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Methods for the Production of Clinically Effective Intermediate- and High-Purity Factor-VIII Concentrates

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SUMMARY. An intermediate- and a high-purity factor-VIII concentrate for clinical use have been prepared on a large scale by cryoethanol precipitation, extraction of the precipitate with tris buffer, and fractionation with polyethylene glycol. With bench-scale fractionation, the intermediate material is 22-fold purified on the average and the mean yield is 63%, while the high-purity factor VIII is 274-fold purified with a mean yield of 62%. With fractionation of 100 l. or more of fresh frozen plasma, the intermediate material shows a 30% yield and 14–30-fold purification; the high-purity factor VIII shows an 18% yield and 125–350-fold purification using 5.8 g/100 ml polyethylene glycol (PEG). A yield of nearly 30% should be possible with PEG, 4–5 g/100 ml. Both factor-VIII preparations are stable for over a year in the lyophilized state at 4°C. Other plasma proteins can be fractionated from the residual plasma by routine Cohn procedures.

Early attempts to isolate factor VIII from normal human plasma for clinical use in haemophiliacs were only partially successful (Pohle & Taylor, 1937; Lozner *et al*, 1939; Lozner & Taylor, 1939). Shortly thereafter, the classical Cohn method for ethanol fractionation of human plasma (method 6) was introduced (Cohn *et al*, 1946). Cohn Fraction 1, made by this procedure, contained about 5-fold purified factor VIII as well as fibrinogen (Edsall *et al*, 1944; Minot *et al*, 1945; Van Creveld & Mochtar, 1962).

More potent, clinically useful, 7–20-fold purified preparations were produced by various modifications of the ethanol fractionation procedure, including extraction with glycine-ethanol-citrate solution (Table I) on a small scale (Blombäck & Blombäck, 1956; Blombäck *et al*, 1960). A large-scale preparation was described by McMillan *et al* (1961). Also introduced were precipitation with 3% ethanol (Magnussen, 1963), and ethanol precipitation from plasma containing ε-aminocaproic acid (EACA) and glycine (Johnson *et al*, 1964a). The latter method was used by the American National Red Cross (ANRC) for the preparation of factor VIII referred to in Table I as ANRC Low-Purity (EACA-glycine).

Ethyl ether was used in place of ethanol at the Lister Institute of Preventive Medicine, Elstree, England, to prepare 10–17-fold purified factor VIII (Kekwick *et al*, 1946; Kekwick & Mackay, 1954; Kekwick & Wolf, 1957). Phosphate-citrate buffers were used to fractionate 30–50-fold purified bovine and porcine concentrates containing 5000–8000 factor-VIII u/g protein (Bidwell, 1955a, b) later made commercially.

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An intermediate-purity factor-VIII concentrate (about 20-fold purified) prepared by glycine precipitation (Webster *et al*, 1965) was commercially available until superseded by a concentrate of higher purity. Cryoprecipitation of factor VIII from single units of fresh-frozen plasma is a simple, practical procedure that can be carried out by any blood bank (Pool *et al*, 1964; Pool & Shannon, 1965). The resulting material, which is 6–25-fold purified (Table I), has proved useful in many countries.

TABLE I. Purification, yield and batch size of ANRC* routine preparations of factor VIII

Factor-VIII preparation	*Times purified*	*% yield*	*Batch size and source material*
Fresh-frozen plasma (FFP)†	1	80	1 donation
ANRC-Blombäck method (low-purity, 1961–65)	7–20	30	Small, FP only‡
ANRC low-purity (EACA-Glycine, 1962–65)	7–15	30	Medium, FFP
Cryoprecipitate (Pool)	6–25	35 (60§)	1 donation FFP
ANRC intermediate-purity (ethanol-cryoprecipitated, 1965 onwards)	14–30 (22§)	30 (63§)	Large, FFP
ANRC high-purity (precipitated with PEG, 1965 onwards)	125–350‖ (274§)	18¶ (62§)	Large, FFP
ANRC very-high-purity (reprecipitated with PEG, 1967 onwards)	800	—	Large, FFP

* ANRC = American National Red Cross.
† Fresh-frozen plasma (FFP) factor VIII *c* 0.8 u/ml.
‡ Fresh plasma (FP) factor VIII *c* 1.0 u/ml.
§ Bench-scale preparation (mean).
‖ Soluble in 2.5 ml but usually reconstituted in 8.0 ml.
¶ Prepared with 5.8% PEG (see Table VII).

Concentrates have been prepared by fractionation with a variety of different materials: 33-fold purified factor VIII with aliphatic amino acids (Wagner *et al*, 1964); 40–100-fold purified concentrate with tannin (Simonetti *et al*, 1961); 200-fold purified material, with Fuller's earth and ethanol (Wagner *et al*, 1957). Material over 1000-fold purified was prepared by chromatography on ECTEOLA or DEAE cellulose (Van Creveld *et al*, 1959; Michael & Tunnah, 1963, 1966). Unfortunately, most of these preparations are relatively impure, or toxic, or have a very short half-disappearance time *in vivo*, or the methods are difficult to reproduce.

Earlier reports from this laboratory (Johnson *et al*, 1966a, b, 1967, 1969) showed that clinically effective intermediate-purity concentrate (14–30-fold purified—Table I) can be produced by simultaneous ethanol- and cryo-precipitation of factor VIII from the melting fresh-frozen plasma followed by extraction of the precipitate with tris buffer and adsorption of factors II, VII, IX and X on aluminium hydroxide (Fig 1). This material is remarkably soluble and stable, and can be made on a large scale.

Albertsson (1960) first used polyethylene glycol (PEG) (Union Carbide Co., Tarrytown, N.Y.) in a two-phase system to separate macromolecules and showed that its effect depended primarily on the molecular weight and concentration of the protein. He also noted the importance of pH in the separation of plasma proteins in these systems. Later experiments by

others showed that PEG was effective in the fractionation of plasma proteins, particularly fibrinogen, gamma-globulin and albumin (Polsen *et al*, 1964; Chun *et al*, 1967).

