DEVELOPMENT SYSTEMS FOR EFFICIENT
SEPARATION OF FACTOR VIII FROM HUMAN
FRESH FROZEN PLASMA

Monday 23rd November
Groningen, The Netherlands

A REPORT FOR THE SNBTS

BY
C Prowse, B Griffin & P Foster
1. PLAN OF REPORT AND AIMS OF MEETING

(a) The aim of the meeting was to form an ad hoc working group to investigate methods of improving the extraction of factor VIII from donor blood. It arose from comments made to Dr Lane at the Haemonetics meeting in Geneva earlier this year and was organised jointly by Drs Lane, Rock and Smit-Sibinga, to follow on from the Groningen annual seminar on blood components. In practice the meeting was largely concerned with the effects of heparin addition on factor VIII production.

(b) This report summarises data presented at the meeting but does not include any data already published. This is summarised in the attached report. Dr Smit-Sibinga will be producing a printed publication of the proceedings in the near future (after correction of tape transcripts by the individual participants) for circulation to the participants.

(c) The meeting was introduced by Drs Smit-Sibinga, Rock and Britten. It was pointed out a region of 10% population could probably not produce much more than 5,000 litres of fresh frozen plasma for fractionation. Concentrate produced at 20% yield from this might just meet current factor VIII requirements, although these vary considerably from country to country, eg the Bonn haemophilic centre may use more concentrate per year than the United States. The use of SAG and substitution of cryo-depleted for whole clinical plasma might increase the available material but the big area for development was improved treatment and processing of plasma at and after donation. The above donation rate also yields 100 kg albumin per 10^7 (based on 25 g from one litre). The annual factor VIII requirement of haemophiliacs varies between countries from 2,800 to 1,100,000 units.

(d) Dr Rock briefly described her technique for production of factor VIII from heparinised plasma and suggested this might improve yields by three mechanisms.

1. increased plasma VIII activity;
2. improved cryoprecipitability;
3. permits purification using the cold-heparin precipitation technique.

(e) The Irish have adopted Dr Rock’s method for intermediate purity concentrate production and obtain a 67% yield of VIII from plasma (Table 1). With a population of 3 x 10^7 Dr Hanratty calculates 4,500 litres of fresh plasma (300 donations/week) would yield 3 x 10^9 of factor VIII as concentrate sufficient to treat their 107 severe haemophiliacs on home therapy (23,000 u/wk) and allow a bit over for the 181 less severe haemophiliacs. They currently collect 2,700 donations a week but Dr Rock’s procedure requires centre session donations.

(f) Dr Lane reported only 20% of donations in England and Wales (which only give 28,300 donations/10^6) are processed to fresh frozen plasma for fractionation. He is aiming to get 60% of donations along this path and hopes the devoted wedge pack will help in this and if achieved this would yield 200 metric tonnes of fresh plasma per year. At present yields, even this would not allow self-sufficiency in factor VIII, but if a yield of 67% could be obtained self-sufficiency would be on. Plasmapheresis would also improve the situation.

(g) Dr Britten when asked what a haemophiliac would like in a factor VIII preparation suggested low volume (230 u/ml), effective, easy to administer and store and sufficient quantity (possibly 20,000 u/yr would be insufficient) as requirements.
2. BLOOD COLLECTION

(a) Dr Smit-Sibinga described the current multiple plastic bag system and suggested a donation rate of 50,000/year giving 25,000 donations of fresh plasma could yield $2.7 \times 10^3$ factor VIII by current methods or $4 \times 10^3$ by the recently developed technique. Dr Lane described the wedge pack system designed to satisfy the regulatory authorities and meet the requirements of Good Manufacturing Practice. He hoped that this bag directed towards fractionation plasma would increase production and allow addition of additives to plasma that were unrelated to non-fractionation components. Dr Prowse described the pigtail system and gave evidence that it was possible to use with bacterial safety and then described various possibilities for optional and dedicated additive packs.

(b) Both Dr Rock and Dr Smit-Sibinga hinted that a commercial sterile docking device might soon be available. They would not say which company was producing this but Fenwall would seem the most likely possibility.

