of this invention to the same patient 13 days apart gave no evidence of sensitization or antigenicity.

TABLE VI

Dose: 290 ml. (2,030 units) started 11:10 a.m., finished 12:25 p.m.

Spec. No.	Date	Time	Elapsed time		Clotting factors (percent)			
			Hours	Minutes	II	VII	IX	X
0		11:00 a.m.	0	0	75	64	1.0	195
1		12:45 p.m.	0	20	144	165	54.2	1200
2		1:10 p.m.	0	45	136	122	49.2	1213
3		2:20 p.m.	1	55	123	83	46.0	193
4		3:30 p.m.	3	5	105	87	44.5	170
5		4:25 p.m.	4	00	105	103	48.5	176
6		5:30 p.m.	5	5	110	76	39.2	185
7		8:05 p.m.	7	40	107	75	43.5	183
8		10:30 p.m.	10	5	106	62	29.0	183
9		2:00 a.m.	13	35	94	48	24.3	125
10		3:35 a.m.	20	10	100	40	13.4	148
11		11:20 a.m.	22	55	100	33	12.1	151
12		2:30 p.m.	26	5	79	34	10.4	147
13		4:35 p.m.	28	10	101	35	10.8	132
14		10:05 a.m.	45	40	99	55	9.1	130
15		1:00 p.m.	48	35	101	51	9.6	138
16		4:30 p.m.	52	5	106	58	9.2	141
17	2/25/67	10:15 a.m.	69	50	95	37	7.0	117
18	2/26/67	11:00 a.m.	94	35	92	39	5.9	121
19	2/27/67	11:14 a.m.	118	50	85	42	3.35	97
20	2/28/67	2:10 p.m.	143	45	80	47	2.9	98

¹ Predicted factor IX=56.4%.

The complex of this invention was tested for and passed all requirements of this USP XVII assay for depressor substances. The results are shown in Table IV.

TABLE IV

Dose	Change in Mean Arterial Pressure
0.05 mcg/Kg. Histamine* i.v.	-35
0.10 mcg/Kg. Histamine* i.v.	-50
0.15 mcg/Kg. Histamine* i.v.	-55
0.10 mcg/Kg. Histamine* i.v.	-50
0.10 mcg/Kg. Histamine* i.v.	-50
Invention sample 0.2 cc/Kg. i.v.	-5
0.10 mcg/Kg. Histamine* i.v.	-40
Invention sample 0.2 cc/Kg. i.v.	-5
0.10 mcg/Kg. Histamine* i.v.	-40

(Invention sample diluted to 3.0 cc)

*(Free Base)

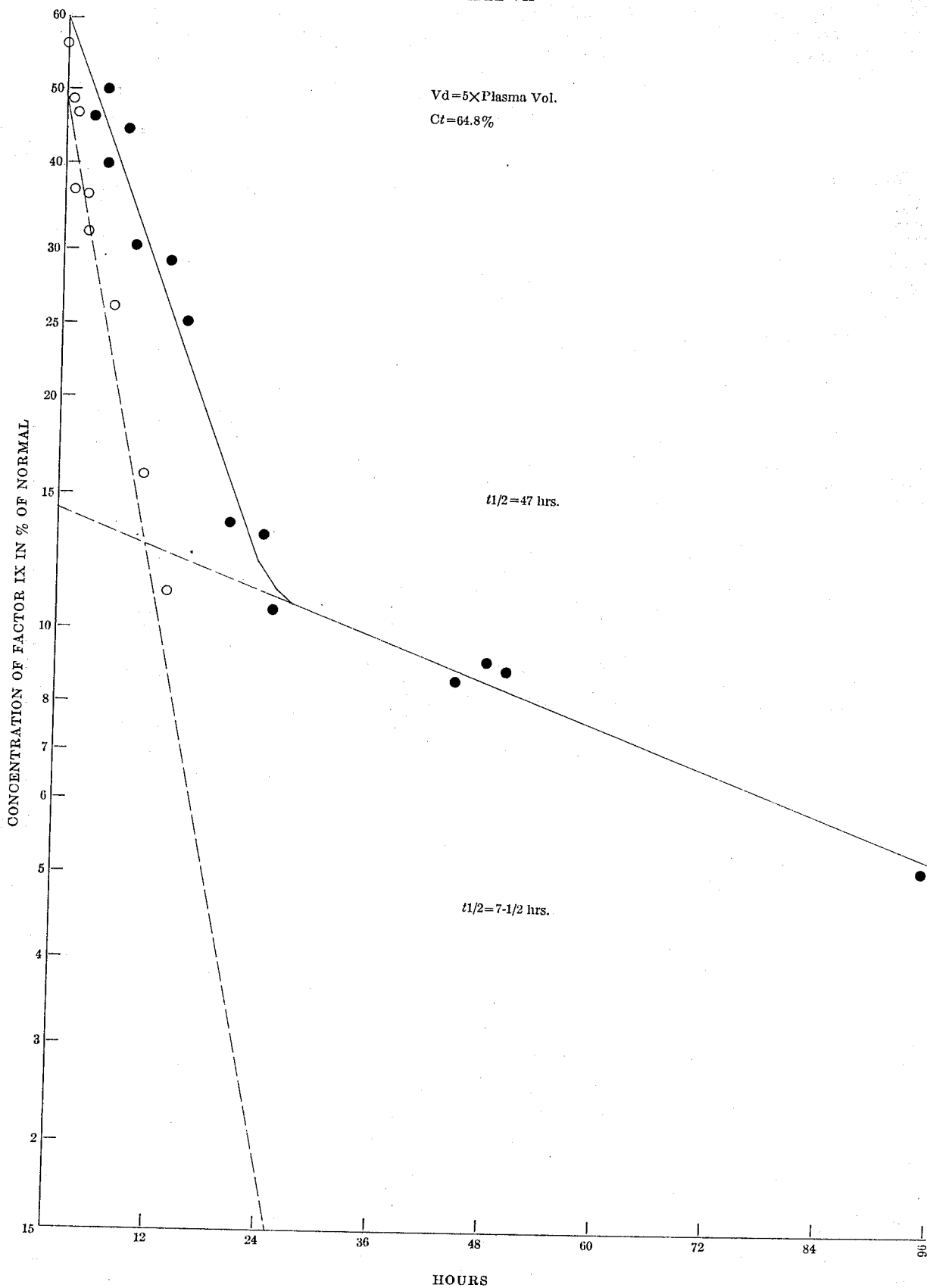
The thromboplastin-thrombin assay above described gave the following results when a sample of the complex of this invention was tested:

The post-infusion disappearance curve of Factor IX is shown on the following page. The curve is typically bi-phasic; T $\frac{1}{2}$ of the first component is 7½ hours; T $\frac{1}{2}$ of the second component is 47 hours. The post-infusion half life of the Factor IX in different patients appears to vary from about 20 hours to about 50 hours.

Reliable coagulation assays have been collected for the four coagulation components following administration of eight different lots to each of two or more patients. In those patients who would be considered fairly normal (including bleeding patients, but excluding those exhibiting fibrinolysis or widely shifting plasma volumes), the administration of one unit per pound of body weight results in approximately a 4 per cent rise in Factors II, VII, IX, and X. The post-infusion rise of Factor X is generally a little higher than that of the other three factors.

The product of this invention has been used by 14 clinical investigators in the treatment of emergency

TABLE VII

 $V_d = 5 \times \text{Plasma Vol.}$ $Ct = 64.8\%$ 

situations involving over 65 patients. These are patients who had either a permanent congenital deficiency of either Factor VII or IX or X, or who had a temporary acquired deficiency of all four factors (II, VII, IX and X); and who were either highly reactive to plasma or could not tolerate the volume of plasma which would be required; and who were either suffering a serious bleeding episode or who required protection while undergoing major emergency surgery.

The mode of administering the product of this invention will be readily known by physicians. Very generally the lyophilized product is reconstituted with water for injection to make an isotonic solution which is injected intravenously to the patient.

The disclosures and description herein are purely illustrative and are not intended to be in any sense limiting to change that might suggest themselves to those skilled in the art without departing from the spirit and scope of the invention.

What is claimed is:

1. A sterile lyophilized storage stable intravenous injectable concentrate of citrated human plasma for controlling bleeding in hemophilia, free of heparin, thrombin, the activated form of Factor X, depressor activity and anticomplement activity, and containing the coagulation Factors II, VII, IX, X in non-activated form, in proportions substantially the same as in human plasma, and having a Factor IX reconstituted potency of at least about 2,000 percent that of normal plasma and a Factor IX specific activity of at least about 0.5 clinical unit per milligram protein and a Factor IX post-infusion biological half-life of about 20-50 hours.

2. A plasma concentrate according to claim 1 having a Factor IX specific activity of at least about 0.9 clinical

cal units per mg. protein.

3. A mixture of a plasma concentrate according to claim 1 and a NaCl-citrate electrolyte buffer.

4. A process for the production of a storage stable plasma concentrate of the mixture of the Factors II, VII, IX and X coagulation components of human plasma in non-activated form, free of heparin, thrombin, the activated form of Factor X, depressor activity and anticomplement activity, which comprises the steps of applying Cohn Supernatant I, Method 6 from unmodified citrated human plasma onto an ion exchange resin consisting essentially of cross-linked dextran chains with diethylaminoethyl groups attached by ether linkages to the glucose units of the polysaccharide chains, and adsorbing thereon said coagulation components of the plasma; selectively eluting the column with ammonium bicarbonate solution of a pH of from about 7.3 to about 8.2 and increasing molarity; separating the eluate fraction containing coagulation components; freezing the separated eluate fraction and removing the ammonium bicarbonate therefrom by lyophilizing the frozen fraction.

5. The process of claim 4 wherein the ammonium bicarbonate solution eluting the fraction containing the coagulation activity is about 0.75 M.

6. The process of claim 4 wherein the resin containing the adsorbed coagulation components is washed free of ceruloplasmin with ammonium bicarbonate solution of up to 0.3 molarity and the coagulation components are eluted with ammonium bicarbonate solution of about 0.75 M until the Factor IX is eluted.

7. The process according to claim 6 wherein the eluate fraction containing coagulation activity is eluted at a pH of about 7.6-7.8.

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