Additional studies in our laboratory (Johnson *et al*, 1970) indicated that the precipitation of our intermediate-purity concentrate with PEG of 4000 or 6000 mol wt (Fig 1) resulted in a

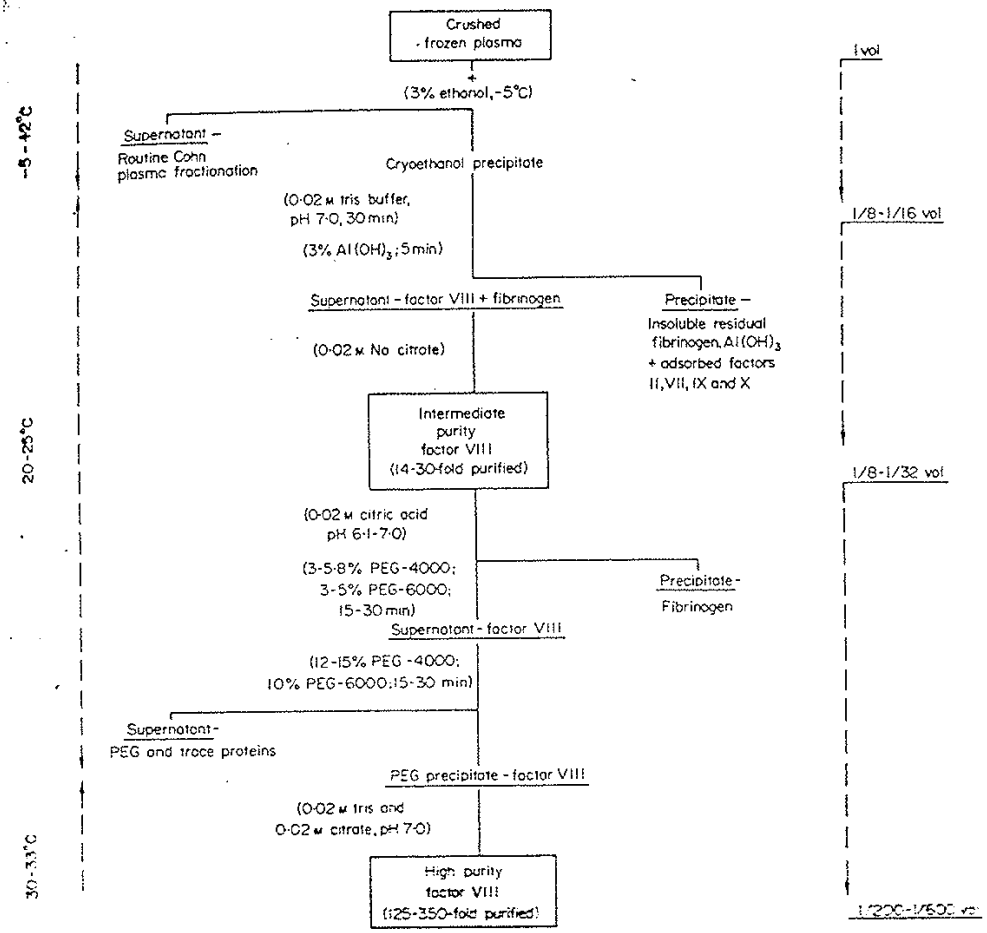

Fig 1. Fractionation of intermediate- and high-purity factor VIII from fresh-frozen plasma. PEG: polyethylene glycol.

concentrate purified 125–350-fold (Table I) which can be prepared in large amounts and has proved effective in the treatment of haemophiliacs (Johnson *et al*, 1971). This high-purity concentrate also serves as starting material for an ultra-high-purity concentrate (purified about 10 000-fold) for biochemical studies (Johnson *et al*, 1967). Others subsequently confirmed the practicability of the PEG step in factor-VIII fractionation, using it to produce material purified approximately 150-fold which is also concentrated with glycine (Brinkhous *et al*,

1968). Similar material is now available commercially (Hemophil; Hyland Laboratories, Los Angeles, Calif.).

The present communication will be concerned with detailed methods for bench- and plant-scale preparation of both the intermediate- and the high-purity concentrates.

MATERIALS AND METHODS

Plasma and Other Starting Materials for Fractionation

Whole blood, 450 ml, was collected from normal donors into a double plastic bag containing acid-citrate-dextrose, formula A (ACD-A) as anticoagulant, 67.5 ml (ACD-A: monohydrated citric acid, 0.8 g; dihydrated trisodium citrate, 2.2 g; monohydrated dextrose, 2.45 g; water to 100 ml; Mollison, 1967). The collection time was limited to 8 min. The blood was centrifuged in the cold at 5000 *g* as soon as possible after collection. The plasma was expressed into a satellite bag, immediately frozen in dry ice, and stored at $-30°C$. (If immediate centrifugation is not feasible, the blood may be kept at 4°C for at least 4–6 hr. We have not yet determined the maximal storage period at 4°C consistent with a good yield.)

Frozen plasma can be stored approximately 1–3 mth without perceptible changes, but after that time factor VIII and fibrinogen tend to form large aggregates which probably cause some of each to precipitate prematurely during fractionation and interfere with Millipore filtration, resulting in a lower yield of the final product. Plasma frozen for more than 2–3 mth will be called 'slightly denatured' with regard to the behaviour of the factor VIII and fibrinogen during the fractionation procedure. This term will also be applied to fresh-frozen, intermediate-purity concentrate stored for more than 1 week in the frozen state or lyophilized, since either preparation undergoes changes which affect fractionation. As described below, this type of starting material may require modification of the fractionation method.

The term 'moderately denatured' will refer to the factor VIII and fibrinogen in frozen cryoprecipitate prepared from large volumes of plasma during commercial fractionation. This material has a greater tendency to precipitate prematurely during fractionation than the slightly denatured plasma and therefore requires extensive modification of the fractionation method.

Observations and results relating to large-scale fractionation refer to factor VIII prepared under the direction of Mr Clayton Bloom by the Blood Products Division of E. R. Squibb and Sons from batches of 40 l. or more of plasma.

Polyethylene glycol. PEG of 4000 or 6000 mol wt was supplied by Union Carbide Co., Chemicals Division, Tarrytown, New York, U.S.A.

Assay Methods and Tests of Purity

Protein assay. The protein content was assayed by the biuret method (Layne, 1957), and also calculated from the nitrogen contents as determined by gas chromatography (Carbon-Hydrogen-Nitrogen Analyzer, Model 185, F and M Scientific Co., Avondale, Pa.). The standards for nitrogen were: (1) cystine, N = 11.66% (No. 143b, U.S. Department of Commerce, National Bureau of Standards Reference Materials), and (2) crystallized bovine plasma albumin, N = 15.8% (Lot No. A 70112, Armour Pharmaceutical Co., Kankakee, Ill.).

Fibrinogen assay. The fibrinogen content (clottable protein) was assayed by a method previously described (Johnson *et al*, 1964b).