(c) Dr Robinson did not attend but Dr Rock gave a short talk on plasmapheresis. She has been using the Haemonetics 50 but has recently taken delivery of 20 of the new model VS50 which can collect platelet poor plasma (lower count than on 50) or platelet rich plasma which can then be processed to concentrate and platelet poor plasma. (3 to 4 units of platelets and 500 ml plasma per run). She now collects plasma using a 1:13 to 1:15 volume ratio of ACD anticoagulant by metered addition near the needle. Although not yet tried there is also the possibility of DDAVP treatment and heparin placement in the plasma collection bag. She did say the use of 1:15 ACD yields proportionately more VIII than the usual 1:8 volume ratio.

(d) It was mentioned that the Swiss firm DOLTRON are developing a device for metered addition of anticoagulant to blood during normal donation. A flow meter monitors the donor line and controls anticoagulant addition near the venepuncture site by a roller pump. Dr Pflugshaupt stated this device improved plasma levels of factor VIII (C, KNG and RI CoF) reduces FpA levels and gives improved platelet quality (ADP aggregation) compared to normal CPD collection.

(e) Comments were invited on the effect of platelet levels in plaspheresis plasma on VIII fractionation. Dr Smith said that such plasma had a slightly higher count than normal donor plasma, although these were small platelets and had little effect on VIII yield or solubility. Dr Rock stated the new VS5 machine produced plasma with a lower count than the Haemonetics 50. Dr Ennleistle said the Oxford centre gave plaspheresis plasma a second spin to remove some residual platelets.

(f) Dr Pflugshaupt presented data on the fibrinopeptide A (FpA) levels in donor plasma as a measure of the quality of blood collection. Initially blood was collected in Nessel's anticoagulant and assayed in a double antibody RIA using 5.5mM Tris, 0.1M NaCl, 0.1% w/v ovalbumin as buffer. Fibrinogen was removed from samples, not by dialysis or bentonite adsorption, but by dilution in 0.5M acetate pH5.0 followed by heat precipitation at 85°C for 30 min and room temperature for 1 min. This allowed 350 samples (0.05ml) to be assayed per day in a 24 hr assay. Initial comparison of Nessel's anticoagulant in tubes with collection in normal donor bags with and without mixing showed it was possible to obtain basal values in the latter:
<table>
<thead>
<tr>
<th>Tube</th>
<th>Unmixed Bag</th>
<th>Mixed Bag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fpa</td>
<td>3ng/ml</td>
<td>2.9 &amp; 47.3</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

Assay of Fpa in routine plasma donations then showed

Fpa <30ng/ml n = 1014; Fpa >30ng/ml n = 171 (levels up to 400ng/ml)
with a wide distribution.

Following the second series carried out (after instruction of donor attendants on mixing during donation and the reasons for it) gave:

Fpa <7ng/ml n = 1021; Fpa >7ng/ml n = 158
with a much narrower distribution.

Subsequent processing of these plasmas in groups of varying Fpa content showed that poorly collected material (high Fpa) was more difficult to process and of low solubility, although the VIII content was not affected by cryoprecipitate.

(Table 2)

(g) Dr Hanratby stated that heparin addition within 30 minutes of donation to ACD plasma improved VIII yields after processing but addition 6 hours after donation was too late. Heparin addition was therefore limited to central sessions (or would require a centrifuge and separation at sessions).

(h) Dr Rock stated, but did not present data, that citrate addition to plasma removes calcium from VIII within 30 minutes and destabilised VIII. At 20 min the levels of ionised calcium were as low as they are going to be, but readdition of calcium can restore VIII activity (Weiss has published data saying such addition only prevents further decay). Addition of calcium to the void volume peak of gel-filtered factor VIII does not lead to reactivation, presumably as it is too late. She has also tried to re-activate plasma with other metal ions and has some evidence manganese and Zinc will substitute for calcium. In a paper in press she suggests zinc re-activates by a conformational/steric effect as the kinetics do not fit an enzymatic process.

(i) In the absence of Dr Blagdon, Dr Myllyla pointed out SAG collection increased the volume of plasma available from each donation and that Dr Blagdon had presented evidence (see attached report) that heparin addition improved the stability and cryoprecipitability of factor VIII in plasma. On the phone Dr Blagdon stated his results were based on collection of donations in Fenwall heparin packs (final 4u/ml blood) followed by addition of a CDP-SAG mix to the separated red cells. This does not tie in completely with reports of the Cambridge RDG meeting of this year.