Antihaemophilic factor assays. One unit of factor VIII is equivalent to the average factor-VIII activity in 1 ml of pooled, fresh, normal, male plasma obtained from whole blood anticoagulated with ACD-A in the proportion 9 vol to 1 vol. Factor-VIII activity has been measured by three methods (Table II). In the first, a two-stage procedure based on the thromboplastin generation test (TGT) described by Biggs *et al* (1955), five dilutions each of the sample and control were prepared and minimal clotting times, based on actual experiments to determine the optimal incubation times, were recorded for each dilution. The factor IX in dilute human serum was activated with a celite system or by purified contact factor as a source of activated XI (XIa) (Nossel, 1964). Factor V was obtained from fresh-frozen,

TABLE II. Methods used for assaying factor VIII

Assay method	*Thromboplastin generation mixture (TGM)*	*Incubation time and temperature*	*Assay mixture*
Modified two-stage*	0.1 ml haemolysate 0.1 ml $Al(OH)_3$-adsorbed VIII-deficient plasma 0.1 ml human serum 0.1 ml sample dilution 0.1 ml 0.025 M-$CaCl_2$	37°C; 8–25 min	0.1 ml TGM 0.1 ml 0.025 M $CaCl_2$ 0.1 ml substrate plasma
Division of Biologics Standards (National Institutes of Health) two-stage†	0.1 ml inosithin 0.1 ml human serum 0.1 ml bovine serum 0.1 ml 0.025 M-$CaCl_2$	37°C; 5 min	0.1 ml TGM 0.1 ml 0.025 M-$CaCl_2$ 0.1 ml substrate plasma
Modified partial thromboplastin time one-stage†	0.1 ml platelin + contact factor§ 0.1 ml sample dilution 0.1 ml VIII-deficient plasma	37°C; no pre-incubation	0.3 ml TGM 0.1 ml 0.05 M-$CaCl_2$

* End-point observed visually.
† End-point monitored with Fibrometer.
§ Platelin reconstituted with diluted purified contact factor.

deprothrombinized, factor-VIII deficient plasma (McMillan *et al*, 1961). The saline-diluted partial thromboplastin (phospholipid) consisted of washed, lysed, human red blood cells, and the major buffer was 0.05 M imidazole. This system appears to give accurate assays for plasma and intermediate-purity factor VIII, as judged by *in vivo* recovery studies, but the values for high-purity factor VIII are abnormally high.

Another TGT procedure was used in which factors IX, X and XI were obtained from dilute human serum and factor V from bovine serum (Ware & Seegers, 1948; Pool & Robinson, 1959; D. L. Aronson, personal communication). Inosithin, a soybean lipid (Associated Concentrates, Woodside, N.Y.), was used as the partial thromboplastin, and 0.05 M imidazole-buffered saline (0.065 M-NaCl) as buffer. Five minutes was arbitrarily selected as a single pre-

incubation or thromboplastin generation time with this procedure, since most of the factor-VIII activation was complete within that period.

Although the second TGT was the method of choice, it was generally used with a third method, a modified partial thromboplastin time (PTT), both types of assay being used to counter-check the activity of the high-purity factor VIII. In the PTT assay, which has long been in clinical use (Langdell *et al*, 1953; Hardisty & MacPherson, 1962; Ingram, 1964; Simone *et al*, 1967), the factor IX was activated with purified human contact factor, the phospholipid generally employed was Platelin (Warner-Chilcott Laboratories, Morris Plains, N.J.). The buffer was made by dissolving 1.4714 g of sodium diethylbarbiturate and 0.9714 g of sodium acetate trihydrate in 50 ml of water, then adding 50 ml of 0.1 N-HCl and 900 ml of 0.145 M-NaCl to a final concentration of 0.007 M diethylbarbiturate and 0.007 M acetate, at 0.18 ionic strength. The second and third assay methods were adapted for use with a Fibrometer (Bioquist Division of Becton Dickinson Co.) to permit more objective reading of the end point. To our surprise, this equipment or equivalent was found to be essential for optimally reproducible results.

We assayed one reference standard twice daily, a house standard made by suitable dilutions of ANRC intermediate-purity factor VIII (Lot 564) calibrated to match the activity of the pooled plasma standard of the NIH Division of Biologics Standards (DBS); we also assayed the DBS standard itself once daily.

Acrylamide-gel electrophoresis. Preparations containing factor VIII were subjected to disc electrophoresis on acrylamide gel (Ornstein, 1964; Davies, 1964) in a Canalco disc electrophoresis apparatus (Canal Industries, Bethesda, Md). Discontinuous electrophoresis was run at pH 9.5 (Williams & Reisfeld, 1964) for 2 hr at 2.5 mA per tube, using 5% acrylamide gel. The gels were stained with amido black 10B (Fisher Scientific Co., New York, N.Y.).

Preparation of Intermediate-Purity Factor VIII

Ethanol-cryoprecipitation of plasma. The plastic satellite bag containing fresh-frozen human plasma is softened by storage at − 5 to 0°C for 4–8 hr, or in a water bath at 30°C for 3–5 min. For small-scale production (1–40 l.), the softened plasma is crushed in the bag with a plastic mallet, and the resulting slush is transferred to a stainless-steel container immersed in a water bath at 0°C. For large-scale production (40–400 l.), the frozen plasma may be crushed mechanically in an ice crusher and transferred to a fully jacketed stainless-steel tank. Immediately thereafter, ice-cold ethanol, 53.3%, is added over a 15-min period, with gentle mixing of the crushed plasma, to a final ethanol concentration of 3%.

The addition of about 0.05 ml of capryl alcohol per litre of plasma prevents the formation of foam. When bench-scale batches of frozen plasma are processed, melting is facilitated by directing a stream of warm air over the surface. With moderate volumes of plasma (20–100 l.), fluid at 6°C is circulated through a stainless-steel coil heat-exchanger. The coil is repositioned at intervals to maintain maximal contact with the floating, unmelted layer and prevent dissolution of the cryoprecipitate by further warming of the already melted plasma. With volumes over 100 l., fluid prewarmed to 20–30°C is circulated through the jacket of the tank while the plasma is mixed gently with a stainless-steel stirrer at 20–40 rpm. In either procedure the temperature of the melted plasma should not exceed 2°C. In bench-scale fractionation, the precipitate is collected by centrifugation with a swinging bucket centrifuge at

5000 *g*. In the large-scale method, the fine factor-VIII-containing precipitate which has been passed through a colander is separated by two sequential centrifugations in a Sharples centrifuge (Model EY16, Pennsalt Chemicals Corp.) at 30 l.hr (using a No. 0.093 injection nozzle expanded to $\frac{1}{8}$ in.) and added to the precipitate previously collected in the colander. The supernatant plasma is refrozen pending further fractionation by the Cohn method.

Extraction with tris buffer solution. The white, tenacious ethanol cryoprecipitate may be washed with 8% ethanol at −5°C to obtain a 15–22% increase in purity at this stage (Table III). Since factor VIII obtained by the cryoethanol method may precipitate in the cold but is relatively stable at room temperature, later fractionation steps are carried out at this temperature. The precipitate is extracted gently with a Vibromixer (Model E1, Chemapex Inc., Hoboken, N.J.) for 30 min at 20°C with 125 ml of 0.02 M tris solution, pH 7.0, for each litre of starting plasma.

TABLE III. Comparative purity and yield of intermediate-purity factor VIII prepared on a large- and a bench-scale with and without washing of cryoethanol precipitate

	Factor-VIII activity of starting plasma used for calculation of yield and purity	*No. of batches*	*Times purified (mean ± SD)*	*% yield (mean ± SD)*
Washed bench-scale (Laboratory)	1.0 u/ml assumed	40	22 ± 8	63 ± 12
Washed large-scale (Commercial)	1.0 u/ml assumed	57	20 ± 10	32 ± 16
Unwashed large-scale (Commercial)	1.0 u/ml assumed	30	17 ± 5	34 ± 12
Unwashed (Commercial)	As found by assay	25	17 ± 10	42 ± 21

All cryoethanol precipitates were extracted with 0.02 M tris buffer, pH 7.0. Where precipitates were washed, the wash solution was 6% ethanol in 0.02 M tris buffer, pH 7.0, at 0°C.

Treatment with $Al(OH)_3$ gel. The residual precipitate, which contains many proteins including fibrinogen, is removed by passage through a single layer of nylon cloth (mesh count 134 × 143, Schwartzenbach Huben Co.). The factor-VIII-rich supernatant is adsorbed with 30 ml of $Al(OH)_3$ gel per litre of tris supernatant. After mixing for 5 min, the supernatant is centrifuged in a Sharples centrifuge with an $\frac{1}{8}$ in. nozzle to remove the $Al(OH)_3$ gel with absorbed proteins, including plasma coagulation factors II, VII, IX and X, and the remaining undissolved precipitate. It is then filtered through a microfibre glass disc prefilter (type AP20, Millipore Corp.) to remove any remaining $Al(OH)_3$ gel particles, and 0.5 M trisodium citrate is added to a final concentration of 0.02 M after this step. At this time the citrated factor VIII may be further purified (see below) or sterilized by Millipore filtration and lyophilized for clinical use (intermediate-purity factor VIII).

Preparation of High-Purity Factor VIII

Purification with PEG-4000 or PEG-6000. The citrated factor VIII from the previous stage

is used without sterilization. Before the PEG is added, the tris-extracted factor-VIII supernatant is acidified by slowly adding 0.02 M citric acid to a final pH of 6.1. In the pH range 6.0–6.5, a final concentration of PEG-6000, 3–4 g/100 ml, or of PEG-4000, 4–5 g/100 ml, precipitates most of the fibrinogen without removing more than 20% of the factor VIII. The resulting large flocculent precipitate is easily removed from the factor-VIII-rich supernatant by centrifugation at not less than 5000 *g* for 5 min.

Concentration of factor VIII by PEG. The previous PEG concentration of the supernatant (3–5 g/100 ml) is raised to 10 g/100 ml (with PEG-6000) or 12 g/100 ml (with PEG-4000) to precipitate and concentrate the factor VIII and other residual proteins, and the mixture is centrifuged for 10 min at 6000 *g*. The supernatant is discarded. In the bench procedure, some of the PEG remaining on the walls of the centrifuge cup is removed manually with sterile gauze; most of the remainder and that which is on top of the precipitate is removed by one or two washes with 8% ethanol-tris solution at – 5°C or lower. However, washing must not be vigorous enough to agitate the precipitate or dislodge it from the container walls. The washed precipitate may be subsequently redissolved in approximately 1/200th or less of the original plasma volume with 0.02 M tris–0.02 M citrate buffer, pH 7.0; it is brought to 30°C and sterile-filtered through a Millipore sandwich filter or separate filters, consisting of a prefilter and 8.0, 3.0, 1.2, 0.45 and 0.22 μ pore size filters. The factor-VIII solution is then immediately shell frozen and lyophilized.

RESULTS

Ethanol-Cryoprecipitation and Tris Extraction

Using Pool's cryoprecipitation method (Pool *et al*, 1964) for not less than 5 l. of pooled plasma (pilot-plant scale), we obtained a concentrate with a yield of about 35% of the factor-VIII activity in the starting plasma. The addition of 3% ethanol raised the yield to about 70% (Fig 2). The yield of material prepared by commercial-scale cryoprecipitation (about 1000 l.)

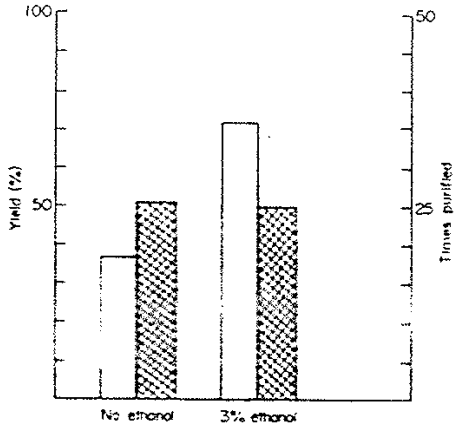

FIG 2. Yield and purity of intermediate-purity factor VIII prepared by bench-scale cryoprecipitation of plasma thawed at 1°C with and without added ethanol. Open column shows yield; hatched column shows times purified.

was only 15% without ethanol but nearly 30% with it. After this step, some contaminating proteins may be removed by washing the ethanol cryoprecipitate with 8% ethanol in 0.02 M tris, pH 7.0, at −5°C—a desirable step if the intermediate-purity concentrate is intended for clinical use in the United States and possibly other countries where it must meet specific purification requirements.

Although the intermediate-purity concentrate was initially made by dissolving the cryo-ethanol precipitate in physiological saline, it soon became evident that further purification, solubility and stability could be achieved by extracting the factor VIII. After testing various solutions and buffers for the extraction step, e.g. tris, imidazole, saline, distilled water, glycine and glycine with citrate, it was found that factor VIII of optimal yield and purity was obtained with low-ionic-strength tris (Table IV).

TABLE IV. Effect of various buffers used in bench-scale extraction of cryoethanol precipitate on the purity and yield of intermediate-purity factor VIII

Buffer	*% yield*	*Factor VIII (u/mg protein)*	*Times purified*
0.02 M tris	77	1.14	76
0.05 M sodium citrate + 0.01 M glycine	67	0.19	13
0.01 M-NaCl	57	1.16	82
0.02 M imidazole	48	1.07	55
H_2O	46	0.38	27
0.02 M glycine	32	0.36	20
0.02 M-NaCl	28	1.08	63

The extraction was generally performed at pH 7.0.