3. ASSAY PROBLEMS

(a) Jim Smith pointed out the problems of trying to introduce improved methods for VIII collection on a large scale. With good statistically valid assays performed in a symmetrical design with replicate samples the confidence limits may be 90–100% and require 10 runs to reach significance. In sharing development workload between laboratories the differences between laboratories and 1- and 2-stage assays (the latter are probably easier for heparinised samples – see Table 3) must also be taken into account. This
will be helped by use of appropriate concentrate or plasma standards (NOT local normal pools) and adoption of an arbitrary convention for standards used to assay cryoprecipitate and cryosupernatant. He also suggested that assuming of any freeze-dried samples between participating laboratories would help and suggested expressing yields as mW/ml starting plasma, rather than as a percentage would reduce some of the potential variation. Paired samples can help considerably. Longitudinal studies require special attention to standards. If plasma is to be assayed this could be done on fractionation plasma by frozen core, crushed snow or cryoprecipitate suspended in supernatant stages which would not necessarily be equivalent.

(b) The Oxford laboratory have shown 9 in/ml heparin in plasma does not interfere in the 2-stage assay due to its adsorption to alumina and the dilutions involved. Using this assay they found no difference in the VIII content of blood collected in trisodium citrate, heparin or citrate and heparin (Table 1). (Note however that the 2-stage assay takes about 20 min and involves dilution in a citrate-saline buffer.)

(c) Dr Rock stated she had produced a pamphlet describing exactly how she carries out the one-stage VIII assay on heparinised samples by protamine neutralisation. I have written requesting a copy of this.

(d) Dr Morgensthalter at Dr Duckert's suggestion had avoided using protamine for neutralisation as he had seen a German paper (? by Zimmerman) which stated protamine excess may result in overestimation of VIII. He had found addition of 12 µg/ml protamine to plasma containing 0.45 u/ml VIII resulted in an activity of ~0.6 u/ml. Dr Hanraty also stated that his laboratory had difficulty assaying samples other than his final product, and that this might be due to excess protamine causing overestimation. Dr Morgensthalter had added heparin at 0, 1, 2, 3, 4 and 5 u/ml to pre-assayed concentrate and found neutralisation with Polybrene gave complete neutralisation and no interference between 0.1 and 0.6 mg/ml. Higher polybrene levels resulted in underestimation. At >6u/ml heparin complete neutralisation was not obtained, although an apparent plateau of activity was seen between 0.1 and 0.6 mg/ml Polybrene. For plasma samples the region of neutralisation without interference was reduced by 0.1 to 0.2 mg/ml Polybrene.

(e) Dr Over had tried using General Diagnostics Hepasorb beads to remove heparin from plasma but had had problems with frequent invalid assays (not-parallel with some standards).

(f) Dr Rock stated her produce had a RAG/CAG ratio close to 1.0 suggesting little VIII:C degradation in process but she had avoided using CAG assays to monitor purification as CAG and C assays can diverge (eg during clotting or on binding to phospholipid).

4. FACTOR VIII IN FRESH PLASMA AND HEPARIN

(a) In answer to my question "Do you know of any data, apart from your own, which shows collection in heparin results in elevated levels of VIII in fresh plasma?" Dr Rock stated that Dr Holst and Dr Wickerhauser had obtained similar results to her using her methods exactly. She did not show any data and it is not published. The experience in Holland, the UK and Switzerland (see below and attached report) is of no benefit at this fresh plasma stage.
(b) Dr Smith-Sibinga's published data and Dr Over's results using Heparin to remove heparin (when it works) show no difference in VIII between fresh citrate and heparin plasma.

(c) Dr Morgenthaler found little if any difference in VIII levels of fresh plasma of donations collected in a paired manner in ACD or ACD plus 5 u/ml heparin and 3mM calcium (final concentrations in blood with heparin/Ca++) addition at completion of donation ie <30 min).

(d) Oxford (2 stage assay) and Dr Blagdon (? which assay) find no difference in fresh plasma levels of VIII after collection in heparin, heparin + citrate or citrate (Table 3 and attached report).

(e) Oxford have compared VIII levels in fresh liquid and fresh frozen-thawed heparin and CPD plasma and find no appreciable differences (all 96-100% of fresh liquid heparin plasma).