TABLE V. Effect of temperature during extraction on the yield and specific activity of intermediate-purity factor VIII

No. of expts.	*Extraction temp. (°C)*	*Factor VIII yield (%)*	*Specific activity (Factor VIII u/mg protein)*	*Times purified*
5	15	22	0.17	12
5	20	54	0.33	23
5	25	47	0.54	38

The optimal temperature for extraction of the cryoprecipitate from fresh-frozen plasma was 25°C (Table V). However, 20°C proved more suitable for processing partially denatured starting materials such as frozen cryoprecipitate or fresh-frozen plasma stored over 2 mth. The lower temperature permitted extraction of 50–75% of the undenatured factor VIII and fibrinogen while preventing solubilization of most of the moderately denatured, highly aggregated factor VIII and fibrinogen. Although it was possible to fractionate those less desirable, partly denatured starting materials, the factor-VIII yield of the final product was reduced.

The optimal extraction time appeared to be 30 min with use of fresh-frozen plasma an 0.02 M tris at 25°C at a given setting of the Vibromixer. A longer extraction period resulte in no higher a yield of factor VIII which, in fact, actually dropped in some instances. More over, the concentrate contained increased amounts of contaminating proteins (Johnson *et a* 1970).

Plasma coagulation factors II, VII, IX and X were adsorbed with $Al(OH)_3$ gel, to stabiliz the final product by preventing the formation of minute amounts of thrombin that migh labilize the factor VIII or clot the fibrinogen. However, we attempted to remove from the ex tract no more than 95–97% of these factors (based upon the total amount normally present i plasma), since the gel also adsorbs the factor VIII when present in a high enough concentra tion to remove them completely. The gel is added to the citrate-free extraction buffer tc prevent solubilization of the aluminium as aluminium citrate. Citrate, which stabilizes factor VIII (Hynes *et al*, 1969) and fibrinogen, is added after the $Al(OH)_3$ step, for the same reason. Lyophilized intermediate-purity concentrate in this form is stable for more than 1 yr at 4°C (Table VI).

TABLE VI. Stability studies on intermediate-purity factor VIII stored at 4°C and 25°C

Storage temp.	*No. of lots*	*Months stored (mean and range)*	*Factor VIII (u/ml) (mean ± SD and range)*	
			Initial	*Final*
4°C	6	16 (11–18)	2.49 + 0.68 (1.54–3.30)	3.06 ± 0.57 (2.34–3.70)
4°C and 25°C*	10	16 (14–18)	2.68 ± 0.71 (1.50–3.78)	2.25 ± 0.61 (1.20–2.94)

The differences between initial and final factor-VIII activity were not significant in either condition of storage.

* Temperature was maintained at 4°C for about three-quarters of storage period, and at 25°C for remaining time.

The intermediate material made in our laboratory by cryoethanol precipitation, ethanol wash, tris extraction and $Al(OH)_3$ adsorption showed a mean purification of 22-fold, and the mean yield was about 63% when calculated against a theoretical value of 1.0 u factor VIII/ml for the starting plasma (Table III). Factor VIII made by a similar fractionation method in the Blood Products Division of E. R. Squibb and Sons in batches of over 40 l. was purified about 20-fold and the mean yield was about 32% when the starting plasma was assigned a theoretical value of 1.0 u factor VIII/ml. This disparity in yield is partially due to the larger scale of the procedure, and partly to the fact that the assigned 1.0 u/ml is considerably higher than the actual value. The mean yield *in vitro* for the lots was 42% as compared with 32% and 34% on the basis of a theoretical factor-VIII activity of 1.0 u/ml (Table III). When the same fractionation method was used on the large scale without the cryoethanol precipitate washing step, the concentrate was purified 17.4-fold.

Actual factor-VIII levels of the starting plasma immediately before fractionation were obtained for 25 lots of intermediate-purity concentrate made by the large-scale procedure from 1966 to 1968. This was done by melting the frozen plasma in the tubes connecting each main blood collection bag to its satellite bag and pooling equal volumes for assay. Assay of

factor-VIII levels in frozen plasma used for large-scale fractionation over a 3-yr period (31 pools) revealed activity of 0.39–1.62 u/ml with a mean of 0.8 u/ml.

First PEG Precipitations

PEG may be added as a concentrated aqueous solution or as dry flakes. There is little increase in volume with the dry flakes, an advantage in large-scale production, but the flakes require at least 15 min of vigorous mixing to dissolve completely.

The hydrogen ion concentration is an important variable in the selective precipitation of plasma proteins by PEG (Albertsson, 1960; Polson *et al*, 1964). Thus, more fibrinogen and other contaminating proteins were selectively precipitated from the tris-extracted factor-VIII supernatant as the pH was progressively lowered from 7.5 to 6.0 with citric acid, resulting in significantly higher purification (Table VII). The citrate buffer provides the needed H^+

TABLE VII. Effect of pH variation on yield and purity of high-purity factor VIII fractionated from intermediate-purity (20-fold) factor VIII with PEG-4000

pH	*Factor-VIII yield (%)*	*Times purified*
6.0	80	440
6.5	70	280
7.0	60	220
7.5	20	80

ions as well as greater pH control. A pH of 6.1–6.2 was considered optimal when the starting material was undenatured fresh-frozen plasma, or intermediate-purity concentrate which had become denatured during long-term storage in the frozen state or after lyophilization, and a higher pH is essential with moderately denatured frozen cryoprecipitate. These materials may also become slightly denatured by slow freezing of the cryoethanol precipitate, or allowing it to aggregate too long in the cold (more than 4–6 hr) before tris extraction. Excessive denaturation causes the fibrinogen and factor VIII to precipitate below pH 6.3 and at unusually low PEG concentrations, with a resulting decrease in yield during this step. The optimal pH with undenatured fresh-frozen plasma as the starting material was 6.1, compared with pH 6.5–7.0 for moderately denatured material.

Several polyethylene glycols in the range of mol wt 400–6000 were tested in factor-VIII fractionation; the PEG concentration required for optimal purification varied inversely with the molecular weight (Fig 3). Under the particular test conditions, the purification appeared to be higher with PEG-4000, but over 5.2% of the polymer resulted in a decrease in yield (Fig 4).

When PEG-4000 was used in the optimal concentration of 4–5 g/100 ml non-denatured, fresh-frozen plasma as starting material (Fig 4), the factor VIII had an overall yield of 62% and was purified 274-fold on the average (Table VIII). The data in Fig 4 indicate that maximal purification with a high yield resulted from use of PEG 5 g/100 ml with either fresh-frozen or slightly denatured plasma. The high purification obtained when PEG 4 g/100 ml was used with plasma was attributed to the high proportion of 'old' plasma fractionated, i.e. stored

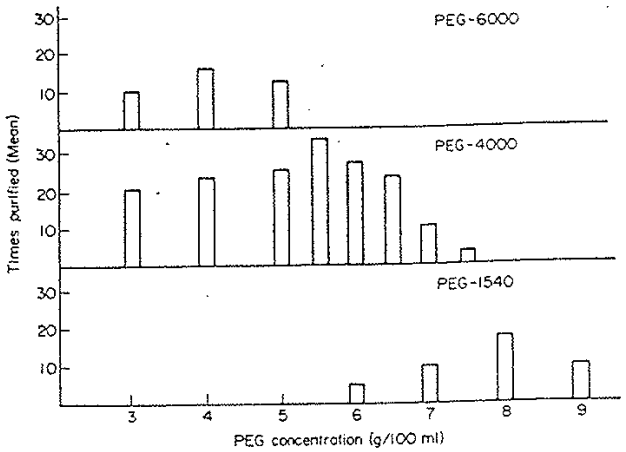

FIG 3. Additional purification of high-purity factor VIII produced by bench-scale fractionation of intermediate-purity concentrate using PEG of different molecular weights and various concentrations. Final purification = purification of intermediate material (22-fold) multiplied by additional purification obtained with PEG step.

more than 2 mth. With slightly denatured intermediate-purity concentrate as starting material, the yield was in some instances slightly decreased but the purity was moderately increased.

When the degree of purification of the high-purity concentrate made from fresh-frozen plasma was plotted against that of the intermediate-purity concentrate used in its preparation (100 pairs), the data indicated a definite relationship between the two ($r = 0.61$, $P < 0.001$; Fig 5). It should be emphasized that this correlation was found despite the varying levels of PEG (3–5 g/100 ml) used in the fractionation procedure, and appears to minimize the importance of the concentration of PEG used in the fractionation of fresh-frozen plasma. As shown by Fig 4, however, the purity of the high-purity material was far more dependent upon the PEG concentration when the starting material was a slightly denatured, lyophilized, intermediate-purity concentrate.

The first PEG precipitate consists mainly of partially purified fibrinogen and is excellent as a starting material for very-high-purity fibrinogen.

Factor-VIII Concentration by PEG

As indicated, PEG-6000 10 g/100 ml or PEG-4000 12 g/100 ml was used in the next step to precipitate and concentrate the factor VIII. The precipitate and container were washed in a solution of 8% ethanol and 0.02 M tris at −5°C (PEG, unlike factor VIII, is readily soluble in ethanol). With PEG of increasing mol wt and concentration, the solution becomes more viscous, making the polymer more difficult to remove. The lowest effective mol wt and concentration should therefore be used (Fig 3). A low pH also seems important for maximal recovery of factor VIII in the second PEG precipitation (Table VIII).

The average purification for 94 batches of high-purity factor VIII made by the bench procedure was 274-fold (Table VIII) and about 248 fold for 29 batches made by the large-scale procedure. Using sufficient PEG to obtain optimal purification in the first step (4–5

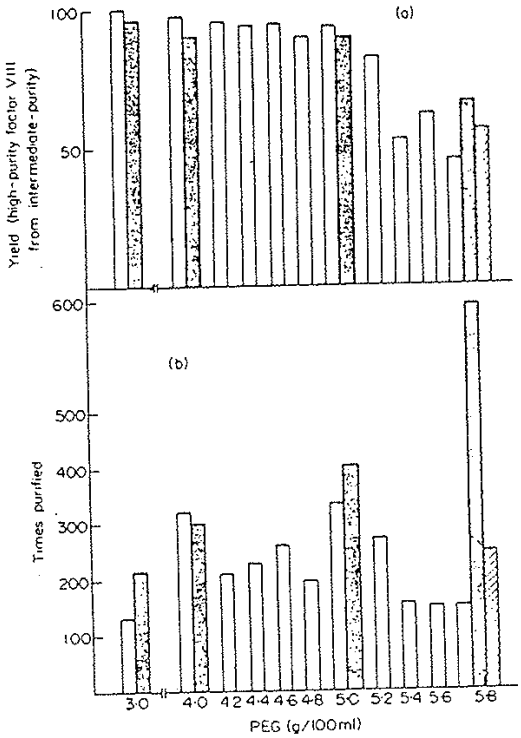

FIG 4. (a) Relation between % yield of high purity factor VIII and concentration of PEG used in fractionation (mean values for 150 fractionations). High-purity factor VIII prepared from fresh-frozen plasma shown in clear columns; from lyophilized intermediate-purity material in solid columns; and large-scale preparation from fresh-frozen plasma in hatched columns. (b) Relation between purification of high-purity factor VIII and the PEG concentration used in fractionation (mean values for 150 fractionations). High-purity factor VIII prepared from fresh-frozen plasma shown in clear columns; from lyophilized intermediate-purity material in solid columns; and large-scale preparation from fresh-frozen plasma in hatched columns. The high purification obtained when 4 g/100 ml PEG was used with plasma was attributed to the high proportion of 'old' plasma fractionated, i.e. stored more than 2 mth.

TABLE VIII. Comparative purification and yield of high-purity factor VIII prepared on bench scale with 4–5% PEG and on large scale with 5–8% PEG

	No. of batches	*Purification (mean ± SD)*	*Yield (mean ± SD)*
Bench scale prepared in laboratory.(1–5 l.)	94	274 ± 156	62 ± 15
Large scale (40–400 l.)	29	248 ± 112	18 ± 7

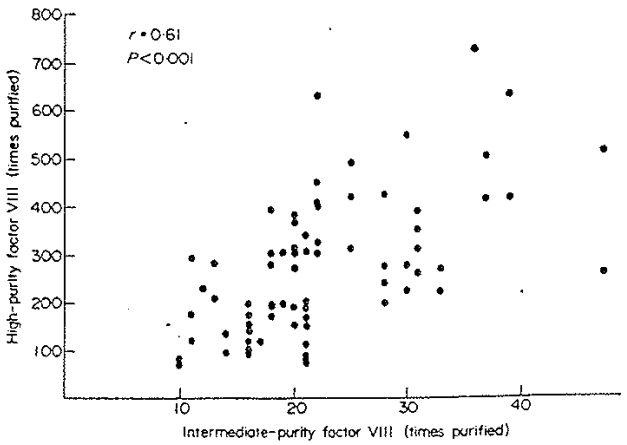

FIG 5. Correlation between purity of high-purity factor VIII and its starting material, intermediate-purity factor VIII (prepared with 4.0–5.0 g/100 ml PEG).

g/100 ml), we obtained factor VIII with a yield of 62% by the bench method, calculated on an assumed activity in the starting plasma of 1.0 u/ml. A higher concentration of PEG (5.8 g/100 ml) with 'native' plasma as starting material in a large-scale procedure resulted in a material of lower yield and purity (Fig 4), but higher purity was achieved with slightly denatured starting material.