(f) In answer to the question "Is calcium heparin any better than sodium?" Dr Rock said she had only tried the sodium salt but had added increments of calcium to sodium heparin plasma without any benefit.

(g) Nobody knew what the optimal heparin concentration for collection was although Dr Rock had tried different concentrations for their effect on VIII stability. Dr Holst thought 1 u/ml was sufficient.

(h) Dr Blumenstock asked if other glycosaminoglycans could substitute for heparin. He and Dr Rock undertook to investigate the effects of such compounds on fibronectin.

(i) On request Dr Smith showed a summary of his published data that CPD is better than ACD plasma for VIII preservation:

<table>
<thead>
<tr>
<th>IU/Kg Plasma</th>
<th>ACD</th>
<th>CPD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single donor plasma</td>
<td>710</td>
<td>805</td>
<td>10</td>
</tr>
<tr>
<td>Frozen plasma cores</td>
<td>710</td>
<td>810</td>
<td>10</td>
</tr>
<tr>
<td>Cryoprecipitate (bulk)</td>
<td>325</td>
<td>367</td>
<td>8</td>
</tr>
<tr>
<td>Intermediate purity concentrate</td>
<td>211</td>
<td>256</td>
<td>8</td>
</tr>
</tbody>
</table>

5. HEPARIN AND PLASMA VIII STABILITY

(a) Creared found little difference in VIII lost during 6 hour storage at 20°C as plasma between heparin and trisodium citrate. They do find some benefit of heparin over CPD collection on VIII stability in blood or plasma stored at 4°C or 30°C for 24 hours (Table 3). Dr Blagdon has similar results (see attached report).

(b) Dr Morgenthaler (polybrene/1 stage assay) found some minor benefit of immediate addition of 5u/ml heparin and 3mM Calcium to ACD blood or plasma curing storage for 12 to 24h at 4°C, but little, if any, benefit of such addition during storage for 12 to 18 h at 30°C. (experiments on ~10 split donations).
6. HEPARIN AND FRACTIONATION OF PLASMA FACTOR VIII

6A Cryoprecipitation

(a) Dr Rock stated that heparin addition resulted in a cryoprecipitate with an increased protein (as well as factor VIII) content, possibly due to heparin interaction with fibrinogen and fibronectin. She also said unlike other methods, hers allowed all plasma factor VIII to be accounted for after processing. We would disagree, both local thaw-siphoning and bulk continuous thawing lead to a sum of cryoprecipitate and cryosupernatant factor VIII very close to the original plasma content.

(b) Dr Blagdon confirms the benefit of heparin over CPD in increasing the VIII content of cryoprecipitate (see attached report).

(c) Dr Pflugshaupt in fractionating pools of different FpA content showed heparin addition (unstated at what stage or concentration) improved the stability of his final product but had little effect on the yield of his process (Table 2).

(d) Heparin, if it is to be of benefit, must be added before freezing plasma.

6B Further Processing

(a) See 6Ac above and Table 2 for Dr Pflugshaupt's results.

(b) Dr Rock stated she is now processing cryoprecipitate (containing about 10% of plasma fibrinogen) pools of 6-20 units by resolution in heparin buffer and cold precipitation for 1 hour at 4°C and this gives a product with about 1% of the plasma protein and lower levels of isoagglutinins than cryoprecipitate. For heparin plasma: yield 800 u VIII/litre at 1 u VIII/mg protein. CPD plasma and heparin: yield 660 u VIII/litre (62%) at 0.8 - 0.9 u/mg.

For heparin plasma she stated the initial cryoprecipitate cannot be drained completely as some clotting occurs (Dr Snit-Sibinga does drain at this stage without problems). For heparin-CPD plasma this does not occur and Dr Hanratty confirms this. With Dr Wickerhauser, Dr Rock has also further processed cryoprecipitate by 11% PEG precipitation to give a high purity product at 530 u/litre.

(c) Dr Hanratty described the Irish adaptation of Dr Rock's method. This is described in Table 1 and gives a product of ~0.8 u VIII/mg protein at ~700 u/litre. Their first three batches gave much poorer yields (~300 u/litre) and this is now thought to be due to (i) heparin addition too late; (ii) difficulties with assay in presence of protamine or heparin excess; (iii) insufficient care in the first cryoprecipitation step.