With an assigned factor-VIII activity of 1.0 u/ml for pooled starting plasma, mean yield for the high-purity concentrate prepared on a large scale was 18%. This figure was derived from actual production runs with PEG 5.8 g/100 ml, and involved the sacrifice of about one-half of the factor-VIII activity of the intermediate-purity material used during PEG fractionation, to obtain a higher purification (Fig 4). If a concentration of PEG expected to give an optimal yield (4–5 g/100 ml) were to be used in the first PEG precipitation, a yield close to 30% could be expected even on the large scale since, with this PEG concentration, the mean yield of the high-purity factor VIII was 90–95% of the activity of the intermediate-purity material used in its preparation.

Lyophilized high-purity factor VIII is stable at 4°C for over 1 yr (Table IX). Stability at higher temperatures is being tested.

TABLE IX. Stability studies on high-purity factor VIII stored at 4°C

No. of lots	*Months stored (mean and range)*	*Assayed by thromboplastin generation test (factor VIII u/ml) (mean ± SD and range)*	
		Initial	*Final*
15	11 (6–23)	108 ± 40 (22–156)	132 ± 48 (17–204)

The differences between the initial and final activity were not significant.

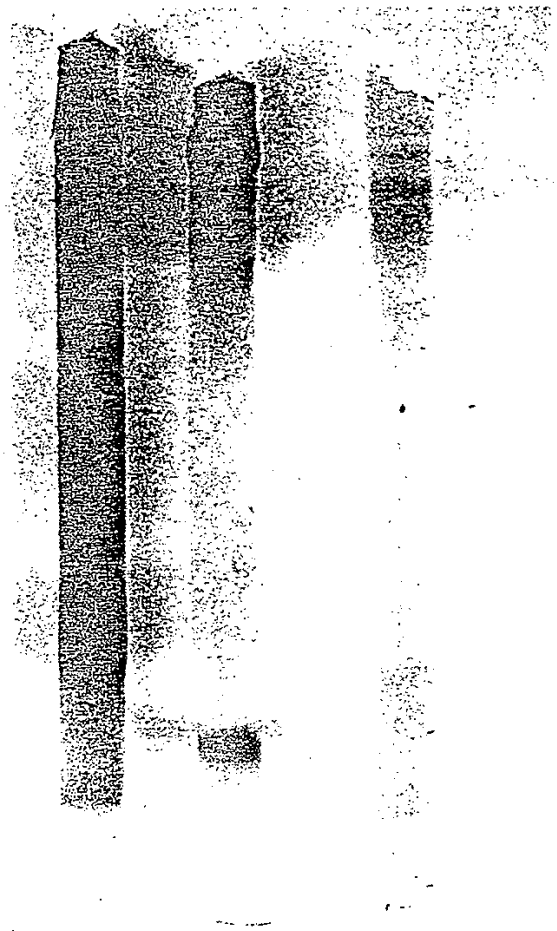

FIG 6. Discontinuous acrylamide gel electrophoresis (pH 9.5) with various materials containing 1 u of factor VIII: normal plasma (left), intermediate-purity concentrate (middle) and high-purity concentrate (right).

(*Facing p 14*)

In some instances, the intermediate-purity concentrate was put through two successive PEG fractionations, i.e. purification and precipitation with PEG, then re-solution and another purification and precipitation. In this experiment, the tris-extracted starting material was purified about 20-fold with a specific activity of 0.3 u/mg protein; when this material was processed further by PEG fractionation, the resulting concentrate was purified 350-fold with > 5 u/mg protein; after a second fractionation with PEG, the product was purified > 800-fold with > 10 u/mg protein (Table I). After the second precipitation, the yield was often considerably reduced.

Acrylamide-gel electrophoresis of 1 u factor VIII contained in plasma, intermediate- and high-purity factor VIII, respectively, appears in Fig 6. Three heavily stained major bands and two minor bands were usually evident in the intermediate material; two lightly stained major and two minor bands were found in the high purity material; one, due to fibrinogen, accounted for about 20% of the total protein (Table X).

TABLE X. Fibrinogen in high-purity factor VIII

Lot No.	*Fibrinogen (%)*
874	14
893	24
908	16
913	37
921	19
927	19
939	23
950	25
981	20
Mean	22
SD	±7

DISCUSSION

Two practical fractionation procedures are presented for making stable, soluble factor-VIII concentrates of intermediate or high purity for clinical or experimental use. Both preparations were made on a bench scale, then adapted to large-scale production (40–400 l.).

Cryoethanol precipitation, as used in the first fractionation step for both materials, probably depends largely on salvaging and preserving the factor-VIII-containing cryoprecipitate which forms during both freezing and thawing of the plasma (Pool *et al*, 1964). Thus, the yield of factor VIII in cryoprecipitate from a single plasma bag, which is potentially high in the hands of an expert technician (Pool & Shannon, 1965), is decreased by large-scale production (R. Rosenfield, personal communication) but may be partially restored by adding alcohol to the frozen slush in the large-scale cryoethanol procedure; these data seem to support the theory that the mechanism is primarily one of enhanced salvage and preservation of the cryoprecipitate. A relatively uniform effect was achieved with the addition of 1–4% ethanol

but higher alcohol concentrations precipitated inordinate amounts of other proteins, reducing the purity of the final product. Magnussen (1963) showed that 3% ethanol alone precipitated factor VIII from the unfrozen plasma supernatant. Addition of 7% ethanol, as in the Blombäck method, resulted in less stable material, suggesting the precipitation of a destructive substance or inhibitor with the factor VIII at the higher ethanol concentrations.

A difficult problem in the economics of producing factor-VIII concentrates arises from the fact that factor VIII prepared by cryoethanol precipitation on a large scale has a much lower yield (34%) than on a bench scale (63%). A similar reduction in yield was found with large-scale production of cryoprecipitate. Increasing the efficiency of these steps could do a great deal to reduce the cost of treatment of haemophilic patients.