(d) Dr Snit-Sibinga's variation of Dr Rock's process has been published and yields ~700 u/litre at 1 u VIII/mg protein. The following additional information was given. Prior to the second precipitation, which is carried out in a 300 ml Fenwall transfer pack for 2 hours at 0°C, the product is the same colour as plasma. After this step it is colourless and contains a few strands (? fibrin). The Dutch remove this on a 170u filter, Dr Rock by centrifugal washing. After this the product easily redissolves at 37°C. Relative to starting plasma the Dutch product contains 0.34% albumin, 15% immunoglobulin and 11% fibrinogen. The original blood heparin is distributed as follows (from each 450 ml donation + 1500u heparin):
350 ml red cell concentrate: 0.8 IU heparin/ml
234 ml cryosupernatant: 2.5 IU heparin/ml
10 ml product: 9 IU heparin/ml

(e) Dr Sakariassen mentioned a new high purity product produced by Dr Bloedhoek and Kabi which retains the ability to correct the bleeding time in von Willebrand's patients. This is apparently made by alumina adsorption of cryoprecipitate followed by treatment with heparin-agarose to remove fibrinogen.

(f) In the Abstracts (I have a copy) of the seminar on "congenital bleeding problems - haemophilia home care" held the previous week in Groningen, Dr. Feldman mentions a new Armour factor VIII product - "Factorate Generation II", with a potency of 30-40 IU VIII/ml and purity ~3 IU/mg protein with a C:G ratio ~1.0 and a solution half life of ~100 days.

(g) The German process of pasteurising factor VIII is apparently carried out in 24 glycols, 30% w/w sucrose which must presumably be removed after pasteurisation. The overall yield is low (? 5u/litre).

7. OTHER PROTEINS

7A. von Willebrand Factor (vWF)

(a) Dr Sakariassen described his Baumgartner model for assessing vWF by binding of 125I-labelled platelets to subendothelium from human cadaver renal or umbilical arteries. Using dextran 40, albumin, cryosupernatant plasma, or von Willebrand's disease plasma as perfusate about 25% of normal plasma platelet adherence levels are obtained and can be corrected by addition of purified vWF. Using 125I-vWF he found a correlation between sub-endothelial binding of vWF and the plasma level of this protein. Treatment of von Willebrand's patients with DDAVP showed a good correlation of bleeding time correction with the activity of patients plasma in this platelet adhesion assay of vWF. He has tested a variety of VIII products in this assay and finds only three of activity: freeze-dried cryo, Kabi fraction I-0 and the new Kabi high purity product, all of which show a slow moving peak in crossed immunoelectrophoresis for VIII:Ag (1st dimension SDS-agarose). Other intermediate purity and high purity products were not active in his assay, which did not give the same results as the ristocetin cofactor aggregation assay. However, he found gelfiltration fractionation of purified vWF gave material of slightly lower molecular weight with activity in his assay. Very high molecular weight is therefore not a prerequisite for vWF activity and it is possible such activity resides on a particular polypeptide chain in factor VIII. He also has preliminary evidence that the vWF receptor on subendothelium is fibronectin (? + fibrinogen).

7B. Fibronectin

(a) Dr. Blumenstock showed a slide (Figure 1) of the functional domains in fibronectin. These might be used to separate factor VIII from fibronectin. He purifies fibronectin from plasma by 2 cycles of chromatography on gelatin-agarose, washing and elution with 7M urea. This yields a homogenous product active in a liver slice phagocytosis assay. This is not however the whole story as, in this assay fibronectin-deficient plasma contains a potent inhibitor of fibronectin action. Thus purified fibronectin might not be a suitable clinical product. He also mentioned the curious effects of heparin on the function of the reticuloendothelial system in animal models, low doses potentiate activity, but high doses inhibit it.
(b) The use of cryoprecipitate to correct fibronectin deficiency in trauma patients in the Eastern United States now presents a fair proportion of its total use. Without good evidence some centres are prospectively treating all trauma and sepsicaemia cases in casualty with 10 to 20 units of cryoprecipitate. Dr Blumenstock has a clinical trial in progress and results should be available in a few months. Cryoprecipitate contains about ten times the fibronectin level of plasma.