Temperature control during thawing and handling of the precipitate is a very important determinant of the yield. If the temperature of the mixture is kept at 0–2°C, the thawing rate of the crushed fresh-frozen plasma (slush) may be varied widely. During the considerable handling required in large-scale production, however, the finely divided cryoprecipitate may be extensively dispersed, with partial solution and a reduction in yield; conversely, a significantly higher yield has been routinely achieved by the minimal handling which obtains in the small-scale procedure.

In an attempt to determine the maximal yield of cryoprecipitate from plasma, we repeated the cycle three to five times: freezing the plasma, thawing it, and removing the precipitate. A total of 70–90% of the factor VIII was normally precipitated under these conditions, most of it in the first cryoprecipitation. These observations, and our earlier findings that ultra-high-purity factor VIII is markedly aggregated when prepared by agarose-gel chromatography or sucrose gradient ultracentrifugation, probably representing different weights or populations of molecules (Johnson *et al*, 1967), together with the ultracentrifuge data of Weiss & Kochwa (1970), led us to believe that this variation may be due to different degrees of aggregation and/or conformational differences in the molecule. If this premise is valid, the potential factor-VIII yield from plasma may well be limited by the different forms of factor VIII which result in part from presently available methods of purification, and may be expected to remain limited unless an entirely new fractionation method is developed for isolating a less aggregated, more 'native' product.

The effect of cryoprecipitation on the factor VIII and contaminating fibrinogen in the concentrate is very important since both tend to aggregate in the cold: during prolonged storage of frozen plasma or intermediate-purity concentrate, during cryoprecipitation in the cold, during prolonged processing in the cold (4°C), or with use of frozen cryoprecipitate. Excessive aggregation may cause great difficulty in the differential fractionation of factor VIII and fibrinogen, and in Millipore filtration before lyophilization, reducing both the yield and purity of the final concentrate. For these reasons and for convenience, we have used less-aggregated, fresh-frozen, native plasma whenever possible and carried out the fractionation procedure at room temperature after the initial cryoethanol precipitation step.

With slightly denatured fresh-frozen plasma (e.g. stored over 3 mth), frozen intermediate-purity material (stored over 1 week), or lyophilized intermediate-purity material as starting material, a PEG concentration of 4–5 g/100 ml is recommended to obtain a high yield. If the desideratum is high purity rather than high yield, the optimal PEG concentration is 5.0–5.8 g/100 ml. With moderately denatured, frozen cryoprecipitate as the starting material, the

markedly aggregated factor VIII and fibrinogen are excluded from the final product by decreasing the extraction temperature from 25°C to 20°C, raising the pH from 6.1 to 7.0 during the first PEG precipitation, reducing the PEG concentration during this step to 3–4.5 g/100 ml and carefully avoiding low temperatures after collecting the cryoprecipitate. Detailed steps in the fractionation of moderately denatured material will be described in a later publication.

These differences in starting materials and required modifications in fractionation methods suggest that the factor-VIII and fibrinogen molecules undergo a series of complex, imperceptible changes starting immediately after blood is taken for transfusion. It is also evident that the amount of denaturation occurring prior to fractionation depends on numerous factors, e.g. the conditions for drawing blood at each blood bank; the techniques, care and time involved in the freezing, processing, storing and transporting of plasma; as well as the handling and storage of this material after it reaches the fractionating centre or manufacturer. To obtain the best possible results, each fractionator may have to tailor his methods to the particular quality of plasma he usually receives from blood banks for processing, and the duration and temperature of storage.

Several investigators have successfully separated factor VIII from most of the fibrinogen (e.g. Wagner & Thelin, 1957; Bidwell *et al*, 1966; Johnson *et al*, 1967; Hershgold *et al*, 1967). While our present high-purity clinical material is not free of fibrinogen, the clottable protein has been reduced to *c* 20%.

Although approximately 0.02 M tris buffer appears optimal for factor-VIII extraction, a buffer of lower ionic strength may denature the product and cause a spurious increase in factor-VIII activity on assay *in vitro*, not evident on infusion *in vivo*, which we have attributed to nonspecific conformational changes in the molecule. We and other investigators have observed this spurious increase in activity with various buffers, during the initial phases of heat denaturation (Thelin, 1968), and during fractionation of ultra-high-purity factor VIII (Michael & Tunnah, 1963, 1966; Veder, 1966).

Acrylamide-gel electrophoresis has revealed the presence of many proteins including fibrinogen and albumin in both ANRC concentrates but, as might be expected, fewer proteins have been demonstrated and at lower concentration in the high-purity material. We observed a definite decrease in purity of the intermediate-purity factor VIII with elimination of the initial cryoprecipitate wash step, but it was our impression that the subsequent high-purity material was even more soluble because of the retention of some albumin.

In our earlier studies we prepared highly purified factor VIII for intramuscular injection, using excessive amounts of PEG at a considerable sacrifice in yield to obtain a preparation which could be concentrated to at least 100–200 u/ml. We later found that the TGT assay using red cell haemolysate did not accurately represent the yield of the high-purity factor VIII, which was still lower than we had expected with the high PEG concentration. We now feel that the concentration should not exceed 5 g/100 ml for optimal yield under the conditions of large-scale production, notably the varying age of the fresh-frozen plasma, temperature during storage, the length of time the cryoethanol precipitate is kept at 0°C or lower, and the considerable amount of handling required to isolate the factor VIII. Other factors significantly affecting the potential yield include: the factor-VIII content of the fresh-frozen starting plasma; the temperature, duration and intensity of extraction; the rate of adding

citric acid to the intermediate-purity material to reduce the pH during the first PEG precipitation (e.g. excessive amounts or too rapid addition may cause premature precipitation); the care used in harvesting the final PEG precipitate; and the temperature of the reconstituted concentrate during Millipore filtration (30–33°C). The concentration of factor VIII in the starting plasma depends upon several factors, such as proper blood collection methods (Perkins *et al*, 1962; Preston & Barr, 1964), the mixing rate during collection, the use of plastic bags, and the time elapsing between collection and chilling of the blood and freezing of the plasma. The expected factor-VIII content of plasma frozen within 4 hr after collection is 0.7–0.8 u/ml; the theoretical level of 1.0 u/ml in pooled fresh-frozen plasma is rarely encountered.

The methods employed in the large-scale preparation of these two concentrates permit collection of the starting fresh-frozen plasma in various cities simultaneously or at different times, to be sent to one or more central points for processing. Since the frozen plasma can be stored at low temperatures, preferably −30°C or lower, for 1–3 mth without significant loss of activity, most regional blood banks can accumulate this material and send it in appropriately large batches to central fractionation plants for economic production. In addition, the high-purity concentrate is highly soluble and can be reconstituted in a small volume. The small size of the container and stability of the product make it easy to transport, to store the vials in a home refrigerator, or to carry on the patient's person.

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