(c) Dr Blumenstock states glycosaminoglycans, other than heparin do not bind purified fibronectin (but see attached report).

(d) Dr Sakariassan said DDAVP infusion does not influence plasma fibronectin but does increase VIIIIR:Ag, particularly the higher multimers (as observed in platelets is ~25 x 10^7 daltons.)

(e) Dr Smit-Sibinga stated that the supernatant from the second precipitation step in the Dutch process for intermediate purity factor VIII production does not contain detectable levels of fibronectin.

8. REGULATORY ASPECTS

(a) Dr Smit-Sibinga outlined the WHO requirements for sterility, acute toxicity and pyrogenicity of products. He is currently checking these on his product.

(b) Dr Hanratty stated the Irish product met these requirements.

(c) Dr Holst outlined Hyland’s experience with blood products and heparin over a number of years.

(i) They have collected plasmapheresis plasma (15,000 litres/year) in heparin for some years for clinical chemistry controls. They are currently investigating its suitability for whole blood collection.

(ii) They have for a number of years added heparin during VIII processing after the initial cryoprecipitation. It is possible that heparin addition to plasma might alter the results of Cohn fractionation and it would be necessary to ascertain where the heparin ends up. They had some experience of this from the 1950s.

(iii) Heparin in the final product presents little problem as some factor IX products currently contain it. All that is required is that the product is labelled with the heparin content. Effects on haemostasis in haemophiliacs might have to be investigated.

In essence the regulatory authorities would want to know:

(i) In vitro equivalents of heparin in the product.

(ii) Animal toxicity studies on downstream products made from heparin/plasma.

(iii) In vivo efficacy of the heparinised product.

9. FUTURE PLANS

(a) Dr Hanratty is starting a clinical trial of the Irish product very soon. He has a stock of 50 vials of his product. Dr Rock is doing studies in haemophilic dogs with Dr J Dodds.
(b) Dr Rock and Dr Blumenstock are to look into the contribution made by fibronectin to the effects Dr Rock observes during her factor VIII process.

(c) Dr Lane stated that for the next 3 years the Oxford PFL will act as a pilot scale facility (≤100 Kg processes) and could be used as a basis for investigations arising from the meeting.

(d) An ad hoc committee of Drs Smit-Sibinga, Rock and Lane was formed. They will transcribe the meeting proceedings and circulate it to participants. The aim is "to develop systems for efficient fractionation of factor VIII" and hoped that the meeting would form the basis of a working group which would involve pooling data and sharing the workload in this area. They proposed to inform the WPH, International Red Cross and ISBT they were doing this (which might help with some funding) and prepare a worklist of potential areas of interest to be circulated with a call for volunteers. It was intended to have some form of continuous review and to hold regular meetings (next one possibly at Budapest ISBT congress). Possible areas of interest might be:

(i) Assay problems.
(ii) Different mechanisms of heparin action.
(iii) Fibronectin.
(iv) Platelet effects.
(v) Additive mixtures.
(vi) Red cell storage additives.
(vii) Cold precipitation processing.
(viii) Transfusion Centre logistics to ensure fast separation and freezing.

This is obviously a wider remit than the heparin process which formed a large part of the meeting.

PERSONAL COMMENTS

(a) How does the 2-stage assay citrate-saline buffer diluent affect results?

(b) Why does heparin plasma not clot when neutralised eg with protamine?

(c) Is there an easy way to remove heparin from samples? (heparinase, Polybrene-agarose?)

(d) How much heparin is needed and is the calcium better than the sodium salt?

(e) Can DDAVP and SAG + CPD be combined with the heparin processing?

(f) Will other glycosaminoglycans substitute for heparin at any stage?

(g) How soon, how much, what kind and what route of heparin and at what temperature?

(h) Does heparin really increase factor VIII levels in fresh plasma or only increase stability?

(i) It seems fairly well accepted heparin improves stability and process yield of Factor VIII.

(j) Should blood be collected in heparin and CPD (SAG) added to red cells or collect in CPD and add heparin to plasma.
(k) Can the method be used in 3rd World Countries?

(1) Is outdated plasma a potential source of clinically usable/effective fibronectin?

Albert Farrugia is already thinking of looking at c, d and f. Dr. Lane knows this and may ask us to undertake this work for the group.

The Swiss are probably attempting preparation of fibronectin from outdated cryoprecipitate and should soon be doing clinical trials.
TABLE 1

DUBLIN METHOD (DR HANRATTY)

- Aim to meet national needs with intermediate purity material.

1. Collect blood in Fenwall triple ACD pack. (7dCPR)

2. Add 4 ml heparin (from Fenwall, gives 1-2u/ml in plasma) and freeze plasma at -83°C.

3. When 150 donations stock.

4. Thaw in 35 minutes with 48 donations per Grant bath like Edinburgh's. (74°C)

5. Spin off cryo in pre-cooled centrifuge.

6. Replace bags in Grant bath and siphon off supernatant into third pack using tube strippers, leaving minimal fluid (~2ml).

7. Immediately add 3 ml heparin-buffer (Rock's) to cryo (cannot freeze at this stage).

8. In sterile suite in laminar flow hood pool 25 donations into Sorval centrifuge bottle (n = 6).

9. Place these in Tecan bath (with ping-pong balls) at 0°C (v important) for 2h.

10. Spin in precooled Sorval centrifuge 7,000 g, 10 min and aspirate supernatant.

11. Resuspend precipitate in 165 ml buffer (as Dutch technique) for flask.

12. Pool all in one flask (~1000 ml) and ampoule as 100 x 10 ml vials (25 ml) and lyophilise.

13. From each test 4 vials for sterility, others for pyrogen and toxicity (all OK).

<table>
<thead>
<tr>
<th>Plasma (pre-heparin)</th>
<th>Final Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume (ml)</td>
<td>30,080</td>
</tr>
<tr>
<td>Total VIIIu</td>
<td>32,250 (1.08u/ml)</td>
</tr>
<tr>
<td>Yield (%)</td>
<td>100</td>
</tr>
<tr>
<td>Total Protein (g/l)</td>
<td>67</td>
</tr>
<tr>
<td>Specific activity u/mg</td>
<td>0.016</td>
</tr>
<tr>
<td>Purification</td>
<td>x 1</td>
</tr>
</tbody>
</table>

Comments

1. Still cannot get satisfactory assays on intermediate process samples due to heparin (assays by Dublin haemophilia centre).

2. Heparin must be added early (? within 30 min), 6 hours is too late.

3. Some evidence excess protamine sulphate causes overestimation of VIII.

4. Good and careful technique in initial cryoprecipitation is essential (first three batches gave yields 24, 18 and 28% due to poor attention to detail).
### TABLE 2

**SWISS DATA (DR PFLUGSHAUPP)**

- relating fibrinopeptide A content of plasma to VIII fractionation.

#### A. Freeze Dried Cryoprecipitate

<table>
<thead>
<tr>
<th>FP A (ng/ml ± 2SD)</th>
<th>Number of Donations</th>
<th>VIII:C (u/ml)</th>
<th>Solubility (min)</th>
<th>Stability (number clotted after 24h at 20°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 2.2 ± 0.24</td>
<td>8</td>
<td>3.05</td>
<td>5.47</td>
<td>1 of 8</td>
</tr>
<tr>
<td>2. 21.2 ± 6.55</td>
<td>9</td>
<td>2.97</td>
<td>4.28</td>
<td>0 of 9</td>
</tr>
<tr>
<td>3. 183 ± 151</td>
<td>8</td>
<td>3.01</td>
<td>11.57*</td>
<td>4 of 8*</td>
</tr>
</tbody>
</table>

#### B. Concentrate

<table>
<thead>
<tr>
<th>FP A (ng/ml)</th>
<th>&lt;30</th>
<th>&lt;30</th>
<th>&gt;30</th>
<th>&lt;7</th>
<th>&lt;7</th>
<th>&gt;7</th>
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<tbody>
<tr>
<td>Heparin addition</td>
<td>NO</td>
<td>YES</td>
<td>NO</td>
<td>NO</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>u/ml VIII:C in</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starting cryo</td>
<td>4.2</td>
<td>3.0</td>
<td>5.0</td>
<td>3.9</td>
<td>3.9</td>
<td>3.0</td>
</tr>
<tr>
<td>Pre-lyophilisation</td>
<td>23.5</td>
<td>17.0</td>
<td>(clotted)</td>
<td>19.0</td>
<td>22.5</td>
<td>12.0</td>
</tr>
<tr>
<td>After soln.</td>
<td>21.8</td>
<td>17.3</td>
<td>-</td>
<td>26.5</td>
<td>23.7</td>
<td>14.3</td>
</tr>
<tr>
<td>In soln 24h</td>
<td>33.0</td>
<td>17.5</td>
<td>-</td>
<td>25.3</td>
<td>20.0</td>
<td>13.8</td>
</tr>
<tr>
<td>In soln 48h</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.8</td>
<td>19.0</td>
<td>-</td>
</tr>
</tbody>
</table>

**Consequently:**

1) poor plasma, i.e. high FP A, gives poor processing
2) heparin addition (u/ml ? at what stage) improves stability
TABLE 3

OXFORD DATA

Collect blood in 1/10 volume of 50 u/ml heparin or 3.8% citrate or both.

- adjust VIII potency for plasma protein content to allow for anticoagulant differences (in practice this adjustment can be obtained from the volumes of blood and anticoagulant, and the donors haematocrit).

- assay VIII:C against donations collected in citrate-only by two-stage assay at one hour and six hours after collection (stored at 20°C as plasma)

- each experiment was a pool of 3 donations each split between the three anticoagulants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>1 Hour</th>
<th>6 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heparin/citrate vs citrate</td>
<td>0.95</td>
<td>0.95</td>
</tr>
<tr>
<td>heparin vs citrate</td>
<td>0.99</td>
<td>1.01</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heparin/citrate vs citrate</td>
<td>0.97</td>
<td>1.00</td>
</tr>
<tr>
<td>heparin vs citrate</td>
<td>1.12</td>
<td>1.25</td>
</tr>
</tbody>
</table>

**Demonstrating**

- little, if any, benefit of heparin on fresh plasma VIII:C.

- possible minor benefit of heparin on VIII stability.

- no effect of 5u/ml heparin in two-stage assay. (\( \log_{10} \approx 0.1 \) in plasma)

The latter may be explained partly by the demonstration that heparin (anti-Xa assay) and antithrombin III are removed during the alumina adsorption (80 to 90% and ~98% respectively) used in the two stage assay.

**Calculation shows:**

<table>
<thead>
<tr>
<th>ASSAY</th>
<th>2 Stage</th>
<th>1 Stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alumina adsorption</td>
<td>removes heparin</td>
<td>-</td>
</tr>
<tr>
<td>Working dilution</td>
<td>128 to 512</td>
<td>10 to 100</td>
</tr>
<tr>
<td>Assay dilution</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Final heparin (from 5u/ml in blood)</td>
<td>(10^{-3})u/ml</td>
<td>(10^{-1})u/ml</td>
</tr>
<tr>
<td>ATIII in assay</td>
<td>NOT</td>
<td>0.2u/ml</td>
</tr>
</tbody>
</table>
Yield of factor VIII in plasma and cryoprecipitate relative to fresh heparin plasma (9u/ml) corrected for anticoagulant volume differences by assay of plasma protein.

(a) In fresh plasma before and after freezing by 2-stage assay
- heparin and CPD very similar - all 96 - 100%.

(b) Yield of VIII:C (2-stage) in blood or plasma stored at different temperatures for 24h (4 donors) as % of fresh heparin plasma.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Heparin (u/ml Blood)</th>
<th>CPD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Blood</td>
<td>4°C</td>
<td>55%</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>85%</td>
</tr>
<tr>
<td>Plasma</td>
<td>4°C</td>
<td>90%</td>
</tr>
<tr>
<td></td>
<td>20°C</td>
<td>90%</td>
</tr>
</tbody>
</table>

- Low yield in 4°C blood due to cryoprecipitate lost in red cells as rewarming blood at 24h restores yield to plasma levels.

- Assay of PKA with S-2302 shows no difference in contact activation between samples collected in heparin or CPD.
FIGURE I

FUNCTIONAL DOMAINS IN FIBRONECTIN

FIBRIN
COLLAGEN (CROSS LINKING ACTIN) BOUNDING HEPARIN BINDING
S. AUREUS

GLYCOSAMINOGLYCANS, OTHER THAN HEPARIN (BUT WHICH ONES), DO NOT BIND TO PURIFIED FIBRONECTIN